Secretion processes as a limiting factor of protein production in *Bacillus*

DISSERTATION

zur Erlangung des Doktorgrades

der Naturwissenschaften

(Dr. rer. nat.)

im Fachbereich Chemie

der Philipps-Universität Marburg

vorgelegt von

Manuel Strach, M. Sc.

geboren in Herdecke, Deutschland

Marburg an der Lahn,
September 2023

Die vorliegende Dissertation wurde von März/ 2019 bis September/ 2023 am Fachbereich Chemie/ SYNMIKRO der Philipps-Universität Marburg unter Leitung von Prof. Dr. Peter L. Graumann angefertigt.

Vom Fachbereich Chemie der Philipps-Universität Marburg (Hochschulkennziffer 1180) als Dissertation angenommen am _____

Erstgutachter:	Prof. Dr. Peter L. Graumann Fachbereich Chemie, Philipps-Universität Marburg
Zweitgutachter:	Prof. Dr. Martin Thanbichler Fachbereich Biologie, Philipps-Universität Marburg

Tag der Disputation: _____

Eidesstattliche Erklärung

Ich erkläre, dass eine Promotion noch an keiner anderen Hochschule als der Philipps-Universität Marburg, Fachbereich Chemie, versucht wurde.

Hiermit versichere ich, Manuel Strach, dass ich die vorliegende Dissertation:

Secretion processes as a limiting factor in protein production in *Bacillus*

selbstständig, ohne unerlaubte Hilfe Dritter angefertigt und andere als die in der Dissertation angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten oder unveröffentlichten Schriften entnommen sind, habe ich als solche kenntlich gemacht. Dritte waren an der inhaltlich-materiellen Erstellung der Dissertation nicht beteiligt; insbesondere habe ich hierfür nicht die Hilfe eines Promotionsberaters in Anspruch genommen. Kein Teil dieser Arbeit ist in einem anderen Promotions- oder Habilitationsverfahren verwendet worden. Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Marburg, 27.09.2023	
Ort/Datum	Unterschrift (Vor- und Nachname)

Summary

Protein secretion involves several important sequential steps. First, proteins to be secreted must be recognized and their translocation-competent conformation must be ensured. This is followed by the overcoming of two barriers, the cell membrane and the cell wall. The active transport across the membrane can occur by several well-studied mechanisms, the most notably of them are known as "general secretory" (Sec) and "twin-arginine translocation" (Tat). For the passage through the cell wall, on the other hand, understanding is still almost completely lacking.

In this work, I investigated this process, using super-resolution fluorescence microscopy to visualize AmyE-mCherry during secretion in *Bacillus subtilis* and *Bacillus licheniformis*. The overexpressed fusion protein localized as distinct foci in the cell envelope, which were mostly lost upon degradation of the bacterial cell wall through treatment with lysozyme. I could also show that AmyE is released from the cells at discrete zones, similar to the localization of fluorescently labeled AmyE as foci inside the envelope. High-level protein secretion peaked at the transition from exponential growth to the stationary phase and appears to be restricted to a subpopulation of cells, which presumably is also the case for general protein secretion. Time lapse experiments revealed the AmyE-mCherry foci to be statically positioned throughout several minutes, in contrast to the lateral mobility of Sec-machinery associated membrane proteins SecA and SecDF, labeled with mNeonGreen. Interestingly, the AmyE-mCherry foci displayed considerable fluctuations of fluorescence intensities within a minutes-time-scale, suggesting visualized diffusion of proteins along the passage through the cell walls meshwork. This idea of diffusion is supported by recent AFM Imaging results of *B. subtilis*, revealing a heterologous cell wall structure with deep pores its peptidoglycan surface.

For large parts of industrial biotechnology, the secretion of microbially produced enzymes and proteins into the culture supernatant is of enormous relevance, due to the lower costs for subsequent processing associated with this method as compared to the disruption of the producing cells. Studies investigating secretion efficiency in *Bacillus* species, have revealed numerous influencing factors.

Since the bacterial cell wall is often overlooked in the search for secretion bottlenecks, I targeted autolysins that can affect cell wall thickness and the density of the meshwork. While absence of LytD had little effect on secretion, deletion of *lytC* and *lytF* significantly impaired AmyE transport to the outside of the cell. By introducing additional genes encoding the autolysins LytC and LytF or the cell wall hydrolase PBP5 (*dacA*), I was able to improve secretion by up to 200%. These findings suggest that cell wall permeability for secreted proteins is modulated by autolysin activity.

Flotillins, which are thought to form functional membrane microdomains (FMM) in *B. subtilis*, are often linked with secretion, although the nature of this connection is not exactly clear. To approach this subject, I used a Δ*yuaG* (FloT) deletion strain with reduced AmyE secretion and showed that the addition of the membrane fluidizer benzyl alcohol could recover the AmyE secretion level of the wild type. This result indicates, that flotillins affect protein secretion in *B. subtilis* through the ability to improve membrane fluidity. Furthermore, I was able to double the efficiency of AmyE secretion of *B. subtilis* by introducing an additional gene encoding FloT.

Zusammenfassung

Die Proteinsekretion umfasst mehrere wichtige aufeinander folgende Schritte. Zunächst müssen die zu sekretierenden Proteine erkannt und ihre translokations-kompetente Konformation gewährleistet werden. Danach müssen zwei Barrieren überwunden werden, die Zellmembran und die Zellwand. Der aktive Transport durch die Membran kann durch mehrere gut untersuchte Mechanismen erfolgen, insbesondere durch den sogenannten "general secretory" (Sec) und den "twin-arginine translocation" (Tat). Für die Passage durch die Zellwand hingegen fehlt das Verständnis noch fast vollständig.

In dieser Arbeit untersuchte ich diesen Prozess in *Bacillus subtilis* und *Bacillus licheniformis*, indem ich AmyE-mCherry während der Sekretion, mittels superhochauflösender Fluoreszenz-mikroskopie, sichtbar machte. Das überproduzierte Fusionsprotein lokalisierte als deutliche Foci in der Zellhülle, welche beim Abbau der bakteriellen Zellwand, durch Behandlung mit Lysozym, größtenteils verloren gingen. Ich konnte zeigen, dass AmyE an bestimmten Zonen aus den Zellen freigesetzt wird, die Ähnlichkeit zu den Foci von fluoreszenzmarkiertem AmyE in der Hülle aufweisen. Die hochperformante Proteinsekretion erreichte ihren Höhepunkt beim Übergang vom exponentiellen Wachstum zur stationären Phase und scheint auf eine Teilpopulation von Zellen beschränkt zu sein, was vermutlich auch für die native Proteinsekretion zutrifft. Zeitrafferexperimente zeigten, dass die AmyE-mCherry-Foci über mehrere Minuten hinweg statisch fixiert blieben, im Gegensatz zur hohen lateralen Mobilität der Sec-Maschinen-assoziierten Membranproteine SecA und SecDF, die mit mNeonGreen markierten wurden. Interessanterweise zeigten die AmyE-mCherry-Foci erhebliche Fluoreszenzintensitätsschwankungen innerhalb von Minuten, was auf eine sichtbare Diffusion des Proteins entlang der Passage durch die Zellwandmatrix hindurch schließen lässt. Diese Annahme von Diffusion wird durch jüngste AFM-Imaging-Ergebnisse von *B. subtilis* unterstützt, die eine heterologe Zellwandstruktur mit tiefen Poren in der Oberfläche des Peptidoglykans zeigen.

Für weite Teile der industriellen Biotechnologie ist die Sekretion von mikrobiell produzierten Enzymen und Proteinen in den Kulturüberstand von enormer Bedeutung, da diese Methode im Gegensatz zum Aufschluss der produzierenden Zellen, mit geringeren Kosten für die Weiterverarbeitung verbunden ist. Untersuchungen zur Sekretionseffizienz bei *Bacillus*-Vertretern haben zahlreiche Einflussfaktoren enthüllt.

Da die bakterielle Zellwand bei der Suche nach Sekretionsengpässen oft übersehen wird, habe ich mich auf Autolysine konzentriert, welche die Zellwanddicke und die Dichte des Maschenwerks beeinflussen können. Während das Fehlen von LytD nur geringe Auswirkungen auf die Sekretion hatte, beeinträchtigte die Deletion von LytC und LytF den Transport von AmyE in die Umgebung der Zelle erheblich. Durch die Einführung zusätzlicher Gene, die für die Autolysine LytC und LytF oder die Zellwandhydrolase PBP5 (*dacA*) kodieren, konnte ich die Sekretion um bis zu 200% verbessern. Diese Ergebnisse deuten darauf hin, dass die Durchlässigkeit der Zellwand für sekretierte Proteine durch die Autolysin-Aktivität moduliert wird.

Flotilline, von denen man annimmt, dass sie in *B. subtilis* funktionelle Membranmikrodomänen (FMM) bilden, werden oft mit der Sekretion in Verbindung gebracht, obwohl die Art dieser Verbindung noch unklar ist. Um sich diesem Thema zu nähern, habe ich einen Δ*yuaG* (FloT)-Deletionsstamm mit verminderter AmyE-Sekretion verwendet und konnte zeigen, dass die Zugabe des Membranfluidisators Benzylalkohol, das Wildtyp-Sekretionsniveau wiederherstellen kann. Dieses Ergebnis deutet darauf hin, dass Flotilline die Proteinsekretion in *B. subtilis* durch ihre Fähigkeit zur Verbesserung der Membranfluidität beeinflussen. Außerdem konnte ich die Effizienz der AmyE-Sekretion von *B. subtilis* verdoppeln, indem ich ein zusätzliches Gen einführte, das für FloT kodiert.

Contents

List of figures

Introduction

Article I

Manuscript I

Manuscript II

List of tables

Aritcle I

Manuscript I

Manuscript II

General Material and Methods

Abbreviations

%	percentage
°C	degree Celsius
AFM	atomic force microscopy
atm	atmosphere
ATP	adenosine triphosphate
BF	brightfield
bp	base pair
c-di-GMP	cyclic dimeric guanosine monophosphate
CWP	covalently attached protein
DNA	deoxyribonucleic acid
DRM	detergent-resistant membrane
DSM	detergent-soluble membrane
EDTA	ethylenediaminetetraacetic acid
EMCCD	electron-multiplying-charge-coupled device
EPS	extracellular polymeric substances
FMM	functional membrane microdomains
g	gram
GC	guanine-cytosine
GlcNAc	N-acetylglucosamine
GRAS	generally regarded as safe
h	hour
i.e.	in essence
IMP	integral membrane protein
IPTG	isopropyl-β-d-thiogalactopyranoside
kDa	kilo dalton
l	liter
LB	lysogeny broth

Lpo	lipoprotein cofactors
LTA	lipoteichoic acid
M	molar
mA	milliampere
MCS	multiple cloning site
mg	milligram
ml	milliliter
mM	millimolar
mNG	mNeonGreen
ms	milliseconds
MurNAc	N-acetylmuramic acid
NAG	N-acetyl-glucosamine
nm	nanometer
OD	optical density
PBP	penicillin-binding protein
PBS	phosphate buffered saline
PCR	polymerase-chain reaction
PG	peptidoglycan
pH	potential of hydrogen
PL	phospholipid
PMF	proton motive force
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec/ s	seconds
Sec-pathway	general secretory pathway
SIM	structured illumination microscopy
SMT	single molecule tracking

sp	signal peptide
SQD	squared displacement analyses
UDP	uridine diphosphate
V	volt
w/v	weight per volume
wt	wild type
WTA	wall teichoic acid
YFP	yellow fluorescent protein
µg	microgram
µl	microliter
µm	micrometer

1 Introduction

1.1 Biotechnology

Long before their discovery, microorganisms were used to preserve foods such as milk, fruits and vegetables, and to produce others like cheese, bread, pickled foods and vinegar. (Demain *et al*., 2017). It was not until the late 17th century, that Antonie van Leeuwenhoek reported to the Royal Society of London, seeing tiny moving organisms (*animalcules*), which he found via microscopy in a sample of his dental plaque (Dobell, 1932). This moment is widely regarded as the first discovery of microorganisms, that opened the door to a whole new science. However, the biotechnological importance of these organisms was still overlooked until Pasteur concluded in 1858, that fermentation is a living process driven by yeast (Pasteur, 1858). Since that point, compounds derived from microorganisms have been utilized in industries like agriculture, food and medicine (Sanchez *et al*., 2012; Dias *et al*., 2012).

Now, bio-pharma, industrial biotech and agricultural biotech account for nearly 70% of the global biotech market, with a size estimated to be around 295 billion dollars (Martin *et al*., 2021). These industries are shaped by countless products, proteins and enzymes derived from microorganisms. Examples of agricultural biotechnology are the potent antifungal glycolipids ieodoglucomide C and ieodoglycolipid, isolated from marine bacterium *Bacillus licheniformis*, which act against common plant pathogenic fungi like *Aspergillus niger* and *Rhizoctonia solani* (Tareq *et al*., 2015). Hydrolytic enzymes such as proteases, amylases and lipases account for the majority of the global industrial enzyme production (Kahled *et al*., 2022). Dominant in that market are proteases, which are used in numerous processes in the food and feed industry, (Olempska-Beer *et al*., 2006) for the production of leather goods (Lageiro *et al*., 2007) and numerous other areas (Rao *et al*., 1998; Gupta *et al*., 2002; Haki & Rakshit, 2003). Representing the second largest group, amylases and cellulases play a role in the starch-, textile-, detergent- and baking-industries (Godfrey & Reichelt, 1982; Kirk *et al*., 2002). Streptomyces is of particular importance for the pharma industry, since many antibiotics have been derived from this genus: streptomycin from *Streptomyces griseus* (Waksman *et al*., 1946) chloramphenicol from *S. venezuelae* (Duggar, 1948) or from *S. aureofaciens* (Ehrlich *et al*., 1947). Also, in the fight against cancer biotechnology is relevant. Geldanamycin is a benzoquinone ansamycin compound derived from *S. hygroscopicus var. geldanus* which acts as an anticancer agent in multiple myeloma, breast, and prostate cancer (Singh *et al*., 2010, Gorska *et al*., 2012). Biotechnology is even used to tackle environmental problems, by creating bioplastic via compounds produced by fungi and bacteria (Degli *et al*., 2021). Furthermore, the breakthrough of the CRISPR/Cas system (Jinek *et al*., 2012), which was awarded the Nobel Prize in chemistry 2020, has multiple far-reaching applications in agriculture and biotechnology. Numerous emerging biotechnologies based on CRISPR–Cas aim to increase plant yield, quality, disease resistance (Zhu *et al*., 2020) or improve multiplexed engineering and high throughput screening (Ding *et al*., 2020).

Therapeutic and FDA-approved compounds are mainly produced by *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris* (Ferrer-Miralles et al., 2009). The most commonly used production hosts for proteins would be *E. coli* and gram-positive members of the genus *Bacillus* (Westers *et al*., 2004). In 2007, about 60% of all commercially produced enzymes were produced with different *Bacillus* hosts (Fu *et al*., 2007). The genus *Bacillus* is economically very important and is used for the production of various molecules and enzymes for the food, pharmaceutical, environmental and agricultural industries (Su, *et al*., 2020; Soltani *et al*., 2019; Sumi *et al*., 2015; Vary *et al*., 2007). For example, the alkaline serine proteases produced by the species *B. clausii, B. amyloliquefaciens* and *B. halodurans*, represent the main detergent enzymes on the market (Schallmey *et al*., 2004; Saeki *et al*., 2007). Also, *B. megaterium* and *B. stearothermophilus* produce the extracellular enzyme α-galactosidase, with broad applications in beet sugar, pulp and paper industries, soya food and animal feed processing (Gote *et al*., 2004; Patil *et al*., 2010). Among the *Bacillus* species, *B. thuringiensis* is widely used within the agricultural industry due to its production of insecticidal parasporal crystals (Höfte and Whiteley, 1989; Schnepf *et al*., 1998), which result in the release of protoxin proteins upon ingestion by insects (Höfte and Whiteley, 1989; Gill *et al*., 1992). However, in the field of biotechnology, certain species are preferred because they have excellent fermentation properties and can produce a variety of heterologous proteins, toxin-free and in high yields, such as *B. subtilis, B. amyloliquefaciens,* and *B. licheniformis* (Pham *et al*., 2019). Among these, *B. subtilis* is most extensively studied species due to its flexibility during genetic engineering and its fermentation and secretion capacity, which allows protein production of several grams per liter on an industrial scale (Schallmey *et al*., 2004; Pham *et al*., 2019). In addition, its ability to adapt to varying environmental conditions as well as its classification as “generally regarded as safe” (GRAS) has contributed tremendously to its success in the industrial platform (Baysal & Yildiz, 2017).

1.2 Bacterial Secretion

Due to production with strong controllable promoters being, in general, more effective in *E. coli* (Schallmey *et al*., 2004), it might come as a surprise that *Bacillus* species are often preferred for production. One of the main reasons is the utilization of the extracellular secretion systems of *Bacillus* species, while *E. coli*, mostly uses intracellular production strategies that require expensive and often difficult purification processes (Pham *et al*., 2019). As a consequence, many attempts have been made for *E. coli* to target recombinant proteins to the culture medium (Fakruddin *et al*., 2013). Protein secretion was improved for example via the co-expression of bacteriocin release protein (BRP) (Rahman *et al*., 2005; Beshay *et al*., 2007), the fusion as 'passenger' proteins linked to YebF which is naturally secreted (Zhang *et al*., 2006) and through exploration of the autotransporter pathway for virulence factors (Jong *et al*., 2010). Although high level secretion by *E. coli* is not impossible, it is generally still problematic (Choi & Lee, 2004; Yoon *et al*., 2010). The outer membrane hinders the secretion of proteins into the extracellular environment often leading to accumulation of the expressed proteins in the periplasm as inclusion bodies (Mergulhao & Monteiro, 2004). To gain access to the intracellularly produced proteins, cells must be digested, which can be elaborate and expensive (Sørensen & Mortensen, 2005; Graumann & Premstaller, 2006). The cytoplasm is a reducing environment that hinders the formation of disulfide bonds, so the secretion of proteins can also lead to better folding conditions, which prevents the formation of inclusion bodies (Li *et al*., 2004; Van Dijl and Hecker, 2013). As a Gram-positive bacterium, *B. subtilis* lacks an outer membrane and is able to secrete the enzymes straight into the medium, which allows for an easy recovery of purified proteins and less expensive down-stream processing (Zweers *et al*., 2008).

It has been estimated, that bacteria secrete 5–10% of the proteins encoded on their chromosomes (Holland, 2004). Proteins that are transported to the periplasm, cell envelope, or membrane are also often referred to as "secreted proteins," although the term is primarily used to describe proteins that are secreted into the environment. Further exploration of bacterial secretion within this work will be focused on the secretion into the surrounding. For bacteria, secretion is an essential process necessary for numerous purposes, such as the interaction with the surrounding, export of antibiotics and virulence factors, cell-cell interactions, biofilm formation and accessibility of nutrients. Toxins and antibacterial agents are used by many microorganisms to secure their habitat and gain a competitive edge against other microorganisms (Cornforth & Foster, 2015). Earliest and most famous example of this is penicillin G produced and secreted by the fungus *Penicillium notatum*, which was discovered by Fleming in 1929. Further examples of secreted antibacterial substances are cephalosporin C from *Cephalosporium acremonium* (Newton & Abraham, 1955) which interferes with cell wall synthesis (Tipper & Strominger, 1968), erythromycin from *Saccharopolyspora erythraea,* which inhibits protein production by binding to the 50s subunit of the bacterial ribosome (Haight & Finland, 1952) and vancomycin from *Amycolatopsis orientalis* which is inhibiting proper cell wall synthesis in Gram-positive bacteria (Hammes & Neuhaus, 1974). Various pathogenic microorganisms can use their secretion systems to manipulate the host and establish a replicative niche by secreting virulence factors. In the case of *Mycobacterium*

tuberculosis, secreted virulence factors can assist the bacterium to adapt physiologically and metabolically in the hostile host environment or to disrupt the host signaling network (Forrellad *et al*., 2013; Sharma *et al*., 2017). The capsule of *B. anthracis* is also a virulence factor, that inhibits bacterial phagocytosis during infection and is comprised of multiple secreted components (Makino *et al*., 2002). Another area in which secretion plays a role are biofilms, as formed by microorganisms such as *B. subtilis* (Branda *et al*., 2001). Biofilms are multicellular microbial communities of cells, embedded in a matrix of secreted extracellular polymeric substances (EPS) (Wingender *et al*., 1999). Microbial EPS are biopolymers consisting of proteins and nucleic acids (Frølund *et al*., 1996; Nielsen *et al*., 1997; Dignac *et al*., 1998) as well as amphiphilic compounds including (phospho)-lipids (Neu, 1996; Takeda *et al*., 1998). Bacteria secrete EPS to contribute to the formation of microbial aggregates (Geesey, 1982), gain a protective barrier and additional benefits like better retention of water (Donlan, 2002). Also, *B. subtilis* reduces surface tension through the secretion of surfactants, which allows for gliding over solid surfaces, furthering the swarming-motility reach (Kinsinger *et al*., 2003).

Since organic nutrients in the environment such as amino acids, nucleic acids, and sugars are often present as oligomers too large to be imported, they must be partially digested outside the cell by secreted enzymes (Cezairliyan & Ausubel, 2017). Examples include the secretion of the α-amylase AmyE by *B. subtilis* to digest large starch molecules (Ingle & Erickson, 1978) and the secretion of multiple proteases by *Pseudomonas aeruginosa* to utilize large proteins as a nutrient source (Van Delden *et al*., 1998). Since bacteria commonly live in diverse communities, this process creates possibilities for other neighboring organisms. Increasing the accessibility of nutrients in the surrounding environment by producing and secreting proteases (Diggle *et al.,* 2007; Sandoz *et al.,* 2007) or iron-scavenging siderophores (Griffin *et al.,* 2004), provides opportunities for non-producers to exploit these public goods for a fitness benefit (Asfahl & Schuster, 2017).

Bacterial cells can respond via quorum sensing to cell-population density, as well as integrate environmental cues of nutrient accessibility to regulate and optimize secretion strategies (Schuster and Greenberg 2006; Venturi 2006; Srivastava and Waters 2012).. In the 1970s, Khokhlov *et al.* discovered a small molecule, the so-called A-factor, which is secreted by a Streptomycete, that autoinduces both sporulation and antibiotic production, upon accumulation in the medium. A number of studies have revealed several other communicatory molecules with biological effects similar to that of the A-factor, secreted into the medium (Voloshin & Kaprelyants, 2004). Furthermore, Gram-negative bacteria with type VI secretion systems (T6SSs), are capable of transporting effector proteins from one bacterium to another in a contact-dependent manner, which is believed to play a role in bacterial communication and interactions in the environment (Russell *et al*., 2014).

For *B. subtilis* most proteins are secreted via the general secretion pathway or the twin-arginine pathway, but there are also ABC transporters and non-classical secretion.

1.2.1 ABC Transporter

ATP-binding cassette (ABC) transporters are one of the largest superfamilies of membrane transporters and can be found in all domains of life (Holland *et al*., 2003; Rismondo & Schulz, 2021). Bacteria, archaea, fungi, plants and human parasites all have ABC transporters that catalyze essential functions (Leprohon *et al*., 2011; Rea, 2007; Martinoia *et al*., 2002) and are often called traffic ATPases (Ames *et al*., 1990). Besides the conserved ATP binding motif (Fath & Kolter, 1993), all bacterial ABC transporters share two transmembrane domains (TMDs), two nucleotide binding domains (NBDs) and a substrate-binding site (Song *et al*., 2015). The TMDs are typically composed of twelve transmembrane helices, which form the translocation pathway across the membrane bilayer and also contain the substrate-binding site (Beis, 2015). The two NBDs, which can bind and hydrolyze ATP (Locher, 2016), contain multiple stretches of highly conserved sequences (Higgins *et al*., 1986, 1990; Decottignies & Goffeau, 1997), in contrast to the diverse translocator units of the TMDs (Hollenstein *et al*., 2007). Since the majority of ABC transporters are highly specific, most feature a substrate binding protein (SBP), additionally to NBDs and TMDs, for the capturing and transfer of substrates to the transporter (Boos, 1999; Berntsson *et al*., 2010; Maqbool *et al*., 2015). Interestingly, SBPs are typically soluble and periplasmic in Gram-negative bacteria, and anchored to the membrane via an N-terminal hydrophobic lipid extension in Gram-positive bacteria (Quentin *et al*., 1999). For the actual translocation across the membrane, ABC transporters have to pump substrates against a chemical gradient, via ATP hydrolysis as the driving force. Once ATP is hydrolyzed by the NBDs, conformational changes are transmitted from the NBDs to the TMDs, which leads to a reorientation of the substrate-binding site from an inward-facing to an outward-facing conformation, resulting in the translocation across the membrane (Ward *et al*., 2007; Dawson & Locher, 2006; Seeger & van Veen, 2009; Locher, 2016).

During the sequencing of the *B. subtilis* genome, more and more ABC transporters have been identified in a relatively short time (Fath & Kolter, 1993; Kunst *et al*., 1997; Linton & Higgins, 1998). Genome analyses have revealed 78 ABC transporters in *B subtilis*, which can be split into 38 importers and 40 exporters (Quentin *et al*., 1999). However, there is potentially a high degree of redundancy (Orelle *et al*., 2019) as bacteria, such as *B. subtilis*, *S. aureus* and *E. coli* carry up to 30 putative drug efflux pumps (Hassan *et al*., 2007; Paulsen *et al*., 2001). ABC transporters can translocate a variety of molecules across the cell membrane in an ATP-dependent manner. This includes toxic substances (Wong *et al*., 2014; Seeger *et al*., 2009), the uptake of nutrients (Davidson *et al*., 2008; Cui *et al*., 2011) and the translocation of building blocks for cell-wall assembly (Raetz *et al*., 2007; Ruiz *et al*., 2008; Cuthbertson *et al*., 2010). ABC transport systems contribute not only to the translocation of proteins, but are also involved in the transport of cyclic β-glucans (Roset *et al*., 2004; Breedveld & Miller, 1994), polysaccharides (Feng *et al*., 2004), sialic acid (Nsahlai *et al*., 2003) and a wide variety of small peptide signaling molecules and bacteriocins (Dirix *et al*., 2004; Young & Holland, 1999). Others possess regulatory functions such as FtsEX of *B. subtilis*, which activates the D,L-endopeptidase CwlO (Meisner *et al*., 2013) or *opuB* and *opuC* operons, which mediate osmoprotection (Kappes *et al*., 1999).

1.2.2 Non-classical Secretion

Facilitated by the availability of the genome sequence of *B. subtilis* (Kunst *et al*., 1997), exported proteins are usually identified and characterized by featuring a signal peptide (Tjalsma, *et al*., 2000). Most frequently, extracellular proteins are secreted either by the Sec-pathway, the Tat-pathway or in some instances via an ABC transporter. Nevertheless, multiple proteomic analyses of the secretome in various bacteria have revealed a number of proteins in the extracellular environment, that were previously characterized as cytosolic, lacking any form of signal peptides (Tjalsma *et al*., 2004; Antelmann *et al*., 2006; Wang *et al*., 2016). Among these non-classically secreted proteins are fibrinogen-binding protein A and enolase of *Listeria monocytogenes* (Schaumburg *et al*., 2004; Dramsi *et al*., 2004), glucose-6-phosphate isomerase of *Streptococcus agalactiae* (Hughes *et al*., 2002), glutamine synthetase of *Mycobacterium tuberculosis* (Harth *et al*., 1997) and the pullulanase PulA of *B. subtilis* (Zhen *et al*., 2021). The long-held assumption was, that detection of these proteins in the extracellular environment had to be attributed to cell lysis. However, the works of Yang *et al*., 2011 and *Ebner et al*., 2016 could prove this assumption wrong and confirm that non-classical secretion is a general phenomenon *B. subtilis* and *S. aureus* respectively. Speculations for the mechanism include specific loosening of the cell membrane (Pasztor *et al*., 2010), some unknown specific protein channels (Ebner *et al*., 2017) or membrane vesicles (Wang *et al*., 2013). Despite a lot of speculation, the mechanism of the non-classical secretion pathway has not yet been identified (Jiang *et al*., 2022). Reports have shown that the transport of some of these proteins seems to be coupled with their multimer state (Zhao *et al*., 2017), conformational motifs (Yang *et al*., 2018) or translation stress (Morra *et al*., 2018). Additional investigations indicate, that the N-terminal and/or C-terminal sequences of proteins appear to be essential for non-classical secretion in *E. coli* (Gao *et al*., 2016; Xin, *et al*., 2019) and *B. subtilis* (Pan *et al*., 2016; Niu *et al*., 2021), even though no form of signal peptide was found. Interestingly, proteins are likely exported as a single unit via the non-classical secretion system (Zhao *et al*., 2017) and always concentrate in the cell poles and septum during secretion (Kang *et al*., 2020). Despite the usually low secretion levels of recombinant proteins using the non-classical secretion pathway (Chen *et al*., 2016), recently, the Sec-pathway dependent secretion of γ-CGTase could be increased by expression without the signal peptide, utilizing non-classical secretion (Jiang *et al*., 2022).

1.2.3 Sec-Pathway

The general secretion pathway (Sec) is essential for viability and is ubiquitously found in all domains of life, being conserved in bacteria, archaea, and eukaryotes (Papanikou *et al*., 2007, Bolhuis *et al*., 2004; Rothman & Orci, 1992). Together with the twin arginine translocation (Tat) pathway, it is the most commonly used bacterial secretion system to transport proteins across the cytoplasmic membrane (Green & Mecsas, 2016). The Sec system primarily translocates proteins in their unfolded state (Harwood & Cranenburgh, 2008) and consists of a protein targeting component, a motor

protein and a membrane integrated conducting channel, called the SecYEG translocase (Papanikou *et al*., 2007).

Secretory proteins are identified via intrinsic signals that govern their transport and localization in the cell, called signal peptides, a discovery that was awarded the Nobel Prize in Physiology or Medicine in 1999. The signal peptides of the Sec system are hydrophobic N-terminal extensions of pre-proteins that allow them to be recognized by the export machinery (Chatzi *et al*., 2013; von Heijne, 1990) and delay early folding in the cytoplasm (Tjalsma *et al*., 2000). *B. subtilis* exhibits a great diversity of signal peptides with more than 100 different sequences identified, so optimal signal peptides are often unique for different proteins (Brockmeier *et al*., 2006; Petersen *et al*., 2011). While sequences may vary, signal peptides share common structural features, such as a positively charged N-terminus, followed by a hydrophobic core and a short cleavage region (von Heijne, 1990; Harwood & Cranenburgh, 2008). The selective recognition of the signal peptide and the subsequent transport to the SecYEG channel in the membrane can be achieved in different ways (Fig. 1.1). The ATPase SecA primarily recognizes the pre-protein after release from the ribosome (Hartl *et al*., 1990; Chun and Randall, 1994) and binds the N-terminal signal sequences via a shallow groove within its preprotein-binding domain (Gelis *et al*., 2007; Grady *et al*., 2012). The pre-protein is then transferred by SecA to the translocon, where SecA can bind with high affinity to the cytosolic loops of SecY (Douville *et al*., 1995; Mori and Ito, 2006). In addition, SecA can also directly bind the ribosome (Huber *et al*., 2011, Singh *et al*., 2014) to facilitate co-translational substrate recognition (Karamyshev and Johnson, 2005; Steinberg *et al*., 2018), which was observed for multiple translocated proteins (Huber *et al*., 2017; Rawat *et al*., 2015; Wang *et al*., 2017).
While the ATPase SecA is found exclusively in bacteria and chloroplasts (Pohlschröder *et al*., 1997; Pohlschröder *et al.*, 2005), the signal recognition particle (SRP) is universally conserved and essential in almost all cells (Lütcke, 1995; Dalbey *et al.*, 2017). SRP binds to the ribosome at the same binding site as SecA and many other ribosome-associated chaperones and processing factors (Kramer *et al*., 2009; Denks *et al*., 2017; Knüpffer *et al*., 2019). After binding to the ribosome, SRP can recognize and bind the signal peptide of a nascent chain in a co-translational manner (Shan & Walter, 2005; Akopian *et al*., 2013). SRP, which consists of multiple proteins and RNA (Struck *et al*., 1989; Nakamura *et al*., 1999), then targets the ribosome-associated nascent chains to the membrane-bound SRP receptor FtsY and ultimately to the SecYEG translocase complex (Kuhn *et al*., 2017; Angelini *et al*., 2005; Macao et al., 1997).

Although the translocation process in *B. subtilis* and *E. coli* is principally very similar (Tjalsma *et al*., 2004), the latter possesses an additional chaperone called SecB, which can support the translocation competence of the unfolded pre-protein (Collier *et al*., 1988; Huang *et al*., 2016). SecB can bind to a small number of secretory proteins (Bechtluft *et al*., 2010) and cooperates with SecA to facilitate their translocation (Fekkes *et al*., 1998; Zhou & Xu, 2003). The chaperone CsaA is assumed to serve as a SecB homolog in *B. subtilis,* as it can directly interact with SecA and precursor proteins to influence their secretory efficiency (Müller *et al*., 2000; Shapova & Paetzel, 2007).

However, it was found not to bind to the conserved SecB-binding domain in SecA (Müller *et al*., 2000) and it is still unclear whether CsaA can recognize signal peptides (Yan & Wu, 2017).

In its minimal form, the Sec translocase consists of the SecYEG protein-conducting channel, consisting of the proteins SecY, SecE and SecG (Manting *et al*., 2000). SecY, the major subunit of this complex, is forming a clamshell-like structured pore, with two halves of transmembrane segments, to open the lipid bilayer (van den Berg *et al*., 2004; Gumbart & Schulten, 2007). SecE stabilizes the structure of SecY in a crucial way, as SecY has been reported to be rapidly degraded by the membrane protease FtsH in the absence of SecE (Kihara *et al*., 1995; a Nijeholt *et al*., 2013). In contrast to SecY and SecE, the subunit SecG is not essential to the cell, but stabilizes the closed SecY channel (Belin *et al*., 2015) and leads to protein transport defects upon deletion (Nishiyama *et al*., 1994). Together they form the SecYEG translocon complex, an hourglass-shaped pore in the cell membrane (Van den Berg *et al*., 2004). Additional to its functional monomeric state (Kedrov *et al*., 2011), SecYEG seems to form dimers (Breyton *et al*., 2002; Bessonneau *et al*., 2002) and higher oligomers or other heteromeric complexes (Schulze et al., 2014; Komar et al., 2016), which likely allow the Sec-translocase to adapt to a wide variety of different substrates and to different physiological conditions. Structural analysis of SecYEG revealed that in its resting state, the exit site of the hourglass pore is sealed by an α-helical plug that folds back into the channel (Park & Rapoport, 2011; Tam *et al*., 2005), which prevents ion leakage (Maillard *et al*., 2007). The ATPase SecA is often described as the motor that drives the translocation event (Cooper *et al*., 2008; Gupta *et al*., 2020), since it provides the energy for this process through repetitive ATP hydrolysis cycles (Knyazev *et al*., 2018). Upon binding of SecA to SecY, conformational changes are initiated that open up the channel of SecYEG and displace the plug domain (Zimmer *et al*., 2008; Li *et al*., 2016). SecA then directs the preprotein in a stepwise manner into the pore (Economou & Wickner, 1994, du Plessis *et al*., 2011).

It is noteworthy that there are additional factors that contribute to efficient protein secretion. The chaperone PrsA, located on the outer surface of the cytoplasmic membrane, assists maturation and folding of exported proteins (Kontinen *et al*., 1991; Kontinen & Sarvas, 1993). RasP is an intramembrane protease that can impact secretion of different proteins (Neef *et al*., 2017, Neef *et al*., 2020) through the degradation of cleaved signal peptides (Saito *et al*., 2011) and SecDF, associated with the SecYEG translocon, aids secretion using the proton motive force (PMF) (Tsukazaki *et al*., 2011) and is proposed to pull out the precursor protein from the SecYEG channel into the periplasm via repeated conformational transitions (Tsukazaki, 2018).

Following the secretion through the SecYEG translocon, the signal peptide of the pre-protein is removed by a signal peptidase. *B. subtilis* encodes genes for five type I signal peptidases (*sipT*, *sipS*, *sipU*, *sipV* and *sipW*) (van Rosmaalen *et al*., 2004) and one type II signal peptidase (*lspA*) (Prágai *et al*., 1997), of which only SipS and SipT appear to be essential (Tjalsma *et al*., 1998). When the signal peptide is cleaved, the pre-protein is released from the translocation machinery for folding or further trafficking (Auclair *et al*., 2012).

SecYEG also cooperates with the insertase YidC by forming a SecDF stabilized complex (Samuelson *et al*., 2000) to facilitate membrane protein insertion (Sachelaru *et al*., 2013; Sachelaru *et al*., 2017; Petriman *et al*., 2018). Although the mechanistic details of the YidC-SecYEG-dependent protein insertion remain elusive at the molecular level (Polasa *et al*., 2022), the protein chain likely is transferred from SecYEG to YidC through a lateral gate, followed by insertion via hydrophobic pressure (Dalbey *et al*., 2014; Kleinbeck & Kuhn, 2021). Alternatively, YidC can insert membrane proteins independently of SecYEG (Serek *et al*., 2004), when pre-proteins are delivered to the insertase via SRP and FtsY (Welte *et al*., 2012) or by binding the translating ribosome directly (Kedrov *et al*., 2013).

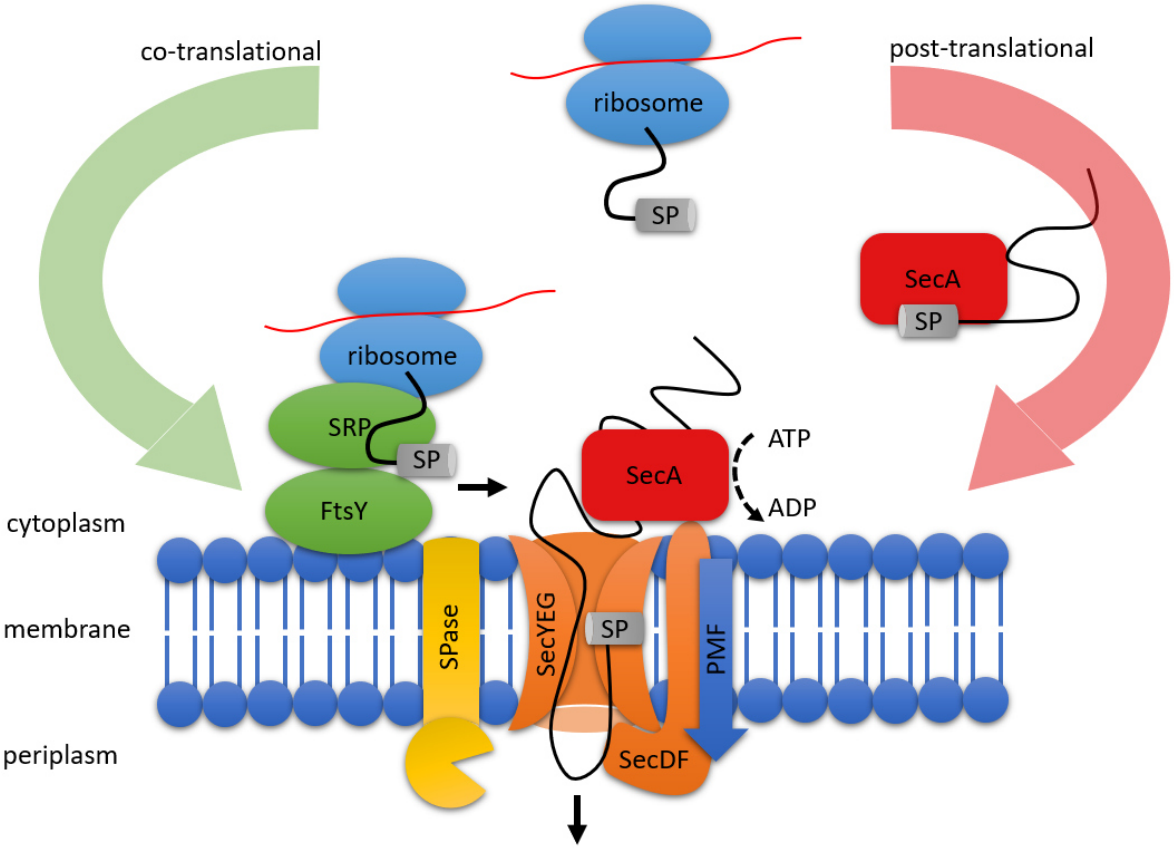


Figure 1.1: Schematic depiction of the Sec pathway. The signal recognition particle (SRP) binds to the ribosome to recognize and bind the signal peptide (SP) of a nascent chain in a co-translational manner. The translating ribosome is then brought to the SecYEG translocon through an interaction between SRP and its membrane receptor FtsY. The ATPase SecA primarily recognizes the pre-protein post-translationally, after release from the ribosome. It binds the N-terminal SP and transfers the pre-protein to the SecYEG translocon, where SecA then binds to SecY and initiates ATP-driven secretion through the hourglass-shaped pore of SecYEG. SecDF is associated with the SecYEG translocon and aids secretion via the proton motive force (PMF). Following the secretion through the SecYEG translocon, the signal peptide of the pre-protein is removed by a signal peptidase (SPase).

1.2.4 Twin-arginine translocation system

In the early 1990's, it was noticed that a subset of polypeptides could be translocated through the thylakoid membrane of chloroplasts independently of ATP hydrolysis and instead relied solely on the proton gradient (Mould & Robinson, 1991). This translocation was facilitated by the twin-arginine translocation system (Tat), named after the characteristic twin-arginine (RR) motif of the N-terminal signal peptide, which predestines a substrate to be exported from the cytoplasm by the Tat-pathway (Chaddock *et al*., 1995; Berks, 1996). The Tat system is found in a variety of organisms (Palmer *et al*., 2012), such as thylakoids (Hynds *et al*., 1998), archaea (Pohlschröder *et al*., 2005), Gram-positive (Jongbloed *et al*., 2002) and Gram-negative bacteria (Müller, 2005). While the general secretion pathway (Sec) can only translocate proteins in an unfolded state, the Tat pathway stands out by its specialization in the export of fully folded proteins, which often require a cofactor insertion or immediate oligomerization (Palmer *et al*., 2005; Berks, 2015; Frain *et al*., 2019). The number of Tat-dependent cargo proteins ranges from over 100 in *Streptomyces* species (Schaerlaekens *et al*., 2001, 2004; Widdick *et al*., 2006; Joshi *et al*., 2010), to roughly 30 proteins in *E. coli* and *Salmonella* (Palmer *et al*., 2010) and only a few in *B. subtilis* (Goosens *et al*., 2013) and *Staphylococcus aureus* (Yamada *et al*., 2007; Biswas *et al*. 2009), while in *Lactobacillus* species, none are known (Bolotin *et al*., 2001; Kleerebezem *et al*., 2003). Among the few proteins targeted by the Tat system in *B. subtilis*, is the Rieske iron-sulfur protein QcrA, which is translocated into the cytoplasmic membrane (Goosens *et al*., 2014). YkuE, a metallophosphoesterase, is directed to the cell wall (Monteferrante *et al*., 2012) and the hemoprotein EfeB both to the membrane-cell wall interface as well as the extracellular milieu (Miethke *et al*., 2013).

The core components identified in *E. coli* that make up the translocase are TatA, TatB, and TatC (Sargent *et al*., 1998; Sargent *et al*., 1999; Palmer *et al*., 2005). Intriguingly, certain TatA proteins, like the TatA proteins from *B. subtilis*, were demonstrated to be bifunctional and replace both *E. coli* TatA and TatB in the TatA-B-C system (Blaudeck *et al*., 2005; Barnett *et al*., 2008; Barnett *et al*., 2011). As a result, *B. subtilis* lacks the third component TatB, that is important to Tat systems of Gram-negative bacteria and thylakoids (Jongbloed *et al*., 2006; Barnett *et al*., 2009). Its core translocase TatAyCy is composed of only two necessary components, the constitutively expressed TatAy and TatCy proteins (Fig. 1.2) (Jongbloed *et al*., 2000; Pop *et al*., 2002). A second translocase namely TatAdCd, consisting of TatAd and TatCd, is detectable only under phosphate starvation conditions to enable secretion of the phosphodiesterase PhoD (Jongbloed *et al*., 2000; Jongbloed *et al*., 2004). Whereas TatC is a relatively large integral membrane protein (28–28.9 kDa) with six transmembrane domains (Nolandt *et al*., 2009; Rollauer *et al*., 2012), the TatA component is smaller (6–7.4 kDa) and has only one N-terminal transmembrane domain (Yen *et al*., 2002; Lange *et al*., 2007).

Despite almost three decades of research, the mechanism for the twin-arginine translocation system is not entirely understood (Frain *et al*., 2019; Palmer & Stansfeld, 2020). However, what is currently agreed upon is that the process is initiated when a substrate with the correct RR-signal peptide

interacts with a docking complex composed of TatC and a TatA-like protein (Bolhuis *et al*., 2001; Kneuper *et al*., 2012; Whitaker *et al*., 2012). Once the cargo protein has docked, the translocation complex is formed and a large number of TatA proteins are recruited to the translocation site in a proton-motive force-dependent manner (Mori & Cline 2002; Alami *et al*., 2003; Dabney-Smith *et al*., 2006). As for the actual mechanism of translocation through the membrane via the Tat pathway, multiple models have been proposed (Hao *et al*., 2022). In one model, multiple TatAs are forming a pore around the incoming transport substrate by inserting the TMHs into the membrane (Gohlke *et* al., 2005; Frain *et al*., 2019). Another model assumes that, under the influence of all Tat subunits, the substrate itself, and the driving proton motive force, the membrane thins locally until the bilayer collapses and lipid-lined toroidal pores form through which the substrates cross the membrane (Brüser & Sanders, 2003; Hou *et al*., 2018; Asher & Theg *et* al., 2021). After translocation through the cytoplasm membrane, the signal peptide is cleaved by a signal peptidase to release the mature protein (Jongbloed *et al*., 2004; Lüke *et al*., 2009; Dalbey *et al*., 2012).

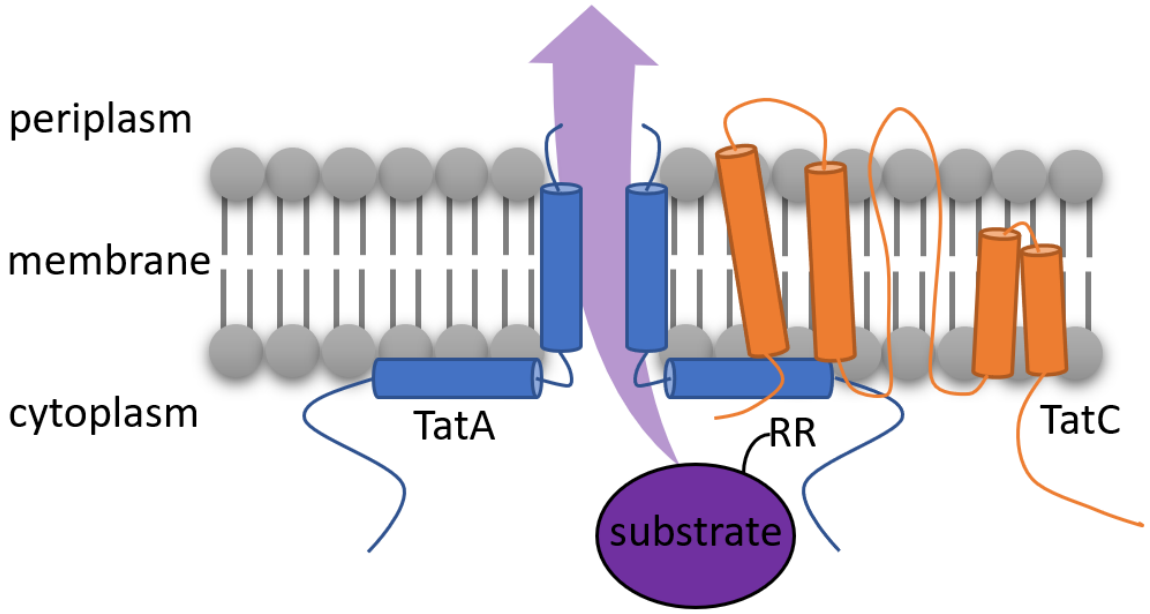


Figure 1.2: Schematic depiction of the twin-arginine translocation system (Tat) in *B. subtilis*. The translocation process is initiated when a substrate with the correct twin-arginine-(RR)-signal interacts with a docking complex composed of TatC and a TatA-like protein. Multiple TatAs are forming a proteinaceous pore around the incoming transport substrate, by inserting the TMHs into the membrane, through which the substrates cross the membrane.

1.3 Cell wall

Bacteria can be classified into two different groups, Gram-positive and Gram-negative, based on an ancient staining technique developed by Hans Christian Gram in the 19th century (Gram, 1884). Gram-negative bacteria are characterized generally by a thin cell wall layer that is covered by an inner cytoplasmic membrane and an additional outer membrane (Matias *et al*. 2003; Vollmer and Höltje 2004), while Gram-positive bacteria exhibit a thick cell wall without an additional outer membrane (Shockman *et al*., 1983; Beveridge & Matias, 2006). Despite exemptions from the rules, e.g. cyanobacteria, which share characteristic features of both groups (Hoiczyk & Hansel, 2000), both stain and classification are still in widespread use. The bacterial cell wall is a sieve-like meshwork mainly consisting of peptidoglycan, whose structure was first recognized by Wolfhard Weidel and his group at the Max Planck Institute for Biology in Tübingen (Weidel & Primosigh, 1958). The peptidoglycan sacculus constitutes a rigid layer, that is essential for bacteria to maintain their characteristic shapes (Young, 2003) and to withstand the high internal turgor pressure that can reach 5 atm in Gram-negative and up to 50 atm in Gram-positive bacteria (Archibald *et al*., 1993; Seltmann & Holst, 2002). Peptidoglycan consists of a polysaccharide backbone with β-(1,4) glycosidically linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) molecules (Schleifer & Kandler, 1972), which is attached to an oligopeptide chain consisting of L-Ala-D-Glu-L-meso-diaminopimelic acid-D-Ala-D-Ala (Atrih *et al*., 1999). While the cell wall of Gram-negative bacteria like *E. coli* was determined to be between 3 to 8 nm (Yao *et al*., 1999; Leduc *et al*., 1989), the Gram-positive cell wall consists of several layers and is 30 - 100 nm thick, 30 – 40 nm in *B. subtilis* (Rohde, 2019; Beveridge & Murray, 1979; Graham & Beveridge, 1994). Combining AFM with size exclusion chromatography revealed that the inner surface of *B. subtilis* cell walls consist of ca. 50-nm-wide glycan chains running circumferentially around the short axis with a length of up to 200 μm (Hayhurst *et al*. 2008, Turner *et al*., 2018). With the emerging method of electron microscopy, the peptidoglycan cell wall could be visualized for the first time (Mudd *et al*., 1941; Salton & Horne 1951). Further advances have allowed visualizations of the cell wall in an unprecedented resolution, revealing a disordered peptidoglycan landscape characterized by large and deep pores (Fig. 1.3) (Tank *et al*., 2021; Pasquina-Lemonche *et al*., 2020).

The *B. subtilis* cell wall is negatively charged due to the presence of teichoic acids (Sonnenfeld *et al*., 1985; Hyyryläinen *et al*., 2007), which are essential for maintaining cell shape in rod-shaped bacteria (Swoboda *et al*., 2010). They are unique to Gram-positive bacteria and can be differentiated into wall teichoic acids (WTAs) which are covalently attached to peptidoglycan and membrane anchored lipoteichoic acids (LTAs) (Neuhaus & Baddiley, 2003, Ward, 1981). Teichoic acids are considered one of the major contributors to envelope structure and function, since these polymers can collectively account for up to 60% of the mass of the Gram-positive cell wall (Silhavy *et al*., 2010). In addition to teichoic acids, the cell wall surface is decorated with a variety of proteins, some of which are anchored in the membrane, and others are covalently attached to or associated tightly with peptidoglycan (Scott & Barnett, 2006). Among them are various peptidoglycan synthases like the penicillin-binding proteins (PBPs), Mtg monofunctional glycosyltransferases and shape,

elongation, division and sporulation (SEDS) proteins (Bhavsar & Brown, 2006; Meeske *et al*., 2016).

Since the cell wall is so essential, almost every single step of its biosynthesis pathway is targeted by at least one antibiotic (Bugg *et al*., 2011; Schneider & Sahl, 2010). Old pulse-labeling studies suggested an inside-to-out-side flux of wall material, where new wall is synthesized and integrated along the cytoplasmic membrane and older cell wall material is removed from the exterior by autolysins (Merad *et al*., 1989; Pooley, 1976). It has since been calculated for this autolysin-mediated cell wall turn-over to affect as much as 50% of cell wall material per generation, which is then recycled by the cells (Park & Uehara 2008; Reith & Mayer 2011; Borisova *et al*., 2016). The steady degradation and resynthesis of the peptidoglycan envelope allows the bacterial cell wall to balance between flexibility and rigidness (Vermassen *et al*., 2019).

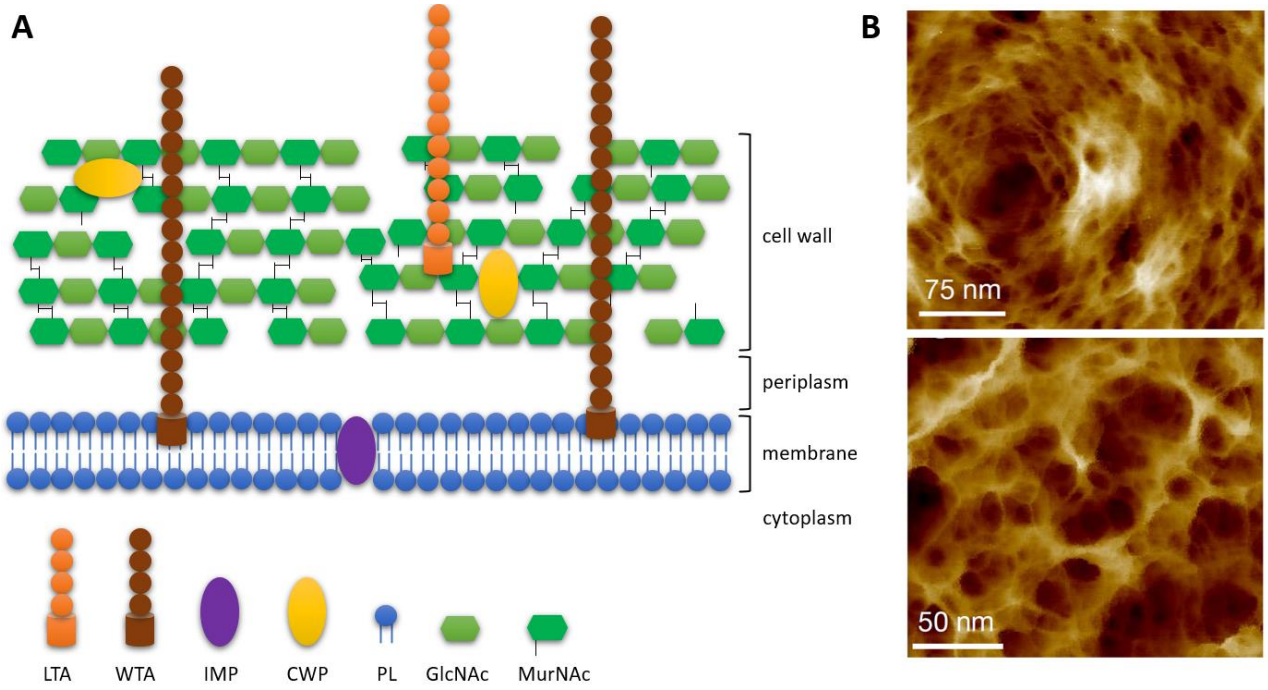


Figure 1.3: Depiction of the *B. subtilis* cell wall. A: Cross section model of a Gram-positive cell envelope; LTA: lipoteichoic acid; WTA: wall teichoic acid; IMP: integral membrane protein; CWP: covalently attached protein; PL: phospholipid; GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid. B: AFM images showing the peptidoglycan surface of living *B. subtilis* cells, adapted from Pasquina-Lemonche *et al*., 2020.

1.4 Autolysin

Autolysins are bacteriolytic enzymes that digest the cell wall peptidoglycan of the bacteria that produce them (Shockman & Hölltje, 1994), whereas other peptidoglycan hydrolases are not classified as autolysins, if they cannot cause cell lysis on their own (Foster, 1994). Although potentially lethal, it appears that autolysins are universal among all bacteria that possess peptidoglycan (Shockman *et al*., 1996; Shockman *et al*., 1994). Initially, only a few autolytic enzymes were known (Young, 1966; Brown & Young, 1970), but genome analysis revealed the presence of 35 definite or predicted autolysins in *B. subtilis* that cluster in 11 different protein families (Kunst *et al*., 1997; Smith *et al*., 2000). The abundance of autolysins leads to a great number of functional redundancies (Smith *et al*., 1996). For example, for each amide bond in the peptidoglycan, there are several amidases capable of hydrolyzing the bond (Walter & Mayer, 2019). Since autolysins are potentially lethal, their expression and activity must be tightly regulated. The flagellar motility and chemotaxis sigma factor σ^D (Márquez *et al*., 1990) controls expression of the major vegetative autolysin genes *lytC* and *lytD*, together with the minor vegetative endopeptidase gene *lytF*, with a peak in activity at the start of stationary phase (Kuroda & Sekiguchi, 1993; Margot *et al*., 1994, Margot *et al*., 1999). Additionally, it seems like the autolysins are post-translationally regulated, as multiple reports of *B. subtilis* strains with inactivated protease genes, exhibiting increased susceptibility to autolysis, indicate (Cho *et al*., 2004; Stephenson *et al*., 1999; Coxon *et al*., 1991). Physical and chemical factors like alkaline medium (Jolliffe *et al*., 1983) or excess of monovalent cations (Svarachorn *et al*., 1989) can also influence autolytic activity.

Autolysins are involved in numerous cellular processes including sporulation, cell separation, cell shape, cell wall turn-over, motility and many more (Smith *et al*., 2000). In the differentiation processes of sporulation, the autolysins SleB and CwlJ are required for spore germination (Chirakkal *et al*., 2002), while LytH is involved in maturation of the spore cortex (Horsburgh *et al*., 2003). The release of spores occurs through lysis of the mother cell, a step that involves numerous autolysins, such as CwlB, CwlC (Shida *et al*., 2001), LytC, CwlC and CwlH (Nugroho *et al*., 1999; Smith and Foster, 1995). Like the separation of spore and mother cell, the cell separation during vegetative growth requires autolytic activity (Blackman et al., 1998). The two genes encoding autolysins that are known to play a key role in cell separation are *lytC* and *lytF*, since their inactivation leads to chain formation, especially in the Δ*lytF* mutant (Ohnishi *et al*., 1999; Chen *et al*., 2009). LytF, together with three other D,L-endopeptidases (LytE, CwlO, and CwlS) is also involved in cell morphology maintenance, with varied roles e.g. in cell elongation (Hashimoto *et al*., 2018). Investigations of cell wall turn-over using pulse-chase experiments were able to show, that inactivation of LytC leads to a sharp decrease in the turn-over rate of the cell wall (Margot *et al*., 1994; Blackman *et al*., 1998). While LytD inactivation alone has no effect, it will further reduce turn-over in a Δ*lytC* background (Margot *et al*., 1994). Autolysins also play a role in bacterial motility, as *B. subtilis* cells lacking LytC and LytD activity exhibit greatly diminished swarming motility (Blackman et al., 1998; Rashid et al., 1993). Likewise, the less well studied lytic transglycosylase CwlQ was found to be essential for swarming motility (Sanchez *et al*., 2021).

Interestingly, autolysins are also associated with phenomena such as cannibalism, which is a social behavior occurring in *B. subtilis* populations during the early stages of sporulation (González-Pastor, 2011). The cannibalistic toxin SDP (Liu *et al*., 2010), rapidly collapses the protonmotive force of other cells and induces autolysin mediated lysis (Lamsa *et al*., 2012).

Among the many cell wall hydrolases, the N-acetylmuramoyl-L-alanine amidase LytC and the N-acetyl-glucosaminidase LytD are the most significant autolysins, accounting for around 95% of the autolytic activity of the cell (Kuroda & Sekiguchi, 1991; Lazarevic *et al*., 1992). LytC is an N-acetylmuramoyl-L-alanine amidase (Kuroda & Sekiguchi, 1991), which localizes uniformly in the cell envelope and hydrolyzes the linkage of N-acetylmuramoyl-L-alanine in peptidoglycan (Fig. 1.4) (Yamamoto *et al*., 2003). As one of the two major autolysins in *B. subtilis*, LytC is involved in a variety of cell functions like sporulation (Smith & Foster, 1995), swarming motility (Yamamoto *et al*., 2003), cell separation and cell wall turn-over (Blackman *et al*., 1998). LytD is an endo-β-N-acetyl-glucosaminidase that forms a homodimer (Margot *et al*., 1994) and localizes at the cell separation sites and both cell poles, similar to LytF (Yamamoto *et al*., 2003). First described as an endolysin, LytD can cleave the bacterial cell wall at the covalent bond between the N-acetylglucosamine (GlcNAc) and the N-acetylmuramie acid (MurNAc) of the glycan chain (Rogers *et ai,* 1980). LytF, a γ-D-glutamate *meso*-diaminopimelate muropeptidase, acts as the primary autolysin involved in vegetative daughter cell separation (Margot *et al*., 1999, Chen *et al*., 2009). Immunofluorescence microscopy shows LytF localizing at the pole and the septum dependent on the presence of WTAs, which inhibit the sidewall localization (Yamamoto *et al*., 2008). LytF cuts the peptidoglycan γ-D-glutamate-*meso*-diaminopimelate bond, but alone has only a minor cell wall lytic activity *in vitro* (Ohnishi *et al*., 1999, Margot *et al*., 1999). LytE and CwlO are D,L-endopeptidases that can hydrolyze the linkage of d-γ-glutamyl-*meso*-diaminopimelic acid in peptidoglycan (Yamaguchi *et al*., 2004; Ishikawa *et al*., 1998). LytE localizes to the septum and poles, but also like CwlO, at the lateral wall in a helix-like manner (Hashimoto *et al*., 2012; Kasahara *et al*., 2016). While the single knockout mutants of either autolysin are viable, a double deletion of *lytE* and *cwlO* is lethal, indicating similar functions (Bisicchia *et al*., 2007). As LytE interacts with MreBH (Carballido-López *et al*., 2006) and CwlO with Mbl (Dominguez-Cuevas *et al*., 2013), they influence two of the three actin homologs in *B. subtilis*. Expression of both LytE and CwlO is regulated by the WalR-WalK two-component signaling system, with the ability to sense peptidoglycan cleavage products, generated by LytE and CwlO and up- or downregulate their activity accordingly (Dobihal *et al*., 2019).

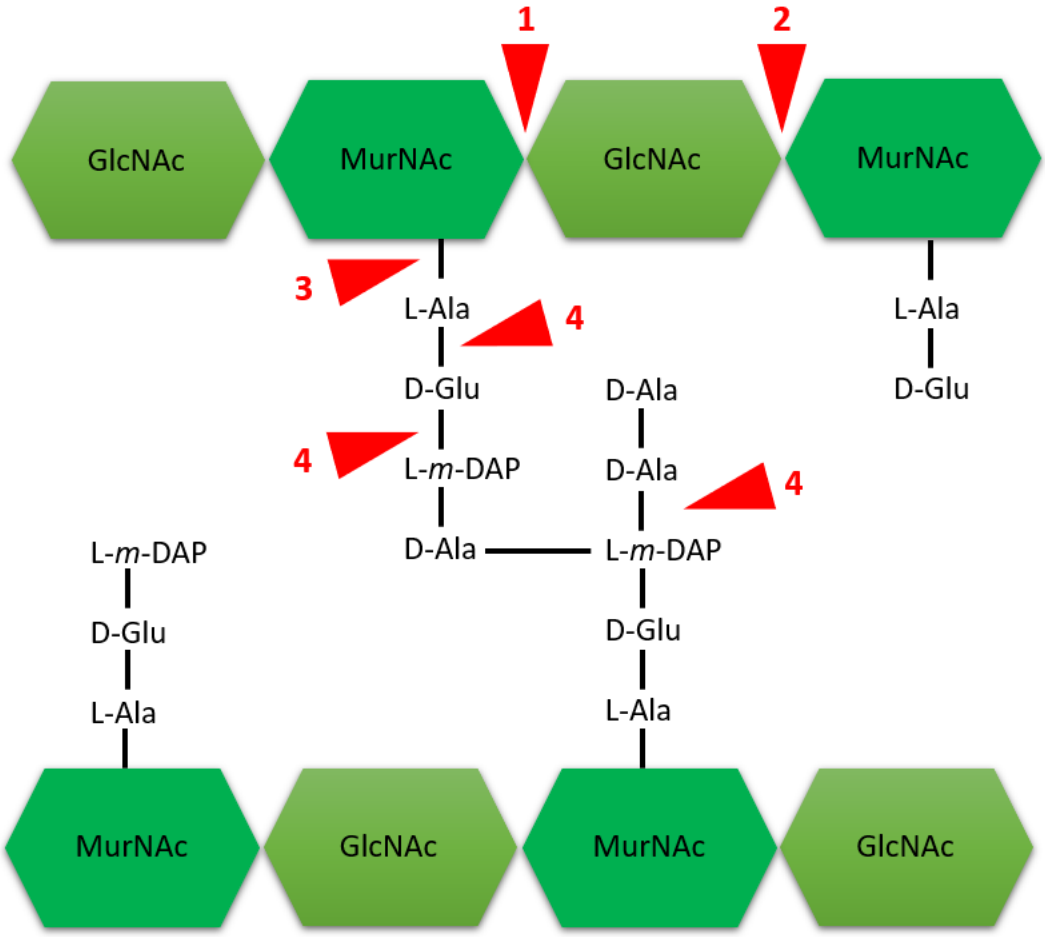


Figure 1.4: Simplified schematic structure of mature *B. subtilis* peptidoglycan cell wall. Examples of each type of bond attacked: 1. glucosaminidase (LytD), 2. muramidase/lytic transglycosylase (CwlQ), 3. amidase (LytC) and 4. endopeptidase (LytF) are indicated by arrows on each structure.

1.5 Flotilline

The common theory to describe membrane organization and behavior is called fluid mosaic model, which suggests that the membrane behaves like a two-dimensional fluid with heterogeneous lateral mobility of the membrane components (Singer & Nicolson, 1972; Jacobson *et al*., 1995). A variety of lipid species, that are present in the cell membrane, tend to segregate laterally into discrete regions, and likewise do proteins that concentrate preferentially in specific membrane domains (Yeagle *et al*., 2004; Matsumoto *et al*., 2006). Through visualization tools like the cardiolipin-specific dye NAO (10-N-nonyl acridine orange), it was shown that the region of the cell pole is especially enriched in cardiolipins (Romantsov *et al*., 2007), whereas other negatively charged lipids appear to localize in a helical pattern along the longitudinal axis (Muchova *et al*., 2010). Membrane proteins of many organisms, from bacteria to humans also frequently show non-uniform localization patterns (Lingwood & Simons, 2010; Simons & Gerl, 2010). Eukaryotic cells organize a variety of proteins related to signal transduction and membrane trafficking into discrete cholesterol- and sphingolipid-enriched microdomains or rafts (Simons & Ikonen, 1997) through the affinity of the membrane proteins for specific heterogeneously localized lipids (Levental *et al*., 2010).

Flotillins were first discovered in the cell membrane of eukaryotes (Schulte *et al*., 1997), but have since been established as an evolutionarily conserved class of proteins found across all organisms (Hinderhofer *et al*., 2009). A lot of research has been dedicated to their numerous functions in eukaryotes, where they play a role in endocytosis (Doherty & McMahon, 2009), cytoskeleton rearrangements (Ludwig *et al*., 2010), cell-cell adhesion (Resnik *et al*., 2011), nutrient uptake (Ge *et al*., 2011) and cell signaling (Staubach *et al*., 2011). They affect so many different aspects of the cell, because flotillins act as scaffolding proteins in membrane domains, providing a platform for the recruitment of multiprotein complexes (Langhorst *et al*., 2005; Ludwig *et al*., 2010; Resnik *et al*., 2011). Members of the family of Flotillin proteins are known to accumulate in detergent-resistant microdomains (DRMs) of eukaryotic membranes and generally serve as a lipid raft marker (Bickel *et al*., 1997; Browman *et al*., 2007; Borner *et al*., 2005). Genes encoding flotillins or flotillin-like proteins are ubiquitous and can be found in genomes of metazoans, plants, fungi and bacteria suggesting a fundamental well conserved function (Rivera-Milla *et al*., 2006; Bramkamp & Lopez, 2015).

As for the bacterial membrane, several investigations increased the evidence for the existence of non-homogenous lipid distribution (Renner *et al*., 2011; Mukhopadhyay, *et al*., 2008; Muchova *et al*., 2010). *B. subtilis* encodes two flotillin homologs, FloT and FloA, which like their eukaryotic counterparts have also been detected in DRM fractions (López & Kolter *et al*., 2010). The protein family of flotillins is characterized by an N-terminal transmembrane domain, the flotillin domain and the conserved SPFH domain, named after stomatin, prohibitin, flotillin and HflC/K, (Browman *et al*., 2007; Hinderhofer *et al*., 2009). Besides FloA and FloT, YdjI was identified in *B. subtilis* as a putative flotillin, containing the SPFH-domain (Cozy *et al*., 2012). But YdjI does not co-localize with the other flotillins and other than FloA and FloT, YdjI was found exclusively in the detergent-

soluble membrane (DSM) fraction (Scholz *et al*., 2021). The main flotillin in *B. subtilis* is FloT, while the functions of FloA are partially redundant (Lopez & Kolter, 2010). Both, the continuously expressed FloA and FloT, which is increasingly expressed upon entry into the stationary phase, are genetically regulated by the sigma factor σ^W (Huang *et al*., 1999), which in turn is triggered by membrane stressors (Petersohn *et al*., 2001; Wiegert *et al*., 2001). FloA and FloT represent integral membrane proteins, that localize in defined focal structures independent of each other, within the cell membrane and move in a highly dynamic manner (Donovan & Bramkamp, 2009; Dempwolff *et al*., 2016). Interestingly flotillin operon structures harboring genes encoding for proteins with NfeD domains is observed all over the prokaryotic kingdom (Hinderhofer *et al*., 2009), which leads to the assumption that a conserved interaction between these two proteins is probable. While it was demonstrated that the NfeD protein YuaF, colocalizes with FloT and influences its behavior, the NfeD protein YqeZ and FloA seem to act independently (Dempwolff *et al*., 2012).

The exact functions of bacterial flotillins are not yet clearly understood. In *B. subtilis* the absence of one of the flotillins only leads to mild defects like a delay in the differentiation process of sporulation and cell-widening (Donovan & Bramkamp, 2009). Whereas the absence of both flotillins results in loss of proper cell shape, cell division defects (Dempwolff *et al*., 2012), altered membrane fluidity (Lee *et al*., 2012; Bach and Bramkamp, 2013) and defective signaling events during the transition into the biofilm growth style (Lopez & Kolter, 2010). Also, an excess of flotillins can lead to defects, namely the considerable shortening of cells, which is in part caused by an unusual stabilization of the raft-associated protease FtsH (Yepes *et al*., 2012; Mielich-Süss *et al*., 2013). While it is established for eucaryotic flotillins to act as scaffolding proteins in defined membrane domains, it is eagerly disputed, whether their bacterial counterparts fulfill a similar role. Flotillins were co-isolated from DRM fractions with NfeD proteins, the signaling receptor KinC, cell wall synthesis enzyme Pbp5, secretory protein SecY and many more, leading to the hypothesis of flotillin organized lipid rafts or functional membrane microdomains (FMMs), which harbor these proteins (Lopez & Kolter, 2010; Bach & Bramkamp, 2013; Bramkamp & Lopez, 2015; Lopez & Koch, 2017). KinC for example was found to co-localize and interact in a beneficial way with FloT (Lopez & Kolter, 2010; Schneider *et al*., 2015). This interaction was disturbed in cells lacking YisP, a suspected squalene synthase (Lopez & Kolter, 2010), associated with synthesis pathways of lipids needed for bacterial raft formation (Jermy, 2010). However, subsequent studies suggested YisP to act as a phosphatase, with no squalene catalytic activity, catalyzing the formation of farnesol instead (Feng *et al*., 2014). Furthermore, super resolution microscopy showed that the flotillins and other proteins found in DRMs do not colocalize and have different dynamic behaviors (Dempwolff et al., 2016), making it unlikely that FMMs are regions in the membrane that offer a favorable environment in which these membrane proteins are continuously present and active. Others question the method of DRM extraction, arguing that it is an artificial process that does not reflect native membrane organization (Brown, 2006; Scholz *et al*., 2021). Additionally, flotillins appear to be required for the formation of regions with increased fluidity, which are the counterparts to the FMMs (Zielinska *et al*., 2020). While flotillins are often linked with SecY and it has been observed that protein secretion

is reduced in strains lacking flotillins (Bach & Bramkamp, 2013), there is a need for further investigation, since the cause is unclear.

1.6 Aims of research

Secretion is an essential process of bacteria and highly relevant for the biotechnological production of proteins. Although this area has been the subject of extensive research, many aspects such as the passage through the cell wall or the role of flotillins have not yet been studied. The key aim of this work was to develop an understanding of the location and dynamics of secretion including and focusing on cell wall passage, using super-resolution fluorescence microscopy. Furthermore, the influence of flotillins and autolysins on protein secretion in *Bacillus* will be investigated in this project and the possibility of improving secretion is going to be explored.

2 Article and Manuscripts

Personal contribution to published work:

The majority of this work has been published in one article and two manuscripts:

Article

Manuel Strach, Felicitas Koch, Svenja Fiedler, Klaus Liebeton, Peter L. Graumann

Protein secretion zones during overexpression of amylase within the Gram-positive cell wall

I designed and carried out all experiments except for the single molecule tracking, which was performed by Felicitas Koch and Svenja Fiedler. I wrote and revised the manuscripts during the publishing process together with Prof. Dr. Peter L. Graumann and the support of Dr. Klaus Liebeton. I created all figures and tables.

Manuscript I

Manuel Strach, Klaus Liebeton, Peter L. Graumann

The Influence of cell wall hydrolases on amylase secretion in *Bacillus subtilis*

I designed and carried out all experiments. Furthermore, I wrote the majority of the manuscript with the support of Prof. Dr. Peter L. Graumann and Dr. Klaus Liebeton and created all figures and tables.

Manuscript II

Manuel Strach, Klaus Liebeton, Peter L. Graumann

The Influence of flotillins on amylase secretion in *Bacillus subtilis*

I designed and carried out all experiments. Furthermore, I wrote the majority of the manuscript with the support of Prof. Dr. Peter L. Graumann and Dr. Klaus Liebeton and created all figures and tables.

2.1 Article

Strach *et al. BMC Biology*
https://doi.org/10.1186/s12915-023-01684-1

BMC Biology

RESEARCH ARTICLE Open Access

Protein secretion zones during overexpression of amylase within the Gram-positive cell wall

Manuel Strach[1], Felicitas Koch[1], Svenja Fiedler[1], Klaus Liebeton[2] and Peter L. Graumann[1*]

Abstract

Background Whereas the translocation of proteins across the cell membrane has been thoroughly investigated, it is still unclear how proteins cross the cell wall in Gram-positive bacteria, which are widely used for industrial applications. We have studied the secretion of α-amylase AmyE within two different *Bacillus* strains, *B. subtilis* and *B. licheniformis*.

Results We show that a C-terminal fusion of AmyE with the fluorescent reporter mCherry is secreted via discrete patches showing very low dynamics. These are visible at many places within the cell wall for many minutes. Expression from a high copy number plasmid was required to be able to see these structures we term"secretion zones". Zones corresponded to visualized AmyE activity on the surface of cells, showing that they release active enzymes. They overlapped with SecA signals but did not frequently co-localize with the secretion of ATPase. Single particle tracking showed higher dynamics of SecA and of SecDF, involved in AmyE secretion, at the cell membrane than AmyE. These experiments suggest that SecA initially translocates AmyE molecules through the cell membrane, and then diffuses to a different translocon. Single molecule tracking of SecA suggests the existence of three distinct diffusive states of SecA, which change during AmyE overexpression, but increased AmyE secretion does not appear to overwhelm the system.

Conclusions Because secretion zones were only found during the transition to and within the stationary phase, diffusion rather than passive transport based on cell wall growth from inside to outside may release AmyE and, thus, probably secreted proteins in general. Our findings suggest active transport through the cell membrane and slow, passive transition through the cell wall, at least for overexpressed proteins, in bacteria of the genus *Bacillus*.

Keywords Protein secretion, Gram-positive cell wall, Amylase, SecA, Bacterial cell biology

*Correspondence:
Peter L. Graumann
graumanp@uni-marburg.de; peter.graumann@synmikro.unimarburg.de
[1] Centre for Synthetic Microbiology (SYNMIKRO) and Fachbereich Chemie, Philipps-Universität Marburg, Marburg 35032, Germany
[2] BRAIN Biotech AG, Darmstädter Str. 34-36, Zwingenberg 64673, Germany

Background

Members of the genus *Bacillus* are famous for their use in industrial production of exoenzymes, and are widely used in biotechnological applications. Protein secretion is a two-step process, involving transport across the cell membrane, and passage through the several-layered peptidoglycan (PG) cell wall. While the prior is relatively well-understood [1, 2], the latter path is essentially unclear.

It has been estimated that 10% of the encoded *B. subtilis* proteins are secreted [3], to make extracellular

polymers available for nutrient uptake, with α-amylase being one with the widest economic importance [4]. While the twin-arginine translocation (Tat) pathway requires transported proteins to be folded [5], most proteins are secreted in an unfolded state via the general secretory (Sec) pathway [6]. Proteins destined for the Sec-pathway have an N-terminal signal peptide that delays folding in the cytoplasm [7]. The SecY, SecE, and SecG proteins together form the translocon complex SecYEG, an hourglass-shaped pore in the cell membrane with a constricted ring in the center [8]. Another component often described as the "motor" that drives translocation is the ATPase SecA [9]. SecA can interact with both the pre-protein to be secreted and SecYEG [10] as it catalyzes the translocation of the polypeptide chain through ATP binding and hydrolysis [1]. Additionally, the stabilizing protein SecDF plays an important role in maintaining a high capacity of protein secretion [11, 12]. To be released from the membrane the signal peptide of the preprotein has to be removed by a signal peptidase [13]. The two major signal peptidases recognizing the signal peptide of secreted proteins are SipS and SipT [14]. Furthermore, there are secretion-assisting factors like the membrane-associated, chaperone-like lipoprotein PrsA [6]. PrsA is crucial for efficient secretion of a number of exoproteins like amylases [15].

After overcoming the membrane, the passage through the cell wall is the next barrier for extracellular proteins. The Gram-positive cell wall has been described to form a sieve-like meshwork, which allows diffusion of proteins up to a molecular weight of 25–50 kDa [16]. However, secreted enzymes have a range of sizes between 15 and 70 kDa, such as amylases, lipases, and proteases, and the release from the cell wall was described as the rate-limiting step in the secretion of the α-amylase from *B. subtilis* [17, 18]. The passage of large proteins through multiple layers of peptidoglycan would require a pore-forming transport system, or otherwise heterogeneous meshwork sizes to allow for diffusion passages. Indeed, large cavities within the cell wall and the heterogeneous density of PG strands have been visualized using atomic force microscopy [19, 20]. Such discontinuities within the PG would be compatible with passages for larger proteins through the cell envelope. However, the passage of proteins through the cell wall is still an unresolved question.

The cell wall protects the cell against environmental stress, from bursting due to internal turgor pressure and is responsible for cell shape [21]. The Gram-positive cell wall consists of several layers and is 30–40 nm thick in *B. subtilis* [22, 23]. The main component of the cell wall is the peptidoglycan, which consists of a polysaccharide backbone with β-(1,4) glycosidically linked N-acetylglucosamine (GlnNAc) and N-acetylmuramic acid (MurNAc) molecules [24]. Attached to the lactyl group of N-acetylmuramic acid is an oligopeptide chain, which in most bacteria, including *Bacillus subtilis* and *Escherichia coli*, consists of L-Ala–D-Glu–L-meso-diaminopimelic acid–D-Ala–D-Ala [25].

Cell wall synthesis of the multilayered Gram-positive cell wall is thought to occur at the cell membrane, with the release of the oldest strands to the cell periphery, and thus in an "inside-out" mode. In a first step, the soluble PG precursor consisting of a pentapeptide bound to UDP-MurNAc is synthesized in the cytoplasm. In the second phase, the linkage to undecaprenyl phosphate in the cytoplasmic membrane is catalyzed, forming the lipid-linked monosaccharide peptide lipid I [26]. Subsequently, the glycosyltransferase MurG ligates a N-acetylglucosamine (NAG) residue to lipid I generating the lipid-bound disaccharide-pentapeptide precursor, lipid II [27]. In a next step, lipid II is transported across the plasma membrane to the outside by the flippase MurJ [28]. In the final stage of cell wall biosynthesis, lipid II is polymerized and cross-linked by RodA and penicillin-binding proteins (PBPs) [29, 30]. In contrast to the Gram-negative cell wall, the Gram-positive cell wall possesses so-called wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), which largely determine the charge of the cell wall. Their D-alanyl residues with free amino groups neutralize the negative charge of phosphates [31], making the cell wall more positively charged and influencing the folding and stability of secreted proteins by modulation of the availability of ions like, e.g., calcium [32].

Visualization of protein secretion and the components of the secretion machinery has previously been studied up until the membrane barrier [33]. We aim to advance the understanding of the location and dynamics of secretion including and focusing on cell wall passage. While we failed to visualize AmyE localization in the cell wall under native conditions, we were able to observe localized accumulation in the cell wall upon overproduction of AmyE, using two *Bacillus* species: *B. subtilis* and *B. licheniformis*. We argue that observed zones of protein secretion reflect genuine native regions of low density in the cell wall that allow for the diffusion of large proteins through the PG network.

Results

Secretion of active AmyE-mCherry in *B. subtilis* cells

It has been described that mCherry can be used as a fluorescent reporter outside of the bacterial cell, e.g., within the periplasm of Gram-negative bacteria [34]. We sought to establish whether mCherry can be used as a reporter to visualize the secretion of proteins from *Bacillus* cells. We generated a fusion of AmyE-mCherry at the original gene locus and observed only very weak fluorescence that

could not be spatially resolved due to the relatively high back ground fluorescence of *B. subtilis* in the red channel (data not shown). We resolved to using a high copy plasmid expressing AmyE-mCherry, which allowed yielding high concentrations of AmyE in the culture supernatant. We reasoned that as AmyE is heavily produced as well as secreted it might reveal its path across the cell envelope.

Figure 1 shows that AmyE-mCherry when expressed from a plasmid, under the control of the strong constitutive HpaII promoter [35], is largely detected as a full-length protein (72.6+26.7 kDa=99.3 kDa) in *B. subtilis* cells, and to a large extent also in *B. licheniformis*, although here, degradation is quite extensive. As will become apparent below, expression at a single cell level is very heterogeneous, but expression levels of the entire population were quite similar between biological replicates (Fig. 1A).

In order to verify that AmyE-mCherry is secreted into the medium, we performed fluorescence assays of cultures grown to the transition to stationary phase (for a time course of secretion, see Fig. 2B). Cells expressing AmyE from the native gene copy only showed weak background fluorescence, while cells expressing AmyE-mCherry from a plasmid displayed fluorescence. The strongest fluorescence was observed in the culture supernatant.

When AmyE-mCherry was expressed as a polypeptide lacking the signal sequence, thus eliminating translocation across the cell membrane, fluorescence was reduced to background. In *B. licheniformis*, a very similar situation was observed. However, in the absence of a signal sequence, clearly higher fluorescence was detected in the cells, but also in the culture supernatant, pointing to differences in the potential to fold and maintain the reporter protein in the cytoplasm between *B. subtilis* and *B. licheniformis* (Fig. 1B). These findings suggest that while leaderless AmyE-mCherry accumulates within *B. licheniformis* cells, and is partially released, possibly by cell lysis, it seems either not to be produced or be degraded in the cytoplasm of *B. subtilis*. Thus, fluorescence found in the supernatant of *Bacillus* cultures depends on the signal sequence of AmyE-mCherry, showing that the fusion protein is efficiently translocated across the cell membrane as well as across the cell wall. To prove that it is not only mCherry that is secreted, we determined amylase activity from culture supernatants. Higher amylase activity was determined for cells that produce AmyE from the plasmid than for wild type cells (Fig. 1C). Amylase activity was lower for cells overproducing AmyE-mCherry, indicating that the fusion either reduces AmyE activity or reduced its efficiency of secretion. In any event, AmyE-mCherry activity was higher than in wild type cells, proving that AmyE-mCherry is efficiently secreted across the *B. subtilis* or *B. licheniformis* cell envelope and sufficiently stable for the following analysis.

AmyE-mCherry is produced during the exponential phase but is secreted only by a subpopulation of cells while entering the stationary phase

We next determined the expression profile of *Bacillus* cells overproducing AmyE-mCherry growing in a liquid culture based on the fluorescence determined for the whole culture, i.e., cell-associated and in the culture supernatant. Fluorescence was observed in the culture starting with cells entering the stationary phase, and continued to be well detectable in stationary cells (Fig. 2A). This is in line with reports of secretory processes usually commencing as cells transit from exponential into the stationary phase [36], but does not fit the idea that the expression of *amyE-mCherry* is driven by a strong constitutive promoter, rather than by a stationary phase-induced promoter. We therefore performed a time course of AmyE-mCherry secretion over the entire growth curve of *B. subtilis* and *B. licheniformis* cells by measuring the amylase activity in the culture supernatant. Figure 2C and D shows that for *B. subtilis*, AmyE-mCherry secretion peaks at the transition phase, while for *B. licheniformis*, activity is highest during stationary growth. Western Blot analyses showed that for *B. subtilis*, amylase secretion as measured by the extracellular activity (Fig. 2C) remained relatively constant throughout the growth phases (Fig. 2E and F). In *B. licheniformis* AmyE-mCherry synthesis peaked at mid exponential phase (Fig. 2E), while AmyE-mCherry activity in the supernatant increased up to the stationary phase (Fig. 2C), showing that AmyE-mCherry produced during exponential phase is not efficiently secreted, while this is the case as cells enter stationary phase.

We next sought to analyze the percentage of cells showing AmyE-mCherry fluorescence using fluorescence microscopy. During the mid-exponential phase, we found 4 to 5% of cells showing AmyE-mCherry-associated fluorescence in the red channel (Fig. 3A and B). Intriguingly, fluorescence was not found within the cytosol of cells, or throughout the entire cell periphery, but was observed in a punctate pattern within the cell envelope, for both, *B. subtilis* as well as for *B. licheniformis*. These findings suggest that AmyE-mCherry accumulates at the cell membrane, and/or within the cell wall, but not within the cytosol. The number of cells showing AmyE-mCherry puncta increased during the transition phase to 23% and 18% for *B. subtilis* cells and *B. licheniformis* cells, respectively (Fig. 2C). During the stationary phase, we observed an average of 34% of *B. licheniformis* cells showing AmyE-mCherry signals, while only 19% for *B. subtilis* cells (Fig. 2D). Thus, high-level protein secretion, as judged

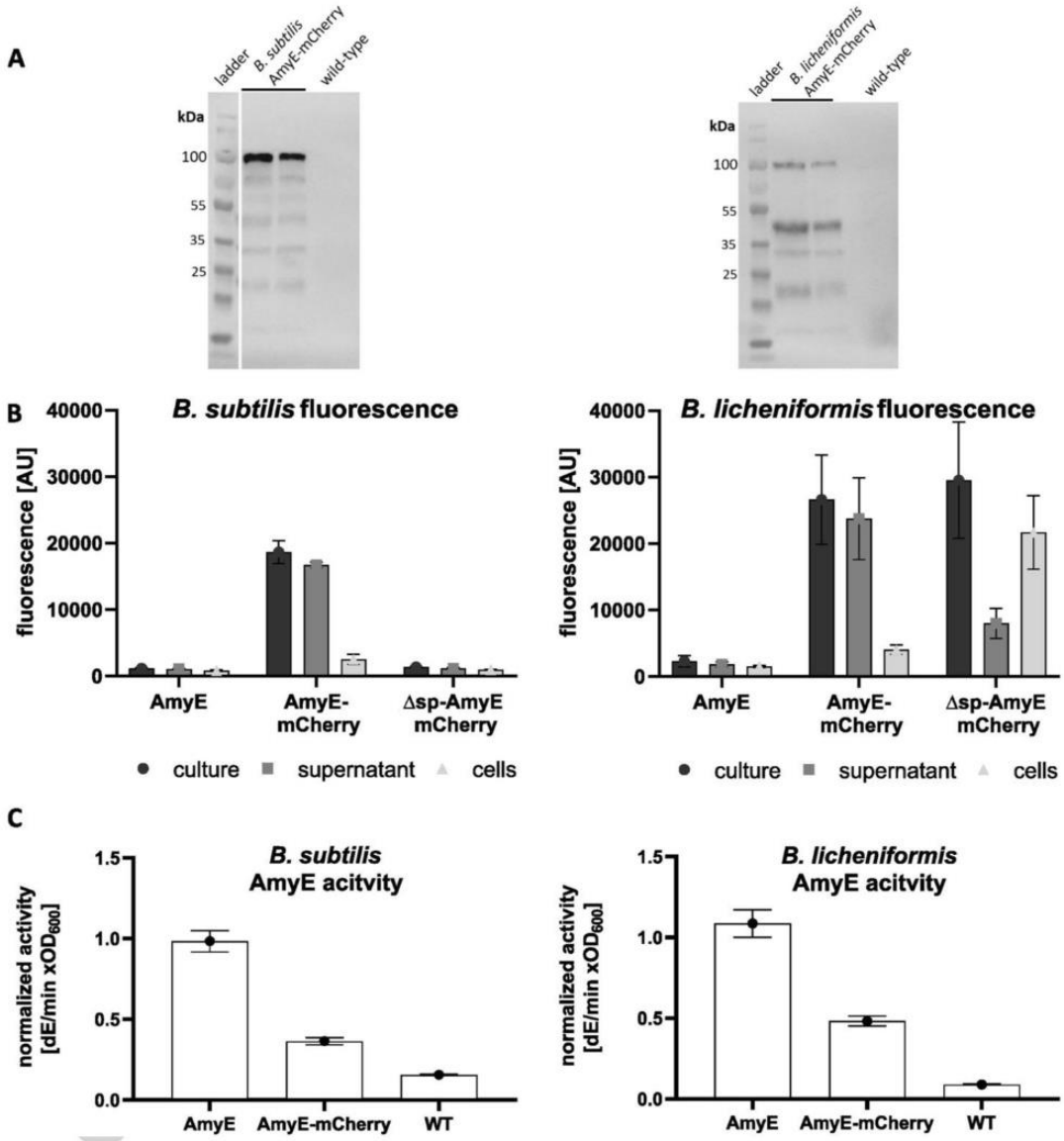


Fig. 1 AmyE-mCherry is efficiently secreted from *B. subtilis* and *B. licheniformis* cells, but also detectable in a cell-associated from. **A** Western blot showing the presence of AmyE-mCherry fusion protein (calculated Mw: 99.3 kDa) in cell lysates of *B. subtilis* and *B. licheniformis* after 16 h of growth (note that duplicates are shown for assessment of reproducibility) using polyclonal antibodies against mCherry; **B** fluorescence measurement of the whole culture, supernatant, or separated cells; and **C** amylase activity in culture supernatant. AmyE, plasmid-based expression of *amyE*; AmyE-mCherry, plasmid-based expression of the reporter construct; WT, native genomic expression of *amyE* (**C**). Δsp-AmyE-mCherry represents a variant without signal peptide in **B**

from the visualization of fluorescent signals, occurs in a highly heterogeneous manner at the cell population level. Because low-level secretion of amylase, which we were unable to detect using epifluorescence microscopy, might occur throughout the cell surface, general protein secretion might also take place throughout the cell envelope.

However, we would like to suggest that it is unlikely that general protein secretion follows a different path than high-level secretion of overproduced proteins.

To further test the idea of heterogeneous secretion, we imaged cells expressing the AmyE-mCherry fusion lacking the signal peptide (sp) for secretion. As shown in Fig. 3C), cells grown to the transition into stationary phase showed non-homogeneous fluorescence, frequently associated with the periphery of the cell, but also fluorescence within the cytosol, instead of puncta at the cell periphery for full-length AmyE-mCherry. Western Blot analyses revealed that sp-less AmyE-mCherry was more prone to degradation than the full-length version (Fig. 3D). Interestingly, also, expression occurred in a heterogeneous manner, as 47%±9% of *B. subtilis* cells showed fluorescence, as opposed to 19% showing foci for secreted AmyE-mCherry, and 56%±16% of *B. licheniformis* cells possessed fluorescent signal, compared with 34% showing full-length AmyE-mCherry foci. These data reveal that while only about 50% of cells produced AmyE-mCherry (as deduced from the non-secreted version), only a subpopulation showed accumulation of AmyE-mCherry at the cell periphery. Our data also suggest that AmyE-mCherry is rapidly secreted out of the cytosol, to accumulate within either the periplasm or, more probably, in the cell wall, as deduced from the formation of fluorescent foci.

AmyE-mCherry is statically positioned in the cell envelope

We next investigated if the observed peripheral location of AmyE-mCherry foci is due to AmyE-mCherry being slowly translocated through the cell membrane, or to AmyE-mCherry being present within the cell wall. The observation of discrete foci strongly argues against accumulation within the periplasm, because the protein would rapidly equilibrate within this space, as is, e.g., the case of taken-up DNA in competent *B. subtilis* cells [37]. To address this question, we visualized the fusion protein in cells during the transition phase, with or without treatment with lysozyme, which degrades the bacterial cell wall by cutting within the glycan strands. Figure 4 shows that more than 90% of cells treated with lysozyme lost rod shape and became sphaeroplasts upon treatment with lysozyme. While 23±2% of non-treated *B. subtilis* cells (or 18±6% *B. licheniformis* cells) showed envelope-associated AmyE-mCherry fluorescence, only 6±3% of cells (or 3±2%) retained signals after exposure to lysozyme. We cannot distinguish if fluorescence seen in such sphaeroplasted cells is due to residual patches of peptidoglycan, or due to fusion proteins still attached to the cell membrane (via SecA and the translocon). Thus, envelope-associated signals of AmyE-mCherry are to a large extent due to the presence of intact PG layers, indicating that visible AmyE-mCherry accumulations at various positions along the cell wall represent protein molecules associated with the cell wall. However, our experiments do not rule out that there are AmyE-mCherry molecules within the periplasm, because our imaging does not capture motion of quickly diffusing molecules.

AmyE dynamics differ from those of SecA or from SecDF

Intrigued by the observation that AmyE-mCherry fluorescence was recognized as punctate foci in two different *Bacillus* species, we set out to study the dynamics of the passage of the fusion protein through the cell wall. Because peptidoglycan synthesis is expected to occur from inside/out [38–41], and the wall to be rather rigid, we expected low lateral mobility of AmyE-mCherry foci. We employed structured illumination microscopy (SIM), reaching doubled resolution relative to conventional light microscopy. Time-lapse experiments revealed that AmyE-mCherry foci remain statically positioned throughout many minutes (Fig. 5A). As opposed to this, even a slow-diffusing membrane protein (forming large clusters) such as flotillin FloT diffuses throughout the entire cell membrane of *B. subtilis* cells in time scale of 1.5 min [42]. This finding suggests that AmyE-mCherry, after being transported across the cell membrane, does not move through the entire cell wall, even when produced in high amounts, but moves through defined positions, or diffuses to and accumulates in defined positions. These data suggest that secretion of overexpressed amylase through the PG layers occurs within secretion zones, rather than throughout the cell wall. We tracked the position of foci relative to the long axis of cells using particle tracking. We found a speed of 77.6 nm shift per frame (i.e., 60 s) for *B. licheniformis* and a slightly lower speed of 69.6 nm for *B. subtilis*. To set this into relation, we tracked

(See figure on next page.)

Fig. 2 Dependence of cell density and fractions of cells producing AmyE-mCherry on growth phases. **A** Growth curves of *B. subtilis* and *B. licheniformis* incubated at 37 °C for 21 h. **B** Kinetics of mCherry fluorescence of the whole culture (cell-associated plus culture supernatant) normalized to cell density. AmyE-mCherry production in *B. subtilis* and *B. licheniformis* during 10 h of growth. **C** Amylase activity in culture supernatant of *B. subtilis* and *B. licheniformis*. **D** Growth curves of *B. subtilis* and *B. licheniformis* incubated at 37 °C for 10 h. **E** Western blot showing the presence of AmyE-mCherry fusion protein (calculated Mw: 99.3 kDa) in cell lysates of *B. subtilis* and *B. licheniformis*. **F** SDS PAGE gel is shown as a loading control

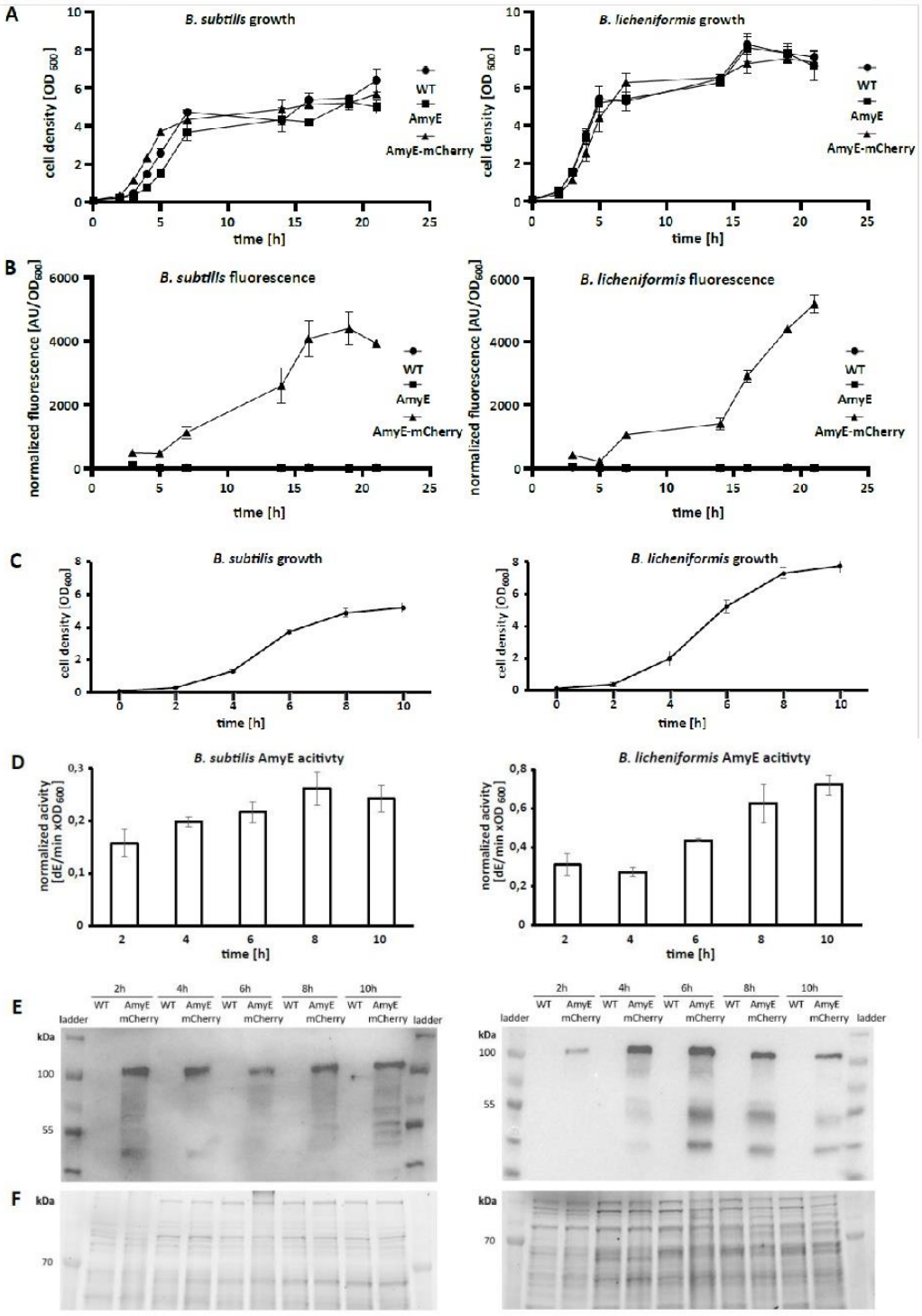


Fig. 2 (See legend on previous page.)

a SecA-mNeonGreen fusion expressed from the original gene locus in *B. subtilis* PY79 cells, as a single copy of SecA in the cell (Additional file 1: Fig. S1). While *secA* is an essential gene in *B. subtilis*, cells grew indistinguishable from wild type cells, showing that the SecA-mNeonGreen fusion can functionally replace the wild type SecA copy. We also tracked a SecDF-NeonGreen fusion in the same genetic background. SecDF has been reported to play an important role in AmyE secretion, while not being essential for cell viability [43]. Amylase activity in culture supernatant was similar in cells expressing SecDF-NeonGreen or the native copy of SecDF, indicating that the fusion was also largely functional (Additional file 2: Fig. S2). SecA-NeonGreen formed discrete foci at the cell membrane in *B. subtilis* cells (a higher number than AmyE-mCherry), as has been described before for a SecA-GFP fusion [33], and these had a mean shift of 127.2 nm/frame, almost twice as fast as AmyE-mCherry (Fig. 5B). Note that our analyses do not capture single SecA molecules diffusing between foci, which contain several rather than single molecules. SecDF-NeonGreen even showed a speed of 157.6 nm per frame (Fig. 5B), more than two-fold higher than AmyE-mCherry. Thus, cytosolic and membrane proteins involved in AmyE secretion will come and go to and from AmyE secretion zones, i.e., to the SecYEG translocons involved in AmyE translocation across the cell membrane, while AmyE will continue to vertically diffuse through the wall towards the exterior of cells. Of note, the distinct localization patterns of SecA and of SecDF did not visually differ between wild type cells and cells overexpressing AmyE-mCherry (Additional file 3: Fig. S3).

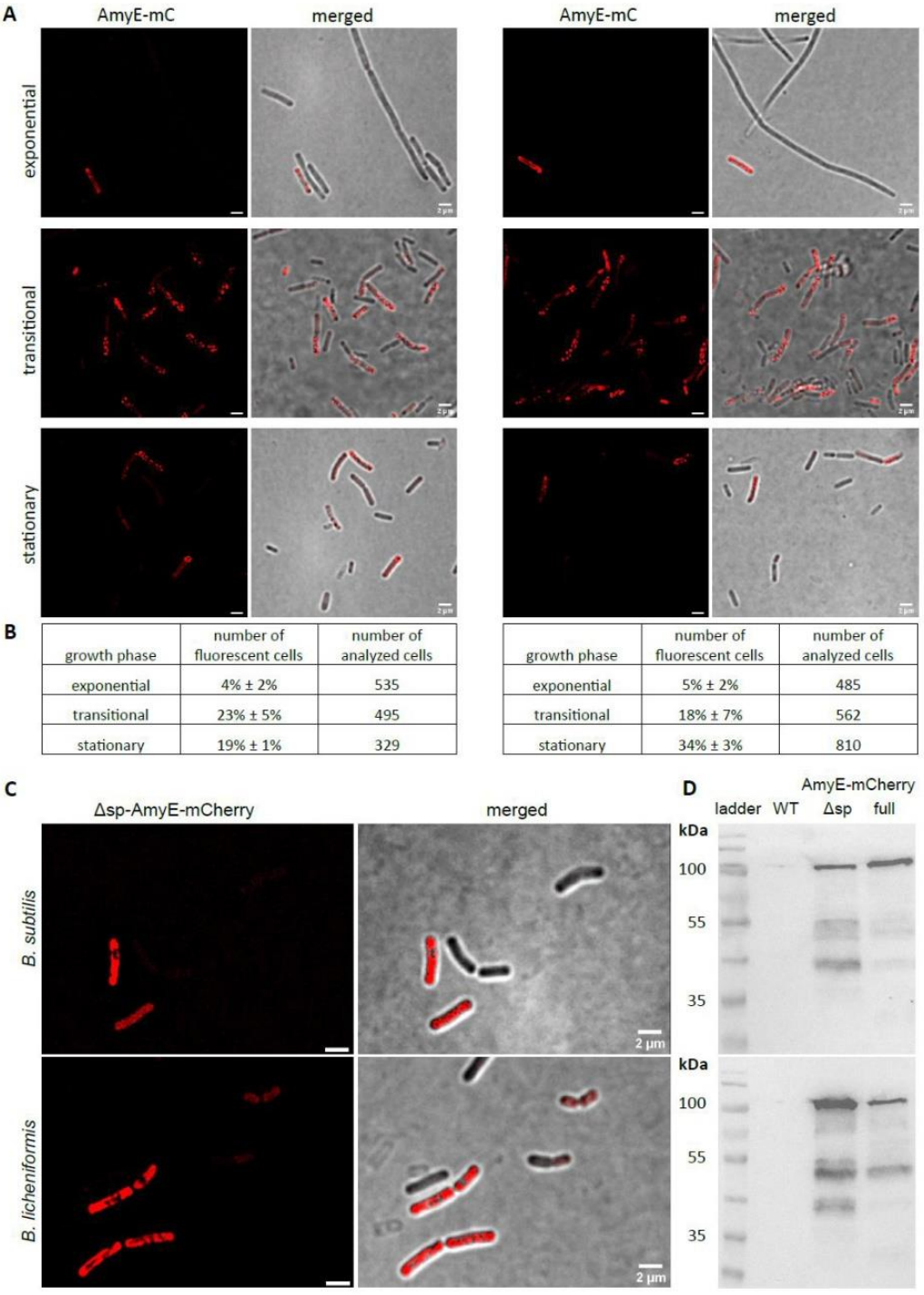
AmyE secretion zones show fluctuations within a minutes-time scale

The cell wall has been estimated to have a thickness of 30 to 100 nm in Gram-positive bacteria [23]. The prevalence of many stationary AmyE-mCherry foci (remaining static for up to 30 min) suggests that AmyE-mCherry slowly diffuses through the lateral cell wall at several loci. When we analyzed time courses of AmyE-mCherry foci, we noticed that a considerable proportion of foci (25%) showed noticeable fluctuations in fluorescence intensity. Figure 6 (and Additional file 4: Fig. S4) shows an example of *B. licheniformis* cells containing 2 foci that show strong fluctuations in fluorescence intensity. In order to rule out that fluctuations are caused by a shift in the focal plane or fluctuations in background fluorescence, we correlated fluorescence throughout cells with focal fluorescence intensity. Figure 6B shows that fluctuations in focal fluorescence were largely independent of general fluctuations, and also much larger in intensity (as analyzed by converting into arbitrary units). Gain or loss of fluorescence could be observed between 1-min intervals, suggesting that secretion zones can gain or lose fluorescent AmyE molecules within 60-s intervals. On the other hand, slow-moving SecA-mNeonGreen and SecDF-mNeongreen foci did not show such fluctuations (Additional file 5: Fig. S5). It must be kept in mind that especially for *B. licheniformis* cells, degradation products for AmyE-mCherry were observed (Fig. 1A), so fluctuations could also include proteolytic events. We interpret these data to support the notion of discrete zones within the cell wall that allow the passage of almost 100-kDa molecules within a time frame that is way below the scale of the 25 min for the cell cycle of cells growing in rich medium, not accounting for the fact that the cells are strongly slowing down growth during the transition into stationary phase. In this respect, a passive transport of secreted proteins through the meshwork of the murein sacculus by the incorporation of new peptidoglycan-precursors on the inner side of the cell wall and the gradual displacement of older glycan-strands to the outside as proposed by Kemper et al. [44] seems improbable.

To test for the spatiotemporal involvement of SecA and SecDF in AmyE secretion, we analyzed the co-localization of SecA-mNeonGreen or SecDF-mNeonGreen fusions with AmyE-mCherry in cells during the transition phase. SecDF foci are found at almost all sites within the cell membrane (Fig. 7) and thus might show a high degree of co-localization with AmyE secretion zones by default. However, using the program Fiji [45], we quantified 18 ±3% spatial overlap between SecDF and AmyE, showing that AmyE-mCherry signals frequently lacked overlap with SecDF fluorescence. For SecA, the pattern of localization was more punctate (Fig. 7). While SecA could be found in all cells, AmyE-mCherry foci were not, in agreement with the

(See figure on next page.)

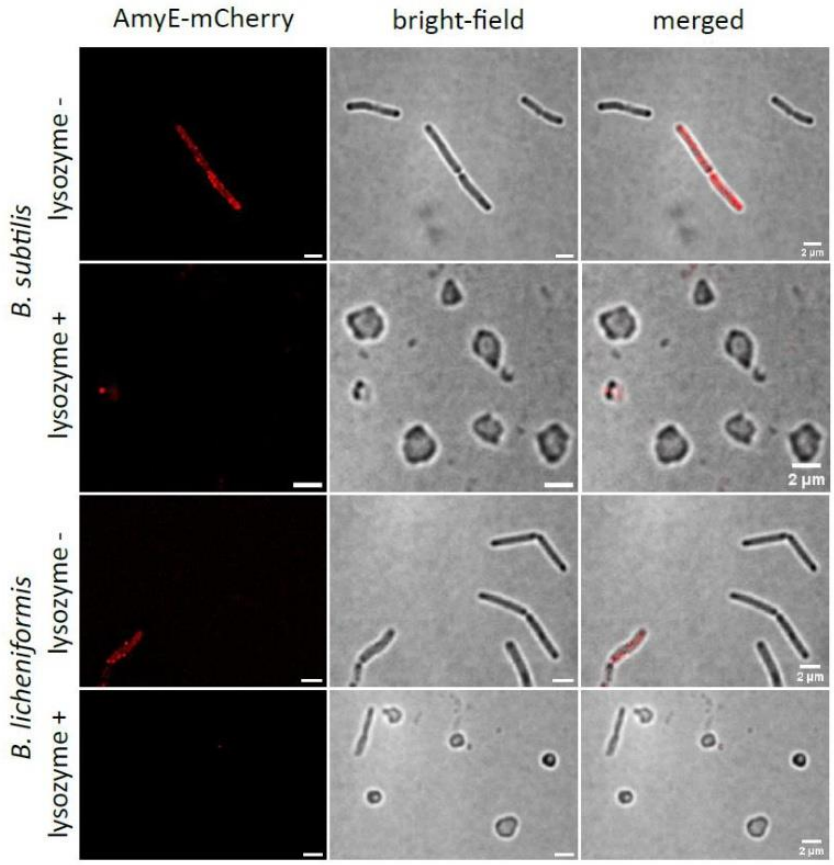
Fig. 3 Fraction of cells showing AmyE-mCherry signals in different growth phases. **A** Structured illumination microscopy (SIM) imaging of *B. subtilis* (left) and *B. licheniformis* cells (right) showing AmyE-mCherry fluorescence during different growth phases. **B** Table showing percentage and number of analyzed cells. **C** The variant Δsp-AmyE-mCherry without signal peptide accumulates in the cytosol. Structured illumination microscopy (SIM) imaging of *B. subtilis* (top) and *B. licheniformis* cells (bottom) showing Δsp-AmyE-mCherry fluorescence during the transitional growth phase. (**D**) Western blot showing the presence of Δsp-AmyE-mCherry (calculated Mw: 96.4 kDa) *and* AmyE-mCherry fusion protein (calculated Mw: 99.3 kDa) in cell lysates of *B. subtilis* (top) and *B. licheniformis* (bottom) after 16 h of growth



growth phase	number of fluorescent cells	number of analyzed cells
exponential	4% ± 2%	535
transitional	23% ± 5%	495
stationary	19% ± 1%	329

growth phase	number of fluorescent cells	number of analyzed cells
exponential	5% ± 2%	485
transitional	18% ± 7%	562
stationary	34% ± 3%	810

Fig. 3 (See legend on previous page.)



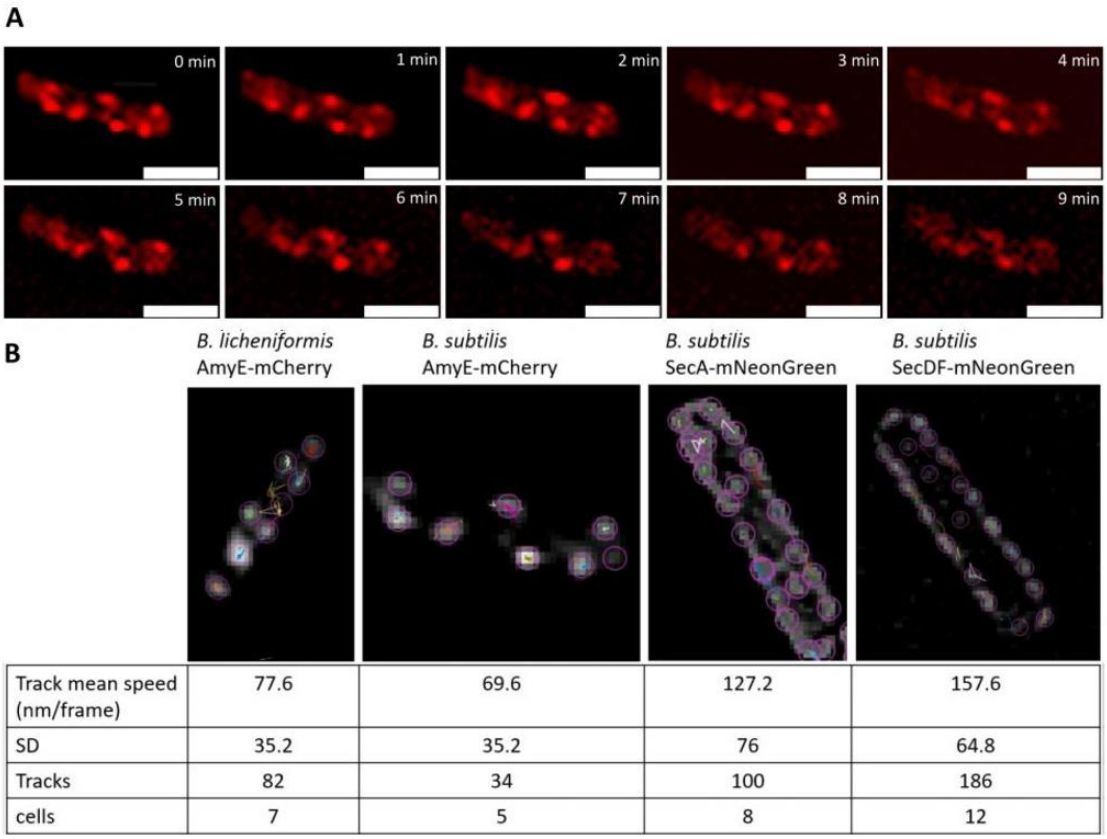
	lysozyme	number of fluorescent cells	number of analyzed cells
B. subtilis	-	23 ± 2%	195
	+	6 ± 3%	501
B. licheniformis	-	18 ± 6%	290
	+	3 ± 2%	652

Fig. 4 SIM imaging of AmyE-mCherry in *B. subtilis* and *B. licheniformis* in the transitional growth phase upon spheroplasting treatment with lysozyme. Cells displaying fluorescent signal were counted and normalized to the number of all analyzed cells

heterogeneity of secretion zones observed (Fig. 3). This finding rules out that population-heterogeneity of secretion zones might be due to heterogeneous expression of SecA or of SecDF.

While there is some co-localization of AmyE-mCherry and SecA-mNeonGreen foci, as expected from the essential nature of SecA within the secretion process, many AmyE-mCherry foci were observed lacking a SecA-mNeonGreen signal (Fig. 7). We quantified 19±4% spatial overlap between both signals, revealing that secretion zones frequently lack an associated ATPase at the corresponding cytosolic site.

These observations are in agreement with the higher dynamics of SecA- mNeonGreen foci compared to AmyE-mCherry. SecA appears to accumulate at sites corresponding to AmyE translocation zones, but to diffuse to other sites once translocation across the SecYEG translocon has been achieved, or possibly even during the translocation of a single AmyE molecule. The data are also compatible with the idea that many AmyE molecules



Track mean speed (nm/frame)	77.6	69.6	127.2	157.6
SD	35.2	35.2	76	64.8
Tracks	82	34	100	186
cells	7	5	8	12

Fig. 5 AmyE-mCherry foci remain statically positioned. **A** Time-lapse SIM experiments of a *B. subtilis* cell expressing AmyE-mCherry. Scale bar 2 µm. **B** Single particle tracking of SIM time-lapse images via TrackMate. SIM imaging for 10–30 min of AmyE-mCherry, SecDF-mNeonGreen, and SecA-mNeonGreen fusion proteins in *B. subtilis* and AmyE-mCherry in *B. licheniformis*

are translocated into the cell wall, where they diffuse towards the outside of the cell wall, constrained by the cell wall meshwork, and be released into the culture medium. Strong fluctuations of AmyE-mCherry fluorescence within secretion zones suggest that subsequently to loss of fluorescence via dissociation into the medium, new molecules can be translocated into a secretion zone, pointing to their long-lived nature, relative to assembly/disassembly of SecA foci at the cell membrane.

Our data support the hypothesis that AmyE molecules accumulate in secretion zones within the cell wall and are released from these in a minutes' time scale, as we corrected for a general loss or gain of fluorescence by fluctuations during image acquisition.

AmyE is released from the cells at discrete zones

Our idea of secretion zones within the *Bacillus* cell wall implies that as amylase transits through the PG layers, it also emerges in defined zones from the cell envelope. To test this idea, we added fluorescently-labeled starch ("bodipy-starch") to cells, an amylase substrate that develops fluorescence as it is degraded into monomers. Additional file 6: Fig. S6 shows that fluorescence can be detected on only 1% of *B. subtilis* cells carrying solely the native copy of AmyE, also in a punctate manner. Overexpression of AmyE-mCherry gives rise to 18% and 25% of cells of *B. subtilis* and *B. licheniformis*, respectively, showing punctate fluorescent signals during the transition phase, 30 min after incubation with the substrate (Fig. 8A and B). Note that longer incubation with the substrate (e.g., 60 min) resulted in homogeneous staining of the cell surface (Fig. 8A), in agreement with a diffusion of AmyE molecules out of the secretion zones and dispersal over the whole cell surface. These experiments indicate that as AmyE-mCherry enters secretions zones at the lower (inner) level of the cell wall, it exits the cell wall, as witnessed by its activity, at similarly discrete zones. These data show that active enzyme exits from discrete patches,

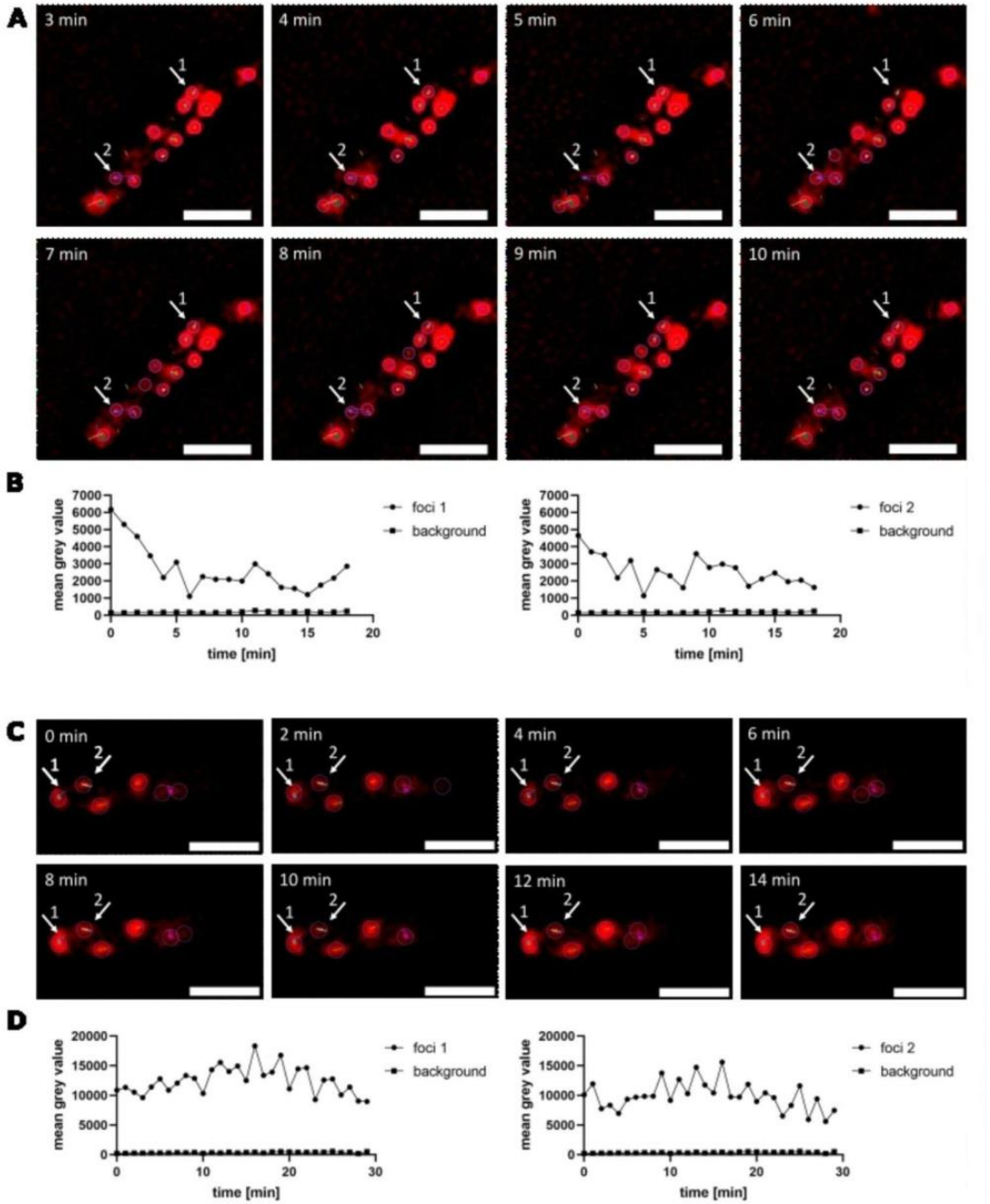


Fig. 6 AmyE-mCherry foci showing intensity fluctuations over time. SIM time-lapse images of AmyE-mCherry in **A** *B. licheniformis* and **C** in *B. subtilis*, showing each two foci that fluctuate in fluorescence intensity. **B**, **D** Fluorescence intensity analysis over time of the two foci and the background. Scale bar 2 µm

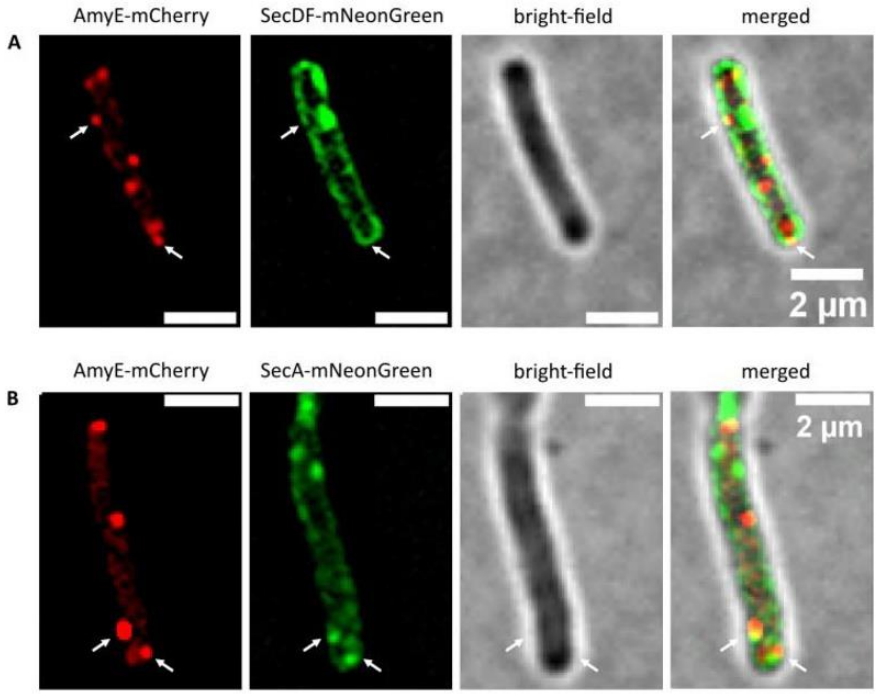


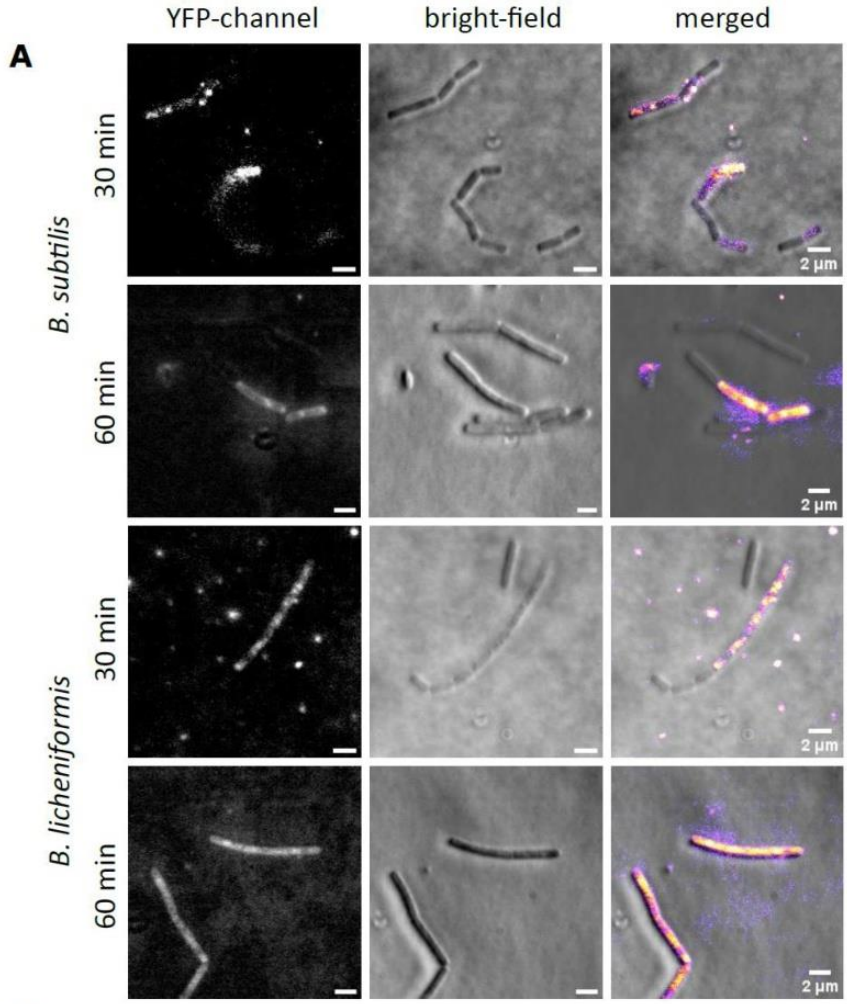
Fig. 7 SIM imaging of *B. subtilis* cells co-expressing AmyE-mCherry and mNeonGreen fusion. **A** Localization of AmyE-mCherry and SecDF-mNeonGreen with a shared area of 18% (3% SD). **B** Localization of AmyE-mCherry and SecA-mNeonGreen with a shared area of 19% (4% SD)

ruling out that AmyE-mCherry accumulation within secretion zones is entirely based on the accumulation of aggregated proteins within the cell wall. Unfortunately, due to technical difficulties with the fluorophores, we did not succeed in co-localizing AmyE-mCherry and bodipy-starch foci.

Taken together, our data support the idea of areas of a diameter of 125 nm or less within the *Bacillus* cell wall, which allow the passage of many AmyE-mCherry molecules. We favor the view that this also holds true for the secretion of proteins produced at wild type-level, i.e., not overexpressed molecules, in agreement with the finding of Campo et al. who found foci of AmyQ being secreted using immunofluorescence microscopy [33].

Expression of AmyE leads to considerable changes in SecA dynamics at a single molecule level

Foci observed for SecA-mNeonGreen described above imply the presence of several molecules within close spatial proximity, because single, diffusing molecules can not be resolved by epifluorescence or SI(M) microscopy. In order to better understand the SecA-driven process of AmyE secretion, we performed single molecule tracking (SMT) using the SecA-mNeonGreen fusion [46]. SMT was performed as described before [47, 48]. SecA-mNeonGreen molecules were visualized using 20 ms stream acquisition, in cells grown to the transition phase (see movie S1). Using Squared Displacement Analyses (SQD) we found that the observed distribution of tracks was best fitted assuming three different populations of SecA-mNeonGreen molecules without overfitting of data (Fig. 9A, note that SMTracker 2.0 uses Bayesian Information Criterion and other tests to avoid overfitting artifacts). Figure 9C displays the size of populations and their corresponding average diffusion constants from the data shown in Fig. 9A. Populations observed either moved with 0.7 $\mu m^2\ s^{-1}$, a value compatible for a large, freely diffusing cytosolic protein (SecA forms a dimer in solution [49–53]), or with 0.15 $\mu m^2\ s^{-1}$, in the range of freely diffusing ribosomal subunits [54], or with 0.04 $\mu m^2\ s^{-1}$ (Table 1). This extremely slow mobility has been proposed to account for the SRP system bound to the ribosome nascent chain complex as well as to the SecYEG translocon [55], or for translating ribosomes [54, 56]. According to this interpretation, about 21% of SecA is temporarily engaged in a secretion complex, while 50% diffuse through the cell and/or along the membrane bound to a substrate, and about 28% are freely diffusing, unbound SecA dimers. The three populations we observed are entirely compatible with data obtained by



B

B. subtilis	number of fluorescent cells	number of analyzed cells
WT	1 ± 1%	383
AmyE	18 ± 9%	634

B. licheniformis	number of fluorescent cells	number of analyzed cells
WT	0 ± 1%	522
AmyE	25 ± 10%	916

Fig. 8 Localization of AmyE in *B. subtilis* and *B. licheniformis* cells determined by its activity. **A** Cells were mounted on agarose slides for immobilization of cells and incubated for 30 and 60 min, respectively, at 37 °C with starch-BODIPY-FL. Fluorescence is produced by hydrolysis of this substrate when AmyE is secreted to the outer level of the cell wall. **B** Cells from three independent experiments displaying fluorescent signal were counted and normalized to the number of all analyzed cells

SMT on SecA from *E. coli* [57]. During high AmyE secretion activity, SecA-mNeonGreen trajectories became shorter (Fig. 9A). The slow mobile "static" fraction of SecA-mNeonGreen increased from 20.9 to 29%, i.e., by 39%, while the medium "mobile" fraction remained relatively stable, and accordingly, the freely diffusing population decreased (Fig. 9C). These data suggest that most SecA molecules are bound to a substrate and in search of a translocon, and upon increased synthesis of AmyE, engagement with the translocon is increased, but free SecA is still available. In approximation to static engagement with a translocon, we determined the average dwell time from the number of molecules staying within a radius of 106 nm (three times our localization error) for a given time. We determined about 300 ms for this time, no matter if AmyE was produced at wild type-level, or from the plasmid (Table 1, Fig. 9G). Note that we are underestimating dwell times due to bleaching during imaging. The probability of dwell events could only be explained by using two exponential decay curves (Fig. 9G), suggesting that under wild type expression conditions, 78% of molecules have an average dwell time of 240 ms (τ_1), and 22% of 450 ms (τ_2). The latter fraction likely corresponds to molecules being bound to a translocon, in very good agreement with the population of static molecules (Fig. 9C, 21%); short dwell times can arise from stochastically occurring slow diffusion events. In cells carrying the plasmid overproducing AmyE, dwell times remained very similar (Table 1), but the number of molecules showing extended dwell times (τ_2, 430 ms) increased to 29%, again closely reflecting changes in population size of the static molecules (Fig. 9C). These finding suggest that while the time SecA spends on transport of molecules remains the same, more SecA molecules are engaged in transport events during AmyE overexpression.

When tracks were sorted into different populations, and tracks of the slow mobile "static" fraction were projected into a medium-sized cell of 3×1 μm, most molecules were found close to the cell poles, or in the cell center, which is very similar to the localization of translating ribosomes [54, 58, 59] (Fig. 9C). Upon expression of plasmid-encoded AmyE, the pattern of localization of static SecA molecules changed, in that more sites along the lateral cell membrane showed high density of tracks (Fig. 9C).

SMT of SecDF also suggested the presence of three populations (Fig. 9B), of which the static population showed a milder increase upon overproduction of AmyE (Fig. 9D). The pattern of localization of static tracks became more uniform when cells expressed AmyE from plasmid (Fig. 8F, note the different scaling of the heat maps). Thus, SecDF also showed changes in single molecule dynamics during AmyE overproduction, but not as strongly as SecA.

These data support the idea that SecA exchanges between transport events at SecYEG translocons, in a time scale of few hundreds of milliseconds, in stark contrast to long-lived AmyE secretion zones, supporting the view that many AmyE molecules are transported into secretion zones by a highly dynamic population of SecA molecules.

Discussion

We show that high-level secretion of amylase AmyE in two *Bacillus* species leads to an accumulation of molecules at discrete zones within the cell wall. The finding that amylase activity at the surface of *B. subtilis* cells can also be observed to follow a discrete, patch-like pattern shows that active amylase molecules traverse the cell wall via such zones, which we term "secretion zones". Release of high levels of amylase-mCherry from cells was detectable by enzyme and fluorescence assays of culture supernatants, supporting the idea that we have visualized the passage of largely active amylase molecules across the cell envelope. Fluorescence measurements indicate that passage through the wall may take place at a minutes' time scale, possibly occurring in a pulse-like manner, and confirming the duration of cell wall passage of an amylase determined by pulse chase experiments [17, 18]. These results are in agreement with the idea of motion of amylase molecules across the cell wall via (passive) diffusion, via structures within the cell wall that allow diffusion of proteins even larger than 50 kDa.

(See figure on next page.)

Fig. 9 Single molecule tracking of SecA and SecDF. **A** Jump distance analyses of SecA-mNeonGreen (SecA-mNG) according to SQD analyses. The left panels represent wild type cells, right panels describe cells carrying the AmyE expression plasmid. The solid lines represent slow diffusing/static molecules, dotted lines are fits for medium-mobile molecules, dashed lines represent the fast-mobile population. **B** same as A for SecDF-mNeonGreen (mNG). **C** Bubble plots showing results from fitting of three populations by SQD analysis of single molecule tracks for SecA-mNeonGreen, size of bubbles corresponds to population size, diffusion constants are given on the *y*-axis, and bars in bubbles represent fitting errors. **D** bubble plot for SecDF-mNeonGreen data. **E** Heat map of static tracks of SecA-mNeonGreen projected into a 3×1 μm large cell, **F** similar as **E** for SecDF-mNeonGreen. **F** Plot of the probability density function of events of molecules staying within a radius of 106 nm for a certain amount of time (shown on the *X*-axis). The exponential decay curve can be explained by assuming two populations, one with a shorter (green curve) and one with a longer average dwell time (red curve), as stated in the inset

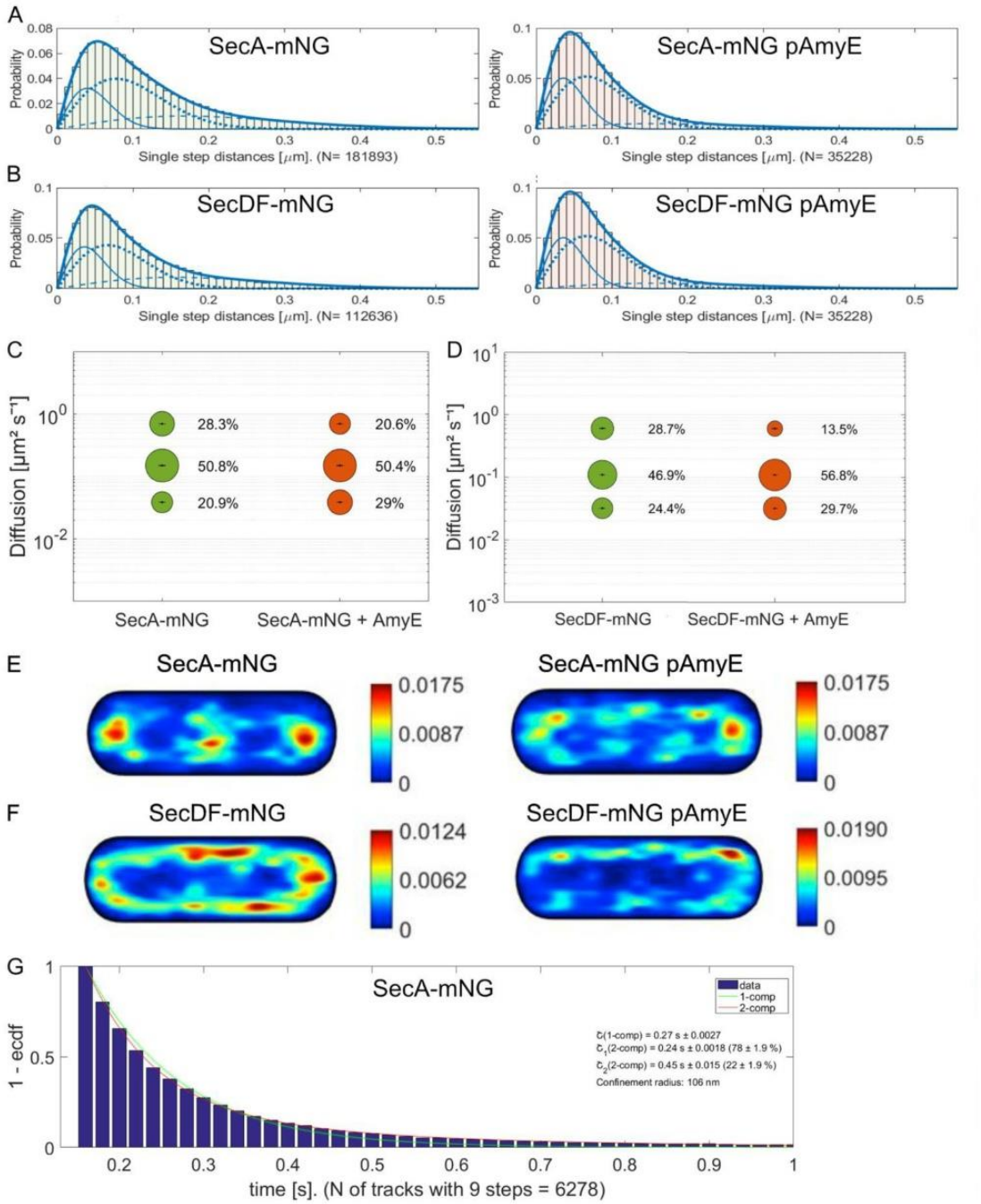


Fig. 9 (See legend on previous page.)

Table 1 SMT data from SecA-mNeonGreen

	B. subtilis BG214 SecA-mNG	*B. subtilis* BG214 SecA-mNG AmyE	*B. subtilis* BG214 SecDF-mNG	*B. subtilis* BG214 SecDF-mNG AmyE
Number of cells	188	194	161	83
Average cell length [μm]	2.7	2.8	2.6	2.7
Number of *tracks*	18,455	11,603	11,556	3566
$Population_{static}$ [%]	20.9±0.001	29.0±0.001	24.4±0.002	29.7±0.002
$Population_{mobile}$ [%]	50.8±0	50.4±0	46.9±0.001	57.0±0.001
$Population_{free}$ [%]	28.3±0.001	20.6±0.001	28.7±0.002	13.0±0.002
D_{static} [$\mu m^2 s^{-1}$]	0.04±0	0.04±0	0.03±0	0.03±0
D_{mobile} [$\mu m^2 s^{-1}$]	0.15±0	0.15±0	0.11±0	0.11±0
D_{free} [$\mu m^2 s^{-1}$]	0.70±0.001	0.70±0.001	0.60±0.002	0.60±0.002
R^2	1	1	1	1
Average dwell time τ [s]	0.297±0.004	0.301±0.005	0.306±0.004	0.288±0.005
τ_1 (2-comp.) [s]	0.24±0.002	0.23±0.002	0.25±0.001	0.23±0.003
Fraction τ_1 [%]	78.2±1.95	70.9±1.98	80.7±1.36	71.8±4.01
τ_2 (2-comp.) [s]	0.45±0.015	0.43±0.012	0.48±0.013	0.39±0.017
Fraction τ_2 [%]	21.8±1.95	29.1±1.98	19.3±1.36	28.2±4.01

Our analyses have to be viewed in the light of some caveats. Massive enzyme activity measured in the supernatant indicates the release of a large number of non-aggregated and functional molecules, however, we cannot rule out that observed fluorescence signals contain aggregates of some misfolded AmyE-mCherry molecules, which may traverse the cell wall slower than folded proteins. We have observed that fluorescence intensities in secretion zones fluctuate considerably, which could be due to the accumulation of aggregates (that do not prevent large-scale protein secretion), but could also indicate the accumulation of folded molecules at sites that represent bottlenecks for diffusion in the cell wall, i.e., a traffic jam for diffusing molecules through passages with differing diameters. It must also be kept in mind that maturation of mCherry requires several minutes, such that the fluctuating increases and decreases of fluorescence likely include maturation kinetics of mCherry. Keeping these caveats in mind, our findings of a large quantify of folded and active amylase at the transition to the stationary phase strongly support the idea of diffusion of even large, folded molecules through the cell wall, as opposed to transport via the synthesis of peptidoglycan layers from inside to outside. The existence of foci for AmyE-mCherry, at least during protein overexpression, within the cell wall of low GC firmicutes suggests that there might be cavities extending into pore-like structures within the wall that extend perpendicular to the cell circumference, allowing for the passage of AmyE (70 kDa) and larger proteins, maybe involving diffusion-based through the meshwork.

On average, the wall as a sieve-like meshwork of PG allows for the unhindered passage of up to 25 kDa proteins [16]. Our data confirm the suggestion that the wall is a barrier to the passage of AmyE, although it has also protein folding supportive characteristics [17]. However, its obstructive features for the passage of large proteins are not homogeneous, but discontinuous, likely including areas of lower meshwork density. Such structures have been hinted at by recent AFM visualization of the *B. subtilis* cell wall [19, 20]. Thus, the multilayered PG envelope of firmicutes efficiently counteracts high intracellular turgor, but appears to leave many spots for passage of proteins. We show that levels of AmyE-mCherry fluorescence change within a minute time frame, independent of fluorescence bleaching, showing a decrease as well as an increase. Assuming that bleaching-independent fluctuations in fluorescence of discrete signals reflect changes of numbers of amylase molecules within a secretion zone over time, this would be consistent with constrained diffusion of a protein along a passage through a meshwork (of a thickness of about 30–40 nm). It slows down free diffusion through a solution, which occurs in a time frame of milliseconds for nanometer distances [60]. As such, secretion zones appear to allow for faster diffusion as opposed to the typically pictured homogeneous PG meshwork. A good control for these ideas would be to track single amylase-fluorescent protein fusions. Unfortunately, in our hands, *B. subtilis* cells show excessive background fluorescence in SMT experiments using strong 561 nm laser excitation, precluding single molecule tracking experiments in the red channel at present.

On the other hand, we found efficient amylase secretion to culture supernatants only using mCherry as fluorescent reporter, but not YFP or a yellow/green fluorescent protein we have successfully employed for SMT experiments before. Thus, definite proof for our hypothesis of secretion zones acting via passive molecule diffusion awaits further proof.

A further caveat in our analyses is that AmyE-mCherry signals could only be discerned from back ground fluorescence in the red fluorescence channel during overproduction of AmyE-mCherry from a high copy number plasmid. Thus, it could be argued that the accumulation of AmyE within discrete zones in the cell wall is an artifact of protein overproduction. We suggest that this is not the case, based on the following considerations: we observed secretion zones at the transition to the stationary phase, when *B. subtilis* is known to secrete a multitude of proteins, from proteases to lipases, including several sugar-polymer-degrading enzymes [61]. It is possible, but unlikely that overexpression of one of these proteins disturbs or overwhelms the entire system, because we did not observe noticeable changes in growth of cells that could point to a stress situation. At the time of visual identification of secretion zones, the transition to stationary growth, cell wall synthesis had stopped, or was at least strongly slowing down [41]. We propose that increased synthesis of AmyE allowed us to track the path of molecules, as opposed to low production level, which does not allow tracking the passage of fewer molecules versus back ground fluorescence. Fluctuations of AmyE-mCherry fluorescence also suggest that secretion zones are not clogged up with overproduced AmyE molecules, but allow for an oscillating passage of many molecules, including bursts of release and phases of re-accumulation, through gaps in the PG structure, including the mentioned analogy to molecular traffic jams.

Heterogeneity of transcriptional expression of genes is a well-established phenomenon in bacteria [62], as well as a share of labor between cells growing in a biofilm [63]. While some cells provide energy to generate extracellular matrix in biofilms, others engage in competence development or spore formation or remain mobile and ensure the dispersing of cells from biofilms [64]. Production of antibiotics has been shown to occur in a heterogeneous manner [65], and even DNA repair enzymes can be found in only a subset of exponentially growing cells, leading to the heterogeneity of DNA damage response, in this case, based on extremely low numbers of molecules per cell [66]. Likewise, c-di-GMP signaling components of *B. subtilis* cells are found to be absent in a considerable subpopulation of cells, due to the low abundance of proteins within the network [67]. The mentioned phenomena of heterogeneity notwithstanding, we were surprised to see that the overproduction of AmyE-mCherry follows a very strong pattern of heterogeneity, with a maximum of 23% of cells showing AmyE-mCherry secretion zones during the transition phase, and 34% during the stationary phase. Heterogeneity was observed as cells entered stationary growth, but was not based on heterogeneity of SecA expression in cells. Interestingly, about 50% of cells showed intracellular accumulation of an AmyE-mCherry fusion lacking a signal peptide, indicating that only half of the population actively expresses the protein. Indeed, for plasmid-based production of proteins in *B. megaterium,* fluctuating plasmid abundance was observed, which resulted in population heterogeneity [68]. In any event, much less than 50% of cells showed AmyE-mCherry foci, suggesting that in spite of enzyme production, not all cells efficiently secrete the protein. This is backed up by time course experiments, in which we found that during the exponential phase, AmyE is being synthesized by *B. subtilis* as well as by *B. licheniformis* cells, while amylase activity is mostly detectable in culture supernatants at the transition to stationary phase. Interestingly, AmyE-mCherry lacking a signal peptide did not accumulate in a homogeneous manner within the cytosol, but showed strong membrane association. These observations suggest that synthesis of AmyE-mCherry occurs in a membrane-proximal manner. This is supported by the lack of any cytosolic fluorescence for full-length AmyE-mCherry, which is apparently secreted as it is synthesized, and only accumulates in the cytosol in the absence of a secretion-signal.

In addition to larger cavities within the cell wall observed from isolated cell walls [20], secretion zones may increase in size and number as cells turn off cell wall synthesis, in a heterogeneous manner. Zones containing larger pore sizes of the peptidoglycan meshwork may put cells at risk of bursting due to internal turgor. A culture entering stationary phase may thus be evolved to allow for a minority of cells going at risk of dying, in order to provide large amounts of extracellular enzymes for the rest of the population.

AmyE secretion zones did not show lateral mobility within the cell, in agreement with the presence of immobile structures within the cell wall that allow for molecule passage. While SecA also showed the formation of focal assemblies at the cell membrane, these showed higher lateral dynamics than AmyE secretion zones, and likewise, SecDF showed much higher dynamics at the cell membrane. These data are in agreement with our observation that SecA and SecDF co-localized with AmyE-mCherry foci in less than 20% of the cells showing red fluorescence, suggesting that SecA molecules move between SecYEG translocons (for which we have so far failed to generate functional fusions), transporting AmyE

molecules across the cell membrane. Within the (pseudo) periplasm, molecules may diffuse laterally into pores until they find a site that is wide enough to allow for their passage to the outside. This would imply that smaller molecules can move through the cell wall at more sites than larger ones, assuming a variety of different meshwork sizes within the wall.

In order to obtain a better spatiotemporal resolution of SecA dynamics, we employed single molecule tracking. As was described for *E. coli* SecA [57], we found three populations of SecA molecules having strongly different average diffusion constants. These populations can be best explained by molecules actively transporting secreted proteins at the translocon (about 20%), SecA molecules having bound cargo in search of a translocon (about 50%), and freely diffusing SecA dimers (30%). Upon overproduction of AmyE, the slow mobile population increased to about 30%, the medium mobile fractions remained constant, and the freely diffusing molecule decreased to 20%, suggesting that more SecA molecules are engaged in active transport, but that there is still a substantial pool of free SecA molecules to allow for efficient general protein secretion to continue. Interestingly, average dwell times of SecA did not change, but the population of about 20% of molecules showing a longer dwell time increased to about 30% upon AmyE overproduction, suggesting that average transport times remain constant (as well as exchange of SecA molecules between translocons), but the number of molecules dwelling at the translocon increases.

Conclusions

Overall, our data support the findings of heterogeneity within the cell wall [19, 20], showing that a subpopulation of cells secretes overproduced amylase molecules at discrete zones, allowing proteins to move through the wall, in a manner compatible with Brownian motion. This would also explain why a putative machinery allowing active or directed transport of proteins through the *Bacillus* cell wall has never been identified. In contrast to slow AmyE dynamics, SecA shows high turnover at SecYEG translocons and becomes more engaged during AmyE overproduction, but is not overwhelmed with additional AmyE secretion during overproduction. Thus, protein secretion in *Bacilli* appears to be a two-tier process, active membrane transport, and cell wall passage, involving very different time scales of protein motion.

Methods

Bacterial strains and plasmids

The B. subtilis strain used was PY79 (a derivative of Bacillus 168), and the *B. licheniformis* MC28 and MC26 strains were provided by B.R.A.I.N. Biotech AG (Zwingenberg, Germany) (Additional file 7: Table S1). MC26 was used as a control strain. Bacillus strains were grown at 37 °C overnight on nutrient agar plates using commercial nutrient broth LB solidified by the addition of 1% (w/v) agar. Overnight cultures in tubes were inoculated from a fresh agar plate and incubated overnight at 37 °C and 200 rpm. Day cultures in 100 ml shake flasks with 10 ml media were inoculated to a cell density of OD_{600} of 0.1 in LB from the overnight cultures and then incubated at 37 °C and 200 rpm.

For the visualization of the secreted protein α-amylase AmyE, the mCherry gene was cloned via Gibson Assembly in frame to *amyE* in plasmid pM11K_amyEBs provided by the B.R.A.I.N. AG (Zwingenberg, Germany). This plasmid provides the HpaII-promoter [35] to drive the expression of *amyE* and a high copy number pUB110-like replicon. The C-terminal fusion includes an 8-amino acid linker (KLGSGSGS). This non-integrating plasmid carries a kanamycin resistance for selection with 25 μg/ml kanamycin in *Bacillus*. The plasmid is available, upon reasonable request, after signing a Material Transfer Agreement.

The fusion of SecA and SecDF to mNeonGreen was cloned into the pSG1164 vector containing a sequence encoding monomeric NeonGreen [69] and a flexible 14-amino acid linker (GPGLSGLGGGGGSL). For this purpose, at least 500 bp of the 3`end of the desired gene (excluding the stop codon) was amplified by polymerase-chain reaction (PCR) using *B. subtilis* PY79 gDNA as template, oligonucleotides (Additional file 8: Table S2), Phusion DNA polymerase, and deoxynucleotide solution (both from New England Biolabs, NEB). The resulting PCR product was integrated into the plasmid via the Gibson Assembly cloning system (New England Biolabs-NEB). The pSG1164 plasmid integrates at the native locus of the corresponding gene by a single-crossover event, creating a C-terminal fusion [70].

Structured illumination microscopy (SIM)

Samples taken typically at the transitional growth phase were mounted on ultrapure-agarose slides dissolved in LB (1%) for immobilization of cells prior to image acquisition. For localization experiments, image Z-stacks (~100 nm steps) were acquired using bright-field (BF) image acquisition (transmitted light) or illumination microscopy (SIM) with a ZEISS ELYRA PS.1 setup (Andor EMCCD camera, 80 nm 1.15 size; 3× rotations and 5× phases per z-slice; with an excitation wavelength 561 nm at 15% intensity or 488 nm at 10% intensity; ZEISS alpha Plan-Apochromat 100x/NA 1.46 Oil DIC M27 objective). SIM reconstructions were processed using ZEN-Black software by ZEISS. ImageJ2/FIJI version 1.52p was used for visualization and image

processing [45, 71, 72]. No automatic features like autofocus or drift correction were used. For time-lapse imaging, the acquisition time was set to 1 min. SIM reconstructions were manually cropped in axial and lateral dimensions, depending on the plausibility of cellular positions, using the "Duplicate"-function. Signal not connected to the cells was considered to be a background and was therefore in most cases eliminated. For single-particle tracking, spots were identified with the LoG Detector of TrackMate v6.0.1 [73], implemented in Fiji 1.53 q, an estimated diameter of 0.5 µm and sub-pixel localization activated. Spots were merged into tracks via the Simple LAP Tracker of TrackMate, with a maximum linking distance of 500 nm, one frame gap allowed, and a gap closing max distance of 800 nm.

Generation of protoplasts

Bacillus cells in the transitional growth phase were treated according to the protocol of Chang and Cohen [74] to obtain protoplasts. During the process, kanamycin was added to the media to maintain the AmyE-mCherry plasmid. Imaging of the cells before and after the incubation with lysozyme was performed by SIM microscopy.

Starch-BODIPY-FL staining

For this experiment, the streptococcal SpeB protocol for *Streptococcus* by Rosch and Caparon [75] was adapted to *Bacillus*. Strains were cultivated in LB medium at 37 °C and 200 rpm with the addition of 25 µg/ml kanamycin until the transitional growth phase. The culture was pelleted at 4000 rpm for 2 min and resuspended in fresh LB containing 1% of the "DQ starch substrate stock solution" (1 mg/ml, EnzChek Ultra Amylase Assay Kit, Invitrogen Detection Technologies, Carlsbad, CA, USA). Cells were mounted on ultrapure-agarose slides dissolved in LB (1%) for immobilization of cells and incubated for 30 min at 37 °C.

Imaging was performed via epi-fluorescence microscopy, using a Nikon Eclipse Ti-E, Nikon Instruments Inc with a CFI Apochromat objective (TIRF 100× oil, NA 1.49) and an EMCCD camera (ImagEM X2 EM-CCD, Hamamatsu Photonics KK). The samples were illuminated with Nikon C-HGFIE Intensilight (Precentered Fiber Illuminator) and the YFP-channel filter cube ET 500/20, T 515 LP, ET 535/30. Images were processed with MetaMorph (version 2.76), and ImageJ [45].

Phadebas test for amylase activity

For the quantification of α-amylase activity in the culture supernatant, the Phadebas Amylase Test (Phadebas AB, Uppsala, Sweden) was used. One Phadebas tablet was dissolved in a 20-ml buffer solution (0.1 M acetic acid, 0.1 M potassium acetate, 5 mM calcium chloride, pH 5). Overnight cultures of *Bacillus* were centrifuged at 14,000 rpm for 2 min in a microfuge, 20 µl supernatant was mixed with 180 µl substrate solution and incubated for 10 min at 37 °C and 1000 rpm in a thermomixer (Eppendorf Thermomixer comfort). The reaction was stopped by the addition of 60 µl 1 M sodium hydroxide. The reaction tubes were centrifuged and the absorption of 100 µl of the supernatant was measured at 620 nm via a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). Activities were corrected for dilution and normalized to the cell density (OD_{600}) of the culture.

Immunoblotting

Thirty milliliters of a culture in the transitional growth phase was pelleted and resuspended in 3 ml buffer (100 mM NaCl, 50 mM EDTA, 5 mg/ml Lysozyme). Cells were incubated at 37 °C until lysis, which was observed visually. Samples were incubated at 95 °C with sodium dodecyl sulfate (SDS) loading buffer for 5 min. Proteins were separated by polyacrylamide®gel electrophoresis (PAGE) on a 10% mini-PROTEAN TGX precast gel (Bio-Rad, CA, USA) at 140 V and 300 mA for 1 h. Gels were transferred onto cellulose membranes using transfer-buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% EtOH, pH 9.8) at 25 V, 500 mA for 1 h. Membranes were blocked for 1 h using blocking-buffer (PBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) and incubated with diluted (1:10,000) rabbit polyclonal antiserum (Sigma-Aldrich) against mCherry overnight. Subsequently, membranes were washed three times with PBS for 5 min each and incubated with goat-anti-Rabbit-IgG, peroxidase-conjugated (1:10.000) for 1 h (Sigma-Aldrich). Before detection of proteins, the membranes were washed three times as described®before. Detection was performed using an Immobilon Forte Western membrane substrate (Merck KGA, Darmstadt, Germany) according to the manfacturer's protocol. Protein marker Thermo Scientific™ PageRuler Prestained Protein Ladder was used.

Single molecule tracking

Individual molecules were tracked using a custom-made slim-field setup on an inverted fluorescence microscope (Nikon Eclipse Ti-E, Nikon Instruments Inc.). An EMCCD camera (ImagEM X2 EM-CCD, Hamamatsu Photonics KK) was used to ensure high-resolution detection of the emission signal, resulting in a calculated resolution of the position of the molecule down to 20 nm. The central part of a 514-nm laser diode (max power 100 mW, TOPTICA Beam Smart) was used with up to 20% of the intensity (about 160 W cm^{-2} in the image plane) to excite samples, fused to mNeonGreen (using laser filter set BrightLine 500/24, dichroic mirror 520 and BrightLine 542/27),

by focusing the beam onto the back focal plane of the objective. A CFI Apochromat objective (TIRF 100×Oil, NA 1.49) was used in the setup. For the analysis, a video of 3000 frames at 20 ms was recorded, of which the last 1000 frames were used for the analysis. Software Oufti [76] was used to set the necessary cell meshes. Utrack [77] was employed for the automatic determination of molecule trajectories. Data analysis was carried out using the software SMTracker 2.0 [47, 48].

Abbreviations

AFM	Atomic force microscopy
ATP	Adenosine triphosphate
BF	Brightfield
bp	Base pair
c-di-GMP	Cyclic dimeric guanosine monophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMCCD	Electron-multiplying-charge-coupled device
GlnNAc	N-acetylglucosamine
LB	Lysogeny broth
LTA	Lipoteichoic acid
mNG	MNeonGreen
MurNAc	N-acetylmuramic acid
NAG	N-acetyl-glucosamine
OD	Optical density
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase-chain reaction
PG	Peptidoglycan
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Sec pathway	General secretory pathway
SIM	Structured illumination microscopy
SMT	Single molecule tracking
sp	Signal peptide
SQD	Squared displacement analyses
wt	Wild type
WTA	Wall teichoic acid
YFP	Yellow fluorescent protein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12915-023-01684-1.

Additional file 1: Fig. S1. Western blot showing the presence of SecDF-mNeonGreen and SecA-mNeonGreen fusion proteins.

Additional file 2: Fig. S2. Amylase activity in the medium.

Additional file 3: Fig. S3. SIM imaging showing that SecDF and SecA mNeonGreen localization is not affected by AmyE overproduction in *B. subtilis*.

Additional file 4: Fig. S4. Analysis of fluctuating AmyE-mCherry foci in two *B. licheniformis* cells.

Additional file 5: Fig. S5. SecA-mNeonGreen and SecDF-mNeonGreen foci do not show intensity fluctuations over time.

Additional file 6: Fig. S6. Localization of AmyE in *B. subtilis* and *B. licheniformis* cells determined by its activity.

Additional file 7: Table S1. Strains used in this study.

Additional file 8: Table S2. Primers used in this study.

Additional file 9: Movie S1. Showing real time motion of single SecA-mNeonGreen molecules within live *B. subtilis* cells. Cells can be discerned by their weak background fluorescence, shown are 6 cells, 3 of which grow in a chain. 20 ms stream acquisition, movie speed 50 frames/s.

Acknowledgements
We gratefully acknowledge help with SIM microscopy by Sven Holtrup and Maximilian Greger from SYNMIKRO, Marburg University. This work is dedicated to Richard Losick in celebration of his 80th birthday.

Authors' contributions
MS performed all experiments, except those for Fig. 9; FK and SF performed all experiments shown in Fig. 9; MS, KL, and PLG wrote the manuscript; KL and PLG conceived of the study and obtained funding for the work. KL and PLG supervised experiments. All authors read and approved the final manuscript.

Funding
This work was supported by the Bundesministerium für Bildung und Forschung (BMBF, Program NatLifE).

Availability of data and materials
All data are shown in the manuscript. Raw single molecule data are provided under FAIR standards at http://dx.doi.org/10.17192/fdr/111.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 14 December 2022 Accepted: 16 August 2023

References

1. Driessen AJM, Nouwen N. Protein translocation across the bacterial cytoplasmic membrane. Ann Rev Biochem. 2008;77(1):643–67.
2. Tsirigotaki A, De Geyter J, Sostaric N, Economou A, Karamanou S. Protein export through the bacterial Sec pathway. Nat Rev Microbiol. 2017;15(1):21–36.
3. Holland IB. Translocation of bacterial proteins–an overview. Biochem Biophys Acta. 2004;1694(1–3):5–16.
4. Jeon E-J, Jung J-H, Seo D-H, Jung D-H, Holden JF, Park C-S. Bioinformatic and biochemical analysis of a novel maltose-forming α-amylase of the GH57 family in the hyperthermophilic archaeon Thermococcus sp. CL1. Enzyme Microb Technol. 2014;60:9–15.
5. Frain KM, van Dijl JM, Robinson C. The twin-arginine pathway for protein secretion. EcoSal Plus. 2019;8(2). https://doi.org/10.1128/ecosalplus.ESP-0040-2018.
6. Harwood CR, Cranenburgh R. Bacillus protein secretion: an unfolding story. Trends Microbiol. 2008;16(2):73–9.
7. Tjalsma H, Bolhuis A, Jongbloed JD, Bron S, van Dijl JM. Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. Microbiol Mol Biol Rev. 2000;64(3):515–47.
8. van den Berg B, Clemons WM, Collinson I, Modis Y, Hartmann E, Harrison SC, Rapoport TA. X-ray structure of a protein-conducting channel. Nature. 2004;427(6969):36–44.
9. Gupta R, Toptygin D, Kaiser CM. The SecA motor generates mechanical force during protein translocation. Nat Commun. 2020;11(1):3802.
10. Kang Z, Yang S, Du G, Chen J. Molecular engineering of secretory machinery components for high-level secretion of proteins in Bacillus species. J Ind Microbiol Biotechnol. 2014;41(11):1599–607.

11. Bolhuis A, Broekhuizen CP, Sorokin A, van Roosmalen ML, Venema G, Bron S, Quax WJ, van Dijl JM. SecDF of *Bacillus subtilis*, a molecular Siamese twin required for the efficient secretion of proteins. J Biol Chem. 1998;273(33):21217–24.
12. Tsukazaki T. Structure-based working model of SecDF, a proton-driven bacterial protein translocation factor. FEMS Microbiol Lett. 2018;365(12):fny112.
13. van Roosmalen ML, Geukens N, Jongbloed JD, Tjalsma H, Dubois JY, Bron S, van Dijl JM, Anne J. Type I signal peptidases of Gram-positive bacteria. Biochim Biophys Acta. 2004;1694(1–3):279–97.
14. Tjalsma H, Noback MA, Bron S, Venema G, Yamane K, van Dijl JM. *Bacillus subtilis* contains four closely related type I signal peptidases with overlapping substrate specificities. Constitutive and temporally controlled expression of different sip genes. J Biol Chem. 1997;272(41):25983–92.
15. Vitikainen M, Pummi T, Airaksinen U, Wahlström E, Wu H, Sarvas M, Kontinen VP. Quantitation of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of α-amylase in *Bacillus subtilis*. J Bacteriol. 2001;183(6):1881–90.
16. Demchick P, Koch AL. The permeability of the wall fabric of *Escherichia coli* and *Bacillus subtilis*. J Bacteriol. 1996;178(3):768–73.
17. Leloup L, Haddaoui EA, Chambert R, Petit-Glatron MF. Characterization of the rate-limiting step of the secretion of *Bacillus subtilis* alpha-amylase overproduced during the exponential phase of growth. Microbiology (Reading). 1997;143(Pt 10):3295–303.
18. Haddaoui E, Chambert R, Petit-Glatron MF, Lindy O, Sarvas M. *Bacillus subtilis* alpha-amylase: the rate limiting step of secretion is growth phase-independent. FEMS Microbiol Lett. 1999;173(1):127–31.
19. Turner RD, Mesnage S, Hobbs JK, Foster SJ. Molecular imaging of glycan chains couples cell-wall polysaccharide architecture to bacterial cell morphology. Nat Commun. 2018;9(1):1263.
20. Pasquina-Lemonche L, Burns J, Turner RD, Kumar S, Tank R, Mullin N, Wilson JS, Chakrabarti B, Bullough PA, Foster SJ, et al. The architecture of the Gram-positive bacterial cell wall. Nature. 2020;582(7811):294–7.
21. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harbor Persp Biol. 2010;2(5):a000414.
22. Graham LL, Beveridge TJ. Structural differentiation of the *Bacillus subtilis* 168 cell wall. J Bacteriol. 1994;176(5):1413–21.
23. Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev. 2003;67(4):686–723.
24. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev. 1972;36(4):407–77.
25. Atrih A, Bacher G, Allmaier GN, Williamson MP, Foster SJ. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. J Bacteriol. 1999;181(13):3956–66.
26. Chung BC, Zhao J, Gillespie RA, Kwon D-Y, Guan Z, Hong J, Zhou P, Lee S-Y. Crystal structure of MraY, an essential membrane enzyme for bacterial cell wall synthesis. Science. 2013;341(6149):1012–6.
27. Men H, Park P, Ge M, Waller S. Substrate synthesis and activity assay for MurG. J Am Chem Soc. 1998;120(10):2484–5.
28. Sham L-T, Butler EK, Lebar MD, Kahne D, Bernhardt TG, Ruiz N. Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. Science. 2014;345(6193):220–2.
29. Lebar MD, Lupoli TJ, Tsukamoto H, May JM, Walker S, Kahne D. Forming cross-linked peptidoglycan from synthetic gram-negative Lipid II. JAm Chem Soc. 2013;135(12):4632–5.
30. Graumann PL. Cytoskeletal elements in bacteria. Annu Rev Microbiol. 2007;61:589–618.
31. Perego M, Glaser P, Minutello A, Strauch MA, Leopold K, Fischer W. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in Bacillus subtilis. Identification of genes and regulation. J Biol Chem. 1995;270(26):15598–606.
32. Hyyryläinen H-L, Vitikainen M, Thwaite J, Wu H, Sarvas M, Harwood CR, Kontinen VP, Stephenson K. d-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of Bacillus subtilis. J Biol Chem. 2000;275(35):26696–703.
33. Campo N, Tjalsma H, Buist G, Stepniak D, Meijer M, Veenhuis M, Westermann M, Müller JP, Bron S, Kok J, et al. Subcellular sites for bacterial protein export. Mol Microbiol. 2004;53(6):1583–99.
34. Dinh T, Bernhardt TG. Using superfolder green fluorescent protein for periplasmic protein localization studies. J Bacteriol. 2011;193(18):4984–7.
35. Zyprian E, Matzura H. Characterization of signals promoting gene expression on the *Staphylococcus aureus* plasmid pUB110 and development of a gram-positive expression vector system. DNA. 1986;5(3):219–25.
36. Herbort M, Klein M, Manting EH, Driessen AJ, Freudl R. Temporal expression of the Bacillus subtilis secA gene, encoding a central component of the preprotein translocase. J Bacteriol. 1999;181(2):493–500.
37. Burghard-Schrod M, Kilb A, Kramer K, Graumann PL. Single molecule dynamics of DNA receptor ComEA, membrane permease ComEC and taken up DNA in competent *Bacillus subtilis* cells. J Bacteriol. 2022:jb0057221.
38. Workman SD, Strynadka NCJ. A slippery scaffold: synthesis and recycling of the bacterial cell wall carrier lipid. J Mol Biol. 2020;432(18):4964–82.
39. Scheffers DJ, Pinho MG. Bacterial cell wall synthesis: new insights from localization studies. Microbiol Mol Biol Rev. 2005;69(4):585–607.
40. Tank RK, Lund VA, Kumar S, Turner RD, Lafage L, Pasquina Lemonche L, Bullough PA, Cadby A, Foster SJ, Hobbs JK. Correlative super-resolution optical and atomic force microscopy reveals relationships between bacterial cell wall architecture and synthesis in *Bacillus subtilis*. ACS Nano. 2021;15(10):16011–8.
41. Angeles DM, Scheffers DJ. The cell wall of *Bacillus subtilis*. Curr Issues Mol Biol. 2021;41:539–96.
42. Dempwolff F, Schmidt FK, Hervas AB, Stroh A, Rosch TC, Riese CN, Dersch S, Heimerl T, Lucena D, Hulsbusch N, et al. Super resolution fluorescence microscopy and tracking of bacterial flotillin (Reggie) paralogs provide evidence for defined-sized protein microdomains within the bacterial membrane but absence of clusters containing detergent-resistant proteins. PLoS Genet. 2016;12(6):e1006116.
43. Neef J, van Dijl JM, Buist G. Recombinant protein secretion by *Bacillus subtilis* and *Lactococcus lactis*: pathways, applications, and innovation potential. Essays Biochem. 2021;65(2):187–95.
44. Kemper MA, Urrutia MM, Beveridge TJ, Koch AL, Doyle RJ. Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. J Bacteriol. 1993;175(17):5690–6.
45. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012;9(7):676–82.
46. Strach M, Koch F, Fiedler S, Liebeton K, Graumann PL. Raw data for single molecule tracking using SecA-mNeonGreen and SecDF-mNeonGreen. data_UMR. 2022. https://doi.org/10.17192/fdr/17111.
47. Rotter DAO, Heger C, Oviedo-Bocanegra LM, Graumann PL. Transcription-dependent confined diffusion of enzymes within subcellular spaces of the bacterial cytoplasm. BMC Biol. 2021;19(1):183.
48. Oviedo-Bocanegra LM, Hinrichs R, Rotter DAO, Dersch S, Graumann PL. Single molecule/particle tracking analysis program SMTracker 2.0 reveals different dynamics of proteins within the RNA degradosome complex in *Bacillus subtilis*. Nucleic Acids Res. 2021;49(19):e112.
49. Driessen AJ. SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. Biochemistry. 1993;32(48):13190–7.
50. Wowor AJ, Yan Y, Auclair SM, Yu D, Zhang J, May ER, Gross ML, Kendall DA, Cole JL. Analysis of SecA dimerization in solution. Biochemistry. 2014;53(19):3248–60.
51. Schenk K, Hervas AB, Rosch TC, Eisemann M, Schmitt BA, Dahlke S, Kleine-Borgmann L, Murray SM, Graumann PL. Rapid turnover of DnaA at replication origin regions contributes to initiation control of DNA replication. PLoS Genet. 2017;13(2):e1006561.
52. Schibany S, Hinrichs R, Hernandez-Tamayo R, Graumann PL. The major chromosome condensation factors Smc, HBsu, and Gyrase in *Bacillus subtilis* operate via strikingly different patterns of motion. mSphere. 2020;5(5):e00817-20.
53. Kumar M, Mommer MS, Sourjik V. Mobility of cytoplasmic, membrane, and DNA-binding proteins in *Escherichia coli*. Biophys J. 2010;98(4):552–9.
54. Sanamrad A, Persson F, Lundius EG, Fange D, Gynna AH, Elf J. Single-particle tracking reveals that free ribosomal subunits are not excluded from the *Escherichia coli* nucleoid. Proc Natl Acad Sci USA. 2014;111(31):11413–8.
55. Mayer B, Schwan M, Oviedo-Bocanegra LM, Bange G, Thormann KM, Graumann PL. Dynamics of bacterial signal recognition particle at a single molecule level. Front Microbiol. 2021;in press.

56. Bakshi S, Siryaporn A, Goulian M, Weisshaar JC. Superresolution imaging of ribosomes and RNA polymerase in live *Escherichia coli* cells. Mol Microbiol. 2012;85(1):21–38.
57. Seinen AB, Spakman D, van Oijen AM, Driessen AJM. Cellular dynamics of the SecA ATPase at the single molecule level. Sci Rep. 2021;11(1):1433.
58. Lewis PJ, Thaker SD, Errington J. Compartmentalization of transcription and translation in *Bacillus subtilis*. EMBO J. 2000;19(4):710–8.
59. Sattler L, Graumann PL. Real-time messenger RNA dynamics in *Bacillus subtilis*. Front Microbiol. 2021;12:760857.
60. Trovato F, Tozzini V. Diffusion within the cytoplasm: a mesoscale model of interacting macromolecules. Biophys J. 2014;107(11):2579–91.
61. Nijland R, Kuipers OP. Optimization of protein secretion by *Bacillus subtilis*. Recent Pat Biotechnol. 2008;2(2):79–87.
62. Graumann PL. Different genetic programmes within identical bacteria under identical conditions: the phenomenon of bistability greatly modifies our view on bacterial populations. Mol Microbiol. 2006;61(3):560–3.
63. Veening JW, Smits WK, Kuipers OP. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol. 2008;62:193–210.
64. Lopez D, Vlamakis H, Kolter R. Biofilms. Cold Spring Harb Perspect Biol. 2010;2(7):a000398.
65. Dewachter L, Fauvart M, Michiels J. Bacterial heterogeneity and antibiotic survival: understanding and combatting persistence and heteroresistance. Mol Cell. 2019;76(2):255–67.
66. Uphoff S, Lord ND, Okumus B, Potvin-Trottier L, Sherratt DJ, Paulsson J. Stochastic activation of a DNA damage response causes cell-to-cell mutation rate variation. Science. 2016;351(6277):1094–7.
67. Kunz S, Tribensky A, Steinchen W, Oviedo-Bocanegra L, Bedrunka P, Graumann PL. Cyclic di-GMP signaling in *Bacillus subtilis* is governed by direct interactions of diguanylate cyclases and cognate receptors. mBio. 2020;11(2):e03122-19.
68. Munch KM, Muller J, Wienecke S, Bergmann S, Heyber S, Biedendieck R, Munch R, Jahn D. Polar fixation of plasmids during recombinant protein production in *Bacillus megaterium* results in population heterogeneity. Appl Environ Microbiol. 2015;81(17):5976–86.
69. Shaner NC. Fluorescent proteins for quantitative microscopy: important properties and practical evaluation. Methods Cell Biol. 2014;123:95–111.
70. Lewis PJ, Marston AL. GFP vectors for controlled expression and dual labelling of protein fusions in *Bacillus subtilis*. Gene. 1999;227(1):101–10.
71. Linkert M, Rueden CT, Allan C, Burel JM, Moore W, Patterson A, Loranger B, Moore J, Neves C, Macdonald D, et al. Metadata matters: access to image data in the real world. J Cell Biol. 2010;189(5):777–82.
72. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, Eliceiri KW. Image J2: ImageJ for the next generation of scientific image data. BMC Bioinformatics. 2017;18(1):529.
73. Tinevez JY, Perry N, Schindelin J, Hoopes GM, Reynolds GD, Laplantine E, Bednarek SY, Shorte SL, Eliceiri KW. TrackMate: an open and extensible platform for single-particle tracking. Methods. 2017;115:80–90.
74. Chang S, Cohen SN. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol Gen Genet. 1979;168(1):111–5.
75. Rosch J, Caparon M. A microdomain for protein secretion in Gram-positive bacteria. Science. 2004;304(5676):1513–5.
76. Paintdakhi A, Parry B, Campos M, Irnov I, Elf J, Surovtsev I, Jacobs-Wagner C. Oufti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. Mol Microbiol. 2016;99(4):767–77.
77. Jaqaman K, Loerke D, Mettlen M, Kuwata H, Grinstein S, Schmid SL, Danuser G. Robust single-particle tracking in live-cell time-lapse sequences. Nat Methods. 2008;5(8):695–702.

Publisher's Note

Supplementary Material

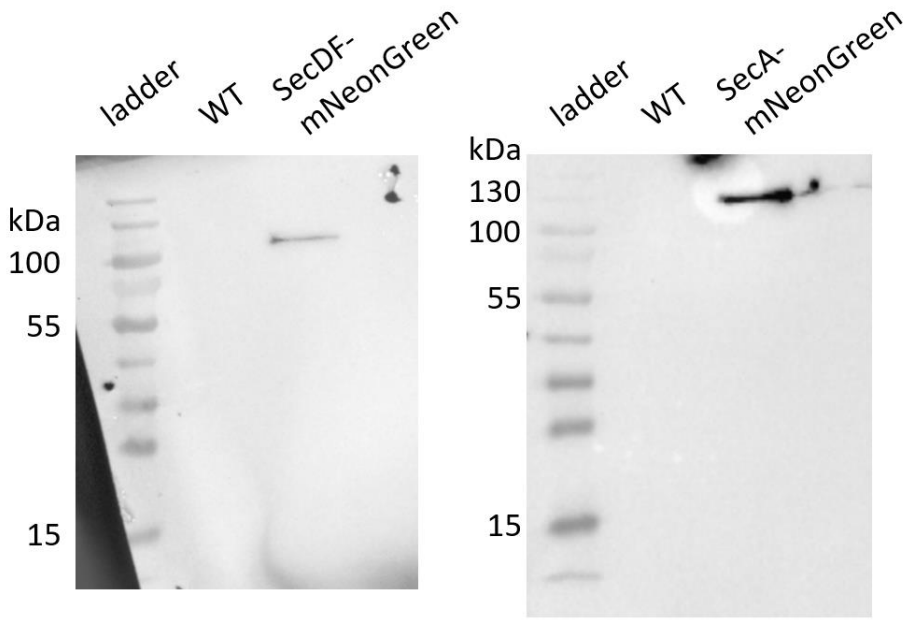


Fig. S1 Western blot showing the presence of SecDF-mNeonGreen and SecA-mNeonGreen fusion proteins (calculated Mw: 100 kDa and 120 kDa respectively) in cell lysates of *B. subtilis* and after 16 h of growth using polyclonal antibodies against mNeonGreen.

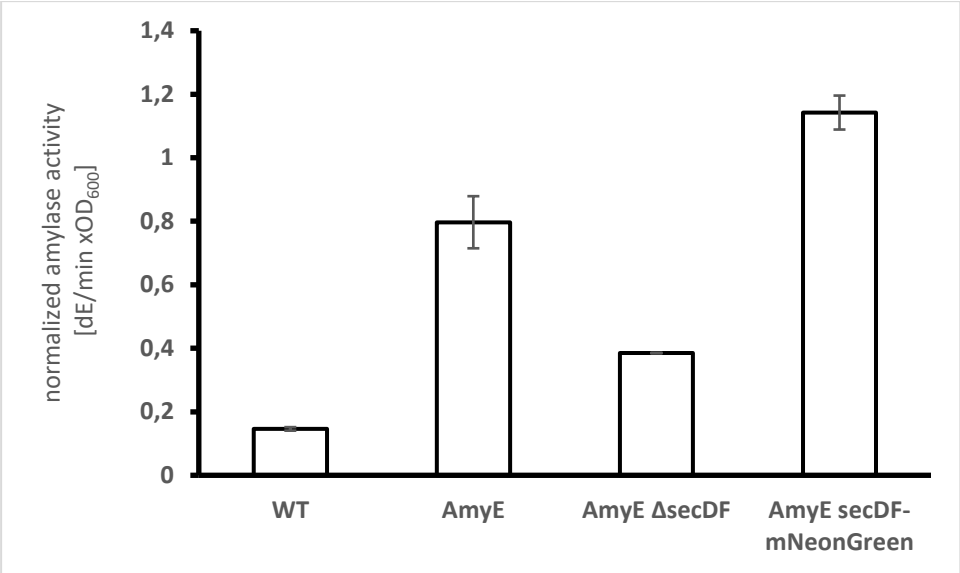


Fig. S2 Amylase activity in the medium analyzed via Phadebas test shows a reduction of secreted AmyE in the *B. subtilis* Δ*secDF* strain. The fusion of mNeonGreen to SecDF has no negative impact on the AmyE secretion.

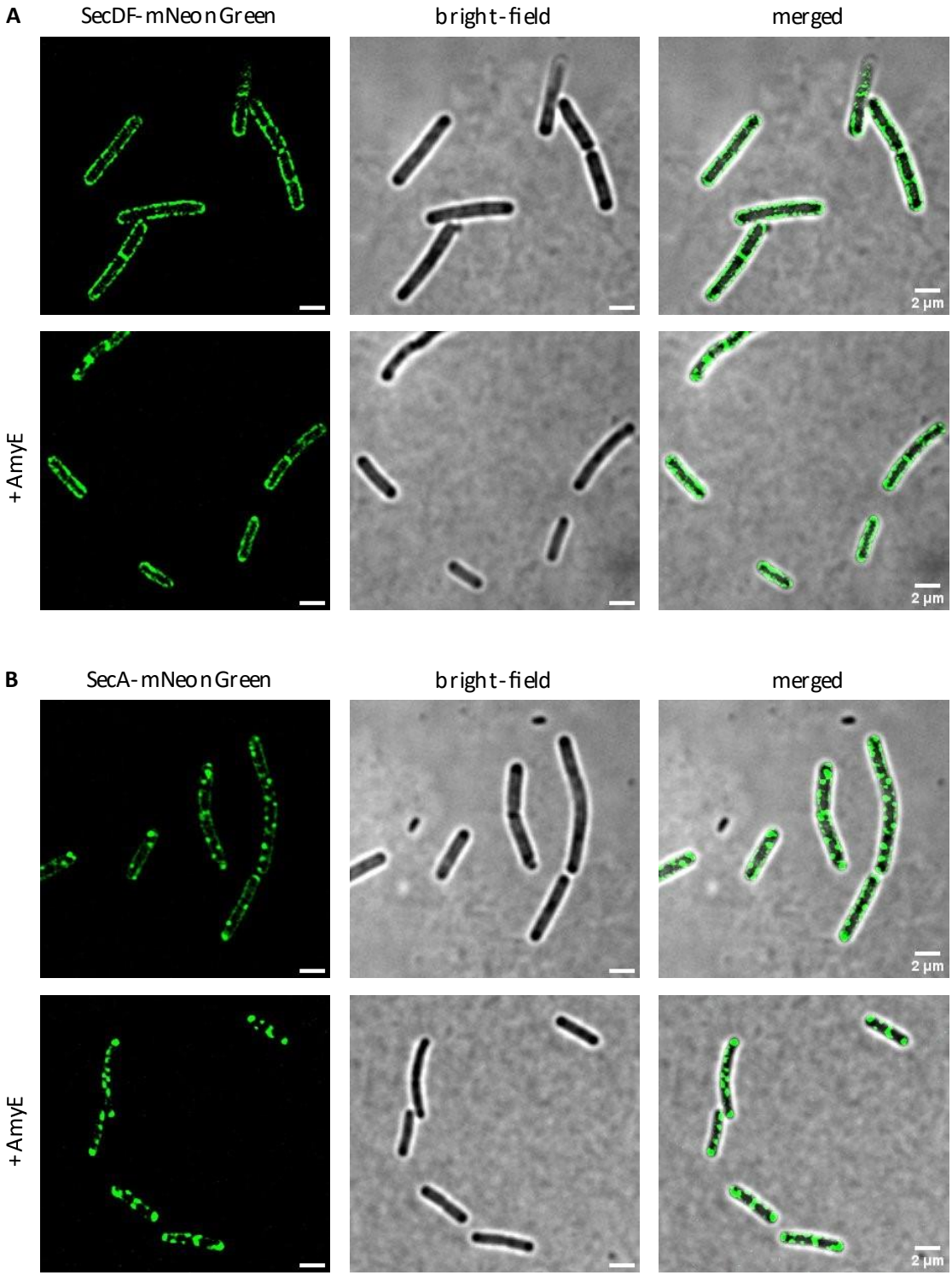


Fig. S3 SecDF and SecA mNeonGreen localization is not affected by AmyE overproduction in *B. subtilis*. (A) Localization of SecDF-mNeonGreen with and without additional plasmid-based overexpression of AmyE (B) Localization of SecA-mNeonGreen with and without additional plasmid-based overexpression of AmyE.

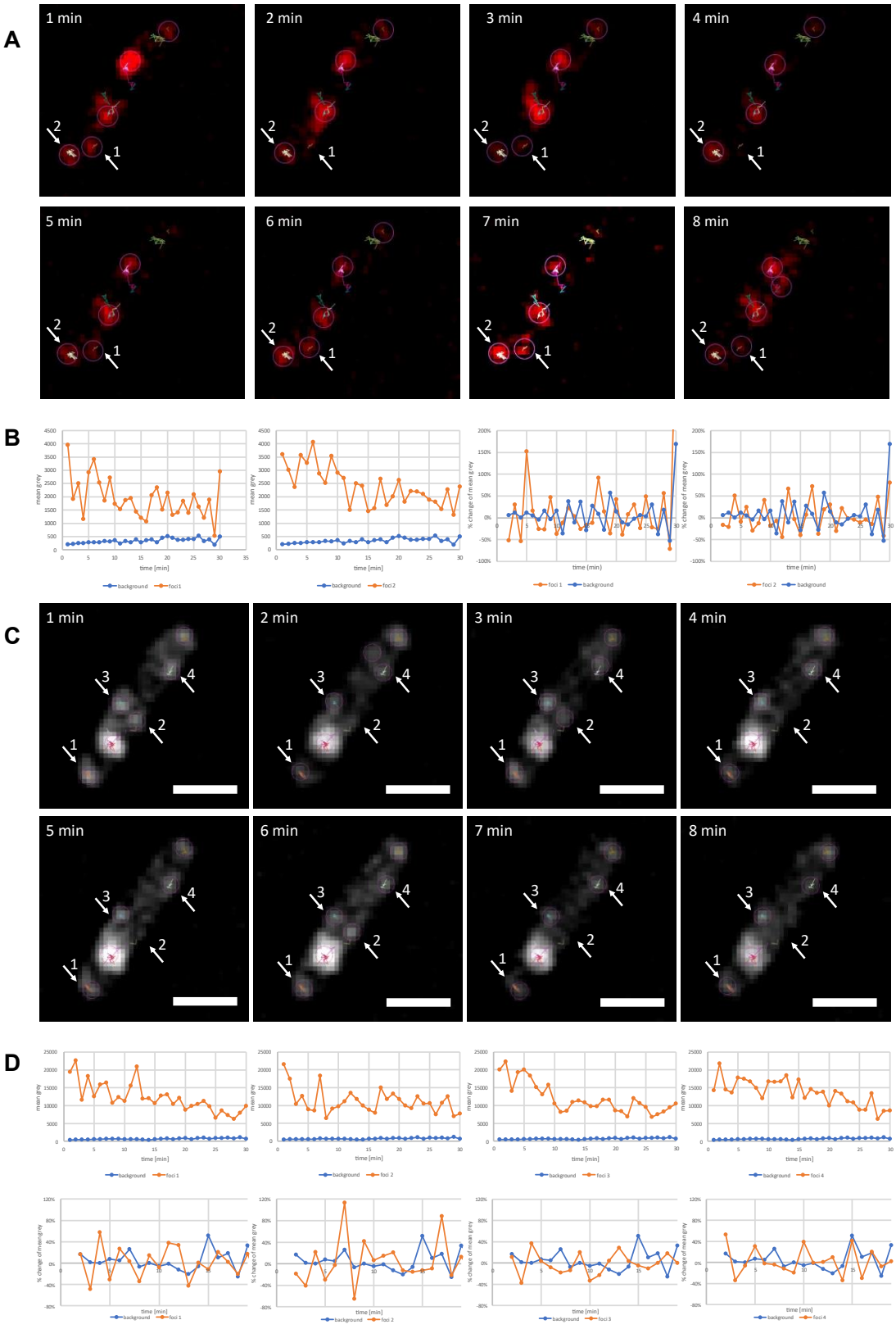


Fig. S4 Analysis of fluctuating AmyE-mCherry foci in two *B. licheniformis* cells. (**A, C**) SIM time lapse images showing cells with AmyE-mCherry foci fluctuating in fluorescence intensity over time. (**B, D**) Fluorescence intensity analysis of the foci confirming the fluctuation against the background and calculation of the change in fluorescence intensity. Scale bars 2 µm.

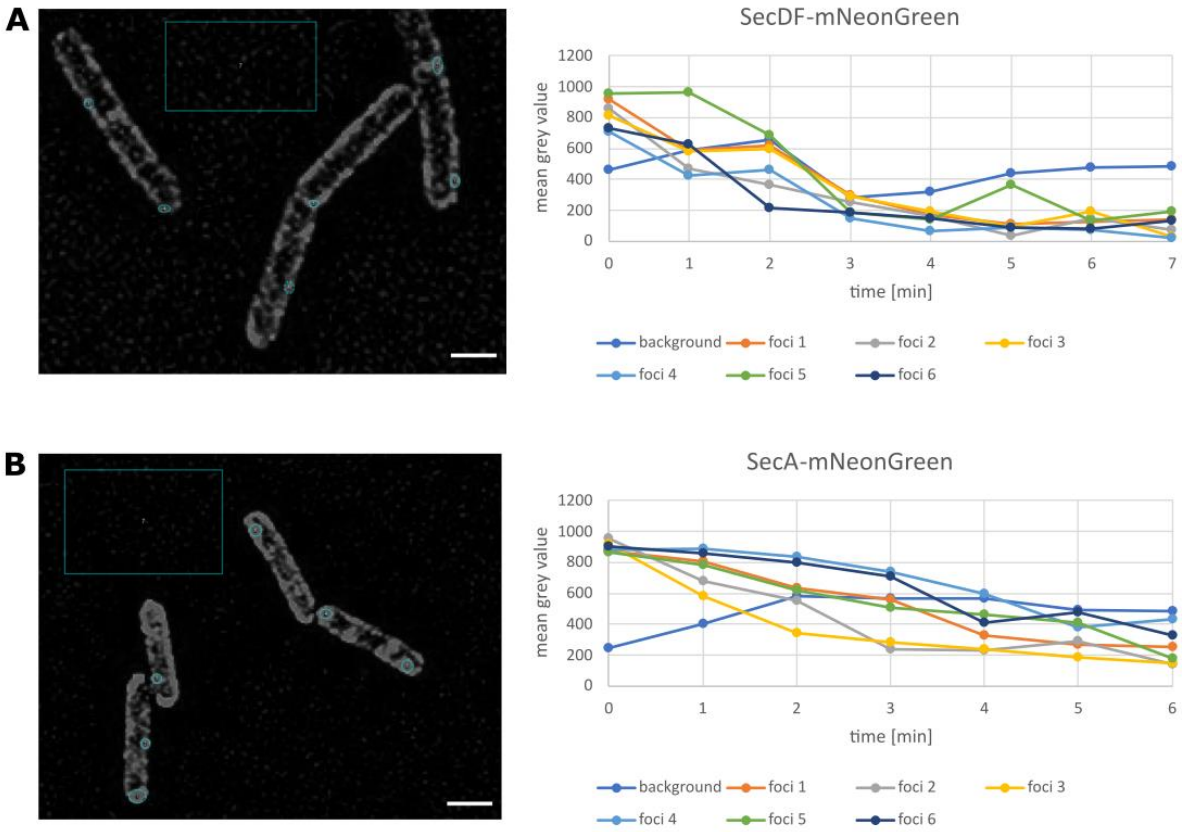


Fig. S5 SecA-mNeonGreen and SecDF-mNeonGreen foci showing no intensity fluctuations over time. SIM time lapse images of SecDF-mNeonGreen in (**A**) and SecA-mNeonGreen (**B**) in *B. subtilis,* and fluorescence intensity analysis over time of 6 picked foci and the background. Scale bars 2 µm.

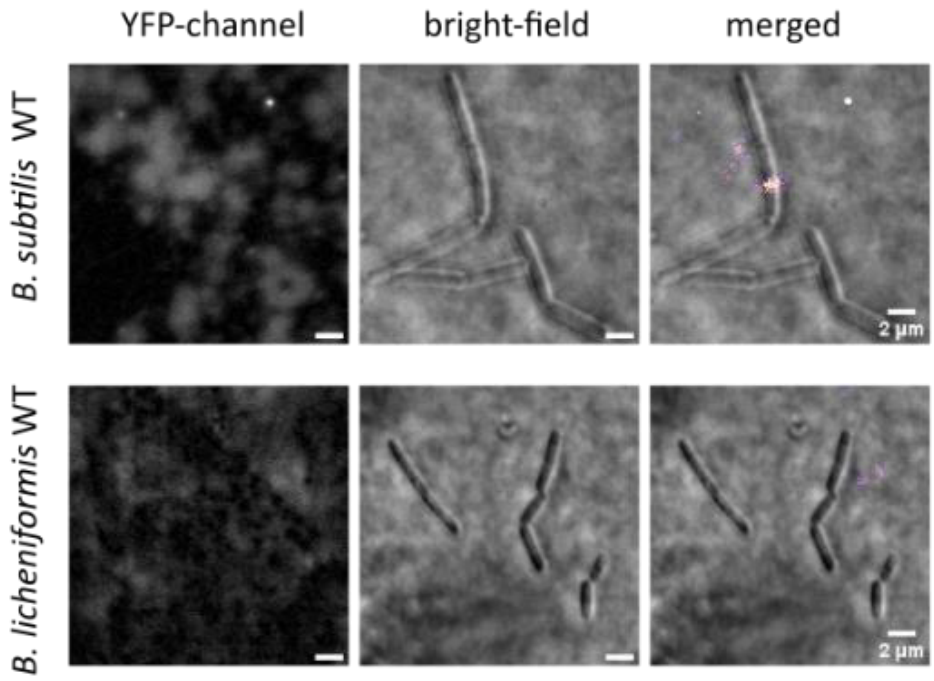


Fig. S6 Localization of AmyE in *B. subtilis* and *B. licheniformis* cells determined by its activity. Cells without plasmid-based expression of AmyE referred to as WT (wild type), showing virtually no fluorescence signal produced by hydrolysis of starch-BODIPY-FL.

2.2 Manuscript I

This manuscript is part of an invention disclosure. The results are currently investigated to assess their patentability. Therefore, premature efforts to publish the contents of the manuscript have been suspended.

The Influence of cell wall hydrolases on amylase secretion in *Bacillus subtilis*

Manuel Strach[1], Klaus Liebeton[2], Peter L. Graumann[1]*

[1]Centre for Synthetic Microbiology (SYNMIKRO) and Fachbereich Chemie, Philipps-Universität Marburg, 35032 Marburg, Germany

[2]BRAIN Biotech AG, Darmstädter Str. 34-36, 64673 Zwingenberg, Germany

Abstract

Bacillus subtilis is a Gram-positive model organism that is also widely used for industrial protein production, due to its high capacity of secretion. Although many aspects of the secretion process have been thoroughly investigated, the passage of the cell wall and the proteins that affect it have received little attention. Therefore, we have studied the influence of some of the most prominent cell wall hydrolases on protein secretion in the *Bacillus subtilis* PY79 strain. We constructed deletion strains and inducible overexpression systems of the major autolysins LytC, LytD, LytF, and of the D-alanyl-D-alanine carboxypeptidase PBP5 (*dacA*). Effects on secretion were assessed by following the secretion of the amylase AmyE, expressed from a high copy number plasmid. Examination of the deletion strains showed a significant decrease in AmyE secretion in Δ*lytC* and Δ*lytF* mutants. Upon increasing the expression level of LytC, LytF, and PBP5 by expression of a second gene copy from the endogenous *amyE* locus, plasmid-derived AmyE secretion was significantly increased and in the case of PBP5 even doubled. Our findings support the hypothesis that especially the secretion of large proteins like AmyE in *B. subtilis* is limited by the exclusion size of the murein meshwork, which can be overcome by the expression level of cell wall-modulating enzymes.

Introduction

Members of the genus *Bacillus* are famous for their use in the industrial production of exoenzymes, and are widely used in biotechnological applications (Harwood, 1992; Schallmey *et al*., 2004; Danilova & Sharipova, 2020). Protein secretion is a two-step process, involving transport across the cell membrane, and passage through the several-layered peptidoglycan (PG) cell wall. Previously the influence on secretion of many secretion-associated factors has been investigated *e.g.*, signal peptides (Degering *et al*., 2010), chaperones (Wahlström *et al*., 2003; Chen *et al*., 2015), teichoic acid D-alanylation (Hyyryläinen *et al*., 2000) and proteases (Stephenson & Harwood, 1998). The Gram-positive cell wall has been described to form a sieve-like meshwork, which is easier passable for small proteins (Demchick & Koch, 1996). The effects of cell wall hydrolases on secretion are mostly examined in combination with other regulators or proteins (Berger *et al*., 2011; Zobel *et al*., 2015) or focus on growth effects rather than modulation of the cell wall (Ren *et al*., 2022). Despite a reported decrease in cell wall permeability of mutants with reduced autolytic activity (Williamson & Ward 1981), the possibility of increasing permeability through additional genes of autolysins is yet to be explored.

Cell wall hydrolases are found in all bacteria with peptidoglycan, *Bacillus subtilis* alone encodes as many as 35 different ones. They are very likely regulated by exogenous proteases as *B. subtilis* strains with inactivated protease genes become highly susceptible to autolysis (Stephenson *et al*., 1999, Cho *et al*., 2004). LytC is an N-acetylmuramoyl-L-alanine amidase with three cell wall binding (CWB) repeat regions at the N-terminus and a catalytic region at the C-terminus (Kuroda & Sekiguchi, 1991, Yamamoto *et al*., 2003). It localizes uniformly and hydrolyzes the linkage of N-acetylmuramoyl-L-alanine in peptidoglycan (Yamamoto *et al*., 2003). LytD is an endo-β-N-acetyl-glucosaminidase that forms a homodimer (Margot *et al*., 1994). It is predicted to have an N-terminal SH3B cell wall binding domain but further details about the localization are currently not known (Bateman *et al*., 2022). Initially described as an endolysin, LytD can cleave the bacterial cell wall at the covalent bond between the N-acetylglucosamine (GlcNAc) and the N-acetylmuramie acid (MurNAc) of the glycan chain (Rogers *et al,* 1980). LytF an α γ-D-glutamate *meso*-diaminopimelate muropeptidase, that acts as the primary autolysin involved in vegetative daughter cell separation (Margot *et al*., 1999, Chen *et al*., 2009). It consists of an N-terminal LysM cell wall binding domain and a catalytic region at the C-terminus (Margot *et al*., 1999). Immunofluorescence microscopy shows LytF localizing at the pole and the septum dependent on the absence of WTAs, which inhibit the sidewall localization (Yamamoto *et al*., 2008). LytF cuts the peptidoglycan γ-D-glutamate-*meso*-diaminopimelate bond but on its own has only a minor cell wall lytic activity *in vitro* (Ohnishi *et al*., 1999, Margot *et al*., 1999).

Transcription of three major autolysins *lytC*, *lytD* and *lytF* is controlled by the alternative sigma factor σ^D (Márquez *et al*., 1990). LytD and LytC account for ~ 95% of the autolytic activity of the cell (Smith *et al*., 2000). Both LytC and LytD play a role in cell separation, swarming motility and wall turnover (Blackman *et al*., 1998). LytC also lyses the mother cell at the end of sporulation (Smith & Foster, 1995).

The gene *dacA* encodes for the low-molecular-weight (low-MW) penicillin-binding protein PBP5 and is the major D-alanyl-D-alanine carboxypeptidase of vegetative *B. subtilis* cells (Lawrence & Strominger, 1970). In native conditions, the protein forms a homodimer with a C-terminal membrane anchor (Skoog *et al*., 2011, Pratt *et al*., 1986). Fluorescence microscopy showed that PBP5 localizes at the septum and in concentrated spots along the lateral cell wall (Scheffers *et al*., 2004). Because PBP5 can cleave the D-Ala-D-Ala from the pentapeptide of the peptidoglycan, a *dacA* knockout mutation leads to a substantial increase in muropeptides with a pentapeptide side chain. Interestingly, overexpression of PBP5 in the Gram-negative bacterium *Escherichia coli* has been shown to increase the secretion of amylase K, suggesting that altered cell wall crosslinking could affect bacterial protein secretion in general (Yang *et al*., 2022).

Results

Missing autolysins negatively impact secretion of an overproduced protein

In this study, we aimed to examine the impact of cell wall hydrolases on α-amylase secretion in *B. subtills*. We, therefore, constructed single deletion mutants of the cell wall modifying enzymes LytC, LytD, LytF (hereafter collectively named cell wall hydrolase) and PBP5 (*dacA*) (cell wall modifier) in combination with AmyE overexpression. All these enzymes are capable to hydrolyze peptidoglycan or its precursors, influencing cell separation, swarming motility, autolysis, and crosslinking of glucan strands. The absence of these proteins can influence the structure of the cell wall, which is one of the two structural hurdles for the passage of secretory proteins from the cytoplasm to the extracellular space.

In this study, the commonly used 72.6 kDa α-amylase AmyE was selected as a model secreted protein to quantify secretion efficiency of different strains. Here AmyE is expressed through the non-integrating plasmid pM11K_*amyEBs* provided by the B.R.A.I.N. AG (Zwingenberg, Germany). To analyze the effects of the different mutations of cell wall hydrolases on the amount of secreted AmyE the α-amylase activity was determined by the Phadebas method (Pen *et al*., 1992). Interestingly despite having only minor lytic activity and being mostly responsible for cell separation, LytF had a significant effect on the secretion of AmyE (Fig. 1). In the Δ*lytF* strain, α-amylase activity detected in the media was reduced by 15% compared to the control strain. The Absence of LytC had a major impact on the extracellular appearance of AmyE. The deletion resulted in a 50% loss of amylase activity in the supernatant suggesting that the *lytC* gene product had the strongest effect on protein secretion in *B. subtilis* (Fig. 1). No significant impact of the *lytD* mutation could be detected.

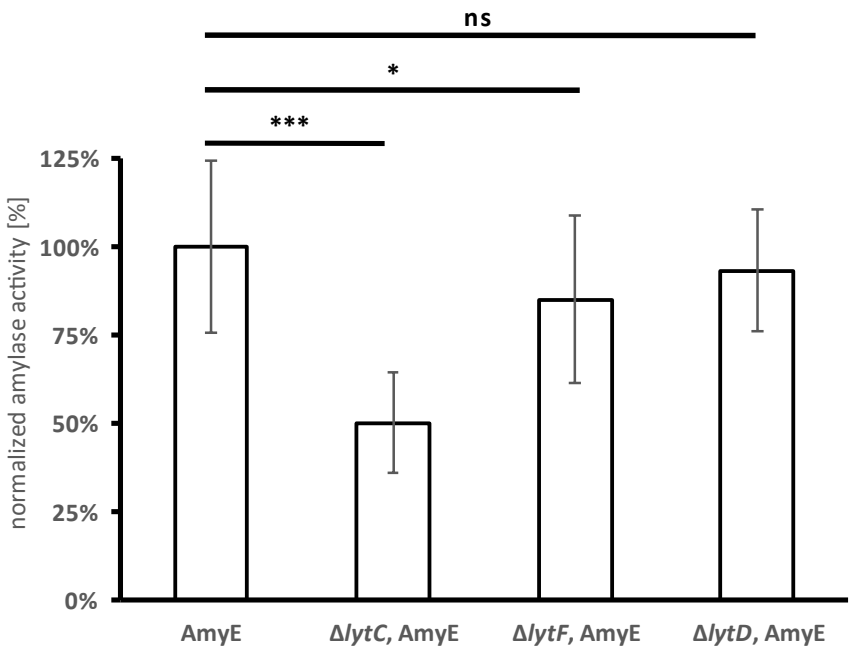


Figure 1: *A*mylase activity in a culture supernatant of *B. subtilis* PY79 mutants Δ*lytC*, Δ*lytF,* Δ*lytD.* AmyE: strain with plasmid-based expression of AmyE. Significance of differences was estimated via two-tailed T-test. AmyE, Δ*lytC* and Δ*lytF*: n = 34; Δ*lytD*: n = 12.

Increased levels of cell wall hydrolases can improve secretion

In order to verify the effects of these cell wall hydrolases on protein secretion, *B. subtilis* further experiments regarding the enzymes were conducted. Based on the results generated through the deletion strains (Fig. 1), we focused on *lytC*, *lytF*, and *dacA* in the following experiments. Additional cell wall hydrolase genes under the control of inducible promoters were ectopically inserted into the native *amyE*-locus of *B. subtilis*. The genes *lytF* and *dacA* were expressed using the IPTG-inducible promoter $P_{hyper\text{-}spank}$ while *lytC* was expressed using the xylose-inducible promoter P_{xyl}. Strains containing additional cell wall hydrolase genes and the AmyE overexpressing plasmid were investigated for the impact on protein secretion. To this end, extra hydrolase gene expression was induced by addition of 0.5 mM IPTG or 0.25% (v/v) xylose respectively, 3 h after inoculation. Secretion efficiency was evaluated after 8 h of growth, through assessment of the amylase activity in the media.

Our results show that upon induction of *lytF* expression, the amylase activity in culture supernatants was increased by 70%, indicating the detection of elevated AmyE secretion levels in *B. subtilis* (Fig. 2). In the absence of promoter induction, no significant increase in amylase activity was found in the supernatant.

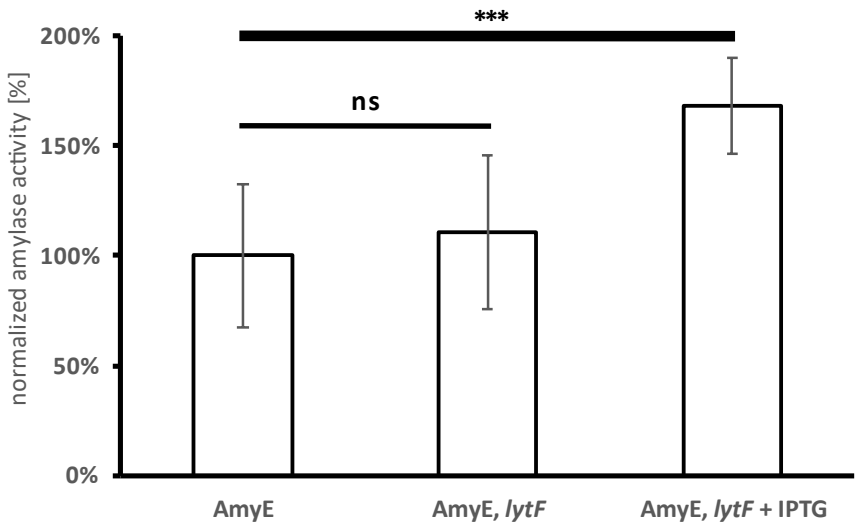


Figure 2: Amylase activity in culture supernatant of *B. subtilis* PY79 with *lytF* overexpression. AmyE: strain with plasmid-based expression of AmyE. *lytF*: strain with *amyE::*$P_{hyper\text{-}spank}$*-lytF*. IPTG indicates the addition of 0.5 mM of this inductor. Significance of differences was estimated via two-tailed T-test. n = 10.

While testing the *B. subtilis* strain containing the extra inducible *lytC* gene, we found strongly raised levels of amylase activity in the culture supernatant. Upon inducing the expression of the additional *lytC* gene through xylose, the detected amylase activity was increased by 150%. (Fig. 3). Interestingly, the introduction of an additional *lytC* gene at the *amy*-site without inducing expression, resulted in a ~70% increase in amylase activity compared to the control strain.

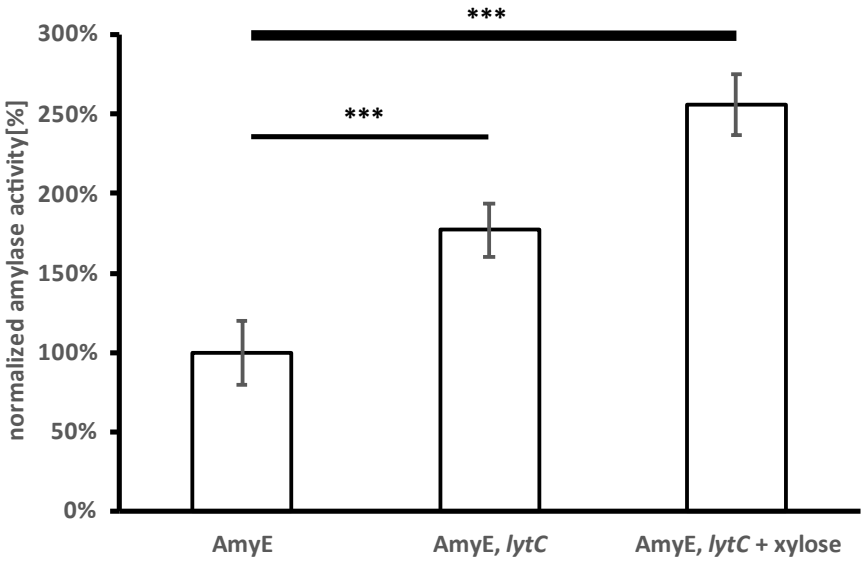


Figure 3: Phadebas assay of amylase activity in culture supernatant of *B. subtilis* PY79 with an extra ectopic *lytC* gene. AmyE: strain with plasmid-based expression of AmyE. *lytC*: strain with *amyE::*P_{xyl}*-lytC*. Xylose indicates the addition of 0.25% (v/v) of this inductor. Significance of differences was estimated via two-tailed T-test. AmyE: n = 24; *lytC*: n = 28, *lytC* + xylose: n = 12.

Similar to *lytC*, the introduction of an additional *dacA* gene without its induction resulted in a ~70% increase in amylase activity in the supernatant compared to the control strain (Fig. 4). The induction of the additional *dacA* by 0.5 mM IPTG improved the secretion by additional 50% compared to the non-induced cells. It seems that the extra PBP5 produced through the leakiness of the $P_{hyper\text{-}spank}$ promoter is sufficient to more than double the secretion capacity for AmyE of *B. subtilis*. With the α-amylase activity in the supernatant not increasing as much upon IPTG induction, a saturation effect possibly sets in.

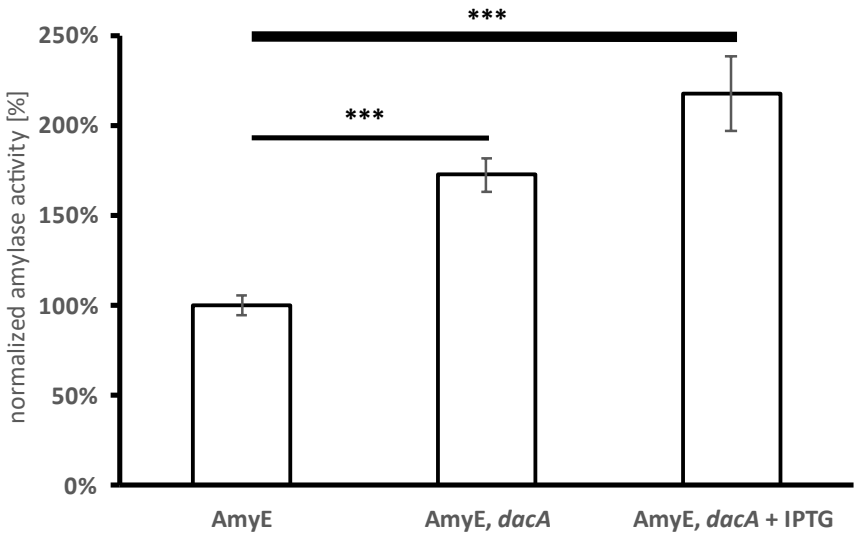


Figure 4: Phadebas assay of amylase activity in culture supernatant of *B. subtilis* PY79 with an extra *dacA* gene. AmyE: strain with plasmid-based expression of AmyE. *dacA*: strain with *amyE::*$P_{hyper\text{-}spank}$*-dacA*. If marked, 0.5 mM IPTG was added to the culture. Significance of differences was estimated via two-tailed T-test. n = 12.

Discussion

Autolysins are often considered when exploring strategies to improve bacterial protein production in an industrial context (Ren *et al*., 2022). These bacteriolytic enzymes can hydrolyze the cell wall peptidoglycan of the bacteria that produce them (Shockman & Hölltje, 1994) and are found in high abundance (Smith *et al*., 1996). The Among the many cell wall hydrolases, the N-acetylmuramoyl-L-alanine amidase LytC and the N-acetyl-glucosaminidase LytD are the most significant autolysins (Rogers *et al*., 1984; Margot *et al*., 1994), accounting for around 95% of the autolytic activity of the cell (Kuroda & Sekiguchi, 1991; Lazarevic *et al*., 1992). LytF, an α γ-D-glutamate *meso*-diaminopimelate muropeptidase is also a very interesting autolysin, since it acts as the primary autolysin involved in vegetative daughter cell separation (Margot *et al*., 1999, Chen *et al*., 2009).

PBP5 (*dacA*) is the major DD-carboxypeptidase in *B. subtilis*, cleaving the C-terminal D-Ala-D-Ala peptide bond of the stem peptide (Lawrence & Strominger, 1970). The absence of the terminal D-Ala in the stem peptide prevents the formation of a crosslinking peptide bridge between the glycan strands. In this work, we could show that, an increased PBP5 expression level results in a substantial

increase in secretion efficiency of AmyE. Considering the cell wall modulating effects of PBP5, we advocate that the extra enzyme activity reduced the number of cross-linkable stem peptides, which increased the peptidoglycan mesh size and thus made the cell wall more permeable for the secreted amylase. Interestingly, overexpression of *dacA* in the Gram-negative bacterium *Escherichia coli* has been shown to increase amylase K yield in the supernatant (Yang *et al*, 2022). This positive effect was attributed to increased permeability of the outer membrane (Yang *et al*, 2022), which represents a greater hurdle for secretion in Gram-negative bacteria like *E. coli*, than their thin cell wall (Wandersman, 1992; Mergulhao *et al*., 2004)

To further examine the impact of cell wall modifying enzymes on α-amylase secretion in *B. subtills*, we constructed single deletion mutants of *lytC*, *lytD* and *lytF* in combination with AmyE overexpression. While the deletion of *lytF* had a mild impact, the *lytC* deletion resulted in a 50% loss of amylase activity in the supernatant. No significant impact of the *lytD* mutation could be detected. Notably, it is hard to measure the effect of Δ*lytD*, as LytC has been reported to be able to compensate for this deletion (Blackman, 1998). These results suggest that missing autolysins have a detrimental effect on protein secretion, since their autolytic activity influences the properties of the cell wall, which represents a barrier for secreted proteins. This idea is supported by the fact that strains with impaired cell wall hydrolase activity exhibit a thicker cell wall than wild type strains (Fan *et al*., 1971). A thicker cell wall could be reasonably considered an increased hindrance for protein secretion and consequently should lead to decreased secretion levels. Additionally, work by Williamson and Ward in 1981 provides evidence that reduced autolytic activity in *B. subtilis* as well as *Streptococcus pneumoniae* will lead to a decreased permeability of the cell wall.

In order to investigate the impact of increased autolytic activity on AmyE secretion, additional copies of cell wall hydrolase genes *lytC* and *lytF* under the control of inducible promoters were introduced into *B. subtills*. While *lytF* was under the control of the $P_{hyper\text{-}spank}$ promoter, *lytC* expression was controlled by the xylose-inducible promoter P_{xyl}. Since *lytC* encodes for the major cell wall hydrolase LytC, which can lead to autolysis (Garcia *et al*., 1999; Smith *et al*., 1995), we chose to express this gene via P_{xyl} as it leads to weaker expression levels and less leakiness in comparison to $P_{hyper\text{-}spank}$ (Vavrová *et al*. 2010). We demonstrated that the induction of the additional *lytF* gene via IPTG resulted in a 70% increase in amylase activity in the culture supernatant. Upon inducing the expression of the additional *lytC* gene through xylose, the detected amylase activity was increased by 150%. This represents an increased secretion efficiency of AmyE in *B. subtilis* as a result of elevated autolysin expression. Our findings imply that the permeability of the cell wall for secreted proteins can be modified through up or down-regulating the activity of certain autolysins.

This represents a novelty as autolysins are usually considered an impediment to secretion rather than beneficial (Ren *et al*., 2022). Since autolysins are bacteriolytic enzymes that digest the cell wall peptidoglycan, they can lead to cell lysis and are thought to be responsible for a significant reduction in cell biomass and the associated limitation of product expression and fermentation efficiency (Westers *et al*., 2003). While older studies suggest that inactivation of the main autolysins LytC,

LytD or LytF does not affect cell growth (Margot & Karamata, 1992; Margot *et al*., 1994), our experiments show a slightly increased OD_{600} with the Δ*lytC* strain (data not shown). However, this did not translate into an overall positive effect on the secretion. Contrary to our findings, two reports show that reducing cell autolysis in *B. subtilis* via deletion of *lytC*, leads to increased secretion of recombinant proteins, by maintaining more stable growth over a longer period of time (Kabisch *et al*., 2013; Wang *et al*., 2014). However, Wang *et al*. used a mutant with multiple additional deletions for the cannibalism factors *skfA*, *sdpC*, as well as the prophage gene *xpf*, which hinders the precise assessment of the Δ*lytC*-specific effects on secretion. Also, the reported increase in biomass was not accounted for, in the evaluation of secretion levels in both studies.

Studies supporting our findings on the benefits of autolysins for secretory processes report the inactivation of LytC and/or LytD impedes secretion of proteins by *B. subtilis* (Smith *et al*., 2000). Also, Zobel *et al*., 2015 observed a strong reduction of the enniatin secretion in a Δ*lytC* mutant. Interestingly another study investigating a *B. subtilis* mutant with reduced protease activity, found increased levels of autolytic activity and secretion (Fahnestock and Fisher 1987), providing early evidence of a positive connection. Additional work confirmed that autolysins are regulated by protease activity in *B. subtilis*, as a mutant with 8 deleted exported proteases exhibited a higher quantity of the dominant autolysins LytC, LytD, LytE and LytF (Kodama *et al*., 2007).

In this work, we demonstrate a novel approach to utilize induced expression of specific autolysins to improve secretion efficiency of AmyE, an example of a large secreted protein with a molecular weight of nearly 60 kDa.

Material & methods

Bacterial strains and plasmids

The B. subtilis strain used was PY79 (derivative of Bacillus 168) shown in table T1. *Bacillus* strains were grown at 37°C overnight on nutrient agar plates using commercial nutrient broth LB solidified by addition of 1% (w/v) agar. Overnight cultures in tubes were inoculated from a fresh agar plate and incubated overnight at 37°C and 200 rpm. Day cultures in 100 ml shake flasks with 10 ml media were inoculated to a cell density of OD_{600} of 0.1 in LB from the overnight cultures and then incubated at 37°C and 200 rpm.

For the analysis of α-amylase AmyE secretion, the plasmid pM11K_*amyEBs* provided by the B.R.A.I.N. AG (Zwingenberg, Germany) was used. This plasmid provides the HpaII-promoter (Zyprian & Matzura, 1986) to drive the expression of *amyE* and a high copy number pUB110-like replicon. This non-integrating plasmid carries a kanamycin resistance for selection with 25 µg/ml kanamycin in *Bacillus*. The plasmid is available, upon reasonable request, after signing a Material Transfer Agreement.

The *B. subtilis* PY79 deletion strains were created via gDNA transfer from *B. subtilis* 168 deletion strains, obtained from the *Bacillus* Genetic Stock Center (BGSC) (Columbus, Ohio, https://bgsc.org/)

(Koo *et al.*, 2017). To create clean deletions the kanamycin resistance cassette was removed (pDR 244 Cre-lox) following the method of Koo *et al*., 2017.

To generate isopropyl-b-D-thiogalactoside-inducible copies of *lytF* and *dacA* at the amylase locus, the genes were PCR amplified from PY79 gDNA and cloned via Gibson Assembly downstream of hyperspank promoter in plasmid pDR111 (a kind gift from D. Rudner, Harvard Medical School). To generate a xylose-inducible *lytC* copy at the amylase locus, the gene was PCR amplified from PY79 gDNA and cloned via Gibson Assembly downstream of xylose promoter in plasmid pSG1191 (Feucht and Lewis, 2001).

Phadebas test for amylase activity

For the quantification of α-amylase activity in the culture supernatant, the Phadebas Amylase Test (Phadebas AB, Uppsala, Sweden) was used. The principle behind the Phadebas test, is the release of a water-soluble blue dye after starch microspheres with the dye cross-linked to the starch is digested by amylase enzymes. One Phadebas tablet was dissolved in 20 ml buffer solution (0.1 M acetic acid, 0.1 M potassium acetate, 5 mM calcium chloride, pH 5). Cultures of *Bacillus* in the stationary growth phase were centrifuged at 14000 rpm for 2 minutes in a microfuge, 20 µl supernatant was mixed with pre-warmed 180 µl substrate solution and incubated for 10 min at 37°C and 1000 rpm in a thermomixer (Eppendorf Thermomixer comfort). The reaction was stopped by the addition of 60 µl 1 M sodium hydroxide. The reaction tubes were centrifuged and the absorption of 100 µl of the supernatant was measured at 620 nm via a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). Activities were corrected for dilution and normalized to the cell density (OD_{600}) of the culture. Experiments were performed at least as biological triplicates with additional technical replicates, the total number of which is stated as “n”.

Table 1 Strains used in this study

Strain	*Relevant features*	*Reference of source*
B. subtilis PY79	Wild type	Richard Losick Harvard University
B. subtilis PY79	pM11K_*amyEBs*	This study
B. subtilis PY79	Δ*lytC*	This study
B. subtilis PY79	Δ*lytD*	This study
B. subtilis PY79	Δ*lytF*	This study
B. subtilis PY79	Δ*lytC,* pM11K_*amyEBs*	This study
B. subtilis PY79	Δ*lytD,* pM11K_*amyEBs*	This study
B. subtilis PY79	Δ*lytF,* pM11K_*amyEBs*	This study
B. subtilis PY79	*amyE::*$P_{hyper\text{-}spank}$*-lytF*	This study
B. subtilis PY79	*amyE::*$P_{hyper\text{-}spank}$*-dacA*	This study
B. subtilis PY79	*amyE::*P_{xyl}*-lytC*	This study
B. subtilis PY79	*amyE::*$P_{hyper\text{-}spank}$*-lytF,* pM11K_*amyEBs*	This study
B. subtilis PY79	*amyE::*$P_{hyper\text{-}spank}$*-dacA,* pM11K_*amyEBs*	This study
B. subtilis PY79	*amyE::*P_{xyl}*-lytC,* pM11K_*amyEBs*	This study

Author contribution

MS has performed all experiments, and co-wrote the manuscript. KL and PLG supervised experiments, conceived of the study, and co—wrote the manuscript.

References

Atrih, A., Bacher, G., Allmaier, G., Williamson, M. P., & Foster, S. J. (1999). Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *Journal of bacteriology*, *181*(13), 3956-3966.

Bateman, A., Martin, M. J., Orchard, S., Magrane, M., Ahmad, S., Alpi, E., ... & UniProt Consortium. (2022). UniProt: the Universal Protein Knowledgebase in 2023. *Nucleic Acids Research*.

Blackman, S. A., Smith, T. J., & Foster, S. J. (1998). The role of autolysins during vegetative growth of *Bacillus subtilis* 168. *Microbiology*, *144*(1), 73-82.

Chen, J., Fu, G., Gai, Y., Zheng, P., Zhang, D., & Wen, J. (2015). Combinatorial Sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: identification of bottlenecks by systematic gene overexpression. *Microbial Cell Factories*, *14*(1), 1-15.

Chen, R., Guttenplan, S. B., Blair, K. M., & Kearns, D. B. (2009). Role of the σD-dependent autolysins in *Bacillus subtilis* population heterogeneity. *Journal of bacteriology, 191*(18), 5775-5784.

Cho, H. Y., Yukawa, H., Inui, M., Doi, R. H., & Wong, S. L. (2004). Production of minicellulosomes from Clostridium cellulovorans in *Bacillus subtilis* WB800. *Applied and Environmental Microbiology*, *70*(9), 5704-5707.

Danilova, I., & Sharipova, M. (2020). The practical potential of bacilli and their enzymes for industrial production. *Frontiers in microbiology*, *11*, 1782.

Degering, C., Eggert, T., Puls, M., Bongaerts, J., Evers, S., Maurer, K. H., & Jaeger, K. E. (2010). Optimization of protease secretion in *Bacillus subtilis* and *Bacillus licheniformis* by screening of homologous and heterologous signal peptides. *Applied and environmental microbiology*, *76*(19), 6370-6376.

Fahnestock, S. R., & Fisher, K. E. (1987). Protease-deficient *Bacillus subtilis* host strains for production of Staphylococcal protein A. *Applied and environmental microbiology*, *53*(2), 379-384.

Fan, D. P., & Beckman, M. M. (1971). Mutant of *Bacillus subtilis* demonstrating the requirement of lysis for growth. *Journal of bacteriology*, *105*(2), 629-636.

Feucht, A., and Lewis, P. J. (2001). Improved plasmid vectors for the production of multiple fluorescent protein fusions in *Bacillus subtilis*. Gene 264, 289–297. doi: 10.1016/s0378-1119(01)00338-9

Garcia, P., González, M. P., García, E., García, J. L., & López, R. (1999). The molecular characterization of the first autolytic lysozyme of Streptococcus pneumoniae reveals evolutionary mobile domains. *Molecular microbiology*, *33*(1), 128-138.

Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends in biotechnology*, *10*, 247-256.

Hyyryläinen, H. L., Vitikainen, M., Thwaite, J., Wu, H., Sarvas, M., Harwood, C. R., ... & Stephenson, K. (2000). D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of *Bacillus subtilis*. *Journal of Biological Chemistry*, *275*(35), 26696-26703.

Kabisch, J., Thürmer, A., Hübel, T., Popper, L., Daniel, R., & Schweder, T. (2013). Characterization and optimization of *Bacillus subtilis* ATCC 6051 as an expression host. *Journal of biotechnology*, *163*(2), 97-104.

Kodama, T., Endo, K., Ara, K., Ozaki, K., Kakeshita, H., Yamane, K., & Sekiguchi, J. (2007). Effect of *Bacillus subtilis spo0A* mutation on cell wall lytic enzymes and extracellular proteases, and prevention of cell lysis. *Journal of bioscience and bioengineering*, *103*(1), 13-21.

Koo, B. M., Kritikos, G., Farelli, J. D., Todor, H., Tong, K., Kimsey, H., ... & Gross, C. A. (2017). Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell systems*, *4*(3), 291-305.

Kuroda, A., & Sekiguchi, J. (1991). Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. *Journal of bacteriology*, *173*(22), 7304-7312.

Lawrence, P. J., & Strominger, J. L. (1970). Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: XVI. The reversible fixation of radioactive penicillin G to the D-alanine carboxypeptidase of *B. subtilis*. *Journal of Biological Chemistry*, *245*(14), 3653-3666.

Margot, P., & Karamata, D. (1992). Identification of the structural genes for N-acetylmuramoyl-L-alanine amidase and its modifier in *Bacillus subtilis* 168: inactivation of these genes by insertional mutagenesis has no effect on growth or cell separation. *Molecular and General Genetics MGG*, *232*, 359-366.

Margot, P., Mauël, C., & Karamata, D. (1994). The gene of the N-acetylglucosaminidase, a *Bacillus subtilis* 168 cell wall hydrolase not involved in vegetative cell autolysis. *Molecular microbiology*, *12*(4), 535-545.

Margot, P., Pagni, M., & Karamata, D. (1999). *Bacillus subtilis* 168 gene *lytF* encodes a γ-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, σD. *Microbiology*, *145*(1), 57-65.

Márquez, L. M., Helmann, J. D., Ferrari, E., Parker, H. M., Ordal, G. W., & Chamberlin, M. J. (1990). Studies of sigma D-dependent functions in *Bacillus subtilis*. *Journal of bacteriology*, *172*(6), 3435-3443.

Mergulhao, F. J. M., Taipa, M. A., Cabral, J. M. S., & Monteiro, G. A. (2004). Evaluation of bottlenecks in proinsulin secretion by Escherichia coli. *Journal of biotechnology*, *109*(1-2), 31-43.

Ohnishi, R., Ishikawa, S., & Sekiguchi, J. (1999). Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of *Bacillus subtilis*. *Journal of bacteriology*, *181*(10), 3178-3184.

Pratt, J. M., Jackson, M. E., & Holland, I. B. (1986). The C terminus of penicillin-binding protein 5 is essential for localisation to the E. coli inner membrane. *The EMBO journal*, *5*(9), 2399-2405.

Ren, K., Wang, Q., Hu, M., Chen, Y., Xing, R., You, J., ... & Rao, Z. (2022). Research Progress on the Effect of Autolysis to *Bacillus subtilis* Fermentation Bioprocess. *Fermentation*, *8*(12), 685.

Rogers, H. J., Perkins, H. R., & Ward, J. B. (1980). The bacterial autolysins. In *Microbial cell walls and membranes* (pp. 437-460). Springer, Dordrecht.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of Bacillus species for industrial production. *Canadian journal of microbiology*, *50*(1), 1-17.

Scheffers, D. J., Jones, L. J., & Errington, J. (2004). Several distinct localization patterns for penicillin-binding proteins in *Bacillus subtilis*. *Molecular microbiology*, *51*(3), 749-764.

Skoog, K., Bruzell, F. S., Ducroux, A., Hellberg, M., Johansson, H., Lehtiö, J., ... & Daley, D. O. (2011). Penicillin-binding protein 5 can form a homo-oligomeric complex in the inner membrane of Escherichia coli. *Protein Science*, *20*(9), 1520-1529.

Smith, T. J., & Foster, S. J. (1995). Characterization of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. *Journal of bacteriology*, *177*(13), 3855-3862.

Smith, T. J., Blackman, S. A., & Foster, S. J. (2000). Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. *Microbiology*, *146*(2), 249-262.

Stephenson, K., & Harwood, C. R. (1998). Influence of a cell-wall-associated protease on production of α-amylase by *Bacillus subtilis*. *Applied and Environmental Microbiology*, *64*(8), 2875-2881.

Stephenson, K., Bron, S., & Harwood, C. R. (1999). Cellular lysis in *Bacillus subtilis*; the affect of multiple extracellular protease deficiencies. *Letters in applied microbiology*, *29*(2), 141-145.

Vavrová, Ľ., Muchová, K., & Barák, I. (2010). Comparison of different *Bacillus subtilis* expression systems. *Research in microbiology*, *161*(9), 791-797.

Wahlström, E., Vitikainen, M., Kontinen, V. P., & Sarvas, M. (2003). The extracytoplasmic folding factor PrsA is required for protein secretion only in the presence of the cell wall in *Bacillus subtilis*. *Microbiology*, *149*(3), 569-577.

Wandersman, C. (1992). Secretion across the bacterial outer membrane. *Trends in Genetics*, *8*(9), 317-322.

Wang, Y., Chen, Z., Zhao, R., Jin, T., Zhang, X., & Chen, X. (2014). Deleting multiple lytic genes enhances biomass yield and production of recombinant proteins by *Bacillus subtilis*. *Microbial cell factories*, *13*(1), 1-11.

Westers, H., Dorenbos, R., Van Dijl, J. M., Kabel, J., Flanagan, T., Devine, K. M., ... & Quax, W. J. (2003). Genome engineering reveals large dispensable regions in *Bacillus subtilis*. *Molecular Biology and Evolution*, *20*(12), 2076-2090.

Williamson, R., & Ward, J. B. (1981). Deficiency of autolytic activity in *Bacillus subtilis* and Streptococcus pneumoniae is associated with a decreased permeability of the wall. *Microbiology*, *125*(2), 325-334.

Yamamoto, H., Kurosawa, S. I., & Sekiguchi, J. (2003). Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. *Journal of bacteriology*, *185*(22), 6666-6677.

Yamamoto, H., Miyake, Y., Hisaoka, M., Kurosawa, S. I., & Sekiguchi, J. (2008). The major and minor wall teichoic acids prevent the sidewall localization of vegetative DL-endopeptidase LytF in *Bacillus subtilis*. *Molecular microbiology*, *70*(2), 297-310.

Yang, H., Wang, H., Wang, F., Zhang, K., Qu, J., Guan, J., ... & Chen, X. (2022). Efficient extracellular production of recombinant proteins in E. coli via enhancing expression of dacA on the genome. *Journal of Industrial Microbiology and Biotechnology*, *49*(4), kuac016.

Zobel, S., Kumpfmüller, J., Süssmuth, R. D., & Schweder, T. (2015). *Bacillus subtilis* as heterologous host for the secretory production of the non-ribosomal cyclodepsipeptide enniatin. *Applied microbiology and biotechnology*, *99*, 681-691.

Zyprian, E. & Matzura, H. (1986). Characterization of signals promoting gene expression on the *Staphylococcus aureus* plasmid pUB110 and development of a gram-positive expression vector system. *DNA* **5**, 219-225, doi:10.1089/dna.1986.5.219

2.3 Manuscript II

This manuscript is part of an invention disclosure. The results are currently investigated to assess their patentability. Therefore, premature efforts to publish the contents of the manuscript have been suspended.

The Influence of flotillins on amylase secretion in *Bacillus subtilis*

Manuel Strach[1], Klaus Liebeton[2], Peter L. Graumann[1]*

[1]Centre for Synthetic Microbiology (SYNMIKRO) and Fachbereich Chemie, Philipps-Universität Marburg, 35032 Marburg, Germany

[2]BRAIN Biotech AG, Darmstädter Str. 34-36, 64673 Zwingenberg, Germany

Abstract

Bacillus subtilis is a Gram-positive model organism that is also widely used for industrial protein production, due to its high capacity of secretion. Although flotillins have a known connection to the translocase, a strategy to improve protein secretion via flotillins has yet to be explored. Therefore, we have studied the influence of FloT on protein secretion in the *Bacillus subtilis* PY79 strain. We constructed a *yuaG* deletion strain and a strain with an IPTG inducible overexpression system *amyE::*$P_{hyper\text{-}spank}$*-yuaFG.* Effects on secretion were assessed by following the secretion of the amylase AmyE, expressed by a constitutive promoter from a high copy number plasmid. The FloT deletion strain showed a significant decrease in AmyE secretion. By adding 0.1% benzyl alcohol to the media, which has been shown to mitigate the reduction in membrane fluidity of flotillin deletion strains, the secretion capacity was recovered to the level of the control strain. Upon increasing the expression level, plasmid-derived AmyE secretion was doubled. Our findings indicate that the reduction in membrane fluidity of the Δ*yuaG* strain is responsible for its decreased AmyE secretion and expression of additional FloT improves translocation efficiency.

Introduction

Flotillins were first discovered in eukaryotes (Schulte *et al*., 1997), where they play a role in a wide variety of membrane dynamics (Doherty & McMahon, 2009; Ludwig *et al*., 2010; Resnik *et al*., 2011; Ge *et al*., 2011). Flotillin-like proteins form detergent-resistant microdomains (DRMs) or lipid rafts in eukaryotic cells and act as scaffolding proteins (Langhorst *et al*., 2005; Browman *et al*., 2007; Bickel *et al*., 1997; Babuke & Tikkanen, 2007). In *Bacillus subtilis* the flotillin-like proteins FloT and FloA can also be found in DRMs fractions (López and Kolter, 2010) and are often assumed to organize functional membrane microdomains (FMMs) (García-Fernández *et al*., 2017; López and Kolter, 2010). Flotillins are characterized by a central SPFH domain (named after the proteins Stomatin, Prohibitin, Flotillin and HflK C), an N-terminal transmembrane domain and the flotillin domain (Hinderhofer *et al*., 2009; Dempwolff *et al*., 2012). The main flotillin of *B. subtilis* is FloT expressed via the *yuaG* gene, while *yqfA* encodes the second flotillin FloA, whose functions are partially redundant (Lopez & Kolter, 2010). FloA is constitutively expressed, whereas FloT is expressed primarily during stationary growth, cell wall stress and sporulation (Schneider *et al*., 2015; Huang *et al*., 1999; Nicolas *et al*., 2012). Both proteins localize in defined focal structures independent of each other, within the cell membrane and move in a highly dynamic and random manner (Dempwolff *et al*., 2016). The flotillins play a role in sporulation processes (Donovan & Bramkamp, 2009), cell shape maintenance and cell division (Dempwolff *et al*., 2012). Through deletion and overproduction of flotillins, it has been shown, that they control the membrane fluidity (Lee *et al*., 2012; Bach & Bramkamp, 2013) in a way that influences other proteins, like MreB (Zielińska *et al*., 2020).

Flotillins are often linked with secretion, since translocase components like SecY appear to be integrated within the flotillin clusters in the membrane (Bach & Bramkamp, 2013; Dempwolff *et al*., 2016). While it has been observed that protein secretion is reduced in strains lacking flotillins (Bach & Bramkamp, 2013), the cause is unclear and the possibility that flotillins could have a beneficial effect on secretion has never been explored. This project aims to further investigate the effects of flotillins on secretion and the novel strategy of using FloT to enhance protein secretion in *B. subtilis*.

Results

Since FloT is considered the main flotillin of *B. subtilis* and the functions of FloA are partially redundant, we focused on FloT in our experiments. To analyze the effects of flotillins on secretion, the α-amylase activity of a *yuaG* deletion strain was determined via the Phadebas method (Pen *et al*., 1992). We compared the amylase activity in the media of the deletion mutant *B. subtilis* Δ*yuaG* PY79 with that of a control strain after 20 h of incubation. Both strains were overproducing AmyE via the pM11K_amyEBs plasmid. Our results show that missing the FloT protein leads to less amylase activity in the supernatant, due to a reduced secretion capacity (Fig.1).

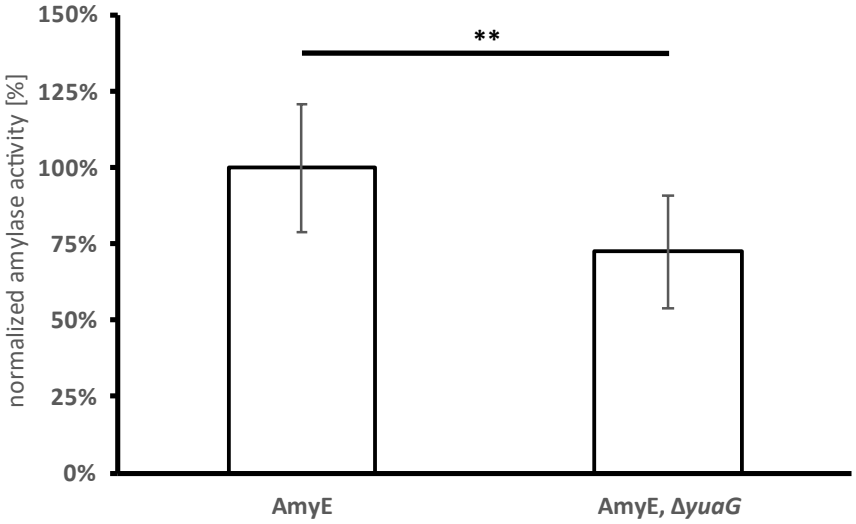


Figure 1: Phadebas assay of amylase activity in culture supernatant of *B. subtilis* PY79 mutant Δ*yuaG*. AmyE: strain with plasmid-based expression of amyE. Significance of differences was estimated via two-tailed T-test: n = 9.

To further investigate the mechanisms responsible for the negative effects of the *yuaG* deletion on secretion, we looked to restore functions of FloT in the deletion strain. Zielińska *et al*., 2020 have shown that a Δ*yuaG* mutation in *B. subtilis* PY79 leads to reduced membrane fluidity. But they could compensate for this by adding 0.1% benzyl alcohol, a membrane fluidizer, to the medium. If the reduction of AmyE secretion in the Δ*yuaG* mutant is related to the decreased membrane fluidity, the effect may be compensated through addition of benzyl alcohol.

First, we determined a benzyl alcohol concentration that doesn't affect the growth of the AmyE overproducing *B. subtilis* strains. Different concentrations of benzyl alcohol were added to cultures of the wild type with and without AmyE overproduction and the *yuaG* mutant with AmyE overproduction. The impact on growth was analyzed by measuring the optical density (OD) of the cultures after 20 h of incubation. While no negative effects on growth were observed at concentrations of 0.1% and 0.2% benzyl alcohol, 0.5% resulted in serious growth inhibition (Fig.2).

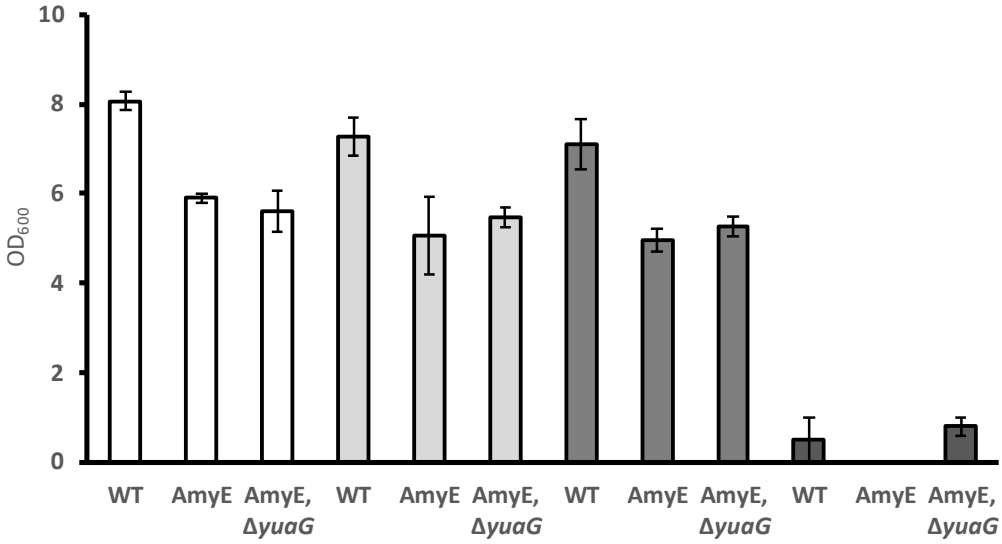


Figure 2: Effects of various benzyl alcohol concentrations on the growth of *B. subtilis*. Growth was measured by optical density (OD) at 600 nm after 20 h incubation. Benzyl alcohol concentrations added to the growth media are shown as 0% 0.1% 0.2% 0.5%; n = 6.

We investigated whether the addition of the membrane fluidizer benzyl alcohol could recover the negative impact of the *yuaG* deletion on secretion. After 2 h of initial growth in LB (OD of ~ 0.5 reached), 0.1% benzyl alcohol was added to the cultures, followed by an additional 18 h of incubation. Subsequently, the amylase activity in the media was determined. Our results show that the addition of benzyl alcohol to the Δ*yuaG* mutant strain, could in fact recover the AmyE secretion level to that of the control strain (Fig. 3). This leads to the assumption that the negative impact of the *yuaG* deletion on secretion is caused by a reduction in membrane fluidity.

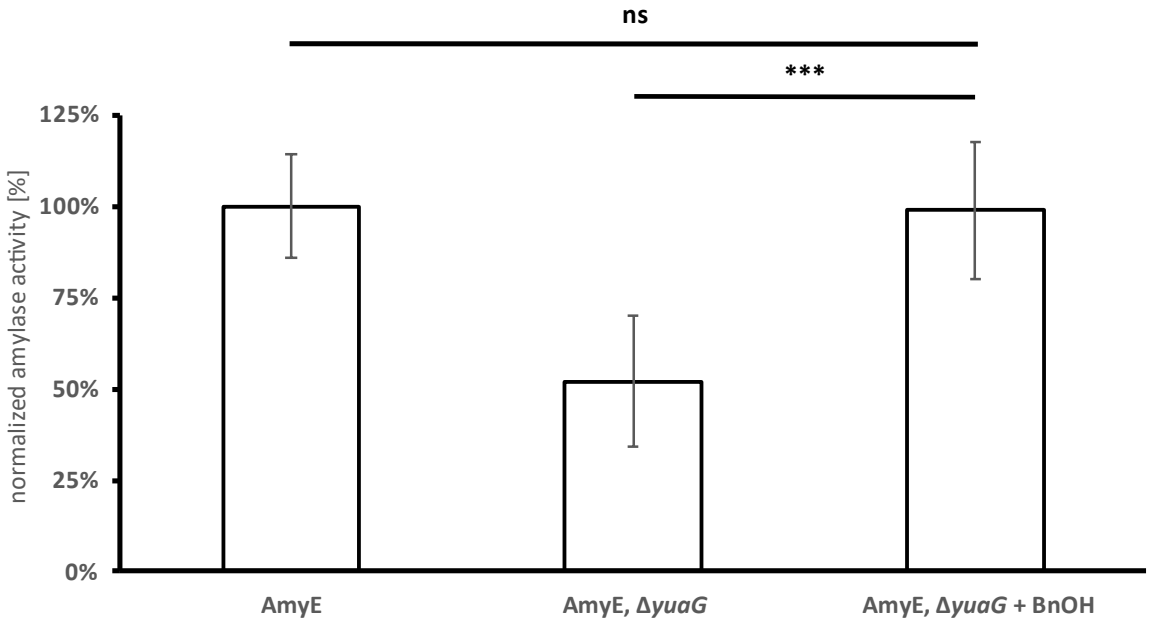


Figure 3: Phadebas assay of amylase activity in culture supernatant of *B. subtilis* PY79 mutant Δ*yuaG*. AmyE: strain with plasmid-based expression of AmyE. If marked, 0.1% benzyl alcohol (BnOH) was added to the culture Significance of differences was estimated via two-tailed T-test: AmyE: n = 22; Δ*yuaG* 17; Δ*yuaG* + BnOH: n = 24.

Here we investigate the impact of additional and inducible *yuaG* gene on AmyE secretion in *B. subtilis*. To study this we introduced an Isopropyl-β-D-thiogalactopyranosid (IPTG) inducible copy of *yuaFG* into the original AmyE locus creating the *B. subtilis* PY79 *amyE::*$P_{hyper\text{-}spank}$*-yuaFG* strain. Since *yuaG* is placed within an operon structure including the NfeD domain-harboring gene *yuaF* and the two proteins influence each other's localization behavior (Dempwolff *et al*., 2012), we decided to use the *yuaFG* combination for our experiments.

Interestingly just the introduction of the additional *yuaFG*, without the IPTG induction, resulted in the amylase activity in the supernatant to double compared to the control (Fig.4). Overproducing FloT by induction with 1 mM IPTG did not result in further improvement of secretion processes. This leads us to believe that the additional FloT protein expressed via the leakiness of the $P_{hyper\text{-}spank}$ promoter system was enough to saturate the beneficial effect FloT can have on the secretion of AmyE.

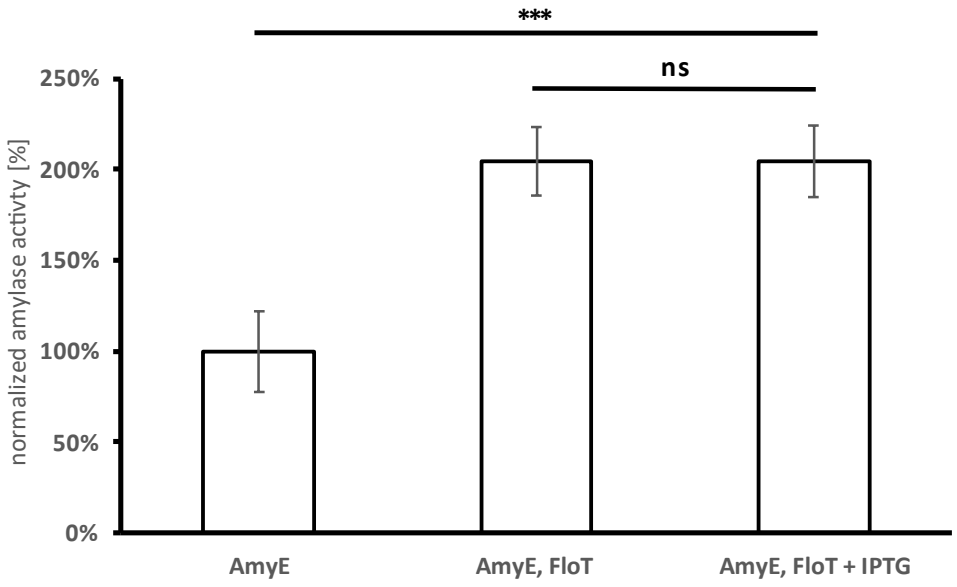


Figure 4: Phadebas assay of amylase activity in culture supernatant of *B. subtilis* PY79 an extra ectopic *yauG* gene. AmyE: strain with plasmid-based expression of AmyE. FloT: strain with *amyE::*$P_{hyper\text{-}spank}$*-yuaFG* If marked, 1 mM IPTG was added to the culture Significance of differences was estimated via two-tailed T-test. AmyE: n = 24; FloT n = 22

Discussion

Flotillins play a role in sporulation processes (Donovan & Bramkamp, 2009), cell shape maintenance and cell division (Dempwolff *et al*., 2012). They are often linked with secretion, since FloT and the translocase component SecY were co-eluted in DRM fractions (Bach & Bramkamp, 2013; Dempwolff *et al*., 2016). Previously Bach & Bramkamp (2013) showed a general reduction of protein secretion in flotillin deletion strains Δ*yuaG,* Δ*yqfA* and Δ*yuaG;*Δ*yqfA*. Our data contributes evidence that flotillins play a large role in influencing secretion processes. We showed that the AmyE secretion of a Δ*yuaG,* deletion strain is significantly decreased. But secretion was recovered to the level of the control by addition of membrane fluidizer BuOH in the same way Zielińska *et al*. (2020) elevated the reduced membrane fluidity of the mutant strain back to the wild type level. Through deletion and overproduction of flotillins, it has been shown, that flotillins impact membrane fluidity (Lee *et al*., 2012; Bach & Bramkamp, 2013) in a way that influences other proteins, for example, MreB (Zielińska *et al*., 2020).

Flotillins are commonly believed to act as scaffolding proteins in defined membrane domains for other proteins also found in DRM fractions (Lopez & Kolter, 2010; Lopez & Koch, 2017). In a recent study the SPFH domain, which is sufficient to form multimers (Kuwahara *et al*., 2009), was used to anchor enzymes into FMMs to improve their production capacity (Lv *et al*., 2020). Thus, it can be speculated that flotillins can affect secretion with this domain in a similar manner. The SecYEG translocon could be anchored into FMMs or its multimerization could be encouraged. But as it was shown in *in vitro* experiments, that only a single SecYEG translocon is necessary for a translocation event to occur (Kedrov *et al*., 2011; Taufik *et al*., 2013), and the benefit of potential multimerization is not certain. Furthermore, other publications show that flotillins and other proteins found in DRMs do not colocalize and have different movement dynamics (Dempwolff *et al*., 2016).

However, another interaction in which flotillins may be indirectly involved in is possibly more impactful. It was suggested some time ago, that the creation of a specific lipid environment might be necessary for translocation (Hendrick & Wickner, 1991). SecA anchors in the lipid bilayer interface with its amphipathic N-terminal helix, which activates SecA for high affinity binding to the translocon (Lill *et al*., 1990; Breukink *et al*., 1992; Koch *et al*., 2016). Here the membrane composition is of great importance, since unsaturated fatty acids (UFAs) cause loose packing of lipid head groups, where the N-terminal amphipathic helix of SecA docks. Kamel *et al*. (2022) could recently increase protein secretion *in vitro* by an artificial membrane, that was less tightly packed and more fluid. The UFAs used, promoted SecA binding to the membrane, which stimulated SecA:SecYEG-mediated protein translocation. In a similar manner, FloT could indirectly improve SecA binding, through its contribution to membrane fluidity and thereby positively affecting protein secretion. A FloT deletion mutant exhibiting a more rigid cell membrane should therefore feature an impaired SecA binding leading to the decreased secretion we reported. FMMs on the other hand are enriched in isoprenoid lipids (García-Fernández *et al*., 2017; López and Kolter, 2010) and display increased order and thickness compared with the surrounding bilayer (van Tilburg *et al*., 2022). So, it is unlikely that FMMs are regions in the membrane that offer a favorable environment

for secretion processes involving SecA (*e.g.* secretion of AmyE). As it was shown in this work, the negative effect on secretion of missing FloT can be compensated for, by addition of a membrane fluidizer. Therefore, it is likely that the effect stems from the reduced membrane fluidity of the Δ*yuaG* mutant.

In a novel approach, we used additional FloT expression to influence secretion in a beneficial manner. Upon introduction of an additional *yuaFG*, controlled by the $P_{hyper\text{-}spank}$ promoter, we observed a doubling of the AmyE secretion capacity. Increased levels of FloT in the PY79_ *amyE::*$P_{hyper\text{-}spank}$*-yuaFG* strain may consequently lead to more membrane fluidity, which would improve secretion through the SecA:SecYEG interaction.

While the Δ*yuaG* mutation leads to changes in the lipid order, no changes in the overall fatty acid composition of the membranes have been detected (Zielinska *et al*., 2020). The mechanism by which FloT influences membrane fluidity is currently not entirely understood.

Material & methods

Bacterial strains and plasmids

The B. subtilis strain used was PY79 (derivative of Bacillus 168) shown in table T1. Bacillus strains were grown at 37°C overnight on nutrient agar plates using commercial nutrient broth LB solidified by addition of 1% (w/v) agar. Overnight cultures in tubes were inoculated from a fresh agar plate and incubated overnight at 37°C and 200 rpm. Day cultures in 100 ml shake flasks with 10 ml media were inoculated to a cell density of OD_{600} of 0.1 in LB from the overnight cultures and then incubated at 37°C and 200 rpm.

For the analysis of α-amylase AmyE secretion, the plasmid pM11K_amyEBs provided by the B.R.A.I.N. AG (Zwingenberg, Germany) was used. This plasmid provides the HpaII-promoter (Zyprian & Matzura, 1986) to drive the expression of *amyE* and a high copy number pUB110-like replicon. This non-integrating plasmid carries a kanamycin resistance for selection with 25 µg/ml kanamycin in *Bacillus*. The plasmid is available, upon reasonable request, after signing a Material Transfer Agreement.

To generate an isopropyl-b-D-thiogalactoside-inducible copy of *yuaFG* at the amylase locus, the genes were PCR amplified from PY79 gDNA and cloned via Gibson Assembly downstream of hyperspank promoter in plasmid pDR111 (a kind gift from D. Rudner, Harvard Medical School).

Phadebas test for amylase activity

For the quantification of α-amylase activity in the culture supernatant, the Phadebas Amylase Test (Phadebas AB, Uppsala, Sweden) was used. One Phadebas tablet was dissolved in 20 ml buffer solution (0.1 M acetic acid, 0.1 M potassium acetate, 5 mM calcium chloride, pH 5). Overnight cultures of *Bacillus* were centrifuged at 14000 rpm for 2 minutes in a microfuge, 20 µl supernatant was mixed with 180 µl substrate solution and incubated for 10 min at 37°C and 1000 rpm in a

thermomixer (Eppendorf Thermomixer comfort). The reaction was stopped by addition of 60 µl 1 M sodium hydroxide. The reaction tubes were centrifuged and the absorption of 100 µl of the supernatant was measured at 620 nm via a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). Activities were corrected for dilution and normalized to the cell density (OD_{600}) of the culture. IPTG and/or benzyl alcohol was added 2-3 h (OD ~0,5) after inoculation of the cultures.

Table 1 Strains used in this study

Strain	*Relevant features*	*Reference of source*
B. subtilis PY79	Wild type	Richard Losick Harvard University
B. subtilis PY79	pM11K_amyEBs	This study
B. subtilis PY79	Δ*yuaG*	Dempwolff *et al*., 2012
B. subtilis PY79	Δ*yuaG,* pM11K_amyEBs	This study
B. subtilis PY79	*amyE::*$P_{hyper\text{-}spank}$*-yuaFG,* pM11K_amyEBs	This study

Author contribution

MS has performed all experiments, and co-wrote the manuscript. KL and PLG supervised experiments, conceived of the study, and co—wrote the manuscript.

References

Babuke, T., & Tikkanen, R. (2007). Dissecting the molecular function of reggie/flotillin proteins. *European journal of cell biology*, *86*(9), 525-532.

Bach, J. N., & Bramkamp, M. (2013). Flotillins functionally organize the bacterial membrane. *Molecular microbiology*, *88*(6), 1205-1217.

Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., & Lodish, H. F. (1997). Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *Journal of Biological Chemistry*, *272*(21), 13793-13802.

Breukink, E., Demel, R. A., De Korte-Kool, G., & De Kruijff, B. (1992). SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. *Biochemistry*, *31*(4), 1119-1124.

Browman, D. T., Hoegg, M. B., & Robbins, S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. *Trends in cell biology*, *17*(8), 394-402.

Dempwolff, F., Möller, H. M., & Graumann, P. L. (2012). Synthetic motility and cell shape defects associated with deletions of flotillin/reggie paralogs in *Bacillus subtilis* and interplay of these proteins with NfeD proteins. *Journal of bacteriology*, *194*(17), 4652-4661.

Dempwolff, F., Schmidt, F. K., Hervás, A. B., Stroh, A., Rösch, T. C., Riese, C. N., ... & Graumann, P. L. (2016). Super resolution fluorescence microscopy and tracking of bacterial flotillin (Reggie) paralogs provide evidence for defined-sized protein microdomains within the bacterial membrane but absence of clusters containing detergent-resistant proteins. *PLoS genetics*, *12*(6), e1006116.

Doherty, G. J., & McMahon, H. T. (2009). Mechanisms of endocytosis. *Annual review of biochemistry*, *78*(1), 857-902.

Donovan, C., & Bramkamp, M. (2009). Characterization and subcellular localization of a bacterial flotillin homologue. *Microbiology*, *155*(6), 1786-1799.

García-Fernández, E., Koch, G., Wagner, R. M., Fekete, A., Stengel, S. T., Schneider, J., ... & Lopez, D. (2017). Membrane microdomain disassembly inhibits MRSA antibiotic resistance. *Cell*, *171*(6), 1354-1367.

Ge, L., Qi, W., Wang, L. J., Miao, H. H., Qu, Y. X., Li, B. L., & Song, B. L. (2011). Flotillins play an essential role in Niemann-Pick C1-like 1-mediated cholesterol uptake. *Proceedings of the National Academy of Sciences*, *108*(2), 551-556.

Hendrick, J. P., & Wickner, W. (1991). SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the Escherichia coli plasma membrane. *Journal of Biological Chemistry*, *266*(36), 24596-24600.

Hinderhofer, M., Walker, C. A., Friemel, A., Stuermer, C. A., Möller, H. M., & Reuter, A. (2009). Evolution of prokaryotic SPFH proteins. *BMC Evolutionary Biology*, *9*(1), 1-18.

Huang, X., Gaballa, A., Cao, M., & Helmann, J. D. (1999). Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor, σW. *Molecular microbiology*, *31*(1), 361-371.

Kamel, M., Löwe, M., Schott-Verdugo, S., Gohlke, H., & Kedrov, A. (2022). Unsaturated fatty acids augment protein transport via the SecA: SecYEG translocon. *The FEBS journal*, *289*(1), 140-162.

Kedrov, A., Kusters, I., Krasnikov, V. V., & Driessen, A. J. (2011). A single copy of SecYEG is sufficient for preprotein translocation. *The EMBO journal*, *30*(21), 4387-4397.

Koch, S., de Wit, J. G., Vos, I., Birkner, J. P., Gordiichuk, P., Herrmann, A., ... & Driessen, A. J. (2016). Lipids activate SecA for high affinity binding to the SecYEG complex. *Journal of Biological Chemistry*, *291*(43), 22534-22543.

Kuwahara, Y., Unzai, S., Nagata, T., Hiroaki, Y., Yokoyama, H., Matsui, I., ... & Hiroaki, H. (2009). Unusual thermal disassembly of the SPFH domain oligomer from Pyrococcus horikoshii. *Biophysical journal*, *97*(7), 2034-2043.

Langhorst, M. F., Reuter, A., & Stuermer, C. A. O. (2005). Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. *Cellular and Molecular Life Sciences CMLS*, *62*(19), 2228-2240.

Lee, Y. H., Kingston, A. W., & Helmann, J. D. (2012). Glutamate dehydrogenase affects resistance to cell wall antibiotics in *Bacillus subtilis*. Journal of bacteriology, 194(5), 993-1001.

Lill, R., Dowhan, W., & Wickner, W. (1990). The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell*, *60*(2), 271-280.

López, D., & Kolter, R. (2010). Functional microdomains in bacterial membranes. *Genes & development*, *24*(17), 1893-1902.

Ludwig, A., Otto, G. P., Riento, K., Hams, E., Fallon, P. G., & Nichols, B. J. (2010). Flotillin microdomains interact with the cortical cytoskeleton to control uropod formation and neutrophil recruitment. *Journal of Cell Biology*, *191*(4), 771-781.

Lv, X., Zhang, C., Cui, S., Xu, X., Wang, L., Li, J., ... & Liu, L. (2020). Assembly of pathway enzymes by engineering functional membrane microdomain components for improved N-acetylglucosamine synthesis in *Bacillus subtilis*. *Metabolic Engineering*, *61*, 96-105.

Mielich-Süss, B., Schneider, J., & Lopez, D. (2013). Overproduction of flotillin influences cell differentiation and shape in *Bacillus subtilis*. *MBio*, *4*(6), e00719-13.

Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., ... & Noirot, P. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, *335*(6072), 1103-1106.

Resnik, N., Sepčić, K., Plemenitaš, A., Windoffer, R., Leube, R., & Veranič, P. (2011). Desmosome assembly and cell-cell adhesion are membrane raft-dependent processes. *Journal of Biological Chemistry*, *286*(2), 1499-1507.

Schneider, J., Klein, T., Mielich-Süss, B., Koch, G., Franke, C., Kuipers, O. P., ... & Lopez, D. (2015). Spatio-temporal remodeling of functional membrane microdomains organizes the signaling networks of a bacterium. *PLoS genetics*, *11*(4), e1005140.

Schulte, T., Paschke, K. A., Laessing, U., Lottspeich, F., & Stuermer, C. A. (1997). Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development*, *124*(2), 577-587.

Taufik, I., Kedrov, A., Exterkate, M., & Driessen, A. J. (2013). Monitoring the activity of single translocons. *Journal of Molecular Biology*, *425*(22), 4145-4153.

Tavernarakis, N., Driscoll, M., & Kyrpides, N. C. (1999). The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. *Trends in biochemical sciences*, *24*(11), 425-427.

van Tilburg, A. Y., Warmer, P., van Heel, A. J., Sauer, U., & Kuipers, O. P. (2022). Membrane composition and organization of *Bacillus subtilis* 168 and its genome-reduced derivative mini *Bacillus* PG10. *Microbial biotechnology*, *15*(5), 1633-1651.

Zhang, Y.-M., and Rock, C. O. (2008). Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. 6, 222–233.

Zielińska, A., Savietto, A., de Sousa Borges, A., Martinez, D., Berbon, M., Roelofsen, J. R., ... & Scheffers, D. J. (2020). Flotillin-mediated membrane fluidity controls peptidoglycan synthesis and MreB movement. *Elife*, *9*, e57179.

3 General Discussion

Bacillus subtilis, a model organism for Gram-positive bacteria, is widely known for its capacity as a host for expression and secretion of various industrially relevant proteins (Pham *et al*., 2019). This proteobacteria is highly valued for advantages like easy and inexpensive culturing methods, high cell densities, GRAS status and efficient secretion processes due to the absence of an outer membrane. Of the known secretory routes, the Sec-pathway can direct the majority of secretory proteins to the growth medium (Green & Mecsas, 2016). The path of a protein to the outside of the cell is a process that can be divided into multiple stages, from the gene to the passage through the membrane and the cell wall. For almost every step in the production and secretion, bottlenecks are investigated and approaches aimed at optimization are pursued.

Signal peptides, of which there are well over 100 in *B. subtilis* (Brockmeier *et al*., 2006), enable proteins to be recognized by the export machinery (von Heijne, 1990). To optimize this part of the secretion process, extensive high-throughput screening approaches were pursued to find the ideal signal peptide, which lead to an improved yield of several target proteins (Degering *et al*., 2010). It is however impossible to predict which signal peptide would result in better secretion of a given extracellular target protein (Degering *et al*., 2010). Since SecA can bind the signal peptide of a pre-protein and transfer it to the translocon (Gelis *et al*., 2007; Douville *et al*., 1995), it is also a popular research target. Secretion of some proteins was improved via truncation of SecA's not well conserved C-terminal (Kakeshtia *et al*., 2010) and elevated levels of SecA have been reported to enhance the yield of secreted levansucrase in *B. subtilis* (Leloup *et al*., 1999). Furthermore, a hybrid protein comprised of SecA from *B. subtills* and SecB from *E. coli* was able to improve protein export of SecB-dependent proteins in *B. subtilis.* The SecYEG protein-conducting channel, consisting of the proteins SecY, SecE and SecG, is facilitating the translocation through the membrane (Manting *et al*., 2000). Increasing the amount of translocons in the membrane to boost translocation has been tried multiple times with varying success. While some report a considerable increase in protein secretion (Mulder *et al*., 2013), others observed only marginal benefits (Chen *et al*., 2015) or even a reduction in secretion efficiency upon *secYEG* overexpression (Ma *et al*., 2018). Following the translocation through the membrane, the signal peptide of the secreted protein is removed by a signal peptidase. The remnant signal peptides left behind are then digested by proteases like SppA (Ichihara *et al*., 1984). Interestingly, Cai *et al*., 2017 demonstrated that protein secretion dramatically decreased in a *sppA* deficient strain, whereas extracellular protein yields were significantly increased in a strain overexpressing SppA, identifying an unexpected bottleneck. Since foreign proteins are often sensitive to degradation by the extracellular proteases of the host, the strategy of generating protease-deficient *B. subtilis* strains was able to increase the yield of multiple heterologous secreted proteins (Stephenson & Harwood, 1998; Zhang *et al*., 2005). In another approach to the same problem, Chen *et al*. (2015) overexpressed the periplasmic chaperone PrsA to facilitate and support the folding of secreted proteins, resulting in less degradation and an increased secretion yield. Additionally, the microenvironment of the cell wall plays an important role in influencing protein folding. Teichoic acids make up a large portion of the cell wall (Beveridge *et al*., 1982) and

determine its charge to a large extent (Perego *et al*., 1995). The *dlt*-operon is responsible for the D-alanylation of teichoic acids, which results in the neutralization of the negatively charged phosphates of the teichoic acids, with free amino groups, making the cell wall more positively charged (Perego *et al*., 1995, Lambert *et al*., 1975). Thus, deletion of the *dlt*-operon leads to a more negatively charged cell wall that allows further binding of metal cations (Beveridge *et al*., 1980; Petit-Glatron *et al*., 1993), which increases the yield of many secreted proteins, that depend on cations for folding (Hyyryläinen *et al*., 2000; Thwaite *et al*., 2002).

3.1 Cell wall hydrolases and secretion

Following the membrane, the cell wall is the next barrier for secreted extracellular proteins to overcome. The Gram-positive cell wall has been described to form a sieve-like meshwork, which allows diffusion of proteins up to a molecular weight of 25 kDa (Demchick & Koch, 1996). This is not problematic for smaller (5 - 6 kDa) secreted antimicrobial peptides such as EpeX and sublancin (Butcher *et al*., 2007; Paik *et al*., 1998) or the extracellular nuclease NucB with a molecular weight of only 14.8 kDa (Akrigg, 1978). However, numerous secreted proteins would be considered too large to easily pass through the cell wall, exceeding the threshold of 25 kDa, such as the alkaline protease AprE at 39 kDa (Stahl & Ferrari 1984) or the α-amylase AmyE with 72.6 kDa (Yang *et al*., 1983). Although many aspects of the secretion process have been thoroughly investigated, the passage through the cell wall and the proteins that affect it have received not as much attention (Forster & Marquis, 2012).

Autolysins are bacteriolytic enzymes that digest the cell wall (Shockman & Hölltje, 1994) and are universally found in all bacteria that possess peptidoglycan (Shockman et al., 1996; Shockman *et al*., 1994). Among the many cell wall hydrolases, the N-acetylmuramoyl-L-alanine amidase LytC and the N-acetyl-glucosaminidase LytD are the most significant autolysins, accounting for around 95% of the autolytic activity of the cell (Kuroda & Sekiguchi, 1991; Lazarevic *et al*., 1992). LytC is involved in a variety of cell functions like sporulation (Smith & Foster, 1995), cell separation, cell wall turn-over (Blackman *et al*., 1998) and together with LytD, swarming motility (Yamamoto *et al*., 2003). LytF is an α γ-D-glutamate *meso*-diaminopimelate muropeptidase, that acts as the primary autolysin involved in vegetative daughter cell separation (Margot *et al*., 1999, Chen *et al*., 2009). Also involved in shaping the cell wall structure is the major D-alanyl-D-alanine carboxypeptidase PBP5 (*dacA)* of vegetative *B. subtilis* cells (Lawrence & Strominger, 1970). PBP5 can cleave the C-terminal D-Ala-D-Ala peptide bond of the peptidoglycan stem peptide, which prevents the formation of a crosslinking peptide bridge between the glycan strands (Lawrence & Strominger, 1970).

This work investigates the role of cell wall hydrolyzing enzymes and their impact on secretion efficiency in the Gram-positive model organism *B. subtilis*. Since the meshwork of the cell wall represents a hurdle in the secretion process, we hypothesized that cell wall hydrolyzing activity

should lead to a more permeable barrier and thus increase secretion. To test this hypothesis, deletion mutants of the main autolysins LytC, LytD and LytF were created and the effect of the missing cell wall hydrolyzing activity on secretion was analyzed. For this purpose, the commonly used α-amylase AmyE was selected as a model secreted protein, expressed through a non-integrating plasmid. While the Δ*lytD* mutation showed no significant impact on AmyE secretion, both Δ*lytF* and Δ*lytC* resulted in a significant loss of 15% and 50% amylase activity in the supernatant. Although results regarding Δ*lytF* and Δ*lytC* are in line with our hypothesis, the question arises why no significant impact on secretion was detected for Δ*lytD*. The abundance of autolysins leads to a great number of functional redundancies (Smith *et al*., 1996). This is also the case for LytD, which makes it challenging to measure any effect of Δ*lytD*, given that *lytC* can compensate for this deletion (Blackman, 1998). Overall, the results show that the deletion of genes encoding autolysins and the consequently reduced cell wall hydrolyzing activity leads to a diminished secretion efficiency of AmyE in *B. subtilis*.

Contrary to these findings, two reports show that reducing autolysis in *B. subtilis* via deletion of *lytC* can lead to increased secretion of recombinant proteins, by maintaining more stable growth over a longer period of time (Kabisch *et al*., 2013; Wang *et al*., 2014). Since the elevated biomass of the mutant strains was not considered in these assessments, it can be concluded that the increased secretion was caused by higher cell densities producing more product protein, while secretion processes were not improved. Other studies suggest that inactivation of the main autolysins LytC, LytD or LytF does not affect cell growth (Margot & Karamata, 1992; Margot *et al*., 1994), indicating that the positive effects on growth could be dependent on additional factors. Furthermore, inactivation of LytC has also been reported to impede the secretion of proteins by *B. subtilis*, supporting our findings (Smith *et al*., 2000; Zobel *et al*., 2015). Here, an OD-normalized secretion assessment was applied, accounting for possible effects on growth. This approach was also used in this work. Our hypothesis is further substantiated by the fact that strains with impaired cell wall hydrolase activity exhibit a thicker cell wall than wild type strains (Fan *et al*., 1971). A thicker cell wall could be reasonably considered an increased hindrance for protein secretion and consequently should lead to decreased secretion efficiency. Confirming this concept is the work of Williamson and Ward (1981), who reported that reduced autolytic activity in *B. subtilis* as well as *Streptococcus pneumoniae* will lead to a decreased permeability of the cell wall.

Subsequently, we wondered whether an increase in cell wall hydrolyzing activity through the overexpression of corresponding enzymes would improve the secretion capacity of cells. Additional cell wall hydrolase genes under the control of inducible promoters were ectopically inserted into the native *amyE*-locus of *B. subtilis*. The genes *lytF* and *dacA* were expressed using the IPTG-inducible promoter $P_{hyper\text{-}spank}$ while *lytC* was expressed using the xylose-inducible promoter P_{xyl}. Since autolysins are bacteriolytic enzymes that digest the cell wall peptidoglycan, their overexpression can potentially lead to cell lysis. Previous studies demonstrated unproblematic overexpression of *lytF* (Margot *et al*., 1999) and *dacA* (Scheffers *et al*., 2004), so expression through $P_{hyper\text{-}spank}$ was considered feasible without risking cell lysis. Overexpression of *lytC* on the other hand can

reportedly result in excessive autolysis (Wang *et al*., 2018), hence the P_{xyl} promoter system was selected, as it leads to weaker expression levels and less leakiness in comparison to $P_{hyper\text{-}spank}$ (Vavrová *et al*. 2010). Expression was induced by addition of 0.5 mM IPTG or 0.25% (v/v) xylose respectively, 3 h after inoculation. Upon induction of the expression, strongly increased amylase activity was detected in the supernatant in all three cases. Additional expression of *lytF* resulted in a 70% increase of AmyE secretion, while 150% was detected for *lytC* and 225% for *dacA*. These results show a tremendous improvement in secretion performance and strengthen our hypothesis that increased cell wall hydrolytic activity leads to a more permeable cell wall and facilitates passage for secreted proteins.

Multiple reports of *B. subtilis* strains with inactivated protease genes, exhibiting increased susceptibility to autolysis indicate that autolysins are post-translationally regulated (Cho *et al*., 2004; Coxon *et al*., 1991). Primarily responsible for this control of autolytic activity in *B. subtilis are the* proteases NprE and AprE (Stephenson *et al*., 1999). Notably, another study investigating a *B. subtilis* mutant with reduced protease activity, found increased levels of autolytic activity and secretion (Fahnestock & Fisher 1987). This case provides early evidence for the positive connection between increased autolytic activity and improved secretion, described in this work.

Interestingly, the additionally introduced *lytC* and *dacA* genes also have had positive effects on AmyE secretion even without induction, whereas no such effect was detected for *lytF*. It is reasonable to assume that the observed effect was caused by the leakiness of the implemented promoter systems. The additional enzymes produced this way may already have been sufficient to increase the secretory capacity for AmyE in the *B. subtilis* strains. The additional *lytF* expressed through leakiness without IPTG induction has not produced a similar effect. Deletion and overexpression of *lytF* impacted protein secretion to a lesser extent compared to the other enzymes, indicating that small amounts of *lytF* expressed through promoter leakiness would also influence secretion to a lesser extent.

Many factors might influence cell wall permeability, such as electrostatic interactions (Ou & Marquis, 1970), the average length of glycan chains (Vollmer *et al*., 2008) and even the size of the secreted protein (Leloup *et al*., 1997). Levansucrase (50 kDa) is translocated twice as fast, as the larger α-amylase (69 kDa) (Leloup *et al*., 1997). Additionally, 20% of the peptide chains are cross-linked in *B. subtilis* (Ward, 1973; Atrih *et al*.,1998; Hayhurst *et al*., 2008), which probably also influences cell wall permeability. Considering the cell wall modulating effects of PBP5, we advocate that the extra enzyme activity reduced the number of cross-linkable stem peptides, which increased the peptidoglycan mesh size and thus made the cell wall more permeable for the secreted amylase. Interestingly, overexpression of *dacA* in the Gram-negative bacterium *Escherichia coli* has also been shown to increase amylase yield in the supernatant (Yang *et al*, 2022). Although the positive effect in that case was attributed to increased permeability of the outer membrane and it remains unclear how the outer membrane was influenced by PBP5.

LytF was shown to be responsible for cell separation leading to single cells when expressed rather than long chains (Ohnishi *et al*., 1999; Chen *et al*., 2009). This is potentially relevant for its effect on secretion since it is speculated for *S. pyogenes* that protein transport across the cell wall might be more efficient at sites of bacterial division (Rosch & Caparon, 2004; Forster & Marquis, 2012). Overexpression of *lytF* results in less chain formation and more single cells, providing more free division sites from which to secrete, potentially explaining the beneficial effect of LytF on secretion. However, in this work it was shown through fluorescence microcopy, that AmyE is not increasingly secreted at the division sites in *B. subtilis*, contradicting this theory. Immunofluorescence microscopy showed LytF localizing at the pole and the septum dependent on the presence of WTAs, which inhibit the sidewall localization (Yamamoto *et al*., 2008). Though, it is unclear whether this confined localization of LytF is still maintained upon overexpression.

Alterations in cell wall permeability could be investigated, as well as the effects of other cell wall hydrolyzing enzymes such as LytE (Margot *et al*., 1998) and PBP4a (*dacC*) (Pedersen *et al*., 1998). These investigations could further verify our ideas regarding the mechanism responsible for the increased secretion efficiency by additional cell wall hydrolysis activity. Since autolysins are bacteriolytic enzymes that digest the cell wall peptidoglycan, they can lead to cell lysis and are therefore usually associated with a reduction in cell biomass and fermentation efficiency (Westers *et al*., 2003). Therefore, it is possible that increased autolysis may counteract the beneficial effects of cell wall hydrolases on secretion efficiency during fermentation and additional protective measures for osmotic stabilization should be considered.

Most research investigating possible improvements to protein secretion through the cell wall is focused on protein folding (Stephenson *et al*., 1998; Forster & Marquis 2012) either through chaperones like PrsA (Vitikainen *et al*., 2001; Hyyrylainen *et al*., 2010) or the net charge attracting divalent metal cations (Perego *et al*., 1995; Hyyrylainen *et al*., 2010). Despite mutants with reduced autolytic activity reportedly exhibiting a decreased cell wall permeability (Williamson & Ward 1981), the possibility of increasing permeability through additional genes of autolysins was yet to be explored. In this work, we showed that secretion of AmyE, an example of a large secreted protein with a size of 72.6 kDa, can be increased by induced expression of a second gene copy of the cell wall hydrolases LytC, LytF or PBP5. The resulting improvements of up to 225% increased secretion represents an overwhelming success of a novel strategy. As this approach seemingly improves cell wall permeability, a moderate increase of autolysin activity may be applicable for the enhancement of secretion of any protein of interest. Furthermore, cell wall hydrolases are found in all bacteria with peptidoglycan, thus a great potential to extend the approach can be anticipated.

3.2 Flotillins and secretion

Flotillins were first discovered in the cell membrane of eukaryotes (Schulte *et al*., 1997), but have since been established as an evolutionarily conserved class of proteins found across all organisms (Hinderhofer *et al*., 2009). Flotillins are characterized by their conserved SPFH domain (Browman *et al*., 2007) and play a role in sporulation processes (Donovan & Bramkamp, 2009), cell shape maintenance and cell division (Dempwolff *et al*., 2012). In the case of *B. subtilis* there have been two flotillin homologs identified, FloT (*yuaG*) and FloA (*yqfA*), which were co-isolated from DRM fractions together with the secretory protein SecY (Lopez & Kolter, 2010). Based on this association, it has been suggested that flotillins affect protein secretion.

The main flotillin in *B. subtilis* is FloT, while the functions of FloA are partially redundant (Lopez & Kolter, 2010). To explore the effects of FloT on protein secretion a *B. subtilis* Δ*yuaG* strain with plasmid-based AmyE overproduction was constructed. Our results show that the absence of the FloT protein leads to a decreased secretory capacity, as evidenced by lower amylase activity in the supernatant. This solidifies the previous findings of Bach & Bramkamp 2013, who showed a general reduction of protein secretion in the flotillin deletion strains Δ*yuaG,* Δ*yqfA* and Δ*yuaG*Δ*yqfA*. In order to gain information about the mechanisms involved, we aimed to raise the protein secretion of the mutant strain back to wild type levels. Zielińska *et al*. (2020) were able to compensate for reduced membrane fluidity in the Δ*yuaG* mutant by adding 0.1% of the membrane fluidizer benzyl alcohol. This compound can anchor in the membrane via its benzyl ring, which leads to an increase in membrane fluidity without changing the lipid composition (Chin & Goldstein, 1977; Goldstein, 1984). Consequently, we investigated if the addition of benzyl alcohol could recover the negative impact of the Δ*yuaG* deletion on secretion. Our results show that the addition of this membrane fluidizer could in fact recover the AmyE secretion level of the wild type, in the Δ*yuaG* mutant strain. Since the lowered secretion efficiency could be compensated by the addition of the membrane fluidizer benzyl alcohol, we propose that the negative impact of the Δ*yuaG* deletion on secretion is caused by a reduction in membrane fluidity.

Furthermore, it was reported that overexpression of FloT and NfeD2 resulted in an altered membrane fluidity (Lee *et al*., 2012). Therefore, we pursued the approach of introducing an additional and inducible *yuaG* gene into *B. subtilis*, to improve protein secretion. Interestingly just the introduction of the additional gene, without the induction, resulted in the amylase activity in the supernatant doubling compared to the wild type. Overproducing FloT by induction with 1 mM IPTG did not result in further improvement of secretion processes. This leads us to believe that the additional FloT protein expressed via the leakiness of the $P_{hyper\text{-}spank}$ promoter system was sufficient to fully exhaust the beneficial effect of FloT on the secretion of AmyE. Considering the results gained via the deletion strains, we suggest that FloT regulates membrane fluidity which can be enhanced through additional FloT to improve protein secretion. However, it must be considered that while the reduction of membrane fluidity upon deletion of *yuaG* has been demonstrated, the enhancement of membrane fluidity through additional FloT was not tested for. To clarify this part of the hypothesis,

the membrane fluidity of the *B. subtilis amyE::P*$_{hyper\text{-}spank}$*-yuaFG* strain could be determined in future experiments via the LAURDAN fluorescent dye (Parasassi *et al*., 1997; Harris *et al*., 2002).

Since eucaryotic flotillins are established to act as scaffolding proteins in defined membrane domains (Langhorst *et al*., 2005; Ludwig *et al*., 2010; Resnik *et al*., 2011), the view that their bacterial counterparts must fulfill a similar role is very common (Lopez & Kolter, 2010; Lopez & Koch, 2017). Like their eukaryotic counterparts, the two flotillin homologs of *B. subtilis*, FloT and FloA, have also been detected in DRM fractions along with NfeD proteins, signaling receptor KinC, cell wall hydrolase PBP5, secretory protein SecY and many more, supporting the lipid raft or functional membrane microdomain (FMM) hypothesis (Lopez & Kolter, 2010; Bach & Bramkamp, 2013; Bramkamp & Lopez, 2015; Lopez & Koch, 2017). FMMs are enriched in isoprenoid lipids (García-Fernández *et al*., 2017; Lopez & Kolter, 2010) and display increased order and thickness compared with the surrounding bilayer (van Tilburg *et al*., 2022). Bacterial flotillins do not recruit specific, more rigid lipids, such as hopanoids and carotenoids, associated with FMMs, but are themselves being recruited as flotillins preferentially bind to these lipids and oligomerize in the corresponding domains (Bramkamp & Lopez, 2015; García-Fernández *et al*., 2017). However, super-resolution microscopy showed that the flotillins and other proteins found in DRMs do not colocalize and have different dynamic behaviors (Dempwolff *et al*., 2016). Others question the method of DRM extraction, arguing that it is an artificial process that does not reflect native membrane organization (Brown, 2006; Scholz *et al*., 2021). Additionally, flotillins appear to be required for the formation of regions with increased fluidity, which are the counterpart to the rigid and less fluid FMMs (Zielinska *et al*., 2020). Our results demonstrate that the connection between FloT and protein secretion is most likely based on the flotillin's effect on membrane fluidity. However, we cannot rule out the possible involvement of lipid rafts, as benzyl alcohol can show strong effects on membrane organization and especially the formation of lipid rafts (Peters, 2008). There are various raft-acting drugs, typically characterized as those that decrease membrane fluidity, induce ordering and lead to lipid raft formation and others that increase membrane fluidity, induce disordering and lead to lipid raft disruption (Tsuchiya & Mizogami, 2020). These raft-acting drugs could be used to further investigate the connections of FloT, secretion and FMMs, as they are reported to severely affect raft-associated protein localization (Hering *et al*., 2003). Although it would be difficult to separate effects specific to FMMs from the general influence on membrane fluidity.

Membrane fluidity depends on its lipids with a special importance on the fatty acid composition. While saturated fatty acid acyl chains can be tightly packed, unsaturated fatty acid's rigid kink of the cis double bond leads to much poorer chain packing due to the steric hindrance (Mansilla *et al*., 2004; Vigh *et al*., 1998; de Mendoza & Cronan, 1983). In *E. coli* low levels of anionic phospholipids in the membrane, like phosphatidylglycerol and cardiolipin, reduce membrane fluidity (Nenninger *et al*., 2014) and weaken SecYEG translocon stability and integrity (Ryabichko *et al*., 2020). Correspondingly secretion of AmyE, as well as other proteins, can be greatly reduced by inhibiting fatty acid synthesis in microorganisms (Paton, 1979). Furthermore, a strain with a modulated

membrane composition resulting in an enhanced membrane fluidity showed improved membrane insertions of membrane proteins (Kanonenberg *et al*., 2019). SecA anchors in the lipid bilayer interface with its amphipathic N-terminal helix, which activates SecA for high affinity binding to the translocon (Lill *et al*., 1990; Koch *et al*., 2016). Here the membrane composition is of great importance since unsaturated fatty acids cause loose packing of lipid head groups, where the N-terminal amphipathic helix of SecA docks (Breukink *et al*., 1992). Kamel *et al*. (2022) could recently enhance protein secretion *in vitro* via an artificial membrane with increased levels of unsaturated fatty acids. In this study, the less tightly packed and more fluid membrane promoted SecA binding to the membrane, which stimulated SecA:SecYEG-mediated protein translocation. The presence of flotillins reportedly enhanced the fluidity of a model membrane and *in vivo*, the membranes of flotillin-mutant cells are less fluid (Zielińska *et al*., 2020). Along with our findings, this leads us to believe that the positive impact of additional FloT on AmyE secretion is likely a result of increased membrane fluidity, which in turn results in a stimulated SecA:SecYEG-mediated protein translocation through a promoted SecA binding to the membrane.

However, no changes in the overall fatty acid composition of the membranes have been detected in the Δ*yuaG* mutants, suggesting fluidity was impacted through changes in the lipid ordering (Zielinska *et al*., 2020). Also, it should be noted that while the phospholipid composition of the *B. subtilis* membrane remains relatively unchanged during growth (Gidden *et al*., 2009), FloT is increasingly expressed upon entry into the stationary phase (Huang *et al*., 1999). This growth phase marks also the beginning of an enhanced secretion capacity, as shown in this work, further reinforcing the favorable connection of flotillins and protein secretion. The mechanism by which FloT influences membrane fluidity is currently not known. Nevertheless, increased membrane mobility should improve SecA binding, even if it is not caused by increased unsaturated fatty acid levels. However, further testing needs to be conducted in order to confirm whether the hypothesis of FloT indirectly affecting SecA binding is correct. Admittedly, since membrane fluidity affects so many processes, it is not that simple to determine what exactly leads to the observed effects. Interestingly, even the diffusion of membrane-targeted mRNAs is affected by membrane fluidity, altering the translation of inner membrane proteins (Bergmiller *et al*., 2022).

3.3 The cell wall passage

Most proteins intended for secretion feature an N-terminal signal peptide that enables targeting by the secretion system (Tjalsma *et al*., 2000) and are translocated in an unfolded state via the general secretory (Sec) pathway (Harwood & Cranenburgh, 2008). Here they are transported through the cytoplasmic membrane via the hourglass-shaped translocon complex SecYEG, (van den Berg *et al*., 2004), a process catalyzed by SecA (Driessen & Nouwen, 2008). On the other side, the signal peptide of the secreted protein is removed by a signal peptidase and the protein is released from the membrane (van Roosmalen *et al*., 2004). After overcoming the membrane, the passage through the cell wall is the next barrier for extracellular proteins which was described as a rate-limiting step in the secretion of the α-amylase in *B. subtilis* (Leloup *et al*., 1997; Haddaoui *et al*., 1999). Whereas the translocation of proteins across the cell membrane has been thoroughly investigated, it is still unclear how proteins cross the cell wall in Gram-positive bacteria.

Since there is no evidence of channels facilitating active transport of proteins across the cell wall, several different hypotheses have been proposed. One of them is based on the link between flagella and the secretion of proteins. Disruption of the *flgE* gene, encoding a flagellar hook protein in *B. subtilis*, caused inhibition of mobility and a striking 200–300% increase in α-amylase production yield (Fehler *et al*., 2022). Also, the P_{class2} and P_{class3} promoters associated with flagellar genes in *S. typhimurium* (Chilcott & Hughes, 2000) are continuously regulated in response to protein secretion rates (Brown *et al*., 2008). These indicators lead to the speculation that proteins could be released from the (damaged) flagella, using the breach in the cell wall necessary for the flagella (Tjalsma *et al*., 2004). Also, passive leakage of secreted proteins through damaged surface areas or leakage sites has been reported by Tao *et al*. (2020). Although this phenomenon closely resembles lysis and is consequently perceived as such (Osamura *et al*., 2023). As suggested by the location of the Sec translocon in *S. pyogenes*, protein transport across the cell wall might be more efficient at sites of bacterial division (Rosch & Caparon, 2004). Naturally, it is also possible in *B. subtilis* or other bacteria, that the poles are involved in secretion, although this has not been substantiated with further evidence. Another hypothesis revolves around the turn-over of the Gram-positive cell wall, which can reach a rate of 50% per generation during growth (Koch and Doyle, 1985). The inside-to-outside model suggests that autolysins hydrolyze and remove the oldest wall material from the outer surface, to allow newly synthesized peptidoglycan to expand and become stress-bearing (Graham & Beveridge, 1994). Cell wall-bound proteins are as a result of cell wall turn-over simply released into the surrounding (Antelmann *et al*., 2002). Hence the idea, that secreted proteins may be incorporated into the newly synthesized cell wall, to be later released with old peptidoglycan through the turn-over effect. Also, common is the view that the cell wall allows proteins to traverse the cell wall by simple diffusion, since it is considered a porous peptidoglycan structure or a sieve-like meshwork (Demchick & Koch, 1996). To investigate the process of passage through the cell wall, we have studied the secretion of α-amylase AmyE within two different *Bacillus* strains, *B. subtilis* and *B. licheniformis*.

3.3.1 Protein secretion zones

To advance the understanding of the location and dynamics of secretion, focusing on cell wall passage, we sought to visualize this process using fluorescence microscopy. During secretion, the cytosol and membrane are followed by the oxidizing environment of the periplasm, which promotes disulfide bridge formations (Missiakas & Raina, 1997), impairing folding of fluorophores with cysteine residues and preventing fluorescence (Feilmeier *et al*., 2000). Therefore, we used the fluorophore mCherry, which does not feature any cysteine (Shaner *et al*., 2004) and is described as foldable and fluorescent in the bacterial periplasm (Dinh & Bernhardt 2011). Since amylases represent one of the most important enzyme groups within the field of biotechnology, they are commonly used as model secreted proteins in scientific research (Naidu & Saranraj, 2013; Yan & Wu, 2017). We generated a fusion of AmyE-mCherry expressed via constitutive promoter from a high copy plasmid in *B. subtilis* and *B. licheniformis*. Microscopy revealed punctate fluorescent AmyE-mCherry signals associated with the periphery of the cells, during the transitional growth phase. As the fusion is targeted by the Sec-system, it remains in an unfolded, secretion-competent state, until translocated out of the cytosol. This indicates that the fluorescent signal detected is localized either in the periplasm or cell wall. When cells expressing AmyE-mCherry were treated with lysozyme, they lost their cell wall and were forming spheroplasts, while the detected fluorescence diminished drastically. This further suggests that the detected signal originates from AmyE-mCherry molecules that are on their way out of the cells. However, with the spatial resolution available, it was not possible to clearly distinguish between signals in the cell wall and in the periplasm. The periplasmic space, first visualized by transmission electron cryo-microscopy (CryoTEM) in *B. subtilis* (Matias & Beveridge, 2005), contains proteins, small molecules, and membrane-anchored lipoteichoic acids (Reichmann & Grundling, 2011; Matias & Beveridge, 2008). It has been previously suggested that the periplasm is gel-like and diffusion opportunities are limited due to molecular crowding (Hahn *et al*., 2021). However, recently it has been shown that DNA taken up by *B. subtilis* cells during the state of competence can freely diffuse or at least move with high velocity through the periplasm (Burghard-Schrod *et al*., 2022). Consequently, the observation of discrete foci strongly argues against accumulation within the periplasm, since the protein would diffuse freely in this space and not form foci. However, ultimately our experiments cannot rule out, that some visualized AmyE-mCherry molecules are within the periplasm.

When evaluating these results, it must be considered, that the AmyE-mCherry fusion used to visualize secretion processes is overproduced from a plasmid. Therefore, the fluorescent foci shown could possibly be the result of accumulation of aggregated proteins. As we see massive activity of AmyE-mCherry in culture supernatants, it is unlikely that the foci are aggregated protein, but suggests that foci contain active amylase-mCherry fusions. The foci could also be interpreted as evidence of an overburdened secretion process, but since the cells do not show any defects or abnormal growth, we safely assume that they are not overly stressed and the secretion system is not overwhelmed. To further verify the results indicating that AmyE passes the cell wall in different secretion zones, we used microscopy in combination with starch-BODIPY-FL. This labeled

substrate becomes fluorescent upon “digestion” by an amylase, in this case AmyE. Coating *B. subtilis* cells with this substrate allows for the visualization of secreted AmyE emerging from the cell wall. Imaging shows that active enzyme exits from discrete patches, ruling out that AmyE-mCherry accumulation within secretion zones is entirely based on accumulation of aggregated proteins within the cell wall. The idea of secretion zones within the *Bacillus* cell wall implies that when amylase transits through the PG layers in defined zones, it should also emerge similarly from the cell envelope. That this holds true was demonstrated and visualized using the starch-BODIPY-FL substrate.

Furthermore, our results are in agreement with the findings of Campo *et al*. (2004) who also reported foci of AmyQ being secreted using immunofluorescence microscopy. Since in this case native amylase expression levels were used, it is unlikely that the AmyE-mCherry secretion zones are the result of overexpression. We propose that increased synthesis of AmyE allowed us to track the path of molecules, as opposed to a low production level, which does not allow tracking the passage of fewer molecules versus background fluorescence. Also, we favor the view that this also holds true for the secretion of proteins produced at wild type-level, i.e., not overexpressed molecules. Visualization of secreted proteins during their passage through the cell wall in living *B. subtilis* cells, represents a great novelty and fills an important knowledge gap.

3.3.2 Secretion is influenced by the growth phase

Early investigations of bacterial growth described different phases, 1. lag phase, 2. acceleration phase, 3. exponential phase, 4. retardation or transitional phase, 5. stationary phase and 6. phase of decline (Lane-Claypon, 1909; Monod, 1949). Coupled with the growth state of the cells, bacteria undergo a series of changes that affect morphology, metabolism (Buchanan, 1918; Clark & Ruehl, 1919) and gene expression (Klumpp *et al*., 2009). In *B. subtilis* examples of growth phase specific systems are cell division during exponential growth (Edwards & Errington, 1996), regulation of competence and motility during the transitional growth phase (Strauch & Hoch, 1993) and spore formation in the stationary growth phase (Errington, 2003).

Despite the fact that AmyE-mCherry is expressed via a constitutive promoter system, we noticed differences in secretion levels of the fusion over the growth period. Naturally, we were interested in determining at what time the protein is secreted in *B. subtilis* and *B. licheniformis.* So, the expression profile of *Bacillus* cells overproducing AmyE-mCherry during the growth cycle were determined, based on Western blots and fluorescence levels and amylase activity in the culture supernatant. The results showed that AmyE-mCherry is released at a higher rate at the transition into and during the stationary phase. Interestingly, the Western blots indicated a normal and constant expression of AmyE-mCherry via the constitutive promoter system. Therefore, although AmyE-mCherry is produced during the exponential phase, it is not efficiently secreted, whereas this is the case as soon as the cells enter the stationary phase. Our results fit the literature regarding increased secretion in

the transitional growth phase. Cells of both *Bacillus subtilis* and *Bacillus licheniformis* reportedly secrete the highest amount of proteins in the onset stationary growth phase (Voigt *et al*., 2008), which is also true for AmyE specifically (Hamada *et al*., 1967). Possibly related, this is also the point of maximum *secA* expression (Herbort *et al*., 1999), which would facilitate secretion events. In the stationary growth phase, the cell wall degradation is slowed down (Doyle *et al*., 1988), the peptidoglycan synthesis is down-regulated (Lam *et al*., 2009) and cell wall turn-over is negligible (Glaser & Lindsay 1977). In consideration of these circumstances, the hypothesis of a passage through the cell wall facilitated by the cell wall turn-over becomes much less likely for secreted proteins. Instead, these results give rise to the idea that a series of changes at the exit from exponential growth, possibly involving modification of the cell wall, is required for efficient high-level protein secretion and possibly normal protein secretion.

3.3.3 Subpopulation-dependent secretion

Heterogeneity in transcriptional expression of genes is a well-established phenomenon in bacteria (Graumann, 2006). In fact, populations of genetically identical *B. subtilis* usually comprise numerous distinct cell types. In addition to spores, cells can become genetically competent, motile, produce extracellular matrix or degradative enzymes, or secrete toxins that allow them to cannibalize their neighbors (Lopez *et al*., 2008). Also, the production of antibiotics has been shown to occur in a heterogeneous manner (Dewachter *et al*., 2019) and even DNA repair enzymes can be found in only a subset of exponentially growing cells, leading to heterogeneity of DNA damage response, based on extremely low numbers of molecules per cell (Uphoff *et al*., 2016). Likewise, cyclic-di-GMP signaling components of *B. subtilis* cells are found to be absent in a considerable subpopulation of cells, due to low abundance of proteins within the network (Kunz *et al*., 2020).

We sought to analyze the proportion of cells displaying AmyE-mCherry fluorescence using fluorescence microscopy. Interestingly, overproduction of AmyE-mCherry followed a very strong pattern of heterogeneity, with a maximum of 23% of cells showing AmyE-mCherry secretion zones during the transition phase, and 34% during stationary phase. Although antibiotics were used at all times to select for the overexpression plasmid, loss of the plasmid by a subpopulation could also account for the observed heterogeneity. Indeed, for plasmid-based production of proteins in *B. megaterium,* fluctuating plasmid abundance was observed, which resulted in population heterogeneity (Münch *et al*., 2015). Also, a recent review suggested that sigma factor σ^D may play a role in the overproduction of α-amylases which may be subpopulation dependent (Yan & Wu, 2019). This sigma factor may help to hold back the proteases HtrA and HtrB, which can degrade α-amylases (Darmon et al., 2002; Ploss et al., 2016).

The heterogeneity of cells displaying AmyE-mCherry signal was surprising because the heterogeneous expression pattern did not match the labeled components of the secretory machinery SecA and SecDF, which were detected homogeneously in the culture. However, despite the

homogeneous occurrence, widely varying numbers on the cellular concentration of SecA have been reported, ranging from 37 up to 13,000 SecA copies per cell (Oliver & Beckwith, 1982; Akita *et al*., 1991; Or *et al*., 2002; Seinen *et al*., 2021). Some of the variances of SecA numbers are likely related to the different methods used to evaluate the figures, e.g., radiolabeling, quantitative immunoblotting and microscopic single-molecule tracking. Although, large cell-to-cell differences are also found within the individual assessments, indicating some inherent heterogeneity of SecA molecule count. As SecA drives secretion (Cooper *et al*., 2008), cells with a particularly large amount of SecA would be expected to be capable of higher secretion levels. In the case of levansucrase, an increased level of SecA has been reported to significantly enhance the secretion yield (Leloup *et al*., 1999). However, another study found only a marginal improvement in AmyE secretion upon s*ecA* overexpression (Chen *et al*., 2015).

Naturally, the question arises as to how large a proportion of cells express AmyE-mCherry to begin with. By visualizing the AmyE-mCherry fusion lacking its signal peptide, we showed that about 50% of cells exhibit intracellular accumulation of an AmyE-mCherry fusion lacking a signal peptide, indicating that only half of the population actively expresses the protein. When considering these results, we come to the conclusion that not all cells that do express AmyE can efficiently secrete the protein. Interestingly, AmyE-mCherry lacking a signal peptide did not accumulate in a homogeneous manner within the cytosol, but often displayed membrane association. These observations suggest that synthesis of AmyE-mCherry occurs in a membrane-proximal manner. Translating ribosomes and mRNAs of membrane proteins have been previously localized close to the cell membrane and the polar regions of the cells (Lewis *et al*., 2000; Nevo-Dinur *et al*., 2011). These findings substantiate the hypothesis that proteins are synthesized close to their intended location (Buxbaum *et al*., 2015). Western Blot analyses revealed that AmyE-mCherry proteins missing the signal peptide were more heavily degraded than the full-length version of the fusion. Seemingly, proteins lacking the signal peptide are more prone to proteolysis within the cytosol than full-length proteins which are likely to be rapidly secreted.

We suspect that the subpopulation capable of efficient secretion exhibits a more permeable cell wall, which allows the overexpressed AmyE-mCherry to pass through the cell wall more easily. The cell wall protects the cell against environmental stress, from bursting due to internal turgor pressure and is responsible for cell shape (Silhavy *et al*., 2010). A culture entering stationary phase may thus be evolved to allow for a subpopulation of cells bears the risk of a more porous cell wall, in order to provide large amounts of extracellular enzymes for the rest of the population. Share of labor between cells is a well-studied phenomenon in bacteria. A clear example of this is the ability of *B. subtilis* to produce and secrete large quantities of extracellular proteases in only a fraction of cells when cultures reach the stationary phase (Veening *et al*., 2008). Although only a subpopulation expends energy for the production and secretion of these exoproteases, the whole community benefits. The small peptides derived from protein degradation can be used and metabolized by all cells, regardless of whether they belong to the subpopulation that actually produced the exoproteases (Msadek, 1999). Furthermore, the expression of both the *eps* and *yqxM* operons, responsible for

exopolysaccharide-rich (EPS) matrix production, follows reportedly a heterogeneous pattern (Chai *et al*., 2008; Vlamakis *et al*., 2008). Although the production of the extracellular matrix is observed in only a subpopulation, all the cells that form the biofilm are encased in this extracellular matrix and can benefit from it.

The results revealed that about 50% of cells produce AmyE-mCherry, as deduced from the non-secreted version of the fusion. And furthermore, only a subpopulation showed accumulation of AmyE-mCherry foci in the cell envelope. The data also suggests that AmyE-mCherry is rapidly secreted out of the cytosol, to accumulate within the periplasm and the cell wall, as deduced from the formation of fluorescent foci.

3.3.4 Dynamics of Secretion

The secretion machinery in *B. subtilis* is composed of a multitude of components (Simonen & Palva, 1993). The SecYEG translocon as well as the motor protein SecA seem to be organized in specific clusters in the membrane (Campo *et al*., 2004). Super-resolution microscopy revealed, that while a some SecA molecules are cytosolic, the protein is predominantly membrane-associated (Seinen *et al*., 2021). Another protein involved in secretion is SecDF, which is evenly distributed over the membrane (Rubio *et al*., 2005). SecA as well as SecY are dynamically moving through the cell membrane, forming assembling and disassembling oligomers, suggesting secretory events (Dajkovic *et al*., 2016; Koch *et al*., 2021).

While the localization and dynamic of membrane-associated components of the secretion machinery are well researched, this is not the case for secreted proteins. Therefore, we utilized time lapse imaging to track dynamic movement of AmyE-mCherry and the components of secretion machinery SecA and SecDF. Unfortunately, a functional SecYEG fusion could not be generated for this task. Presumably, a fusion with a fluorophore creates spatial interferences with interaction partners, as others have reportedly been unsuccessful as well (Dajkovic *et al*., 2016). The experiments showed that AmyE-mCherry foci remain statically positioned for many minutes and do not display lateral mobility within the cell. This supports our view, that the fusion proteins are in the process of passing through the cell wall and are not highly mobile or still connected to the membrane-bound secretion machinery. Even slow-diffusing membrane proteins forming large clusters such as the flotillin FloT, diffuse throughout the entire cell membrane of *B. subtilis* cells in a time-scale of 1.5 minutes (Dempwolff *et al*., 2016). SecA-mNeonGreen also showed the formation of focal assemblies, but displayed much higher lateral dynamics than the AmyE secretion zones. Likewise, SecDF showed much higher dynamics, moving in the cell membrane, than the AmyE-mCherry fusion. These data are in agreement with our observation that SecA and SecDF co-localized with AmyE-mCherry foci in less than 20% of the cells showing both fluorescence signals. Furthermore, the distribution pattern of SecDF and SecA fusions was not affected by overexpression of AmyE. The hypothesis that protein transport might be more efficient at sites of bacterial division, is based on the location of the

Sec translocon at those sides in *S. pyogenes* (Rosch and Caparon, 2004). However, the localization data obtained in this work regarding Sec-machinery components and AmyE, contradict this idea and lead to the conclusion that the results from *S. pyogenes* cannot be transferred to *B. subtilis*.

In order to obtain a better spatiotemporal resolution of SecA dynamics, we employed single molecule tracking. Corresponding to previous studies (Seinen *et al*., 2021), we found three populations of SecA molecules having strongly different average diffusion constants. These populations can be best explained by molecules actively transporting secreted proteins at the translocon (~20%), SecA molecules having bound cargo in search of a translocon (~50%), and freely diffusing SecA dimers (~30%). SecA can penetrate into lipid monolayers with its N-terminal amphipathic helix (Breukink *et al*., 1992), which primes SecA for high-affinity binding to SecYEG (Koch *et al*., 2016). The binding to the large SecYEG translocon likely leads to the reduced average diffusion constant. The highly dynamic behavior supports a model in which SecA diffuses along the membrane surface in between SecYEG translocons to facilitate transportation of AmyE molecules. Upon overproduction of AmyE, the slow mobile population increased to about 30%, while the freely diffusing molecules decreased to 20% and the medium mobile fraction remained constant. This indicates that more SecA molecules are involved in active transport, but that there is still a substantial pool of free SecA molecules to enable efficient general protein secretion. Therefore, we propose, that the secretion system is not overloaded by the overexpression of AmyE and may reflect a state reasonably similar to the native condition. Interestingly, average dwell times of SecA did not change, suggesting that average transport times, as well as exchange of SecA molecules between translocons, remain constant, but the number of molecules dwelling at the translocon increased. Cytosolic and membrane proteins involved in AmyE secretion across the cell membrane will come and go to the involved SecYEG translocons, while AmyE will continue to vertically diffuse through the wall towards the exterior of cells.

3.3.5 Protein secretion through the cell wall

The first reported observation of protein transport across the cell wall involved the secretion of α-amylase and an undefined protease by *B. amyloliquefaciens* (Gould *et al*., 1975). It was discovered that transport of these two enzymes would continue for approximately 15 min after blocking de novo protein synthesis with chloramphenicol. This phenomenon appears to be independent of translocation by the Sec translocon, as ATPase inhibition had no impact. Furthermore, these detected enzymes were absent in protoplasts, leading to the conclusion that secreted proteins accumulate on the trans side of the cell membrane and that transport of these proteins across the cell wall is restricted.

The fluorescence measurements performed in this work indicate, that passage through the wall may take place at a minutes-time-scale, possibly occurring in a pulse-like manner. These results are confirming the duration of cell wall passage of an amylase determined by previous pulse-chase

experiments (Lepoup *et al*., 1997; Haddaoui *et al*., 1999). The prevalence of many stationary AmyE-mCherry foci, which could remain static for up to 30 minutes, suggests that AmyE-mCherry slowly diffuses through the lateral cell wall at several loci. When analyzing time courses of AmyE-mCherry foci, we found that a considerable quantity of foci showed noticeable fluctuations in fluorescence intensity. Since the intensity of the foci not only decreased but also increased bleaching should not be considered as a possible cause for this effect. And as the minor fluctuations of the background fluorescence did not match the fluctuations of AmyE-mCherry foci, fluctuations of the excitation light were also ruled out. Maturation of mCherry, which must occur after membrane passage, requires several minutes, such that the fluctuating increase and decrease of fluorescence that was observed in a minutes-time-scale possibly includes maturation kinetics of mCherry. Additionally, it must be kept in mind that degradation products for AmyE-mCherry were detected via Western-blot analysis, therefore the observed fluctuations could also include proteolytic events. But amylase activity as well as mCherry fluorescence was detected in the supernatant of strains expressing full-length AmyE-mCherry, indicating that the functional fusion is successfully secreted to the outside of the cells. We show that levels of AmyE-mCherry fluorescence change within a minute time frame, independent of fluorescence bleaching, showing decrease as well as increase. We assume that these fluctuations in fluorescence of discrete signals reflect changes in numbers of amylase molecules within a secretion zone over time. This would be consistent with the constrained diffusion of a protein along a passage through a meshwork of a thickness of about 30-40 nm. The cell wall slows down free diffusion through a solution, which would normally occur in a time frame of milliseconds for nanometer distances (Trovato & Tozzini, 2014). Our findings suggest that AmyE-mCherry, after being transported across the cell membrane, diffuses and accumulates through defined positions in the cell wall, when produced in high amounts. However, the obstructive features for the passage of large proteins are not homogeneous, but discontinuous, including areas of lower meshwork density. Such structures have been hinted at by recent AFM visualization of the *B. subtilis* cell wall (Turner *et al*., 2018; Pasquina-Lemonche *et al*., 2020). Here, molecules may diffuse laterally into pores until they find a site that is wide enough to allow for their passage to the outside. This would imply that smaller molecules can move through the cell wall at more sites than larger ones, assuming a variety of different meshwork sizes within the wall. Judging from the dimensions derived from crystallization of *B. subtilis* α-amylase (Mizuno *et al*., 1993; Kagawa *et al*., 2003), a minimal pore size of the wall to allow passage of an amylase molecule would be in the range of at least 7.5 nm. Pores have been described to account for 5% of the total cell wall volume of *B. megaterium* and were found to have a mean pore radius of 12.5 nm (Scherrer *et al*., 1977). Pasquina-Lemonche *et al*., 2020 revealed even pores of up to 60 nm in diameter in the *B. subtilis* cell wall. Note that these pores in the cell wall are not tunnels, but random connections to the outside, that do not have to be straight and most probably are not. Diffusion through the cell wall, facilitated by these pores, does not represent directed movement so pulse-like accumulations and releases likely occur at narrow bottlenecks.

The question arises, where the pores are coming from and how they are formed. Although cell wall synthesis is directed by the MreB protein family (Garner *et al*., 2011; Dersch *et al*., 2020), a perfect and seamlessly-organized meshwork does not emerge. A combination of AFM, SIM and STORM revealed a characteristic irregular banding or striped pattern of newly synthesized peptidoglycan (Tank *et al*., 2021). The observed gaps and pores may provide a template that can subsequently be filled in, by envelope-spanning class-A penicillin-binding proteins (aPBPs) and lipoprotein cofactors (Lpos) (Pasquina-Lemonche *et al*., 2020; Vigouroux *et al*., 2020), which act as repair enzymes and are activated at sites with defects or large pores in the cell wall (Typas *et al*., 2012; Cho *et al*., 2016). However, recent data suggest that the filling of pores happens randomly, so that large pores are not preferentially filled (Tank *et al*., 2021). Thus, pores in the cell wall could arise simply by chance, during synthesis. Alternatively, enzymes with peptidoglycan hydrolyzing activity are involved in the formation of pores in the cell wall. Bacterial growth requires constant remodeling of the peptidoglycan meshwork, which is mediated by cell wall-anchored autolysins (Jolliffe *et al*., 1981; Blackman *et al*., 1998), predominantly LytC and LytD (Kuroda & Sekiguchi, 1991; Lazarevic *et al*., 1992). LytC is an N-acetylmuramoyl-L-alanine amidase (Kuroda & Sekiguchi, 1991), which localizes uniformly in the cell envelope and hydrolyzes the linkage of N-acetylmuramoyl-L-alanine in peptidoglycan (Yamamoto *et al*., 2003). The sigma factor σ^D controls expression of the major vegetative autolysin genes *lytC, lytD* and *lytF*, with a peak in activity at the start of the stationary phase (Margot *et al*., 1994, Margot *et al*., 1999). Interestingly, this peak in autolysin expression corresponds to the growth phase with elevated AmyE secretion we reported in this work. Furthermore, a majority of cells do not seem to express these autolysins. Studying expression patterns of the σ^D-dependent autolysins LytC, LytD, and LytF, Chen *et al*. (2009) found these enzymes predominantly in the same subpopulation that expressed the flagellar filament. Here again, a connection to our work can be found, since we were similarly able to detect strong secretion of AmyE-mCherry only in a subpopulation of the *B. subtilis* culture. Authors of a recent review suggested a probable link between heterogeneous amylase secretion and σ^D-driven gene expression and wondered whether the two involve the same subpopulation (Yan & Wu, 2019). Investigating this idea further would be an interesting project for the future.

Murosomes with lytic activity punch wall perforations (pores) into the peripheral cell wall at the start of cell separation in *S. aureus* (Giesbrecht *et al*., 1998; Giesbrecht *et al*., 1998). This is an interesting example for the capability of bacteria to use autolytic enzymes to create cell wall pores for a beneficial or necessary effect. Furthermore, a protein secretion system for chitinase utilizing a holin membrane protein in tandem with the cell wall-editing enzyme ChiX was recently described for the Gram-negative *Serratia marcescens* (Palmer *et al*., 2021). ChiX is a peptidoglycan hydrolase with L-Ala D-Glu endopeptidase activity, whose cell wall-editing activity is absolutely critical for chitinase secretion (Owen *et al*., 2018). Another example in which the protein secretion is linked with autolytic activity can be found in *Salmonella enterica* serovar Typhi. Here the secretion of Typhoid toxin relies on the muramidase TtsA, which was also encoded at the typhoid toxin locus (Hodak & Galan, 2013).

Fluctuations of AmyE-mCherry fluorescence suggest that secretion zones allow for an oscillating passage of many molecules, including bursts of release and phases of re-accumulation, through gaps in the PG structure. Thus, the multilayered PG envelope of firmicutes efficiently counteracts high intracellular turgor, but appears to leave many spots for passage of proteins. While highly speculative, it is possible that the cell wall hydrolyzing activity of the enzyme might mediate the secretion of substrate proteins from the periplasm into the cell interior (Fig. 3.1)

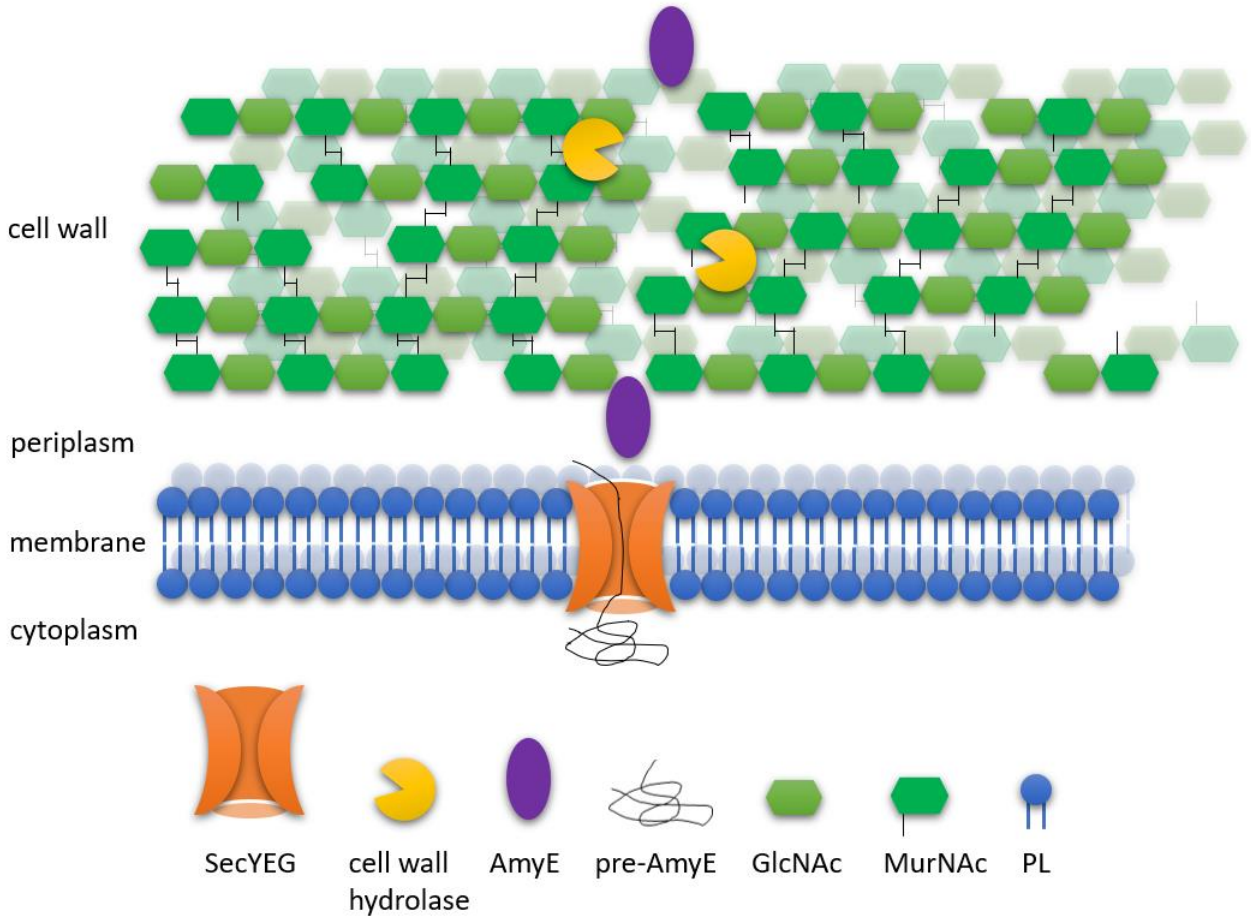


Figure 3.1: Schematic depiction of secreted AmyE molecules passing through a pore in the cell wall the cell wall.

4 General Material and Methods

4.1 Kits and chemicals

In this work, the standard chemicals were purchased from AppliChem GmbH (Darmstadt, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Sigma-Aldrich Chemie GmbH/ Merck KGaA (Taufkirchen/ Darmstadt, Germany) and GE HealthCare GmbH (Frankfurt am Main, Germany). Enzymes, DNA polymerases, reagents for Gibson assembly (molecular cloning), and DNA and protein standards were supplied by New England Biolabs GmbH (Frankfurt am Main, Germany). The plasmid extraction was performed with the Monarch Plasmid Miniprep kit by New England Biolabs GmbH (Frankfurt am Main, Germany) or the GeneJET Plasmid Miniprep Kit by Thermo Fischer Scientific Co. (St. Louis, MO, USA) and the extraction of genomic DNA with innuPREP DNA extraction kit by Analytic Jena GmbH (Jena, Germany). Kits for PCR purification and gel extractions were performed using kits from Qiagen GmbH (Hilden, Germany).

4.2 Bacterial strains

E. coli strain DH5α (Woodcock *et al*., 1989) was used for the construction and propagation of plasmids. All *B. subtilis* strains constructed during this work are derivatives of the wild type PY79 (Youngman *et al*., 1983). All *B. licheniformis* strains used during this work were kindly provided by B.R.A.I.N. Biotech AG (Zwingenberg, Germany).

4.3 Bacterial growth media

E. coli, B. subtilis and *B. licheniformis* cells were grown in Luria-Bertani (LB) medium or on solid plates containing 1.5% agar (w/v) at 37°C. The LB medium was mixed with the antibiotic required for the appropriate selection. Incubation of the liquid culture was then performed at 200 rpm and a temperature of 37°C. Cultivation on growth plates was also performed at a temperature of 37°C. Prior to inoculation in liquid media from frozen stock, *B. subtilis* strains were streaked on LB 1.5 % (w/v) agar plates. The growth was monitored by measuring the optical density at 600 nm. Activation of inducible promoters was accomplished by addition of the appropriate amount of xylose or isopropanol-b-D-thiogalactopyranoside (IPTG). For microscopy examinations, the growth temperature was reduced to 30°C. In case of single molecule tracking, cells were grown in $S7_{50}$ minimal medium prior to microscopy.

Table 1: Antibiotics and supplements that were used in this study

Name	**Final concentration**
Ampicillin	100 µg/ ml
Chloramphenicol	5 µg/ ml
Kanamycin	50 µg/ ml
Spectinomycin	100 µg/ ml
Tetracycline	25 µg/ml
D-Xylose	up to 0.5% (w/v)
IPTG	0.25 mM

Table 2: Bacterial growth media

Medium	**Composition**	**Final concentration**
LB medium	Trypton	10 g/l
	Yeast extract	5 g/l
	NaCl	10 g/l
	Agar-Agar	15 g/l
	pH 7.0 ± 0.2	
$S7_{50}$ medium	10 x S750 salts	1 x
	100 x S750 metals	1 x
	D-glucose	1 % (w/v)
	Glutamic acid	0.5 mM
	Casamino acids	0.004% (w/v)
10 x $S7_{50}$ salts	MOPS	500 mM
	$(NH_4)_2SO_4$	100 mM
	$KH2PO_4$	50 mM
	adjusted pH to 7.0 (KOH)	
100 x $S7_{50}$ metals	$MgCl_2$	0.2 M
	CaCl2	70 mM
	MnCl2	5 mM
	ZnCl2	0.1 mM
	Thiamine-HCL	0.01% (w/v)
	HCl	2 mM
	$FeCl_3$	0.5 mM

4.4 Preparation of chemically competent *E. coli* DH5α cells and transformation

E. coli cells were grown in LB medium at 37°C to an optical density of OD_{600} 0.5. After entering the desired exponential growth phase, cells were incubated on ice for 30 minutes. This was followed by pelleting the cells at 4°C and 5000 rpm for 10 minutes. The resulting supernatant was discarded

and the cell pellet was resuspended in 5 mL of competent buffer (0.1 M $CaCl_2$, 15% Glycerol). For storage, cells were aliquoted into 1.5 mL Eppendorf tubes with 150 µl each. Competent cells were deep-frozen in liquid nitrogen and stored at -80°C.

For transformation, DNA was added to the cells before thawing them on ice for 20 minutes. Then the cells were exposed to a heat shock of 42°C for 2 minutes and again incubated on ice for 10 minutes. After the incubation on ice, pre-warmed LB medium was added and the cells were incubated for 1 hour at 37°C and 200 rpm. Subsequently, the culture was spread on an LB solid plate containing the appropriate antibiotic and incubated overnight at 37°C to select for successful transformation.

4.5 Preparation and transformation of competent *B. subtilis* cells

At the transition from exponential to stationary phase, a subpopulation of *B. subtilis* is naturally able to take up exogenous DNA and incorporate this DNA into its chromosome (Albano *et al*., 1987). Laboratory strains of *B. subtilis* such as strain PY79 and strain 168, are renowned for high-frequency natural transformation (Zafra *et al*., 2012; Konkol *et al*., 2013). For transformation of *B. subtilis*, cells were grown overnight in liquid LB at 37°C and 200 rpm. For the 1x Modified Competence Medium (MCM) (Spizizen, 1958), 1 ml 10x MC medium was mixed with 8.7 ml sterile H_2O and 0.333 ml M $MgSO_4$. 10 ml MCM medium was inoculated via the overnight culture to an OD_{600} of 0.1 and incubated at 37°C and 200 rpm. When cultures reached an OD_{600} of 1.3-1.5, plasmid or genomic DNA was added to 1 ml of cell suspension. After further incubation at 37°C and constant shaking for at least 1-2 hours, the culture was spread on an LB solid plate containing the appropriate antibiotic and incubated overnight at 37°C to select for successful transformation.

Table 3: 10x MC competence medium

Composition	**Final concentration (250 ml)**
K_2HPO_4 x 3 H_2O	35.1 g
KH_2PO_4	13.1 g
D-Glucose	50 g
Potassium glutamate	5 g
Casein hydrolysate	2.5 g
Sodium citrate (300 mM)	25 ml
ferric ammonium citrate (22 mg/ml)	2.5 ml
adjusted pH to 7.0	

Mixed components were sterile filtrated and stored at -20°C.

4.6 Preparation and transformation of competent *B. licheniformis* cells

All *B. licheniformis* strains proved by B.R.A.I.N. Biotech AG (Zwingenberg, Germany) were equipped with the competence-inducing plasmid pMM*comK* (Hoffmann *et al*., 2010). ComK is the key regulator responsible for the development of genetic competence in *Bacillus*, controlling the transcription of all genes involved in DNA binding, processing, uptake, and homologous recombination of DNA (Haijema *et al*., 1996; Hamoen *et al*., 2003). The pMM*comK* plasmid carrying the *comK* gene under the control of the P_{xyl} promoter allowing for xylose inducible expression of the key regulator, resulting in highly competent strains (Hoffmann *et al*., 2010).

For the transformation of *B. licheniformis*, cells carrying the pMM*comK* plasmid were grown overnight in liquid LB with 25 µg/ml tetracycline at 37°C and 200 rpm. A 60 ml main culture (LB with 25 µg/ml tetracycline) was inoculated with the overnight culture to an OD_{600} of 0.1 and incubated at 37°C and 200 rpm until an OD_{600} of 0.5 was reached. The main culture was divided into two 30 ml cultures, and each was transferred into separate Erlenmeyer flasks. In culture A, ComK expression was induced with addition of 0.25% xylose, whereas culture B served as the uninduced negative control. Both of the cultures (A and B) were incubated for an additional 3 h. The successfully induced competence in culture A was determined on the basis of a lower OD_{600} in comparison to the negative control culture B, with a targeted gap of >1. After competence was successfully induced, cells of culture A were pelleted at 4°C and 5000 rpm for 10 minutes and resuspended in 2 ml supernatant. After the addition of 15% Glycerol, competent cells were deep-frozen in liquid nitrogen and stored at -80°C.

For the transformation, 50 µl competent cells were transferred to a tube containing DNA and 300 µl LB media containing 25 µg/ml tetracycline and 0.25% xylose. The cells were incubated for 30 min at 37°C and 200 rpm. Then the cells were pelleted at 14000 rpm for 1 minute, resuspended in 500 µL pre-warmed SOC media and incubated for an additional 30 min at 37°C and 200 rpm. After incubation, cells were spread on two LB solid plates (250 µl each) containing the appropriate antibiotic and incubated overnight at 37°C to select for successful transformation. Since the pMM*comK* plasmid is not integrated into the genome, subsequent addition of tetracycline to the media is required to avoid loss of the plasmid, if it is still needed.

Table 4: SOC media

Composition	**Final concentration**
bacto-tryptone	2%
Bacto-yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM
adjusted pH to 7.0 (NaOH)	

4.7 Polymerase chain reaction (PCR)

PCR is a method that allows DNA segments to be specifically amplified *in vitro* (Mullis *et al*., 1986). The PCR process consists of several cycles, each consisting of three different steps (denaturation, hybridization and elongation), that take place in a thermal cycler. One PCR reaction was usually composed of 1x buffer (5x Phusion® High-Fidelity reaction buffer, NEB), 50-100 ng chromosomal DNA, 200 mM dNTPs, 0.5 mM of each oligonucleotide (primer) and 0.02 U/µl polymerase (Phusion® High-Fidelity DNA Polymerase, NEB).

Following analysis by agarose gel electrophoresis, DNA fragments were purified from enzymes, nucleotides and salts using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Table 5: PCR cycling program

Steps	°C	time	
Initial denaturation	98	1 min	
Denaturation	98	20 sec	
Annealing	55-65	30 sec	30x
Extension	72	30 sec/ kb	
Final extension	72	5 min	
Hold	4	Pause	

4.8 Isolation of chromosomal DNA

Chromosomal DNA was isolated using the innuPREP DNA extraction kit by Analytic Jena GmbH (Jena, Germany). Cells from a 2-ml overnight culture of Bacillus were pelleted at 14000 rpm for 1 min, and then chromosomal DNA was isolated according to the manufacturer's protocol.

4.9 Isolation of plasmid DNA

Plasmids that were amplified by *E. coli* cells were isolated using the method described in the Monarch Plasmid Miniprep kit by New England Biolabs GmbH (Frankfurt am Main, Germany) following the manufactures instructions.

For isolation of plasmid DNA from *Bacillus*, 2 ml of an overnight culture was pelleted at 14000 rpm for 1 min and resuspended in 250 µL SET buffer. Then 50 µl lysozyme (stock 40 mg/ml) was added and carefully mixed. Cells were incubated at 37 °C for 20-30 min, followed by the addition of 250 µl of lysis buffer from the GeneJET plasmid miniprep kit (Thermo Fischer Scientific). From this step, plasmid DNA was isolated according to the manufacturer's protocol.

Table 6: SET Puffer

Composition	**Final concentration**
Saccarose	25%
EDTA	50 mM
Tris	50 mM
adjusted pH to 8.0	

4.10 DNA sequencing

To verify correct cloning and mutagenesis of genes or gene fragments, DNA sequencing was performed by the following companies: GATC Biotech (Konstanz, Germany) or Eurofins Genomics (Ebersberg, Germany). Purified plasmids were provided in a concentration of 50-100 ng/µl and oligonucleotides according to the corresponding companies´ instructions.

4.11 Molecular cloning

For the construction of plasmids, the Gibson Assembly technique (Gibson *et al*., 2009) was applied, using the commercially available Gibson assembly by New England Biolabs GmbH (Frankfurt am Main, Germany).

For the cloning at the original locus, the pSG1164 plasmid (Lewis & Marston, 1999) was used. The last 500 base pairs from the gene of interest (without the stop codon) were inserted into the multiple cloning site (MCS) of the plasmid to create a C-terminal fluorescence protein fusion. The resulting

vector allows homologous recombination via a single Campbell-type integration at the original locus.

For the overexpression of certain genes, the vectors pSG1191 and pDR111 were used. The pSG1191 has been originally designed for the generation of N-terminal fluorescent protein fusions that integrate into the chromosome by double homologous crossover at the *amyE* locus of *B. subtilis* (Feucht & Lewis, 2001). Expression of integrated genes can be controlled from the xylose-inducible P_{xyl} promoter. In this work, the gene encoding the fluorescent protein was removed from the vector by the RF-cloning method (Van Den Ent & Löwe, 2006; Unger *et al*., 2010) to allow xylose-inducible expression of genes without creating fusion proteins. The pDR111 plasmid is a derivative of the $P_{hyper\text{-}spank}$ plasmid pJQ43 (Quisel *et al*., 2001) that allows expression of integrated genes under the control of the LacI-repressible, isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible promoter $P_{hyper\text{-}spank}$. The plasmid contains an additional *lacO* binding site to achieve better repression in the absence of the inducer IPTG and similar to pSG1191 *amyE* homologous regions for double crossover at the *amyE* locus of *B. subtilis*. For the integration in *B. licheniformis* the regions homologous to *amyE* were replaced for regions homologous to *amyS* from *B. licheniformis*, allowing for integration into the chromosome by double homologous crossover at the *amyS* locus of *B. licheniformis*. Successful integration of these plasmids was confirmed by loss of amylase function.

4.12 SDS-PAGE

SDS-PAGE was performed to separate proteins according to their molecular mass under denaturating conditions. A culture at the desired growth phase was pelleted and resuspended in a corresponding amount of lysis buffer for a 10-fold concertation of cells. Samples were incubated at 37°C until lysis was visually observed. Samples were mixed with 4x SDS loading buffer, boiled for 10 minutes at 95 °C and loaded on the gel. The visualization of samples was carried out with self-prepared polyacrylamide gels of 10% or 12% (v/v) polyacrylamide gels obtained from BIO-RAD (Mini-PROTEAN® TGX™ Precast Protein Gels). Gels were prepared according to Laemmli (1970) using the Mini-PROTEAN® system (BIO-RAD). Electrophoresis was carried out in a Mini-PROTEAN® Tetra Cell with 100-140 V (Electrophoresis power supply, Consort EV243). PageRuler™ Plus from Thermo Scientific was used as protein ladder.

Table 7: 4x SDS loading buffer

Composition	**Final concentration**
Tris-HCl pH 6,8	50 mM
SDS	8% (w/v)
Glycerin	40% (v/v)
β-mercaptoethanol	20% (v/v)
bromophenol blue	0.01% (w/v)

Table 8: Lysis buffer

Composition	Final concentration
NaCl	100 mM
EDTA	50 mM
Lysozyme (added fresh)	5 mg/ml

4.13 Western blotting and immunodetection

A western blot with subsequent immunostaining was performed to specifically detect proteins of interest. In this procedure, the proteins, previously separated by an SDS-PAGE, are transferred onto a nitrocellulose membrane for further detection. After the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane (pore size 2 µm; 82 mm thick; Protran BA83, Whatman™, GE Healthcare), using the Trans-Blot Turbo Transfer System (Bio-Rad) according to the manufacturer's protocol. To this end, three layers of Whatman papers soaked in transfer buffer, a nitrocellulose membrane soaked in transfer buffer, the SDS-PAGE gel and another three layers of soaked Whatman papers were placed on top of each other inside the blotting apparatus. The proteins were visualized using rabbit polyclonal antiserum binding to the protein of interest and secondary peroxidase-conjugated goat-anti-Rabbit-IgG (dilution 1:10.000) for detection signal amplification (Sigma-Aldrich). Prior to detection, the nitrocellulose membrane was incubated in blocking buffer (PBS, 0.1%, Tween-20, 5% w/v nonfat dry milk) for 1 h under constant shaking, followed by incubation overnight with the first antiserum diluted in blocking solution. After incubation, three washing steps with PBS (3 x 10 min) followed, the incubation with the secondary antibody for 1 h and three final washing steps (3 x 10 min) with PBS. Detection was performed using an Immobilon® Forte Western membrane substrate (Merck KGA, Darmstadt, Germany) and signal detection via the ChemiDoc™ MP Imaging System (BIO-RAD). Proteins were visualized by chemiluminescence, as a consequence of enzymatic luminol oxidation by the IgG coupled horseradish peroxidase.

Table 9: Transfer buffer

Composition	Final concentration
Tris	48 mM
glycine	39 mM
SDS	1.3 mM
EtOH	20%
adjusted pH to 9.8	

4.14 Structured Illumination Microscopy (SIM)

Cell samples were diluted to an OD_{600} of ~0.5 and mounted on ultrapure-agarose slides dissolved in LB (1%) for immobilization of cells prior to image acquisition. For localization experiments, image Z-stacks (~100 nm steps) were acquired using brightfield (BF) image acquisition (transmitted light) or illumination microscopy (SIM) with a ZEISS ELYRA PS.1 setup (Andor EMCCD camera, 80 nm 1.15 size; 3× rotations and 5× phases per z-slice; with an excitation wavelength 561 nm at 15% intensity or 488 nm at 10% intensity; ZEISS alpha Plan-Apochromat 100x/NA 1.46 Oil DIC M27 objective). SIM reconstructions were processed using ZEN-Black software by ZEISS. ImageJ2/FIJI version 1.52p was used for visualization and image processing (Schindelin *et al*., 2012; Linkert *et al*., 2010; Rueden *et al*., 2017). No automatic features like autofocus or drift correction were used. For time lapse imaging the acquisition time was set to 1 minute. SIM reconstructions were manually cropped in axial and lateral dimensions, depending on the plausibility of cellular positions, using the "Duplicate"-function. Signal not connected to the cells was considered to be background and was therefore in most cases eliminated. For single-particle tracking, spots were identified with the LoG Detector of TrackMate v6.0.1(Tinevez *et al*., 2017), implemented in Fiji 1.53 q, an estimated diameter of 0.5 µm and sub-pixel localization activated. Spots were merged into tracks via the Simple LAP Tracker of TrackMate, with a maximum linking distance of 500 nm, one frame gaps allowed, and a gap closing max distance of 800 nm.

4.15 Generation of protoplasts

Bacillus cells in the transitional growth phase were treated according to the protocol of Chang & Cohen (1979) to obtain protoplasts. Cells were grown in PAB medium at 37°C and 200 rpm to a cell density of 1 x 10^8 cfu/ml or approx. OD_{600} 1. The culture was then pelleted at 5000 rpm for 10 minutes and resuspended in a corresponding amount of SMMP media for a 10-fold concertation of cells. After addition of 2 mg/ml lysozyme, the cells were incubated at 37 °C and 55 rpm for 2 h to obtain >90% protoplasts. During the process, antibiotics were added to the media to maintain the selection pressure for plasmids. Imaging of the cells before and after the incubation with lysozyme was performed by SIM microscopy.

Table 10: PAB media

Composition	**Final concentration**
Beef extract	1.5 g/l
Yeast extract	1.5 g/l
Peptone	5 g/l
D-glucose	1 g/l
NaCl	3.5 g/l
K_2HPO_4	3.68 g/l
KH_2PO_4	1.32 g/l

Table 11: 2x SMM buffer

Composition	Final concentration
sucrose	1 M
maleate	0.04 M
$MgCl_2$	0.04 M
adjusted pH to 6.5	

Table 12: SMMP media

Composition	Final concentration
2x SMM Puffer	50%
4x PAB Medium	50%

4.16 Microscopy with starch BODIPY-FL

For the microscopy of cells with starch BODIPY-FL, a protocol adapted for *Bacillus* from Rosch & Caparon (2005) was used. Strains were cultivated in LB medium at 37°C and 200 rpm mixed with the antibiotic required for the appropriate selection until the transitional growth phase. The culture was pelleted at 4000 rpm for 2 min, the resulting supernatant was discarded and the pellet was resuspended in fresh LB containing 1% of the "DQ starch substrate stock solution" (1 mg/ml, EnzChek Ultra Amylase Assay Kit, Invitrogen Detection Technologies, Carlsbad, CA, USA). Cells were mounted on ultrapure-agarose slides dissolved in LB (1%) for immobilization of cells and incubated for 30 minutes at 37°C.

Imaging was performed via epi-fluorescence microscopy, using a Nikon Eclipse Ti-E, Nikon Instruments Inc with a CFI Apochromat objective (TIRF 100× oil, NA 1.49) and an EMCCD camera (ImagEM X2 EM-CCD, Hamamatsu Photonics KK). The samples were illuminated with Nikon C-HGFIE Intensilight (Precentered Fiber Illuminator) and the YFP-channel filter cube ET 500/20, T 515 LP, ET 535/30. Images were processed with MetaMorph (version 2.76), and ImageJ (Rueden *et al*., 2017).

4.17 Phadebas test

For the quantification of α-amylase activity in the culture supernatant, the Phadebas Amylase Test (Phadebas AB, Uppsala, Sweden) was used. One Phadebas tablet was dissolved in 20 ml Phadebas buffer solution. A culture of *Bacillus,* usually grown overnight, was pelleted at 14000 rpm for 2 min, 20 µl supernatant was mixed with 180 µl the prepared Phadebas buffer solution and incubated for 10 min at 37°C and 1000 rpm in a thermomixer (Eppendorf Thermomixer comfort). The reaction

was stopped by addition of 60 µl 1 M sodium hydroxide. The reaction tubes were centrifuged and the absorption of 100 µl of the supernatant was measured at 620 nm via a microplate reader (Tecan Infinite 200 PRO, Tecan, Switzerland). The activity values were corrected for dilution and normalized to the cell density (OD_{600}) of the culture.

Table 13: Phadebas buffer

Composition	**Final concentration**
acetic acid	0.1 M
potassium acetate	0.1 M
calcium chloride	5 mM
adjusted pH to 5	

References

a Nijeholt, J. A. L., de Keyzer, J., Prabudiansyah, I., & Driessen, A. J. (2013). Characterization of the supporting role of SecE in protein translocation. *FEBS letters*, *587*(18), 3083-3088.

Akita, M., Shinkai, A., Matsuyama, S. I., & Mizushima, S. (1991). SecA, an essential component of the secretory machinery of Escherichiacoli, exists as homodimer. *Biochemical and biophysical research communications*, *174*(1), 211-216.

Akopian, D., Shen, K., Zhang, X., & Shan, S. O. (2013). Signal recognition particle: an essential protein-targeting machine. *Annual review of biochemistry*, *82*, 693-721.

Akrigg, A., (1978). Purification and properties of a manganese-stimulated deoxyribonuclease produced during sporulation of Bacillus subtilis. *Biochemical Journal*, *172*(1), 69-76.

Alami, M., Lüke, I., Deitermann, S., Eisner, G., Koch, H. G., Brunner, J., & Müller, M. (2003). Differential interactions between a twin-arginine signal peptide and its translocase in Escherichia coli. *Molecular cell*, *12*(4), 937-946.

Albano, M., Hahn, J., & Dubnau, D. (1987). Expression of competence genes in Bacillus subtilis. *Journal of bacteriology*, *169*(7), 3110-3117.

Ames, G. F. L., Nikaido, K., Wang, I. X., Liu, P. Q., Liu, C. E., & Hu, C. (2001). Purification and characterization of the membrane-bound complex of an ABC transporter, the histidine permease. *Journal of bioenergetics and biomembranes*, *33*, 79-92.

Angelini, S., Deitermann, S., & Koch, H. G. (2005). FtsY, the bacterial signal-recognition particle receptor, interacts functionally and physically with the SecYEG translocon. *EMBO reports*, *6*(5), 476-481.

Antelmann, H., Van Dijl, J. M., Bron, S., & Hecker, M. (2006). Proteomic survey through secretome of Bacillus subtilis. *Methods of biochemical analysis*, *49*, 179.

Antelmann, H., Yamamoto, H., Sekiguchi, J., & Hecker, M. (2002). Stabilization of cell wall proteins in Bacillus subtilis: a proteomic approach. *Proteomics*, *2*(5), 591-602.

Archibald, A. R., Hancock, I. C., & Harwood, C. R. (1993). Cell wall structure, synthesis, and turn-over. *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, 379-410.

Asfahl, K. L., & Schuster, M. (2017). Social interactions in bacterial cell–cell signaling. *FEMS Microbiology Reviews*, *41*(1), 92-107.

Asher, A. H., & Theg, S. M. (2021). Electrochromic shift supports the membrane destabilization model of Tat-mediated transport and shows ion leakage during Sec transport. *Proceedings of the National Academy of Sciences*, *118*(12), e2018122118.

Atrih, A., Bacher, G., Allmaier, G., Williamson, M. P., & Foster, S. J. (1999). Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *Journal of bacteriology*, *181*(13), 3956-3966.

Auclair, S. M., Bhanu, M. K., & Kendall, D. A. (2012). Signal peptidase I: cleaving the way to mature proteins. *Protein Science*, *21*(1), 13-25.

Bach, J. N., & Bramkamp, M. (2013). Flotillins functionally organize the bacterial membrane. *Molecular microbiology*, *88*(6), 1205-1217.

Barnett, J. P., Eijlander, R. T., Kuipers, O. P., & Robinson, C. (2008). A minimal Tat system from a gram-positive organism: a bifunctional TatA subunit participates in discrete TatAC and TatA complexes. *Journal of Biological Chemistry*, *283*(5), 2534-2542.

Barnett, J. P., Lawrence, J., Mendel, S., & Robinson, C. (2011). Expression of the bifunctional *Bacillus subtilis* TatAd protein in Escherichia coli reveals distinct TatA/B-family and TatB-specific domains. *Archives of microbiology*, *193*, 583-594.

Barnett, J. P., van der Ploeg, R., Eijlander, R. T., Nenninger, A., Mendel, S., Rozeboom, R., ... & Robinson, C. (2009). The twin-arginine translocation (Tat) systems from *Bacillus subtilis* display a conserved mode of complex organization and similar substrate recognition requirements. *The FEBS journal*, *276*(1), 232-243.

Baysal, O., & Yildiz, A. (2017). Bacillus subtilis: an industrially important microbe for enzymes production. *EC Microbiol*, *5*(4), 148-156.

Bechtluft, P., Nouwen, N., Tans, S. J., & Driessen, A. J. (2010). SecB—a chaperone dedicated to protein translocation. *Molecular BioSystems*, *6*(4), 620-627.

Beis, K. (2015). Structural basis for the mechanism of ABC transporters. *Biochemical Society Transactions*, *43*(5), 889-893.

Belin, D., Plaia, G., Boulfekhar, Y., & Silva, F. (2015). Escherichia coli SecG is required for residual export mediated by mutant signal sequences and for SecY-SecE complex stability. *Journal of bacteriology*, *197*(3), 542-552.

Benke, A., Olivier, N., Gunzenhäuser, J., & Manley, S. (2012). Multicolor single molecule tracking of stochastically active synthetic dyes. *Nano letters*, *12*(5), 2619-2624.

Bergmiller, T., Krasnopeeva, E., Sarikas, S., Nikolic, N., & Guet, C. C. (2022). Real-time dynamics of individual chemoreceptor mRNA molecules reveals translation hotspots at the inner membrane of Escherichia coli. *bioRxiv*, 2022-12.

Berks, B. C. (1996). A common export pathway for proteins binding complex redox cofactors?. *Molecular microbiology*, *22*(3), 393-404.

Berks, B. C. (2015). The twin-arginine protein translocation pathway. *Annual review of biochemistry*, *84*, 843-864.

Berntsson, R. P. A., Smits, S. H., Schmitt, L., Slotboom, D. J., & Poolman, B. (2010). A structural classification of substrate-binding proteins. *FEBS letters*, *584*(12), 2606-2617.

Beshay, U., Miksch, G., Friehs, K., & Flaschel, E. (2007). Increasing the secretion ability of the kil gene for recombinant proteins in Escherichia coli by using a strong stationary-phase promoter. *Biotechnology letters*, *29*, 1893-1901.

Bessonneau, P., Besson, V., Collinson, I., & Duong, F. (2002). The SecYEG preprotein translocation channel is a conformationally dynamic and dimeric structure. *The EMBO journal*, *21*(5), 995-1003.

Beveridge, T. J., & Matias, V. R. (2006). Ultrastructure of gram-positive cell walls. *Gram-Positive Pathogens*, 3-11.

Beveridge, T. J., & Murray, R. G. (1980). Sites of metal deposition in the cell wall of Bacillus subtilis. *Journal of bacteriology*, *141*(2), 876-887.

Beveridge, T. J., & Murray, R. G. E. (1979). How thick is the *Bacillus subtilis* cell wall?. *Current Microbiology*, *2*, 1-4.

Beveridge, T. J., Forsberg, C. W., & Doyle, R. J. (1982). Major sites of metal binding in Bacillus licheniformis walls. *Journal of bacteriology*, *150*(3), 1438-1448

Bhavsar, A. P., & Brown, E. D. (2006). Cell wall assembly in Bacillus subtilis: how spirals and spaces challenge paradigms. *Molecular microbiology*, *60*(5), 1077-1090.

Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., & Lodish, H. F. (1997). Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. *Journal of Biological Chemistry*, *272*(21), 13793-13802.

Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., ... & Devine, K. M. (2007). The essential YycFG two-component system controls cell wall metabolism in Bacillus subtilis. *Molecular microbiology*, *65*(1), 180-200.

Biswas, L., Biswas, R., Nerz, C., Ohlsen, K., Schlag, M., Schäfer, T., ... & Götz, F. (2009). Role of the twin-arginine translocation pathway in Staphylococcus. *Journal of bacteriology*, *191*(19), 5921-5929.

Blackman, S. A., Smith, T. J., & Foster, S. J. (1998). The role of autolysins during vegetative growth of *Bacillus subtilis* 168. *Microbiology*, *144*(1), 73-82.

Blaudeck, N., Kreutzenbeck, P., Muller, M., Sprenger, G. A., & Freudl, R. (2005). Isolation and characterization of bifunctional Escherichia coli TatA mutant proteins that allow efficient Tat-dependent protein translocation in the absence of TatB. *Journal of Biological Chemistry*, *280*(5), 3426-3432.

Bolhuis, A. (2004). The archaeal Sec–dependent protein translocation pathway. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *359*(1446), 919-927.

Bolhuis, A., Mathers, J. E., Thomas, J. D., Barrett, C. M., & Robinson, C. (2001). TatB and TatC form a functional and structural unit of the twin-arginine translocase from Escherichia coli. *Journal of Biological Chemistry*, *276*(23), 20213-20219.

Bolhuis, A., Tjalsma, H., Smith, H. E., De Jong, A., Meima, R., Venema, G., ... & Van Dijl, J. M. (1999). Evaluation of bottlenecks in the late stages of protein secretion in Bacillus subtilis. *Applied and Environmental Microbiology*, *65*(7), 2934-2941.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., ... & Sorokin, A. (2001). The complete genome sequence of the lactic acid bacterium Lactococcus lactis ssp. lactis IL1403. *Genome research*, *11*(5), 731-753.

Boos, W. (1999). Periplasmic binding protein-dependent ABC transporters. *Eshrichia coli and Salmonera typhimurium*, 1175-1209.

Bordusa, F. (2002). Substrate mimetics in protease catalysis: characteristics, kinetics, and synthetic utility. *Current Protein and Peptide Science*, *3*(2), 159-180.

Borisova, M., Gaupp, R., Duckworth, A., Schneider, A., Dalügge, D., Mühleck, M., ... & Mayer, C. (2016). Peptidoglycan recycling in Gram-positive bacteria is crucial for survival in stationary phase. *MBio*, *7*(5), e00923-16.

Borner, G. H., Sherrier, D. J., Weimar, T., Michaelson, L. V., Hawkins, N. D., MacAskill, A., ... & Dupree, P. (2005). Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. *Plant physiology*, *137*(1), 104-116.

Bosch, P. J., Corrêa, I. R., Sonntag, M. H., Ibach, J., Brunsveld, L., Kanger, J. S., & Subramaniam, V. (2014). Evaluation of fluorophores to label SNAP-tag fused proteins for multicolor single-molecule tracking microscopy in live cells. *Biophysical journal*, *107*(4), 803-814.

Bramkamp, M., & Lopez, D. (2015). Exploring the existence of lipid rafts in bacteria. *Microbiology and Molecular Biology Reviews*, *79*(1), 81-100.

Branda, S. S., González-Pastor, J. E., Ben-Yehuda, S., Losick, R., & Kolter, R. (2001). Fruiting body formation by Bacillus subtilis. *Proceedings of the National Academy of Sciences*, *98*(20), 11621-11626.

Breedveld, M. W., & Miller, K. J. (1994). Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiological reviews*, *58*(2), 145-161.

Breukink, E., Demel, R. A., De Korte-Kool, G., & De Kruijff, B. (1992). SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. *Biochemistry*, *31*(4), 1119-1124.

Breyton, C., Haase, W., Rapoport, T. A., Kühlbrandt, W., & Collinson, I. (2002). Three-dimensional structure of the bacterial protein-translocation complex SecYEG. *Nature*, *418*(6898), 662-665.

Brockmeier, U., Caspers, M., Freudl, R., Jockwer, A., Noll, T., & Eggert, T. (2006). Systematic screening of all signal peptides from Bacillus subtilis: a powerful strategy in optimizing heterologous protein secretion in Gram-positive bacteria. *Journal of molecular biology*, *362*(3), 393-402.

Browman, D. T., Hoegg, M. B., & Robbins, S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. *Trends in cell biology*, *17*(8), 394-402.

Brown, D. A. (2006). Lipid rafts, detergent-resistant membranes, and raft targeting signals. *Physiology*, *21*(6), 430-439.

Brown, J. D., Saini, S., Aldridge, C., Herbert, J., Rao, C. V., & Aldridge, P. D. (2008). The rate of protein secretion dictates the temporal dynamics of flagellar gene expression. *Molecular microbiology*, *70*(4), 924-937.

Brown, W. C., & Young, F. E. (1970). Dynamic interactions between cell wall polymers, extracellular proteases and autolytic enzymes. *Biochemical and Biophysical Research Communications*, *38*(4), 564-568.

Brüser, T., & Sanders, C. (2003). An alternative model of the twin arginine translocation system. *Microbiological research*, *158*(1), 7-17.

Buchanan, R. E. (1918). Life phases in a bacterial culture. *The Journal of Infectious Diseases*, 109-125.

Bugg, T. D., Braddick, D., Dowson, C. G., & Roper, D. I. (2011). Bacterial cell wall assembly: still an attractive antibacterial target. *Trends in biotechnology*, *29*(4), 167-173.

Burghard-Schrod, M., Kilb, A., Krämer, K., & Graumann, P. L. (2022). Single-Molecule Dynamics of DNA Receptor ComEA, Membrane Permease ComEC, and Taken-Up DNA in Competent *Bacillus subtilis* Cells. *Journal of Bacteriology*, *204*(3), e00572-21.

Butcher, B. G., Lin, Y. P., & Helmann, J. D. (2007). The yydFGHIJ operon of *Bacillus subtilis* encodes a peptide that induces the LiaRS two-component system. *Journal of bacteriology*, *189*(23), 8616-8625.

Buxbaum, A. R., Haimovich, G., & Singer, R. H. (2015). In the right place at the right time: visualizing and understanding mRNA localization. *Nature reviews Molecular cell biology*, *16*(2), 95-109.

Cai, D., Wang, H., He, P., Zhu, C., Wang, Q., Wei, X., ... & Chen, S. (2017). A novel strategy to improve protein secretion via overexpression of the SppA signal peptide peptidase in Bacillus licheniformis. *Microbial Cell Factories*, *16*(1), 1-10.

Cai, D., Wang, H., He, P., Zhu, C., Wang, Q., Wei, X., ... & Chen, S. (2017). A novel strategy to improve protein secretion via overexpression of the SppA signal peptide peptidase in Bacillus licheniformis. *Microbial Cell Factories*, *16*(1), 1-10.

Campo, N., Tjalsma, H., Buist, G., Stepniak, D., Meijer, M., Veenhuis, M., ... & Jongbloed, J. D. (2004). Subcellular sites for bacterial protein export. *Molecular microbiology*, *53*(6), 1583-1599.

Carballido-López, R., Formstone, A., Li, Y., Ehrlich, S. D., Noirot, P., & Errington, J. (2006). Actin homolog MreBH governs cell morphogenesis by localization of the cell wall hydrolase LytE. *Developmental cell*, *11*(3), 399-409.

Cezairliyan, B., & Ausubel, F. M. (2017). Investment in secreted enzymes during nutrient-limited growth is utility dependent. *Proceedings of the National Academy of Sciences*, *114*(37), E7796-E7802.

Chaddock, A. M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R. G., Klösgen, R. B., & Robinson, C. (1995). A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the delta pH-dependent thylakoidal protein translocase. *The EMBO journal*, *14*(12), 2715-2722.

Chang, S., & Cohen, S. N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Molecular and General Genetics MGG*, *168*(1), 111-115.

Chatzi, K. E., Sardis, M. F., Karamanou, S., & Economou, A. (2013). Breaking on through to the other side: protein export through the bacterial Sec system. *Biochemical Journal*, *449*(1), 25-37.

Chen, J., Fu, G., Gai, Y., Zheng, P., Zhang, D., & Wen, J. (2015). Combinatorial Sec pathway analysis for improved heterologous protein secretion in Bacillus subtilis: identification of bottlenecks by systematic gene overexpression. *Microbial Cell Factories*, *14*(1), 1-15.

Chen, J., Zhao, L., Fu, G., Zhou, W., Sun, Y., Zheng, P., ... & Zhang, D. (2016). A novel strategy for protein production using non-classical secretion pathway in Bacillus subtilis. *Microbial cell factories*, *15*, 1-16.

Chen, R., Guttenplan, S. B., Blair, K. M., & Kearns, D. B. (2009). Role of the σD-dependent autolysins in *Bacillus subtilis* population heterogeneity. *Journal of bacteriology, 191*(18), 5775-5784.

Chilcott, G. S., & Hughes, K. T. (2000). Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli. *Microbiology and Molecular Biology Reviews*, *64*(4), 694-708.

Chin, J. H., & Goldstein, D. B. (1977). Electron paramagnetic resonance studies of ethanol on membrane fluidity. *Advances in Experimental Medicine and Biology*, *85*, 111-122.

Chirakkal, H., O'Rourke, M., Atrih, A., Foster, S. J., & Moir, A. (2002). Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. *Microbiology*, *148*(8), 2383-2392.

Cho, H., Wivagg, C. N., Kapoor, M., Barry, Z., Rohs, P. D., Suh, H., ... & Bernhardt, T. G. (2016). Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. *Nature microbiology*, *1*(10), 1-8.

Cho, I. H., Choi, E. S., Lim, H. G., & Lee, H. H. (2004). Purification and characterization of six fibrinolytic serine-proteases from earthworm Lumbricus rubellus. *BMB Reports*, *37*(2), 199-205.

Choi, J. H., & Lee, S. (2004). Secretory and extracellular production of recombinant proteins using Escherichia coli. *Applied microbiology and biotechnology*, *64*, 625-635.

Chun, S. Y., & Randall, L. L. (1994). In vivo studies of the role of SecA during protein export in Escherichia coli. *Journal of bacteriology*, *176*(14), 4197-4203.

Clark, P. F., & Ruehl, W. H. (1919). Morphological changes during the growth of bacteria. *Journal of bacteriology*, *4*(6), 615-629.

Collier, D. N., Bankaitis, V. A., Weiss, J. B., & Bassford Jr, P. J. (1988). The antifolding activity of SecB promotes the export of the E. coli maltose-binding protein. *Cell*, *53*(2), 273-283.

Cooper, D. B., Smith, V. F., Crane, J. M., Roth, H. C., Lilly, A. A., & Randall, L. L. (2008). SecA, the motor of the secretion machine, binds diverse partners on one interactive surface. *Journal of molecular biology*, *382*(1), 74-87.

Cornforth, D. M., & Foster, K. R. (2015). Antibiotics and the art of bacterial war. *Proceedings of the national academy of sciences*, *112*(35), 10827-10828.

Coxon, R. D., Harwood, C. R., & Archibald, A. R. (1991). Protein export during growth of Bacillus subtilis: the effect of extracellular protease deficiency. *Letters in applied microbiology*, *12*(3), 91-94.

Cui, J., & Davidson, A. L. (2011). ABC solute importers in bacteria. *Essays in biochemistry*, *50*, 85-99.

Cuthbertson, L., Kos, V., & Whitfield, C. (2010). ABC transporters involved in export of cell surface glycoconjugates. *Microbiology and Molecular Biology Reviews*, *74*(3), 341-362.

Dabney-Smith, C., Mori, H., & Cline, K. (2006). Oligomers of Tha4 organize at the thylakoid Tat translocase during protein transport. *Journal of Biological Chemistry*, *281*(9), 5476-5483.

Dajkovic, A., Hinde, E., MacKichan, C., & Carballido-Lopez, R. (2016). Dynamic organization of SecA and SecY secretion complexes in the B. subtilis membrane. *PLoS One*, *11*(6), e0157899.

Dalbey, R. E., Kuhn, A., Zhu, L., & Kiefer, D. (2014). The membrane insertase YidC. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1843*(8), 1489-1496.

Dalbey, R. E., Wang, P., & van Dijl, J. M. (2012). Membrane proteases in the bacterial protein secretion and quality control pathway. *Microbiology and molecular biology reviews*, *76*(2), 311-330.

Davidson, A. L., Dassa, E., Orelle, C., & Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiology and molecular biology reviews*, *72*(2), 317-364.

Dawson, R. J., & Locher, K. P. (2006). Structure of a bacterial multidrug ABC transporter. *Nature*, *443*(7108), 180-185.

de Mendoza, D., & Cronan Jr, J. E. (1983). Thermal regulation of membrane lipid fluidity in bacteria. *Trends in Biochemical Sciences*, *8*(2), 49-52.

Decottignies, A., & Goffeau, A. (1997). Complete inventory of the yeast ABC proteins. *Nature genetics*, *15*(2), 137-145.

Degering, C., Eggert, T., Puls, M., Bongaerts, J., Evers, S., Maurer, K. H., & Jaeger, K. E. (2010). Optimization of protease secretion in Bacillus subtilis and Bacillus licheniformis by screening of homologous and heterologous signal peptides. *Applied and environmental microbiology*, *76*(19), 6370-6376.

Degli Esposti, M., Morselli, D., Fava, F., Bertin, L., Cavani, F., Viaggi, D., & Fabbri, P. (2021). The role of biotechnology in the transition from plastics to bioplastics: An opportunity to reconnect global growth with sustainability. *FEBS open bio*, *11*(4), 967-983.

Demain, A. L., Vandamme, E. J., Collins, J., & Buchholz, K. (2017). History of industrial biotechnology. *Industrial biotechnology: microorganisms*, *1*, 1-84.

Demchick, P., & Koch, A. L. (1996). The permeability of the wall fabric of Escherichia coli and Bacillus subtilis. *Journal of bacteriology*, *178*(3), 768-773.

Dempwolff, F., Möller, H. M., & Graumann, P. L. (2012). Synthetic motility and cell shape defects associated with deletions of flotillin/reggie paralogs in Bacillus subtilis and interplay of these proteins with NfeD proteins. *Journal of bacteriology*, *194*(17), 4652-4661.

Dempwolff, F., Schmidt, F. K., Hervás, A. B., Stroh, A., Rösch, T. C., Riese, C. N., ... & Graumann, P. L. (2016). Super resolution fluorescence microscopy and tracking of bacterial flotillin (Reggie) paralogs provide evidence for defined-sized protein microdomains within the bacterial membrane but absence of clusters containing detergent-resistant proteins. *PLoS genetics*, *12*(6), e1006116.

Denks, K., Sliwinski, N., Erichsen, V., Borodkina, B., Origi, A., & Koch, H. G. (2017). The signal recognition particle contacts uL23 and scans substrate translation inside the ribosomal tunnel. *Nature microbiology*, *2*(4), 1-10.

Dersch, S., Mehl, J., Stuckenschneider, L., Mayer, B., Roth, J., Rohrbach, A., & Graumann, P. L. (2020). Super-resolution microscopy and single-molecule tracking reveal distinct adaptive dynamics of MreB and of cell wall-synthesis enzymes. *Frontiers in Microbiology*, *11*, 1946.

Dewachter, L., Fauvart, M., & Michiels, J. (2019). Bacterial heterogeneity and antibiotic survival: understanding and combatting persistence and heteroresistance. *Molecular cell*, *76*(2), 255-267.

Diao, L., Dong, Q., Xu, Z., Yang, S., Zhou, J., & Freudl, R. (2012). Functional implementation of the posttranslational SecB-SecA protein-targeting pathway in Bacillus subtilis. *Applied and environmental microbiology*, *78*(3), 651-659.

Dias, D. A., Urban, S., & Roessner, U. (2012). A historical overview of natural products in drug discovery. *Metabolites*, *2*(2), 303-336.

Diggle, S. P., Griffin, A. S., Campbell, G. S., & West, S. A. (2007). Cooperation and conflict in quorum-sensing bacterial populations. *Nature*, *450*(7168), 411-414.

Dignac, M. F., Urbain, V., Rybacki, D., Bruchet, A., Snidaro, D., & Scribe, P. (1998). Chemical description of extracellular polymers: implication on activated sludge floc structure. *Water Science and Technology*, *38*(8-9), 45-53.

Ding, X., Yin, K., Li, Z., Lalla, R. V., Ballesteros, E., Sfeir, M. M., & Liu, C. (2020). Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay. *Nature communications*, *11*(1), 4711.

Dinh, T., & Bernhardt, T. G. (2011). Using superfolder green fluorescent protein for periplasmic protein localization studies. *Journal of bacteriology*, *193*(18), 4984-4987.

Dirix, G., Monsieurs, P., Marchal, K., Vanderleyden, J., & Michiels, J. (2004). Screening genomes of Gram-positive bacteria for double-glycine-motif-containing peptides. *Microbiology*, *150*(5), 1121-1126.

Dobell (1932). Antony van Leeuwenhoek and his 'Little animals'. Dover Publications, New York, NY.

Dobihal, G. S., Brunet, Y. R., Flores-Kim, J., & Rudner, D. Z. (2019). Homeostatic control of cell wall hydrolysis by the WalRK two-component signaling pathway in Bacillus subtilis. *Elife*, *8*, e52088.

Doherty, G. J., & McMahon, H. T. (2009). Mechanisms of endocytosis. *Annual review of biochemistry*, *78*, 857-902.

Domínguez-Cuevas, P., Porcelli, I., Daniel, R. A., & Errington, J. (2013). Differentiated roles for MreB-actin isologues and autolytic enzymes in B acillus subtilis morphogenesis. *Molecular microbiology*, *89*(6), 1084-1098.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerging infectious diseases*, *8*(9), 881.

Donovan, C., & Bramkamp, M. (2009). Characterization and subcellular localization of a bacterial flotillin homologue. *Microbiology*, *155*(6), 1786-1799.

Douville, K., Price, A., Eichler, J., Economou, A., & Wickner, W. (1995). SecYEG and SecA are the stoichiometric components of preprotein translocase. *Journal of Biological Chemistry*, *270*(34), 20106-20111.

Doyle, R. J., Chaloupka, J., & Vinter, V. (1988). Turnover of cell walls in microorganisms. *Microbiological reviews*, *52*(4), 554-567.

Dramsi, S., Bourdichon, F., Cabanes, D., Lecuit, M., Fsihi, H., & Cossart, P. (2004). FbpA, a novel multifunctional Listeria monocytogenes virulence factor. *Molecular microbiology*, *53*(2), 639-649.

Dreyer, R., Pfukwa, R., Barth, S., Hunter, R., & Klumperman, B. (2023). The Evolution of SNAP-Tag Labels. *Biomacromolecules*, *24*(2), 517-530.

Driessen, A. J., & Nouwen, N. (2008). Protein translocation across the bacterial cytoplasmic membrane. *Annu. Rev. Biochem.*, *77*, 643-667.

Du Plessis, D. J., Nouwen, N., & Driessen, A. J. (2011). The sec translocase. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1808*(3), 851-865.

Duggar, B.M. (1948) Aureomycin: a product of the continuing search for new antibiotics. *Ann. N. Y. Acad. Sci.,* 51, 177 – 181.

Feucht, A., & Lewis, P. J. (2001). Improved plasmid vectors for the production of multiple fluorescent protein fusions in Bacillus subtilis. *Gene*, *264*(2), 289-297.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison III, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods*, *6*(5), 343-345.

Haddaoui, E., Chambert, R., Petit-Glatron, M. F., Lindy, O., & Sarvas, M. (1999). Bacillus subtilis α-amylase: the rate limiting step of secretion is growth phase-independent. *FEMS microbiology letters*, *173*(1), 127-131.

Hahn, J., DeSantis, M., & Dubnau, D. (2021). Mechanisms of transforming DNA uptake to the periplasm of Bacillus subtilis. *MBio*, *12*(3), 10-1128.

Haight, T. H., & Finland, M. (1952). The antibacterial action of erythromycin. *Proceedings of the Society for Experimental Biology and Medicine*, *81*(1), 175-183.

Haijema, B. J., Van Sinderen, D., Winterling, K., Kooistra, J., Venema, G., & Hamoen, L. W. (1996). Regulated expression of the dinR and recA genes during competence development and SOS induction in Bacillus subtilis. *Molecular microbiology*, *22*(1), 75-85.

Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *Bioresource technology*, *89*(1), 17-34.

Hamada, N., Yamamoto, T., & Fukumoto, J. (1967). α-Amylase formation and calcium metabolism of Bacillus subtilis. *Agricultural and Biological Chemistry*, *31*(1), 1-6.

Hammes, W. P., & Neuhaus, F. C. (1974). On the mechanism of action of vancomycin: inhibition of peptidoglycan synthesis in Gaffkya homari. *Antimicrobial agents and chemotherapy*, *6*(6), 722-728.

Hamoen, L. W., Venema, G., & Kuipers, O. P. (2003). Controlling competence in Bacillus subtilis: shared use of regulators. *Microbiology*, *149*(1), 9-17.

Hao, B., Zhou, W., & Theg, S. M. (2022). Hydrophobic mismatch is a key factor in protein transport across lipid bilayer membranes via the Tat pathway. *Journal of Biological Chemistry*, *298*(7).

Harris, F. M., Best, K. B., & Bell, J. D. (2002). Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1565*(1), 123-128.

Harth, G., & Horwitz, M. A. (1997). Expression and Efficient Export of Enzymatically Active Mycobacterium tuberculosis Glutamine Synthetase in Mycobacterium smegmatis and Evidence That the Information for Export is Contained within the Protein. *Journal of Biological Chemistry*, *272*(36), 22728-22735.

Hartl, F. U., Lecker, S., Schiebel, E., Hendrick, J. P., & Wickner, W. (1990). The binding cascade of SecB to SecA to SecYE mediates preprotein targeting to the E. coli plasma membrane. *Cell*, *63*(2), 269-279.

Harwood, C. R., & Cranenburgh, R. (2008). Bacillus protein secretion: an unfolding story. *Trends in microbiology*, *16*(2), 73-79.

Hashimoto, M., Matsushima, H., Suparthana, I. P., Ogasawara, H., Yamamoto, H., Teng, C., & Sekiguchi, J. (2018). Digestion of peptidoglycan near the cross-link is necessary for the growth of Bacillus subtilis. *Microbiology*, *164*(3), 299-307.

Hashimoto, M., Ooiwa, S., & Sekiguchi, J. (2012). Synthetic lethality of the lytE cwlO genotype in *Bacillus subtilis* is caused by lack of D, L-endopeptidase activity at the lateral cell wall. *Journal of bacteriology*, *194*(4), 796-803.

Hassan, K. A., Skurray, R. A., & Brown, M. H. (2007). Active export proteins mediating drug resistance in staphylococci. *Microbial Physiology*, *12*(3-4), 180-196.

Hayhurst, E. J., Kailas, L., Hobbs, J. K., & Foster, S. J. (2008). Cell wall peptidoglycan architecture in Bacillus subtilis. *Proceedings of the National Academy of Sciences*, *105*(38), 14603-14608.

Hendrick, J. P., & Wickner, W. (1991). SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the Escherichia coli plasma membrane. *Journal of Biological Chemistry*, *266*(36), 24596-24600.

Herbort, M., Klein, M., Manting, E. H., Driessen, A. J., & Freudl, R. (1999). Temporal expression of the *Bacillus subtilis* secA gene, encoding a central component of the preprotein translocase. *Journal of bacteriology*, *181*(2), 493-500.

Hering, H., Lin, C. C., & Sheng, M. (2003). Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *Journal of Neuroscience*, *23*(8), 3262-3271.

Hinderhofer, M., Walker, C. A., Friemel, A., Stuermer, C. A., Möller, H. M., & Reuter, A. (2009). Evolution of prokaryotic SPFH proteins. *BMC Evolutionary Biology*, *9*, 1-18.

Hodak, H., & Galan, J. E. (2013). A Salmonella Typhi homologue of bacteriophage muramidases controls typhoid toxin secretion. *EMBO reports*, *14*(1), 95-102.

Hoelzel, C. A., & Zhang, X. (2020). Visualizing and Manipulating Biological Processes by Using HaloTag and SNAP-Tag Technologies. *ChemBioChem*, *21*(14), 1935-1946.

Hoffmann, K., Wollherr, A., Larsen, M., Rachinger, M., Liesegang, H., Ehrenreich, A., & Meinhardt, F. (2010). Facilitation of direct conditional knockout of essential genes in Bacillus licheniformis DSM13 by comparative genetic analysis and manipulation of genetic competence. *Applied and environmental microbiology*, *76*(15), 5046-5057.

Höfte, H., & Whiteley, H. (1989). Insecticidal crystal proteins of Bacillus thuringiensis. *Microbiological reviews*, *53*(2), 242-255.

Hoiczyk, E., & Hansel, A. (2000). Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *Journal of bacteriology*, *182*(5), 1191-1199.

Holland, I. B. (2004). Translocation of bacterial proteins—an overview. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1694*(1-3), 5-16.

Holland, I. B., Cole, S. P., Kuchler, K., & Higgins, C. F. (Eds.). (2003). *ABC proteins: from bacteria to man*. Elsevier.

Hollenstein, K., Dawson, R. J., & Locher, K. P. (2007). Structure and mechanism of ABC transporter proteins. *Current opinion in structural biology*, *17*(4), 412-418.

Horsburgh, G. J., Atrih, A., & Foster, S. J. (2003). Characterization of LytH, a differentiation-associated peptidoglycan hydrolase of *Bacillus subtilis* involved in endospore cortex maturation. *Journal of bacteriology*, *185*(13), 3813-3820.

Hou, B., Heidrich, E. S., Mehner-Breitfeld, D., & Brüser, T. (2018). The TatA component of the twin-arginine translocation system locally weakens the cytoplasmic membrane of Escherichia coli upon protein substrate binding. *Journal of Biological Chemistry*, *293*(20), 7592-7605.

Huang, C., Rossi, P., Saio, T., & Kalodimos, C. G. (2016). Structural basis for the antifolding activity of a molecular chaperone. *Nature*, *537*(7619), 202-206.

Huang, X., Gaballa, A., Cao, M., & Helmann, J. D. (1999). Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor, σW. *Molecular microbiology*, *31*(1), 361-371.

Huber, D., Jamshad, M., Hanmer, R., Schibich, D., Döring, K., Marcomini, I., ... & Bukau, B. (2017). SecA cotranslationally interacts with nascent substrate proteins in vivo. *Journal of bacteriology*, *199*(2), e00622-16.

Huber, D., Rajagopalan, N., Preissler, S., Rocco, M. A., Merz, F., Kramer, G., & Bukau, B. (2011). SecA interacts with ribosomes in order to facilitate posttranslational translocation in bacteria. *Molecular cell*, *41*(3), 343-353.

Hughes, M. J., Moore, J. C., Lane, J. D., Wilson, R., Pribul, P. K., Younes, Z. N., ... & Santangelo, J. D. (2002). Identification of major outer surface proteins of Streptococcus agalactiae. *Infection and immunity*, *70*(3), 1254-1259.

Hynds, P. J., Robinson, D., & Robinson, C. (1998). The sec-independent twin-arginine translocation system can transport both tightly folded and malfolded proteins across the thylakoid membrane. *Journal of Biological Chemistry*, *273*(52), 34868-34874.

Hyyryläinen, H. L., Pietiäinen, M., Lunden, T., Ekman, A., Gardemeister, M., Murtomäki-Repo, S., ... & Kontinen, V. P. (2007). The density of negative charge in the cell wall influences two-component signal transduction in Bacillus subtilis. *Microbiology*, *153*(7), 2126-2136.

Hyyryläinen, H. L., Vitikainen, M., Thwaite, J., Wu, H., Sarvas, M., Harwood, C. R., ... & Stephenson, K. (2000). D-Alanine substitution of teichoic acids as a modulator of protein folding and stability at the cytoplasmic membrane/cell wall interface of Bacillus subtilis. *Journal of Biological Chemistry*, *275*(35), 26696-26703.

Ichihara, S., Beppu, N., & Mizushima, S. (1984). Protease IV, a cytoplasmic membrane protein of Escherichia coli, has signal peptide peptidase activity. *Journal of Biological Chemistry*, *259*(15), 9853-9857.

Ingle, M. B., & Erickson, R. J. (1978). Bacterial α-amylases. *Advances in Applied Microbiology*, *24*, 257-278.

Ishikawa, S., Hara, Y., Ohnishi, R., & Sekiguchi, J. (1998). Regulation of a new cell wall hydrolase gene, cwlF, which affects cell separation in Bacillus subtilis. *Journal of bacteriology*, *180*(9), 2549-2555.

Jacobson, K., Sheets, E. D., & Simson, R. (1995). Revisiting the fluid mosaic model of membranes. *Science*, *268*(5216), 1441-1442.

Jermy, A. (2010). Bacterial lipid rafts discovered. *Nature Reviews Microbiology*, *8*(11), 756-756.

Jiang, Z., Xie, X., Li, Z., Ban, X., Gu, Z., Tang, X., ... & Li, C. (2022). Regulation of Cell Membrane Permeability Enhanced the Non-Classical Secretion of γ-Cyclodextrin Glycosyltransferase in Bacillus subtilis. *Journal of Agricultural and Food Chemistry*.

Jiang, Z., Zhang, L., Zhou, W., Li, H., Li, Y., Qin, W., ... & Gao, B. (2023). The rational modification of the secretion pathway: The bidirectional grinding strategy on signal peptide and SecA in Bacillus subtilis. *Biochemical Engineering Journal*, *191*, 108769.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *science*, *337*(6096), 816-821.

Jolliffe, L. K., Doyle, R. J., & Streips, U. N. (1981). The energized membrane and cellular autolysis in Bacillus subtilis. *Cell*, *25*(3), 753-763.

Jolliffe, L. K., Langemeier, S. O., & Doyle, R. J. (1983). Hydrogen ion control of autolysin-dependent functions in Bacillus subtilis. *Microbios*, *38*(153-154), 187-194.

Jones, P. M., & George, A. M. (2011). Molecular-dynamics simulations of the ATP/apo state of a multidrug ATP-binding cassette transporter provide a structural and mechanistic basis for the asymmetric occluded state. *Biophysical journal*, *100*(12), 3025-3034.

Jong, W. S., Saurí, A., & Luirink, J. (2010). Extracellular production of recombinant proteins using bacterial autotransporters. *Current opinion in biotechnology*, *21*(5), 646-652.

Jongbloed, J. D., Antelmann, H., Hecker, M., Nijland, R., Bron, S., Airaksinen, U., ... & Braun, P. G. (2002). Selective contribution of the twin-arginine translocation pathway to protein secretion in Bacillus subtilis. *Journal of Biological Chemistry*, *277*(46), 44068-44078.

Jongbloed, J. D., Grieger, U., Antelmann, H., Hecker, M., Nijland, R., Bron, S., & Van Dijl, J. M. (2004). Two minimal Tat translocases in Bacillus. *Molecular microbiology*, *54*(5), 1319-1325.

Jongbloed, J. D., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., ... & Müller, J. (2000). TatC is a specificity determinant for protein secretion via the twin-arginine translocation pathway. *Journal of Biological Chemistry*, *275*(52), 41350-41357.

Jongbloed, J. D., van der Ploeg, R., & van Dijl, J. M. (2006). Bifunctional TatA subunits in minimal Tat protein translocases. *Trends in microbiology*, *14*(1), 2-4.

Joshi, M. V., Mann, S. G., Antelmann, H., Widdick, D. A., Fyans, J. K., Chandra, G., ... & Palmer, T. (2010). The twin arginine protein transport pathway exports multiple virulence proteins in the plant pathogen Streptomyces scabies. *Molecular microbiology*, *77*(1), 252-271.

Kabisch, J., Thürmer, A., Hübel, T., Popper, L., Daniel, R., & Schweder, T. (2013). Characterization and optimization of *Bacillus subtilis* ATCC 6051 as an expression host. *Journal of biotechnology*, *163*(2), 97-104.

Kagawa, M., Fujimoto, Z., Momma, M., Takase, K., & Mizuno, H. (2003). Crystal structure of *Bacillus subtilis* α-amylase in complex with acarbose. *Journal of bacteriology*, *185*(23), 6981-6984.

Kahled, E., Fouad, M. F., Badawi, M. H., El-Rahim, A., Mohamed, W., Shawky, H., & Moawad, H. (2022). Thermostable Protease, Amylase and Lipase Enzymes of Thermophilic Bacteria Isolated from Egyptian Hot Springs. *Egyptian Journal of Chemistry*, *65*(10), 225-238.

Kakeshtia, H., Kageyama, Y., Ara, K., Ozaki, K., & Nakamura, K. (2010). Enhanced extracellular production of heterologous proteins in *Bacillus subtilis* by deleting the C-terminal region of the SecA secretory machinery. *Molecular biotechnology*, *46*, 250-257.

Kamel, M., Löwe, M., Schott-Verdugo, S., Gohlke, H., & Kedrov, A. (2022). Unsaturated fatty acids augment protein transport via the SecA: SecYEG translocon. *The FEBS journal*, *289*(1), 140-162.

Kang, Q., & Zhang, D. (2020). Principle and potential applications of the non-classical protein secretory pathway in bacteria. *Applied microbiology and biotechnology*, *104*(3), 953-965.

Kang, Z., Yang, S., Du, G., & Chen, J. (2014). Molecular engineering of secretory machinery components for high-level secretion of proteins in Bacillus species. *Journal of Industrial Microbiology and Biotechnology*, *41*(11), 1599-1607.

Kanonenberg, K., Royes, J., Kedrov, A., Poschmann, G., Angius, F., Solgadi, A., ... & Schmitt, L. (2019). Shaping the lipid composition of bacterial membranes for membrane protein production. *Microbial cell factories*, *18*, 1-12.

Kappes, R. M., Kempf, B., Kneip, S., Boch, J., Gade, J., Meier-Wagner, J., & Bremer, E. (1999). Two evolutionarily closely related ABC transporters mediate the uptake of choline for synthesis of the osmoprotectant glycine betaine in Bacillus subtilis. *Molecular microbiology*, *32*(1), 203-216.

Karamyshev, A. L., & Johnson, A. E. (2005). Selective SecA association with signal sequences in ribosome-bound nascent chains: a potential role for SecA in ribosome targeting to the bacterial membrane. *Journal of Biological Chemistry*, *280*(45), 37930-37940.

Kasahara, J., Kiriyama, Y., Miyashita, M., Kondo, T., Yamada, T., Yazawa, K., ... & Yamamoto, H. (2016). Teichoic acid polymers affect expression and localization of dl-endopeptidase LytE required for lateral cell wall hydrolysis in Bacillus subtilis. *Journal of Bacteriology*, *198*(11), 1585-1594.

Ke, N., Landgraf, D., Paulsson, J., & Berkmen, M. (2016). Visualization of periplasmic and cytoplasmic proteins with a self-labeling protein tag. *Journal of bacteriology*, *198*(7), 1035-1043.

Kedrov, A., Kusters, I., Krasnikov, V. V., & Driessen, A. J. (2011). A single copy of SecYEG is sufficient for preprotein translocation. *The EMBO journal*, *30*(21), 4387-4397.

Kedrov, A., Sustarsic, M., de Keyzer, J., Caumanns, J. J., Wu, Z. C., & Driessen, A. J. (2013). Elucidating the native architecture of the YidC: ribosome complex. *Journal of molecular biology*, *425*(22), 4112-4124.

Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., & Johnsson, K. (2003). A general method for the covalent labeling of fusion proteins with small molecules in vivo. *Nature biotechnology*, *21*(1), 86-89.

Khokhlov, A. S., Anisova, L. N., Tovarova, I. I., Kleiner, E. M., Kovalenko, I. V., Krasilnikova, O. I., ... & Pliner, S. A. (1973). Effect of A-factor on the growth of asporogenous mutants of Streptomyces griseus, not producing this factor. *Zeitschrift für allgemeine Mikrobiologie*, *13*(8), 647-655.

Kihara, A., Akiyama, Y., & Ito, K. (1995). FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proceedings of the National Academy of Sciences*, *92*(10), 4532-4536.

Kikuchi, Y., Itaya, H., Date, M., Matsui, K., & Wu, L. F. (2008). Production of Chryseobacterium proteolyticum protein-glutaminase using the twin-arginine translocation pathway in Corynebacterium glutamicum. *Applied microbiology and biotechnology*, *78*, 67-74.

Kinsinger, R. F., Shirk, M. C., & Fall, R. (2003). Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. *Journal of bacteriology*, *185*(18), 5627-5631.

Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current opinion in biotechnology*, *13*(4), 345-351.

Kleerebezem, M., Boekhorst, J., Van Kranenburg, R., Molenaar, D., Kuipers, O. P., Leer, R., ... & Siezen, R. J. (2003). Complete genome sequence of Lactobacillus plantarum WCFS1. *Proceedings of the National Academy of Sciences*, *100*(4), 1990-1995.

Kleinbeck, F., & Kuhn, A. (2021). Membrane Insertion of the M13 Minor Coat Protein G3p Is Dependent on YidC and the SecAYEG Translocase. *Viruses*, *13*(7), 1414.

Klumpp, S., Zhang, Z., & Hwa, T. (2009). Growth rate-dependent global effects on gene expression in bacteria. *Cell*, *139*(7), 1366-1375.

Kneuper, H., Maldonado, B., Jäger, F., Krehenbrink, M., Buchanan, G., Keller, R., ... & Palmer, T. (2012). Molecular dissection of TatC defines critical regions essential for protein transport and a TatB–TatC contact site. *Molecular microbiology*, *85*(5), 945-961.

Knüpffer, L., Fehrenbach, C., Denks, K., Erichsen, V., Petriman, N. A., & Koch, H. G. (2019). Molecular mimicry of SecA and signal recognition particle binding to the bacterial ribosome. *MBio*, *10*(4), e01317-19.

Knyazev, D. G., Kuttner, R., Zimmermann, M., Sobakinskaya, E., & Pohl, P. (2018). Driving forces of translocation through bacterial translocon SecYEG. *The Journal of membrane biology*, *251*, 329-343.

Koch, A. L., & Doyle, R. J. (1985). Inside-to-outside growth and turnover of the wall of gram-positive rods. *Journal of theoretical biology*, *117*(1), 137-157.

Koch, S., de Wit, J. G., Vos, I., Birkner, J. P., Gordiichuk, P., Herrmann, A., ... & Driessen, A. J. (2016). Lipids activate SecA for high affinity binding to the SecYEG complex. *Journal of Biological Chemistry*, *291*(43), 22534-22543.

Koch, S., Seinen, A. B., Kamel, M., Kuckla, D., Monzel, C., Kedrov, A., & Driessen, A. J. (2021). Single-molecule analysis of dynamics and interactions of the SecYEG translocon. *The FEBS Journal*, *288*(7), 2203-2221.

Kodama, T., Endo, K., Ara, K., Ozaki, K., Kakeshita, H., Yamane, K., & Sekiguchi, J. (2007). Effect of *Bacillus subtilis spo0A* mutation on cell wall lytic enzymes and extracellular proteases, and prevention of cell lysis. *Journal of bioscience and bioengineering*, *103*(1), 13-21.

Kolberg, K., Puettmann, C., Pardo, A., Fitting, J., & Barth, S. (2013). SNAP-tag technology: a general introduction. *Curr. Pharm. Des*, *19*(30), 5406-5413.

Komar, J., Alvira, S., Schulze, R. J., Martin, R., Lycklama a Nijeholt, J. A., Lee, S. C., ... & Collinson, I. (2016). Membrane protein insertion and assembly by the bacterial holo-translocon SecYEG–SecDF–YajC–YidC. *Biochemical journal*, *473*(19), 3341-3354.

Konkol, M. A., Blair, K. M., & Kearns, D. B. (2013). Plasmid-encoded ComI inhibits competence in the ancestral 3610 strain of Bacillus subtilis. *Journal of Bacteriology*, *195*(18), 4085-4093

Kontinen, V. P., & Sarvas, M. (1993). The PrsA lipoprotein is essential for protein secretion in *Bacillus subtilis* and sets a limit for high-level secretion. *Molecular microbiology*, *8*(4), 727-737.

Kontinen, V. P., Saris, P., & Sarvas, M. (1991). A gene (*prsA*) of *Bacillus subtilis* involved in a novel, late stage of protein export. *Molecular microbiology*, *5*(5), 1273-1283.

Kramer, G., Boehringer, D., Ban, N., & Bukau, B. (2009). The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nature structural & molecular biology*, *16*(6), 589-597.

Kuhn, A., Koch, H. G., & Dalbey, R. E. (2017). Targeting and insertion of membrane proteins. *EcoSal Plus*, *7*(2).

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G. O., Azevedo, V., ... & Yoshikawa, H. (1997). The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature*, *390*(6657), 249-256.

Kunz, S., Tribensky, A., Steinchen, W., Oviedo-Bocanegra, L., Bedrunka, P., & Graumann, P. L. (2020). Cyclic di-GMP signaling in *Bacillus subtilis* is governed by direct interactions of diguanylate cyclases and cognate receptors. *Mbio*, *11*(2), 10-1128.

Kuroda, A., & Sekiguchi, J. (1991). Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. *Journal of bacteriology*, *173*(22), 7304-7312.

Laemmli, U. K. (1970). SDS gel electrophoresis. *Nature (Londong)*, *227*, 680685.

Lageiro, M. M., Moura, M. J., Reis, A., & Costa-Ferreira, M. J. (2007). Microbial proteases application in leather industry. *Journal of Biotechnology*, *2*(131), S239-S240.

Lam, H., Oh, D. C., Cava, F., Takacs, C. N., Clardy, J., de Pedro, M. A., & Waldor, M. K. (2009). D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science*, *325*(5947), 1552-1555.

Lambert, P. A., Hancock, I. C., & Baddiley, J. A. M. E. S. (1975). Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. *Biochemical Journal*, *151*(3), 671-676.

Lamsa, A., Liu, W. T., Dorrestein, P. C., & Pogliano, K. (2012). The *Bacillus subtilis* cannibalism toxin SDP collapses the proton motive force and induces autolysis. *Molecular microbiology*, *84*(3), 486-500.

Lane-Claypon, J. E. (1909). Multiplication of bacteria and the influence of temperature and some other conditions thereon. *Epidemiology & Infection*, *9*(2), 239-248.

Lange, C., Müller, S. D., Walther, T. H., Bürck, J., & Ulrich, A. S. (2007). Structure analysis of the protein translocating channel TatA in membranes using a multi-construct approach. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1768*(10), 2627-2634.

Langhorst, M. F., Reuter, A., & Stuermer, C. A. O. (2005). Scaffolding microdomains and beyond: the function of reggie/flotillin proteins. *Cellular and Molecular Life Sciences CMLS*, *62*, 2228-2240.

Lawrence, P. J., & Strominger, J. L. (1970). Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: XVI. The reversible fixation of radioactive penicillin G to the D-alanine carboxypeptidase of *B. subtilis*. *Journal of Biological Chemistry*, *245*(14), 3653-3666.

Lazarevic, V., Margot, P., Soldo, B., & Karamata, D. (1992). Sequencing and analysis of the *Bacillus subtilis* lytRABC divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier. *Journal of general microbiology*, *138*(9), 1949-1961.

Leduc, M., Fréhel, C., Siegel, E., & Van Heijenoort, J. (1989). Multilayered distribution of peptidoglycan in the periplasmic space of Escherichia coli. *Microbiology*, *135*(5), 1243-1254.

Lee, Y. H., Kingston, A. W., & Helmann, J. D. (2012). Glutamate dehydrogenase affects resistance to cell wall antibiotics in Bacillus subtilis. *Journal of bacteriology*, *194*(5), 993-1001.

Leloup, L., Driessen, A. J., Freudl, R., Chambert, R., & Petit-Glatron, M. F. (1999). Differential dependence of levansucrase and α-amylase secretion on SecA (Div) during the exponential phase of growth of Bacillus subtilis. *Journal of bacteriology*, *181*(6), 1820-1826.

Leloup, L., Haddaoui, E. A., Chambert, R., & Petit-Glatron, M. F. (1997). Characterization of the rate-limiting step of the secretion of *Bacillus subtilis* α-amylase overproduced during the exponential phase of growth. *Microbiology*, *143*(10), 3295-3303.

Leprohon, P., Légaré, D., & Ouellette, M. (2011). ABC transporters involved in drug resistance in human parasites. *Essays in biochemistry*, *50*, 121-144.

Levental, I., Grzybek, M., & Simons, K. (2010). Greasing their way: lipid modifications determine protein association with membrane rafts. *Biochemistry*, *49*(30), 6305-6316.

Lewis, P. J., & Marston, A. L. (1999). GFP vectors for controlled expression and dual labelling of protein fusions in Bacillus subtilis. *Gene*, *227*(1), 101-109.

Lewis, P. J., Thaker, S. D., & Errington, J. (2000). Compartmentalization of transcription and translation in Bacillus subtilis. *The EMBO Journal*, *19*(4), 710-718.

Li, L., Park, E., Ling, J., Ingram, J., Ploegh, H., & Rapoport, T. A. (2016). Crystal structure of a substrate-engaged SecY protein-translocation channel. *Nature*, *531*(7594), 395-399.

Li, W., Zhou, X., & Lu, P. (2004). Bottlenecks in the expression and secretion of heterologous proteins in Bacillus subtilis. *Research in microbiology*, *155*(8), 605-610.

Lill, R., Dowhan, W., & Wickner, W. (1990). The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell*, *60*(2), 271-280.

Lingwood, D., & Simons, K. (2010). Lipid rafts as a membrane-organizing principle. *science*, *327*(5961), 46-50.

Linkert, M., Rueden, C. T., Allan, C., Burel, J. M., Moore, W., Patterson, A., ... & Swedlow, J. R. (2010). Metadata matters: access to image data in the real world. *Journal of Cell Biology*, *189*(5), 777-782.

Linton, K. J., & Higgins, C. F. (1998). The Escherichia coli ATP-binding cassette (ABC) proteins. *Molecular microbiology*, *28*(1), 5-13.

Liu, W. T., Yang, Y. L., Xu, Y., Lamsa, A., Haste, N. M., Yang, J. Y., ... & Dorrestein, P. C. (2010). Imaging mass spectrometry of intraspecies metabolic exchange revealed the cannibalistic factors of Bacillus subtilis. *Proceedings of the National Academy of Sciences*, *107*(37), 16286-16290.

Locher, K. P. (2016). Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nature structural & molecular biology*, *23*(6), 487-493.

Lopez, D., & Koch, G. (2017). Exploring functional membrane microdomains in bacteria: an overview. *Current opinion in microbiology*, *36*, 76-84.

López, D., & Kolter, R. (2010). Functional microdomains in bacterial membranes. *Genes & development*, *24*(17), 1893-1902.

Lopez, D., Vlamakis, H., & Kolter, R. (2008). Generation of multiple cell types in Bacillus subtilis. *FEMS microbiology reviews*, *33*(1), 152-163.

Ludwig, A., Otto, G. P., Riento, K., Hams, E., Fallon, P. G., & Nichols, B. J. (2010). Flotillin microdomains interact with the cortical cytoskeleton to control uropod formation and neutrophil recruitment. *Journal of Cell Biology*, *191*(4), 771-781.

Lüke, I., Handford, J. I., Palmer, T., & Sargent, F. (2009). Proteolytic processing of Escherichia coli twin-arginine signal peptides by LepB. *Archives of microbiology*, *191*, 919-925.

Lütcke, H. (1995). Signal recognition particle (SRP), a ubiquitous initiator of protein translocation. *European journal of biochemistry*, *228*(3), 531-550.

Ma, R. J., Wang, Y. H., Liu, L., Bai, L. L., & Ban, R. (2018). Production enhancement of the extracellular lipase LipA in Bacillus subtilis: effects of expression system and Sec pathway components. *Protein expression and purification*, *142*, 81-87.

Macao, B., Luirink, J., & Samuelsson, T. (1997). Ffh and FtsY in a Mycoplasma mycoides signal-recognition particle pathway: SRP RNA and M domain of Ffh are not required for stimulation of GTPase activity in vitro. *Molecular microbiology*, *24*(3), 523-534.

Maillard, A. P., Lalani, S., Silva, F., Belin, D., & Duong, F. (2007). Deregulation of the SecYEG translocation channel upon removal of the plug domain. *Journal of Biological Chemistry*, *282*(2), 1281-1287.

Makino, S. I., Watarai, M., Cheun, H. I., Shirahata, T., & Uchida, I. (2002). Effect of the lower molecular capsule released from the cell surface of Bacillus anthracis on the pathogenesis of anthrax. *The Journal of infectious diseases*, *186*(2), 227-233.

Mansilla, M. C., Cybulski, L. E., Albanesi, D., & de Mendoza, D. (2004). Control of membrane lipid fluidity by molecular thermosensors. *Journal of bacteriology*, *186*(20), 6681-6688.

Maqbool, A., Horler, R. S., Muller, A., Wilkinson, A. J., Wilson, K. S., & Thomas, G. H. (2015). The substrate-binding protein in bacterial ABC transporters: dissecting roles in the evolution of substrate specificity. *Biochemical Society Transactions*, *43*(5), 1011-1017.

Margot, P., & Karamata, D. (1992). Identification of the structural genes for N-acetylmuramoyl-L-alanine amidase and its modifier in *Bacillus subtilis* 168: inactivation of these genes by insertional mutagenesis has no effect on growth or cell separation. *Molecular and General Genetics MGG*, *232*, 359-366.

Margot, P., Mauël, C., & Karamata, D. (1994). The gene of the N-acetylglucosaminidase, a *Bacillus subtilis* 168 cell wall hydrolase not involved in vegetative cell autolysis. *Molecular microbiology*, *12*(4), 535-545.

Margot, P., Pagni, M., & Karamata, D. (1999). *Bacillus subtilis* 168 gene lytF encodes a γ-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, σD. *Microbiology*, *145*(1), 57-65.

Margot, P., Wahlen, M., Gholamhuseinian, A., Piggot, P., & Karamata, D. (1998). The lytE gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. *Journal of bacteriology*, *180*(3), 749-752.

Márquez, L. M., Helmann, J. D., Ferrari, E., Parker, H. M., Ordal, G. W., & Chamberlin, M. J. (1990). Studies of sigma D-dependent functions in Bacillus subtilis. *Journal of bacteriology*, *172*(6), 3435-3443.

Martin, D. K., Vicente, O., Beccari, T., Kellermayer, M., Koller, M., Lal, R., ... & Dundar, M. (2021). A brief overview of global biotechnology. *Biotechnology & Biotechnological Equipment*, *35*(sup1), S5-S14.

Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, Ü., ... & Schulz, B. (2002). Multifunctionality of plant ABC transporters–more than just detoxifiers. *Planta*, *214*, 345-355.

Matias, V. R., & Beveridge, T. J. (2005). Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. *Molecular microbiology*, *56*(1), 240-251.

Matias, V. R., & Beveridge, T. J. (2008). Lipoteichoic acid is a major component of the *Bacillus subtilis* periplasm. *Journal of bacteriology*, *190*(22), 7414-7418.

Matias, V. R., Al-Amoudi, A., Dubochet, J., & Beveridge, T. J. (2003). Cryo-transmission electron microscopy of frozen-hydrated sections of Escherichia coli and Pseudomonas aeruginosa. *Journal of bacteriology*, *185*(20), 6112-6118.

Matsumoto, K., Kusaka, J., Nishibori, A., & Hara, H. (2006). Lipid domains in bacterial membranes. *Molecular microbiology*, *61*(5), 1110-1117.

Meeske, A. J., Riley, E. P., Robins, W. P., Uehara, T., Mekalanos, J. J., Kahne, D., ... & Rudner, D. Z. (2016). SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature*, *537*(7622), 634-638.

Meisner, J., Montero Llopis, P., Sham, L. T., Garner, E., Bernhardt, T. G., & Rudner, D. Z. (2013). FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in B acillus subtilis. *Molecular microbiology*, *89*(6), 1069-1083.

Merad, T., Archibald, A. R., Hancock, I. C., Harwood, C. R., & Hobot, J. A. (1989). Cell wall assembly in Bacillus subtilis: visualization of old and new wall material by electron microscopic examination of samples stained selectively for teichoic acid and teichuronic acid. *Microbiology*, *135*(3), 645-655.

Mergulhao, F. J., & Monteiro, G. A. (2004). Secretion capacity limitations of the Sec pathway in Escherichia coli. *Journal of microbiology and biotechnology*, *14*(1), 128-133.

Mielich-Süss, B., Schneider, J., & Lopez, D. (2013). Overproduction of flotillin influences cell differentiation and shape in Bacillus subtilis. *MBio*, *4*(6), e00719-13.

Miethke, M., Monteferrante, C. G., Marahiel, M. A., & van Dijl, J. M. (2013). The *Bacillus subtilis* EfeUOB transporter is essential for high-affinity acquisition of ferrous and ferric iron. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1833*(10), 2267-2278.

Missiakas, D., & Raina, S. (1997). Protein folding in the bacterial periplasm. *Journal of bacteriology*, *179*(8), 2465-2471.

Mizuno, H., Morimoto, Y., Tsukihara, T., Matsumoto, T., & Takase, K. (1993). Crystallization and preliminary X-ray studies of wild type and catalytic-site mutant α-amylase from Bacillus subtilis. *Journal of molecular biology*, *234*(4), 1282-1283.

Monod, J. (1949). The growth of bacterial cultures. *Annual review of microbiology*, *3*(1), 371-394.

Monteferrante, C. G., Miethke, M., van der Ploeg, R., Glasner, C., & van Dijl, J. M. (2012). Specific targeting of the metallophosphoesterase YkuE to the Bacillus cell wall requires the twin-arginine translocation system. *Journal of Biological Chemistry*, *287*(35), 29789-29800.

Mori, H., & Cline, K. (2002). A twin arginine signal peptide and the pH gradient trigger reversible assembly of the thylakoid ΔpH/Tat translocase. *The Journal of cell biology*, *157*(2), 205-210.

Mori, H., & Ito, K. (2006). Different modes of SecY–SecA interactions revealed by site-directed in vivo photo-cross-linking. *Proceedings of the National Academy of Sciences*, *103*(44), 16159-16164.

Morra, R., Del Carratore, F., Muhamadali, H., Horga, L. G., Halliwell, S., Goodacre, R., ... & Dixon, N. (2018). Translation stress positively regulates MscL-dependent excretion of cytoplasmic proteins. *MBio*, *9*(1), e02118-17.

Mould, R. M., & Robinson, C. (1991). A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. *Journal of Biological Chemistry*, *266*(19), 12189-12193.

Msadek, T. (1999). When the going gets tough: survival strategies and environmental signaling networks in Bacillus subtilis. *Trends in microbiology*, *7*(5), 201-207.

Muchová, K., Jamroškovič, J., & Barák, I. (2010). Lipid domains in *Bacillus subtilis* anucleate cells. *Research in microbiology*, *161*(9), 783-790.

Mudd, S., Polevitzky, K., Anderson, T. F., & Chambers, L. A. (1941). Bacterial morphology as shown by the electron microscope: II. The bacterial cell-wall in the genus Bacillus. *Journal of Bacteriology*, *42*(2), 251-264.

Mukhopadhyay, R., Huang, K. C., & Wingreen, N. S. (2008). Lipid localization in bacterial cells through curvature-mediated microphase separation. *Biophysical journal*, *95*(3), 1034-1049.

Mulder, K. C., Bandola, J., & Schumann, W. (2013). Construction of an artificial secYEG operon allowing high level secretion of α-amylase. *Protein expression and purification*, *89*(1), 92-96.

Müller, J. P., Ozegowski, J., Vettermann, S., Swaving, J., van Wely, K. H., & Driessen, A. J. (2000). Interaction of *Bacillus subtilis* CsaA with SecA and precursor proteins. *Biochemical Journal*, *348*(2), 367-373.

Müller, M. (2005). Twin-arginine-specific protein export in Escherichia coli. *Research in microbiology*, *156*(2), 131-136.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction, in: *Cold Spring Harbor symposia on quantitative biology*: Cold Spring Harbor Laboratory Press, 263-273.

Münch, K. M., Müller, J., Wienecke, S., Bergmann, S., Heyber, S., Biedendieck, R., ... & Jahn, D. (2015). Polar fixation of plasmids during recombinant protein production in Bacillus megaterium results in population heterogeneity. *Applied and environmental microbiology*, *81*(17), 5976-5986.

Naidu, M. A., & Saranraj, P. (2013). Bacterial amylase: a review. *Int J Pharm Biol Arch*, *4*(2), 274-87.

Nakamura, K., Yahagi, S. I., Yamazaki, T., & Yamane, K. (1999). *Bacillus subtilis* Histone-like Protein, HBsu, Is an Integral Component of a SRP-like Particle That Can Bind theAlu Domain of Small Cytoplasmic RNA. *Journal of Biological Chemistry*, *274*(19), 13569-13576

Neef, J., Bongiorni, C., Goosens, V. J., Schmidt, B., & van Dijl, J. M. (2017). Intramembrane protease RasP boosts protein production in Bacillus. *Microbial Cell Factories*, *16*, 1-9.

Neef, J., Bongiorni, C., Schmidt, B., Goosens, V. J., & van Dijl, J. M. (2020). Relative contributions of non-essential Sec pathway components and cell envelope-associated proteases to high-level enzyme secretion by Bacillus subtilis. *Microbial cell factories*, *19*(1), 1-13.

Nenninger, A., Mastroianni, G., Robson, A., Lenn, T., Xue, Q., Leake, M. C., & Mullineaux, C. W. (2014). Independent mobility of proteins and lipids in the plasma membrane of E scherichia coli. *Molecular microbiology*, *92*(5), 1142-1153.

Neu, T. R. (1996). Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. *Microbiological reviews*, *60*(1), 151-166.

Neuhaus, F. C., & Baddiley, J. (2003). A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiology and molecular biology reviews*, *67*(4), 686-723.

Nevo-Dinur, K., Nussbaum-Shochat, A., Ben-Yehuda, S., & Amster-Choder, O. (2011). Translation-independent localization of mRNA in E. coli. *Science*, *331*(6020), 1081-1084.

Newton, G. G., & Abraham, E. P. (1955). Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid. *Nature*, *175*, 548-548.

Nielsen, P. H., Jahn, A., & Palmgren, R. (1997). Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology*, *36*(1), 11-19.

Nishiyama, K. I., Hanada, M., & Tokuda, H. (1994). Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of Escherichia coli at low temperature. *The EMBO journal*, *13*(14), 3272-3277.

Niu, J., Meng, F., Zhou, Y., Zhang, C., Lu, Z., Lu, F., & Chen, M. (2021). Non-classical secretion of a type I L-asparaginase in Bacillus subtilis. *International Journal of Biological Macromolecules*, *180*, 677-683.

Nolandt, O. V., Walther, T. H., Roth, S., Bürck, J., & Ulrich, A. S. (2009). Structure analysis of the membrane protein TatCd from the Tat system of *B. subtilis* by circular dichroism. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1788*(10), 2238-2244.

Nsahlai, C. J., & Silver, R. P. (2003). Purification and characterization of KpsT, the ATP-binding component of the ABC-capsule exporter of Escherichia coli K1. *FEMS microbiology letters*, *224*(1), 113-118.

Nugroho, F. A., Yamamoto, H., Kobayashi, Y., & Sekiguchi, J. (1999). Characterization of a new sigma-K-dependent peptidoglycan hydrolase gene that plays a role in *Bacillus subtilis* mother cell lysis. *Journal of Bacteriology*, *181*(20), 6230-6237.

Ohnishi, R., Ishikawa, S., & Sekiguchi, J. (1999). Peptidoglycan hydrolase LytF plays a role in cell separation with CwlF during vegetative growth of Bacillus subtilis. *Journal of bacteriology*, *181*(10), 3178-3184.

Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., & DiNovi, M. J. (2006). Food-processing enzymes from recombinant microorganisms—a review. *Regulatory toxicology and Pharmacology*, *45*(2), 144-158.

Oliver, D. B., & Beckwith, J. (1982). Regulation of a membrane component required for protein secretion in Escherichia coli. *Cell*, *30*(1), 311-319.

Or, E., Navon, A., & Rapoport, T. (2002). Dissociation of the dimeric SecA ATPase during protein translocation across the bacterial membrane. *The EMBO journal*, *21*(17), 4470-4479.

Orelle, C., Mathieu, K., & Jault, J. M. (2019). Multidrug ABC transporters in bacteria. *Research in microbiology*, *170*(8), 381-391.

Osamura, T., Takahashi, F., Endo, K., Okuda, M., & Takimura, Y. (2023). Autolysis-induced extracellular production of intracellular carboxylesterase EstGtA2 using multiple-protease-deficient *Bacillus subtilis* strains. *Biochemical Engineering Journal*, 108996.

Ou, L. T., & Marquis, R. E. (1970). Electromechanical interactions in cell walls of gram-positive cocci. *Journal of bacteriology*, *101*(1), 92-101.

Owen, R. A., Fyfe, P. K., Lodge, A., Biboy, J., Vollmer, W., Hunter, W. N., & Sargent, F. (2018). Structure and activity of ChiX: a peptidoglycan hydrolase required for chitinase secretion by Serratia marcescens. *Biochemical Journal*, *475*(2), 415-428.

Paik, S. H., Chakicherla, A., & Hansen, J. N. (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *Journal of Biological Chemistry*, *273*(36), 23134-23142.

Palmer, T., & Stansfeld, P. J. (2020). Targeting of proteins to the twin-arginine translocation pathway. *Molecular microbiology*, *113*(5), 861-871.

Palmer, T., Finney, A. J., Saha, C. K., Atkinson, G. C., & Sargent, F. (2021). A holin/peptidoglycan hydrolase-dependent protein secretion system. *Molecular microbiology*, *115*(3), 345-355.

Palmer, T., Sargent, F., & Berks, B. C. (2005). Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends in microbiology*, *13*(4), 175-180.

Palmer, T., Sargent, F., & Berks, B. C. (2010). The Tat protein export pathway. *EcoSal Plus*, *4*(1).

Pan, X., Yang, Y., Liu, X., Li, D., Li, J., Guo, X., & Zhou, Z. (2016). Secretory expression of a heterologous protein, Aiio-AIO6BS, in *Bacillus subtilis* via a non-classical secretion pathway. *Biochemical and biophysical research communications*, *478*(2), 881-886.

Papanikou, E., Karamanou, S., & Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. *Nature Reviews Microbiology*, *5*(11), 839-851.

Parasassi, T., Gratton, E., Yu, W. M., Wilson, P., & Levi, M. (1997). Two-photon fluorescence microscopy of laurdan generalized polarization domains in model and natural membranes. *Biophysical journal*, *72*(6), 2413-2429.

Park, E., & Rapoport, T. A. (2011). Preserving the membrane barrier for small molecules during bacterial protein translocation. *Nature*, *473*(7346), 239-242.

Park, J. T., & Uehara, T. (2008). How bacteria consume their own exoskeletons (turn-over and recycling of cell wall peptidoglycan). *Microbiology and Molecular Biology Reviews*, *72*(2), 211-227.

Pasteur, L. (1858). Sur la fermentation appelee lactique. *Ann. Chim. Phys*, *3*.

Pasquina-Lemonche, L., Burns, J., Turner, R. D., Kumar, S., Tank, R., Mullin, N., ... & Hobbs, J. K. (2020). The architecture of the Gram-positive bacterial cell wall. *Nature*, *582*(7811), 294-297.

Pasteur, L. (1857). *Mémoire sur la fermentation appelée lactique*. Mallet-Bachelier.

Pasztor, L., Ziebandt, A. K., Nega, M., Schlag, M., Haase, S., Franz-Wachtel, M., ... & Götz, F. (2010). Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. *Journal of Biological Chemistry*, *285*(47), 36794-36803.

Patil, A. G. G., Kumar SK, P., Mulimani, V. H., Veeranagouda, Y., & Lee, K. (2010). α-Galactosidase from Bacillus megaterium VHM1 and Its Application in Removal of Flatulence-Causing Factors from Soymilk. *Journal of Microbiology and Biotechnology*, *20*(11), 1546-1554.

Paton, J. C. (1979). *Studies on the membrane lipids of Bacillus amyloliquefaciens and their relation to extracellular protein secretion* (Doctoral dissertation).

Paulsen, I. T., Chen, J., Nelson, K. E., & Saier Jr, M. H. (2001). Comparative genomics of microbial drug efflux systems. *Journal of molecular microbiology and biotechnology*, *3*(2), 145-150.

Pedersen, L. B., Murray, T., Popham, D. L., & Setlow, P. (1998). Characterization of dacC, which encodes a new low-molecular-weight penicillin-binding protein in Bacillus subtilis. *Journal of bacteriology*, *180*(18), 4967-4973.

Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K., & Fischer, W. (1995). Incorporation of D-Alanine into Lipoteichoic Acid and Wall Teichoic Acid in Bacillus subtilis: Identification of genes and regulation. *Journal of Biological Chemistry*, *270*(26), 15598-15606.

Peters, I. (2008). *Untersuchungen zur Membranfluidität als regulatorisches Element der APP-Prozessierung* (Doctoral dissertation, Frankfurt (Main), Univ., Diss., 2008).

Petersen, T. N., Brunak, S., Von Heijne, G., & Nielsen, H. (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature methods*, *8*(10), 785-786.

Petersohn, A., Brigulla, M., Haas, S., Hoheisel, J. D., Völker, U., & Hecker, M. (2001). Global analysis of the general stress response of Bacillus subtilis. *Journal of bacteriology*, *183*(19), 5617-5631.

Petit-Glatron, M. F., Grajcar, L., Munz, A., & Chambert, R. (1993). The contribution of the cell wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. *Molecular microbiology*, *9*(5), 1097-1106.

Petriman, N. A., Jauß, B., Hufnagel, A., Franz, L., Sachelaru, I., Drepper, F., ... & Koch, H. G. (2018). The interaction network of the YidC insertase with the SecYEG translocon, SRP and the SRP receptor FtsY. *Scientific reports*, *8*(1), 578.

Pham, J. V., Yilma, M. A., Feliz, A., Majid, M. T., Maffetone, N., Walker, J. R., ... & Yoon, Y. J. (2019). A review of the microbial production of bioactive natural products and biologics. *Frontiers in microbiology*, *10*, 1404.

Pohlschröder, M., Giménez, M. I., & Jarrell, K. F. (2005). Protein transport in Archaea: Sec and twin arginine translocation pathways. *Current opinion in microbiology*, *8*(6), 713-719.

Pohlschröder, M., Prinz, W. A., Hartmann, E., & Beckwith, J. (1997). Protein translocation in the three domains of life: variations on a theme. *Cell*, *91*(5), 563-566.

Polasa, A., Hettige, J., Immadisetty, K., & Moradi, M. (2022). An investigation of the YidC-mediated membrane insertion of Pf3 coat protein using molecular dynamics simulations. *Frontiers in Molecular Biosciences*, *9*, 954262.

Pooley, H. (1976). Turn-over and spreading of old wall during surface growth of Bacillus subtilis. *Journal of Bacteriology*, *125*(3), 1127-1138.

Pop, O., Martin, U., Abel, C., & Müller, J. P. (2002). The twin-arginine signal peptide of PhoD and the TatAd/Cd proteins of *Bacillus subtilis* form an autonomous Tat translocation system. *Journal of Biological Chemistry*, *277*(5), 3268-3273.

Prágai, Z., Tjalsma, H., Bolhuis, A., Van Dijl, J. M., Venema, G., & Bron, S. (1997). The signal peptidase II (lsp) gene of Bacillus subtilis. *Microbiology*, *143*(4), 1327-1333.

Quentin, Y., Fichant, G., & Denizot, F. (1999). Inventory, assembly and analysis of *Bacillus subtilis* ABC transport systems. *Journal of molecular biology*, *287*(3), 467-484.

Quisel, J. D., Burkholder, W. F., & Grossman, A. D. (2001). In vivo effects of sporulation kinases on mutant Spo0A proteins in Bacillus subtilis. *Journal of bacteriology*, *183*(22), 6573-6578.

Raetz, C. R., Reynolds, C. M., Trent, M. S., & Bishop, R. E. (2007). Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.*, *76*, 295-329.

Rahman, R. N. Z. R. A., Leow, T. C., Basri, M., & Salleh, A. B. (2005). Secretory expression of thermostable T1 lipase through bacteriocin release protein. *Protein expression and purification*, *40*(2), 411-416.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, *62*(3), 597-635.

Rashid, M. H., Kuroda, A., & Sekiguchi, J. (1993). *Bacillus subtilis* mutant deficient in the major autolytic amidase and glucosaminidase is impaired in motility. *FEMS microbiology letters*, *112*(2), 135-140.

Rawat, S., Zhu, L., Lindner, E., Dalbey, R. E., & White, S. H. (2015). SecA drives transmembrane insertion of RodZ, an unusual single-span membrane protein. *Journal of molecular biology*, *427*(5), 1023-1037.

Rea, P. A. (2007). Plant ATP-binding cassette transporters. *Annu. Rev. Plant Biol.*, *58*, 347-375.

Reichmann, N. T., & Gründling, A. (2011). Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. *FEMS microbiology letters*, *319*(2), 97-105.

Reith, J., & Mayer, C. (2011). Peptidoglycan turn-over and recycling in Gram-positive bacteria. *Applied microbiology and biotechnology*, *92*, 1-11.

Renner, L. D., & Weibel, D. B. (2011). Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes. *Proceedings of the National Academy of Sciences*, *108*(15), 6264-6269.

Resnik, N., Sepčić, K., Plemenitaš, A., Windoffer, R., Leube, R., & Veranič, P. (2011). Desmosome assembly and cell-cell adhesion are membrane raft-dependent processes. *Journal of Biological Chemistry*, *286*(2), 1499-1507.

Rismondo, J., & Schulz, L. M. (2021). Not just transporters: Alternative functions of ABC transporters in *Bacillus subtilis* and *Listeria monocytogenes*. *Microorganisms*, *9*(1), 163.

Rivera-Milla, E., Stuermer, C. A. O., & Málaga-Trillo, E. (2006). Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. *Cellular and Molecular Life Sciences CMLS*, *63*, 343-357.

Rogers, H. J., Perkins, H. R., & Ward, J. B. (1980). The bacterial autolysins. In *Microbial cell walls and membranes* (pp. 437-460). Springer, Dordrecht.

Rohde, M. (2019). The Gram-positive bacterial cell wall. *Microbiology Spectrum*, *7*(3), 7-3.

Rollauer, S. E., Tarry, M. J., Graham, J. E., Jääskeläinen, M., Jäger, F., Johnson, S., ... & Lea, S. M. (2012). Structure of the TatC core of the twin-arginine protein transport system. *Nature*, *492*(7428), 210-214.

Romantsov, T., Helbig, S., Culham, D. E., Gill, C., Stalker, L., & Wood, J. M. (2007). Cardiolipin promotes polar localization of osmosensory transporter ProP in Escherichia coli. *Molecular microbiology*, *64*(6), 1455-1465.

Rosch, J., & Caparon, M. (2004). A microdomain for protein secretion in Gram-positive bacteria. *Science*, *304*(5676), 1513-1515.

Rösch, T. C., Oviedo-Bocanegra, L. M., Fritz, G., & Graumann, P. L. (2018). SMTracker: a tool for quantitative analysis, exploration and visualization of single-molecule tracking data reveals highly dynamic binding of *B. subtilis* global repressor AbrB throughout the genome. *Scientific Reports*, *8*(1), 15747.

Roset, M. S., Ciocchini, A. E., Ugalde, R. A., & Iñón de Iannino, N. (2004). Molecular cloning and characterization of cgt, the Brucella abortus cyclic β-1, 2-glucan transporter gene, and its role in virulence. *Infection and immunity*, *72*(4), 2263-2271.

Rothman, J. E., & Orci, L. (1992). Molecular dissection of the secretory pathway. *Nature*, *355*(6359), 409-415.

Rubio, A., Jiang, X., & Pogliano, K. (2005). Localization of translocation complex components in Bacillus subtilis: enrichment of the signal recognition particle receptor at early sporulation septa. *Journal of bacteriology*, *187*(14), 5000-5002.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC bioinformatics*, *18*, 1-26.

Ruiz, N., Gronenberg, L. S., Kahne, D., & Silhavy, T. J. (2008). Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of Escherichia coli. *Proceedings of the National Academy of Sciences*, *105*(14), 5537-5542.

Russell, A. B., Peterson, S. B., & Mougous, J. D. (2014). Type VI secretion system effectors: poisons with a purpose. *Nature reviews microbiology*, *12*(2), 137-148.

Ryabichko, S., Ferreira, V. D. M., Vitrac, H., Kiyamova, R., Dowhan, W., & Bogdanov, M. (2020). Cardiolipin is required in vivo for the stability of bacterial translocon and optimal membrane protein translocation and insertion. *Scientific reports*, *10*(1), 1-13.

Sachelaru, I., Petriman, N. A., Kudva, R., Kuhn, P., Welte, T., Knapp, B., ... & Koch, H. G. (2013). YidC occupies the lateral gate of the SecYEG translocon and is sequentially displaced by a nascent membrane protein. *Journal of Biological Chemistry*, *288*(23), 16295-16307.

Sachelaru, I., Winter, L., Knyazev, D. G., Zimmermann, M., Vogt, A., Kuttner, R., ... & Koch, H. G. (2017). YidC and SecYEG form a heterotetrameric protein translocation channel. *Scientific reports*, *7*(1), 101.

Saeki, K., Ozaki, K., Kobayashi, T., & Ito, S. (2007). Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. *Journal of bioscience and bioengineering*, *103*(6), 501-508.

Saito, A., Hizukuri, Y., Matsuo, E. I., Chiba, S., Mori, H., Nishimura, O., ... & Akiyama, Y. (2011). Post-liberation cleavage of signal peptides is catalyzed by the site-2 protease (S2P) in bacteria. *Proceedings of the National Academy of Sciences*, *108*(33), 13740-13745.

Salton, M. R. J., Horne, R. W., & Cosslett, V. E. (1951). Electron microscopy of bacteria treated with cetyltrimethylammonium bromide. *Microbiology*, *5*(2), 405-407.

Samuelson, J. C., Chen, M., Jiang, F., Möller, I., Wiedmann, M., Kuhn, A., ... & Dalbey, R. E. (2000). YidC mediates membrane protein insertion in bacteria. *Nature*, *406*(6796), 637-641.

Sanchez, S., Dunn, C. M., & Kearns, D. B. (2021). CwlQ is required for swarming motility but not flagellar assembly in Bacillus subtilis. *Journal of Bacteriology*, *203*(10), e00029-21.

Sanchez, S., Guzmán-Trampe, S., Ávalos, M., Ruiz, B., Rodríguez-Sanoja, R., & Jiménez-Estrada, M. (2012). Microbial natural products. *Natural products in chemical biology*, 65-108.

Sandoz, K. M., Mitzimberg, S. M., & Schuster, M. (2007). Social cheating in Pseudomonas aeruginosa quorum sensing. *Proceedings of the National Academy of Sciences*, *104*(40), 15876-15881.

Sargent, F., Bogsch, E. G., Stanley, N. R., Wexler, M., Robinson, C., Berks, B. C., & Palmer, T. (1998). Overlapping functions of components of a bacterial Sec-independent protein export pathway. *The EMBO journal*, *17*(13), 3640-3650.

Sargent, F., Stanley, N. R., Berks, B. C., & Palmer, T. (1999). Sec-independent protein translocation in Escherichia coli: a distinct and pivotal role for the TatB protein. *Journal of Biological Chemistry*, *274*(51), 36073-36082.

Schaerlaekens, K., Schierová, M., Lammertyn, E., Geukens, N., Anné, J., & Van Mellaert, L. (2001). Twin-arginine translocation pathway in Streptomyces lividans. *Journal of bacteriology*, *183*(23), 6727-6732.

Schaerlaekens, K., Van Mellaert, L., Lammertyn, E., Geukens, N., & Anne, J. (2004). The importance of the Tat-dependent protein secretion pathway in Streptomyces as revealed by phenotypic changes in tat deletion mutants and genome analysis. *Microbiology*, *150*(1), 21-31.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of Bacillus species for industrial production. *Canadian journal of microbiology*, *50*(1), 1-17.

Schaumburg, J., Diekmann, O., Hagendorff, P., Bergmann, S., Rohde, M., Hammerschmidt, S., ... & Kärst, U. (2004). The cell wall subproteome of Listeria monocytogenes. *Proteomics*, *4*(10), 2991-3006.

Scherrer, R., Berlin, E., & Gerhardt. (1977). Density, porosity, and structure of dried cell walls isolated from Bacillus megaterium and Saccharomyces cerevisiae. *Journal of bacteriology*, *129*(2), 1162-1164.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*, *9*(7), 676-682.

Schleifer, K. H., & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological reviews*, *36*(4), 407-477.

Schneider, J., Mielich-Süss, B., Böhme, R., & Lopez, D. (2015). In vivo characterization of the scaffold activity of flotillin on the membrane kinase KinC of Bacillus subtilis. *Microbiology (Reading, England)*, *161*(9), 1871.

Schneider, T., & Sahl, H. G. (2010). An oldie but a goodie–cell wall biosynthesis as antibiotic target pathway. *International Journal of Medical Microbiology*, *300*(2-3), 161-169.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., ... & Dean, D. (1998). Bacillus thuringiensis and its pesticidal crystal proteins. *Microbiology and molecular biology reviews*, *62*(3), 775-806.

Scholz, A. S., Baur, S. S., Wolf, D., & Bramkamp, M. (2021). An Stomatin, Prohibitin, Flotillin, and HflK/C-Domain Protein Required to Link the Phage-Shock Protein to the Membrane in Bacillus subtilis. *Frontiers in microbiology*, 3156.

Schulte, T., Paschke, K. A., Laessing, U., Lottspeich, F., & Stuermer, C. A. (1997). Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. *Development*, *124*(2), 577-587.

Schulze, R. J., Komar, J., Botte, M., Allen, W. J., Whitehouse, S., Gold, V. A., ... & Collinson, I. (2014). Membrane protein insertion and proton-motive-force-dependent secretion through the bacterial holo-translocon SecYEG–SecDF–YajC–YidC. *Proceedings of the National Academy of Sciences*, *111*(13), 4844-4849.

Schuster, M., & Greenberg, E. P. (2006). A network of networks: quorum-sensing gene regulation in Pseudomonas aeruginosa. *International Journal of Medical Microbiology*, *296*(2-3), 73-81.

Scott, J. R., & Barnett, T. C. (2006). Surface proteins of gram-positive bacteria and how they get there. *Annu. Rev. Microbiol.*, *60*, 397-423.

Seeger, M. A., & van Veen, H. W. (2009). Molecular basis of multidrug transport by ABC transporters. *Biochimica Et Biophysica Acta (BBA)-Proteins and Proteomics*, *1794*(5), 725-737.

Seinen, A. B., Spakman, D., van Oijen, A. M., & Driessen, A. J. (2021). Cellular dynamics of the SecA ATPase at the single molecule level. *Scientific Reports*, *11*(1), 1433.

Seltmann, G., & Holst, O. (2002). *The bacterial cell wall*. Springer Science & Business Media.

Serek, J., Bauer-Manz, G., Struhalla, G., van den Berg, L., Kiefer, D., Dalbey, R., & Kuhn, A. (2004). Escherichia coli YidC is a membrane insertase for Sec-independent proteins. *The EMBO journal*, *23*(2), 294-301.

Shan, S. O., & Walter, P. (2005). Co-translational protein targeting by the signal recognition particle. *FEBS letters*, *579*(4), 921-926.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nature biotechnology*, *22*(12), 1567-1572.

Shapova, Y. A., & Paetzel, M. (2007). Crystallographic analysis of *Bacillus subtilis* CsaA. *Acta Crystallographica Section D: Biological Crystallography*, *63*(4), 478-485.

Sharma, A. K., Dhasmana, N., Dubey, N., Kumar, N., Gangwal, A., Gupta, M., & Singh, Y. (2017). Bacterial virulence factors: secreted for survival. *Indian journal of microbiology*, *57*, 1-10.

Shida, T., Hattori, H., Ise, F., & Sekiguchi, J. (2001). Mutational analysis of catalytic sites of the cell wall lytic N-acetylmuramoyl-L-alanine amidases CwlC and CwlV. *Journal of Biological Chemistry*, *276*(30), 28140-28146.

Shockman, G. D., & Barren, J. F. (1983). Structure, function, and assembly of cell walls of gram-positive bacteria. *Annual review of microbiology*, *37*(1), 501-527.

Shockman, G. D., & Höltje, J. V. (1994). Microbial peptidoglycan (murein) hydrolases. In *New comprehensive biochemistry* (Vol. 27, pp. 131-166). Elsevier.

Shockman, G. D., Daneo-Moore, L., Kariyama, R., & Massidda, O. (1996). Bacterial walls, peptidoglycan hydrolases, autolysins, and autolysis. *Microbial drug resistance*, *2*(1), 95-98.

Silhavy, T. J., Kahne, D., & Walker, S. (2010). The bacterial cell envelope. *Cold Spring Harbor perspectives in biology*, *2*(5), a000414.

Simonen, M., & Palva, I. (1993). Protein secretion in Bacillus species. *Microbiological Reviews*, *57*(1), 109-137.

Simons, K., & Gerl, M. J. (2010). Revitalizing membrane rafts: new tools and insights. *Nature reviews Molecular cell biology*, *11*(10), 688-699.

Simons, K., & Ikonen, E. (1997). Functional rafts in cell membranes. *nature*, *387*(6633), 569-572.

Simons, K., & Sampaio, J. L. (2011). Membrane organization and lipid rafts. *Cold Spring Harbor perspectives in biology*, *3*(10), a004697.

Singer, S. J., & Nicolson, G. L. (1972). The Fluid Mosaic Model of the Structure of Cell Membranes: Cell membranes are viewed as two-dimensional solutions of oriented globular proteins and lipids. *Science*, *175*(4023), 720-731.

Singh, B. K. (2010). Exploring microbial diversity for biotechnology: the way forward. *Trends in biotechnology*, *28*(3), 111-116.

Singh, R., Kraft, C., Jaiswal, R., Sejwal, K., Kasaragod, V. B., Kuper, J., ... & Bhushan, S. (2014). Cryo-electron microscopic structure of SecA protein bound to the 70S ribosome. *Journal of Biological Chemistry*, *289*(10), 7190-7199.

Smith, T. J., & Foster, S. J. (1995). Characterization of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. *Journal of bacteriology*, *177*(13), 3855-3862.

Smith, T. J., Blackman, S. A., & Foster, S. J. (1996). Peptidoglycan hydrolases of *Bacillus subtilis* 168. *Microbial Drug Resistance*, *2*(1), 113-118.

Smith, T. J., Blackman, S. A., & Foster, S. J. (2000). Autolysins of Bacillus subtilis: multiple enzymes with multiple functions. *Microbiology*, *146*(2), 249-262.

Soltani, M., Ghosh, K., Hoseinifar, S. H., Kumar, V., Lymbery, A. J., Roy, S., & Ringø, E. (2019). Genus Bacillus, promising probiotics in aquaculture: aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. *Reviews in Fisheries Science & Aquaculture*, *27*(3), 331-379.

Song, Y., Nikoloff, J. M., & Zhan, D. (2015). Improving protein production on the level of regulation of both expression and secretion pathways in Bacillus subtilis. *Journal of microbiology and biotechnology*, *25*(7), 963-977.

Sonnenfeld, J. A. (1985). Shedding light on the Hawthorne studies. *Journal of Organizational Behavior*, *6*(2), 111-130.

Sørensen, H. P., & Mortensen, K. K. (2005). Advanced genetic strategies for recombinant protein expression in Escherichia coli. *Journal of biotechnology*, *115*(2), 113-128.

Spizizen, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences*, *44*(10), 1072-1078.

Srivastava, D., & Waters, C. M. (2012). A tangled web: regulatory connections between quorum sensing and cyclic di-GMP. *Journal of bacteriology*, *194*(17), 4485-4493.

Stahl, M. L., & Ferrari, E. (1984). Replacement of the *Bacillus subtilis* subtilisin structural gene with an in vitro-derived deletion mutation. *Journal of Bacteriology*, *158*(2), 411-418.

Staubach, S., & Hanisch, F. G. (2011). Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert review of proteomics*, *8*(2), 263-277.

Steinberg, R., Knüpffer, L., Origi, A., Asti, R., & Koch, H. G. (2018). Co-translational protein targeting in bacteria. *FEMS microbiology letters*, *365*(11), fny095.

Stephenson, K., & Harwood, C. R. (1998). Influence of a cell-wall-associated protease on production of α-amylase by Bacillus subtilis. *Applied and Environmental Microbiology*, *64*(8), 2875-2881.

Stephenson, K., Bron, S., & Harwood, C. R. (1999). Cellular lysis in Bacillus subtilis; the affect of multiple extracellular protease deficiencies. *Letters in Applied Microbiology*, *29*(2), 141-145.

Stephenson, K., Carter, N. M., Harwood, C. R., Petit-Glatron, M. F., & Chambert, R. (1998). The influence of protein folding on late stages of the secretion of α-amylases from Bacillus subtilis. *FEBS letters*, *430*(3), 385-389.

Strauch, M. A., & Hoch, J. A. (1993). Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Molecular microbiology*, *7*(3), 337-342.

Struck, J. C., Hartmann, R. K., Toschka, H. Y., & Erdmann, V. A. (1989). Transcription and processing of *Bacillus subtilis* small cytoplasmic RNA. *Molecular and General Genetics MGG*, *215*, 478-482.

Su, Y., Liu, C., Fang, H., & Zhang, D. (2020). Bacillus subtilis: a universal cell factory for industry, agriculture, biomaterials and medicine. *Microbial cell factories*, *19*(1), 1-12.

Sumi, C. D., Yang, B. W., Yeo, I. C., & Hahm, Y. T. (2015). Antimicrobial peptides of the genus Bacillus: a new era for antibiotics. *Canadian journal of microbiology*, *61*(2), 93-103.

Svarachorn, A., Shinmyo, A., Tsuchido, T., & Takano, M. (1989). Autolysis of *Bacillus subtilis* induced by monovalent cations. *Applied microbiology and biotechnology*, *30*, 299-304.

Swoboda, J. G., Campbell, J., Meredith, T. C., & Walker, S. (2010). Wall teichoic acid function, biosynthesis, and inhibition. *Chembiochem*, *11*(1), 35-45.

Takeda, M., Nakano, F., Nagase, T., Iohara, K., & Koizumi, J. I. (1998). Isolation and chemical composition of the sheath of Sphaerotilus natans. *Bioscience, biotechnology, and biochemistry*, *62*(6), 1138-1143.

Tam, P. C., Maillard, A. P., Chan, K. K., & Duong, F. (2005). Investigating the SecY plug movement at the SecYEG translocation channel. *The EMBO journal*, *24*(19), 3380-3388.

Tank, R. K., Lund, V. A., Kumar, S., Turner, R. D., Lafage, L., Pasquina Lemonche, L., ... & Hobbs, J. K. (2021). Correlative super-resolution optical and atomic force microscopy reveals relationships between bacterial cell wall architecture and synthesis in bacillus subtilis. *ACS nano*, *15*(10), 16011-16018.

Tao, Z., Fu, G., Wang, S., Jin, Z., Wen, J., & Zhang, D. (2020). Hyper-secretion mechanism exploration of a heterologous creatinase in Bacillus subtilis. *Biochemical Engineering Journal*, *153*, 107419.

Tareq, F. S., Lee, H. S., Lee, Y. J., Lee, J. S., & Shin, H. J. (2015). Ieodoglucomide C and Ieodoglycolipid, New Glycolipids from a Marine-Derived Bacterium Bacillus licheniformis 09IDYM23. *Lipids*, *50*(5), 513-519.

Taufik, I., Kedrov, A., Exterkate, M., & Driessen, A. J. (2013). Monitoring the activity of single translocons. *Journal of Molecular Biology*, *425*(22), 4145-4153.

Tavernarakis, N., Driscoll, M., & Kyrpides, N. C. (1999). The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. *Trends in biochemical sciences*, *24*(11), 425-427.

Thwaite, J. E., Baillie, L. W., Carter, N. M., Stephenson, K., Rees, M., Harwood, C. R., & Emmerson, P. T. (2002). Optimization of the cell wall microenvironment allows increased

production of recombinant Bacillus anthracis protective antigen from B. subtilis. *Applied and environmental microbiology*, *68*(1), 227-234.

Tinevez, J. Y., Perry, N., Schindelin, J., Hoopes, G. M., Reynolds, G. D., Laplantine, E., ... & Eliceiri, K. W. (2017). TrackMate: An open and extensible platform for single-particle tracking. *Methods*, *115*, 80-90.

Tipper, D. J., & Strominger, J. L. (1968). Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: XII. Inhibition of Cross-Linking by Penicillins and Cephalosporins: Studies in Staphylococcus Aureus in vivo. *Journal of Biological Chemistry*, *243*(11), 3169-3179.

Tjalsma, H., Antelmann, H., Jongbloed, J. D., Braun, P. G., Darmon, E., Dorenbos, R., ... & van Dijl, J. M. (2004). Proteomics of protein secretion by Bacillus subtilis: separating the "secrets" of the secretome. *Microbiology and molecular biology reviews*, *68*(2), 207-233.

Tjalsma, H., Bolhuis, A., Jongbloed, J. D., Bron, S., & van Dijl, J. M. (2000). Signal peptide-dependent protein transport in Bacillus subtilis: a genome-based survey of the secretome. *Microbiology and molecular biology reviews*, *64*(3), 515-547.

Tjalsma, H., Bolhuis, A., Van Roosmalen, M. L., Wiegert, T., Schumann, W., Broekhuizen, C. P., ... & Van Dijl, J. M. (1998). Functional analysis of the secretory precursor processing machinery of Bacillus subtilis: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases. *Genes & development*, *12*(15), 2318-2331.

Trovato, F., & Tozzini, V. (2014). Diffusion within the cytoplasm: a mesoscale model of interacting macromolecules. *Biophysical journal*, *107*(11), 2579-2591

Tsuchiya, H., & Mizogami, M. (2020). Interaction of drugs with lipid raft membrane domains as a possible target. *Drug Target Insights*, *14*, 34.

Tsukazaki, T. (2018). Structure-based working model of SecDF, a proton-driven bacterial protein translocation factor. *FEMS microbiology letters*, *365*(12), fny112.

Tsukazaki, T., Mori, H., Echizen, Y., Ishitani, R., Fukai, S., Tanaka, T., ... & Nureki, O. (2011). Structure and function of a membrane component SecDF that enhances protein export. *Nature*, *474*(7350), 235-238.

Turner, R. D., Mesnage, S., Hobbs, J. K., & Foster, S. J. (2018). Molecular imaging of glycan chains couples cell-wall polysaccharide architecture to bacterial cell morphology. *Nature Communications*, *9*(1), 1263.

Typas, A., Banzhaf, M., Gross, C. A., & Vollmer, W. (2012). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*, *10*(2), 123-136.

Unger, T., Jacobovitch, Y., Dantes, A., Bernheim, R., & Peleg, Y. (2010). Applications of the Restriction Free (RF) cloning procedure for molecular manipulations and protein expression. *Journal of structural biology*, *172*(1), 34-44.

Uphoff, S., Lord, N. D., Okumus, B., Potvin-Trottier, L., Sherratt, D. J., & Paulsson, J. (2016). Stochastic activation of a DNA damage response causes cell-to-cell mutation rate variation. *Science*, *351*(6277), 1094-1097.

Van Delden, C., Pesci, E. C., Pearson, J. P., & Iglewski, B. H. (1998). Starvation selection restores elastase and rhamnolipid production in a Pseudomonas aeruginosa quorum-sensing mutant. *Infection and immunity*, *66*(9), 4499-4502.

van den Berg, B., Clemons Jr, W. M., Collinson, I., Modis, Y., Hartmann, E., Harrison, S. C., & Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature*, *427*(6969), 36-44.

Van Den Ent, F., & Löwe, J. (2006). RF cloning: a restriction-free method for inserting target genes into plasmids. Journal of biochemical and biophysical methods, 67(1), 67-74.

van Dijl, J., & Hecker, M. (2013). Bacillus subtilis: from soil bacterium to super-secreting cell factory. *Microbial cell factories*, *12*(1), 1-6.

van Roosmalen, M. L., Geukens, N., Jongbloed, J. D., Tjalsma, H., Dubois, J. Y. F., Bron, S., ... & Anné, J. (2004). Type I signal peptidases of Gram-positive bacteria. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1694*(1-3), 279-297.

van Tilburg, A. Y., Warmer, P., van Heel, A. J., Sauer, U., & Kuipers, O. P. (2022). Membrane composition and organization of *Bacillus subtilis* 168 and its genome-reduced derivative mini *Bacillus* PG10. *Microbial biotechnology*, *15*(5), 1633-1651.

Vary, P. S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W. D., & Jahn, D. (2007). Bacillus megaterium—from simple soil bacterium to industrial protein production host. *Applied microbiology and biotechnology*, *76*, 957-967.

Vavrová, Ľ., Muchová, K., & Barák, I. (2010). Comparison of different *Bacillus subtilis* expression systems. *Research in microbiology*, *161*(9), 791-797.

Veening, J. W., Smits, W. K., & Kuipers, O. P. (2008). Bistability, epigenetics, and bet-hedging in bacteria. *Annu. Rev. Microbiol.*, *62*, 193-210.

Venturi, V. (2006). Regulation of quorum sensing in Pseudomonas. *FEMS microbiology reviews*, *30*(2), 274-291.

Vermassen, A., Leroy, S., Talon, R., Provot, C., Popowska, M., & Desvaux, M. (2019). Cell wall hydrolases in bacteria: insight on the diversity of cell wall amidases, glycosidases and peptidases toward peptidoglycan. *Frontiers in microbiology*, *10*, 331.

Vigh, L., Maresca, B., & Harwood, J. L. (1998). Does the membrane's physical state control the expression of heat shock and other genes?. *Trends in biochemical sciences*, *23*(10), 369-374.

Vigouroux, A., Cordier, B., Aristov, A., Alvarez, L., Özbaykal, G., Chaze, T., ... & van Teeffelen, S. (2020). Class-A penicillin binding proteins do not contribute to cell shape but repair cell-wall defects. *Elife*, *9*, e51998.

Vitikainen, M., Pummi, T., Airaksinen, U., Wahlström, E., Wu, H., Sarvas, M., & Kontinen, V. P. (2001). Quantitation of the capacity of the secretion apparatus and requirement for PrsA in growth and secretion of α-amylase in Bacillus subtilis. *Journal of bacteriology*, *183*(6), 1881-

Vlamakis, H., Aguilar, C., Losick, R., & Kolter, R. (2008). Control of cell fate by the formation of an architecturally complex bacterial community. *Genes & development*, *22*(7), 945.

Voigt, B., Antelmann, H., Albrecht, D., Ehrenreich, A., Maurer, K. H., Evers, S., ... & Hecker, M. (2008). Cell physiology and protein secretion of Bacillus licheniformis compared to Bacillus subtilis. *Microbial Physiology*, *16*(1-2), 53-68.

Vollmer, W., & Holtje, J. V. (2004). The architecture of the murein (peptidoglycan) in gram-negative bacteria: vertical scaffold or horizontal layer (s)?. *Journal of bacteriology*, *186*(18), 5978-5987.

Voloshin, S. A., & Kaprelyants, A. S. (2004). Cell—cell interactions in bacterial populations. *Biochemistry (Moscow)*, *69*, 1268-1275.

von Heijne, G. (1990). The signal peptide. *The Journal of membrane biology*, *115*, 195-201.

Waksman, S. A., Reilly, H. C., & Johnstone, D. B. (1946). Isolation of streptomycin-producing strains of Streptomyces griseus. *Journal of bacteriology*, *52*(3), 393-397.

Walter, A., & Mayer, C. (2019). Peptidoglycan structure, biosynthesis, and dynamics during bacterial growth. *Extracellular sugar-based biopolymers matrices*, 237-299.

Wandersman, C. (1992). Secretion across the bacterial outer membrane. *Trends in Genetics*, *8*(9), 317-322.

Wang, G., Chen, H., Xia, Y., Cui, J., Gu, Z., Song, Y., ... & Chen, W. (2013). How are the non-classically secreted bacterial proteins released into the extracellular milieu?. *Current microbiology*, *67*, 688-695.

Wang, G., Shi, T., Chen, T., Wang, X., Wang, Y., Liu, D., ... & Zhao, X. (2018). Integrated whole-genome and transcriptome sequence analysis reveals the genetic characteristics of a riboflavin-overproducing Bacillus subtilis. *Metabolic engineering*, *48*, 138-149.

Wang, G., Xia, Y., Song, X., & Ai, L. (2016). Common non-classically secreted bacterial proteins with experimental evidence. *Current microbiology*, *72*, 102-111.

Wang, S., Yang, C. I., & Shan, S. O. (2017). SecA mediates cotranslational targeting and translocation of an inner membrane protein. *Journal of Cell Biology*, *216*(11), 3639-3653.

Wang, Y., Chen, Z., Zhao, R., Jin, T., Zhang, X., & Chen, X. (2014). Deleting multiple lytic genes enhances biomass yield and production of recombinant proteins by Bacillus subtilis. *Microbial cell factories*, *13*(1), 1-11.

Ward, A., Reyes, C. L., Yu, J., Roth, C. B., & Chang, G. (2007). Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proceedings of the National Academy of Sciences*, *104*(48), 19005-19010.

Ward, J. B. (1981). Teichoic and teichuronic acids: biosynthesis, assembly, and location. *Microbiological reviews*, *45*(2), 211-243.

Weidel, W., & Primosigh, J. (1958). Biochemical Parallels between Lysis by Virulent Phaǵe and Lysis by Penicillin. *Microbiology*, *18*(2), 513-517.

Welte, T., Kudva, R., Kuhn, P., Sturm, L., Braig, D., Müller, M., ... & Koch, H. G. (2012). Promiscuous targeting of polytopic membrane proteins to SecYEG or YidC by the Escherichia coli signal recognition particle. *Molecular biology of the cell*, *23*(3), 464-479.

Westers, H., Dorenbos, R., Van Dijl, J. M., Kabel, J., Flanagan, T., Devine, K. M., ... & Quax, W. J. (2003). Genome engineering reveals large dispensable regions in Bacillus subtilis. *Molecular Biology and Evolution*, *20*(12), 2076-2090.

Westers, L., Westers, H., & Quax, W. J. (2004). *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1694*(1-3), 299-310.

Whitaker, N., Bageshwar, U. K., & Musser, S. M. (2012). Kinetics of precursor interactions with the bacterial Tat translocase detected by real-time FRET. *Journal of Biological Chemistry*, *287*(14), 11252-11260

Widdick, D. A., Dilks, K., Chandra, G., Bottrill, A., Naldrett, M., Pohlschröder, M., & Palmer, T. (2006). The twin-arginine translocation pathway is a major route of protein export in Streptomyces coelicolor. *Proceedings of the National Academy of Sciences*, *103*(47), 17927-17932.

Wiegert, T., Homuth, G., Versteeg, S., & Schumann, W. (2001). Alkaline shock induces the Bacillus subtilisσW regulon. *Molecular microbiology*, *41*(1), 59-71.

Williamson, R., & Ward, J. B. (1981). Deficiency of autolytic activity in *Bacillus subtilis* and Streptococcus pneumoniae is associated with a decreased permeability of the wall. *Microbiology*, *125*(2), 325-334.

Wingender, J., Neu, T. R., & Flemming, H. C. (1999). What are bacterial extracellular polymeric substances? (pp. 1-19). Springer Berlin Heidelberg.

Wong, K., Ma, J., Rothnie, A., Biggin, P. C., & Kerr, I. D. (2014). Towards understanding promiscuity in multidrug efflux pumps. *Trends in biochemical sciences*, *39*(1), 8-16.

Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., ... & Graham, M. W. (1989). Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic acids research*, *17*(9), 3469-3478.

Wu, X. C., Lee, W. I. L. S., Tran, L., & Wong, S. (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *Journal of bacteriology*, *173*(16), 4952-4958.

Xin, C., Ban, X., Gu, Z., Li, C., Cheng, L., Hong, Y., & Li, Z. (2019). Non-classical secretion of 1, 4-alpha-glucan branching enzymes without signal peptides in *Escherichia coli*. *International journal of biological macromolecules*, *132*, 759-765.

Yamada, K., Sanzen, I., Ohkura, T., Okamoto, A., Torii, K., Hasegawa, T., & Ohta, M. (2007). Analysis of twin-arginine translocation pathway homologue in *Staphylococcus aureus*. *Current microbiology*, *55*, 14-19.

Yamaguchi, H., Furuhata, K., Fukushima, T., Yamamoto, H., & Sekiguchi, J. (2004). Characterization of a new *Bacillus subtilis* peptidoglycan hydrolase gene, yvcE (named cwlO), and the enzymatic properties of its encoded protein. *Journal of bioscience and bioengineering*, *98*(3), 174-181.

Yamamoto, H., Kurosawa, S. I., & Sekiguchi, J. (2003). Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. *Journal of bacteriology*, *185*(22), 6666-6677.

Yamane, K., Bunai, K., & Kakeshita, H. (2004). Protein traffic for secretion and related machinery of *Bacillus subtilis*. *Bioscience, biotechnology, and biochemistry*, *68*(10), 2007-2023.
Yan, S., & Wu, G. (2017). Bottleneck in secretion of α-amylase in *Bacillus subtilis*. *Microbial Cell Factories*, *16*, 1-8.

Yan, S., & Wu, G. (2017). Bottleneck in secretion of α-amylase in *Bacillus subtilis*. *Microbial cell factories*, *16*, 1-8.

Yan, S., & Wu, G. (2019). Proteases HtrA and HtrB for α-amylase secreted from *Bacillus subtilis* in secretion stress. *Cell Stress and Chaperones*, *24*(3), 493-502.

Yang, C. K., Ewis, H. E., Zhang, X., Lu, C. D., Hu, H. J., Pan, Y., ... & Tai, P. C. (2011). Nonclassical protein secretion by *Bacillus subtilis* in the stationary phase is not due to cell lysis. *Journal of bacteriology*, *193*(20), 5607-5615.

Yang, H., Wang, H., Wang, F., Zhang, K., Qu, J., Guan, J., ... & Chen, X. (2022). Efficient extracellular production of recombinant proteins in E. coli via enhancing expression of dacA on the genome. *Journal of Industrial Microbiology and Biotechnology*, *49*(4), kuac016.

Yang, M., Galizzi, A., & Henner, D. (1983). Nucleotide sequence of the amylase gene from *Bacillus subtilis*. *Nucleic Acids Research*, *11*(2), 237-250.

Yang, Y., Li, J., Yu, Q., Hou, J., Gao, C., Li, D., ... & Zhou, Z. (2018). Conformational determinants necessary for secretion of Paecilomyces thermophila β-1, 4-xylosidase that lacks a signal peptide. *AMB Express*, *8*(1), 1-10.

Yao, X., Jericho, M., Pink, D., & Beveridge, T. (1999). Thickness and elasticity of gram-negative murein sacculi measured by atomic force microscopy. *Journal of bacteriology*, *181*(22), 6865-6875.

Yeagle, P. L. (Ed.). (2004). *The structure of biological membranes*. CRC press.

Yen, M. R., Tseng, Y. H., Nguyen, E. H., Wu, L. F., & Saier, M. H. (2002). Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Archives of microbiology*, *177*, 441-450.

Yepes, A., Schneider, J., Mielich, B., Koch, G., García-Betancur, J. C., Ramamurthi, K. S., ... & López, D. (2012). The biofilm formation defect of a *Bacillus subtilis* flotillin-defective mutant involves the protease FtsH. *Molecular microbiology*, *86*(2), 457-471.

Yokoyama, H., & Matsui, I. (2005). A novel thermostable membrane protease forming an operon with a stomatin homolog from the hyperthermophilic archaebacterium Pyrococcus horikoshii. *Journal of Biological Chemistry*, *280*(8), 6588-6594.

Yoon, S. H., Kim, S. K., & Kim, J. F. (2010). Secretory production of recombinant proteins in Escherichia coli. *Recent patents on biotechnology*, *4*(1), 23-29.

Young, F. E. (1966). Autolytic enzyme associated with cell walls of *Bacillus subtilis*. *Journal of Biological Chemistry*, *241*(15), 3462-3467.

Young, J., & Holland, I. B. (1999). ABC transporters: bacterial exporters-revisited five years on. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1461*(2), 177-200.

Young, K. D. (2003). Bacterial shape. *Molecular microbiology*, *49*(3), 571-580.

Youngman, P. J., Perkins, J. B., & Losick, R. (1983). Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. *Proceedings of the National Academy of Sciences*, *80*(8), 2305-2309.

Zafra, O., Lamprecht-Grandío, M., de Figueras, C. G., & González-Pastor, J. E. (2012). Extracellular DNA release by undomesticated *Bacillus subtilis* is regulated by early competence. *PloS one*, *7*(11), e48716.

Zhang, X. Z., Cui, Z. L., Hong, Q., & Li, S. P. (2005). High-level expression and secretion of methyl parathion hydrolase in *Bacillus subtilis* WB800. *Applied and Environmental Microbiology*, *71*(7), 4101-4103.

Zhang, Y.-M., and Rock, C. O. (2008). Membrane lipid homeostasis in bacteria. Nat. Rev. Microbiol. 6, 222–233.

Zhao, L., Chen, J., Sun, J., & Zhang, D. (2017). Multimer recognition and secretion by the non-classical secretion pathway in *Bacillus subtilis*. *Scientific reports*, *7*(1), 44023.

Zhen, J., Zheng, H., Zhao, X., Fu, X., Yang, S., Xu, J., ... & Ma, Y. (2021). Regulate the hydrophobic motif to enhance the non-classical secretory expression of Pullulanase PulA in *Bacillus subtilis*. *International Journal of Biological Macromolecules*, *193*, 238-246.

Zhou, J., & Xu, Z. (2003). Structural determinants of SecB recognition by SecA in bacterial protein translocation. *Nature Structural & Molecular Biology*, *10*(11), 942-947.

Zhu, H., Li, C., & Gao, C. (2020). Applications of CRISPR–Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology*, *21*(11), 661-677.

Zielińska, A., Savietto, A., de Sousa Borges, A., Martinez, D., Berbon, M., Roelofsen, J. R., ... & Scheffers, D. J. (2020). Flotillin-mediated membrane fluidity controls peptidoglycan synthesis and MreB movement. *Elife*, *9*, e57179.

Zimmer, J., Nam, Y., & Rapoport, T. A. (2008). Structure of a complex of the ATPase SecA and the protein-translocation channel. *Nature*, *455*(7215), 936-943.

Zobel, S., Kumpfmüller, J., Süssmuth, R. D., & Schweder, T. (2015). *Bacillus subtilis* as heterologous host for the secretory production of the non-ribosomal cyclodepsipeptide enniatin. *Applied microbiology and biotechnology*, *99*, 681-691.

Zweers, J. C., Barák, I., Becher, D., Driessen, A. J., Hecker, M., Kontinen, V. P., ... & van Dijl, J. M. (2008). Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. *Microbial cell factories*, *7*, 1-20.

Supplements

Single Molecule Tracking of AmyE-SNAP-tag in *B. subtilis*

In this work, we aimed to visualize secretion processes in *Bacillus*. In an early, approach we used the SNAP-tag to create a labeled *amyE*-SNAP fusion protein in *B. subtilis*. The α-amylase AmyE was selected as a model secreted protein, since it is commonly used in scientific research (Naidu & Saranraj, 2013; Yan & Wu, 2017). Secretion of AmyE out of the cytoplasm is facilitated by the Sec-machinery, which translocates proteins in their unfolded state (Harwood & Cranenburgh, 2008). As the oxidizing environment of the periplasm promotes disulfide bridge formations (Missiakas & Raina, 1997), folding of fluorophores with cysteine residues is typically impaired and fluorescence prevented (Feilmeier *et al*., 2000). The SNAP-tag is a small polypeptide based on mammalian O^6-alkylguanine-DNA-alkyltransferase (AGT), that can be self-labeled through addition of a fluorescent substrate, that covalently binds to the SNAP-tag with its benzyl group (Keppler *et al*., 2003; Kolberg *et al*., 2013). As the fluorescent substrate is already folded upon addition, visualization of proteins in the periplasm is reportedly unproblematic (Ke *et al*., 2016). Furthermore, the tag was described as highly specific, allowing even multicolor single molecule tracking (Benke *et al*., 2012).

We employed single molecule tracking (SMT) using the SMTracker tool (Rösch *et al*., 2018), to track and visualize the labeled AmyE-SNAP molecules *in vivo* in *B. subtilis*. The results showed a strong unspecific binding of the used SNAP-Cell 505 substrate (New England BioLabs) to the cell wall of the WT control strain, lacking the SNAP-tag labeled AmyE (Fig. S1.1). Even after several additional washing steps, the control strain displayed strong substrate fluorescence (Fig. S1.1). Several additional steps were taken to mitigate the unspecific binding of the substrate, such as reducing the substrate concentration and using a blocking agent (New England BioLabs). Unfortunately, the objective could not be achieved with this method. It appears that the substrate used in *B. subtilis*, can bind non-specifically to the cell wall or its components such as proteins or teichoic acids. Perhaps this is why, the visualization of proteins via the SNAP-tag method is mainly used by researchers working with eukaryotic cells (Hoelzel & Zhang, 2020). Systematic screening of 22 dyes for optimal performance in single-molecule microscopy revealed that many dyes suffer from either rapid photobleaching or high nonspecific staining (Bosch *et al*., 2014). However, numerous different dyes are available (Dreyer *et al*., 2023), so the problems in this work might not have occurred with the selection of an alternative substrate.

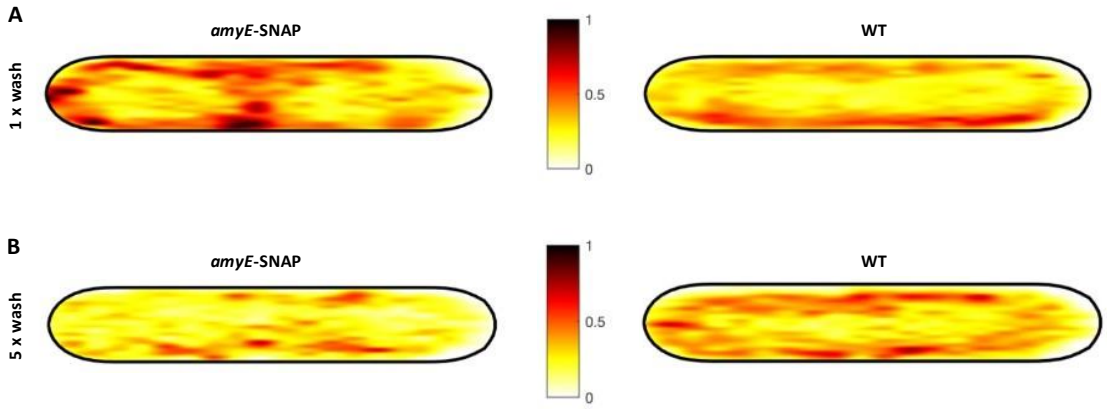


Figure S1.1: Heat maps of single-molecule localization of SNAP-tag substrate binding in *Bacillus subtilis* cells. The distribution of tracks is indicated by a color shift from yellow (low probability) to black (highest probability). A) Fluorescent SNAP-tag substrate localized throughout the cell envelope in both the strain expressing the labeled *amyE*-SNAP fusion protein and the wild-type (WT) control strain. Cells were washed one time with PBS after incubation with the SNAP-tag substrate. B) Cells were washed five time*s* with PBS after incubation with the SNAP-tag substrate.

Effects of *B. subtilis* FloT and PBP5 on secretion in *B. licheniformis*

In this work, we planned to combine the enhanced expression of the main Sec-pathway components with a simultaneous overexpression of flotillins to achieve a positive effect on secretion performance. For this purpose, the *B. licheniformis* strain MC8.4a was used, which is characterized by the co-expression of an artificial operon consisting of the *secY*, *secE* and *secG* genes. This operon is under the control of the *oppA* promoter. An additional copy of *yuaFG*, encoding FloT and NfeD2 from *B. subtilis*, was integrated to the native *amyS* gene locus of *B. licheniformis* MC8.4a under the control of an IPTG- inducible promoter.

Figure S1.2 shows that the additional production of FloT together with co-expression of *secYEG*, did not improve the secretion performance of the *B. licheniformis* strain. Since the additional expression of *yuaFG* in *B. subtilis* resulted in increased AmyE secretion, it is reasonable to assume that the FloT protein from *B. subtilis* does not have the same effects on fluidity and lipid composition of the membrane in *B. licheniformis*.

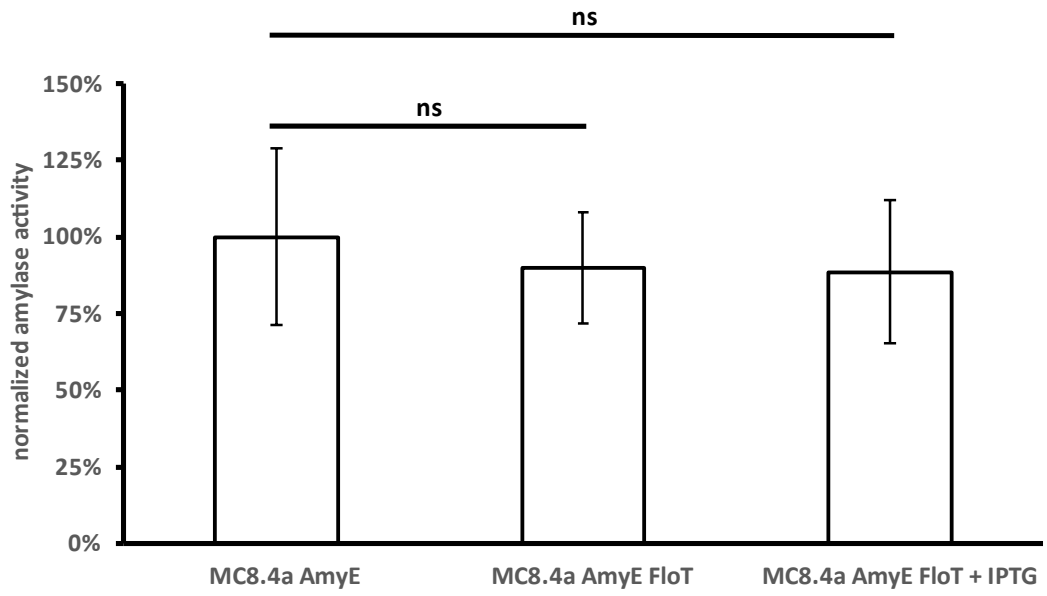


Figure S1.2: Amylase activity in culture supernatant of *B. licheniformis* MC8.4a cells in stationary phase of growth, AmyE: strain with plasmid-based expression of AmyE. FloT: strain with *amyS::P*$_{hyper\text{-}spank}$*-yuaFG*. If marked, 1 mM IPTG was added to the culture. Significance test "two-tailed T-test". Duplicates from biological triplicates are shown.

In addition, *B. licheniformis* strain MC6 was used for the additional expression of FloT. This strain does not show any additional expression of *secYEG*. Again, the additional production of FloT from *B. subtilis* did not result in any improvement of the secretion capacity of AmyE in the *B. licheniformis* strain (Fig. S1.3).

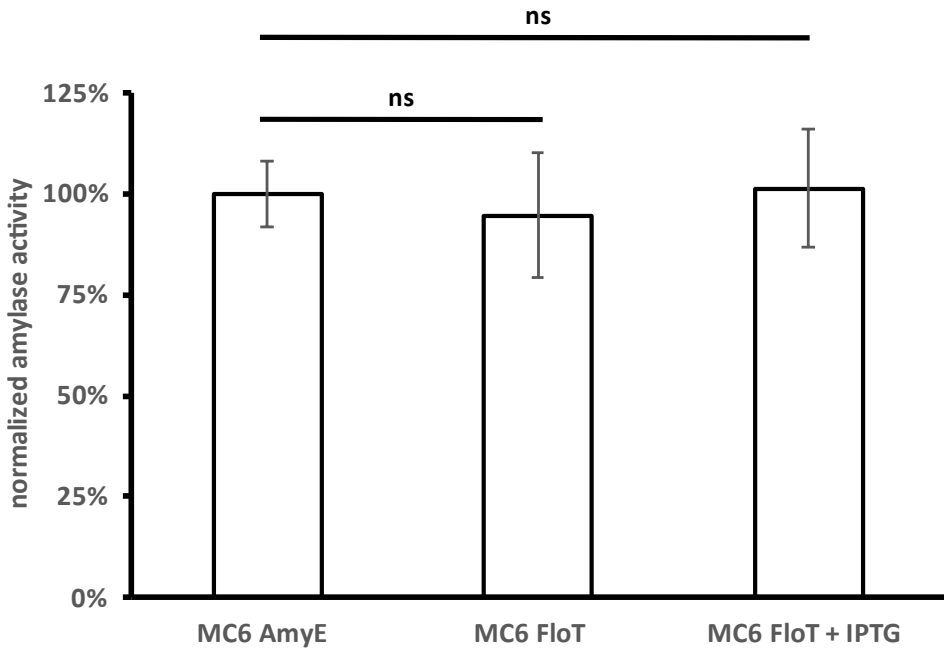


Figure S1.3: Amylase activity in culture supernatant of *B. licheniformis* MC6 cells in stationary phase of growth. AmyE: strain with plasmid-based expression of AmyE. FloT: strain with *amyS::*$P_{hyper\text{-}spank}$*-yuaFG*. If marked, 1 mM IPTG was added to the culture. Significance test "two-tailed T-test". Duplicates from biological triplicates are shown.

It is possible that the FloT from *B. subtilis* is subject to proteolysis when expressed in *B. licheniformis.* As the mechanism by which FloT increases membrane fluidity is currently not completely understood, it is also imaginable that other unknown proteins or systems of *B. subtilis* are involved. Although, it is unlikely since Zielińska *et al*. (2020) showed that the addition of FloT alone can increase membrane fluidity of liposomes *in vitro*. In contrast to the well-known flotillin-like proteins of *B. subtilis* FloA and FloT, the closely related *B. licheniformis* genome contains only one SPFH family member, the BLi02729 protein (Hinderhofer *et al*., 2009). As FloT may not be similar enough to the native flotillin, it perhaps cannot produce the same effect. For this reason, an overexpression of BLi02729 in the extra *secYEG* harboring *B. licheniformis* MC8.4a strain may be more promising.

PBP5 (*dacA*) is the most significant DD-carboxypeptidase in *B. subtilis* cleaving the C-terminal D-Ala-D-Ala peptide bond of the stem peptide (Lawrence & Strominger, 1970) and thereby contributing to control the degree of cell wall cross-linking. Increasing PBP5 activity should reduce the number of cross-linkable stem peptides and a subsequent increase in cell wall mesh size. This would lead to a more permeable cell wall and facilitate the passage through this barrier for secreted protein. With this goal, an additional copy of the *dacA* gene, under the control of an IPTG-inducible promoter, was integrated at the native *amyS* gene locus of *B. licheniformis* MC6.

Although the secretion efficiency in *B. subtilis* was improved in this way, no significant improvement could be achieved in *B. licheniformis* by the additionally expressed *dacA* gene (Fig. S1.4). The reason for this could be a stronger post-translational control of PBP5 by proteases in *B. licheniformis*, so that an increased expression of *dacA* does not lead to increased activity of the protein. Another explanation for the non-significant effect would be that PBP5 from *B. subtilis* may not fulfill the same effectiveness in *B. licheniformis* as a heterogeneously expressed protein. For this reason, an overexpression of the native *dacA* gene of *B. licheniformis*, may be more likely to lead to the intended effect on secretion.

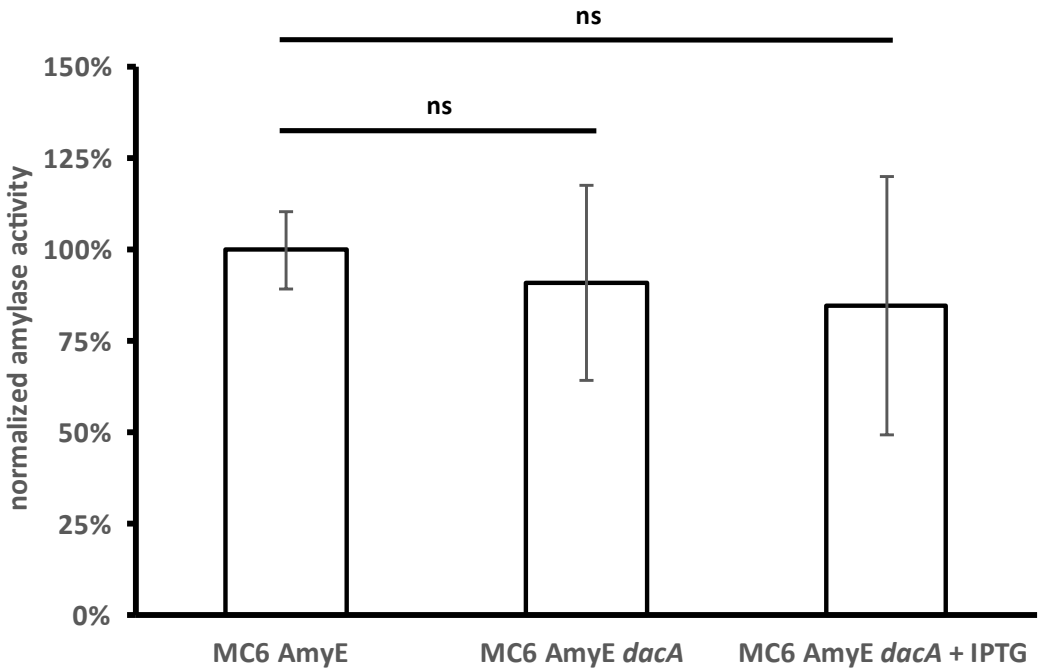


Figure S1.4: cells in stationary phase of growth, AmyE: strain with plasmid-based expression of AmyE. *dacA*: strain with construct *amyS::*$P_{hyper\text{-}spank}$*-dacA*. If marked, 1 mM IPTG was added to the culture. Significance test "two-tailed T-test

Acknowledgements

Als Erstes möchte ich mich bei Prof. Dr. Peter L. Graumann bedanken, für die Möglichkeit, dass ich meine Doktorarbeit in seiner Arbeitsgruppe anfertigen durfte. Ich habe unsere Diskussionen, das ausgewogene Maß an Anleitung und Unabhängigkeit, sowie seine Geduld und Herzlichkeit sehr geschätzt.

Ich möchte mich bei Dr. Klaus Liebeton bedanken, dass er stets Zeit gefunden hat, mit fachlicher Auskunft meine Arbeit zu unterstützen. Des Weiteren danke ich Prof. Dr. Martin Thanbichler, dass er meine Zweitbegutachtung übernommen hat und Prof. Dr. Andreas Seubert dafür, Teil meiner Prüfungskommission zu sein.
Ein weiterer großer Dank geht an die anderen Doktoranden der AG Graumann. Dank euch habe ich ein kameradschaftliches und spannendes wissenschaftliches Umfeld in der AG erlebt. Wegen der fruchtbaren Diskussionen, dem kontinuierlichen gegenseitigen Interesse und der nie abreißenden Bereitschaft zu helfen bin ich jeden Tag gern zur Arbeit gekommen. Mein besonderer Dank gilt dabei Rebecca Hinrichs, Alexandra Kilb, Maximilian Greger, Kristin Velten und Sven Holtrup für jede Art von Unterstützung, Begeisterung und die aufregende Zeit.
Bedanken möchte ich mich auch bei den Frauen hinter den Kulissen der AG Graumann (Frauke Körner, Sabrina Steidl und Antje Schäfer), Dr. Barbara Waidner sowie den ehemaligen Doktoranden der AG Graumann, die ich kennenlernen durfte.

Meine tiefste Dankbarkeit gilt meiner Familie und Freunden, für ihre unbedingte and aufbauende Unterstützung. Besonders möchte mich bei meiner Mutter bedanken, dass sie immer für mich da war und mich unterstützt hat. Ein ganz besonderer Dank gilt auch dir Alena, dafür dass du mit dem nötigen Humor und Verständnis, immer an meiner Seite stehst.

Curriculum vitae

Name:	Manuel Strach
Date of birth:	13.10.1992
Place of birth:	Herdecke

March 2019 – October 2022	Philipps-Universität-Marburg PhD thesis in Biochemistry under the supervision of Prof. Dr. Peter L. Graumann Titel: ”Secretion processes as a limiting factor of protein production in *Bacillus*” Zentrum für Synthetische Mikrobiologie and Department of Chemistry
October 2015 – September 2018	Master of Science Thesis: “Untersuchungen zum Wirkmechanismus des Lipodepsipeptid-antibiotikums Daptomycin” Department: Institut für Pharmazeutische Mikrobiologie (University Bonn) Supervisor: Dr. Fabian Grein
October 2012 – September 2015	Bachelor of Science Thesis: „Alternative Techniken zur Anreicherung und Isolierung Ammoniak oxidierender Bakterien“ Department: Mikrobiologie und Biotechnologie (University Hamburg) Supervisor: PD Dr. Andreas Pommerening-Röser
June 2012	Allgemeine Hochschulreife, Ernst-Barlach-Gymnasium, Unna