Regulation of secretion of the signalling protease PopC in *Myxococcus xanthus*

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Abstract

In response to starvation *Myxococcus xanthus* initiates a developmental program that culminates in fruiting body formation. Completion of this developmental program depends on cell-cellcommunication involving at least two intercellular signals, the A-signal and the C-signal. The contact-dependent intercellular C-signal function to induce and coordinate the two morphogenetic events in fruiting body formation, aggregation and sporulation, temporally and spatially coordinated. The intercellular C-signal is a 17 kDa protein (p17), which is generated by proteolytic cleavage of the full-length 25 kDa *csgA* protein (p25), and is essential for fruiting body formation. p25 and PopC, the protease that cleaves p25, accumulate in the outer membrane and cytoplasm, respectively in vegetative cells. PopC is specifically secreted during starvation. Therefore, restriction of p25 cleavage to starving cells depends on a compartmentalization mechanism that involves the regulated secretion of PopC in response to starvation. In this report, the main focus is on understanding the mechanism underlying regulated secretion of the PopC protease.

We first focused on the identification of proteins required for PopC secretion. PopC lacks a signal peptide and is secreted in an unprocessed form. We report that two incomplete type III secretion systems, a type VI secretion system and type I secretion systems are not involved in PopC secretion. From a collection of mutants generated by random transposon mutagenesis and unable to complete fruiting body formation, we identified seven mutants unable to secrete PopC. None of the insertions were in genes coding for known secretion systems. The mutations were divided into three classes based on the insertion sites. The class I mutation was in a gene cluster largely encoding proteins of unknown function, predicted to localize to the cell envelope, and with a narrow phylogenetic distribution except for a D,D-carboxypeptidase and two Ser/Thr kinases. The class II mutations were in two clusters encoding paralogous proteins of unknown function predicted to localize to the cytoplasm. Several of the class II genes are phylogenetically widely distributed and frequently present in gene clusters linked to genes encoding secretion systems. We speculate that the class I mutation affect a novel type of secretion system involved in PopC

secretion and that the class II mutations either affect proteins with accessory or regulatory functions in PopC secretion.

Next, we focused on elucidating the molecular mechanism underlying the activation of PopC secretion in response to starvation. Our data demonstrate that PopC secretion is controlled at the post-translational level by a regulatory cascade involving the RelA and PopD proteins. Specifically, RelA is required for activation of PopC secretion in response to starvation and PopD, which is encoded in an operon with PopC, interacts directly with PopC and acts as an inhibitor of PopC secretion. On the basis of genetic and biochemical data we suggest that PopC and PopD form a cytoplasmic complex that blocks PopC secretion in the presence of nutrients. In response to starvation, RelA is activated resulting in induction of the stringent response. Activated RelA by an unknown mechanism induces the proteolytic degradation of PopD in the PopC/PopD complex in that way releasing PopC for secretion. On the basis of these data, we suggest that the generation of p17 depends on a two-step proteolytic cascade involving degradation of PopD and, subsequently, the specific cleavage of p25 by PopC.

The current model for intercellular A-signaling in *M. xanthus* proposes that starvation induces the release of extracellular A-signal proteases. These proteases are thought to cleave surface-exposed proteins and extracellular proteins thereby generating the A-signal amino acids and peptides, which serve to measure the density of starving cells early during development. DNA microarray analyses (S. Wegener-Feldbrügge, unpubl.) previously suggested that the primary defect in the *asgA* and *asgB* mutants, which are unable to generate the A-signal, is not a reduced capacity in protein secretion but a reduced expression of genes encoding secreted proteases including *popC*. Here, genetic analyses demonstrated that restored expression of *popCD* rescues development of *asgA* and *asgB* mutants without restoring A-signaling. Thus, ectopic expression of *popCD* leads to a bypass of the requirement for the A-signal during development. We suggest that the inability of *asgA* and *asgB* mutants to undergo development is the result of at least two defects: (i) reduced expression of the genes encoding the A-signal proteases; and, (ii) reduced expression of the *popC* gene.

Zusammenfassung

Unter Nährstoffmangel initiiert *Myxococcus xanthus* ein Differenzierungsprogramm, das die Bildung von multizellulären Fruchtkörpern ermöglicht. Der Ablauf dieses Programms ist abhängig von interzellulären Kommunikationsprozessen und involviert mindestens zwei interzelluläre Signale, das A-Signal und das C-Signal. Das Zellkontakt-abhängige C-Signal induziert und koordiniert die für die Fruchtkörperbildung essentiellen morphogenetischen Prozesse der Aggregation und Sporulation, sowohl räumlich als auch zeitlich. Dieses Signal ist ein 17 kDa Protein (p17), das aus der proteolytischen Spaltung des 25 kDa CsgA-Proteins (p25) hervorgeht, und ist essentiell für die Fruchtkörperbildung. p25 und PopC, die Protease die p25 spaltet, akkumulieren unter vegetativen Bedingungen in der äußeren Membran bzw. im Zytoplasma. Unter Nährstoffmangelbedingungen kommt es dann zu einer spezifischen Sekretion von PopC. Dieser Mechanismus der Kompartimentalisierung garantiert, dass eine Spaltung von p25 nur in hungernden Zellen erfolgen kann. Der Hauptschwerpunkt dieser Arbeit betrifft die Mechanismen, die der regulierten Sekretion der Protease PopC zugrunde liegen.

Zunächst galt es die Proteine zu identifizieren, die für die PopC Sekretion benötigt werden. PopC verfügt über kein Signalpeptid und wird in einer nicht-prozessierten Form sekretiert. In dieser Arbeit wird gezeigt, dass weder zwei unvollständige Typ-III-, noch ein Typ-IV noch ein Typ-I Sekretionssystem an der Sekretion von PopC beteiligt sind.Aus einer Sammlung von Mutanten, die durch zufällige Transposon-Insertionen entstanden sind und keine Fruchtkörper mehr bilden können, wurden sieben Mutanten identifiziert, in denen keine PopC Sekretion mehr stattfand. Keine der dazugehörigen Insertionen waren in Genen lokalisiert, die für bekannte Sekretionssysteme kodieren. Basierend auf den entsprechenden Insertionspositionen wurden drei Klassen definiert:Die Mutation der ersten Klasse war in einem Gencluster lokalisiert, dass überwiegend Proteine unbekannter Funktion kodiert. Diese Proteine lokalisieren vermutlich in der Zellhülle und zeigen, mit der Ausnahme von einer D,D-Carboxypeptidase und zwei Ser/Thr Kinasen, eine eingeschränkte phylogenetische Verbreitung. Die zweite Klasse von Mutationen fand sich in zwei Genclustern, die paraloge

Proteine mit unbekannter Funktion kodieren. Bei diesen Proteinen handelt es sich vermutlich um zytoplasmatische Proteine. Viele Gene dieser 2. Klasse sind phylogenetisch weit verbreitet und finden sich oftmals in Genclustern, die sich in Nachbarschaft mit Sekretionssystem-kodierenden Genen befinden. Wir nehmen an, dass die Klasse 1 Mutation ein neuartiges Sekretionssystem beeinflusst, dass an der PopC Sekretion beteiligt ist. Wir vermuten außerdem, dass die Klasse 2 Mutationen Proteine beeinflussen, die akzessorische oder regulatorische Funktionen bei der PopC Sekretion einnehmen.

Ein weiterer Schwerpunkt dieser Arbeit lag auf dem Mechanismus, mit dem die PopC Sekretion unter Nährstoffmangelbedingungen aktiviert wird. Die vorliegenden Daten demonstrieren, dass die PopC Sekretion auf post-translationaler Ebene durch eine regulatorische Kaskade kontrolliert wird, an der die Proteine RelA und PopD beteiligt sind. Dabei wird RelA für die Aktivierung der PopC Sekretion unter Nährstoffmangelbedingungen benötigt, und PopD, dessen Gen zusammen mit *popC* in einem Operon exprimiert wird, interagiert direkt mit PopC und fungiert dabei als Inhibitor der PopC Sekretion. Auf der Basis genetischer und biochemischer Daten vermuten wir, dass in der Anwesenheit von Nährstoffen PopC und PopD einen zytoplasmatischen Komplex bilden, der eine PopC Sekretion unterbindet. Unter Nährstoffmangel kommt es zu einer RelA-abhängigen Induktion der stringenten Antwort. Durch diese stringente Antwort wird durch einen noch nicht geklärten Mechanismus die proteolytische Degradation von PopD innerhalb des PopC/PopD Komplexes initiiert, wodurch PopC für eine anschließende Sekretion freigesetzt wird. Auf der Basis dieser Daten scheint die Bildung von p17 von einer zweischrittigen proteolytischen Kaskade abzuhängen; zunächst erfolgt eine Degradation von PopD und später die spezifische Spaltung von p25 durch PopC.

Das gegenwärtige Modell des interzellulären A-Signals in *M. xanthus* basiert auf der Annahme, dass unter Nährstoffmangel extrazelluläre A-Signal Proteasen freigesetzt werden. Diese Proteasen sollen dann sowohl Oberflächenproteine als auch extrazelluläre Proteine spalten und dadurch die A-Signal Aminosäuren und Peptide freisetzen, die den Zellen als Sensor der Populationsdichte dienen. DNA-Mikrochip-Analysen (S. Wegener-Feldbrügge, nicht veröffentlicht) ließen vermuten, dass der primäre Defekt von *asgA*- und *asgB*-Mutanten, die kein A-

Signal bilden können, nicht in einer reduzierten Protein Sekretion begründet ist, sondern auf eine reduzierte Expression von Genen, die Proteasen u. a. auch PopD kodieren. In dieser Arbeit konnte gezeigt werden, dass nur durch die wiederhergestellte Expression von *popCD* die Differenzierungsdefekte von *asgA*- und *asgB*-Mutanten aufgehoben werden können, und zwar ohne gleichzeitige Bildung des A-Signals.

Die ektopische Expression von *popCD* umgeht somit während der Differenzierungsphase die A-Signal Abhängigkeit. Wir vermuten, dass die Differenzierungsdefekte der *asgA*- und *asgB*-Mutanten daher zwei Gründe haben: (i) eine reduzierte Expression von Genen, die A-Signal Proteasen kodieren, und (ii) eine reduzierte Expression des *popC* Gens.

Abreviations

ABC	ATP-binding cassette
bp	Base paris
Cm	Chloramphenicol
CTT	Casitone Tris medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Hrs	Hours
IB	Inclusion bodies
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalaktopyranoside
LB	Luria-Bertani medium
MFP	Membrane fusion protein
min	Minutes
Ni-NTA	Nickel-nitriloacetic acid
OD	Optical density
OM	Outer membrane
PI	Protease inhibitors
(p)ppGpp	3’-di-5’-(tri)di-phosphate
RT	Room temperature
SP	Signal peptide
SPS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T1SS – T6SS	Type I secretion system to Type VI secretion system
T4P	Type IV pili
TMH	Transmembrane helix
WT	Wild type

1. Introduction

1.1. The life cycle of *Myxococcus xanthus*

M. xanthus - and Myxobacteria in general - belongs to the delta-subgroup of the proteobacteria. Myxobacteria are typically found in topsoil with only few marine and freshwater myxobacteria having been isolated (Reichenbach, 1999, Velicer & Hillesland, 2008). Soil Myxobacteria grow as saphrophytes on dead organic matter by decomposing degradable polymers or by preying on other microorganisms including bacteria and fungi in what has been described a wolf-pack like manner (Rosenberg & Varon, 1984, Reichenbach, 1999). Predation by *M. xanthus* is conveniently observed in laboratory experiments by placing *M. xanthus* cells adjacent to other microorganisms (Shi & Zusman, 1993, Berleman *et al.*, 2006, Berleman & Kirby, 2007a). However, the importance of Myxobacteria in predation in their natural habitats was only recently illustrated in a study in which living ^{13}C-labeled *Escherichia coli* cells were added to soil samples and used as a proxy for living biomass (Lueders et al., 2006). rRNA stable isotope probing revealed that the ^{13}C-labeled *E. coli* carbon pool was sequestrated mainly by gliding bacteria including Myxobacteria, thus, placing Myxobacteria near or at the top of the microbial food chain.

The lifestyle of *M. xanthus* crucially depends on the ability of cells to display active movement. *M. xanthus* cells move by gliding motility, which is the movement of a rod-shaped cell in the direction of the cell's long axis on a surface and in the absence of a flagellum (Henrichsen, 1972). If present on a solid surface and at a high cell density, *M. xanthus* cells self-organize into three morphologically distinct spatial patterns, spreading colonies, ripples or fruiting bodies (Dworkin, 1996) (Fig. 1). The pattern formed largely depends on the nutritional status of the cells. In the presence of nutrients, the motile, rod-shaped cells grow and divide and form spreading colonies. Cells at the edge of a colony spread coordinately over the surface forming a thin, film-like structure. In the absence of nutrients, the spreading behaviour is constrained and cells initiate a developmental programme that culminates in the formation of multicellular, spore-filled fruiting bodies. Fruiting body formation proceeds in distinct morphological stages that are separated in time and space. The first signs of fruiting body formation are evident after 4-6 hrs of starvation as cells

aggregate to form small aggregation centers. As more cells enter these centers, they increase in size and eventually become mound-shaped. By 24 hrs, the aggregation process is complete and the nascent fruiting bodies each contain approximately 10^5 densely packed cells. Inside the nascent fruiting bodies, the rod-shaped cells undergo morphological and physiological differentiation into spherical myxospores resulting in mature fruiting bodies. Spore-maturation is finished approximately 72 hrs after the onset of starvation. Only 10% of cells undergo sporulation and these cells are those that have accumulated inside the fruiting bodies. Up to 30% of the cells remain outside the fruiting bodies. These cells remain rod-shaped and differentiate to a cell type called peripheral rods (O'Connor & Zusman, 1991b, O'Connor & Zusman, 1991a). Finally, the remaining cells undergo lysis (Rosenbluh et al., 1989). Recently, developmental cell lysis was suggested to reflect programmed cell death and was shown to depend on an unconventional toxin-antitoxin system involving the MazF mRNA interferase (Nariya & Inouye, 2008).

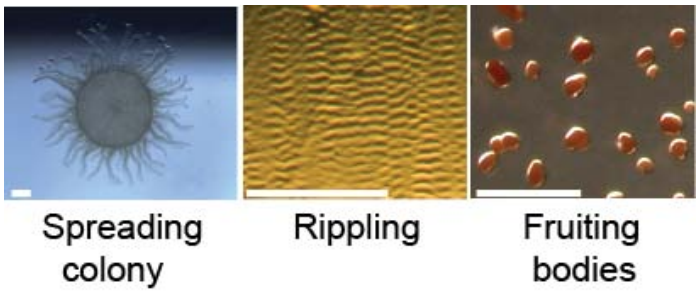

Figure 1. The three cellular patterns formed by *M. xanthus* cells.

Scale bar in left, middle and right panel: 2 mm, 50 µm and 0.2 mm. (Reproduced from (Konovalova *et al.*, 2010a)).

Aggregation and sporulation are the two invariable morphological processes in fruiting body formation. Under less stringent starvation conditions or in the presence of prey (Berleman et al., 2006, Berleman & Kirby, 2007a), fruiting body formation includes a third morphological process referred to as rippling. During rippling cells accumulate in equispaced ridge-like structures separated by troughs of low cell density. The ridge-like structures move coordinately and synchronously as travelling waves over the surface (Shimkets & Kaiser, 1982, Reichenbach, 1965) (for a time lapse movie of rippling cells, see (Welch & Kaiser, 2001)). Microscopic examination of rippling cells has shown that

individual cells essentially oscillate back and forth with no net-movement suggesting that colliding waves reflect each other (Welch & Kaiser, 2001, Sager & Kaiser, 1994, Sliusarenko et al., 2006). Rippling is typically initiated prior to aggregation. Later, during the aggregation process the wave structure disintegrates and cells aggregate into the nascent fruiting bodies.

1.2. Intercellular signalling during fruiting body formation

Fruiting body formation depends extensively on intercellular communication between *M. xanthus* cells. Five intercellular signals have been defined genetically (Hagen et al., 1978, Downard et al., 1993). However, only two of these signals, the A- and C-signals, have been characterized at some details biochemically and functionally. These two systems have different functions. The A-signalling system functions to ensure that fruiting body formation does not initiate unless a sufficiently high number of cells are starving whereas the C-signalling system functions to ensure the correct temporal order of rippling, aggregation and sporulation as well as the spatial coupling of aggregation of cells into fruiting bodies and sporulation of cells that have accumulated inside fruiting bodies.

The A-signal becomes important for development after 2 hrs of starvation (Kuspa *et al.*, 1986). According to the currentmodel, the A-signal consists of two fractions, a heat-stable and a heat-labile fraction. The heat-stable fraction is a mixture of amino acids and their peptides (Kuspa et al., 1992a). Fifteen amino acids have A-signal activity and among them Tyr, Pro, Phe and Leu are the most active. There is no evidence of a specialized A-factor peptide. The peptide fraction of A-signal is a mixture of different peptides and A-signal activity of a peptide is equal to the sum of the A-signal activity of its constituent amino acids. The heat-labile fraction of the A-signal consists of at least two different proteins both of which have protease activity (Plamann et al., 1992). Based on these findings it was suggested that the two A-signal proteases act to produce the *bona fide* A-signal consisting of amino acids and small peptides.

The A-signal functions in a concentration-dependent manner and the amount of A-signal correlates directly with the cell number suggesting that the A-signal acts as a cell-density monitor (Kuspa et al., 1992b). According to the current

model for the functioning of the A-signal, cells during the early stages of starvation secrete a mixture of proteases, which digests surface proteins causing the release of peptides and amino acids that act as the A-signal. If a threshold concentration of A-signal is reached, A-signal is sensed and the expression of developmentally regulated and A-signal dependent genes follows resulting in the progression of the developmental program. Clearly, the A-signalling system is not an analogue of homoserine lactone-based quorum-sensing systems in gram-negative bacteria: As *M. xanthus* uses amino acids as a source of energy, carbon and nitrogen, A-signal at a high concentration supports growth and fail to support development.

Five mutants (*asgA* to *asgE*) that fail to produce A-signal have been isolated (Kuspa & Kaiser, 1989b, Cho & Zusman, 1999, Garza *et al.*, 2000a, Plamann *et al.*, 1994, Plamann *et al.*, 1995, Davis *et al.*, 1995). The corresponding proteins are thought to be components of regulatory pathways important for A-signal synthesis. Noticeably, none of the five *asg* genes encode proteases and the A-signal proteases are still unidentified. Several mutations have been identified that cause a bypass of the A-signal requirement for the expression of A-signal dependent genes (Bowden & Kaplan, 1998, Xu *et al.*, 1998, Kaplan *et al.*, 1991) or for development of *asg* mutants (Dunmire *et al.*, 1999, Tse & Gill, 2002). However, a receptor for the A-signal remains to be identified.

The current model for the molecular nature and function of the A-signal is largely based on analyses of the *asgB* mutant (Kuspa et al., 1992b). Based on analyses of the other mutants with a deficiency in A-signaling (for details, see chapter 4), Diodati et al.have suggested (Diodati et al., 2008) that the A-signal may be a mixture of different signals and that the different *asg* mutants have defects in different components of this “mixed” A-signal.

The C-signal becomes important for fruiting body formation after 6 hrs of starvation (Kroos & Kaiser, 1987b) and is absolutely required for rippling, aggregation and sporulation (Shimkets et al., 1983). C-signal acts in a threshold-dependent manner to induce rippling, aggregation and sporulation (Kim & Kaiser, 1991, Kruse et al., 2001, Li et al., 1992), i.e. rippling is induced at a low threshold, aggregation at an intermediate threshold and at a high threshold level sporulation is induced. C-signal is non-diffusible (Lobedanz

&Søgaard-Andersen, 2003, Shimkets & Rafiee, 1990). In addition C-signal transmission requires active motility and proper cell alignment (Kroos et al., 1988, Kim & Kaiser, 1990c, Kim & Kaiser, 1990b). Based on these observations it has been suggested that C-signal transmission is contact-dependent and involves direct cell-cell contacts. According to this model, C-signal transmission depends on the interaction between C-signal located on one cell and a C-signal-receptor on an adjacent cell. This receptor remains to be identified.

The C-signal is a 17-kDa protein (p17) and its synthesis depends on the *csgA* gene (Kim & Kaiser, 1990d, Kim & Kaiser, 1990a, Lobedanz & Søgaard-Andersen, 2003). *csgA* codes for a 25-kDa protein (p25) (Kruse et al., 2001), which is proteolytically cleaved to generate p17 (Lobedanz & Søgaard-Andersen, 2003). During the proteolytic cleavage of p25, approximately 8 kDa are removed from the N-terminus and p17 corresponds to the C-terminus of p25. p25 as well as p17 are anchored in the outer membrane.

p25 accumulates in vegetative cells, however, it is only cleaved to generate p17 during starvation (Kruse et al., 2001). Recently, the regulatory mechanism restricting p25 cleavage to starving cells was elucidated (Fig. 2). As both p25 and p17 are anchored in the outer membrane and proteolysis of p25 to p17 is blocked by inhibitors of serine proteases (Lobedanz & Søgaard-Andersen, 2003), it was hypothesized that protease responsible for p25 cleavage is a secreted serine protease. A candidate approach led to identification of the subtilisin-like protease PopC as the protease that directly cleaves p25 (Rolbetzki *et al.*, 2008). The mechanism underlying the regulated proteolysis of p25 is based on regulated secretion of PopC. PopC accumulates in the cytoplasm of vegetative cells and is only secreted by starving cells. Therefore, despite the fact that PopC and p25 both accumulate in vegetative cells, they are only present in the same cell compartment in starving cells, thus, restricting p25 cleavage to starving cells. Once secreted, PopC is rapidly degraded and only acts *in cis*. The fast degradation combined with slow secretion of PopC likely ensures the slow accumulation of p17 on the cell surface, which is necessary for the proper function of C-signal as a developmental timer and morphogen.

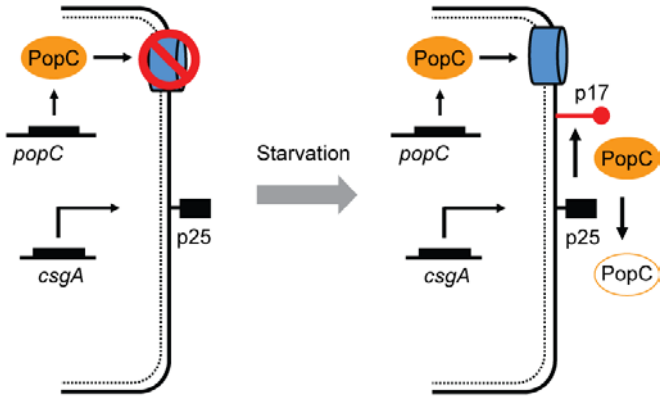

Figure 2. Regulation of p25 cleavage by secretion regulated proteolysis.

In vegetative cells p25 accumulate in the outer membrane and PopC in the cytoplasm. Upon starvation, PopC is secreted by a secretion system (marked in blue) that remains to be identified. Subsequent to secretion of PopC, p25 is cleaved to p17 and PopC rapidly degraded. (Reproduced from (Konovalova et al., 2010a)).

1.3. Protein secretion in Gram-negative bacteria

Bacteria have evolved an extraordinary diversity of mechanisms for protein translocation across the cell envelope to the exterior of cells. This translocation depends on protein complexes referred to as protein secretion systems. The cell envelope of Gram-negative bacteria consists of two membranes, the inner membrane and the outer membrane, separated by a layer of peptidoglycan in the periplasm. Two main strategies exist for protein secretion across the cell-envelope in Gram-negative bacteria (Fig. 3). The so called two-step translocation relies on highly conserved secretion systems, which translocate proteins across the inner membrane, and variety systems that translocate proteins across the outer membrane. In contrast, one-step translocation relies on secretion systems, which form “tunnels” across the cell envelope from the cytoplasm to the cell surface allowing proteins to be translocated from the cytoplasm directly to the outside of cells.

1.3.1. Two-step translocation

There are two distinct and highly conserved systems dedicated to translocating proteins across the inner membrane: the Sec and Tat systems. The Sec system is dedicated to the transport of unfolded proteins. The Sec system consists of the SecYEG proteins, which form an inner membrane channel, the ATPase SecA, the secretion chaperone SecB, and the membrane proteins SecDF and

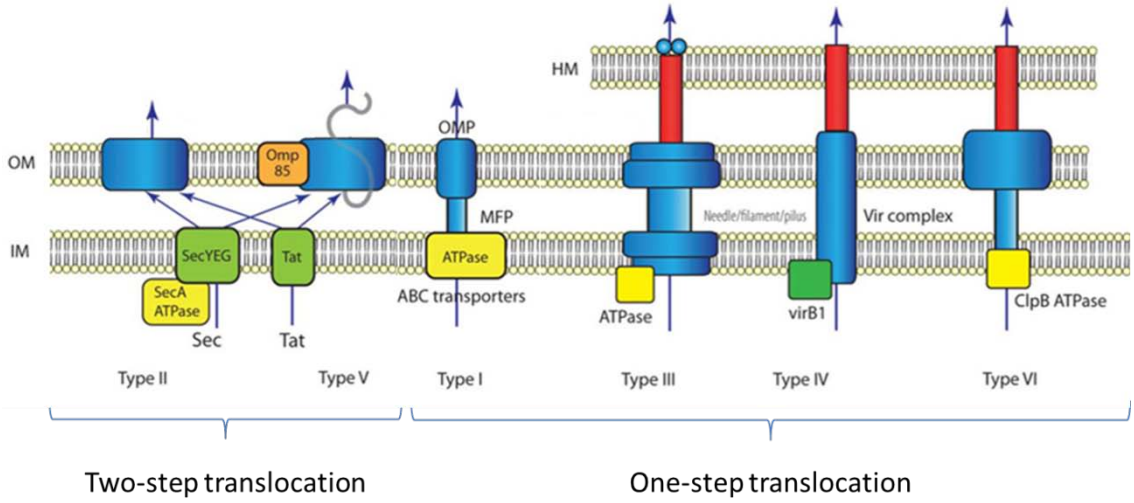

Figure 3. Gram negative bacteria utilize two main strategies for protein secretion cross the cell wall. Figure depicts schematic organization of six known secretion systems (Type I – Type VI) modified from (Tseng et al., 2009) and organized into two groups: two-step and one-step translocation. Inner membrane (IM) and outer membrane (OM) and host cell membrane (HM) are indicated.

YajC the function of which is not well understood (Driessen & Nouwen, 2008). Protein translocation is powered by ATP-hydrolysis by SecA and the proton motive force (Driessen, 1992). Proteins translocated by the Sec-system have a conserved sequence at the N-terminus, referred as the secretion signal peptide, which allows the recognition of these proteins by the SecYEG translocase (Cunningham & Wickner, 1989). The signal peptide consists of three parts, a positively charged N-terminus (N domain), followed by hydrophobic core region (H domain), and a polar region containing the signal peptidase cleavage site (C domain) (Emanuelsson et al., 2007). After translocation across the inner membrane, the signal peptide is removed by the membrane bound signal peptidase I (Paetzel *et al.*, 2002).

The Tat system is dedicated to translocation of fully folded proteins, in many cases complexed to their cofactors (Berks *et al.*, 2003), across the inner membrane. The Tat system consists of the three inner membrane proteins TatA, TatC and TatB and is driven by the proton motive force. Substrates of the Tat system also contain an N-terminal signal peptide with an overall organization similar to that of Sec-signal peptides. However, the Tat signal peptides contain a twin-arginine motif (Bendtsen et al., 2005), hence the name

of the system. The presence of signal peptides in Sec- and Tat-substrates, makes a computational analysis possible in which proteins translocated by these systems can be predicted. Once transported to the periplasm, secreted proteins have several distinct routes to cross the outer membrane via Type II secretion system (T2SS) or Type V secretion system (T5SS).

T2SS - also sometime referred as the general secretory pathway - consists of a core of 10-12 proteins (Filloux, 2004). Several of the proteins of T2SS are highly homologous to proteins of type IV pili (T4P) (Peabody et al., 2003, Planet et al., 2001). Current models propose that T2SS is driven by the cytoplasmic inner-membrane associated ATPase GspE. GspE is thought to promote the assembly of pseudopillins (GspG-K) in the periplasm into a pseudopilus, which is thought to push the protein to be secreted through the outer membrane secretin GspD (Filloux, 2004).

T5SS includes three subfamilies: Autotransporters (T5SSa), two-partner secretion systems (T5SSb), and Oca systems for assembly of cell-surface adhesins (T5SSc) (Desvaux et al., 2004). An autotransporter consists of a single protein with two distinct domains: The passenger domain is the secreted domain and the translocator domain forms an outer membrane pore with a β-barrel structure (Oomen et al., 2004). In the case of two-partner secretion systems, these functions (secreted protein and outer membrane pore) are split into two distinct proteins, referred to as as TpsA and TpsB (Jacob-Dubuisson et al., 2001). Protein members of T5SSc have overall organization as T5SSa, but remain in the outer membrane as surface-attached oligomers (Roggenkamp et al., 2003). No external energy source is required for protein translocation across the outer membrane by T5SS and it has been proposed that the free-energy of folding facilitate the translocation across the outer membrane (Jacob-Dubuisson et al., 2001).

1.3.2. One-step translocation

There are at least four structurally and functionally distinct systems that translocate proteins in a single step from the cytoplasm to the exterior: Type I, III, IV and VI secretion systems (T1SS, T3SS, T4SS and T6SS). Because these systems do not require Sec-(or Tat-) system dependent protein translocation

across the inner membrane, they are often referred to as Sec-independent secretion systems.

T1SS is relatively simple and consists of three different proteins: an ATPase in which the ATP binding cassette (ABC) domain is fused to an inner membrane domain, a membrane fusion protein (MFP) and the outer membrane TolC protein (Andersen et al., 2001). The ABC protein functions as a homodimer and the inner membrane part interacts with MFP (Wang et al., 1991). When this complex interacts with a protein substrate, it recruits the outer membrane component TolC and a continuous channel through the entire cell envelope is formed (Koronakis et al., 2000). Proteins are translocated in an unfolded form (Koronakis et al., 2000) and translation and secretion of proteins by T1SS are thought to be closely coupled (Masi & Wandersman, 2010). A specific secretion signal peptide for T1SS has not been identified. However, it is generally accepted that the C-terminus is important for secretion (Masi & Wandersman, 2010). Moreover, additional signals along the polypeptide are also thought to be important for translocation (Masi & Wandersman, 2010).

T3SS are well-studied in plant- and animal pathogens; however bioinformatics studies have revealed the presence of T3SS genes in non-pathogenic organisms from soil or water environments suggesting that T3SS is widespread among Gram-negative bacteria (Pallen et al., 2005). T3SS is involved in translocation of proteins across the cell envelope into the cytoplasm of a host cell (Cornelis, 2006). T3SS consists of more than 20 proteins, including structural components, a number of regulatory proteins and secretion chaperones (Cornelis, 2006). Most structural T3SS proteins are homologous to components of the flagella assembly apparatus. The key architectural feature of the T3SS is the needle-like structure that projects from the bacterial surface. This needle is positioned on a basal body, which is made of connected ring-like structures that span both membranes (Spreter et al., 2009). T3SS is energized by the proton-motive force while an associated ATPase is important for the disassembly of complexes consisting of secreted substrate and its cognate chaperone (Wilharm et al., 2007). Proteins secreted via T3SS do not have a conserved signal sequence and even the nature of the secretion signal has been a matter of debate, i.e. different groups have suggested that the secretion

signal resides in the 5'-end of the mRNA orin the N-terminus of the secreted protein or involves protein-chaperone interactions (McDermott et al., 2010). T3SS is the paradigm for regulated secretion. T3SS is regulated at different levels, starting with gene expression and assembly of the system to the order and timing of secretion of substrates (Deane et al., 2010). In *Yersinia* spp. at least two environmental stimuli are required for activation of the T3SS. The host's body temperature (37°C) acts as a global environmental signal priming expression of and assembly of T3SS (Cornelis, 2006). However, the secretion system remains closed until contact of the pathogen with the surface of a host cell is established. *In vitro* this activation can be triggered by the removal of Ca^{2+} ions from the growth medium (LCR) (Straley et al., 1993).

T4SS is homologous to bacterial conjugation systems and is involved in translocation of proteins, DNA or DNA-protein complexes across the cell envelope or into the cytoplasm of a host cell (Cascales & Christie, 2003). The prototype T4SS of *Agrobacterium tumefaciense* consists of 12 proteins (Krall et al., 2002), including three inner membrane ATPases (VirB4, VirB11 and VirD4), which are important for secretion and T4SS assembly. Three main structural components VirB7, VirB9 and VirB10 make up the core of the complex spanning from the inner to the outer membrane (Fronzes et al., 2009, Chandran et al., 2009) The major pilin VirB2 together with VirB5 and VirB7 form an extracellular T-pilus (Cascales & Christie, 2003). In addition to the structural components, T4SS involved in DNA translocation also contain proteins involved in DNA processing and recruitment (Gomis-Ruth & Coll, 2006). Proteins secreted via T4SS do not have a conserved signal sequence (Alvarez-Martinez & Christie, 2009). It was proposed that a positively charged C-terminus together with additional intrinsic motifs and possibly accessory proteins are important for recognition of secreted proteins by T4SS (Alvarez-Martinez & Christie, 2009).

The recently identified T6SS is one of the most common secretion systems in Gram-negative bacteria and is involved in a variety of functions including bacterial cell-cell and cell-host interactions, biofilm formation, virulence (Schwarz et al., 2010). T6SS is composed of a core of 13 highly conserved proteins (Boyer et al., 2009). A key feature of the T6SS is a high level of structural similarity between several of its components, such as Hcp, VgrG and

VipAB, and phage-tails proteins (Pukatzki et al., 2007, Bönemann et al., 2009). Based on these observations a model was proposed in which T6SS acts as cell-puncturing device and resembles an “inverted phage-derived injection system” (Bönemann et al., 2010). Activity of T6SS can be monitored by accumulation of the two structural proteins, Hcp and VgrG, in the culture supernatant (Mougous et al., 2006, Zheng & Leung, 2007). However, real secretion substrates remain to be identified for most T6SS systems. In *Pseudomonas aeruginosa* Tse1-3 are secreted by T6SS and are involved in antagonistic cell-cell interactions (Hood et al., 2010). In addition to structural genes, a number of T6SS clusters encode Ser/Thr protein kinases and FHA-domain proteins, which are involved in post-translational regulation of T6SS activity by recruiting the APTase ClpV to the assembled T6SS apparatus (Mougous *et al.*, 2007).

1.4. Scope

As described, PopC accumulates in the cytoplasm of vegetative cells and is selectively secreted during starvation. In the work presented here, we focused on elucidating the molecular mechanism underlying regulated PopC secretion in response to starvation. In the first part of this report, we focused on the identification of proteins important for secretion of PopC. PopC does not contain a signal peptide and it is not known how PopC is secreted (Rolbetzki et al., 2008). Consistent with the lack of a signal peptide, PopC is secreted in an unprocessed form (Rolbetzki et al., 2008). Using a candidate approach, we focused on identification of the secretion system responsible for PopC secretion. Our data suggest that neither T1SS, nor T3SS nor T6SS are responsible for secretion of PopC. Using an unbiased genetic approach in which mutants generated by random transposon mutagenesis and unable to complete fruiting body formation were analyzed for their ability to secrete PopC, we identified four gene clusters, two of which are paralogous, as important for PopC secretion. Interestingly, these gene clusters largely encode proteins of unknown function. In the second part of this report, we established a regulatory cascade involving the RelA and PopD proteins, which control PopC secretion post-translationally in response to starvation. In the third part, we show that the primary defect in *asgA* and *asgB* mutants, which are unable to generate the A-

signal, is not a reduced capacity for protein secretion but a reduced expression of several genes encoding secreted proteases including *popC*. Moreover, we find that ectopic expression of *popC* restores development of *asgA* and *asgB* mutants without restoring A-signaling suggesting that the inability of the *asgA* and *asgB* mutants to undergo development is the result of at least two defects: (i) reduced expression of the genes encoding the A-signal proteases, and, (ii) reduced expression of the *popC* gene.

2. Proteins of unknown function are required for PopC secretion

2.1. Results

2.1.1. Bioinformatic analysis of putative secretion systems in *M. xanthus*

Protein secretion has so far not been studied in details in *M. xanthus*. To explore the genomic potential in *M. xanthus* for protein secretion, we first performed a genome-wide computational screen for protein secretion systems.

M. xanthus contains all the systems for translocation of unfolded proteins over the cytoplasmic membrane to the periplasm and for the integration of membrane proteins into the cytoplasmic membrane (Table 1), for a review see (Driessen & Nouwen, 2008). In the Sec system, *M. xanthus* is only missing the chaperone SecB; however, SecB is reportedly dispensable (Driessen & Nouwen, 2008). Moreover, *M. xanthus* contains a signal recognition particle and its receptor as well as the membrane protein insertase YidC.

The twin-arginine translocation (Tat) pathway is used to translocate folded protein across the cytoplasmic membrane (Berks *et al.*, 2003). *M. xanthus* contains all three components (TatA, TatB and TatC) of this system (Kimura *et al.*, 2006) (Table 1). An *M. xanthus tatBC* deletion mutant is viable and displays a pleiotropic phenotype with a decreased growth rate, an inability to form spore-filled fruiting bodies, and altered surface properties (Kimura et al., 2006). The majority of the predicted Tat-substrates are hypothetical proteins, oxidoreductases, peptidases and lipoproteins (Kimura et al., 2006).

To help proteins cross the cytoplasmic membrane, *M. xanthus* contains a single gene encoding a signal peptidase I, which process non-lipoprotein substrates that are exported by the SecYEG pathway or the Tat pathway, and four signal peptidase II, which cleave off signal peptides of lipoproteins exported by the Sec pathway, for review see (Paetzel *et al.*, 2002).

Type II secretion systems (T2SS) represent amajor pathway for translocation of proteins from the periplasm across the outer membrane (Filloux, 2004). T2SS consist of a core of 10-12 proteins (Filloux, 2004). The *M. xanthus* genome contains an intact T2SS system and most of the components of this system are

Table 1. Proteins of Sec system, Sec-dependent systems and Tat system encoded by the *M. xanthus* genome[1]

Gene	Component	Function & comments
Sec system and Sec-dependent systems		
Not found	SecB	Protein export chaperone
MXAN5345	SecA	Protein translocase, ATPase
MXAN3319	SecY	Protein translocase
MXAN3071	SecE	Protein translocase; identified using hidden Markov model.
MXAN2818	SecG	Protein translocase
MXAN4691	SecD	SecYEG protein translocase auxillary subunit
MXAN4690	SecF	SecYEG protein translocase auxillary subunit
MXAN4692	YajC	SecYEG protein translocase auxillary subunit
MXAN4854	Ffh	Signal recognition particle
Remains to be identified	4.5S RNA	Signal recognition particle
MXAN5735	FtsY	Signal recognition particle-docking protein
MXAN7509	YidC	Membrane protein insertase
Tat system		
MXAN5905	TatB	Protein translocase
MXAN5904	TatC	Protein translocase
MXAN2960	TatA	Protein translocase
Signal peptidases[2]		
MXAN3509	Signal peptidase I	Cleavage of type I signal peptides
MXAN0368	Signal peptidase II	Cleavage of type II signal peptides
MXAN0369	Signal peptidase II	Cleavage of type II signal peptides
MXAN3930	Signal peptidase II	Cleavage of type II signal peptides
MXAN3944	Signal peptidase II	Cleavage of type II signal peptides

[1]*M. xanthus* genes were identified using three strategies: Information in the JCVI *M. xanthus* database, BLASTp searches of the *M. xanthus* genome using either protein sequences from *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces coelicolor* or hidden Markov models. [2] The prepilin peptidase GspO is mentioned in the context of the T2SS and a T4P prepilin peptidase (PilD, MXAN5779) is found in the T4P gene cluster. (Reproduced from (Konovalova *et al.*, 2010a))

encoded in a single gene cluster (MXAN2504-MXAN2515) (Table 2).*M. xanthus*does not contain genes encoding GspN and GspM; however these two components are dispensable for the function of T2SS (Filloux, 2004). A GspO prepilin peptidase is encoded in a small gene cluster, which also encodes paralogs of GspD and GspE (MXAN3105-MXAN3107) (Table 2). In addition, the genome contains at least four genes encoding orphan paralogs of GspC, GspE and GspG (Table 2). Several of the proteins of T2SS are highly homologous to proteins of T4P (Peabody et al., 2003, Planet et al., 2001). All the genes for T4P are localized to a single gene cluster (MXAN5771-MXAN5788) with the exception of the *tgl* gene (MXAN3084), which encodes an outer membrane lipoprotein required for assembly of the outer membrane

Table 2. Proteins of type II secretion systems encoded by *M. xanthus* genome

***M. xanthus* gene**	**Component**	**Function & comments**
MXAN2504	GspL	Integral cytoplasmic membrane protein
MXAN2505	GspK	Minor pseudopilin
MXAN2506	GspJ	Minor pseudopilin
MXAN2507	GspI	Minor pseudopilin
MXAN2508	GspH	Minor pseudopilin
MXAN2509	GspG	Major pseudopilin
MXAN2510	GspG	Major pseudopilin
MXAN2512	GspF	Integral cytoplasmic membrane protein
MXAN2513	GspE	Secretion ATPase
MXAN2514	GspD	Outer membrane secretin
MXAN2515	GspC	Integral cytoplasmic membrane protein
MXAN3105[2]	GspO	Prepilin peptidase
MXAN3106[2]	GspD	Outer membrane secretin
MXAN3107[2]	GspE	Secretion ATPase
MXAN2332	GspC	Integral cytoplasmic membrane protein; orphan
MXAN2658	GspE	Secretion ATPase; orphan
MXAN3824	GspG	Major pseudopilin; orphan
MXAN7176	GspE	Secretion ATPase; orphan

[1]*M. xanthus* genes were identified using two strategies: Information in the JCVI *M. xanthus* database and BLASTp searches of the *M. xanthus* genome using either protein sequences from *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces coelicolor*.(Reproduced from (Konovalova *et al.*, 2010a))

secretin PilQ (Nudleman *et al.*, 2006) and four orphan paralogs of the PilT secretion ATPase (MXAN1995, MXAN0415, MXAN6705 and MXAN6706) (Clausen *et al.*, 2009).

The alternatives to the two-step secretion of proteins to the outside of cells using the Sec/Tat pathway in combination with the T2SS are the type I, III, IV and VI secretion systems (Economou *et al.*, 2006). These systems transfer proteins from the cytoplasm directly to the cell exterior or into the cytoplasm of a eukaryotic cell in one-step mechanisms. The *M. xanthus* genome encode all these systems with the exception of a type IV secretion system.

The type I secretion system (T1SS), often referred to as ATP-binding cassette (ABC) secretion systems or ABC protein exporters, generally consist of two inner membrane proteins, an ATPase (the ATP binding cassette protein, ABC protein), a membrane fusion protein (MFP) and the outer membrane TolC protein (Andersen et al., 2001). T1SS have general structural similarity to ABC transporters, which are involved in the import and export of a wide variety of different compounds (Young & Holland, 1999), thus, making it difficult to predict

how many T1SS the *M. xanthus* encodes. The *M. xanthus* genome encodes at least 73 transporter type ABC proteins (Table S1) and 15 TolC-like proteins (Table 3). Based on the genetic organization of the genes encoding the ABC proteins, the *M. xanthus*genome likely codes for 60 ABC transporters. Among them, 22 are likely to be importers based on presence of genes encoding for periplasmic binding proteins.

Table 3. TolC paralogs encoded by the *M. xanthus* genome

TolC gene	Genetic organization[2]
MXAN0985	###0983 0984 0985###
MXAN0990	###0988 0989 0990###
MXAN1791[1]	### 1788 1789 1790 1791 ###
MXAN3424	### 3422 # 3424###
MXAN3431	### 3431###
MXAN3447	###3447 3448 3449#
MXAN3744[1]	### 3744###
MXAN3905	### 3903 3904 3905 # #3908 3909 3910 3911###
MXAN4176[1]	### 4172 4173 4174 4175 4176 ###
MXAN4198[1]	### 4198 4199 4200 4201###
MXAN5030[1]	### 5030 5031 5032 ###
MXAN6176	###6176 6177 6178###
MXAN6487[1]	### 6487 ###
MXAN7238[1]	### 7238 7239 7240 ###
MXAN7436	###7436 7437 7438###

[1] These TolC paralogs are predicted to be part of T1SS based on sequence analyses. [2] Genetic organization: Genes encoding TolC-, ABC-, MFP- or efflux pump proteins are indicated in red, yellow, green and blue, respectively. Genes are indicated by their MXAN numbers. # indicates a gene not encoding TolC-, ABC-, MFP- or efflux pump proteins. (Reproduced from (Konovalova *et al.*, 2010a))

Type III secretion systems (T3SS) support protein export from the bacterial cytoplasm across the periplasm and outer membrane directly to either the extracellular space or the cytosol of eukaryotic cells and are routinely described as specific to pathogenic bacteria (Cornelis, 2006). However, bioinformatics studies have revealed the presence of T3SS genes in non-pathogenic bacteria suggesting that T3SS may have functions not related to pathogenesis (Pallen et al., 2005). We found two gene clusters in the *M. xanthus* genome encoding subunits of T3SS. Both gene clusters are highly degenerate and none of them seem to encode an intact T3SS (Fig. 4). Cluster I (MXAN2434-MXAN2464)

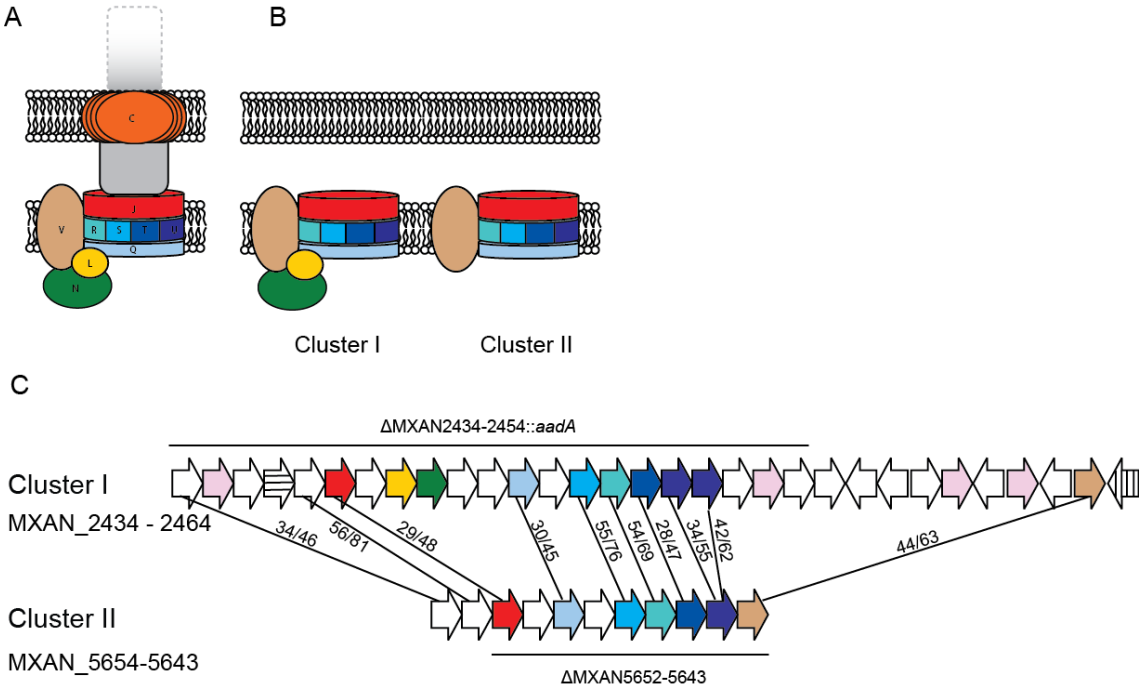

Figure 4. Putative type III secretion system in *M. xanthus.*

(A) Generic T3SS system with the localization of YscL and the nine core proteins conserved in all T3SS. Nomenclature of the proteins is the indicated letter preceded by Ysc (*Yersinia* spp nomenclature). (B) Potential structure of the T3SS encoded by gene cluster I and II in *M. xanthus*; colour code is as in A. (C) Genetic organisation of the two T3SS gene clusters in *M. xanthus*; colour code is as in A. In the connections list the % identity/similarity between paralogs. Notice that no gene is annotated as MXAN5653. Genes are not drawn to scale. The gene replacement ΔMXAN2434-2454::*aadA* in SA3407 and the in-frame deletion in ΔMXAN5652-5643 in SA3448is indicated. (Modified from (Konovalova *et al.*, 2010a))

(Table 4; Fig. 4) encodes eight of the nine proteins that are highly conserved in T3SS - the missing protein being the outer membrane secretin (YscC in the *Yersinia* spp. nomenclature). The remaining eight proteins include those making up the cytoplasmic C-ring, the inner membrane MS ring and the associated export apparatus. Thus, this system is missing the genes encoding the outer membrane ring and the needle structure. Cluster I encodes several proteins containing TPR repeats and one containing a FHA domain. Proteins with these domains have been reported to function as T3SS chaperones and regulators (Pallen *et al.*, 2003, Pallen *et al.*, 2002). Cluster II (MXAN5643-MXAN5654) (Table 5; Fig. 4) consists of 11 genes and also only encodes the proteins that would make up the C-ring, the MS ring and the export apparatus. Importantly, this cluster does not encode the ATPase (YscN in the *Yersinia* spp. nomenclature) that energizes protein export. Thus, both clusters lack the proteins that would make up the secretion apparatus in the outer membrane

Table 4. Proteins encoded by T3SS gene cluster I in the *M. xanthus*[1]

Gene	Component [2]	Function & comments
MXAN2434		Hypothetical conserved in Myxococcales
MXAN2435		TPR repeat containing protein
MXAN2436		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2437		RNA polymerase sigma factor, FliA/WhiG/SigD family
MXAN2438	YscI?	Hypothetical conserved in Myxococcales with low homology to YscI
MXAN2439	YscJ[3]	Lipoprotein in cytoplasmic membrane; component of MS ring
MXAN2440		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2441	YscL	May tether YscN ATPase to export channel
MXAN2442	YscN[3]	Type III secretion system ATPase
MXAN2443		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2444	YscP?	Low homology to YscP
MXAN2445	YscQ[3]	Likely makes up cytoplasmic C-ring
MXAN2446		Hypothetical
MXAN2447	YscR[3]	Basal structure, cytoplasmic membrane protein
MXAN2448	YscS[3]	Basal structure, cytoplasmic membrane protein
MXAN2449	YscT[3]	Basal structure, cytoplasmic membrane protein
MXAN2450	YscU[3]	Basal structure, cytoplasmic membrane protein
MXAN2451	YscU[3]	Basal structure, cytoplasmic membrane protein
MXAN2452		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2453		TPR repeat containing protein
MXAN2454		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2455		Hypothetical
MXAN2456		Hypothetical
MXAN2457		Hypothetical
MXAN2458		Hypothetical conserved in *Stigmatella aurantiaca*
MXAN2459		TPR repeat containing protein
MXAN2460		Hypothetical
MXAN2461		TPR repeat containing protein
MXAN2462		Hypothetical
MXAN2463	LcrD/YscV[3]	Basal structure, cytoplasmic membrane protein
MXAN2464		FHA domain containing protein

[1] *M. xanthus* genes were identified using two strategies: Information in the JCVI *M. xanthus* database and BLASTp searches of the *M. xanthus* genome using protein sequences from *Yersinia spp.*[2] Nomenclature is based on that of *Yersinia* spp. (Cornelis, 2006).[3] Proteins that are universally conserved in T3SS (Cornelis, 2006).(Reproduced from (Konovalova *et al.*, 2010a))

and the needle structure. Consistently, there are no reports in the literature on *M. xanthus* cells containing needle structures protruding from the cell surface. It is not clear if these two gene clusters encode functional protein secretion/translocation systems. However, it is intriguing that despite the

apparent degeneracy of the two gene clusters, the components present precisely match those making up the part of T3SS that allows protein translocation across the cytoplasmic membrane. Therefore, it remains a possibility that these two systems are specifically optimized to the needs of *M. xanthus* and involved in protein translocation only over the cytoplasmic membrane. Alternatively, both systems could function together with one or more of the three secretins of T2SS (Table 2) and T4P. The order of conserved genes in the two clusters is largely conserved (Fig. 4) and similar to that of T3SS gene clusters in other organisms, thus, making it difficult to determine if the two clusters are connected by a gene duplication event. However, the very different identity/similarity values between paralogs in the two clusters (Fig. 4) suggest that the two gene clusters are not connected by a gene duplication event.

Type VI secretion systems (T6SS) also allow the direct translocation of proteins from the bacterial cytoplasm to the extracellular space or to the cytosol of eukaryotic cells (Filloux *et al.*, 2008, Cascales, 2008). These systems have mostly been analyzed in pathogenic bacteria and are, therefore, also commonly referred to as virulence factors. Bioinformatics analyses have, however, shown

Table 5. Proteins encoded by T3SS gene cluster II in the *M. xanthus*[1]

Gene	Component2	Function & comments
MXAN5643	LcrD/YscV[3]	Basal structure, cytoplasmic membrane protein
MXAN5644	YscU[3]	Basal structure, cytoplasmic membrane protein
MXAN5645	YscT[3]	Basal structure, cytoplasmic membrane protein
MXAN5646	YscS[3]	Basal structure, cytoplasmic membrane protein
MXAN5647	YscR[3]	Basal structure, cytoplasmic membrane protein
MXAN5648		Hypothetical
MXAN5649	YscQ[3]	Likely makes up cytoplasmic C-ring
MXAN5650		Hypothetical
MXAN5651	YscJ[3]	Lipoprotein in cytoplasmic membrane; component of MS ring
MXAN5652	YscI?	Hypothetical conserved in Myxococcales with low homology to YscI
MXAN5654		Hypothetical conserved in Myxococcales

[1] *M. xanthus* genes were identified using two strategies: Information in the JCVI *M. xanthus* database and BLASTp searches of the *M. xanthus* genome using protein sequences from *Yersinia spp.*[2] Nomenclature is based on that of *Yersinia* spp. (Cornelis, 2006).[3] Proteins that are universally conserved in T3SS (Cornelis, 2006).(Reproduced from (Konovalova *et al.*, 2010a))

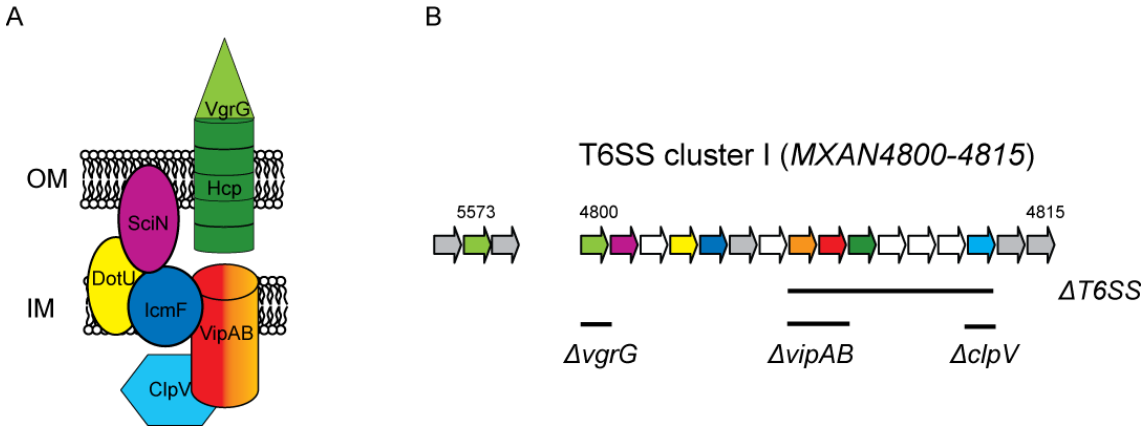

Figure 5.T6SS in *M. xanthus*.

(A) Schematic of generic T6SS system. The suggested localization of eight core proteins, which are also present in *M. xanthus*, is indicated. (B) T6SS gene cluster in *M. xanthus*. The color coding refers to the proteins in (A). White arrows indicate genes encoding core proteins of the T6SS and for which the localization is not known. Grey arrows indicate genes encoding proteins which are not T6SS core proteins. Arrows indicate the direction of transcription. The in-frame deletion ΔT6SS (MXAN4807-4813) in SA3410, ΔVgrG (MXAN4800) in SA3437, ΔT6SS (MXAN4807-4808)in SA3462, ΔclpV (MXAN4813) in SA3416 are indicated. Genes are not drawn to scale.

Table 6. Proteins of the type VI secretion system encoded in the *M. xanthus* genome

Gene	Component[1]	Trivial name & comments
MXAN5573	COG3501/VC_A0123	VgrG
MXAN4800	COG3501/VC_A0123	VgrG
MXAN4801	COG3521/VC_A0113	
MXAN4802	COG3522/VC_A0114	
MXAN4803	COG3455/VC_A0115	DotU
MXAN4804	COG3523/VC_A0120	IcmF
MXAN4805		Conserved hypothetical
MXAN4806	COG3515/VC_A0119	
MXAN4807	COG3516/VC_A0107	VipA
MXAN4808	COG3517/VC_A0108	VipB
MXAN4809	COG3157/VC_A0017	Hcp
MXAN4810	COG3518/VC_A0109	
MXAN4811	COG3519/VC_A0110	
MXAN4812	COG3520/VC_A0111	
MXAN4813	COG0542/VC_A0116	ClpV ATPase
MXAN4814		Hypothetical
MXAN4815		Conserved hypothetical

[1] Core proteins of T6SS are indicated by their respective COG identity (cluster of orthologous groups of proteins) and the orthologous gene in *Vibrio cholerae* O1 El tor N16961 (Boyer et al., 2009).(Reproduced from (Konovalova *et al.*, 2010a))

that T6SS are widespread also in non-pathogenic bacteria (Boyer et al., 2009, Bingle *et al.*, 2008). The structure of T6SS is still unknown. However, bioinformatics analyses have shown that 13 proteins constitute the core of T6SS (Boyer et al., 2009, Bingle et al., 2008). It was previously noticed that the *M. xanthus* genome contains a single gene cluster for T6SS and that this gene cluster encode all 13 core proteins (Boyer et al., 2009, Bingle et al., 2008) (Table 6, Figure 5) suggesting that this gene cluster codes for a functional T6SS. In addition an orphan VgrG homolog is encoded by MXAN5573, which is located outside the main cluster.

2.1.2. Inactivation of T1SS, T3SS and T6SS do not interfere with secretion of PopC

The *M. xanthus* genome encodes at least 73 ABC proteins (Supplementary Table 1) and 15 TolC proteins (Table 3). Due to the large number of ABC proteins encoded by the *M. xanthus* genome, we focused on the 15 TolC proteins to determine whether a T1SS is involved in PopC secretion.

To determine whether any TolC proteins are important for PopC secretion, we first generated insertion mutations in seven genes which are encoding to TolC proteins that are similar to that of a T1SS. All seven mutants generated fruiting bodies with wild type (WT) morphology and timing and sporulated at WT levels (Table 7). Consistently, these seven mutants secreted PopC at WT levels (Table 7). Moreover, none of the mutants had growth, motility or pigmentation defects (data not shown). Moraledo-Munoz et al. (Moraleda-Munoz *et al.*, 2010) recently reported the inactivation of four gene clusters encoding metal efflux pumps. Each of the four deletions covers a TolC encoding gene (Table 7). Three of these mutants develop normally suggesting that PopC secretion is normal. However, one of the mutants displayed developmental defects (ΔMXAN7436-7438). When we analyzed this mutant, we found that PopC secretion is normal (Table 7). Moreover, Moraledo-Munoz et al. reported that the gene cluster MXAN3447-3449, which encodes a TolC paralog, is likely essential (Table 7). Therefore, we did not test whether TolC encoded by MXAN3447 is essential for PopC secretion. To rule out that any of the three remaining TolC paralogs are involved in PopC secretion, we also inactivated these genes. All three mutants developed normally (Table 7) and secreted WT

Table 7. TolC proteins in *M. xanthus*and their associated phenotypes

TolC gene	Mutation	Development	PopC secretion
MXAN0985	Δ0983-0985[2]	Yes	Not tested
MXAN0990	Δ0988-0990[2]	Yes	Not tested
MXAN1791[1]	1791::pAK1	Yes	Normal
MXAN3424	3424::pAK47	Yes	Normal
MXAN3431	3431::pAK46	Yes	Normal
MXAN3447	Essential[2]	Not applicable	Not applicable
MXAN3744[1]	3744::pAK2	Yes	Normal
MXAN3905	Δ3903-3911::*aadA*[3]	Yes	Normal
MXAN4176[1]	4176::pAK3	Yes	Normal
MXAN4198[1]	4198::pAK4	Yes	Normal
MXAN5030[1]	5030::pAK5	Yes	Normal
MXAN6176	Δ6176-6178[2]	Yes	Not tested
MXAN6487[1]	6487::pAK6	Yes	Normal
MXAN7238[1]	7238::pAK7	Yes	Normal
MXAN7436	Δ7436-7438[2]	No	Normal

[1] These TolC paralogs are predicted to be part of T1SS based on sequence analyses. [2]These mutations and phenotypes were described in (Moraleda-Munoz et al., 2010).[3]*MXAN3905* was inactivated by a gene replacement covering MXAN3903-3911.

levels of PopC (Table 7). Although we cannot rule out that the TolC proteins may function redundantly, these observations suggest that PopC secretion does not depend on a T1SS.

To determine whether any of the two T3SS gene clusters are important for PopC secretion, we generated a gene replacement mutation that covers most of the conserved T3SS genes in cluster I and an in-frame deletion that covers all the conserved T3SS genes in cluster II. Mutation of cluster I, cluster II or both clusters did not cause developmental defects and the three mutants generated fruiting bodies with WT morphology and timing and sporulated at WT levels (data not shown). As expected, based on the lack of developmental defects, all mutants secreted PopC at WT levels (data not shown). Moreover, none of the three mutants had growth, motility or pigmentation defects (data not shown).

To determine whether the T6SS gene cluster is important for PopC secretion, we generated an in-frame mutation that covers seven of the 13 genes encoding core components of T6SS (Fig. 5B): This mutant displayed no growth, motility defects or pigmentation defects (data not shown); however, the mutant was unable to form mature fruiting bodies and was strongly reduced in sporulation (Fig. 6A). Yet, the mutant still secreted PopC at WT levels (data not shown).

Thus, the T6SS or gene(s) covered by the deletion in SA3410 are important for fruiting body formation and sporulation but these defects are not caused by lack of PopC secretion.

The deletion in SA3410 covers seven T6SS genes. To determine whether a functional T6SS or whether individual protein(s) - and not the T6SS *per se* - are important for development, we created in-frame deletions of the genes encoding homologs four core structural components of T6SS (Fig. 5B): VgrG (MXAN4800), which is thought to be located to the tip of the Hcp tube, VipAB (MXAN4807-4808), which have been suggested to act as tail sheaths for the Hcp tube (Bönemann *et al.*, 2010), and ClpV (MXAN4813), which has been suggested to function as an energizing component of the T6SS (Bönemann et al., 2009). Interestingly, all three mutants were able to complete fruiting body formation and sporulation (Fig. 6A). Activity of T6SS can be addressed by following the accumulation of Hcp protein in the culture supernatant (Mougous et al., 2006). To testwhetherinactivation of each of these genes lead to loss of function of T6SS we generated anti-Hcp antibodies and tested cell extracts and supernatants of the corresponding mutants for accumulation of Hcp. Hcp accumulated in the cell extracts of WT cells and in the Δ*vipAB*, Δ*vgrG* and Δ*clpV*mutants but not in SA3410 in which a deletion covers the *hcp* gene (Fig. 6B). We were unable to detect Hcp accumulation in the supernatant of vegetative andstarving WT cells as well as inother mutant strains (Fig. 6B).

2.1.3. Identification of genes required for secretion of PopC

Because the candidate approach to identify genes important for PopC secretion was unsuccessful, we initiated a search for such genes using an unbiased random mutagenesis approach. A *popC* mutant is unable to complete fruiting body formation and is severely reduced in sporulation (Rolbetzki *et al.*, 2008). Therefore, we hypothesized that *M. xanthus* mutants unable to secrete PopC would also be unable to complete fruiting body formation and be severely reduced in sporulation. To isolate *M*. *xanthus* mutants unable to complete fruiting body formation, the WT strain DK1622, was exposed to mutagenesis with the transposon mini-Himar(Kan), which confers resistance to kanamycin.

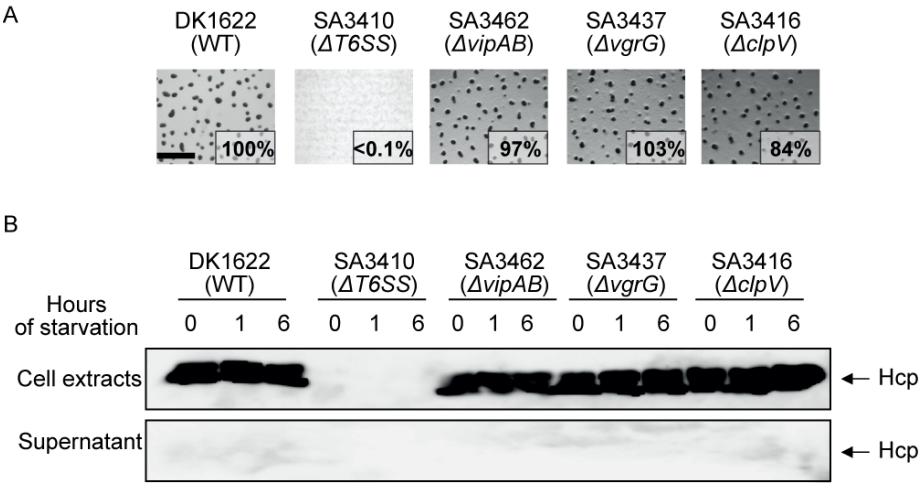

Figure 6.Mutations in T6SS and associated phenotypes.

(A) Developmental phenotype of mutant deleted for the T6SS. Cells of the indicated strains were starved for 120 h on TPM agar. Numbers in each frame indicate the sporulation frequency. Sporulation in DK1622 was set to 100%. Scale bar, 0.1 mm. (B) Hcp accumulation in total cell extracts and supernatant. Cells of the indicated strains were exposed to starvation in the presence of protease inhibitors. Total cell lysates and cell free supernatants were prepared at the indicated time points. Protein isolated from 10^9 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-Hcp antibodies.

Among a total of 10,800 transposon mutants, 115 were unable to complete fruiting body formation. The sites of integration of these 115 mutants were identified by sequencing across the mini-Himar insertion sites. Moreover, the 115 mutants were tested for their ability to secrete PopC. In total, seven mutants were identified, which were severely reduced in PopC secretion. These mutants had similar phenotypes and were unable to aggregate to construct fruiting bodies in submerged culture (Fig. 7A) and on TPM agar as well as on CF agar (data not shown) and were severely reduced in sporulation even after 120 h of starvation (Fig. 7A). All seven mutant strains were indistinguishable from WT cells during vegetative growth with respect to growth, motility and pigmentation (data no shown).

To verify that the mutants were affected in PopC secretion and not in PopC synthesis, WT, a *popC* mutant and the seven mutants were exposed to starvation and PopC accumulation in total cell extracts measured using immunoblotting and PopC secretion to the culture supernatant measured using an ELISA-based assay with PopC-specific antibodies. As shown in Fig. 7BC, all mutants accumulated PopC at WT levels in total cell extracts and all seven mutants were strongly reduced in PopC secretion. As expected based on the

reduced PopC secretion, all seven mutants were severely affected in p25 cleavage to generate p17 (Fig. 7D). Cell fractionation experiments verified that in all seven mutants, p25 was localized to the outer membrane (Fig. 7E). Control experiments with PilQ, PilC and PilB, which localize to the outer membrane, inner membrane and cytoplasm, respectively confirmed that the fractionation procedure functioned properly (Fig. 7E) Thus, all seven mutants display similar phenotypes and are specifically affected in PopC secretion.

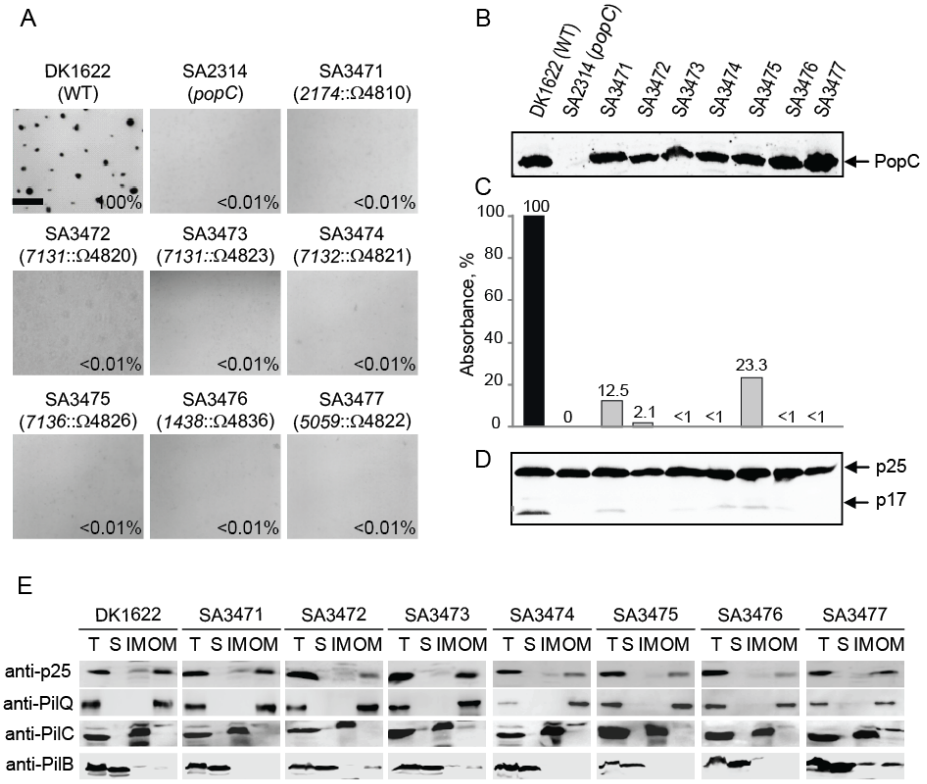

Figure 7. Characterization of mutants blocked in PopC secretion.

(A) Developmental phenotype of mutants blocked in PopC secretion. Cells of the indicated strains were starved for 120 h in submerged culture. Numbers in each frame indicate the sporulation frequency. Sporulation in DK1622 was set to 100%. Scale bar, 0.1 mm.(B) PopC accumulation is normal in mutants unable to secrete PopC. Cells of the indicated strains were starved for 18 h in submerged culture with shaking in the presence of protease inhibitors. Total cell extract from 10^8 cells was added per lane, separated by SDS-PAGE and analyzed by immuno-blotting using antibodies against PopC. (C) Seven mutants are unable to secrete PopC. Cells of the strains indicated in (B) were starved for 18 h in submerged culture with shaking in the presence of protease inhibitors. The cell free supernatant was isolated, concentrated and analyzed for the presence of PopC by ELISA using antibodies against PopC. The PopC-specific signal detected by ELISA in WT cells was set to 100% and the PopC-specific signal detected in the mutants expressed as % of the signal in WT. (D) Mutants unable to secrete PopC are blocked in cleavage of p25. Cells of the strains indicated in (B) were starved for 18 h in submerged culture. Total cell extract from 10^8 cells was added per lane, separated by SDS-PAGE and analyzed by immuno-blotting using antibodies against p25. p25 and p17 are indicated. (E) The PopC secretion mutants are specifically affected in PopC secretion. Total cell extract of vegetative cells (T) of the indicated strains was separated into fractions enriched for soluble (S), inner membrane (IM), and outer membrane (OM) proteins. Protein from 10^8 cells was added per lane and analyzed using antibodies against the outer membrane proteins p25 and PilQ (Bulyha *et al.*, 2009, Nudleman et al., 2006), the inninner membrane protein PilC (Bulyha et al., 2009) and the cytoplasmic protein PilB (Jakovljevic *et al.*, 2008).

The insertions in the seven mutants had occurred in four regions on the *M. xanthus* chromosome, which will be described in the following. mini-Himar(Kan) Ω4810, which we refer to as a class I mutation, had inserted in MXAN2174. The deduced MXAN2174 protein has a size of 150 amino acids and is predicted to localize to the inner membrane based on the presence of three trans-membrane spanning helices (TMH). The MXAN2174 protein does not contain domains of known function, does not share homology with proteins of known function and is only found in members of the order Myxococcales. The developmental defect caused by the miniHimar(Kan) Ω4810 insertion could be due to lack of MXAN2174 function or be caused by a polar effect on the expression of downstream genes (Fig. 8A). Four genes downstream of MXAN2174 are transcribed in the same direction as MXAN2174. MXAN2175 is predicted to encode an inner membrane protein of unknown function and MXAN2175a a periplasmic protein of unknown function both of which are only conserved in the Myxococcales (Table 8). MXAN2176 and MXAN2177 encode Ser/Thr protein kinases (Table 8). To gain further insight into the possible functions of MXAN2174-2177, we also determined the predicted functions of the genes upstream of MXAN2174 (Table 8). The four genes upstream from MXAN2174 encode a fibronectin domain containing protein predicted to localize to the outer membrane (MXAN2170), a widely conserved hypothetical protein (MXAN2171), a hypothetical protein predicted to localize to the inner membrane and only conserved in Myxococcales (MXAN2172), and a D,D-carboxypeptidase (MXAN2173) predicted to localize to the periplasm. MXAN2168 and -2169 encode a transposase and a transposase helper protein respectively.Three additional gene clusters contain paralogs of three or more of the genes in the MXAN2170-2177 gene cluster (Fig. 8A). From the collection of 115 mutants with developmental defects two contain insertions in these gene clusters: mini-Himar(Kan) Ω4809 and Ω4866 are inserted in MXAN1228 and MXAN4841, respectively (Fig. 8A). mini-Himar(Kan) Ω4809 and Ω4866 cause developmental defects but do not block PopC secretion. In addition, paralogs of one or two of the MXAN2170-2177 genes are found in 14 other loci on the *M.xanthus* genome (Fig. 8B). The collection of 115 developmental mutants did

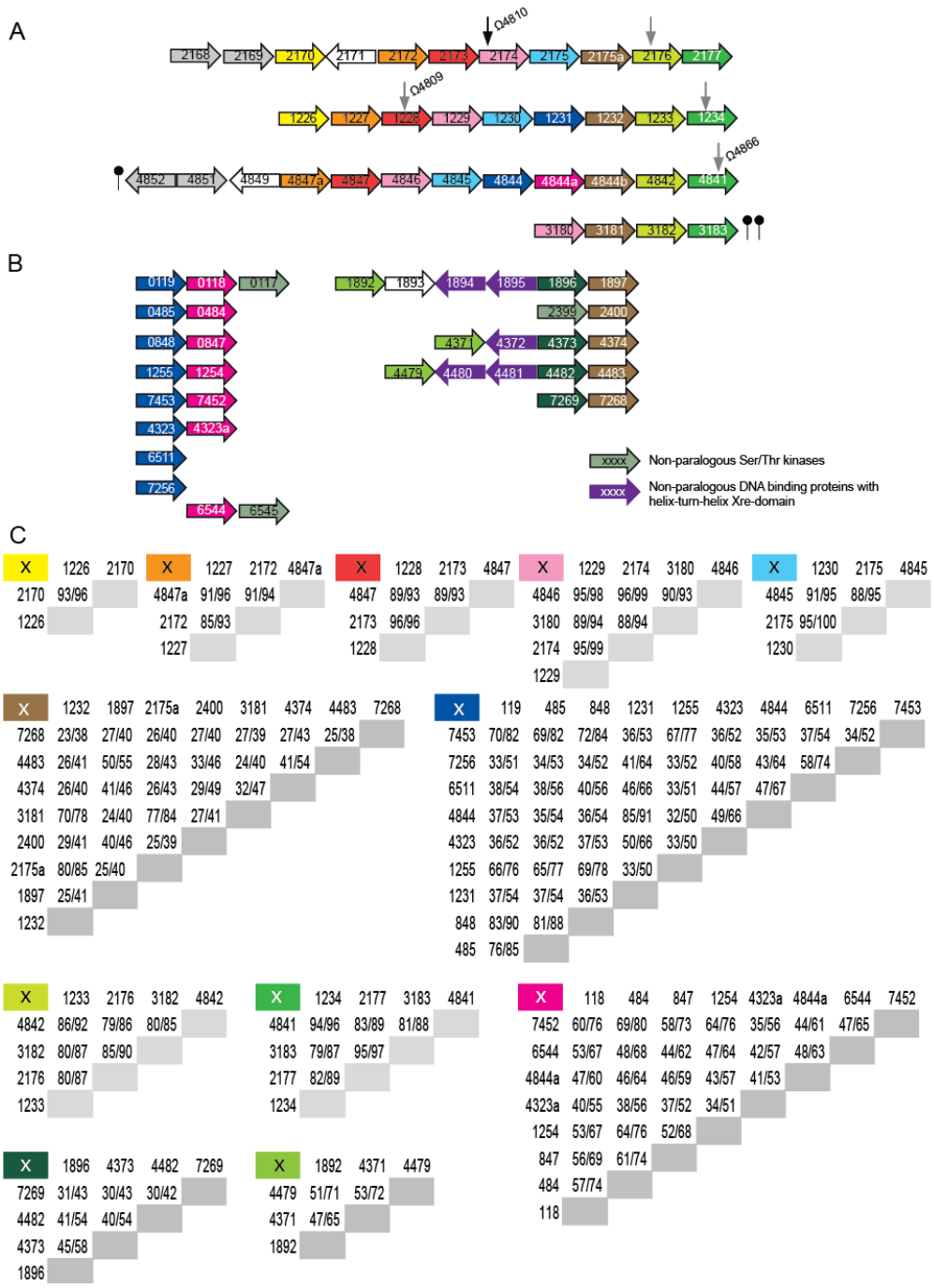

Figure 8. Genetic organization of class I gene clusters.

(A) Genetic organization of the four large class I gene clusters. Paralogs have similar colors. White arrows indicate genes with no paralogs. Grey arrows indicate genes encoding transposases or recombinases. Lollipops indicate tRNA genes. Arrows indicate the direction of transcription. Labeled vertical arrows indicate mini-Himar(Kan) insertions causing a development defect as well as a block in PopC secretion (black) and only causing a developmental defect (grey). Grey, unlabeled vertical arrows indicate genes shown to be required for development (Inouye *et al.*, 2008). It is not known if these genes are required for PopC secretion. Genes are not drawn to scale. (B) Genetic organization of the 14 small class I gene clusters. The color coding is as in (A). Three of the Ser/Thr kinases encoded by these clusters are not paralogs. Three of the clusters encode five DNA binding proteins with an Xre-DNA binding domain. These five proteins are not paralogs. Genes are not drawn to scale. (C) Identities and similarities of proteins encoded by class I gene clusters. The color code shown in the upper left corner refers to the code used in (A) and (B). Numbers to the left and in the upper line refer to gene numbers. For each gene pair, the first number indicates identity and the second similarity.

Table 8. Characteristics of proteins encoded by class I gene clusters

Groups of paralogs	Length (aa)	Predicted function or domainsfor paralogs	Predicted subcellular localization [1]	Phylogenetic distribution [2]
MXAN2170 MXAN1226	1055 1078	Fibronectin domain	OM	Conserved
MXAN2171	322	Conserved hypothetical	Cytoplasmic	Conserved
MXAN2172 MXAN1227 MXAN4847a	119 95 94	Conserved hypothetical	IM (1 TMH)	Myxococcales
MXAN2173 MXAN1228 MXAN4847	178 177 210	D, D-carboxypeptidase	Periplasmic	Conserved
MXAN2174 MXAN1229 MXAN4846 MXAN3180	150 150 150 438	Hypothetical	IM (SP + 3 TMH)	Myxococcales
MXAN2175 MXAN1230 MXAN4845	100 99 99	Hypothetical	IM (SP + 1 TMH)	Myxococcales
MXAN1231 MXAN4844 MXAN0119 MXAN0485 MXAN0848 MXAN1255 MXAN7453 MXAN4323 MXAN6511 MXAN7256	440 440 447 451 448 456 494 436 467 443	Rhs-like protein	IM (SP + 2 TMH)	Conserved
MXAN4849	342	Conserved hypothetical	Cytoplasmic	Conserved
MXAN2175a MXAN1232 MXAN4844b MXAN3181 MXAN1897 MXAN2400 MXAN4374 MXAN4483 MXAN7268	265 287 266 287 318 304 302 309 307	Hypothetical	Periplasmic (SP)	Myxococcales
MXAN4844a MXAN0118 MXAN0484 MXAN0847 MXAN1254 MXAN7452 MXAN4323a MXAN6544	189 190 189 191 189 188 183 240	Hypothetical	Cytoplasmic	Myxococcales
MXAN2176 MXAN1233 MXAN4842 MXAN3182	597 596 596 596	Ser/Thr kinase	Cytoplasmic	Conserved
MXAN2177 MXAN1234 MXAN4841 MXAN3183	405 404 404 384	Ser/Thr kinase	Cytoplasmic	Conserved

[1] Subcellular localization was predicted on the basis of the absence or presence of signal peptides (SP), trans-membrane helices (TMH) using or domains normally associated with a particular subcellular localization.[2]The phylogenetic distribution of proteins is as follows: Proteins labeled conserved have homologs in least one species outside the Myxococcales; proteins labeled Myxococcales have homologs in at least two of the five Myxococcales species with completely sequenced genomes.

not contain mutants with insertions in these genes. We refer to the MXAN2170-2177 genes and the genes in the additional 17 locations as the class I genes.

We refer to the five mini-Himar(Kan) insertions Ω4820 and Ω4823 in MXAN7131, Ω4821 in MXAN7132, Ω4826 in MXAN7136 and Ω4822 in MXAN5059 as the class II mutations. Four of these insertions are in three genes, which are part of a gene cluster extending from MXAN7129-7137 (Fig. 9A). These three genes encode hypothetical proteins (MXAN7131 and MXAN7136) and a conserved hypothetical protein (MXAN7132) (Table 9). All three proteins are predicted to be cytoplasmic. The developmental defect caused by these three insertions could be caused by a lack of the corresponding proteins or by a polar effect on the expression of downstream genes. One gene (MXAN7129) downstream of MXAN7131 is transcribed in the same direction as MXAN7131 and is a paralog of MXAN7136 (Fig. 9A). Seven genes downstream of MXAN7132 are transcribed in the same direction as MXAN7132 and include MXAN7136. With the exception of 7133a, these genes encode hypothetical or conserved hypothetical proteins, which are all predicted to be cytoplasmic (Table 9). MXAN7133a encodes a protein with similarity to 3-ketoacyl- acyl carrier protein (ACP) synthases, which catalyze the condensation reactions in fatty acid biosynthesis (Gottschalk, 1986). However, MXAN7133a lacks the active site Cys residue (Huang *et al.*, 1998). Paralogs of most of the genes in the MXAN7129-7137 gene cluster are found in six additional gene clusters on the *M. xanthus* genome (Fig. 9A). One of these gene clusters is MXAN5066-MXAN5059 and mini-Himar(Kan) Ω4822 is inserted in MXAN5059 (Fig. 9A). MXAN5059 encodes a hypothetical protein predicted to be cytoplasmic and only conserved in *M. xanthus*. The gene downstream of MXAN5059 is transcribed convergently suggesting that MXAN5059 is directly important for PopC secretion. We refer to the genes in these seven clusters as the class II genes.

Finally, mini-Himar(Kan) Ω4836, which we refer to as a class III insertion, is inserted in MXAN1438, which encodes a CinA homolog (Fig. 9C). In *M. xanthus* a second CinA homolog is encoded by MXAN7138, which is located immediately downstream of the MXAN7129-7137 class II gene cluster (Fig. 9AD). In other bacteria, *cinA* is often found in an operon with *recA* (Kaimer &

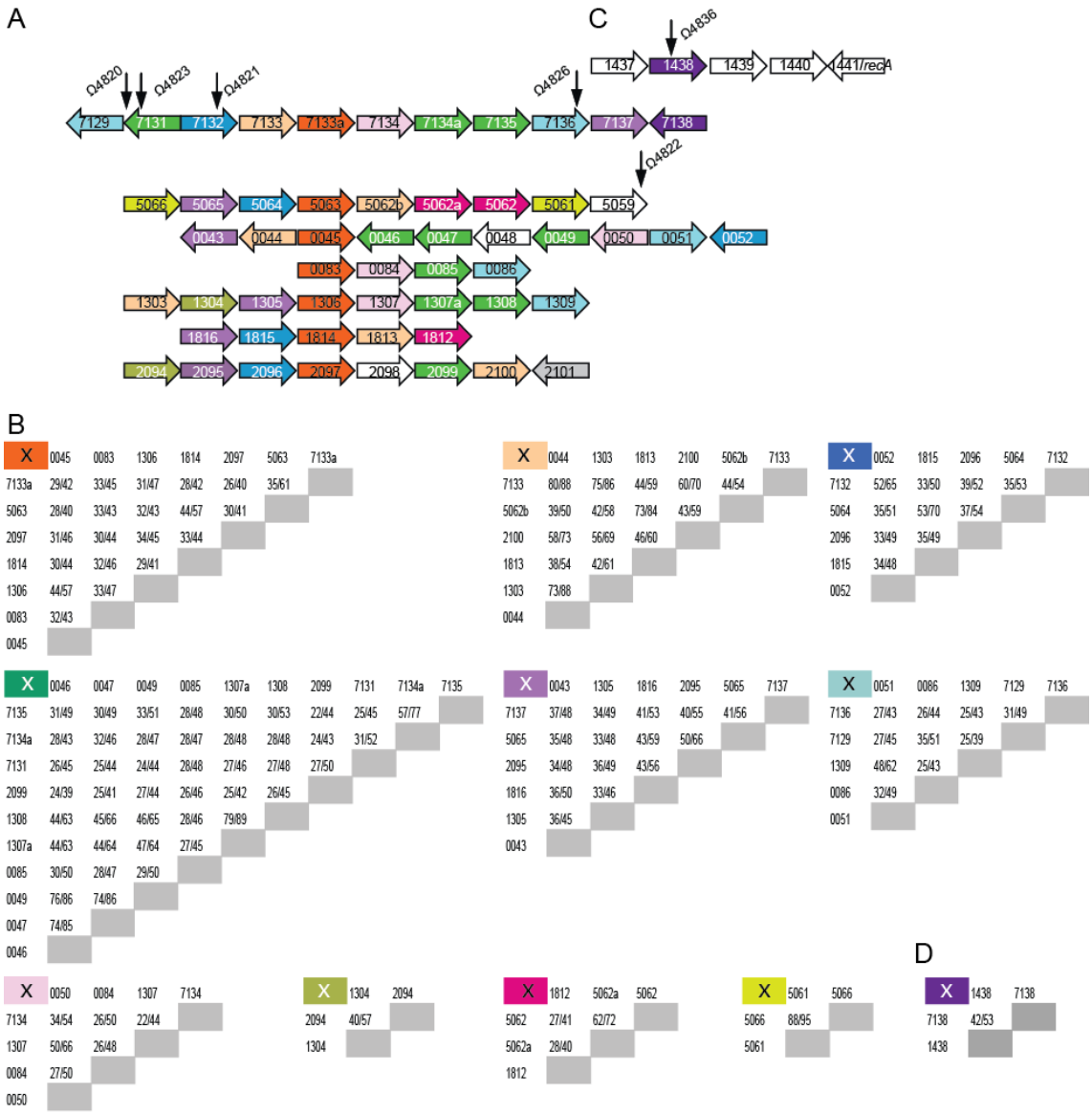

Figure 9. Genetic organization of class II and class III gene clusters.

(A) Genetic organization of the seven class II gene clusters. Paralogs have similar colors. White arrows indicate genes with no paralogs. Grey arrow indicates gene encoding a transposase. Arrows indicate the direction of transcription. Black vertical arrows indicate mini-Himar(Kan) insertions causing a development defect as well as a block in PopC secretion. Genes are not drawn to scale. (B) Identities and similarities of proteins encoded by class II gene clusters in *M. xanthus*. The color code shown in the upper left corner refers to the code used in (A). Numbers to the left and in the upper line refer to gene numbers. For each gene pair, the first number indicates identity and the second number similarity. (C) Genetic organization of the class III gene cluster. Paralogs have the same color. White arrows indicate genes with no paralogs with the exception of MXAN1441/*recA*. Arrows indicate the direction of transcription. Black vertical arrow indicate the mini-Himar(Kan) insertion causing a development defect as well as a block in PopC secretion.(D) Identity and similarity of CinA paralogs encoded by a class II and the class III gene cluster. The color code shown in the upper left corner refers to the code used in (A and C). Numbers to the left and in the upper line refer to gene numbers. For the gene pair, the first number indicates identity and the second number similarity.

Table 9. Characteristics of proteins encoded by class II gene clusters

Groups of paralogs	Length (aa)	Predicted function or domainsfor paralogs	Predicted subcellular localization [1]	Phylogenetic distribution [2]
MXAN7129 MXAN7136 MXAN0086 MXAN1309 MXAN0051	256 275 245 260 261	Hypothetical	Cytoplasmic	MxHahellaLeptotrix
MXAN7131 MXAN7134a MXAN7135 MXAN7255 MXAN0085 MXAN1308 MXAN0047 MXAN0046 MXAN0049	206 198 199 90 195 195 223 212 194	Hypothetical	Cytoplasmic	MxbHahellaLeptotrix
MXAN7132 MXAN0052 MXAN2096 MXAN5064 MXAN1815	356 347 341 336 334	Conserved hypothetical (DUF2169)	Cytoplasmic	Conserved
MXAN7133 MXAN5062b MXAN0044 MXAN1303 MXAN1813 MXAN2100	168 250 154 154 417 128	Conserved hypothetical	Cytoplasmic	Conserved
MXAN7133a MXAN0083 MXAN1306 MXAN0045 MXAN2097 MXAN5063 MXAN1814	401 350 406 376 354 349 363	3-ketoacyl-ACP synthase-like protein	Cytoplasmic	Conserved
MXAN7134 MXAN0050 MXAN1307 MXAN0084	258 294 288 305	Hypothetical	Cytoplasmic	MxHahella
MXAN7137 MXAN5065 MXAN1816 MXAN2095 MXAN1305 MXAN0043	444 435 431 426 440 446	Conserved hypothetical	Cytoplasmic	Conserved
MXAN5061 MXAN5066	393 86	Hypothetical	IM/cytoplasmic [c]	*M. xanthus*
MXAN1812 MXAN5062 MXAN5062a	242 267 252	Hypothetical	Cytoplasmic	Myxococcales
MXAN1304 MXAN2094	178 177	Conserved hypothetical	Cytoplasmic	Conserved
MXAN5059	143	Hypothetical	Cytoplasmic	*M. xanthus*
MXAN0048	118	Hypothetical	Cytoplasmic	*M. xanthus*
MXAN2098	302	Hypothetical	Cytoplasmic	*M. xanthus*

[1]Subcellular localization was predicted on the basis of the absence of signal peptides (SP), trans-membrane helices (TMH) using or domains normally associated with a particular subcellular localization. [2] The phylogenetic distribution of proteins is as follows: Proteins labeled conserved have homologs in all branches of the proteobacteria with the exception of epsilonproteobacteria; proteinslabeledMxHahellaLeptotrix, MxbHahellaLeptotrix, MxHahella, Myxococcales or *M. xanthus* have homologsin *M. xanthus, Hahella chejuensis and Leptotrix cholodnii*, in at least two of the five Myxococcales species with completely sequenced genomes, *H. chejuensis* and *L. cholodnii*,in *M. xanthus* and *H. chejuensis,* in at least two of the five Myxococcales species with completely sequenced genomes, or is only found in *M. xanthus,* respectively. [C] MXAN5061 contains 3 TMH and MXAN5066 does not contain any TMH.

Graumann, 2010). *M. xanthus* encodes two *recA* homologs (MXAN1441 and MXAN1388) and MXAN1441 is located two genes downstream of *cinA*/MXAN1438 (Fig. 9C). The two genes downstream of MXAN1438 are transcribed in the same direction as MXAN1438 (Fig. 9C) and encode a conserved hypothetical proteins predicted to localize to the inner membrane (MXAN1439) and the cytoplasm (MXAN1440).

2.2. Discussion

In this report we focused on the identification of proteins required for PopC secretion using a candidate approach as well as an unbiased genetic approach. Because PopC does not contain a signal peptide and is secreted in an unprocessed form, we initially hypothesized that PopC is secreted by a T1SS, T3SS or a T6SS.

To determine whether a T1SS is important for PopC secretion, we tested 11 mutants each deleted for one out of the 15 TolC-encoding genes in *M. xanthus*. All mutants displayed normal development with the exception of the one deleted for MXAN7436-7438, which encodes a cation efflux pump (Moraleda-Munoz et al., 2010). However, all 11 mutants secrete PopC at WT levels. Munoz-Dorado and co-workers previously showed that inactivation of the three additional *tolC* genes do not interfere with development suggesting that these three TolC paralogs are not important for PopC secretion. Finally, the MXAN3447-3449 gene cluster, which encodes a cation efflux pump, was reported to be essential (Moraleda-Munoz et al., 2010) and the TolC encoded by this cluster (MXAN3447) was not tested for a function in PopC secretion. Although we cannot rule out that the TolC proteins in *M. xanthus* are functionally redundant, these results suggest that PopC is not secreted by a T1SS. Supporting this conclusion, it was recently proposed that translation and secretion of proteins secreted by T1SS are closely coupled (Masi & Wandersman, 2010). We have observed that PopC is secreted in the presence of the inhibitor of translation chloramphenicol (A. Konovalova, unpublished) suggesting that translation and secretion of PopC are uncoupled.

T3SS are often described as specific to pathogenic bacteria, however, bioinformatics analyses have revealed the presence of T3SS genes in non-

pathogenic bacteria suggesting that T3SS may have functions not related to pathogenesis (Pallen et al., 2005). Also, T3SS are normally described as being activated by cell contact to a relevant eukaryotic cell and then specifically injecting effector proteins into this cell. However, in *Yersinia* spp. secretion of effectors to the cell exterior can be triggered by Ca^{2+} chelation (Cornelis, 2006). Therefore, we reasoned that the two incomplete T3SS in *M. xanthus* could in principle be involved in PopC secretion. However, inactivation of both systems neither interfered with development not with PopC secretion providing evidence that these two systems are not required for PopC secretion.

Bioinformatic analyses have shown that T6SS are widespread also in non-pathogenic bacteria (Boyer et al., 2009, Bingle et al., 2008). T6SS have been implicated in many different processes including virulence, host specificity, antagonistic interactions between bacteria and biofilm formation (Jani & Cotter, 2010). Therefore, we reasoned that the T6SS could be involved in PopC secretion. Inactivation of this system results in a failure to develop but not in a failure to secrete PopC. Interestingly, single gene deletionspredicted toinactivate the T6SS did not result in developmental defects. In *Agrobacterium tumefaciens* and *Francisella tularensis* individual T6SS protein but not the T6SS have been reported to be required for virulence (Wu *et al.*, 2008, Barker *et al.*, 2009). It should be noted that Hcp was detected in total cell extracts but not in the supernatant under the conditions tested. Thus, it is not clear whether *M. xanthus* assembles an active T6SS under these conditions. Regardless, we conclude that protein(s) encoded by gene(s) covered by the in-frame deletion used in this work are important for development independently of an active T6SS.

The observation that PopC is secreted in an unprocessed form argues against a model in which it is initially translocated across the inner membrane and then across the outer membrane by the T2SS. The *M. xanthus* genome contains a gene cluster encoding a T2SS. All attempts to inactivate this gene cluster were unsuccessful (data not shown) suggesting that a functional T2SS is essential in *M. xanthus*. The T4P system in *F. tularensis* has been reported to be involved in protein secretion (Hager *et al.*, 2006). Analysis of T4P mutants in *M. xanthus* demonstrated that PopC secretion is independent of this system (data not

shown). In total, our analyses show that neither T3SS nor T6SS nor T4P are important for PopC secretion and our data strongly suggest that a T1SS is also not important for PopC secretion.

To gain further insight into the mechanism of PopC secretion, we isolated transposon mutants unable to secrete PopC. In total, seven mutants blocked in PopC secretion were identified. All seven mutants were able to synthesize and accumulate PopC and, thus, specifically blocked in PopC secretion. Intriguingly, all seven insertions occurred in genes of unknown function and which localize in gene clusters mostly containing other genes of unknown functions.

The class I mutation mini-Himar(Kan) Ω4810 is inserted in MXAN2174 predicted to encode an inner membrane protein of unknown function. MXAN2174 is part of a gene cluster for which paralogs are found in three additional large clusters gene clusters (Fig. 8A) and in 14 smaller gene clusters (Fig. 8B). Insertions in two of the other large gene clusters also caused developmental defects but did not block PopC secretion (Fig. 8A). The predicted functions of the proteins encoded by the class I genes found are as follows (Table 8): MXAN2173 and paralogs are predicted periplasmic proteins similar to D, D-carboxypeptidases, which are involved in peptidoglycan remodelling and generally catalyze the removal of D-alanine from muramyl pentapeptides (Ghosh *et al.*, 2008). mini-Himar(Kan) Ω4809 is inserted in one of these paralogs (MXAN1228). MXAN2174 and paralogs code for predicted inner membrane proteins of unknown function. MXAN1231 and paralogs encode Rhs-like proteins predicted to localize to the inner membrane. Rhs-like proteins are encoded by Rhs elements, which are found ubiquitously in bacteria and often in several copies per genome (Jackson *et al.*, 2009). Rhs elements were initially thought to be DNA rearrangement hotspots (Hill, 1999), however, recent comparative genomics analyses suggest that these elements are not involved in DNA rearrangements (Jackson et al., 2009). All four large gene clusters contain two genes encoding cytoplasmic Ser/Thr kinases. These eight Ser/Thr kinases constitute two groups of each four paralogs (Inouye *et al.*, 2008). mini-Himar(Kan) Ω4866 is inserted in one of these paralogs (MXAN4841). Moreover, among the eight Ser/Thr kinases found in these loci, Inouye and co-workers reported that MXAN1234 and MXAN2176 are required

for fruiting body formation (Inouye et al., 2008) (Fig 8A). Moreover, seven of the small gene clusters encode a total of 10 Ser/Thr kinases (Fig. 8B). These 10 kinases contain two sets of paralogs (MXAN1892, 4371 and 4479) and (MXAN1896, 4373, 4482 and 7269) (Fig. 8C). The remaining five paralogous protein families encoded by the class I genes are of unknown function, however, MXAN2170 and its paralog are predicted to localize to the outer membrane based on the presence of a fibronectin domain. Among the class I paralogs, four have a predicted localization to the inner membrane, two to the periplasm, one to the outer membrane and three to the cytoplasm (Table 8).

It is important to note that we have not determined whether the class I insertion that causes a defect in PopC secretion or the two insertions in class I genes that cause developmental defects do so directly or indirectly by having a polar effect on the expression on downstream genes. We have not determined the operon structure of the class I gene clusters and the distance between the genes in the class I clusters (Table S2), do not allow conclusions about the operon structure. Therefore, our data do not allow us to conclude which of the class I proteins in the MXAN2170-2177 gene cluster are important for PopC secretion. Similarly, we do not know which of the proteins in the MXAN1226-1234 and MXAN4847a-4841 gene clusters are important for development. The observation that the genetic context of the class I genes is largely conserved in the large gene clusters suggests that each gene cluster codes for a set of proteins that function together. Based on the information outlined below, we have as a tentative model that MXAN2170-2177 could possibly encode a secretion system dedicated to secretion of PopC and that the activity of this secretion system is regulated by the two Ser/Thr kinases encoded by this cluster. Similarly, the two other large class I gene clusters could encode secretion systems dedicated to secretion of protein(s) required for development (and which are not PopC). The following lines of evidence support this working model. Firstly, the predicted localization of the class I proteins encoded by the three large gene clusters is to the inner membrane, outer membrane, periplasm and cytoplasm (Table 8). Thus, these gene clusters encode proteins that span the cell envelope as observed in T1SS, T3SS, T4SS and likely also in T6SS. Secondly, each of the three large gene clusters encodes a D, D-

carboxypeptidase and peptidoglycan-modifying enzymes are often associated with trans-envelope transport systems including T2SS, T3SS, T4SS and T4P (Koraimann, 2003). Thirdly, Rhs-like proteins have been linked to secretion: An Rhs-like protein in *Pseudomonas savastanoi* pv. *savastanoi* is important for synthesis and/or secretion of the bacteriocin savastanoicin, which is a 25 kDa protein released to the cell exterior (Sisto *et al.*, 2010). Also, in *E. coli*, normal levels of group 2 capsular polysaccharides depends on RhsA and RhsA has been suggested to be part of a protein complex involved in capsule exopolysaccharide biosynthesis and export (McNulty *et al.*, 2006). Moreover, in *E. coli* several Rhs elements are genetically linked to genes encoding homologs of VgrG and Hcp, which are parts of T6SS (Wang *et al.*, 1998). Fourthly, the activity of the T6SS in *P. aeruginosa* is regulated by a Ser/Thr kinase (Mougous *et al.*, 2007). The phylogenetic distribution of several of the class I genes is relatively narrow (Table 8) suggesting that these potential secretion systems would have a narrow phylogenetic distribution. A precedent for a secretion system with a narrow phylogenetic distribution is PorSS, which has only been found in members of the Bacteroidetes (Sato *et al.*, 2010). Precedents for bacteria containing two or more copies of similar secretion systems are bacteria with two or more T1SS, T3SS or T6SS.

Paralogs of genes in the large class I clusters are also found in smaller gene clusters or as single genes (Fig. 8B). Interestingly, the paralogs in the four large gene cluster are very similar reaching identities and similarities at the protein level of >80%/>90% whereas paralogs encoded by the smaller gene clusters/single genes are more distantly related (Fig. 8C). In this context it is interesting that three of the large gene clusters are flanked by genes encoding transposases, recombinases or tRNAs. Horizontally acquired genetic island are often flanked by such genes (Langille *et al.*, 2010) suggesting that one or more of these gene clusters were acquired by horizontal gene transfer. On the other hand, the high identity and similarity levels of paralogs encoded by the four large gene clusters suggest that these gene clusters were amplified by intragenomic duplication relatively recently. The *M. xanthus* genome encodes approximately 100 Ser/Thr kinases (Goldman *et al.*, 2006). The class I gene clusters contain 51 genes not including genes for Ser/Thr kinases and 18 genes

encoding Ser/Thr kinases (Fig. 8B) suggesting a link between the functions encoded by these gene clusters and regulation of or by Ser/Thr kinases.

The four class II insertions led to the identification of seven gene clusters containing combinations of ten paralogous genes (Fig. 9A).These ten paralogous protein families generally do not share homology with proteins of known function. Likewise, they do not contain domains of known function (Table 9). The exception is MXAN7133a and its paralogs, which are similar to 3-ketoacyl-ACP synthases. However, all seven paralogs lack the active site Cys residue (Huang et al., 1998). The *M. xanthus* genome encodes at least four 3-ketoacyl-ACP synthases with intact active sites (Figure S1). All members of the ten protein families are generally predicted to be localized to the cytoplasm (Table 9). Homologs of five of these proteins are only found in the aquatic betaproteobacterium *Leptothrix cholodnii*, the marine gammaprotebacterium *Hahella chejuensis* or in other myxobacteria (Table 9; Table 10). The remaining five genes are more broadly distributed and found in all branches of the proteobacteria with the exception of the epsilonproteobacteria (Table 10). Interestingly, these five genes are often found in gene clusters and define a core set of class II genes (Table 10). The *M. xanthus* paralogs have relatively low identity and similarity values (Fig. 9B). Moreover, one of the gene clusters is flanked by a gene encoding a transposase (Fig. 9A). These observations may suggest that the lineage specific expansion of these gene clusters in *M. xanthus* involved lateral gene transfer.

What is the function of the MXAN7129-7137 gene cluster and MXAN5059 in PopC secretion? Four insertions in the MXA7129-7137 gene cluster cause a defect in PopC secretion. We do not know whether these insertions have a polar effect on the expression of downstream genes or whether they only affect the genes that they have inserted in. Likewise, we do not know the operon structure of this gene cluster and the distance between the genes in the cluster do not allow us to deduce this structure (Table S3). An insertion in MXAN5059 also caused a defect in PopC secretion. Because the gene downstream of MXAN5059 is transcribed in the direction opposite to that of MXAN5059, we hypothesize that the lack of MXAN5059 is directly responsible for the defect in PopC secretion. MXAN5059 is localized directly downstream of the MXAN5066-

Table 10.Conservation of class II genes in representative proteobacteria and their linkage to T6SS genes.

Species	Class II genes [1]										Phylum	Linked T6SS gene cluster
M. xanthus		83				85	86	84			delta-proteo-bacteria	
		45	44	43		46,7,9	51	50				
		1306	1303	1305	1304	1307a,8	1309					
		1814	1813	1816						1812		
	5064	5063	5062a	5065					5066,1	5062,62a		
	2096	2097	2100	2095	2094	2099						
	7132	7133a	7133	7137		7131,5,4a	7136	7134				
G. sulfurreducens	3186	3181	3182	3180	3179							3162-3177
R. etli	952	953	954	956	957-8						alpha-proteo-bacteria	933-950
R. sphaeroides	3112	3113	3114		3117							3111-3114
A. tumefaciens	3641		3640		3642							3642 (orphan VgrG)
L. cholodnii	4073	4077	4078	4072	4079,80	4075	4074				beta- proteo-bacteria	4053-4092
R. solanaceum	2273	2274	2275	2279	2272							2271 (orphan VgrG)
H. chejuensis	4280	4282	4279	4281	4278	4285-91	4292				gamma- proteo-bacteria	4242-4261
	5704	5705	5708		5703	5707, 20						
	7058	7055	7054	7057		7053						
P. aeruginosa	0097	0098	0099	0101	0096							0085-0095
V.parahaemolyticus	1398		1415		1395							1390-1414
Class II core genes[2]											NA	NA

[1] Genes are color-coded as in Fig. 9. Numbers indicate gene numbers in the various species: *Geobacter sulfurreducens*(Methe *et al.*, 2003), *Rhizobium etli* CIAT 652 (NC_010994), *Rhodobacter sphaeroides* ATCC17029(NC_009050), *A. tumefaciens* C58 (Goodner *et al.*, 2001), *L. cholodnii* SP-6(NC_010524) *Ralstonia solaneceum*(Remenant *et al.*, 2010), *H. chejuensis*(Jeong *et al.*, 2005), *P. aeruginosa*(Stover *et al.*, 2000) and *Vibrio parahaemolyticus* RIMD 2210633(Nasu *et al.*, 2000).[2] Defined as those genes, which are present in more than 50% of the gene clusters.[c] NA, not applicable.

5061 gene cluster (Fig. 9A) suggesting that this gene cluster could also have a function in PopC secretion. Insertions in the remaining five class II gene clusters were not found in our collection of mutants with developmental defects. It is therefore unclear if these gene clusters are important for development.

The predicted localization of the MXAN7129-7137 and MXAN5059 proteins to the cytoplasm suggests that they do not encode a secretion system. It remains a possibility that these proteins are accessory proteins to a secretion system. In this context it is interesting that with the exception of *M. xanthus*, the gene clusters containing the widely conserved core set of five class II genes are constantly genetically linked to genes encoding complete or incomplete T6SS (Table 10) supporting the link established here between one of these gene clusters and protein secretion. Secretion of PopC is a regulated event and induced by starvation (Rolbetzki et al., 2008). Therefore, it is also a possibility that MXAN7129-7137 and possibly also MXAN5066-5061 together with MXAN5059 are involved in regulation of PopC secretion.

Finally, mini-Himar(Kan) Ω4836, which we refer to as a class III insertion, is inserted in MXAN1438, which encodes a CinA homolog. Also for this insertion we do not know if the block in PopC secretion is caused by lack of MXAN1438 or whether the insertion has a polar effect on the expression on downstream genes. CinA has been reported to be a membrane-associated protein involved in recruiting RecA to the membrane during uptake of DNA in *Streptococcus pneumoniae*(Masure *et al.*, 1998). Recently, CinA was also shown to be important for efficient transformation during competence in *Bacillus subtilis* (Kaimer & Graumann, 2010). To our knowledge *M. xanthus*is not able to enter a competence state for DNA uptake, thus, making it unlikely that the two CinA homologs in *M. xanthus* are involved DNA uptake. Interestingly, MXAN7138, which also encodes a CinA homolog, is located downstream from the class II gene cluster MXAN7129-7137 important for PopC secretion (Fig. 9C). Whether MXAN1438 is important for PopC secretion remains to be tested. It remains an open question how CinA/MXAN1438 or the downstream hypothetical proteins could be involved in PopC secretion. In *B. subtilis* completion of DNA replication is essential for initiation of sporulation and DNA damage inhibits initiation of sporulation (Ireton & Grossman, 1994). In order for fruiting body formation and

sporulation to be completed in *M. xanthus*, DNA replication during the first six hours of starvation is important (Rosario & Singer, 2007). To our knowledge it is not known if DNA damage in *M. xanthus* inhibits initiation of fruiting body formation. Based on the connection between CinA proteins in *S. pneumoniae* and *B. subtilis* and DNA metabolism, CinA/MXAN1438 in *M. xanthus* may establish a link between DNA metabolism and the initiation of development and in that way stimulate PopC secretion.

PopC is not the only protein without a signal peptide for which secretion is incompletely understood. The cytolysin A toxin (ClyA) of *E. coli* does not contain a signal peptide, is secreted in an unprocessed form, and secretion is independent of T1SS to T5SS (Wai *et al.*, 2003, Wai *et al.*, 2000). Outer membrane vesicles have been implicated in ClyA release from *E. coli* cells (Wai et al., 2003). In a recent proteomic survey of proteins in outer membrane vesicles released by starving *M. xanthus* cells, PopC was not identified (Kahnt *et al.*, 2010) suggesting that PopC is not released in outer membrane vesicles.

In summary, we have identified several gene clusters largely encoding proteins of unknown function that are important for PopC secretion. We speculate that the genes identified could have two functions: They could be part of a secretion system or they could be regulators of a secretion system, which is activated in response to starvation. The hypotheses laid out here will be tested in future experiments.

3. Identification of a regulatory cascade controlling PopC secretion

3.1. Results

3.1.1 Activation of PopC secretion depends on the RelA protein and is independent of *de novo* protein synthesis

The developmental program that results in fruiting body formation in *M. xanthus* is induced by nutrient starvation, which induces the RelA-dependent stringent response with the accumulation of guanosine 3'-di-5'-(tri)di-phosphate nucleotides [(p)ppGpp] (Singer & Kaiser, 1995). Accumulation of (p)ppGpp is required and sufficient for initiating this developmental program (Singer & Kaiser, 1995, Harris *et al.*, 1998). In the assay for PopC secretion, the cell-free supernatant is isolated from vegetative cells growing in CTT rich medium as well as from cells starved in MC7 buffer. To confirm that PopC secretion is specifically activated in response to starvation as opposed to being a response to changes in buffer composition similarly to that observed for activation of T3SS by low Ca^{2+}(Straley *et al.*, 1993)), we analysed PopC secretion in a Δ*relA* strain (MS1000), which is unable to accumulate (p)ppGpp (Harris et al., 1998).Δ*relA* cells accumulated PopC at levels similar to those observed in WT cells in total cell extractsfrom growing and starving cells (Fig. 10A). As previously reported (Rolbetzki et al., 2008), PopC accumulated in the cell-free supernatant of starving WT cells in the presence of protease inhibitors (Fig. 10B). However, under the same conditions, we were unable to detect PopC in the cell-free supernatant of Δ*relA* cells (Fig. 10B). In a parallel experiment in which cells were starved in the absence of protease inhibitors, p25 cleavage to generate p17 was followed. As expected, p17 accumulated in WT cells during starvation. In agreement with the lack of accumulation of PopC in the supernatant of Δ*relA* cells, p17 accumulation was not observed in Δ*relA*cells (Fig. 10C). Thus, PopC secretion depends on RelA strongly suggesting that activation of PopC secretion is a specific response to starvation.

Because PopC accumulates in the cytoplasm of vegetative cells and is only secreted during starvation, we hypothesized that synthesis of the secretion system responsible for PopC secretion could be restricted to starving cells. To

test this hypothesis, we monitored PopC secretion in the presence of chloramphenicol, an inhibitor of translation. If the secretion system is made during vegetative growth, then cells treated with chloramphenicolfor 10 min prior to starvation and during starvation would be predicted to secrete PopC during starvation. In contrast, if synthesis of the secretion system is induced by starvation, then cells treated as described would be predicted to be unable to secrete PopC.

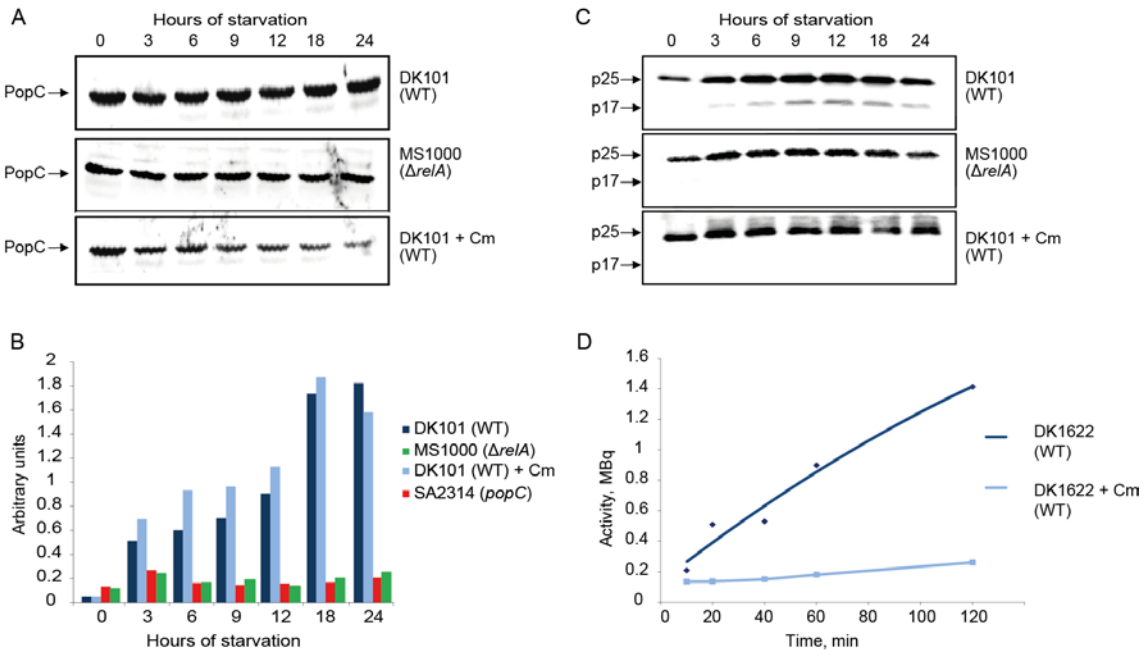

Figure 10. PopC secretion depends on *relA* and independent of *de novo* protein synthesis

(A) PopC accumulation in total cell extracts. Cells of the indicated strains were exposed to starvation in the presence of protease inhibitors and in the presence or absence of 25 µg/ml of chloramphenicol (Cm). Cells treated with chloramphenicol were exposed to 25 µg/ml of chloramphenicol for 10 min prior to starvation as well as during starvation. Total cell lysates were prepared at the indicated time points. Protein isolated from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-PopC antibodies. (B) PopC accumulation in supernatant. Cells were treated as described in (A) and cell free supernatants prepared at the indicated time points. Supernatants from 10^8 cells were analyzed by ELISA with anti-PopC antibodies. (C) Accumulation of p25 and p17 in total cell extracts. Cells were treated as described in (A) but in the absence of protease inhibitors. Total cell lysates were prepared at the indicated time points. Protein isolated from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-p25 antibodies. p25 and p17 are indicated.(D) Effect of chloramphenicol on protein synthesis. Cells of the indicated strains were grown in CTT in the presence of [^{35}S]-Met and [^{35}S]-Cys and in the presence or absence of 25 µg/ml of chloramphenicol. 5×10^7 cells were harvested and radioactivity incorporated into total protein measured.

Initially, we tested if 25µg/ml of chloramphenicol is sufficient to completely block protein synthesis in *M. xanthus*. For this purpose vegetative WT (DK1622) cells growing in CTT rich medium were treated with 25µg/ml of chloramphenicol in the presence of [^{35}S]-Met and [^{35}S]-Cys and incorporation of radioactively labelled amino acids in total cell extracts followed over time. As shown in Fig. 10D, the amount of radioactively labelled amino acids incorporated into untreated cells increased over the course of the experiment whereas cells treated with chloramphenicol displayed strongly reduced incorporation of radioactively labelled amino acids. Thus, chloramphenicol at a concentration of 25µg/ml strongly inhibits protein synthesis.

Next, PopC secretion was monitored in WT cells in the presence or absence of chloramphenicol. In this experiment chloramphenicol was added 10 min prior to initiation of starvation and during starvation. Moreover, protease inhibitors were added to the starving cells to avoid degradation of secreted PopC. As shown in Fig. 10B, PopC secretion in the presence of chloramphenicol follows the same pattern as in the untreated control. In total cell extracts, accumulation of PopC was slightly lower in cells treated with chloramphenicol compared to untreated cells (Fig. 10A) suggesting that PopC is normally synthesized in starving cells. We conclude that PopC secretion does not depend on *de novo* protein synthesis during starvation and that PopC synthesis and secretion are not coupled. Moreover, our data suggest that the system responsible for PopC secretion is present in vegetative cells and that activation of PopC secretion in response to starvation is regulated post-translationally.

In a parallel experiment in which cells were starved in the absence of protease inhibitors, generation of p17 was followed in the presence and absence of chloramphenicol. Interestingly, p17 accumulation could not be detected in cells treated with chloramphenicol (Fig. 10C). These cells accumulated p25 at a reduced level compared to untreated cells. We speculate that this may reflect a coupling between p25 synthesis and cleavage by PopC; alternatively, a proteinaceous factor, which is only synthesized during starvation, is required for PopC to efficiently cleave p25.

3.1.2. PopDinteracts with directly PopC

Because regulation of PopC secretion does not occur at the level of synthesis of the responsible secretion system, we hypothesized that PopC interacts either with a negative regulator that inhibits PopC secretion in vegetative cells or with a positive regulator that stimulates PopC secretion in response to starvation. *popC* is part of a two-gene operon with the downstream *MXAN0207* (henceforth, *popD*) that codes for a 17 kDa protein of unknown function (Fig. 11.) (Rolbetzki et al., 2008). Analysis of the PopD primary sequence suggested that it lacks a signal peptide and trans-membrane helices. Interestingly, PopD is only conserved in *S. aurantiaca*, which also encodes a PopC ortholog. Many proteins secreted via T3SSs interact with specific chaperones and the secreted protein and its cognate chaperone are encoded in the same operon (Galan & Wolf-Watz, 2006). These chaperones are small dimeric proteins that lack ATP-binding and hydrolysis activities. Moreover, they generally do not share primary sequence homology but do share similar tertiary and quaternary structures (Galan & Wolf-Watz, 2006). Based on these analogies between PopD and T3SS chaperones, we investigated the potential role of PopD in PopC secretion.

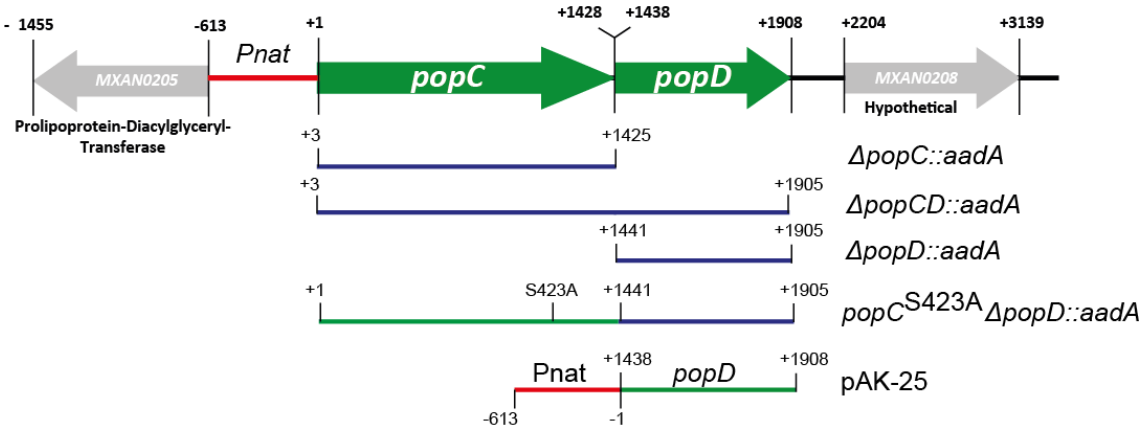

Figure 11.*popCD* locus and constructs used in the genetic analyses.

Arrows indicate direction of transcription and coordinates the start and stop codon of individual genes. *Pnat* indicate the promoter of the *popCD* operon. All coordinates are relative to the start codon of *popC*. The deletions covered in the gene replacement mutations are indicated in blue and with coordinates relative to the start codon of *popC*. The substitution S423A replaces the active site Ser residue in PopC with Ala. In the plasmid pAK-25, the native *popCD* promoter controls the expression of *popD*.

To test whether PopC and PopD interact directly, we generated *E. coli* strains for expression of PopC and PopD in parallel as well as for the separate expression of each of the two proteins. Upon expression in *E. coli*, His6-PopC accumulated in the cell fraction enriched for insoluble proteins (Fig. 12, lane 5-8). This observation is in agreement with previous work showing that His6-PopC forms inclusion bodies when expressed in *E. coli* (Rolbetzki et al., 2008). PopD tagged with the S-tag at the C-terminus (PopD-S) accumulated in the cell fraction enriched for soluble proteins (Fig. 12, lane 1-4). Note that PopD-S is detected in two bands after separation of lysed cells into soluble and insoluble fractions. Mass spectrometry analyses did not reveal differences between PopD-S in these two bands. Thus, we do not know what causes this difference in migration of PopD-S. Importantly, in cells co-expressing His6-PopC and PopD-S, both proteins were detected in the fraction enriched for soluble proteins although a substantial part of His6-PopC still accumulated in the fraction enriched for insoluble proteins (Fig. 12, lane 9-12). The increased solubility of His6-PopC in the presence of PopD-S suggested that the two proteins interact directly.

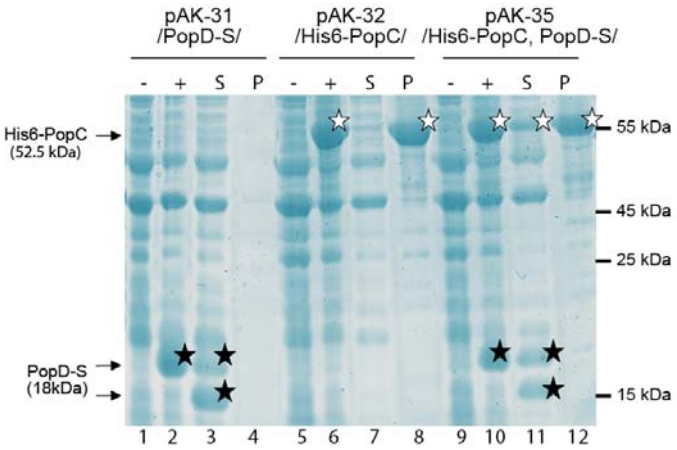

Figure 12. PopC partially accumulates in soluble fraction when co-produced with PopD.

Cells of the *E. coli* Rosetta2 strain containing the indicated plasmids encoding the indicated proteins were grown in LB and expression of the genes encoding PopD-S and His6-PopC induced with 1 mM IPTG at 18°C for 18 hrs. Lanes labelled "-" or "+" contain samples of uninduced and induced cultures, respectively. Following induction cells were lysed by sonication and separated into soluble (S) and insoluble (P) fractions by centrifugation. Proteins from 10^8 cells of the indicated samples were loaded per lane, separated by SDS-PAGE and visualized by Coomassie Brilliant BlueR-250 staining. His6-PopC and PopD-S have calculated molecular masses of 52.5 and 18 kDa, respectively. His6-PopC and PopD-S were identified by mass spectrometry. Positions of His6-PopC and PopD-S are indicated by white and black asterisks, respectively. Positions of molecular markers are indicated to the right. Note that PopD-S is detected in two bands after separation of lysed cells into soluble and insoluble fractions. Mass spectrometry analyses did not reveal differences between PopD-S in these two bands.

To determine whether His6-PopC and PopD-S interact directly to form a soluble complex, we performed pull-down experiments on *E. coli* cell extracts taking advantage of the different tags on His6-PopC and PopD-S. PopD-S bound to a Ni-NTA agarose matrix in a His6-PopC-dependent manner (Fig. 13A) and eluted in the same fractions as His6-PopC (Fig. 13A). In the inverse experiment, His6-PopC bound to an S-protein agarose matrix in a PopD-S dependent manner and was eluted in the same fractions as PopD-S (Fig. 13B). To verify that His6-PopC and PopD-S form a complex we performed size exclusion chromatography on proteins in fraction E3 from the Ni-NTA agarose column. As shown in Fig. 13C, His6-PopC and PopD-S co-eluted in the same fraction (P2) at an elution volume corresponding to an apparent molecular mass of 120 kDa. Thus, His6-PopC and PopD-Sinteract directly forming a soluble complex. The ratio between His6-PopC and PopD-Sin the pull-down experiments and the size of the His6-PopC/PopD-Scomplex in the gel filtration experiment suggest a PopC:PopD stoichiometry of 1:4 in the complex. To determine whether the PopC/PopD complex also exists in *M. xanthus*, we attempted to perform a pull down experiments using anti-PopD antibodies or a C-terminal PopD-Strep tag fusion protein. However, we were unsuccessful in pulling down PopD, most likely, because of its instability in cell extracts (will be discussed in section 3.1.5).

PopC consists of a unique N-terminal extension (residues 1–175; henceforth prosegment) and a C-terminal protease domain (residues 176–475; henceforth protease domain) (Fig. 14A). To determine which part of PopC is important for the interaction with PopD, we generated *E. coli* strains producing His6-prosegment, His6-protease domain and the corresponding strains also expressing PopD-S. As shown in Fig. 2.5B, PopD-S co-purifies with the His6-protease domain but not with the His6-prosegment (Fig. 14C). These findings show that PopD interacts with the protease domain of PopC. Likewise, we found that PopD-S co-purifies with His6-PopC^{S423A} (Fig. 14D), which carries a substitution of the active site Ser423 residue to Ala in the protease domain of PopC and is catalytically inactive (Rolbetzki et al., 2008).

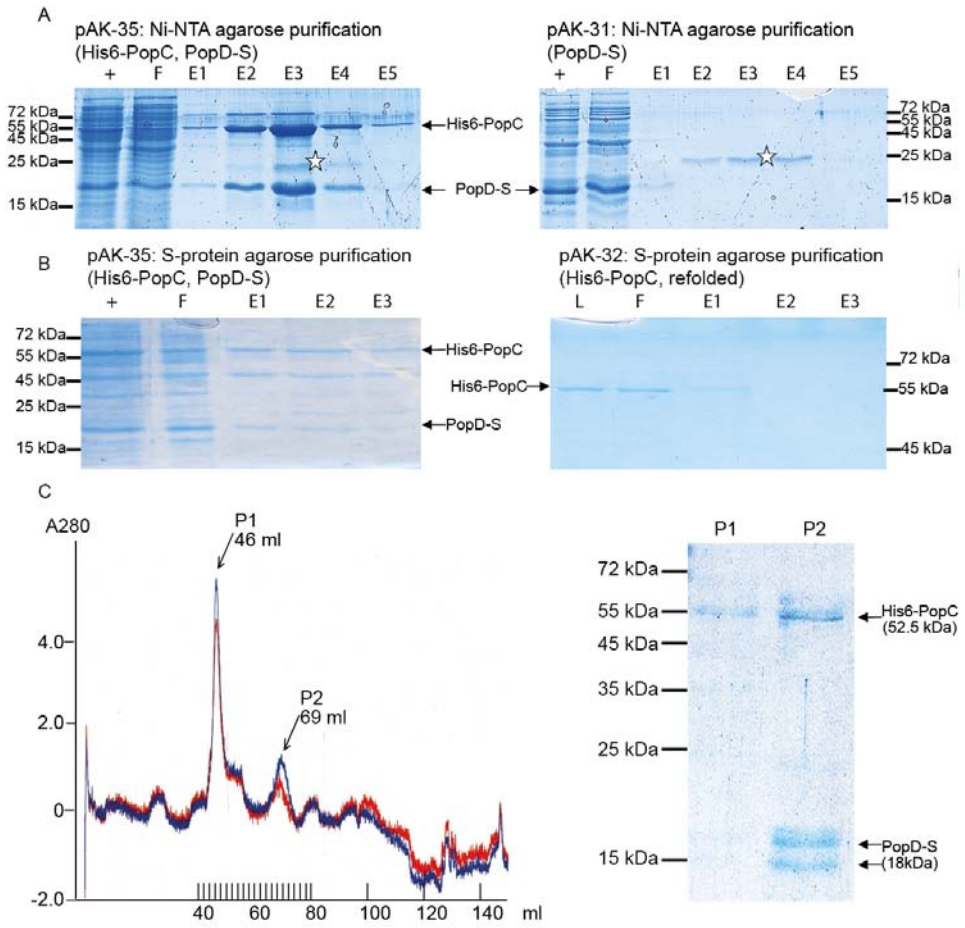

Figure 13. PopC and PopD form a soluble complex.

(A) Ni-NTA agarose affinity chromatography of His6-PopC and PopD-S. Cells of *E. coli* Rosetta2 strains containing the indicated plasmids expressing the indicated proteins were grown and protein synthesis induced as described in Fig. 12. The lanes labeled "+" and F contain proteins in the soluble fraction applied on the Ni-NTA agarose column and the flow-through, respectively. Proteins were eluted with equal volumes of elution buffer containing increasing concentrations of imidazole (50, 100, 150, 200 and 250 mM in E1 to E5, respectively). Samples were separated by SDS-PAGE and visualized by Coomassie Brilliant BlueR-250 staining. His6-PopC and PopD-S were identified by mass spectrometry. Positions of His6-PopC, PopD-S and molecular size markers are indicated. The white asterisk indicates an *E. coli* FKBP-type peptidyl-prolyl cis-trans isomerase protein that binds unspecifically to the Ni-NTA agarose and which was identified by identified by mass spectrometry.

(B) S-protein agarose affinity chromatography of PopD-S and His6-PopC. Cells of *E. coli* Rosetta2 strains containing the indicated plasmids expressing the indicated proteins were grown and protein synthesis induced as described in Fig. 12. In the gel on the right, His6-PopC was purified from inclusion bodies, refolded and applied on the S-protein agarose column (L). Lanes labeled "+" and F contain proteins in the soluble fraction applied on the S-protein agarose column and the flow-through, respectively. Proteins were eluted with equal volumes of elution buffer containing increasing concentrations of NaCl (0.5, 1 and 2M in E1 to E3, respectively). Samples were separated by SDS-PAGE (14% and 8% PAG on left and right, respectively) and visualized by Coomassie Brilliant BlueR-250 staining. His6-PopC and PopD-S were identified by mass spectrometry. Positions of His6-PopC and PopD-S are indicated.

(C) His6-PopC and PopD-S form a soluble complex. His6-PopC and PopD-S were co-expressed, purified as described in (A), protein from fraction E3 applied on a HiLoad 16/60 Superdex 200 column and eluted. Left panel, shows elution profile monitored by A_{280nm}, and right panel, the corresponding SDS–PAGE of aliquots of the peak maxima (indicated with P1 and P2 and their corresponding elution volumes). His6-PopC and PopD-S were identified by mass spectrometry. Positions of His6-PopC, PopD-S and molecular size markers are indicated.

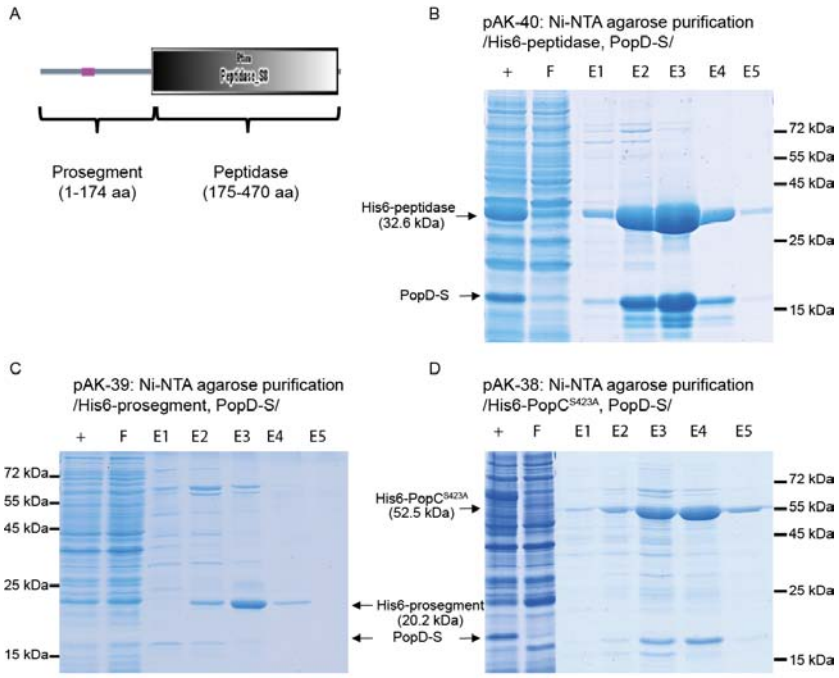

Figure 14. PopD co-purifies with the peptidase domain of PopC.

(A) Schematic representation of PopC domain structure. First and last amino acids of the corresponding domains are indicated. (B-D) Ni-NTA agarose affinity chromatography of different His6-PopC derivatives and PopD-S. Cells of *E. coli* Rosetta2 strains containing the indicated plasmids expressing the indicated proteins were grown and protein synthesis induced as described in Fig. 12. The lanes labeled "+" and F contain proteins in the soluble fraction applied on the Ni-NTA agarose column and the flow-through, respectively. . Proteins were eluted with equal volumes of elution buffer containing increasing concentrations of imidazole (50, 100, 150, 200 and 250 mM in E1 to E5, respectively). Samples were separated by SDS-PAGE and visualized by Coomassie Brilliant BlueR-250 staining. His6-PopC derivatives and PopD-S were identified by mass spectrometry. Positions of His6-PopC derivatives, PopD-S and molecular size markers are indicated. (B) PopD-S co-purifies with peptidase domain of PopC. Positions of His6-peptidase and PopD-S are indicated. (C) PopD-S does not co-purify with prosegment of PopC. Positions of His6-prosegment and PopD-S are indicated. (D) PopD-S co-purifies with His6-PopC^{S423A}. Positions of His6-PopC^{S423A} and PopD-S are indicated.

Because PopD interacts with the PopC protease domain, the effect of PopD on PopC proteolytic activity *in vitro* was investigated. His6-PopC and His6-PopC^{S423A} expressed in the absence of PopD accumulate in inclusion bodies (Rolbetzki et al., 2008). As previously shown (Rolbetzki et al., 2008), refolded His6-PopC cleaves MalE-p25 to generate p17 whereas the active site mutant His6-PopC^{S423} is unable to cleave MalE-p25. Notably, cleavage of MalE-p25 by His6-PopC co-expressed and co-purified with PopD-S on a Ni-NTA matrix was undetectable (Fig. 15). As expected, cleavage of MalE-p25 by His6-PopC^{S423A} co-expressed and co-purified with PopD-S on a Ni-NTA matrix was also undetectable. Thus, these data strongly suggest that PopD blocks PopC protease activity in the PopC/PopD complex.

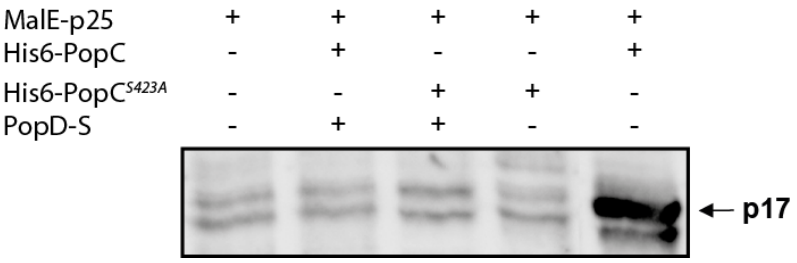

Figure 15. PopD inhibits PopC activity *in vitro*.

Purified MalE-p25 (final concentration 2.5 µM).was incubated alone or with purified His6-PopC, His6-PopC^{S423A}, His6-PopC/PopD-S, or His6-PopC^{S423A}/PopD-S. His6-PopC and His6-PopC^{S423A} were purified from inclusion bodies and refolded. His6-PopC/PopD-S and His6-PopC^{S423A}/PopD-S were purified by NTA affinity chromatography as described in Fig. 13 using elution fraction E3. Proteins were separated by SDS-PAGE and analyzed by immuno-blotting with anti-p25 antibodies. Position of p17 is indicated.

3.1.3. PopD inhibits of PopC secretion and is essential in *csgA*$^{+}$*popC*$^{+}$genetic background

To analyze the function of the PopC/PopD interaction *in vivo*, a genetic analysis was performed (Table 11, Fig. 11). Using several different approaches (in-frame deletion and gene replacement), we repeatedly failed to generate a *popD* mutant in the DK1622 WT strain. However, a gene replacement mutant of *popD* could be generated when a copy of *popD* was expressed from the plasmid pAK-25 at the exogenous phage Mx8 *attB* site (Fig. 11).

Table 11.*popC* or *csgA* mutations suppressgrowth defects caused by Δ*popD*

Strain	Genotype	Viable
DK1622 background		
SA3408	*ΔpopC*	+
-	*ΔpopD::aadA*	-
SA3413	*ΔpopD::aadA//Pnat popD*	+
SA3418	*ΔpopCD::aadA*	+
SA3438	*popC*^{S423A}*ΔpopD::aadA*	+
SA3434	*csgA ΔpopD::aadA*	+
DK101 background		
SA3465	*ΔpopC::aadA*	+
SA3442	*ΔpopD::aadA*	Growth defect
SA3466	*ΔpopD::aadA//Pnat popD*	+
SA3439	*ΔpopCD::aadA*	+
SA3440	*popC*^{S423A}*ΔpopD::aadA*	+
SA3452	*csgAΔpopD::aadA*	+

Next, we tested whether *popD* is essential only in a *popC*$^{+}$ strain. Indeed, a *popCD* gene replacement double mutant was viable, displayed no growth defects (data not shown) and had developmental defects similar to that of the *popC* mutant (Fig. 16). Also, the lethal effect of the Δ*popD::aadA* mutation was suppressed in a strain encoding the active site mutant of PopC *(*PopC^{S423A}) (Table 11). These results are in agreement with the biochemical data indicating that PopD inhibits PopC protease activity and they suggest that an essential function of PopD is to inhibit PopC protease activity.

The only known substrate of PopC is p25, the full-length *csgA* protein. To investigate whether the requirement for PopD to inhibit PopC protease activity is linked to processing of p25 and, thus, C-signalling, we introduced the Δ*popD* mutation in a *popC*$^{+}$, *csgA* mutant. Generation of this mutant was possible (Table 11), the cells displayed no growth defects and were unable to undergo development (Fig. 16). Thus, *popD* is only essential in a *csgA*$^{+}$*popC*$^{+}$ genetic background indicating that an essential function of PopD is to inhibit PopC cleavage of p25, thereby, preventing generation of p17, the active C-signal, in vegetative cells.

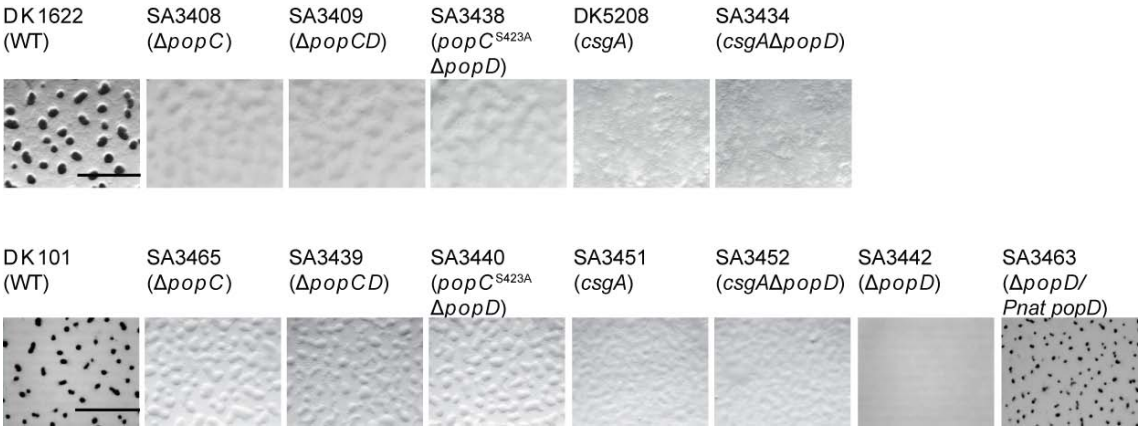

Figure16.Developmental phenotype of corresponding *popC* and *popD* mutants.

10^{8} cells of indicated strains were placed on TPM starvation agar, starved for 120 hours at 32° and and visualized with a Leica MZ8 stereomicroscope. Scale bar, 0.1 mm.

Because it was not possible to generate the Δ*popD* mutation in the DK1622 WT strain, we performed the same genetic analysis in the related WT strain DK101 (Table 11), which was formerly used as a WT strain and is the strain that was used for generation of the Δ*relA* mutation (M. Singer, personal communication). Interestingly, it was possible to generate the Δ*popD* mutation in DK101; however, this mutant displayed severe growth and viability defects (Table 11). Moreover, this mutant failed to develop (Fig. 16). As expected, the growth and viability defects of the Δ*popD* mutant were suppressed by expressing PopD from the plasmid pAK-25 at the exogenous phage Mx8 *attB* site (Table 11). In agreement with the genetic analysis in DK1622, the growth and viability defects caused by the Δ*popD* mutation were suppressed by mutations in *popC* or *csgA* (Table 11).

In vegetative cells PopC and p25 are localized to different cellular compartments: PopC accumulates in the cytoplasm and p25 is localized to the outer membrane (Rolbetzki et al., 2008). The genetic data suggesting that an essential function of PopD is to inhibit PopC cleavage of p25, led us to speculate that PopD, in addition to inhibiting PopC protease activity, may act as a negative regulator of PopC secretion in vegetative cells and that PopC is secreted inappropriately in a Δ*popD* mutant in that way causing the accumulation of p17 and initiation of C-signalling.

To test whether PopD has an effect on PopC secretion, PopC secretion was analysed in the Δ*popD* mutant generated in DK101. If PopD is a negative regulator of PopC secretion, then vegetative cells of the Δ*popD* mutant is expected to secrete PopC resulting in the accumulation of PopC in the supernatant. Likewise, PopC accumulation in the supernatant is expected to occur more rapidly in starving cells of the Δ*popD* mutant than in starving WT cells. Detection of PopC in the supernatant of vegetative cells is technically difficult because *M. xanthus* relies on proteases for growth and it is not possible to grow cells in the presence of protease inhibitors (data not shown). Therefore, we isolated the supernatant of vegetative cells grown in the absence of protease inhibitors, and then immediately added protease inhibitors to avoid degradation of secreted proteins. Under these conditions, a low but

reproducible signal for PopC was detected in the supernatant from a Δ*popD* mutant while the signal in a WT was at the level of the *popC* mutant (Fig. 17A).

Notably, PopC accumulated more rapidly in the supernatant of starving Δ*popD* cells and reached a 2.5-fold higher level compared to the accumulation of PopC in the supernatant of WT cells (Fig. 17A). In parallel, the level of PopC in total cell extracts of the Δ*popD* mutant decreased to almost undetectable at 24 hrs (Fig. 17C).

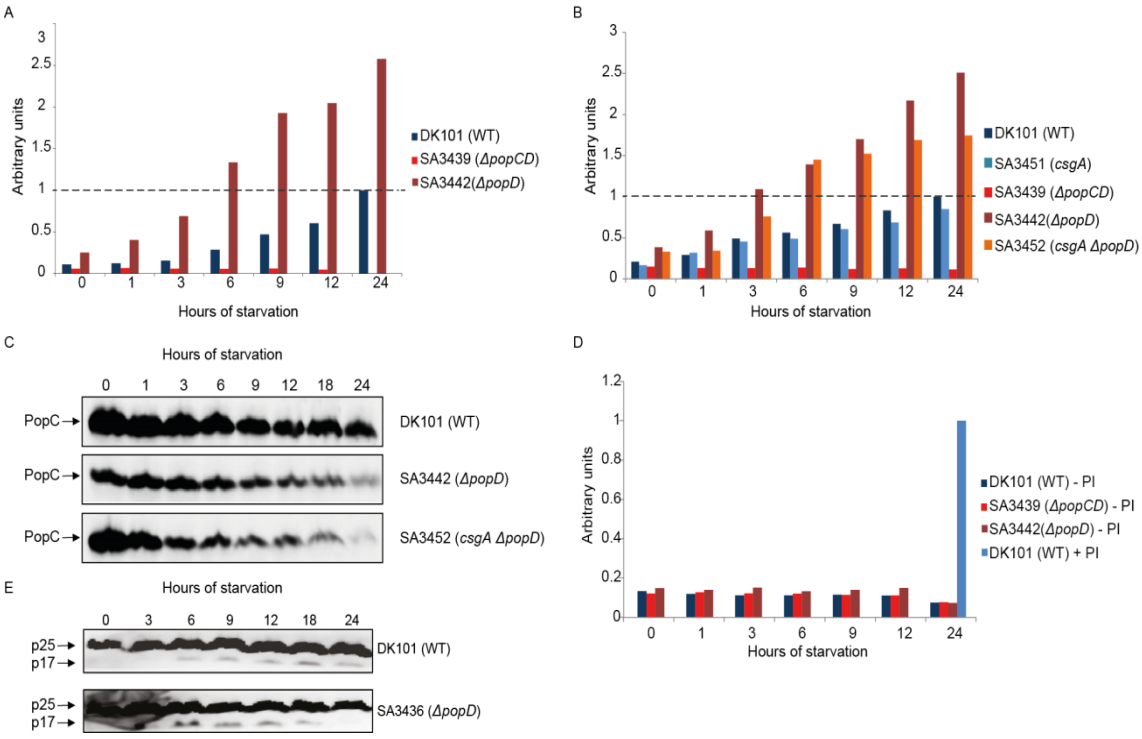

Figure 17. PopD inhibits PopC secretion

(A, B, D) Accumulation of secreted PopC in supernatant. Cells of the indicated strains were exposed to starvation in the presence or absence of protease inhibitors (PI) as indicated. Cell free supernatants were prepared at the indicated time points. Supernatants from 10^8 cells were analyzed by ELISA with anti-PopC antibodies. Level of PopC secreted by WT after 24 hrs is indicated by the dashed line.(A) Secreted PopC accumulates rapidly and at higher levels in the supernatant of the Δ*popD* mutant. (B) Secreted PopC accumulates rapidly and at higher levels in the supernatant of supernatant of the *csgA* Δ*popD* mutant. (C) PopC accumulation in total cell extracts. Cells were treated as described in (A) and total cell lysates prepared at the indicated time points. Protein isolated from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-PopC antibodies. Position of PopC is indicated.(D). Rapid and increased accumulation of secreted PopC in a Δ*popD* mutant is not caused by increased PopC stability. Cells of the indicated strains were exposed to starvation with or without protease inhibitors.(E) Accumulation of p25 and p17 in total cell extracts. Cells of the indicated strains were treated as described in (A) in the absence of protease inhibitors. Total cell lysates were prepared at the indicated time points. Protein from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-p25 antibodies. Positions of p25 and p17 are indicated.

In agreement with the faster and increased secretion of PopC in starving Δ*popD* cells, our preliminary experiments suggest that p17 accumulated earlier in these cells than in WT and with maximum accumulation reached already after 6 hrs of starvation (Fig. 17E). In total, these findings strongly suggest that PopD inhibits PopC secretion in vegetative cells and is involved in the slow secretion of PopC during starvation.

To rule out that the increased accumulation of PopC in the supernatant of the Δ*popD* mutant was caused by cell death/lysis, PopC secretion was determined in the Δ*popD*, *csgA, popC*$^{+}$*,* mutant, which has no growth defects (Table 11). PopC secretion in this mutant was similar to that of the Δ*popD*, *csgA*$^{+}$*, popC*$^{+}$ mutant (Fig. 17B) supporting the notion that PopD inhibits PopC secretion. Finally, to rule out that the increased accumulation of PopC in the supernatant of the Δ*popD* mutant was caused by increased PopC stability, PopC secretion in the Δ*popD* mutant was determined in the absence of protease inhibitors. As shown in Fig. 17D, under these conditions PopC was neither detected in the Δ*popD* mutant nor in WT suggesting that secreted PopC is not more stable in the Δ*popD* mutant compared to WT.

3.1.4. RelA and PopD act in the same pathway to regulate PopC secretion

Our data suggest that RelA is a positive regulator of PopC secretion and that PopD is a negative regulator of PopC secretion. To determine whether RelA and PopD act in the same pathway to regulate PopC secretion, we generated a Δ*relA, ΔpopD* double mutant. As shown in Fig. 18, the PopC secretion defect in the Δ*relA* mutant was partially suppressed in the Δ*relA, ΔpopD* double mutant. These data provide evidence that RelA and PopD act in the same pathway to regulate PopC secretion. Moreover, a simple model that explains all the observations posits that PopD inhibits PopC secretion in vegetative cells. In response to starvation, RelA and stringent response inhibit PopD thereby activating PopC secretion.

3.1.5. PopD is degraded in response to starvation in a RelA-dependent manner.

Because activation of PopC secretion in response to starvation is regulated post-translationally, we hypothesized that PopD could be degraded in response

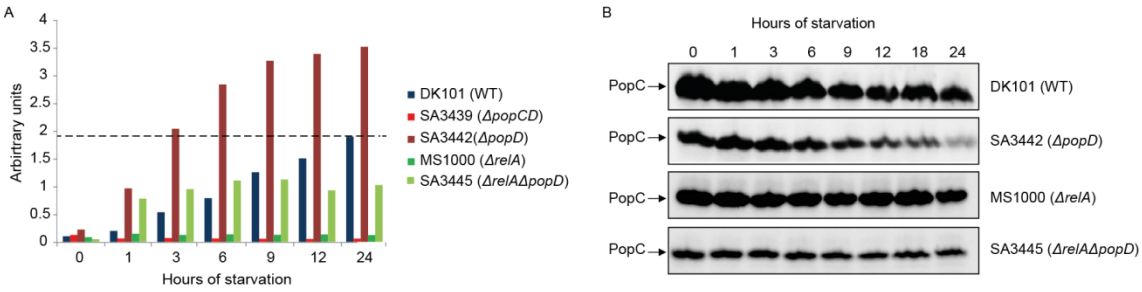

Figure 18. RelA and PopD act in the same pathway to regulate PopC secretion.
(A) Cells of the indicated strains were exposed to starvation in the presence of protease inhibitors and cell free supernatants prepared. Supernatants from 10^8 cells were analyzed by ELISA with anti-PopC antibodies. Level of PopC secreted by WT after 24 hours is indicated by the dashed line.

(B) PopC accumulation in total cell extracts. Cells were treated as described in (A) and total cell lysates prepared at the indicated time points. Protein isolated from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-PopC antibodies. Position of PopC is indicated. Note that the data for DK101, SA3439 are the same as shown in Fig. 2.8.

to starvation resulting in activation of PopC secretion. To test this hypothesis, we attempted to follow PopD protein accumulation during vegetative growth and starvation. In immuno-blots, we were repeatedly unable to detect PopD in total cell extracts as well as in the cell supernatants using two independently generated rabbit, polyclonal α-PopD antibodies or antibodies against the Strep-tag of a functional C-terminal Strep-tagged PopD fusion.

To determine the sensitivity, i.e. detection limit, of the α-PopD antibodies generated against full-length, soluble PopD-His6, we titrated the α-PopD antibodies against PopD-His6 purified from *E. coli* using immuno-blotting. As shown in Figure 19A, the α-PopD antibodies could detect 0.8 ng of purified PopD-His6 protein (corresponding to 280 × 10^8 molecules). In total *M. xanthus* cell extract prepared from 10^8 cells, the α-PopD antibodies cannot detect PopD (data not shown) suggesting that either the number of PopD molecules per cell is <280 or that PopD is highly unstable. As described in section 2.2 our data suggest a PopC:PopD stoichiometry of 1:4 in the PopC/PopD complex. Previously, we estimated that *M. xanthus* cells contain approximately 2000 PopC molecules. Based on our estimate of the stoichiometry of the PopC/PopD complex we would predict that *M. xanthus* contains 8000 PopD molecules per cell. Therefore, it is highly unlikely that inability to detect PopD in *M. xanthus* cell extracts is caused by low antibody sensitivity.

To address whether PopD is unstable in *M. xanthus* cell extracts, PopD-His6 purified from *E. coli* was added to total *M. xanthus* vegetative cell extracts and subsequently SDS-loading buffer was immediately added to stop potential proteolytic reactions. As shown in Fig. 19B, PopD-His6 is degraded when incubated with *M. xanthus* cell extract. At the same time, PopD-His6 was stable when incubated in buffer (Fig. 19B). Based on this experiment we suggest that PopD is rapidly degraded when *M. xanthus* cells extract are prepared and that this instability precludes the detection and analysis of PopD in *M. xanthus* cell extracts using immunological techniques.

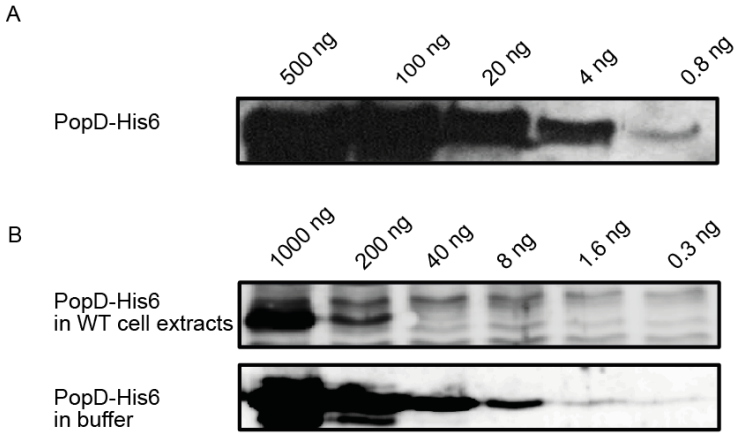

Figure 2.19. PopD is unstable in *M. xanthus* cell extracts.

(A) Determination of the sensitivity, i.e. detection limit, of α-PopD antibodies generated against full-length PopD-His6. The indicated amounts of purified PopD-His6 was loaded on, separated by SDS-PAGE, and analysed by immuno-blotting with anti-PopD antibodies.

(B) Purified PopD-His6 is unstable in the presence of *M. xanthus* WT extract prepared from vegetative cells. The indicated amounts of PopD-His6 were added at room temperature to cell extracts from 10^8 cells prepared from DK101 (WT) and SDS-loading buffer was immediately added to stop reactions. Equal volumes of the samples were analysed as in (A).

Therefore, to study the stability of PopD in response to starvation we used an *in vitro* approach. To this end, purified His6-PopC/PopD-S complex (see 3.1.2) and the control protein MalE purified from *E. coli* were incubated with total cell extracts prepared from vegetative or starving WT and Δ*relA M. xanthus* cells. Whereas MalE and PopC were stable in the presence of all four cell extracts, the PopD level specifically decreased in the presence of extract from starving

WT cells (Fig. 20). Notably, PopD levels did not decrease in the presence of extract from starving Δ*relA* cells Thus, PopD is specifically degraded when incubated with cell extract from starving *M. xanthus* WT cells. These observations suggest that in response to starvation PopD is degraded and that this degradation depends on RelA.

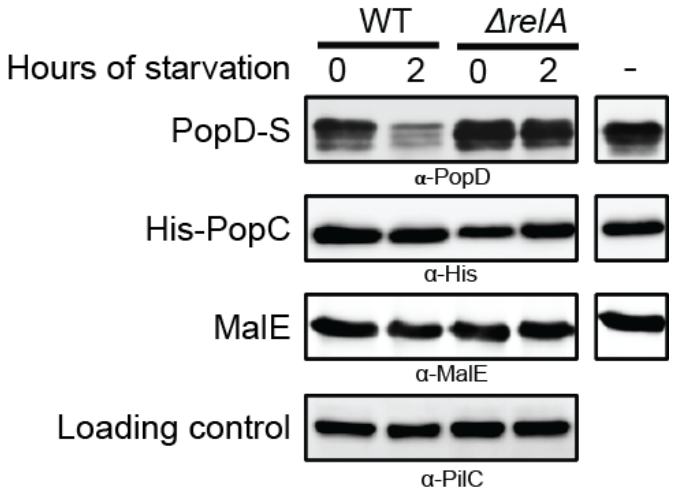

Figure 20. PopD-S is rapidly degraded in WT cell extract from starving cells.

2 µg of purified His6-PopC/PopD-S complex or 1 µg of purified MalE protein were incubated with 7 µg of total cell extract of WT (DK101) or *ΔrelA* (MS1000) cells prepared from vegetative (0 hrs of starvation) or starving (2 hrs of starvation) cells. His6-PopC/PopD-S and MalE were similarly incubated in the absence of cell extract (-). Reaction mixtures were incubated at room temperature for 10 min. Proteins were separated by SDS-PAGE and analysed by immuno-blotting using antibodies against PopD, PopC and MalE as indicated. The immuno-blot with anti-PilC antibodies serves as a loading control for the four cell lysates.

3.1.6. PopC secretion does not depend on LonD protease.

Because activation of PopC secretion depends on degradation of PopD, we searched for candidate proteases responsible for this degradation. The *M. xanthus* genome encodes two Lon proteases, LonD and LonV (Tojo *et al.*, 1993b, Tojo *et al.*, 1993a). LonV is essential (Tojo et al., 1993a) and the LonD protease (also referred to as BsgA; henceforth LonD) was previously shown to be important for development (Gill *et al.*, 1993, Tojo et al., 1993b). Moreover, Lon proteases are known to be allosterically activated in response to starvation (Kuroda *et al.*, 2001). Therefore, we hypothesised that LonD could be involved in PopD degradation, thereby, acting as a positive factor (required) for PopC secretion.

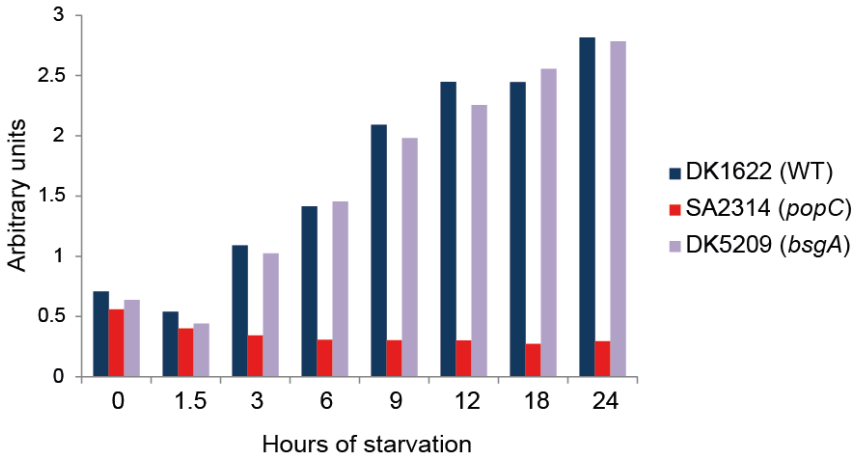

Figure 21. PopC secretion is normal in a *lonD* (*bsgA*) mutant.

Cells of the indicated strains were exposed to starvation in the presence of protease inhibitors and cell free supernatants prepared at the indicated time points. Supernatants from 10^8 cells were analyzed by ELISA with anti-PopC antibodies.

To test this hypothesis we examined PopC secretion in a *lonD* mutant. As shown on Fig. 21 PopC secretion in a *lonD* mutant is similar to that in WT suggesting that LonD is not involved in PopD degradation. Consistently, we previously found that LonD is not required for p17 accumulation (S. Lobedanz, PhD thesis).

3.2. Discussion

We previously showed that p17 generation in *M. xanthus* is limited to starving cells by a mechanism that depends on the regulated secretion of the PopC protease, which cleaves p25 to generate p17. PopC accumulates in the cytoplasm of vegetative cells and is selectively secreted during starvation. In the work presented here, we focused on elucidating the molecular mechanism underlying the activation of PopC secretion in response to starvation. Our *in vivo* data demonstrate that PopC secretion is controlled at the post-translational level by a regulatory cascade involving the RelA and PopD proteins. Specifically, RelA is required for activation of PopC secretion in response to starvation and PopD is an inhibitor of PopC secretion. Moreover, our genetic data suggest that PopD acts downstream of RelA to inhibit PopC secretion and

that, in response to starvation, activated RelA protein alleviates the PopD-mediated inhibition of PopC secretion. Our biochemical data show that PopD interacts directly with PopC forming a complex in which PopC protease activity is blocked. In addition, our biochemical data suggest that RelA, activated in response to starvation, causes the degradation of PopD. On the basis of these data we suggest the following model for regulation of PopC secretion in response to the nutritional status of cells (Fig. 22). In the presence of nutrients, PopC and PopD form a cytoplasmic complex that has two functions (1) to block PopC secretion, and (2) to block PopC protease activity. In response to starvation, RelA is activated and by an unknown mechanism causes the degradation of PopD. This degradation causes the dissociation of the PopC/PopD complex in that way releasing PopC and allowing its secretion. In this model, PopD functions to inhibit PopC protease activity and PopC secretion. We directly showed that PopD inhibits PopC protease activity. The only known substrate of PopC is p25, which localizes to the outer membrane in vegetative cells and, therefore, not accessible to PopC. For these reasons we speculate that the primary function of the PopC/PopD complex is to inhibit PopC secretion.

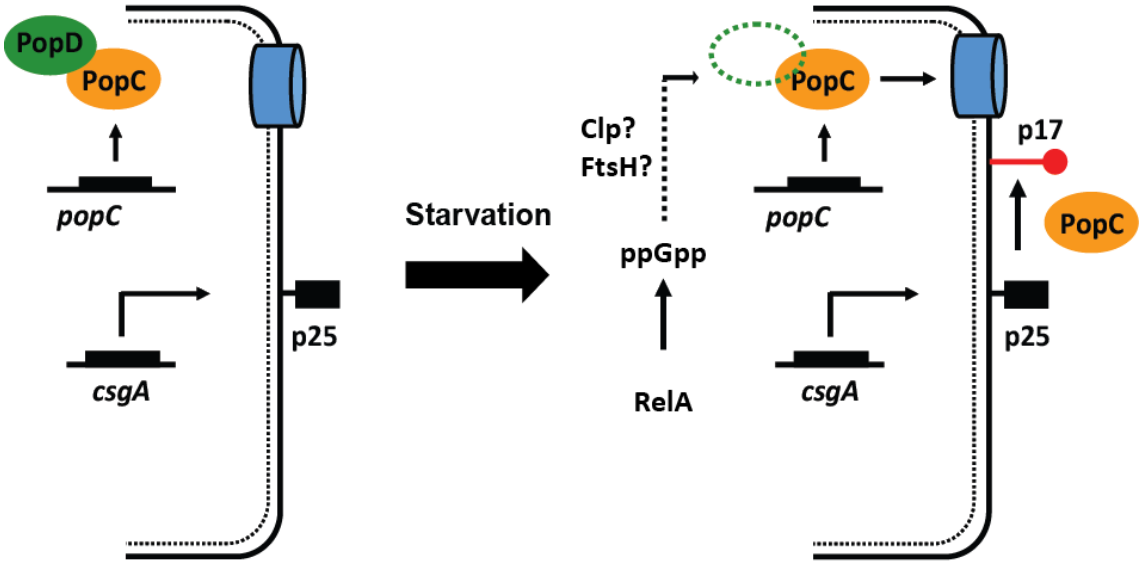

Figure 22. Proposed model for negative regulation of PopC secretion in vegetative cells.

PopD has several features in common with T3SS effector chaperones, which are low molecular weight (15-17 kDa) proteins each of which specifically interact with a cognate effector to regulate its secretion (Feldman & Cornelis, 2003). These chaperones are usually encoded in an operon with the secreted effector. T3SS effector chaperones share little amino acid sequence homology and no predicted conserved domains. However, they have similar three-dimensional structures (Page & Parsot, 2002, Galan & Wolf-Watz, 2006). Similarly, PopD is encoded in an operon with PopC. PopD is a 17 kDa protein with no conserved domains. However, in contrast to T3SS chaperones, which are positive regulators of secretion and are required for secretion, PopD is an inhibitor of PopC secretion and is not required for PopC secretion.

In the model for regulation of PopC secretion, RelA has two key functions, first as a sensor of starvation, and, second to induce PopD degradation. RelA is a ribosome-dependent (p)ppGpp synthetase, which is required and sufficient for initiation of development in *M. xanthus* (Singer & Kaiser, 1995, Harris *et al.*, 1998). RelA is activated in response to the presence of an uncharged tRNA in the A-site of ribosomes resulting in the synthesis of (p)ppGpp, which induces the stringent response (Cashel *et al.*, 1996). (p)ppGpp binds directly to the RNA polymerase (Artsimovitch *et al.*, 2004)causing changes in its transcriptional activity. Thus, the way in which RelA senses starvation and induces changes in transcription are relatively well understood. However, although it has been known for decades that part of the stringent response in *E. coli* (Goldberg & St John, 1976) and *M. xanthus* (Orlowski & White, 1974) is massive proteolysis with degradation of ribosomal protein as the most prominent feature, the molecular mechanisms involved in this proteolysis are still incompletely understood. Kuroda at al. (Kuroda, 2006) showed that in *E. coli* (p)ppGpp triggers the accumulation of polyphosphate, which in turn binds to and activates Lon protease to degrade ribosomal proteins. In *E. coli*, ppGpp was also suggested to bind directly to L-glutamate dehydrogenase in that way regulating its degradation by ClpAP and Lon proteases (Maurizi & Rasulova, 2002). Moreover, it has been shown that (p)ppGpp by an unknown mechanism induces the degradation of the CII repressor of phage λ by host FtsH protease (Slominska *et al.*, 1999). Thus, in all these three cases RelA-proteolysis

depends on (p)ppGpp rather than on activated RelA *per se* and all three cases involves an ATP-dependent protease.

Two questions remain unanswered regarding the RelA-dependent degradation of PopD: the identity of the protease(s) involved and the mechanism by which this protease(s) gains access to PopD. In principle, the RelA-dependent degradation of PopD could have involved the product of a gene induced by the stringent response. However, the observations that cells treated with chloramphenicol prior to and during starvation secrete PopC argue against this possibility. Therefore, by analogy to the examples discussed above, we suggest that the RelA-dependent degradation of PopD depends on (p)ppGpp acting at a post-translational level and an ATP dependent protease. ATP-dependent proteases are also attractive candidates for having a function in PopD degradation as they have unfoldase activity (Striebel *et al.*, 2009). This activity might allow dissociation of the PopC/PopD complex with subsequent degradation of PopD. The *M. xanthus* genome encodes three Clp proteases (two ClpP (MXAN2014 and MXAN6438) and one ClpQ (MXAN3012)), two FtsH proteases (MXAN4333 and MXAN4359) and two Lon proteases (MXAN2017/LonV and MXAN3993/LonD). Among these proteases, only the two Lon proteases have been studied experimentally. LonV is essential for growth (Tojo *et al.*, 1993a) and LonD (also known as BsgA) is essential for development (Tojo *et al.*, 1993b). We showed here that LonD is not required for PopC secretion arguing that LonD is not the protease responsible for PopD degradation. In the context of Lon proteases, it is notable that *M. xanthus* accumulates polyphosphate early during development and that a mutation in the gene encoding polyphosphate kinase-1, which synthesizes polyphosphate reversibly from ATP, causes a developmental defect (Zhang *et al.*, 2005). Our attempts to generate insertion mutation in the three genes coding for Clp proteases failed suggesting that these genes are possibly essential. Finally, it remains an open question how PopD degradation is induced, i.e. does (p)ppGpp activate a protease directly or does (p)ppGpp bind to PopD and/or PopC to make PopD more accessible to a protease. We are currently systematically analyzing whether any of the Clp, Lon or FtsH proteases are

involved in PopD degradation, whether polyphosphate accumulation is essential for PopD degradation, and whether PopD and/or PopC bind (p)ppGpp.

Before the work presented here it was not known whether regulation of PopC secretion involved the starvation-induced synthesis of the secretion system responsible for PopC secretion. PopC lacks a signal peptide and is secreted in an unprocessed form (Rolbetzki et al., 2008). We have shown that PopC is secreted independently of T1SS, T3SS and T6SS in *M. xanthus* (Konovalova *et al.*, 2010b). Moreover, we have identified several gene clusters largely coding for proteins of unknown function that are important for PopC secretion (Konovalova et al., 2010b). The observation that PopC is secreted in cells treated with chloramphenicol strongly suggests that the PopC secretion system is present in vegetative cells. This observation also supports the key function of PopD as a regulator of PopC secretion.

Regulated protein secretion is a well-known phenomenon in T3SS. T3SS secrete three classes of substrates, the subunits of the surface-exposed needle structure, proteins of the translocon that form a pore in the host membrane, and effectors that are transferred directly into the cytoplasm of a eukaryotic host cell (Cornelis, 2006, Galan & Wolf-Watz, 2006). The secretory activity of T3SSs is highly regulated to ensure that the substrates are secreted in the correct order and that the effectors are secreted in response to the appropriate conditions. This regulation can occur at the level of transcription of T3SS genes and involves the secretion of antagonists of transcriptional regulators in response to contact with host cell (Brutinel & Yahr, 2008). Alternatively, regulation of T3SS effector secretion occurs at the post-translational level and involves effector proteins that are present in the bacterial cytoplasm but blocked from being secreted by dedicated chaperones that form complexes with their cognate effector proteins (Galan & Wolf-Watz, 2006). In response to specific signals such as contact to a host-cell or changes in pH a chaperone/effector complex is recruited to the T3SS, dissociated by means of a T3SS specific ATPase, and the effector secreted while the chaperone is reused (Akeda & Galan, 2005). The mechanisms involved in regulating the recruitment of effector/chaperone complexes to the T3SS are largely unknown. However, it was recently shown that in *Salmonella enterica* a “gatekeeper” complex, which blocks the

recruitment of the chaperone/effector complex to the T3SS at low pH, is specifically degraded in response to an increase in pH in that way allowing effector translocation (Yu *et al.*, 2010). Thus, regulation of secretion by a mechanism involving the degradation of a regulatory protein is not specific to PopC or certain T3SS suggesting that it occurs widely.

Surprisingly, lack of PopD specifically causes growth defects in a *popC+csgA+* background, thus, linking the growth defect to processing of p25 and, in that way, C-signaling. Little is known about most upstream components of the C-signal transduction pathway. It has been suggested that C-signal transduction depends on cell-to-cell contacts and involves the interaction between p17 on one cell and a putative receptor on the cell surface of a neighboring cell. The first component in the C-signal transduction pathways is thought to be the DNA-binding response regulator FruA (Ogawa *et al.*, 1996, Ellehauge *et al.*, 1998). Therefore, it is surprising that C-signal transduction is lethal in vegetative cells. Regardless, our data suggest that the most upstream components of the C-signal transduction pathway (e.g. putative receptor and direct downstream targets) may also be present in vegetative cell. Therefore, the sick/lethal phenotype of *popC+csgA+popD-* strains can be a useful genetic tool to study components involved in C-signal accumulation (e.g. PopC and CsgA) as well as C-signal transduction. Mutations in either of these components are expected to suppress the growth defect in a *ΔpopD, popC+csgA+* strain.

4. Ectopic expression of the PopC protease bypasses the requirement for intercellular A-signaling during development

4.1. Results

Initiation of fruiting body formation in *M. xanthus* requires that cells are starving, on a solid surface and at a sufficiently high cell density. The intercellular A-signal has been proposed to function as a population density monitor (Kuspa *et al.*, 1992b). The A-signal consists of two fractions: a heat-stable mixture of amino acids and peptides (Kuspa et al., 1992b, Kuspa *et al.*, 1992a) and a heat-labile fraction containing at least two secreted proteases (Plamann *et al.*, 1992). One of these proteases has a size of 27 kDa and display trypsin-like activity *in vitro*; the second A-signal protease has a size of 10 kDa and displays a specificity *in vitro* different from that of trypsin (Plamann et al., 1992). It has been proposed that the secreted proteases act on surface proteins to generate the heat-stable part of the A-signal (Kuspa et al., 1992a, Plamann et al., 1992). Currently, none of the genes encoding A-signal proteases have been identified. Moreover, none of the genes identified as important for A-signal production encode proteases. Rather these genes encode regulatory proteins: AsgA is histidine protein kinase-like protein (Plamann *et al.*, 1995), AsgB is helix-turn-helix DNA binding protein (Plamann *et al.*, 1994), AsgC is major vegetative sigma factor (Davis *et al.*, 1995), AsgD is a hybrid histidine protein kinase (Cho & Zusman, 1999), and AsgE is an aminohydrolase family protein (Garza et al., 2000b). Accordingly, it has been suggested that the primary defects in most *asg* mutants are in signal transduction pathways important for the release of A-signal proteases (Diodati *et al.*, 2008, Kaplan & Plamann, 1996) and that *asg* mutants have a reduced capacity for protein secretion (Kuspa & Kaiser, 1989b).

To identify genes that are directly or indirectly regulated by AsgA and AsgB, genome-wide expression profiling experiments were previously performed in our group (S. Wegener-Feldbrügge, unpublished) using a *M. xanthus* DNA microarray covering 88% of the 7,380 ORFs on the *M. xanthus* genome. Briefly, *asgA* and *asgB* mutants have strong pleiotropic phenotypes including reduced cohesion, a tan colony colour as opposed to the strong yellow colour of WT colonies, as well as defects in fruiting body formation and sporulation. Based on

the vegetative defects of the *asgA* and *asgB* mutants, it was hypothesized that some – if not all – genes that are directly or indirectly regulated by AsgA and AsgB could be identified by analysing gene expression in these two mutants relative to WT during vegetative growth. Accordingly, total RNA was isolated from mid-exponentially growing WT (DK1622), *asgA* (DK5057) and *asgB* (DK4398) cells. cDNA was prepared, labelled with Cy3 (*asgA* and *asgB* samples) or Cy5 (WT samples), and AsgA/WT samples as well as AsgB/WT samples competitively hybridized to the microarray. A total of three independent biological experiments were performed for all three strains. Genes called to be significantly regulated were selected by a delta value of the Significance Analysis of Microarrays analysis where the false discovery rate became 0% in combination with a 2-fold cutoff and datapoints in at least two out of three biological replicates.

From these DNA microarray analyses, 164 genes were identified that were significantly up- or down-regulated in the *asgA* mutant and 158 genes were identified that were significantly up- or down-regulated in the *asgB* mutant (S. Wegener-Feldbrügge, unpublished; summarized in Fig. 23A). 91 genes were identified as being significantly regulated in both mutants. Out of these 91 genes, 84 displayed similar regulation patterns and the remaining seven were regulated differentially in the *asgA* and *asgB* mutants. The changes in gene expression shared by the *asgA* and *asgB* mutants were asymmetric: 79 genes were expressed at significantly lower levels in the *asgA* and *asgB* mutants, and only five genes were expressed at significantly higher levels in the *asgA* and *asgB*mutants. In total, an extensive overlap exists between the genes directly or indirectly regulated by AsgA and AsgB.

Next, genes similarly regulated in the *asgA* and *asgB* mutants were grouped into functional categories based on their primary annotation, presence of conserved domains and similarity to proteins with known function (Fig. 23B). The three largest of the functional categories are those for hypotheticals, proteases and secondary metabolism. 34 significantly down-regulated and four significantly up-regulated genes encode hypothetical proteins of unknown function. 15 down-regulated genes encode proteases. Interestingly, 12 of these proteases are known to be or predicted to be secreted based on the presence

of signal peptides or trans-membrane helices (Table 12). Eight genes encoding proteins involved in secondary metabolism were also down-regulated and include genes involved inbiosynthesis of DKxanthene pigment, which is the yellow pigment that gives *M. xanthus* colonies their unique yellow colour (Meiser *et al.*, 2006).

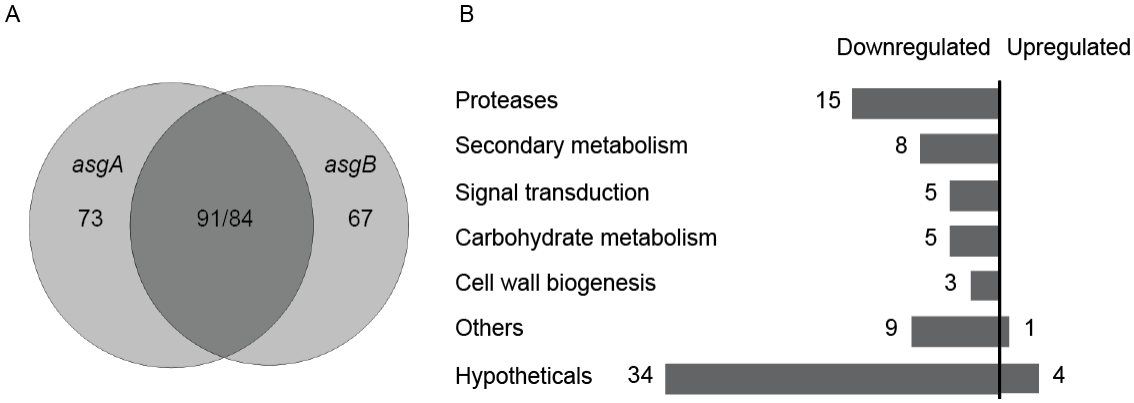

Figure 23. Microarray analysis of *asgA* and *asgB* gene expression.

(A) *asgA* and *asgB* mutants show significant overlap in differentially regulated genes compared to WT. The Venn diagram depicts the number of genes differentially regulated in *asgA* and *asgB* mutants compared to the WT. 91 genes are differentially regulated in the *asgA* and *asgB* mutants 84 of these are similarly regulated. (B) Functional categories of 84 genes similarly regulated in *asgA* and *asgB* mutants. Up- and down-regulated genes shared by the *asgA* and *asgB* mutants were analyzed and grouped into the indicated categories based on primary annotation, the presence of domains of known function and similarity to proteins of known function.

Interestingly, the gene encoding PopC was found to be down-regulated in both *asgA* and *asgB* mutants and this result was further confirmed by immuno-blot analysis (Fig. 24A). PopC is required for development and loss of PopC causes strong development and sporulation defects. To determine whether loss of PopC contributes to the developmental defects of the *asgA* and *asgB* mutants, we cloned the *popCD* genes downstream of the *asg*-independent, constitutively active P_{pilA} promoter in the plasmid pAK-26. Subsequently, pAK-26 was integrated at the Mx8 *attB* site by site-specific recombination in the *asgA* and in the *asgB* strain generating SA3446 and SA3447. As a control, pAK-26 was integrated at the Mx8 *attB* site in the Δ*popCD* strain giving rise to SA3445.

In the three complementation strains containing pAK-26, PopC accumulated in cell extracts at WT levels in vegetative cells (Fig. 24A). It has previously been

Table 12. Proteases downregulated in *asgA* and *asgB* mutants.

Gene	Name	*asgA*[1]	*asgB*[1]	Family[2]	Protease type[2]	Exported[3]
MXAN0206	*popC*	-5.21	-1.242	S8	subtilisin-like serine endopeptidase	Yes (Secreted)[4]
MXAN0538		-2.658	-2.154	M10	matrixin-like metalloendopeptidase	No
MXAN0587		-5.474	-2.269	S1	chymotrypsin-like serine endopeptidase	Yes (SPII)
MXAN0805		-2.617	-1.169	M10	matrixin-like zinc metalloendopeptidases	Yes (SPII, OM)[5]
MXAN1650		-3.759	-2.867	S1	chymotrypsin-like serine endopeptidase	Yes (SPI, OM)[5]
MXAN1967		-4.197	-2.435	S8	subtilisin-like serine endopeptidase	Yes (SPI)
MXAN2790	*prtA*	-2.694	-1.977	S1	trypsin-like serine endopeptidase	Yes (SPII)
MXAN2791	*prtB*	-2.96	-2.237	M57	metalloendopeptidases	Yes (SPII)
MXAN3676		-7.222	-3.336	M36	fungalysin-like metallopeptidase	Yes (N-TMH)
MXAN3964		-3.367	-2.823	S1	Serine endopeptidase	No
MXAN5166		-4.435	-3.602	M28	aminopeptidase	Yes (SPI)
MXAN5392		-3.885	-2.049	M10	matrixin-like zinc metalloendopeptidases	No
MXAN5970		-4.135	-2.49	S8	subtilisin-like serine endopeptidase	Yes (SPI, OM)[5]
MXAN6106	*fibA*	-5.694	-1.878	M4	thermolysin-like metalloendopeptidase	Yes (SPII, ECM)[6]
MXAN6494		-3.087	-2.127	M23	beta-lytic metallopeptidase	Yes (SPI, 1TMD)

[1]log2 ratio of the transcriptional level in comparison to that of WT; [2] protease family and type was predicted based on presence of corresponding domain predicted by pfam (Finn *et al.*, 2010) and MEROPS (Rawlings *et al.*, 2010) databases; [3] Predicted by presence of signal peptides (SPI or SPII and N-terminal transmembrane helix (N-THM)) or transmembrane helixes (TMH) (Juncker *et al.*, 2003, Krogh *et al.*, 2001) or based on experimental evidence: [4] secreted in the supernatant during starvation (Rolbetzki et al., 2008); [5] Proteins found in OM (Kahnt *et al.*, 2010); [6]Proteinfound as component of extracellular matrix (ECM) (Kearns *et al.*, 2002)

shown that *asgA* and *asgB* mutants have severe developmental defects: The *asgA* mutant fails to aggregate into fruiting bodies and to sporulate under all starvation conditions; the *asgB* mutant fails to form mature fruiting bodies buthas some background sporulation, which is relatively high on starvation agar medium and lower under submerged starvation conditions (Kuspa & Kaiser, 1989b). In agreement with these earlier observations, we found that the *asgA* strain (DK5057) was unable to aggregate to form fruiting bodies on TPM agar as well as in submerged culture (Fig. 24BC). Likewise, under both conditions sporulation was strongly reduced (Fig. 24BC). The *asgB* (DK4398) mutants also behaved as previously described and reached a sporulation level of 69% and 27% of the WT on TPM agar and under submerged conditions, respectively. In our next experiment, we tested the three complementation strains for their ability to form fruiting bodies and to sporulate. Strikingly, the constitutive expression of *popCD* restored not only fruiting body formation but also sporulation in the *asgA* and *asgB* mutants (Fig. 24 BC). As expected constitutive expression of *popCD* also restored all developmental defects in the Δ*popCD* mutant (Fig. 24 BC).

The developmental defects of the *asgA* and *asgB* mutants have been linked to their inability to produce the intercellular A-signal (Kuspa et al., 1986). To determine whether ectopic expression of *popCD* restores development in *asgA* and *asgB* mutants at the point at which they are blocked in development, or whether ectopic expression of *popCD* allow the *asgA* or *asgB* mutants to restart development at a later stage in that way bypassing the requirement for the A-signal, we tested whether the ectopic expression of *popCD* in the *asgA* and *asgB* mutants restores A-signaling. A-signal activity is monitored by analyzing the expression of the A-signal-dependent reporter gene *spi* during development (Kuspa et al., 1986). Expression of *spi* is up-regulated after two hrs of starvation in a strictly A-signal dependent manner (Kroos *et al.*, 1986, Kuspa et al., 1986).

Expression of *spi* has conveniently been measured by monitoring levels of β-galactosidase expressed from the *spi*::Tn*5 lac* Ω4521 transcriptional fusion (henceforth *spi*::Ω4521). We followed *spi* expression using qRT-PCR. To this end, total RNA was isolated from vegetative cells as well as from cells starved

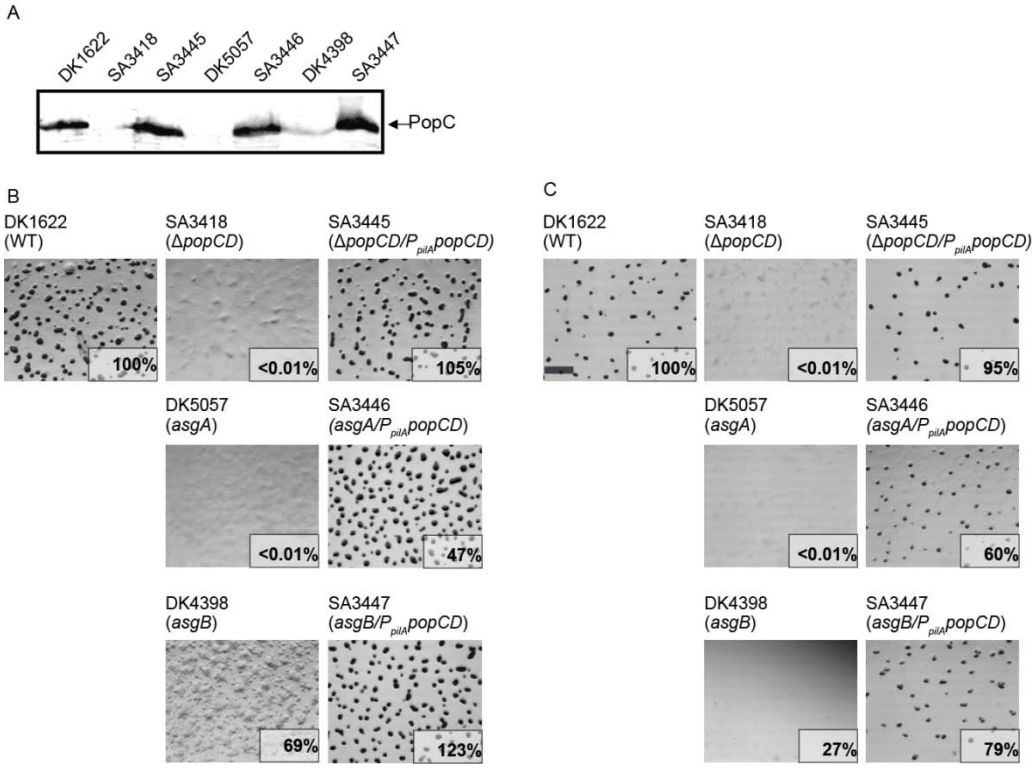

Figure 24. Constitutive expression of *popCD* restores development of *asgA* and *asgB* mutants.

(A) PopC accumulates at WT levels in *asgA/* P_{pilA}*popCD* and *asgB/* P_{pilA}*popCD* complementation strains. Cells of the indicated strains were harvested from vegetatively growing cultures. Protein isolated from 10^8 cells was loaded per lane, separated by SDS-PAGE, and analyzed by immuno-blotting with anti-PopC antibodies. PopC is indicated.(B-C) Fruiting body formation and sporulation are restored in *asgA/* P_{pilA}*popCD* and *asgB/* P_{pilA}*popCD* complementation strains. Cells of indicated strain were starved on TPM starvation agar (B) or in submerged culture (C) for 120 hrs. Numbers in each frame indicate the sporulation frequency. Sporulation in DK1622 was set to 100%. Scale bar, 0.1 mm.

for 3 hrs under submerged conditions. Expression of the *spi* gene was induced approximately 12-fold in WT after 3 hrs of starvation but not in the *asgA* or *asgB* mutants (Fig. 25). These results are similar to those previously observed for *spi*::Ω4521 and therefore qRT-PCR can be used to monitor expression of *spi* gene in this assay. Induction of *spi* expression in response to starvation in the Δ*popCD* mutant as well as its complementation strain was similar to that of WT (Fig. 25). Thus, the Δ*popCD* mutant has no defect in A-signaling. In agreement with these observations, expression of the *spi* gene was not restored in the *asgA/* P_{pilA}*popCD* and *asgB/* P_{pilA}*popCD* strains (Fig. 25). Thus, PopC is not required for A-signal accumulation and, consistently, constitutive expression of *popCD* in *asgA* or *asgB* mutants does not restore A-signaling and *spi* expression. Given that ectopic expression of *popCD* in the *asgA* and *asgB*

mutants does not restore A-signaling and A-signal-dependent gene expression shows that ectopic expression of *popCD* leads to a bypass of the requirement for A-signaling during development.

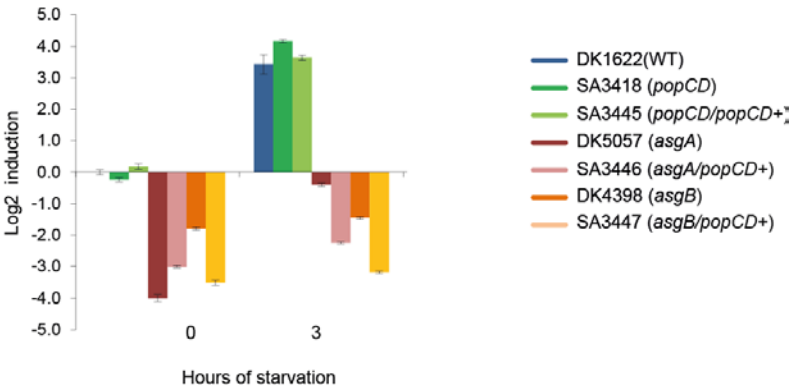

Figure 25. Constitutive expression of *popCD* does not restore expression of *spi*.

Cells of the indicated strains were exposed to starvation in submerged culture. Total RNA was isolated after 0 and 3 hrs of starvation and expression of *spi* analyzed by qRT-PCR. y-axis represents the log2 ratio of the transcriptional level in comparison to that of WT at 0 hr. Error bar indicate the standard deviation between three technical replicates.

Expression of *spi*::Ω4521 as well as fruiting body formation and sporulation in the *asgB* mutant is restored in the A-signal bioassay in which crude A-signal is added to starving *asgB* cells (Kuspa et al., 1986). Given that ectopic expression of *popCD* bypasses the requirement for *asgA* or *asgB* without restoring A-signaling, and given that *popC* expression is strongly reduced in vegetative *asgB* cells, we next asked whether addition of crude A-signal to the *asgB* mutant (DK4398) also restores expression of *popC*. For this purpose we isolated A-signal from the supernatant of WT cells that had been starved for 2 hrs in shaken suspension (Kuspa et al., 1986). Next, purified A-signal was added to cells of the *asgB* mutant starving under submerged conditions. Cells were allowed to develop for 24 hrs and after 0, 3, 6, 12 and 24 hrs of starvation total RNA was isolated. Expression of *spi* and *popC* was followed by qRT-PCR. As shown in Fig. 26A, expression of *spi* in WT cells was induced early during starvation and peaked between 3 and 6 hrs. *spi* expression in the *asgB* mutant was also induced in response to starvation but at lower level than in WT. Importantly, addition of A-signal to the *asgB* cells restored expressions of *spi* to WT levels, thus, confirming that the A-signal bioassay functions properly. In

agreement with previous observations (Rolbetzki et al., 2008), *popC* expression was induced 8-fold in WT cells in response to starvation (Fig. 26B). *popC* expression in the *asgB* mutant was also induced in response to starvation but at lower level than in WT. Importantly, *popC* expression was not restored in the *asgB* mutant by the addition of A-signal.

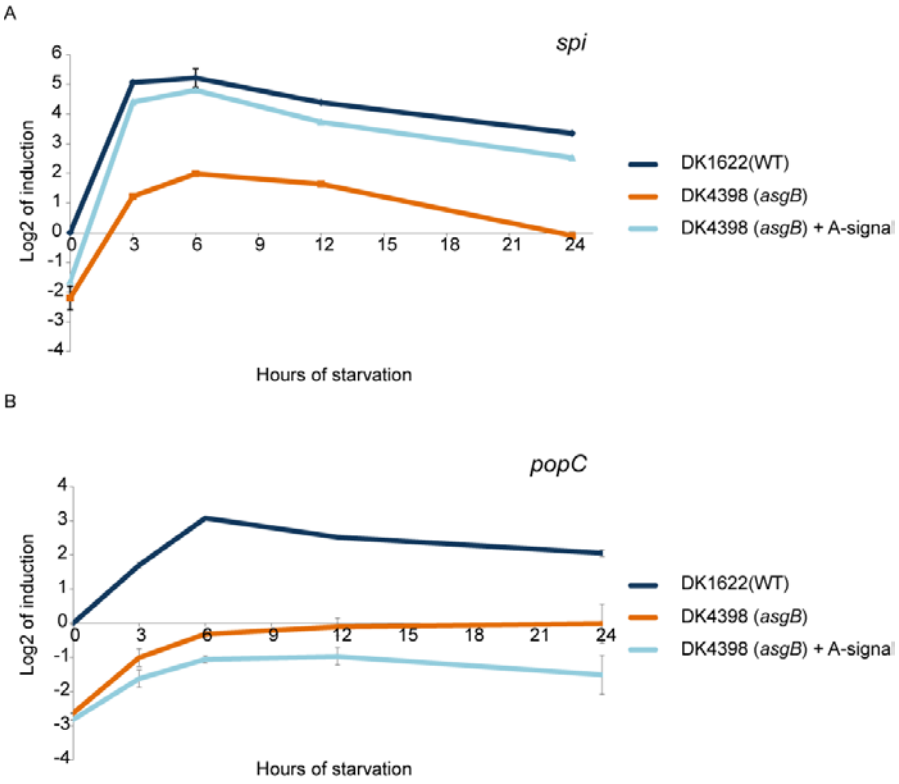

Figure 26. Addition of A-signal does not restore expression of *popC* in *asgB* mutant.

(A-B) Cells of the indicated strains were exposed to starvation in submerged culture in presence or absence of crude A-signal isolated from WT cells starved for 2 hrs. Total RNA was isolated after 0, 3, 6, 12 and 24 hrs of starvation and expression of *spi* and *popC* analyzed by qRT-PCR.y-axis represents the log2 ratio of the transcriptional level in comparison to that of WTat 0 hr. Error bars indicate the standard deviation between three technical replicates.(A) qRT-PCR analysis of expression of *spi*.(B) qRT-PCR analysis of expression of *popC.*

4.2. Discussion

The current model for A-signaling (Kaplan & Plamann, 1996, Diodati et al., 2008) in *M. xanthus* proposes that starvation induces the stringent response resulting in increased cellular levels of (p)ppGpp. Subsequently, (p)ppGpp by an unknown mechanism activates the *asg* genes (*asgABDE*). Activation of the four *asg* genes – again by an unknown mechanism – causes the release of extracellular A-signal proteases. These proteases are thought to digest surface-

exposed proteins and extracellular proteins, thereby, generating the A-signal amino acids and peptides. The concentration of these amino acids is proportional to cell number and, therefore, serves as an intercellular signal to measure cell density. The A-signal amino acids only serve as an intercellular signal in a particular concentration range (10-1000 µM, with optimum concentration at 100 µM) (Kuspa et al., 1992b, Kuspa et al., 1992a). At lower concentrations they fail to support development, and at a higher concentration they support cell growth (Kuspa et al., 1992b, Kuspa et al., 1992a). Notably only a subset of amino acids has A-signal activity (Kuspa et al., 1992b, Kuspa et al., 1992a). Consistently, it has been suggested that the primary defects in most *asg* mutants are in signal transduction pathways important for the release of A-signal proteases (Diodati et al., 2008, Kaplan & Plamann, 1996) and that *asg* mutants have a reduced capacity for protein secretion (Kuspa & Kaiser, 1989b).

Intrigued by the observation that generation of the intercellular C-signal depends on the starvation- and RelA-dependent secretion of the PopC protease, we hypothesized that the *asg* genes could also be involved in PopC secretion. *asgA* and *asgB* mutants have pleiotropic phenotypes. In addition to strong developmental defects, they display defects in vegetative cells with altered colony pigmentation and reduced cell-to-cell cohesion (Kuspa & Kaiser, 1989b) and reduced predation efficiency (Berleman & Kirby, 2007b). Because *asgA* and *asgB* mutants fail to generate the A-signal proteases and because the vegetative phenotypes of *asgA* and *asgB* mutants are associated with cell-surface properties, we carried out global gene expression profiling experiments using total RNA isolated from vegetative cells of the *asgA* and *asgB* mutants to address a possible connection between the *asg* genes and PopC.

Our microarray analyses revealed that a large number of genes display altered expression in vegetative cells of *asgA* and *asgB* mutants. 164 and 158 genes were found to be directly or indirectly regulated by AsgA and AsgB, respectively, among them 91 genes were found to be regulated by both AsgA and AsgB. These findings together with the known vegetative phenotypes of *asgA* and *asgB* mutants suggest that AsgA and AsgB are important for gene expression in vegetative cells and possibly also in starving cells. Importantly, genes coding for proteins of secretion systems were not found to be

significantly down-regulated in the two *asg* mutants. Rather 12 genes encoding proteases predicted to be secreted were down-regulated in both *asg* mutants. These observations strongly suggest that the primary defect of the *asgA* and *asgB* mutants in A-signal generation is not at the level of protein secretion but at the level of gene expression. In other words, the suggested reduced capacity for protein secretion in *asgA* and *asgB* mutants (Kuspa & Kaiser, 1989b) is not caused by a secretion defect *per se* but by reduced expression of genes encoding secreted proteins.

Interestingly, among the down-regulated genes encoding extracellular proteases we identified the *popC* gene. Our analyses demonstrate that restored expression of *popCD* rescued the developmental defect of the *asgA* and *asgB* mutants. Importantly, constitutive expression of *popCD* did not suppress the defects in colony pigmentation and cell-to-cell cohesion. Therefore, constitutive expression of *popCD* specifically suppresses the developmental defects of the *asgA* and *asgB* mutants. Moreover, we found that the expression of the A-signal dependent reporter gene *spi*, which is induced in response to starvation in an A-signal-dependent manner, is not restored by constitutive expression of *popCD* in the *asgA* and *asgB* mutants. Consistently, we also observed that *popCD* is not required for A-signal production. Therefore, constitutive expression of *popCD* does not restore development in *asgA* and *asgB* mutants at the earliest point at which they are blocked in development but allows the *asgA* or *asgB* mutants to restart development at a later stage. In other words, constitutive expression of *popCD* bypasses the requirement for the A-signal for development and, formally, constitutive expression of *popCD* can be classified as a bypass suppressor mutation of *asgA* and *asgB*. Although we have not shown directly that PopC is secreted in the *asgA* and *asgB* mutants expressing *popCD* constitutively, the restored development of these two mutants by constitutive *popCD* expression supports the conclusion that the primary defect in *asg* mutants is the expression of genes coding for secreted proteins rather than in secretion *per se*. We are currently testing whether PopC is secreted in *asgA* and *asgB* mutants expressing *popCD* constitutively.

The developmental defects of the *asgA* and *asgB* mutants have been linked to their inability to produce the intercellular A-signal. Our data strongly suggest

that PopC is not an A-signal protease. In future experiments, it will be analyzed whether development of the *asgA* and *asgB* strains in which *popCD* is constitutively expressed are more sensitive to low-cell densities compared to WT by testing development of these strains at different cell densities. Moreover, our DNA microarray data identified 12 genes encoding proteases suggested to be secreted. This set of proteases represents candidates for being involved in A-signal synthesis.

Several studies have focused on genetic suppressor analyses of *asgA* or *asgB* mutants. The first attempt to isolate *asgB* suppressor used restored expression of *spi*::Ω4521 to screen for suppressor mutations (Kaplan *et al.*, 1991). These analyses identified the *sasA* locus (consists of *rfbABC* operon (MXAN4623-4621) encoding proteins forLPS biosynthesis) and the *sasB* locus (consists of *sasN* (MXAN1244), *sasR* (MXAN1245), and *sasS* (MXAN1249) genes encoding for regulatory proteins important for *spi* expression) (Xu *et al.*, 1998, Yang & Kaplan, 1997, Guo *et al.*, 2000, Guo *et al.*, 1996). Importantly, mutations in the *sas* loci resulted in A-signal independent *spi*::Ω4521 expression and neither restored A-signal production nor development in an *asgB* mutant. Thus, both *sas* mutations are *asgB* bypass suppressor mutations. *sas* mutations also restore *spi*::Ω4521 expression in an *asgA* mutant. A loss-of-function mutation in *spdR*, which encodes an enhancer-binding-protein, was isolated as a suppressor of the developmental defects of an*bsgA* mutant (Hager *et al.*, 2001). However, this suppression is not specific to a *bsgA* mutant because the *spdR* mutation also bypasses an *asgB* mutation (Tse & Gill, 2002). It has been suggested that SpdR is a general inhibitor of development, and, therefore, a mutation in *spdR* results in bypass of several requirements for development including nutrient limitation and mutations in *bsgA* and *asgB* intercellular signaling genes (Hager *et al.*, 2001). Dunmire et al. (Dunmire et al., 1999) isolated suppression mutations of an *asgA* mutant by screening for restored development. These mutations restored development but did not restore A-signal production, thus, also classifying these mutations as bypass suppressors of an *asgA* mutation. These mutations havenot been mapped to our knowledge. Based on the data presented here, we speculate that a mutation causing constitutive expression of *popCD* could be present in this collection of bypass

suppressor mutations. The observation that suppressor mutations that restore A-signal production and development in *asgA* or *asgB* mutants have not been isolated support the notion that the developmental defects in these two mutants are caused by more than one defect.

Our microarray analyses revealed that a large number of genes display altered expression in vegetative cells of *asgA* and *asgB* mutants. Interestingly, genes coding for proteins involved in biosynthesis of the pigment DKxanthene, which is the yellow pigment that gives *M. xanthus* colonies their unique yellow colour (Meiser et al., 2006), were down-regulated. The reduced expression of these genes likely explains the altered colony pigmentation of *asgA* and *asgB* mutants. Mutants unable to synthesize DKxanthene display developmental defects, which can be rescued by addition of purified DKxanthene (Meiser et al., 2006).

On the basis of the data presented here, we suggest that theinability of the *asgA* and *asgB* mutants to undergo development is the result of at least three defects: (i) reduced expression of the genes encoding the A-signal proteases, (ii) reduced expression of the *popC* gene, and, (iii) reduced expression of the DKxanthene gene cluster.

Several questions remain unanswered regarding A-signaling and the phenotypes of *asg* mutants. One question concerns the mechanism of extracellular complementation of *asg* mutants. Sporulation of an *asgB* mutant is rescued by co-development with WT or a mutant of a different extracellular complementation group, e.g. *csgA* (Hagen et al., 1978). Because, *asgB* and *csgA* mutants can rescue each other for development, it was suggested that *csgA* provide A-signal to the *asgB* mutant and the *asgB* mutant provide C-signal to the *csgA* mutant. However, because an *asgB* mutant is reduced in *popC* expression, the *asgB* mutant should also have defect in C-signal accumulation. Our data suggest that addition of A-signal to *asgB* cells does not rescue expression of *popC*. We previously showed that PopC acts in *cis* and a *popC* mutant cannot be rescued by extracellular complementation (Rolbetzki et al., 2008). Therefore, it is unlikely that an *asgB* mutant is rescued by PopC provided by the *csgA* cells. Currently, we cannot exclude that residual expression of *popC* in an *asgB* mutant during development might be sufficient to produce

sufficient C-signal. This question will be addressed in future experiments by careful monitoring of *popC* expression and PopC accumulation as well as secretion as well as C-signal generation at different time points during development of an *asgB* mutant.

The second question is the nature of the A-signal. The model for the A-signal acting as an intercellular signal to measure cell density was based on several lines of evidence. First, A-signal was described as an essential intercellular signal because mutants unable to produce A-signal had strong development defects and these defects could be rescued by co-development (referred to as extracellular complementation) with *asg*+(Kuspa & Kaiser, 1989b, Hagen et al., 1978), by the addition of conditioned supernatants from starving *asg*+ cells (Kuspa *et al.*, 1986) or purified A-signal components (extracellular proteases or amino acids) (Kuspa et al., 1992a, Plamann et al., 1992). These data were mainly collected from studies on the *asgB* mutant. Interestingly, development of an *asgA* mutant is neither rescued by exogenous A-signal nor by its constituent components. Likewise, extracellular complementation of an *asgA* mutant is also very inefficient(Kuspa & Kaiser, 1989). The *asgC* mutant also fails to produce A-signal, yet has only minor developmental defects (Kuspa & Kaiser, 1989). Two recently identified mutants of the A-signal extracellular complementation group also have very different phenotypes. An *asgD* mutant (Cho& Zusman, 1999) undergoes development on stringent starvation medium but not on low-nutrient medium. The developmental defects of an *asgD* mutant are rescued by some (but not all) A-signal amino acids, but in contrast to other *asg* mutants, *asgD* has normal *spi*::Ω4521 expression suggesting normal release of A-signal. An *asgE* mutant (Garza et al., 2000b) has a defect in the production of the heat-labile but not in the heat-stable fraction of A-signal but could not be rescued by addition of proteases (Garza *et al.*, 2000b). Based on these observations, Diodati et al.(Diodati et al., 2008) suggested that the A-signal might be a mixture of different signals and that different *asg* mutants have defects in different components of the A-signal. The experimental approach we used in our analyses, in which gene expression profiles were determined in vegetative cells of *asgA* and *asgB* mutants, maybe also useful in defining the primary defects of other *asg* mutants such *asgD* and *asgE*.

5. Materials and methods

Reagents, Enzymes and Kits

The chemicals and antibiotics used in this were obtained from Roth (Karlsruhe), Sigma (Taufkirchen), Difco (Heidelberg), Invitrogen (Karlsruhe) and Merck (Darmstadt) study unless stated in the text. The DNA markers used in the study were purchased from Bioline (Luckenwalde). The protein ladders were from Fermentas (St. Leon-Rot).

Restriction enzymes, the quick ligase and Antarctic phosphatase were obtained from Fermentas (St. Leon-Rot) or New England Biosciences (Frankfurt a. M.). *Pfu* ultra II® DNA-Polymerase from Stratagene, AccuPrime CG rich DNA Polymerase from Invitrogen (Karlsruhe) was used for cloning PCR reaction. *Taq*-Polymerase in ”Eppendorf® MasterMix“ from Eppendorf (Hamburg) was used for the checking PCR. Buffer J used for PCR reactions was purchased from Epicentre (Hess.-Oldendorf). All oligonucleotides were synthesized by Invitrogen (Karsruhe).

PCR product purification, DNA gel extraction and plasmid purification were performed using the respective Qiagen (Hilden) and Zymo Research (HiSS-Diagnostics, Freiburg) kits. RNeasy Kit from Qiagen (Hilden) was used to purify the RNA from the DNase I digestion reactions. The cDNA Archive kit (ABI) was used to synthesize cDNA from RNA. The PCR product of sequencing reaction was purified with BigDye XTerminator Purification Kit from Applied Biosystems. Genomic DNA was purified with MasterPure™ DNA purification kit from Epicentre (Hess.-Oldendorf).

Anti-protein and anti-peptides polyclonal antibodies were generated by Eurogentec (Seraing, Belgium).

Microbiological methods

Cultivation of bacterial strains

E. coli strains were grown in LB medium (1% Tryptone, 0.5% yeast exact and 1% NaCl, pH 7.0) or on LB agar plate (1.5% agar in LB medium) with relevant antibiotics (Table 13) following standard protocol (Sambrook & Russell, 2001). *E. coli* stains were grown in liquid culture (with 230 rpm shaking) or on the solid

plates at 37°C (or at 30°C for thermosensitive plasmids) under aerobic conditions. Glycerol stocks were made with overnight culture by adding the glycerol to the final concentration of 10% and stored at -80°C.

M. xanthus strains were grown in 1% CTT medium (1% casitone, 10 mM Tris-HCl pH 8.0, 1 mM KH_2PO_4 pH 7.6, 8 mM $MgSO_4$) (Hodgkin & Kaiser, 1977) or on 1% CTT agar plates (1.5% agar in 1% CTT medium) with respective antibiotics and supplied with 1/1000 volume of trace element (1.5% Titriplex III, 1 mM $ZnCl_2$, 1 mM $CuSO_4$, 1 mM $CoCl_2$, 1 mM Na_2MO_4, 1 mM $MnSO_4$, 10 mM $FeSO_4$) at 32°C in the dark. The glycerol stocks were made with the *M. xanthus* culture (OD550 between 0.8 and 1.2) by adding the glycerol to 4% and the mixtures were fast frozen in liquid nitrogen and stored at -80°C. *M. xanthus*strains on the plates were stored at 18°C for one month.

Table 13.Antibiotics used in this study.

Antibiotic	**Final concentration, µg/ml**	
	E. coli	***M. xanthus***
Ampicillin sodium salt	100	
Chloramphenicol	25	
Carbenicillin	100	
Kanamycin sulfate	50	50
Tetracyclin	15	10*
Streptomycin	100	1000
Spectinomycin		800

*Oxytetracycline dihydrate was used for *M. xanthus*.

Table 14.*E. coli* strains used in this study

Strain	**Genotype**	**Reference**
BW25113	K12 Δ*araBAD*, Δ*rhaBAD*	(Datsenko & Wanner, 2000)
Top10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74nupGrecA1 araD139* Δ(*ara-leu*)7697 *galE*15 *galK*16 *rpsL*(Str^R) *endA1* λ^-	Invitrogen
Mach1	Δ*recA*1398 *endA1tonA* Φ80Δ*lacM15* Δ*lacX74hsdR*(r_K^- m_K^+)	Invitrogen
Rosetta 2 (DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pRARE2 (Cam^R)	Novagen
DH5α λpir	*sup E44*, Δ*lacU169* (Φ*lacZ*Δ*M15*), *recA1*, *endA1*, *hsdR17*, *thi-1*, *gyrA96*, *relA1*, λ*pir*	(H.Kaplan, personal communication)

Table 15.*M. xanthus* strains used in this study

Strain	**Genotype**[1]	**Reference**
DK1622	wild type	(Kaiser, 1979)
DK101	wild-type	(Hodgkin & Kaiser, 1977)
DZF1	wild type	(Morrison & Zusman, 1979)
SA2314	*popC*::pAB3	(Rolbetzki et al., 2008)
JMCzc3	DZF1 *ΔMXAN7436-7438*	(Moraleda-Munoz *et al.*, 2010)
ΔrelA	DK101 *ΔrelA*	(M. Singer, personal communication)
DK5057	*asgA*	(Kuspa & Kaiser, 1989b)
DK4398	*asgB*	(Kuspa & Kaiser, 1989b)
DK5208	*csgA*	(Kroos & Kaiser, 1987a)
DK5209	*bsgA*	
SA3400	*MXAN1791*:: pAK1	This study
SA3401	*MXAN3744*:: pAK2	This study
SA3402	*MXAN4176*:: pAK3	This study
SA3403	*MXAN4198*:: pAK4	This study
SA3404	*MXAN5030*:: pAK5	This study
SA3405	*MXAN6487*:: pAK6	This study
SA3406	*MXAN7238::* pAK7	This study
SA3407	*ΔMXAN2434-2454::aadA*	This study
SA3408	Δ*popC*	This study
SA3409	Δ*popCD*	This study
SA3410	*ΔMXAN4807-4813*	This study
SA3412	DK1622/P_{nat}*popD*	This study
SA3413	DK1622 *ΔpopD::aadA*/Pnat *popD*	This study
SA3416	*Δ4813*	This study
SA3418	*ΔpopCD::aadA*	This study
SA3424	*ΔpopCD::aadA*/Pnat *popC popD-Strep tag*	This study
SA3425	*ΔpopCD*/P_{nat}*popCD*	This study
SA3436	*csgA ΔpopD::aadA*	This study
SA3437	*ΔMXAN4800*	This study
SA3438	*popC*^{S423A}*ΔpopD::aadA*	This study
SA3439	DK101 *ΔpopCD::aadA*	This study
SA3440	DK101 *popC*^{S423A}*ΔpopD::aadA*	This study
SA3442	DK101 *ΔpopD::aadA*	This study
SA3443	*asgA*/P_{pilA}*popCD*	This study

SA3444	DK1622/ P_{pilA}*popCD*	This study
SA3445	*ΔpopCD*/ P_{pilA}*popCD*	This study
SA3446	*asgB*/ P_{pilA}*popCD*	This study
SA3448	*ΔMXAN5652-5643*	This study
SA3449	*ΔMXAN2434-2454::aadA, ΔMXAN5652-5643*	This study
SA3451	DK101 *csgA*	This study
SA3452	DK101 *ΔpopD::aadA csgA*	This study
SA3453	DK101 *ΔrelA csgA*	This study
SA3454	DK101 *ΔrelA csgA ΔpopD::aadA*	This study
SA3460	*ΔMXAN3903-3911*::*aadA*	This study
SA3462	*ΔMXAN4807-4808*	This study
SA3463	*MXAN3431::* pAK46	This study
SA3464	*MXAN3424::* pAK47	This study
SA3465	DK101 *ΔpopC::aadA*	This study
SA3466	DK101 *ΔpopD::aadA*/P_{nat}*popD*	This study
SA3471	*MXAN2174*^{127}*::*mini-Himar(Kan)Ω*4810*	This study
SA3472	*MXAN7131*^{536}::mini-Himar(Kan)Ω*4820*	This study
SA3473	*MXAN7131*^{356}*:* mini-Himar(Kan)Ω*4823*	This study
SA3474	*MXAN7132*^{696}*:* mini-Himar(Kan)Ω*4821*	This study
SA3475	*MXAN7136*^{54}*::*mini-Himar(Kan)Ω*4826*	This study
SA3476	*MXAN1438*^{1121}*::* mini-Himar(Kan)Ω*4836*	This study
SA3477	*MXAN5059*^{163}*::*mini-Himar(Kan)Ω*4822*	This study
SA4809	*MXAN1228*^{247}*::*mini-Himar(Kan)Ω*4809*	This study
SA4866	*MXAN4841*^{1085}*::*mini-Himar(Kan)Ω*4866*	This study

[1]Numbers in superscript indicate the mini-Himar(Kan) insertion site relative to the start codon of the relevant gene.

Development assay and spore assay of *M. xanthus*

M. xanthus development was examined on the following three different conditions: TPM agar plates (10 mM Tris-HCl, pH 7.6, 1 mM KH2PO4, pH 7.6, 8 mM MgSO4, 1.5% agar) (Kuner & Kaiser, 1982), CF agar plates (10 mM Tris-HCl, pH 8.0, 1 mM KH2PO4, pH 7.6, 8 mM MgSO4, 0.02% (NH4)2SO4, 0.1% NaPyruvate, 0.2% NaCitrate, 1.5% agar) (Shimkets & Kaiser, 1982), and submerged in MC7 buffer (10 mM MOPS, pH 7.0, 1 mM CaCl2). The stains for the development assay were cultivated in parallel to OD550 0.5~0.9. The cells were harvested and resuspended in MC7 buffer to a calculated density of 5 x 10^9 (unless indicated otherwise) cells/ml. 20 µl aliquots were spotted on CF

agar and TPM agar. For development in submerged culture, concentrated cells were diluted in MC7 to the final density of 6.25 x 10^8 cells/ml. Cell suspension was placed in a 15 mm well in a microtiter dish. Aggregation was followed using a Leica MZ8 stereomicroscope and a Leica IMB/E inverted microscope and visualized using a Leica DFC280 CCD camera.

Spore numbers were determined as the number of spores formed after 120 hrs of starvation by harvesting 5 x 10^8 cells from each of the three different starvation conditions. Cells were placed for 2 hrs at 50°C and briefly sonicated to disperse fruiting bodies. Spores were counted in a haemocytometer (Depth 0.1 mm, Marienfeld). To determine the number of germinating spores, spore solutions were diluted and plated on 1.0% CTT agar plates with CTT softagar (0.75% agar in 1% CTT medium).

A-signal production and bioassay

Crude A-signal was prepared as described previously (Kuspa et al., 1986). Briefly, cells of corresponding strains were grown in liquid CTT to a density of 5 × 10^8 cells/ml. Cells were harvested by centrifugation, and washed in MC7. After wash cells were harvested and resuspended in MC7 buffer to a calculated density of 5 x 10^9 cells/ml and starved for 2 hours at 32°C with shaking. Cells were harvested and cell-free supernatants containing crude A-signal were stored at -80 °C until use.

A-signal was assayed by measuring the restored expression of an asg-dependent *spi* gene in an *asg*- mutant strain DK4398 (*asgB*) (Kuspa & Kaiser, 1989a). For this purpose, exponentially growing cells of DK4398, were harvested by centrifugation and washed in 1 volume of MC7 buffer at room temperature. The cells were resuspended to a calculated density of 3.1 x 10^8 cells/ml in MC7 or crude A-signal. Suspension was distributed into Petri dishes (12 ml per plate) and samples were taken at corresponding time points. Expression of *spi* was measured by qRT-PCR.

Molecular biological methods

Table 16. Primers used in this study

Used for	Primer description	Primer name	Sequence
Generation of insertion mutants			
MXAN1791:: pAK1	forward	AK-29	ATCGCAAGCTTTGATGAGCGATGACCTGCTCCGGTTG
	reverse	AK-30	TAGCGTCTAGAGAGGTGCTGCTCGAACCG
	check forward	AK-15	GATTGACGCTCGCCCTCC
	check reverse	AK-16	GAGTGGAATGACGTCGTCG
MXAN3744:: pAK2	forward	AK-3	ATCGCAAGCTTTGATGACACTACGCAAGCGCCGGAG
	reverse	AK-4	TAGCGTCTAGATAGCCGAAGTACGCCTGCG
	check forward	AK-17	TCATGGCGTGACAGTGGC
	check reverse	AK-18	CGGATCAAACGCGAAGGG
MXAN4176:: pAK3	forward	AK-5	ATCGCAAGCTTTGATGACCCTCCACCAGCACCCAG
	reverse	AK-6	TAGCGTCTAGACGTCTGCTCCAGCGTCAGC
	check forward	AK-19	CAGGCCATGCTGGATGTGG
	check reverse	AK-20	CAACAAGGCGAGCTCAGCG
MXAN4198:: pAK4	forward	AK-7	ATCGCAAGCTTTGATGAAGCGCAGCGCGTTTGGAGC
	reverse	AK-8	TAGCGTCTAGAGCGGGCTCGTCATTGAACC
	check forward	AK-21	TCTGGCCCAGGCAACGAC
	check reverse	AK-22	TCAGGTCCTCCTGGGCCAC
MXAN5030:: pAK5	forward	AK-9	TAGCGTCTAGAGCGGGCTCGTCATTGAACC
	reverse	AK-10	ATCGCAAGCTTTGATGAGGTAACGACCTGGGACGAGG
	check forward	AK-23	ATGGCATCCCCTTCCGTC
	check reverse	AK-24	ACACACTCCAGATGGCCACG
MXAN6487:: pAK6	forward	AK-11	ATCGCAAGCTTTGATGACATCACCCGCAACTCCATCG
	reverse	AK-12	TAGCGTCTAGACGCCGTCCCACAACGTCCAG
	check forward	AK-25	CCTACGCTGACCCTGGAGG
	check reverse	AK-26	GGTGAGGCGATGGGACTCG
MXAN7238:: pAK7	forward	AK-13	ATCGCAAGCTTTGATGATGGGACGAGGCGCTGCAAC
	reverse	AK-14	TAGCGTCTAGATCCAGTTGCTGGCAGTCCCG
	check forward	AK-27	TTCCCGGTGTCCGACCCAC
	check reverse	AK-28	TGAGCCGGTCGTTCTCCTCG
MXAN3431:: pAK46	forward	AK-294	ATCGCAAGCTTTGATGAGGGCGTGGCGCGTCAGAG
	reverse	AK-295	TAGCGTCTAGAAAGCGCCAGCAGCCGGGC
	check forward	AK-296	GACGCAGGGGCACCCGG
	check reverse	AK-297	CCGAGCTCCAGGGGCGTC
MXAN3424:: pAK47	forward	AK-290	ATCGCAAGCTTTGATGACCGGGCTCACCAGCGAGG
	reverse	AK-291	TAGCGTCTAGAAGCCGCCGGACAGGTGGG
	check forward	AK-292	GGACACGGGGGGCACTCC
	check reverse	AK-293	GGCTTGGCTTGCGACCAGC
Generation of in frame deletion mutants			
Δ*popC*	A	AK-60	ATCGCAAGCTTTCTTTCCCGGCCTCGTCTTC

	B	AK-87	CCTGTCTTGATGGACTCCTTTGGAAC
	C	AK-88	GAGTCCATCAAGACAGGCAAGGGCCTGG
	D	AK-61	TAGCGTCTAGACAGGTGGCGGAAGGGCCAC
	E	AK-54	GTTCAGGCCTTCGCGCTC
	F	AK-55	AGGGCTCGGCCACATC
	G	AK-56	ACAGGAAGAGCGCCGTC
	H	AK-57	TGCGGATGGTCTCCGATTC
ΔpopD	A	AK-58	ATCGCAAGCTTCAGTTCAGCCGCCCGGAGAAC
	B	AK-49	ATCATCGTGGAGAGGATGAGGGCCATG
	C	AK-50	ATCATCGTGGATGGACTCCTTTGGAAC
	D	AK-59	TAGCGTCTAGAGCTTCCTGGATTCCAGGC
	E	AK-39	GCGCGCGTCATCCAGTTC
	F	AK-40	GGTGGGACAGGCTGCCC
	G	AK-52	ACGCAGCCCGACTGGATTG
	H	AK-53	CAGCTGGTCCAGCAC
ΔpopCD	A	AK-35	ATCGCGAATTCTCTTTCCCGGCCTCGTCTTC
	B	AK-36	CCTGTCTTGGATGGACTCCTTTGGAAC
	C	AK-50	ATCATCGTGGATGGACTCCTTTGGAAC
	D	AK-51	GAGTCCATCCACGATGATGCCCCTCGAC
	E	AK-54	GTTCAGGCCTTCGCGCTC
	F	AK-40	GGTGGGACAGGCTGCCC
	G	AK-56	ACAGGAAGAGCGCCGTC
	H	AK-53	CAGCTGGTCCAGCAC
ΔMXAN4807-4813	A	AK-68	ATCGCAAGCTTGCTCTCCCTCATGCGACG
	B	AK-93	AGTGCGGTCCGACGCCCGTGGACTTCTG
	C	AK-94	ACGGGCGATGGAGGCGACTGGGACCTC
	D	AK-71	TAGCGTCTAGACGGCAGACCTCCTCATCG
	E	AK-72	GCGTTGCTGGACTCTTCCG
	F	AK-73	GTTGGTGCGCAGCGGCATG
	G	AK-74	CAGCGACATCAAGGGCGAC
	H	AK-75	CGGATGGACTGCGTCATCC
ΔMXAN4800	A	AK-128	ATCGCAAGCTTCACCTGGAGCCAGTCCAACG
	B	AK-129	GAGCGTGCGCTCTCCGGCGGTGAAGGG
	C	AK-130	GCCGGAGAGCGCACGCTCAAGGTGGAC
	D	AK-131	TAGCGTCTAGACGCTGTCCTTGAGCTGGACG
	E	AK-132	GCTGCCGCACCACAATCG
	F	AK-133	GCACTCCACACCCGACCG
	G	AK-134	CAGGACCTGGGTGCCAGC
	H	AK-135	CCCCGTTGTAGACGCTGCC
ΔMXAN4807-4808	A	AK-213	ATCGCTCTAGACCACGTCCTCCGCGCGAGC
	B	AK-214	ATCGCCTGCAGCTCGGTAGGGGCGACGGA
	C	AK-215	ATCGCCTGCAACCTTGTCCCTCGTGGGC
	D	AK-216	TAGCGAAGCTTCGTAACGCCGCCCTGGGTG
	E	AK-211	CGCCTCAACCGCATGGGC
	F	AK-212	CTGGATGGCCTGCCGGATGG

	G	AK-217	GAGCAGGTGGAGCTGCCG
	H	AK-218	CGCGTCCTCGCTCTCACG
ΔMXAN4813	A	AK-118	ATCGCAAGCTTCCTGGAGGGTACGCTGCC
	B	AK-119	CACATGCAGGGGCGTCAGGCGACGGAC
	C	AK-120	CTGACGCCCCTGCATGTGGCCCTGACC
	D	AK-121	TAGCGTCTAGACCTCATCGCCGGAGGTGC
	E	AK-122	TCCAGCCGGGAGCCGAG
	F	AK-123	TGGGGACGGTGGCGCAG
	G	AK-124	GATGCCGCCGCCAGTGG
	H	AK-125	CGGTGACGGCTTCGTCG
ΔMXAN5652-5643	A	AK-223	ATCGCAAGCTTCCTGCTACGTCGCACCCG
	B	AK-224	TTCAGCAAGGCCCTCCAGATTCGCCCC
	C	AK-225	CTGGAGGGCCTTGCTGAATCGCTCCCG
	D	AK-226	TAGCGTCTAGAACAGGGCCGGGCAACTCTC
	E	AK-227	CCAGGCAGCTGACTGCG
	F	AK-228	TCGGGGGTTATCCCTGG
	G	AK-229	GAGGGGAATCATCACCCGC
	H	AK-230	GCACGGCGGTGCTGGAG
Generation of gene replacement mutants			
ΔpopC::aadA	recombination forward	AK-95	CCGGCGTAGCACGGTGGTTCAAGGAGCACACAACCC ATGAATGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-96	CTCCCATGGCGCCGACCCCGGATTCAACCGCCACCG TCATGTGTAGGCTGGAGCTGCTTC
ΔpopD::aadA	recombination forward	AK-97	GGCAAGGGCCTGGCCGTATTCAGGTGACGGTGGCG GTTGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-98	CCCAGACCAGGGGTGCGAGGCGAGGGGCACCCACC GCTATGTGTAGGCTGGAGCTGCTTC
ΔpopCD::aadA	recombination forward	AK-95	CCGGCGTAGCACGGTGGTTCAAGGAGCACACAACCC ATGAATGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-98	CCCAGACCAGGGGTGCGAGGCGAGGGGCACCCACC GCTATGTGTAGGCTGGAGCTGCTTC
	check forward	AK-99	CCATTGCCCCTGTCGCAC
	check reverse	AK-100	AGCCGTCGGTTCTCAGTAG
popC^{S423A}*ΔpopD::aadA*	upstream fragment forwardS423A	AK-220	ACCGTGGGTAGGCGCGTC
	upstream fragment reverse	AK-219	CCGCCACCGTCACCTG
	downstream fragment forward	AK-221	CGGTGGGTGCCCCTCGCC
	downstream fragment reverse	AK-222	GCACAGCCTCACACGCG
	recombination forward	AK-97	GGCAAGGGCCTGGCCGTATTCAGGTGACGGTGGCG GTTGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-98	CCCAGACCAGGGGTGCGAGGCGAGGGGCACCCACC GCTATGTGTAGGCTGGAGCTGCTTC
ΔMXAN2434-2454::aadA	recombination forward	AK-41	TGACCTTTCCGCTGAAAGCAGCGCCTGGAGGAAGCA ATGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-42	TTGCGTCCCTCGGGCCCAGGCGCCGACGCGGTGCG ACTATGTAGGCTGGAGCTGCTTC
	check forward	AK-45	AGCGGCTGATCGCCGAATCG
	check reverse	AK-46	TCACGGGGAAATCTCCTCGG

ΔMXAN3903-3911::*aadA*	recombination forward	AK-181	CAGACGCCCCGCTCACCCAAGCGGTCAGACTCCCCG CTCAAATGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-182	GGCGCTGTCAGTACTCGCAGCTCGGGCGCGGAGGA CTCCTGTAGGCTGGAGCTGCTTC
	check forward	AK-183	GACCGAGCCACCCTGCG
	check reverse	AK-184	CGCCCATCAGCCACAGC
	internal forward	AK-185	CACGGTGGGCACCGCGAC
	internal reverse	AK-186	ACTTGCCCGCGCTGTTGCC
*Δ*MXAN2515-2504::*aadA*	recombination forward	AK-237	TGCCCGCCTGGCCCGCGATGGCACCTGGCTTGCTCA ATGAATGATTCCGGGGATCCGTCGACC
	recombination reverse	AK-238	GGACGGGGGCGAAGACTTCCTGAAGCTTGGCCATGG TCATGTGTAGGCTGGAGCTGCTTC
	check forward	AK-239	CGCTGAGCCGCTCGAAC
	check reverse	AK-240	GTCCTGTCTTGCCCGCC
For protein over-expression in E. coli			
His6-*popC*	forward	AK-164	ATCGCGGATCCGATGAAGTCCTACCTGTTGG
	reverse	AK-160	TAGCGAAGCTTTCATCACCTGAATACGGCCAGG
His6- peptidase	forward	AK-271	ATCGCGGATCCGGAGTGGCCCGACGAGTGC
	reverse	AK-160	TAGCGAAGCTTTCATCACCTGAATACGGCCAGG
His6-prosegment	forward	AK-164	ATCGCGGATCCGATGAAGTCCTACCTGTTGG
	reverse	AK-168	TAGCGAAGCTTTCAGCGCTCGGCCAGCGAG
popD-S	forward	AK-232	ATCGCCATATGAGGGCCATGAGCAGGAAAAATGGC
	reverse	AK-231	ATCGCGGTACCGATGAGGTCGAGGGGCATC
For site directed mutagenesis			
$popC^{S423A}$	forward	AK-241	CGAGCGAATGAGTGGCACGGCGATGGCAGCGCCTTA TGTAGC
	reverse	AK-242	GCTACATAAGGCGCTGCCATCGCCGTGCCACTCATTC GCTCG
For qPCR			
spi	forward	spi for	GGCTGTCTCCCGCTTTCTTC
	reverse	spi rev	TGGATGTCGATCTGATGGTTCT
popC	forward	803F	CCGTTATCGAATCGGAGACC
	reverse	873R	GTGCAGCAGCCACTCCATG
For sequencing and standard PCR check reactions			
M13 for	GTAAAACGACGGCCAGT		
M13 rev	CAGGAAACAGCTATGAC		
ACYCDuetUP1	GGATCTCGACGCTCTCCCT		
DuetDOWN1	GATTATGCGGCCGTGTACAA		
DuetUP2 Primer	TTGTACACGGCCGCATAATC		
T7 Terminator	GCTAGTTATTGCTCAGCGG		
T7	TAA TAC GAC TCA CTA TAG GG		
Arb1 Myxo	GGCCACGCGTCGACTAGTACNNNNNNNNNNGCGAGC		
Arb2 Myxo	GCTCTAGAGGCCACGCGTCGACTAGTAC		
MiniHimar Int-1	GATCTGGGAATCATTTGAAGG		
MiniHimar Ext-1	GGGTTGAGTGTTGTTCCAG		
MiniHimar Int-2	TCTTCTGAGCGGGACTCTGG		
MiniHimar Ext-2	GCTTCCTCGTGCTTTACGGTATCG		
attB left	CGGCACACTGAGGCCACATA		

attB right	GGAATGATCGGACCAGCTGAA
attP left	GGGAAGCTCTGGGTGACGAA
attP right	GCTTTCGCGACATGGAGGA

Amplification of specific DNA-fragments was carried out in 50µL reaction volume in FailSafeTM PCR PreMix Buffer J (Epicentre) with *Pfu* ultra II® DNA-Polymerase (Stratagene). To check integration, colony PCR was performed using Eppendorf® MasterMix (Eppendorf) in a 20µL reaction volume. To amplify resistance cassette for gene replacement, PCR was performed using AccuPrime CG rich DNA Polymerase (Invitrogen).

Standard/Check PCR reaction

Initial denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	5°C below melting temperature	30 sec
Elongation	72°C	1 min per 1kb
Final elongation	72°C	5 min
Hold	4°C	

Walking PCR (Pilhofer et al., 2007)

<u>Specific primer extension, ssDNA synthesis</u>		
Initial denaturation	95°C	3 min
Denaturation	95°C	30 sec
Annealing	56°C	30 sec
Elongation	72°C	4 min
<u>Unspesific binding of random primer, generation of dsDNA</u>		
Denaturation	95°C	30 sec
Annealing	40°C	30 sec
Elongation	72°C	4 min
<u>Specific exponential amplification</u>		
Denaturation	95°C	30 sec
Annealing	56°C	30 sec
Elongation	72°C	4 min
Hold	4°C	

Restriction of DNA was carried out by incubation of 2.5- 5µg DNA with restriction endonucleases for 2 h according to specific requirements for the enzyme.

Ligation reactions were performed with Rapid DNA ligation kit (Fermentas). DNA fragments were ligated into vectors applying a 3 fold molar excess of insert-DNA.

Table 17. Plasmids and cosmids used in this study

Plasmid	Description	Reference
pBJS18	Vector for generation insertion mutants, KanR	(Spratt et al., 1986)
pBJ114	Vector for generation in frame deletion mutants, galK KanR	(Julien et al., 2000)
pSWU30	vector for intergration at the att site, Mx8 attP TetR	(Wu & Kaiser, 1997)
PSW105	pSWU19 containing pilA promotor, KanR	Sebastian Weiss
pRSF Duet-1	vector for coexpression of two target proteins	Novagen
pIJ790	λ-RED (gam, bet, exo), cat, araC, rep101	(Gust et al., 2003)
pIJ778	P1-FRT-oriT-aadA-FRT-P2	(Gust et al., 2003)
pMiniHimar	suicide plasmid containing MiniHimar transposon, KanR	(X. Duan and H.B. Kaplan, personal communication)
Plasmids for generation of insertion mutants		
pAK-1	*MXAN1791* (58-817) in pBJS18	This study
pAK-2	*MXAN3744* (150-860) in pBJS18	This study
pAK-3	*MXAN4176* (292-1062) in pBJS18	This study
pAK-4	*MXAN4198* (176-890) in pBJS18	This study
pAK-5	*MXAN5030* (144-861) in pBJS18	This study
pAK-6	*MXAN6487* (201-937) in pBJS18	This study
pAK-7	*MXAN7238* (206-920) in pBJS18	This study
pAK-46	*MXAN3431*(342-1125) in pBJS18	This study
pAK-47	*MXAN3424* (161-970) in pBJS18	This study
Plasmids forgeneration of in-frame deletions		
pAK-8	*popCD* AD fragment in pBJ114	This study
pAK-9	*popC* AD fragment in pBJ114	This study
pAK-10	*MXAN4807-4813* AD fragment in pBJ114	This study
pAK-11	*MXAN4813* AD fragment in pBJ114	This study
pAK-12	*MXAN4800* AD fragment in pBJ114	This study
pAK-45	*MXAN4807-4808* AD fragment in pBJ114	This study
pAK-36	*MXAN5652-5643* AD fragment in pBJ114	This study
Plasmids for complementation		
pAK-25	Pnat *popD* in pSWU30	This study
pAK-26	P*pilA popCD* inpSW105	This study
pAK-23	Pnat *popCD*-strep tag in pSWU30	This study
Plasmids for protein overexpression		
pAK-31	*popD* in MCSII of pRSF-duet 1	This study
pAK-32	*popC* in MCSI of pRSF- duet 1	This study
pAK-35	*popC* in MCSI and *popD* in MCSII of pRSF-duet 1	This study
pAK-37	$popC^{S423A}$in MCSI of pRSF-duet 1	This study
pAK-38	$popC^{S423A}$ in MCSIand *popD* in MCSII of pRSF-duet 1	This study
pAK-39	prosegment *popC* in MCSI and popD in MCSII of pRSF-duet 1	This study
pAK-40	peptidase *popC* in MCSI and popD in	This study

	MCSII of pRSF-duet 1	
pAK-41	peptidase *popC* in MCSI of pRSF- duet 1	This study
pAK-42	prosegment *popC* in MCSI of pRSF- duet 1	This study
Cosmids for gene replacement		
cAK-1	KN-567-07_G07 *ΔpopC::aadA*	This study
cAK-2	KN-567-07_G07 *ΔpopCD::aadA*	This study
cAK-3	KN-567-07_G07 *ΔpopD::aadA*	This study
cAK-4	KN-567-07_G07 $popC^{S423A}$*ΔpopD::aadA*	This study
cAK-5	KN-567-09_F11 *ΔMXAN2434-2454::aadA*	This study
cAK-11	KN-567-05_B07 *ΔMXAN3903-3011::aadA*	This study
cAK-13	KN-567-07_A05 *ΔMXAN3105-3107::aadA*	This study
cAK-14	KN-567-08_D11 *ΔMXAN2515-2504::aadA*	This study

Transformation of chemocompetent *E. coli*

Appropriate volume of *E. coli* strain (1:100 diluted from overnight culture) was grown up to OD600 0.5~0.7. Cells were harvested (4,700 rpm, 10 min, 4°C), washed twice with half volume of ice cold 50 mM $CaCl_2$ and resuspended in 1/100 volume of ice cold 50 mM $CaCl_2$. 100 µl of competent cells was mixed 100 ng plasmid or 20 µl of ligation product at 4°C for 30 min, and then transferred to 42°C water bath for 2 min, incubated on the ice for 5 min. Cells were recovered for 1 hour in LB and subsequently plated on corresponding antibiotic resistant plates.

Transformation of electrocompetent *E. coli* cells

Appropriate volume of *E. coli* strain (1:100 diluted from overnight culture) was grown up to OD600 0.5~0.7. Cells were harvested (4,700 rpm, 10 min, 4°C), washed with twice with half volume of ice cold 10% (v/v) glycerol and resuspended in 1/100 volume of ice cold 10% (v/v) glycerol. 100 ng of the plasmid or 5µl purified DNA from a ligation reaction were electroporated electrocompetent *E. coli* cells (with 1.8 kV, 25 µF and 200 Ω) Cells were recovered for 1 hour in LB and subsequently plated on corresponding antibiotic resistant plates.

Electroporation of *M. xanthus*

The *M. xanthus* strain for electroporation was grown in the CTT medium to OD550 ~0.5. The cells were harvested (4,700 rpm, 10 min, RT), washed twice with equal volume of H_2O and once with ½ volume of H_2O, and resuspended in

1/100 volume of filtered H_2O. 50 µl competent cells were mixed with 100 ng plasmid (for homologous recombination), 300 ng of cosmid (for gene replacement) or 3 µg chromosomal DNA (for chromosomal transformation) in 0.1 cm ice cold cuvettes. The electroporation was conducted (with 0.65 kV, 25 µF, 400 Ω) and electroporated cells were recovered in CTT medium for 1 to 3 generations and cells were spread on corresponding selective plates overlaid with CTT softagar. The colonies grew up after 4-7 days.

Site-directed mutagenesis

QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used to introduce TCG→GCG (S423A) mutation into *popC* gene of pAK-32 and pAK-35 plasmids to generate pAK-37 and pAK-38 respectively.

RNA preparation from *M. xanthus*

Total RNA was isolated from cell pellets using the hot-phenol method (Overgaard et al., 2006). Briefly, approximately 5 x 10^9 *M. xanthus* cells (2 times more cells from later time points of development) were harvested to a tube containing 1/10 volume of ice-cold stop solution (5% saturated acid phenol (pH <6.0) in 96% ethanol) and spin down (4,700 rpm, 10 min, 4°C). The pellet was resuspended in 600 µl ice cold solution 1 (0.3 M sucrose, 0.01 M NaAc, pH 4.5) and each 300 µl was transferred into 1.5 ml tubes containing 300 µl hot (65°C) solution 2 (2% SDS, 0.01 M NaAc, pH 4.5). The RNA purification was conducted twice with equal volume hot phenol (saturated acid phenol (pH <6.0) at 65°C) extraction, once with acid phenol:chloroform (saturated acid phenol, pH 4.5 : chloroform, 5:1) extraction and once with equal volume of chloroform: isoamyl alcohol (24:1) extraction. RNA was precipitated with 1/10 volume of 3 M NaAc pH 4.5 and 2 volume of 96% ethanol for 20 min at -20°C. The RNA pellet was spin down in microcentrifuge with full speed at 4°C and washed twice with equal volume of ice cold 75% ethanol. The pellet was dried briefly at room temperature and resuspended in 50 µl RNase-free H_2O. The RNA was stored at -80°C.

RNA clean up, cDNA synthesis and qRT-PCR

The purified total RNA was treated with 20 U RNase-free DNase I (Ambion) for 60 min at 37°C. RNA was purified using the RNeasy Mini Kit (QIAGEN). The

absence of DNA was verified by PCR reaction of 32 cycles with Taq polymerase. The presence of PCR products were checked by agarose gel electrophoresis. The above steps were repeated if there was DNA contamination in the RNA sample. The RNA integrity was analyzed by 1% agarose gel electrophoresis. RNA was considered intact, if sharp and clear bands for 23S and 16S rRNA were observed on the gel. 1.0 µg of DNA-free intact total RNA was used as the template to synthesize cDNA with the cDNA Archive kit (ABI) following the recommended protocol.

The qRT-PCR reactions were carried out in a total volume of 25 µl containing 12.5 µl Sybr green PCR Master Mix (ABI), 1 µl of each primer (10 µM), 0.1 µl cDNA and 11.9 µl H_2O. AB 7300 Real time PCR detection system was used for qRT-PCR reactions with standard conditions.The optimal cDNA template dilution used in real-time PCR reactions was chosen which yielded a cycle threshold (Ct) value after 25-30 cycles. Each reaction was performed in triplicate, and the average Ct value from the vegetative WT cells was subtracted from the average Ct values of the tested samples in order to present the expression relative to the vegetative WT sample.

Generation of *M. xanthus* in frame deletion mutants

In-frame deletions of specific genomic regions were generated as previously described (Shi et al., 2008).In brief, approximately 600bp fragments directly up- and downstream of the target gene were amplified by PCR using primers designated as A, B, C and D. The primers A and D contain restriction enzymes for cloning into the plasmid pBJ114. The primers B and C were designed to possess compatible ends which allow fusing the 500bp fragments in a second PCR or having restriction sites for cloning. The fragments AB and CD were used to generate the full-length in-frame deletion fragment either by direct cloning or in a second PCR reaction with primers A and D and the two flanking PCR fragments as templates. Plasmids with error-free inserts were electroporated into *M. xanthus* and kanamycin resistant clones were isolated and checked for the up- or downstream insertions of the plasmid by the PCR. To obtain markerless in frame deletion, counter selection on 2% galactose was used to isolate Gal^RKan^S clones with excised plasmid. These colonies were

screened out and checked by two PCR reactions with the outside primers E and F and the inside primers G and H to verify the in-frame deletion.

Generation of *M. xanthus* insertion mutants

To generate insertion mutants in *M. xanthus*, a central part of the target gene (700bp) was amplified by PCR. The purified PCR product was cloned into the pBGS18 and selected for kanamycin resistance. Insertion mutants were verified by PCR using oligonucleotides specific for the pBGS18 plasmid and a neighboring region up- or downstream of the target gene.

Generation of *M. xanthus* gene replacement mutants

To establish a method for generating large deletions on the *M. xanthus* genome, we adopted a variant of the λ-Red recombineering system, which was developed for generating gene replacements in an ordered cosmid library of *Streptomyces coelicolo*r (Gust et al., 2003). Recombineering is based on bacteriophage λ Red-mediated recombination (Zhang *et al.*, 1998, Datsenko & Wanner, 2000, Murphy *et al.*, 2000). The λ Red recombination system allows the allelic gene replacement in *E. coli* by homologous recombination with a PCR generated selectable marker flanked at both ends with homologous regions of 35 to 50 bp. The system requires the Red-α (*exo*), Red-β (*bet*), and Red-γ (*gam*) proteins of λ to be present in the targeted strain. To adapt this method for *M. xanthus,* an *M. xanthus* cosmid library was generated (GATC, Konstanz, Germany) using the pWEB^{TM} Cosmid Cloning Kit (Epicentre, Madison, USA). A collection of 303 ordered cosmid clones was generated that covers more than 80% of the *M. xanthus* DK1622 genome. The cosmids carry resistance markers for kanamycin and ampicillin. The cosmid containing the target gene(s) is transferred to the *E. coli* recombineering strain BW25113 (Datsenko & Wanner, 2000) containing pIJ790, which contains the three λ Red genes and confers chloramphenicol resistance (Gust et al., 2003). The strain is transformed with a PCR amplified resistance cassette with 39 bp homology regions on both sides corresponding to the flanking regions of the target gene(s). We used the *aadA* gene of pIJ778 (Gust et al., 2003), which confers resistance to spectinomycin and streptomycin, as resistance marker for the gene replacements. Cells of BW25113 with the relevant cosmid were transformed with the appropriate PCR

product and plated on LB agar plates (Sambrook & Russell, 2001) containing kanamycin, ampicillin and streptomycin. Transformants were analyzed by PCR to verify the correct recombination event. Subsequently, the mutant cosmid was electroporated into *M. xanthus* with selection for streptomycin resistance. Clones with a double cross-over between the mutant cosmid and the chromosome were identified by their resistance to streptomycin and sensitivity to kanamycin. Selected clones were verified by PCR. Typically, 75% of streptomycin resistant clones were kanamycin sensitive. Among these clones, 75% typically had the correct replacement.

Random transposon mutagenesis

The transposon mini-Himar(Kan) on the plasmid pMiniHimar, which is a non-replicating plasmid in *M. xanthus* (X. Duan and H.B. Kaplan, personal communication), was introduced into the WT DK1622 by electroporation. Transformants were selectedon the basis of their resistance to kanamycin and individually transferred to a well in a 96-well microtiterdish containing 150 µl of CTT agar and 50 µg kanamycin/ml. After 5 days, cells were transferred to plates containing 150 µL CF agar or TPM agar and their development followed over the next five days. A total of 10,800transformants were isolated and screened for developmental defects. 115 transformants displayed developmental defects. Transposon insertion sites were identified using arbitrary PCR or alternatively transposon with its flanking regions was cloned in *E. coli* DH5α λpir and then sequenced.

In arbitrary PCR, DNA flanking insertion sites were enriched in two rounds of amplification using primers specific to the ends of the transposon element and primers to random sequence, which can anneal to chromosomal sequences flanking the transposon (Caetano-Anolles, 1993). In the first round, a primer unique to the right end of mini-Himar element (Ext1 or Ext2) and arbitrary primer 1 (ARB1) were used in Walking PCR reactions as described previously (Pilhofer *et al.*, 2007, Das *et al.*, 2005). The reactions for the second round of PCR were performed with Int1 or Int2 primer and Arb2 primer which is complementary to the 5' end of the ARB1 primer.

To subclone transposoninsertions, genomic DNA was isolated from vegetative cultures of insertions strains. 3 µg of genomic DNA was cleaved with *Bss*HII for 6 hours at 37°C and restriction enzyme was heat-inactivated at 85°C for 30 min. Digested DNAs was religated with rapid T4 DNA ligase in a total volume of 50 µl at 18°C overnight. DNA was purified from ligation mixture and used for electroporation of electro competent E. coli DH5α λpir cells.

PCR fragments or plasmid DNA were sequenced with primers Int1 and Int2 which complement the ends of the transposable element.

Biochemical methods

Recombinant protein expression in *E. coli*

A relevant construct was transformed into *E. coli* Rosetta 2 (DE3) and a positive colony was inoculated in LB medium with selective antibiotics overnight. Overnight culture was 50-fold diluted into LB with selective antibiotics and was grown till OD600 0.4-0.7 at 37°C. Expression was induced by adding of IPTG to the final concentration of 1mM. Then the culture was further cultivated at 18°C overnight.

To test target protein solubility, 100 ml of induced culture was harvested after induction. The cell pellet was resuspended in 5 ml of Native lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8). Samples were frozen in liquid nitrogen, thawed on ice. Cells were lysed by sonication (4x2 min, 1x 4 min). Lysates were centrifuged 10,000 g for 20 min and supernatant was saved as soluble fraction. The pellet was resuspended into equal volume of lysis buffer and saved as insoluble fraction. The different fractions were examined by SDS-PAGE.

Purification of His-tagged proteins under native conditions

Cell pellet from induced culture was resuspended in Native lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8). Cells were lysed by sonication (4x2 min, 1x 4 min). Lysates were clarified 10,000 g for 20 min and supernatant was mixed with 1-2 ml Ni-NTA slurry gently at 4°C for 30 min. The lysate-Ni-NTA mixture was load on Poly-Prep Chromatography columns (Bio-Rad). After the resin was settled down, the bottom cap was removed and the flow-through was collected. The column was washed with 20x column volume

with wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, pH 8). The protein was eluted with elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) containing different concentrations of imidazole: 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 500 mM. The different fractions were collected, the concentrations were measured (660 nm Protein assay kit, Pierce) and the purity was checked by SDS-PAGE.

Purification of His-tagged PopC from inclusion bodies (IB)

After induction, cells from 500-1,000 ml culture were harvested by centrifugation and pellet was resuspended in 1/10 volume of lysis buffer (100 mM Tris-HCl, 1 mM EDTA, pH 7.0). 1.5 mg of lysozyme/gram of cells was added and cells were lysed for 30 min at RT. Cells were subsequently disrupted by sonication (4x2 min, 1x 4 min). 3 mM of MgCl2 and 250 µg of DNase I was added and incubated for 30 min at RT. 0.5 volume of 60 mM EDTA, 6% Triton X-100, 1.5 M NaCl pH 7.0 was added to the solution and incubated for 30 min at 4 °C. IB were sedimented by centrifugation at 20,000 g (Sorvall SS-34) for 20 min at 4 °C. Pellet was washed in 40 ml of 100 mM Tris-HCl, 20 mM EDTA pH 7.0. IB pellet was stored frozen at -80°C until use.

50 mg of IB was resuspended in 5 ml of 6 M Guanidin-HCl, 100 mM Tris-HCl, 100 mM DTT, 1mM EDTA pH 8 for 2 hours at RT. pH was lowered to pH 3 by addition of 1 M HCl and insoluble debris were removed by centrifugation at 10,000 g for 20 min. DTT was removed by dialysis twice against 500 ml of 4 M Guanidine-HCl, 100 mM Tris-HCl, for 2 hours at RT. Solubilized protein was finally dialyzed against 1 liter of 4 M Guanidin-HCl at 4 °C, ON. Aliquots of protein solution were stored at -80 °C until use.

PopC was refolded by rapid dilution in refolding buffer (0.1 M Tris-HCl pH 8.5, 1 mM DTT, 0.5 M L-arginine-HCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$). RF buffer was exchanged if necessary with A50MC buffer (10 mM MOPS pH 7.2, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM NaCl) by ultrafiltration using a Microcon® Ultracel YM-10 filter (Millipore).

Purification of S-tagged proteins under native conditions

Cell pellet was resuspended in 10 ml of Lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and cells were lysed by sonication (4x2 min, 1x 4 min). Lysates

were clarified at 10,000 g for 20 min and supernatant was mixed with 1 ml S-protein agarose and incubated for 20 min. Column was washed with 10x of native lysis buffer and protein was eluted with Lysis buffer containing increased concentration of NaCl (0.5, 1 and 2M).

Purification of MalE-p25

After induction, cells from 100 ml culture were harvested by centrifugation and pellet was resuspended in Column buffer 1 (20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA) and cell were lysed by sonication (4x2 min, 1x 4 min). Lysates were clarified at 10,000 g for 20 min and supernatant was mixed with 3 ml amylose resin and incubated for 20 min. column was washed with 10x volume of Column buffer 1 and protein was eluted with 5x 0.5 ml of Column buffer 2 (20 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA, 10 mM maltose).

Isolation of secreted proteins

To isolate secreted proteins from vegetative cells, cells were grown in liquid CTT to a density of 5×10^8 cells/ml. Cells were harvested by centrifugation, and supernatants were filtered through a 0.22-µm sterile filter (Millipore). The cell-free supernatants were kept at 4 °C. To isolate secreted proteins from starving cells, cell suspensions at a density of 10^9 per ml were starved at 32°C with shaking. Cell-free supernatant was isolated as described above. Protein from 10^8 cells of each sample was analyzed by immunoblot or ELISA. To identify secreted proteins accumulating in the presence of protease inhibitors, cells were grown and starved as described except that protease inhibitors (Complete Mini, Roche) were added.

Effect of Chloramphenicol on PopC secretion

To test working concentration of chloramphenicol [^{35}S]-Met and [^{35}S]-Cys were used to monitor amino acids incorporation into proteins. For this purpose 26 µl of PRO-MIX L- [^{35}S]- in vitro Cell Labeling Mix (Amersham) was added to 15 ml of vegetative culture of *M. xanthus* in presence of 25 µg/ml of chloramphenicol. 0.1 ml of culture samples were taken at corresponding time points (10, 20, 40, 60 and 120 min) and proteins were precipitated with 10% TCA. Samples were filtered though CF/C glass filters and filters were washed twice with 1 ml of 3%

TCA and with 1 ml of 96% ethanol. Total radioactivity was measured using Beckman LS 6500 Scintillation Counter.

To test an effect of chloramphenicol on PopC secretion cells used for time course in presence of chloramphenicol were exposed to the chloramphenicol(25 µg/ml)for 10 min prior to starvation and during stravation. Secreted proteins were isolated as described above.

Enzyme-linked immunosorbent assay (ELISA)

To analyze PopC accumulation in the supernatant of vegetative and starving cells, supernatant sample of 10^8 cells was sorbed into a well of Nunc-Immuno Maxisorp (Nunc, Denmark) plates at 4 °C overnight. Wells were blocked with TTBS 2% nonfat dry milk (NFDM) for 1 hour at 37°C. Wells were incubated with α-PopC antibodies (1:100 in TTBS 2% NFDM) for 2 hours at RT. Wells were washed 7 times with TTBS and incubated with α-IgG-HPR conjugated (1:10,000 in TTBS 2% NFDM) for 1 hour at RT. Wells were washed 7 times with TTBS. Signal was developed in 100 µL of 1-STEP Ultra TMB-ELISA reagent (Pierce, Rockford, USA) and reaction was allowed to develop for 20 min. Reaction was stopped by adding 50 µL of 1M H_2SO_4 and signal was measured at dual wave length 450/620 nm using Infinite M200 plate reader (Tecan, Switzerland).

Cell fractionation

Cells were grown in liquid CTT. To separate inner membrane and outer membrane proteins from soluble proteins we used the method of (Lobedanz & Søgaard-Andersen, 2003). Briefly, cells were resuspended in 50 mM Tris-HCl (pH 7.6) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany) (protease inhibitors) as recommended by the supplier and lysed by sonication. Cell debris was removed by centrifugation. The supernatants were centrifuged at 45,000× g for 1 hr at 4°C. The resulting supernatants are enriched in soluble proteins. Pellets containing the crude envelope fractions were resuspended in 50 mM Tris-HCl (pH 7.6), 2% Triton X-100 supplemented with protease inhibitors, and subjected to ultracentrifugation as described. The resulting supernatant is enriched in inner membrane proteins, and the pellet is enriched in outer membrane proteins. As controls for proper fractionation, fractions were tested with antibodies against PilB in the cytoplasm

(Bulyha et al., 2009), PilC in the inner membrane (Bulyha et al., 2009), and PilQ in the outer membrane (Bulyha et al., 2009).

In vitro protease assay

Purified refolded PopC protein (6.0 µg) was assayed in A50MC buffer (10 mM MOPS pH 7.2, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 50 mM NaCl) in a reaction mixture (total volume 25 µl) containing MalE-p25 (final concentration 2.5 µM). Reactions were incubated for 24 h at 32 °C and analyzed by immuno-blotting.

SDS Polyacrylamide Gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) was performed to monitor heterologous protein expression and to separate proteins under denaturing conditions. To denature proteins, samples were combined with equal volumes of 2 x Laemmli sample buffer (LSB; 0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and heated at 96°C for 5 min prior to loading the gel. Electrophoresis was performed in Bio-Rad electrophoresis chambers (Bio-Rad, München) at 120 – 150 V in 1 x Trisglycine- SDS running buffer (25mM Tris, 190mM glycine, 0.1% (w/v) SDS). Proteins were visualized by staining in Coomassie brilliant blue R250.

Immunoblot analysis

Corresponding samples were separated on the SDS-PAGE and proteins were transferred to a PROTRAN nitrocellulose membrane (Waltman) with a semi-dry blotting apparatus (Amersham, Biosciences, Munich) with the constant current of 0.8 mA/cm^2. Immunoblot was performed using the standard protocol (Sambrook & Russell, 2001) or using Snap I.D. (Millipore) following manufacturer instructions. The blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Bioinformatics methods

All of the protein and gene sequences were retrieved from JCVI CMG database (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org=gmx) and from NCBI database (http://www.ncbi.nlm.nih.gov). Homology search was performed using BLAST algorithms (Altschul et al., 1990). Multiple sequence alignments were generated using ClustalX (Chenna et al., 2003). The domain analyses were

performed in SMART database (Letunic et al., 2009) and by Pfam database (Finn et al., 2010). Peptidases were recovered from MEROPS database (Rawlings et al., 2010).

Subcellular localization of proteins was predicted with CBS Prediction Servers: SignalP 3.0 (Signal peptide and cleavage sites)(Bendtsen et al., 2004), LipoP 1.0 (Signal peptidase I & II cleavage sites) (Juncker et al., 2003), TatP 1.0 (Twin-arginine signal peptides) (Bendtsen et al., 2005), TMHMM (Transmembrane helices in proteins) (Krogh et al., 2001).

6. Supplementary data

Table S1. ABC proteins in *M. xanthus*

Gene[1]	Primary annotation	Genetic organization[1,2]
0037	putative aliphatic sulfonates ABC transporter, ATP-binding protein	# # # # -0035-0036-0037 # # # #
0250	ABC transporter, ATP-binding protein	# # # # +0249+0250 # # # #
0554	ABC transporter, ATP-binding protein	# # # # -0553-0554 # # # #
0596	ABC transporter, ATP-binding protein	# # # # +0596+0597 # # # #
0629	ABC transporter, ATP-binding protein	# # # # +0629 # # # #
0684	ferric siderophore ABC transporter, ATP-binding protein	# # # #-0684 -0685-0686-0687 # # # #
0721	heme ABC transporter, ATP-binding protein	# # # # +0721+0722 # # # #
0748	ABC transporter, ATP-binding protein/permease protein	# # # # +0748 # # # #
0772	iron ABC transporter, ATP-binding protein	# # # # +0770+0771+0772 # # # #
0968	D-methionine ABC transporter, ATP-binding protein (metN)	# # # # -0966-0967-0968 # # # #
0995	ABC transporter, ATP-binding protein	# # # # +0995 # # # #
1060	ABC transporter, ATP-binding protein	# # # # +1060 # # # #
1097	efflux ABC transporter, permease/ATP-binding protein	# # # # -1097 # # # #
1124	ABC transporter, ATP-binding protein	# # # # +1124 # # # #
1154	FeS assembly ATPase SufC (sufC)	# # # # +1153+ 1154 # # # #
1286	ABC transporter, ATP-binding/permease protein (abcA)	# # # # +1286 # # # #
1321	hemin ABC transporter, ATP-binding protein	# # # # +1319+1320+1321 # # # #
1377	ABC transporter, ATP-binding protein	# # # # -1376-1377 # # # #
1547	ABC transporter, ATP-binding protein	# # # # +1547+1548 +1549 # # # #
1597	ABC transporter, ATP-binding protein	# # # # +1597+1598 # # # #
1789	ABC transporter, ATP-binding protein/peptidase, C39 family	# # # # +1788+ 1789+ 1790+1791 # # # #
1790	ABC transporter, ATP-binding protein	# # # # +1788+ 1789+ 1790+1791 # # # #
2249	proV glycine betaine/L-proline ABC transporter, ATP-binding protein	# # # # +2249 +2250+2251 # # # #
2430	ABC transporter, ATP-binding protein	# # # # -2428 -2429-2430 # # # #
2833	ABC transporter, ATP-binding protein	# # # # -2831-2832-2833# # # #
2853	putative lantibiotic ABC transporter, ATP-binding/permease protein	# # # # -2853 # # # #
2949	cation ABC transporter, ATP-binding protein	# # # # -2948-2949 -# -2951 # # # #
3208	ABC transporter, ATP-binding protein	# # # # -3208-3209 # # # #
3258	heme ABC exporter, ATP-binding protein CcmA (ccmA)	# # # # -3256 -3257-3258 # # # #
3718	ABC transporter, ATP-binding protein	# # # # +3717+3718+3719 # # # #
3908	ABC transporter, ATP-binding protein	# # # # -3903 -3904 -3905+3908+3909 +3910+3911+3912# # #
3911	ABC transporter, ATP-binding protein	# # # # -3903 -3904 -3905+3908+3909 +3910+3911+3912# # #
4074	ABC transporter, ATP-binding protein	# # # # +4074+4075 # # # #

4102	ABC transporter, ATP-binding protein	# # # # **+4102**+4104 # # # #
4174	ABC transporter, ATP-binding protein	# # # # -4172 -4173**-4174**-4175-4176# # # #
4199	putative macrolide efflux ABC transporter, ATP-binding protein	**# # # # +4199 # +4201 # # # #**
4622	o-antigen ABC exporter, ATP-binding protein (rfbB)	# # # # **-4622**-4623 # # # #
4664	oligopeptide/dipeptide ABC transporter, ATP-binding protein	# # # # **-4664 -4665** # # # #
4665	oligopeptide/dipeptide ABC transporter, ATP-binding protein	# # # # **-4664 -4665** # # # #
4716	putative ABC transporter, ATP-binding/permease protein	# # # #-4716 # # # #
4729	lipoprotein releasing system, ATP-binding protein LolD	# # # #**-4729**-4730# # # #
4750	ABC transporter, ATP-binding protein	# # # # -4749-**4750**# # # #
4791	phosphate ABC transporter, ATP-binding protein (pstB)	# # # #-4788-4789 -4790-**4791**# # # #
4818	sulfate ABC transporter, ATP-binding protein (cysA)	# # # #**-4818**-4819 -4820-4821 # # # #
4878	ABC transporter, ATP-binding protein	# # # # **+4878**+4879 # # # #
5167	efflux ABC transporter, permease/ATP-binding protein	# # # # **+ 5167 -5168** # # # #
5168	ABC transporter ATP-binding protein	# # # # **+ 5167 -5168** # # # #
5275	ABC transporter, ATP-binding/permease protein	# # # # **+ 5275 +5276** # # # #
5276	ABC transporter, ATP-binding/permease protein	# # # # **+ 5275 +5276** # # # #
5317	putative daunorubicin resistance ABC transporter, ATP-binding subunit	# # # # -5316-**5317** # # # #
5377	putative sugar ABC transporter, ATP-binding protein	# # # # +5377 +5378**+5379** # # # #
5502	putative amino acid ABC transporter, ATP-binding protein	# # # #**-5502** -5503 # # # #
5584	ABC transporter, ATP-binding protein	# # # #-5583-**5584** # # # #
5699	ABC transporter, ATP-binding protein	# # # # -5698-**5699 # # #** -5702# # # #
5712	ABC transporter, ATP-binding protein	# # # # +5711**+5712**+5713# # # #
5748	putative cell division ABC transporter, ATP-binding protein FtsE	# # # #-5747-**5748** # # # #
5781	efflux ABC transporter, ATP-binding protein PilH (pilH)	# # # #-5780-**5781** # # # #
6003	ABC transporter, ATP-binding protein	# # # # +6000-6001 + 6002**+6003** # # # #
6402	ABC transporter, ATP-binding protein	# # # # **+6402**+6403 # # # #
6475	ABC transporter, ATP-binding protein	**# # # #** -6474-**6475 # # # #**
6553	putative oligopeptide/dipeptide ABC transporter, ATP-binding/permease protein	# # # # +6551+6552+6553**+6554** # # # #
6554	oligopeptide/dipeptide ABC transporter, ATP-binding protein	# # # # +6551+6552+6553**+6554** # # # #
6569	ferrichrome ABC transporter, ATP-binding protein	# # # # -6568-**6569**# # # #
6643	molybdate ABC transporter, ATP-binding protein (modC)	# # # # **-6643**-6644 -6645 # # # #
6661	putative branched-chain amino acid ABC transporter, ATP-binding protein	# # # # **-6661 -6662**-6663 **-**6664-6665 # # # #

6662	branched chain amino acid ABC transporter, ATP-binding protein (livG)	# # # # **-6661 -6662**-6663 **-**6664-6665 # # # #
6765	ABC transporter, ATP-binding protein	**# # # # +6765**+6766 **# # # #**
6827	sodium ABC tranporter ATP-binding protein NatA (natA)	# # # #-6826-**6827**# # # #
6934	ABC transporter, permease/ATP-binding protein	# # # # -6943 # # # #
7144	ABC transporter, ATP-binding protein	# # # #**-7144 -7145**-7146 -7147 # # # #
7145	ABC transporter, ATP-binding protein	# # # #**-7144 -7145**-7146 -7147 # # # #
7225	putative sugar ABC transporter, ATP-binding protein	# # # #**-7225 +7226** # # # #
7226	ABC transporter, ATP-binding protein	# # # #**-7225 +7226** # # # #

[1]Genes are indicated by their MXAN numbers

[2] ABC proteins are highlighted in yellow. Transmembrane domain proteins, periplasmic binding proteins and TolC-homologues are indicated in blue, green and red respectively. Transcription using the lower or upper strand as template is indicated by + and -, respectively. # indicates a gene not encoding an ABC-transporters associated protein.

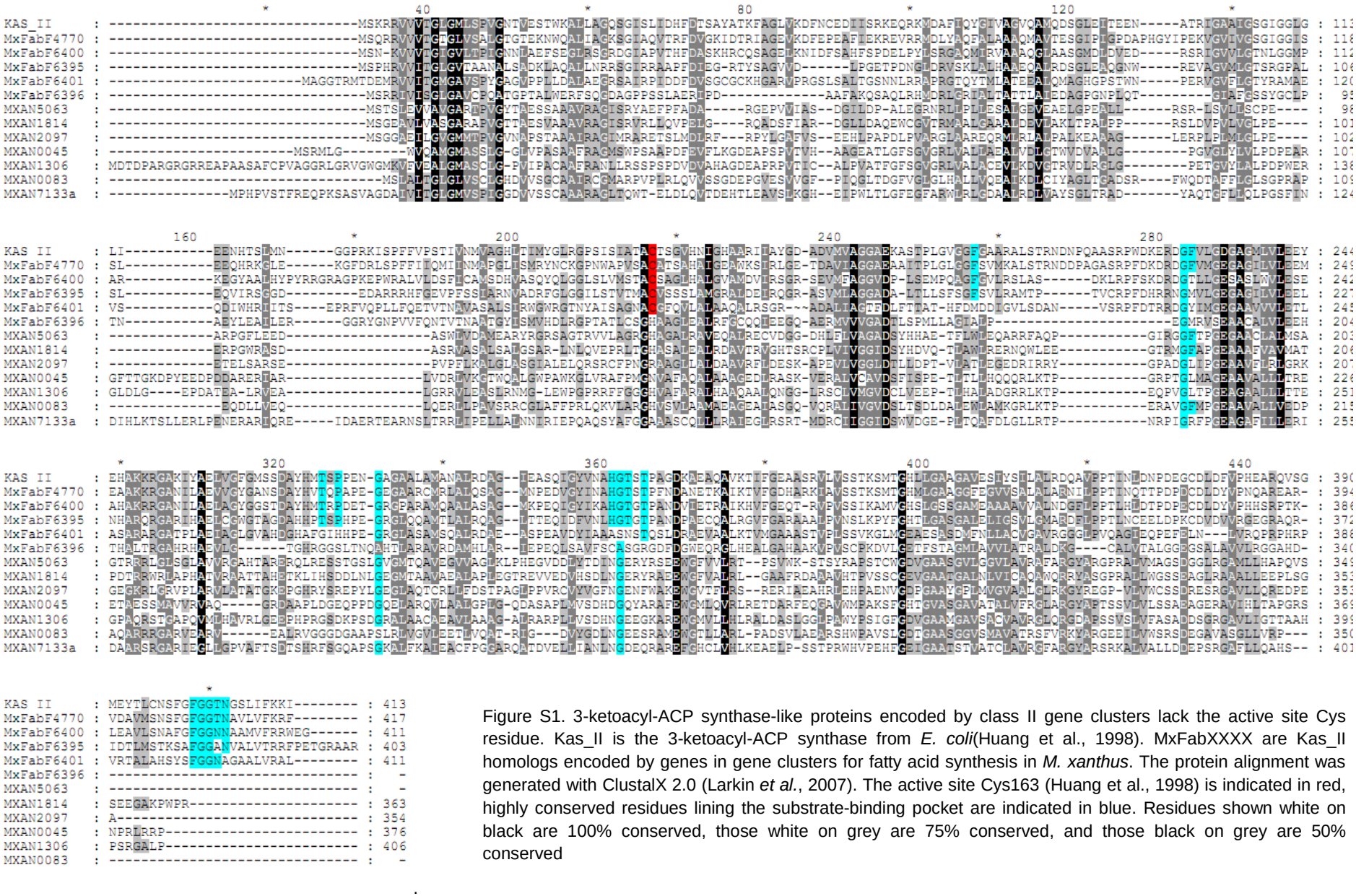

Figure S1. 3-ketoacyl-ACP synthase-like proteins encoded by class II gene clusters lack the active site Cys residue. Kas_II is the 3-ketoacyl-ACP synthase from *E. coli*(Huang et al., 1998). MxFabXXXX are Kas_II homologs encoded by genes in gene clusters for fatty acid synthesis in *M. xanthus*. The protein alignment was generated with ClustalX 2.0 (Larkin *et al.*, 2007). The active site Cys163 (Huang et al., 1998) is indicated in red, highly conserved residues lining the substrate-binding pocket are indicated in blue. Residues shown white on black are 100% conserved, those white on grey are 75% conserved, and those black on grey are 50% conserved

Table S2. Structure of class I gene clusters

Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN2167	+	2505290	2505775	-25	hypothetical protein
MXAN2168	+	2505965	2507503	190	IS21 family transposase
MXAN2169	+	2507687	2508448	184	IS21 family transposition helper protein
MXAN2170	+	2508594	2511761	146	fibronectin type III domain-containing protein
MXAN2171	-	2512352	2513320	591	hypothetical protein
MXAN2172	+	2513516	2513875	196	hypothetical protein
MXAN2173	+	2513872	2514408	-3	D,D-carboxypeptidase
MXAN2174	+	2514421	2514873	13	hypothetical protein
MXAN2175	+	2514870	2515172	-3	hypothetical protein
MXAN2175a	+	2515460	2516261	288	hypothetical protein
MXAN2176	+	2516257	2518050	-4	serine/threonine protein kinase
MXAN2177	+	2518119	2519336	69	putative serine/threonine protein kinase
MXAN2178	-	2519964	2520554	628	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN1225	+	1434602	1435363	2	hypothetical protein
MXAN1226	+	1435370	1438606	7	fibronectin type III domain-containing protein
MXAN1227	+	1440302	1440589	1696	hypothetical protein
MXAN1228	+	1440586	1441119	-3	D,D-carboxypeptidase
MXAN1229	+	1441132	1441584	13	hypothetical protein
MXAN1230	+	1441581	1441880	-3	hypothetical protein
MXAN1231	+	1442248	1443570	368	Rhs-like protein
MXAN1232	+	1444344	1445207	774	hypothetical protein
MXAN1233	+	1445204	1446994	-3	serine/threonine protein kinase
MXAN1234	+	1447087	1448301	93	serine/threonine kinase family protein
MXAN1235	-	1448433	1448711	132	Hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN3179	-	3726448	3727503	1110	hypothetical protein
MXAN3180	+	3726814	3728130	-689	hypothetical protein
MXAN3181	+	3728194	3729057	64	hypothetical protein
MXAN3182	+	3729054	3730844	-3	serine/threonine protein kinase
MXAN3183	+	3730998	3732152	154	serine/threonine protein kinase
Glu tRNA	-	3732282	3732354	130	
Lys tRNA	-	3732378	3732450	24	
MXAN3186	+	3732642	3733172	192	transcription elongation factor GreB
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN4840	-	6057594	6058502	7	pirin family protein

MXAN4841	-	6058815	6060029	313	putative serine/threonine protein kinase
MXAN4842	-	6060122	6061912	93	serine/threonine protein kinase
MXAN4844b	-	6061909	6062709	-3	hypothetical protein
MXAN4844a	-	6063588	6064157	879	hypothetical protein
MXAN4844	-	6064161	6065483	4	Rhs-like protein
MXAN4845	-	6065850	6066149	367	hypothetical protein
MXAN4846	-	6066146	6066598	-3	hypothetical protein
MXAN4847	-	6066611	6067243	13	D,D-carboxypeptidase
MXAN4847a	-	6067144	6067428	-100	hypothetical protein
MXAN4849	+	6068203	6069231	775	hypothetical protein
MXAN4851	-	6070548	6071156	1317	IS3 family transposase OrfA
MXAN4852	+	6071158	6072162	2	phage integrase family site specific recombinase
Leu tRNA	-	6072179	6072264	17	
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN0116	-	134929	136269	54	sigma-54 dependent trancsriptional regulator
MXAN0117	-	136328	139102	59	serine/threonine kinase family protein
MXAN0118	-	139252	139824	150	hypothetical protein
MXAN0119	-	139837	141180	13	Rhs-like protein
MXAN0120	+	141430	141840	250	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN0483	-	541949	543139	20	2-nitropropane dioxygenase family oxidoreductase
MXAN0484	-	543838	544407	699	hypothetical protein
MXAN0485	-	544427	545782	20	Rhs-like protein
MXAN0486	+	546168	546998	386	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN0846	+	962053	962373	28	hypothetical protein
MXAN0847	-	962441	963016	68	hypothetical protein
MXAN0848	-	963017	964363	1	Rhs-like protein
MXAN0849	+	964492	965679	129	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN1251	-	1464257	1464481	62	hypothetical protein
MXAN1254	-	1465231	1465800	750	hypothetical protein
MXAN1255	-	1465816	1467186	16	Rhs-like protein
MXAN1256	-	1467243	1468610	57	DNA repair protein RadA
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN7451	+	9075393	9076208	-3	WecB/TagA/CpsF family glycosyl transferase
MXAN7452	-	9076218	9076784	10	hypothetical protein

MXAN7453	-	9076794	9078278	10	Rhs-like protein
MXAN7454	+	9078380	9079018	102	ECF subfamily RNA polymerase sigma factor
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN4322	+	5319494	5320117	29	thiopurine S-methyltransferase
MXAN4323a	-	5321168	5321756	1051	Hypothetical protein
MXAN4323	-	5321762	5323072	6	Rhs-like protein
MXAN4325	+	5323258	5324187	186	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN6510	+	8031130	8031564	438	DGPF domain-containing protein
MXAN6511	+	8031636	8033039	72	Rhs-like protein
MXAN6512	+	8033049	8033597	10	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN6543	+	8058985	8059995	6	Ser/Thr protein phosphatase family protein
MXAN6544	+	8060180	8060902	185	hypothetical protein
MXAN6545	+	8061019	8062197	117	putative serine/threonine protein kinase
MXAN6546	+	8062240	8063304	43	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN1891	-	2215507	2216085	220	hypothetical protein
MXAN1892	+	2216216	2217388	131	putative serine/threonine protein kinase
MXAN1893	+	2217345	2217782	-43	hypothetical protein
MXAN1894	-	2217974	2218321	192	DNA-binding protein
MXAN1895	-	2218373	2218747	52	DNA-binding protein
MXAN1896	+	2218948	2220936	201	serine/threonine protein kinase
MXAN1897	+	2220966	2221922	30	hypothetical protein
MXAN1898	+	2222547	2224055	625	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN2398	+	2789206	2790369	-3	hypothetical protein
MXAN2399	+	2790555	2792618	186	serine/threonine-protein kinase
MXAN2400	+	2792669	2793583	51	hypothetical protein
MXAN2401	+	2793634	2794416	51	modification methylase
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN4370	-	5376058	5376834	-10	NAD dependent epimerase/dehydratase family protein
MXAN4371	+	5377079	5378248	245	serine/threonine kinase family protein
MXAN4372	-	5378264	5378602	16	DNA-binding protein
MXAN4373	+	5378801	5380642	199	serine/threonine protein kinase

MXAN4374	+	5380684	5381592	42	hypothetical protein
MXAN4375	+	5381747	5382196	155	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN4478	-	5545488	5547335	-3	hypothetical protein
MXAN4479	+	5547428	5548594	93	putative serine/threonine protein kinase
MXAN4480	-	5548617	5549099	23	DNA-binding protein
MXAN4481	-	5549100	5549402	1	DNA-binding protein
MXAN4482	+	5549551	5551503	149	putative serine/threonine protein kinase
MXAN4483	+	5551513	5552442	10	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN7255	-	8856437	8856709	361	hypothetical protein
MXAN7256	+	8856958	8858289	249	Rhs-like protein
MXAN7257	+	8858309	8858920	20	pentapeptide repeat-containing protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN7267	-	8871179	8872648	135	hypothetical protein
MXAN7268	+	8872973	8873896	325	hypothetical protein
MXAN7269	+	8873925	8875784	29	putative serine/threonine protein kinase
MXAN7270	-	8875797	8876987	13	hypothetical protein

Table S3. Structure of class II and III gene clusters.

Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN0042	-	49156	51831	17	hypothetical protein
MXAN0043	-	51872	53212	41	hypothetical protein
MXAN0044	-	53209	53673	-3	hypothetical protein
MXAN0045	+	53785	54915	112	3-ketoacyl-ACP-synthase-like protein
MXAN0046	-	54872	55510	-43	hypothetical protein
MXAN0047	-	55489	56160	-21	hypothetical protein
MXAN0048	-	56126	56482	-34	hypothetical protein
MXAN0049	-	56489	57073	7	hypothetical protein
MXAN0050	-	57099	57983	26	hypothetical protein
MXAN0051	+	58046	58831	63	hypothetical protein
MXAN0052	-	58837	59880	6	hypothetical protein
MXAN0053	+	59886	60509	6	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN0082	+	92915	94909	15	acetyl-CoA carboxylase, biotin carboxylase
MXAN0083	+	95005	96057	96	3-ketoacyl-ACP-synthase-like protein
MXAN0084	+	96070	96987	13	hypothetical protein
MXAN0085	+	96996	97583	9	hypothetical protein
MXAN0086	+	97598	98335	15	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN1811	+	2140770	2142578	116	HEAT repeat-containing PBS lyase
MXAN1812	-	2143618	2144346	1040	hypothetical protein
MXAN1813	-	2144359	2145612	13	hypothetical protein
MXAN1814	-	2145600	2146691	-12	3-ketoacyl-ACP-synthase-like protein
MXAN1815	-	2146693	2147697	2	hypothetical protein
MXAN1816	-	2147726	2149021	29	hypothetical protein
MXAN1817	+	2149251	2149844	230	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN2092	-	2425156	2426520	0	M16 family peptidase
MXAN2094	+	2427372	2427905	852	hypothetical protein
MXAN2095	+	2427917	2429197	12	hypothetical protein
MXAN2096	+	2429216	2430241	19	hypothetical protein
MXAN2097	+	2430243	2431307	2	3-ketoacyl-ACP-synthase-like protein
MXAN2098	+	2431311	2432219	4	hypothetical protein
MXAN2099	+	2432337	2432801	118	hypothetical protein
MXAN2100	+	2432806	2433192	5	hypothetical protein

MXAN2101	-	2433450	2433800	258	IS66 family transposase
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN1302	+	1528290	1529291	938	hypothetical protein
MXAN1303	+	1529365	1529829	74	hypothetical protein
MXAN1304	+	1529849	1530385	20	hypothetical protein
MXAN1305	+	1530403	1531725	18	hypothetical protein
MXAN1306	+	1531620	1532840	-105	3-ketoacyl-ACP-synthase-like protein
MXAN1307	+	1532837	1533703	-3	hypothetical protein
MXAN1307a	+	1533684	1534296	-19	hypothetical protein
MXAN1308	+	1534306	1534893	10	hypothetical protein
MXAN1309	+	1534910	1535692	17	hypothetical protein
MXAN1310	-	1535738	1536523	46	putative lipoprotein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN5058	+	6333433	6334482	37	ribonucleoside-diphosphate reductase, beta subunit
MXAN5059	-	6334942	6335373	460	hypothetical protein
MXAN5061	-	6335798	6336979	425	hypothetical protein
MXAN5062	-	6337175	6337978	196	hypothetical protein
MXAN5062a	-	6337957	6338713	-21	hypothetical protein
MXAN5062b	-	6339149	6339901	436	hypothetical protein
MXAN5063	-	6339850	6340899	-49	3-ketoacyl-ACP-synthase-like protein
MXAN5064	-	6340896	6341906	-3	hypothetical protein
MXAN5065	-	6341913	6343220	7	hypothetical protein
MXAN5066	-	6343593	6343853	373	hypothetical protein
MXAN5067	+	6343884	6344531	31	hypothetical protein
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product
MXAN7128	+	8704254	8704880	176	hypothetical protein
MXAN7129	-	8704893	8705663	13	hypothetical protein
MXAN7131	-	8705689	8706309	26	hypothetical protein
MXAN7132	+	8706371	8707441	62	hypothetical protein
MXAN7133	+	8707516	8708022	75	hypothetical protein
MXAN7133a	+	8708198	8709400	176	3-ketoacyl-ACP-synthase-like protein
MXAN7134	+	8709446	8710222	46	hypothetical protein
MXAN7134a	+	8710222	8710819	0	hypothetical protein
MXAN7135	+	8710927	8711526	108	hypothetical protein
MXAN7136	+	8711541	8712368	15	hypothetical protein
MXAN7137	+	8712453	8713787	85	hypothetical protein
MXAN7138	-	8714144	8714668	357	competence/damage inducible protein CinA
Locus	Strand	Start/Stop	Start/Stop	Intergenic distance	Product

MXAN1436	-	1693660	1694823	41	alpha/beta fold family hydrolase
MXAN1437	+	1694920	1695783	97	CDP-diacylglycerol--serine O-phosphatidyltransferase
MXAN1438	+	1695895	1697160	112	putative competence/damage-inducible protein CinA
MXAN1439	+	1697157	1698689	-3	hypothetical protein
MXAN1440	+	1698735	1699256	46	hypothetical protein
MXAN1441	-	1699253	1700281	-3	RecA protein

7. References

Akeda, Y. & J. E. Galan, (2005) Chaperone release and unfolding of substrates in type III secretion. *Nature***437**: 911-915.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers & D. J. Lipman, (1990) Basic local alignment search tool. *J Mol Biol***215**: 403-410.

Alvarez-Martinez, C. E. & P. J. Christie, (2009) Biological diversity of prokaryotic type IV secretion systems. *Microbiol Mol Biol Rev***73**: 775-808.

Andersen, C., C. Hughes & V. Koronakis, (2001) Protein export and drug efflux through bacterial channel-tunnels *Curr. Opin. Cell Biol.***4**: 412-416.

Artsimovitch, I., V. Patlan, S. Sekine, M. N. Vassylyeva, T. Hosaka, K. Ochi, S. Yokoyama & D. G. Vassylyev, (2004) Structural basis for transcription regulation by alarmone ppGpp. *Cell***117**: 299-310.

Barker, J. R., A. Chong, T. D. Wehrly, J. J. Yu, S. A. Rodriguez, J. Liu, J. Celli, B. P. Arulanandam & K. E. Klose, (2009) The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. *Mol. Microbiol.***74**: 1459-1470.

Bendtsen, J. D., H. Nielsen, G. von Heijne & S. Brunak, (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol***340**: 783-795.

Bendtsen, J. D., H. Nielsen, D. Widdick, T. Palmer & S. Brunak, (2005) Prediction of twin-arginine signal peptides. *BMC Bioinformatics***6**: 167.

Berks, B. C., T. Palmer & F. Sargent, (2003) The Tat protein translocation pathway and its role in microbial physiology. *Adv Microb Physiol***47**: 187-254.

Berleman, J. E., T. Chumley, P. Cheung & J. R. Kirby, (2006) Rippling is a predatory behavior in *Myxococcus xanthus*. *J. Bacteriol.***188**: 5888-5895.

Berleman, J. E. & J. R. Kirby, (2007a) Multicellular development in *Myxococcus xanthus* is stimulated by predator-prey interactions. *J. Bacteriol.***189**: 5675-5682.

Berleman, J. E. & J. R. Kirby, (2007b) Multicellular development in Myxococcus xanthus is stimulated by predator-prey interactions. *J Bacteriol***189**: 5675-5682.

Bingle, L. E., C. M. Bailey & M. J. Pallen, (2008) Type VI secretion: a beginner's guide. *Curr. Opin. Microbiol.***11**: 3-8.

Bönemann, G., A. Pietrosiuk, A. Diemand, H. Zentgraf & A. Mogk, (2009) Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. *Embo J***28**: 315-325.

Bönemann, G., A. Pietrosiuk & A. Mogk, (2010) Tubules and donuts: a type VI secretion story. *Mol Microbiol***76**: 815-821.

Bowden, M. G. & H. B. Kaplan, (1998) The *Myxococcus xanthus* lipopolysaccharide O-antigen is required for social motility and multicellular development. *Mol. Microbiol.***30**: 275-284.

Boyer, F., G. Fichant, J. Berthod, Y. Vandenbrouck & I. Attree, (2009) Dissecting the bacterial type VI secretion system by a genome wide in

silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics***10**: 104.

Brutinel, E. D. & T. L. Yahr, (2008) Control of gene expression by type III secretory activity. *Curr Opin Microbiol***11**: 128-133.

Bulyha, I., C. Schmidt, P. Lenz, V. Jakovljevic, A. Höne, B. Maier, M. Hoppert & L. Søgaard-Andersen, (2009) Regulation of the type IV pili molecular machine by dynamic localization of two motor proteins. *Mol. Microbiol.* **74**: 691–706.

Caetano-Anolles, G., (1993) Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Appl***3**: 85-94.

Cascales, E., (2008) The type VI secretion toolkit. *EMBO Rep.***9**: 735-741.

Cascales, E. & P. J. Christie, (2003) The versatile bacterial type IV secretion systems. *Nat Rev Microbiol***1**: 137-149.

Cashel, M., D. R. Gentry, V. J. Hernandez & D. Vinella, (1996) The stringent response. In: *Escherichia coli* and *Salmonella*: cellular and molecular biology. F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter & H. E. Umbarger (eds). Washington, DC: ASM Press, pp. 1458–1496.

Chandran, V., R. Fronzes, S. Duquerroy, N. Cronin, J. Navaza & G. Waksman, (2009) Structure of the outer membrane complex of a type IV secretion system. *Nature***462**: 1011-1015.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins & J. D. Thompson, (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res***31**: 3497-3500.

Cho, K. & D. R. Zusman, (1999) AsgD, a new two-component regulator required for A-signalling and nutrient sensing during early development of *Myxococcus xanthus*. *Mol. Microbiol.***34**: 268-281.

Clausen, M., V. Jakovljevic, L. Søgaard-Andersen & B. Maier, (2009) High force generation is a conserved property of type IV pilus systems. *J. Bacteriol.*: In press.

Cornelis, G. R., (2006) The type III secretion injectisome. *Nat Rev Micro***4**: 811-825.

Cunningham, K. & W. Wickner, (1989) Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of Escherichia coli. *Proc Natl Acad Sci U S A***86**: 8630-8634.

Das, S., J. C. Noe, S. Paik & T. Kitten, (2005) An improved arbitrary primed PCR method for rapid characterization of transposon insertion sites. *J Microbiol Methods***63**: 89-94.

Datsenko, K. A. & B. L. Wanner, (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA***97**: 6640-6645.

Davis, J. M., J. Mayor & L. Plamann, (1995) A missense mutation in *rpoD* results in an A-signalling defect in *Myxococcus xanthus*. *J. Bacteriol.***18**: 943-952.

Deane, J. E., P. Abrusci, S. Johnson & S. M. Lea, (2010) Timing is everything: the regulation of type III secretion. *Cell Mol Life Sci***67**: 1065-1075.

Desvaux, M., N. J. Parham & I. R. Henderson, (2004) Type V protein secretion: simplicity gone awry? *Curr Issues Mol Biol***6**: 111-124.

Diodati, M. E., R. E. Gill, L. Plamann & H. B. Kaplan, (2008) Initiation and early developmental events. In: Myxobacteria: Multicellularity and differentiation. D. E. Whitworth (ed). Washington, D.C.: ASM Press, pp. 43-76.

Downard, J., S. V. Ramaswamy & K. S. Kil, (1993) Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. *J. Bacteriol.***175**: 7762-7770.

Driessen, A. J., (1992) Precursor protein translocation by the Escherichia coli translocase is directed by the protonmotive force. *Embo J***11**: 847-853.

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

Dunmire, V., L. D. Tatar & L. Plamann, (1999) Genetic suppression analysis of an asgA missense mutation in Myxococcus xanthus. *Microbiology***145 (Pt 6)**: 1299-1306.

Dworkin, M., (1996) Recent advances in the social and developmental biology of the Myxobacteria. *Microbiol. Rev.***60**: 70-102.

Economou, A., P. J. Christie, R. C. Fernandez, T. Palmer, G. V. Plano & A. P. Pugsley, (2006) Secretion by numbers: Protein traffic in prokaryotes. *Mol. Microbiol.***62**: 308-319.

Ellehauge, E., M. Norregaard-Madsen & L. Sogaard-Andersen, (1998) The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in Myxococcus xanthus development. *Mol Microbiol***30**: 807-817.

Emanuelsson, O., S. Brunak, G. von Heijne & H. Nielsen, (2007) Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc***2**: 953-971.

Feldman, M. F. & G. R. Cornelis, (2003) The multitalented type III chaperones: all you can do with 15 kDa. *FEMS MICROBIOL LETT***219**: 151-158.

Filloux, A., (2004) The underlying mechanisms of type II protein secretion. *BBA***1694**: 163-179.

Filloux, A., A. Hachani & S. Bleves, (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology***154**: 1570-1583.

Finn, R. D., J. Mistry, J. Tate, P. Coggill, A. Heger, J. E. Pollington, O. L. Gavin, P. Gunasekaran, G. Ceric, K. Forslund, L. Holm, E. L. Sonnhammer, S. R. Eddy & A. Bateman, (2010) The Pfam protein families database. *Nucleic Acids Res***38**: D211-222.

Fronzes, R., E. Schafer, L. Wang, H. R. Saibil, E. V. Orlova & G. Waksman, (2009) Structure of a type IV secretion system core complex. *Science***323**: 266-268.

Galan, J. E. & H. Wolf-Watz, (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature***444**: 567-573.

Garza, A. G., B. Z. Harris, B. M. Greenberg & M. Singer, (2000a) Control of *asgE* expression during growth and development of *Myxococcus xanthus*. *J. Bacteriol.***182**: 6622-6629.

Garza, A. G., B. Z. Harris, J. S. Pollack & M. Singer, (2000b) The asgE locus is required for cell-cell signalling during *Myxococcus xanthus* development. *Mol Microbiol***35**: 812-824.

Ghosh, A. S., C. Chowdhury & D. E. Nelson, (2008) Physiological functions of D-alanine carboxypeptidases in *Escherichia coli*. *Trends Microbiol.***16**: 309-317.

Gill, R. E., M. Karlok & D. Benton, (1993) *Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling. *J. Bacteriol.***175**: 4538-4544.

Goldberg, A. L. & A. C. St John, (1976) Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annu Rev Biochem***45**: 747-803.

Goldman, B. S., W. C. Nierman, D. Kaiser, S. C. Slater, A. S. Durkin, J. A. Eisen, C. M. Ronning, W. B. Barbazuk, M. Blanchard, C. Field, C. Halling, G. Hinkle, O. Iartchuk, H. S. Kim, C. Mackenzie, R. Madupu, N. Miller, A. Shvartsbeyn, S. A. Sullivan, M. Vaudin, R. Wiegand & H. B. Kaplan, (2006) Evolution of sensory complexity recorded in a myxobacterial genome. *Proc. Natl. Acad Sci. USA***103**: 15200-15205.

Gomis-Ruth, F. X. & M. Coll, (2006) Cut and move: protein machinery for DNA processing in bacterial conjugation. *Curr Opin Struct Biol***16**: 744-752.

Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Qurollo, B. S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo & S. Slater, (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science***294**: 2323-2328.

Gottschalk, G., (1986) *Bacterial metabolism.* Springer Verlag, New York.

Guo, D., M. G. Bowden, R. Pershad & H. B. Kaplan, (1996) The Myxococcus xanthus rfbABC operon encodes an ATP-binding cassette transporter homolog required for O-antigen biosynthesis and multicellular development. *J Bacteriol***178**: 1631-1639.

Guo, D., Y. Wu & H. B. Kaplan, (2000) Identification and characterization of genes required for early Myxococcus xanthus developmental gene expression. *J Bacteriol***182**: 4564-4571.

Gust, B., G. L. Challis, K. Fowler, T. Kieser & K. F. Chater, (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. USA***100**: 1541-1546.

Hagen, D. C., A. P. Bretscher & D. Kaiser, (1978) Synergism between morphogenetic mutants of *Myxococcus xanthus*. *Dev. Biol.***64**: 284-296.

Hager, A. J., D. L. Bolton, M. R. Pelletier, M. J. Brittnacher, L. A. Gallagher, R. Kaul, S. J. Skerrett, S. I. Miller & T. Guina, (2006) Type IV pili-mediated secretion modulates *Francisella* virulence. *Mol. Microbiol.***62**: 227-237.

Hager, E., H. Tse & R. E. Gill, (2001) Identification and characterization of *spdR* mutations that bypass the BsgA protease-dependent regulation of developmental gene expression in *Myxococcus xanthus*. *Mol. Microbiol.***39**: 765-780.

Harris, B. Z., D. Kaiser & M. Singer, (1998) The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. *Genes & Dev.***12**: 1022-1035.

Henrichsen, J., (1972) Bacterial surface translocation: a survey and a classification. *Bacteriol. Reviews***36**: 478-503.

Hill, C. W., (1999) Large genomic sequence repetitions in bacteria: lessons from rRNA operons and Rhs elements. *Res. Microbiol.***150**: 665-674.

Hillesland, K. L., R. E. Lenski & G. J. Velicer, (2007) Ecological variables affecting predatory success in Myxococcus xanthus. *Microb Ecol***53**: 571-578.

Hodgkin, J. & D. Kaiser, (1977) Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*. *Proc. Natl. Acad. Sci. USA***74**: 2938-2942.

Hood, R. D., P. Singh, F. Hsu, T. Guvener, M. A. Carl, R. R. Trinidad, J. M. Silverman, B. B. Ohlson, K. G. Hicks, R. L. Plemel, M. Li, S. Schwarz, W. Y. Wang, A. J. Merz, D. R. Goodlett & J. D. Mougous, (2010) A type VI secretion system of Pseudomonas aeruginosa targets a toxin to bacteria. *Cell Host Microbe***7**: 25-37.

Huang, W., J. Jia, P. Edwards, K. Dehesh, G. Schneider & Y. Lindqvist, (1998) Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E.coli* reveals the molecular architecture of condensing enzymes. *EMBO J.***17**: 1183-1191.

Inouye, S., H. Nariya & J. Munoz-Dorado, (2008) Protein Ser/Thr kinases and phosphatases in *Myxococcus xanthus*. In: Myxobacteria: Multicellularity and differentiation. D. E. Whitworth (ed). Washington, D.C.: ASM Press, pp. 191-210.

Ireton, K. & A. D. Grossman, (1994) DNA-related conditions controlling the initiation of sporulation in *Bacillus subtilis*. *Cell. Mol. Biol. Res.***40**: 193-198.

Jackson, A., G. Thomas, J. Parkhill & N. Thomson, (2009) Evolutionary diversification of an ancient gene family (*rhs*) through C-terminal displacement. *BMC Genomics***10**: 584.

Jacob-Dubuisson, F., C. Locht & R. Antoine, (2001) Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Mol Microbiol***40**: 306-313.

Jakovljevic, V., S. Leonardy, M. Hoppert & L. Søgaard-Andersen, (2008) PilB and PilT are ATPases acting antagonistically in type IV pili function in *Myxococcus xanthus*. *J. Bacteriol.***190**: 2411-2421.

Jani, A. J. & P. A. Cotter, (2010) Type VI secretion: not just for pathogenesis anymore. *Cell Host Microbe***8**: 2-6.

Jeong, H., J. H. Yim, C. Lee, S.-H. Choi, Y. K. Park, S. H. Yoon, C.-G. Hur, H.-Y. Kang, D. Kim, H. H. Lee, K. H. Park, S.-H. Park, H.-S. Park, H. K. Lee, T. K. Oh & J. F. Kim, (2005) Genomic blueprint of *Hahella chejuensis*, a marine microbe producing an algicidal agent. *Nucl. Acids Res.***33**: 7066–7073.

Julien, B., A. D. Kaiser & A. Garza, (2000) Spatial control of cell differentiation in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA***97**: 9098-9103.

Juncker, A. S., H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen & A. Krogh, (2003) Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci***12**: 1652-1662.

Kahnt, J., K. Aguiluz, J. Koch, A. Treuner-Lange, A. Konovalova, S. Huntley, M. Hoppert, L. Søgaard-Andersen & R. Hedderich, (2010) Profiling the outer membrane proteome during growth and development of the social bacterium *Myxococcus xanthus* by selective biotinylation and analyses of outer membrane vesicles. *J. Proteome Res.* **9**: 5197-5208.

Kaimer, C. & P. Graumann, (2010) *Bacillus subtilis* CinA is a stationary phase–induced protein that localizes to the nucleoid and plays a minor role in competent cells. *Arch. Microbiol.***192**: 549-557.

Kaiser, D., (1979) Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA***76**: 5952-5956.

Kaplan, H. B., A. Kuspa & D. Kaiser, (1991) Suppressors that permit A-signal-independent developmental gene expression in *Myxococcus xanthus*. *J. Bacteriol.***173**: 1460-1470.

Kaplan, H. B. & L. Plamann, (1996) A *Myxococcus xanthus* cell density-sensing system required for multicellular development. *FEMS Microbiol. Letters***139**: 89-95.

Kearns, D. B., P. J. Bonner, D. R. Smith & L. J. Shimkets, (2002) An extracellular matrix-associated zinc metalloprotease is required for dilauroyl phosphatidylethanolamine chemotactic excitation in Myxococcus xanthus. *J Bacteriol***184**: 1678-1684.

Kim, S. K. & D. Kaiser, (1990a) C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell***61**: 19-26.

Kim, S. K. & D. Kaiser, (1990b) Cell alignment required in differentiation of *Myxococcus xanthus*. *Science***249**: 926-928.

Kim, S. K. & D. Kaiser, (1990c) Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. *Genes & Dev.***4**: 896-904.

Kim, S. K. & D. Kaiser, (1990d) Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. *Proc. Natl. Acad. Sci. USA***87**: 3635-3639.

Kim, S. K. & D. Kaiser, (1991) C-factor has distinct aggregation and sporulation thresholds during *Myxococcus* development. *J. Bacteriol.***173**: 1722-1728.

Kimura, Y., H. Saiga, H. Hamanaka & H. Matoba, (2006) *Myxococcus xanthus* twin-arginine translocation system is important for growth and development. *Arch. Microbiol.***184**: 387-396.

Konovalova, A., T. Petters & L. Sogaard-Andersen, (2010a) Extracellular biology of Myxococcus xanthus. *FEMS Microbiol Rev***34**: 89-106.

Konovalova, A., S. Wegener-Feldbrügge, S. Lindow, N. Hamann & L. Søgaard-Andersen, (2010b) Proteins of unknown function are required for regulated secretion of the PopC protease in *Myxococcus xanthus*. *In preparation.*

Koraimann, G., (2003) Lytic transglycosylases in macromolecular transport systems of Gram-negative bacteria. *Cell. Mol. Life Sci.***60**: 2371-2388.

Koronakis, V., A. Sharff, E. Koronakis, B. Luisi & C. Hughes, (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature***405**: 914-919.

Krall, L., U. Wiedemann, G. Unsin, S. Weiss, N. Domke & C. Baron, (2002) Detergent extraction identifies different VirB protein subassemblies of the type IV secretion machinery in the membranes of Agrobacterium tumefaciens. *Proc Natl Acad Sci U S A***99**: 11405-11410.

Krogh, A., B. Larsson, G. von Heijne & E. L. Sonnhammer, (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol***305**: 567-580.

Kroos, L., P. Hartzell, K. Stephens & D. Kaiser, (1988) A link between cell movement and gene expression argues that motility is required for cell-cell signaling during fruiting body development. *Genes Dev.***2**: 1677-1685.

Kroos, L. & D. Kaiser, (1987a) Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. *Genes & Dev.***1**: 840-854.

Kroos, L. & D. Kaiser, (1987b) Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. *Genes Dev.***1**: 840-854.

Kroos, L., A. Kuspa & D. Kaiser, (1986) A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev. Biol.***117**: 252-266.

Kruse, T., S. Lobedanz, N. M. S. Berthelsen & L. Søgaard-Andersen, (2001) C-signal: A cell surface-associated morphogen that induces and coordinates multicellular fruiting body morphogenesis and sporulation in *M. xanthus*. *Mol. Microbiol.***40**: 156-168.

Kuner, J. M. & D. Kaiser, (1982) Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. *J. Bacteriol.***151**: 458-461.

Kuroda, A., (2006) A polyphosphate-lon protease complex in the adaptation of Escherichia coli to amino acid starvation. *Biosci Biotechnol Biochem***70**: 325-331.

Kuroda, A., K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, H. Ohtake & A. Kornberg, (2001) Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in E. coli. *Science***293**: 705-708.

Kuspa, A. & D. Kaiser, (1989) Genes required for developmental signalling in *Myxococcus xanthus*: three *asg* loci. *J. Bacteriol.***171**: 2762-2772.

Kuspa, A., L. Kroos & D. Kaiser, (1986) Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. *Dev. Biol.***117**: 267-276.

Kuspa, A., L. Plamann & D. Kaiser, (1992a) Identification of heat-stable A-factor from *Myxococcus xanthus*. *J. Bacteriol.***174**: 3319-3326.

Kuspa, A., L. Plamann & D. Kaiser, (1992b) A-signalling and the cell density requirement for *Myxococcus xanthus* development. *J. Bacteriol.***174**: 7360-7369.

Laemmli, U. K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature***227**: 680-685.

Langille, M. G. I., W. W. L. Hsiao & F. S. L. Brinkman, (2010) Detecting genomic islands using bioinformatics approaches. *Nat. Rev. Micro.***8**: 373-382.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson & D. G. Higgins, (2007) Clustal W and Clustal X version 2.0 *Bioinformatics***23**: 2947-2948.

Letunic, I., T. Doerks & P. Bork, (2009) SMART 6: recent updates and new developments. *Nucleic Acids Res***37**: D229-232.

Li, S., B.-U. Lee & L. J. Shimkets, (1992) *csgA* expression entrains *Myxococcus xanthus* development. *Genes Dev.***6**: 401-410.

Lobedanz, S. & L. Søgaard-Andersen, (2003) Identification of the C-signal, a contact dependent morphogen coordinating multiple developmental responses in *Myxococcus xanthus*. *Genes Dev.***17**: 2151-2161.

Lueders, T., R. Kindler, A. Miltner, M. W. Friedrich & M. Kaestner, (2006) Identification of bacterial micropredators distinctively active in a soil microbial food web. *Appl Environ Microbiol***72**: 5342-5348.

Masi, M. & C. Wandersman, (2010) Multiple signals direct the assembly and function of a type 1 secretion system. *J. Bacteriol.***192**: 3861-3869.

Masure, H. R., B. J. Pearce, H. Shio & B. Spellerberg, (1998) Membrane targeting of RecA during genetic transformation. *Mol Microbiol***27**: 845-852.

Maurizi, M. R. & F. Rasulova, (2002) Degradation of L-glutamate dehydrogenase from Escherichia coli: allosteric regulation of enzyme stability. *Arch Biochem Biophys***397**: 206-216.

McDermott, J. E., A. Corrigan, E. Peterson, C. Oehmen, G. Niemann, E. D. Cambronne, D. Sharp, J. N. Adkins, R. Samudrala & F. Heffron, (2010) Computational prediction of type III and IV secreted effectors in Gram-negative bacteria. *Infect Immun*.

McNulty, C., J. Thompson, B. Barrett, L. Lord, C. Andersen & I. S. Roberts, (2006) The cell surface expression of group 2 capsular polysaccharides in *Escherichia coli*: the role of KpsD, RhsA and a multi-protein complex at the pole of the cell. *Mol. Microbiol.***59**: 907-922.

Meiser, P., H. B. Bode & R. Müller, (2006) The unique DKxanthene secondary metabolite family from the myxobacterium *Myxococcus xanthus* is required for developmental sporulation. *Proc. Natl. Acad Sci. USA***12**: 19128-19133.

Methe, B. A., K. E. Nelson, J. A. Eisen, I. T. Paulsen, W. Nelson, J. F. Heidelberg, D. Wu, M. Wu, N. Ward, M. J. Beanan, R. J. Dodson, R. Madupu, L. M. Brinkac, S. C. Daugherty, R. T. DeBoy, A. S. Durkin, M. Gwinn, J. F. Kolonay, S. A. Sullivan, D. H. Haft, J. Selengut, T. M. Davidsen, N. Zafar, O. White, B. Tran, C. Romero, H. A. Forberger, J. Weidman, H. Khouri, T. V. Feldblyum, T. R. Utterback, S. E. Van Aken, D. R. Lovley & C. M. Fraser, (2003) Genome of *Geobacter sulfurreducens*: Metal reduction in subsurface environments. *Science***302**: 1967-1969.

Moraleda-Munoz, A., J. Perez, A. L. Extremera & J. Munoz-Dorado, (2010) Complexity of the *Myxococcus xanthus* copper response: differential regulation of six heavy metal efflux systems. *Appl. Environ. Microbiol.***76**: 6069-6076.

Morrison, C. E. & D. R. Zusman, (1979) *Myxococcus xanthus* mutants with temperature-sensitive, stage-specific defects: Evidence for independent pathways in development. *J. Bacteriol.***140**: 1036-1042.

Mougous, J. D., M. E. Cuff, S. Raunser, A. Shen, M. Zhou, C. A. Gifford, A. L. Goodman, G. Joachimiak, C. L. Ordonez, S. Lory, T. Walz, A. Joachimiak & J. J. Mekalanos, (2006) A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus. *Science***312**: 1526-1530.

Mougous, J. D., C. A. Gifford, T. L. Ramsdell & J. J. Mekalanos, (2007b) Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nat. Cell Biol.***9**: 797-803.

Murphy, K. C., K. G. Campellone & A. R. Poteete, (2000) PCR-mediated gene replacement in *Escherichia coli*. *Gene***246**: 321-330.

Nariya, H. & M. Inouye, (2008) MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell***132**: 55-66.

Nasu, H., T. Iida, T. Sugahara, Y. Yamaichi, K.-S. Park, K. Yokoyama, K. Makino, H. Shinagawa & T. Honda, (2000) A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. *J. Clin. Microbiol.***38**: 2156-2161.

Nudleman, E., D. Wall & D. Kaiser, (2006) Polar assembly of the type IV pilus secretin in *Myxococcus xanthus*. *Mol. Microbiol.***60**: 16-29.

O'Connor, K. A. & D. R. Zusman, (1991a) Behaviour of peripheral rods and their role in the life cycle of *Myxococcus xanthus*. *J. Bacteriol.***173**: 3342-3355.

O'Connor, K. A. & D. R. Zusman, (1991b) Development in *Myxococcus xanthus* involves differentiation into two cell types, peripheral rods and spores. *J. Bacteriol.***173**: 3318-3333.

Ogawa, M., S. Fujitani, X. Mao, S. Inouye & T. Komano, (1996) FruA, a putative transcription factor essential for the development of *Myxococus xanthus*. *Mol. Microbiol.***22**: 757-767.

Oomen, C. J., P. van Ulsen, P. van Gelder, M. Feijen, J. Tommassen & P. Gros, (2004) Structure of the translocator domain of a bacterial autotransporter. *Embo J***23**: 1257-1266.

Orlowski, M. & D. White, (1974) Intracellular proteolytic activity in developing myxospores of Myxococcus xanthus. *Arch Microbiol***97**: 347-357.

Overgaard, M., S. Wegener-Feldbrugge & L. Sogaard-Andersen, (2006) The orphan response regulator DigR is required for synthesis of extracellular matrix fibrils in Myxococcus xanthus. *J Bacteriol***188**: 4384-4394.

Paetzel, M., A. Karla, N. C. Strynadka & R. E. Dalbey, (2002) Signal peptidases. *Chem Rev***102**: 4549-4580.

Page, A. L. & C. Parsot, (2002) Chaperones of the type III secretion pathway: jacks of all trades. *Mol Microbiol***46**: 1-11.

Pallen, M., R. Chaudhuri & A. Khan, (2002) Bacterial FHA domains: neglected players in the phospho-threonine signalling game? *Trends Microbiol.***10**: 556-563.

Pallen, M. J., S. A. Beatson & C. M. Bailey, (2005) Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perspective. *FEMS Microbiol Rev***29**: 201-229.

Pallen, M. J., M. S. Francis & K. Futterer, (2003) Tetratricopeptide-like repeats in type-III-secretion chaperones and regulators. *FEMS Microbiol. Lett.* **223**: 53-60.

Peabody, C. R., Y. J. Chung, M.-R. Yen, D. Vidal-Ingigliardi, A. P. Pugsley & M. H. Saier Jr., (2003) Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology***149**: 3051-3072.

Pilhofer, M., A. P. Bauer, M. Schrallhammer, L. Richter, W. Ludwig, K. H. Schleifer & G. Petroni, (2007) Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel Two-Step Gene Walking method. *Nucleic Acids Res***35**: e135.

Plamann, L., J. M. Davis, B. Cantwell & J. Mayor, (1994) Evidence that *asgB* encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. *J. Bacteriol.***176**: 2013-2020.

Plamann, L., A. Kuspa & D. Kaiser, (1992) Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*. *J. Bacteriol.***174**: 3311-3318.

Plamann, L., Y. Li, B. Cantwell & J. Mayor, (1995) The *Myxococcus xanthus asgA* gene encodes a novel signal transduction protein required for multicellular development. *J. Bacteriol.***177**: 2014-2020.

Planet, P. J., S. C. Kachlany, R. DeSalle & D. H. Figurski, (2001) Phylogeny of genes for secretion NTPases: identification of the widespread *tadA* subfamily and development of a diagnostic key for gene classification. *Proc. Natl. Acad. Sci. USA***98**: 2503-2508.

Pukatzki, S., A. T. Ma, A. T. Revel, D. Sturtevant & J. J. Mekalanos, (2007) Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad Sci U S A***104**: 15508-15513.

Rawlings, N. D., A. J. Barrett & A. Bateman, (2010) MEROPS: the peptidase database. *Nucleic Acids Res***38**: D227-233.

Reichenbach, H., (1965) Rhythmische vorgänge bei der Schwarmenfaltung von Myxobakterien. *Ber. Deutsch. Bot. Ges.***78**: 102-105.

Reichenbach, H., (1999) The ecology of the myxobacteria. *Env. Microbiol.***1**: 15-21.

Remenant, B., B. Coupat-Goutaland, A. Guidot, G. Cellier, E. Wicker, C. Allen, M. Fegan, O. Pruvost, M. Elbaz, A. Calteau, G. Salvignol, D. Mornico, S. Mangenot, V. Barbe, C. Medigue & P. Prior, (2010) Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. *BMC Genomics***11**: 379.

Roggenkamp, A., N. Ackermann, C. A. Jacobi, K. Truelzsch, H. Hoffmann & J. Heesemann, (2003) Molecular analysis of transport and oligomerization of the Yersinia enterocolitica adhesin YadA. *J Bacteriol***185**: 3735-3744.

Rolbetzki, A., M. Ammon, V. Jakovljevic, A. Konovalova & L. Søgaard-Andersen, (2008) Regulated secretion of a protease activates intercellular signaling during fruiting body formation in *M. xanthus*. *Dev. Cell***15**: 627-634.

Rosario, C. J. & M. Singer, (2007) The *Myxococcus xanthus* developmental program can be delayed by inhibition of DNA replication. *J. Bacteriol.***189**: 8793-8800.

Rosenberg, E. & M. Varon, (1984) Antibiotics and lytic enzymes. In: Myxobacteria: Development and cell interactions. E. Rosenberg (ed). New York: Springer-Verlag, pp. 109-125.

Rosenbluh, A., R. Nir, E. Sahar & E. Rosenberg, (1989) Cell-density-dependent lysis and sporulation of *Myxococcus xanthus* in agarose beads. *J. Bacteriol.***171**: 4923-4929.

Sager, B. & D. Kaiser, (1994) Intercellular C-signaling and the traveling waves of *Myxococcus*. *Genes Dev.***8**: 2793-2804.

Sambrook, J. & D. W. Russell, (2001) *Molecular cloning : a laboratory manual*.Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sato, K., M. Naito, H. Yukitake, H. Hirakawa, M. Shoji, M. J. McBride, R. G. Rhodes & K. Nakayama, (2010) A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc. Natl. Acad Sci. USA***107**: 276-281.

Schwarz, S., R. D. Hood & J. D. Mougous, (2010) What is type VI secretion doing in all those bugs? *Trends Microbiol***18**: 531-537.

Shi, W. & D. R. Zusman, (1993) Fatal attraction. *Nature***366**: 414-415.

Shi, X., S. Wegener-Feldbrugge, S. Huntley, N. Hamann, R. Hedderich & L. Sogaard-Andersen, (2008) Bioinformatics and experimental analysis of proteins of two-component systems in Myxococcus xanthus. *J Bacteriol***190**: 613-624.

Shimkets, L. J., R. E. Gill & D. Kaiser, (1983) Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. *Proc. Natl. Acad. Sci. USA***80**: 1406-1410.

Shimkets, L. J. & D. Kaiser, (1982) Induction of coordinated movement of *Myxococcus xanthus* cells. *J. Bacteriol.***152**: 451-461.

Shimkets, L. J. & H. Rafiee, (1990) CsgA, an extracellular protein essential for *Myxococcus xanthus* development. *J. Bacteriol.***172**: 5299-5306.

Singer, M. & D. Kaiser, (1995) Ectopic production of guanosine penta- and teraphosphate can initiate early developmental gene expression in *Myxococcus xanthus*. *Genes Dev.***9**: 1633-1644.

Sisto, A., M. Cipriani, M. Morea, S. Lonigro, F. Valerio & P. Lavermicocca, (2010) An *Rhs*-like genetic element is involved in bacteriocin production by *Pseudomonas savastanoi* pv. *savastano*. *Antonie Van Leeuwenhoek*: 1-13.

Sliusarenko, O., J. Neu, D. R. Zusman & G. Oster, (2006) Accordion waves in *Myxococcus xanthus*. *Proc. Natl. Acad. Sci. USA***103**: 1534-1539.

Slominska, M., P. Neubauer & G. Wegrzyn, (1999) Regulation of bacteriophage lambda development by guanosine 5'-diphosphate-3'-diphosphate. *Virology***262**: 431-441.

Spratt, B. G., P. J. Hedge, S. te Heesen, A. Edelman & J. K. Broome-Smith, (1986) Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene***41**: 337-342.

Spreter, T., C. K. Yip, S. Sanowar, I. Andre, T. G. Kimbrough, M. Vuckovic, R. A. Pfuetzner, W. Deng, A. C. Yu, B. B. Finlay, D. Baker, S. I. Miller & N. C. Strynadka, (2009) A conserved structural motif mediates formation of the periplasmic rings in the type III secretion system. *Nat Struct Mol Biol***16**: 468-476.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. L. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R.

L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. S. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory & M. V. Olson, (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature***406**: 959-964.

Straley, S. C., G. V. Plano, E. Skrzypek, P. L. Haddix & K. A. Fields, (1993) Regulation by Ca2+ in the Yersinia low-Ca2+ response. *Mol Microbiol***8**: 1005-1010.

Striebel, F., W. Kress & E. Weber-Ban, (2009) Controlled destruction: AAA+ ATPases in protein degradation from bacteria to eukaryotes. *Curr Opin Struct Biol***19**: 209-217.

Tojo, N., S. Inouye & T. Komano, (1993a) Cloning and nucleotide sequence of the Myxococcus xanthus lon gene: indispensability of lon for vegetative growth. *J Bacteriol***175**: 2271-2277.

Tojo, N., S. Inouye & T. Komano, (1993b) The lonD gene is homologous to the lon gene encoding an ATP-dependent protease and is essential for the development of Myxococcus xanthus. *J Bacteriol***175**: 4545-4549.

Tse, H. & R. E. Gill, (2002) Bypass of A- and B-signaling requirements for *Myxococcus xanthus* development by mutations in spdR. *J. Bacteriol.***184**: 1455-1457.

Tseng, T. T., B. M. Tyler & J. C. Setubal, (2009) Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. *BMC Microbiol***9 Suppl 1**: S2.

Velicer, G. J. & K. L. Hillesland, (2008) Why cooperate? The ecology and evolution of myxobacteria. In: Myxobactera: Multicellularity and differentiation. D. E. Whitworth (ed). Washington, D.C.: ASM Press, pp. 17-40.

Wai, S. N., B. Lindmark, T. Soderblom, A. Takade, M. Westermark, J. Oscarsson, J. Jass, A. Richter-Dahlfors, Y. Mizunoe & B. E. Uhlin, (2003) Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell***115**: 25-35.

Wai, S. N., M. Westermark, J. Oscarsson, Y. Mizunoe & B. E. Uhlin, (2000) Localization and export of the ClyA cytotoxin in *Escherichia coli*. *Med. Microbiol. Immunol.***189**: 51-.

Wang, R. C., S. J. Seror, M. Blight, J. M. Pratt, J. K. Broome-Smith & I. B. Holland, (1991) Analysis of the membrane organization of an Escherichia coli protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins. *J Mol Biol***217**: 441-454.

Wang, Y.-D., S. Zhao & C. W. Hill, (1998) Rhs elements comprise three subfamilies which diverged prior to acquisition by *Escherichia coli*. *J. Bacteriol.***180**: 4102-4110.

Welch, R. & D. Kaiser, (2001) Pattern formation and traveling waves in myxobacteria: Experimental demonstration. *Proc. Natl. Acad. Sci. USA***98**: 14907-14912.

Wilharm, G., S. Dittmann, A. Schmid & J. Heesemann, (2007) On the role of specific chaperones, the specific ATPase, and the proton motive force in type III secretion. *Int J Med Microbiol***297**: 27-36.

Wu, H.-Y., P.-C. Chung, H.-W. Shih, S.-R. Wen & E.-M. Lai, (2008) Secretome analysis uncovers an Hcp-family protein secreted via a Type VI secretion system in *Agrobacterium tumefaciens*. *J. Bacteriol.***190**: 2841-2850.

Wu, S. & D. Kaiser, (1997) Regulation of expression of the pilA gene in Myxococcus xanthus. *J. Bacteriol.***179**: 7748-7758.

Xu, D., C. Yang & H. B. Kaplan, (1998) *Myxococcus xanthus sasN* encodes a regulator that prevents developmental gene expression during growth. *J. Bacteriol.***180**: 6215-6223.

Yang, C. & H. B. Kaplan, (1997) Myxococcus xanthus sasS encodes a sensor histidine kinase required for early developmental gene expression. *J Bacteriol***179**: 7759-7767.

Young, J. & I. B. Holland, (1999) ABC transporters: bacterial exporters-revisited five years on. *BBA***1461**: 177-200.

Yu, X. J., K. McGourty, M. Liu, K. E. Unsworth & D. W. Holden, (2010) pH sensing by intracellular Salmonella induces effector translocation. *Science***328**: 1040-1043.

Zhang, H., N. N. Rao, T. Shiba & A. Kornberg, (2005) Inorganic polyphosphate in the social life of Myxococcus xanthus: motility, development, and predation. *Proc Natl Acad Sci U S A***102**: 13416-13420.

Zhang, Y., F. Buchholz, J. P. Muyrers & A. F. Stewart, (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.***20**: 123-128.

Zheng, J. & K. Y. Leung, (2007) Dissection of a type VI secretion system in Edwardsiella tarda. *Mol Microbiol***66**: 1192-1206.

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List of publications

Konovalova, A., S. Wegener-Feldbrügge, S. Lindow, N. Hamann & L. Søgaard-Andersen, (2010) Proteins of unknown function are required for regulated secretion of the signalling protease PopC in *Myxococcus xanthus*. *Submitted*.

Konovalova, A., T. Petters & L. Sogaard-Andersen, (2010) Extracellular biology of *Myxococcus xanthus*. *FEMS Microbiol Rev***34**: 89-106.

Kahnt, J., K. Aguiluz, J. Koch, A. Treuner-Lange**, A. Konovalova**, S. Huntley, M. Hoppert, L. Sogaard-Andersen & R. Hedderich, (2010) Profiling the outer membrane proteome during growth and development of the social bacterium *Myxococcus xanthus* by selective biotinylation and analyses of outer membrane vesicles. *J Proteome Res***9**: 5197-5208.

Rolbetzki, A., M. Ammon, V. Jakovljevic, **A. Konovalova**& L. Sogaard-Andersen, (2008) Regulated secretion of a protease activates intercellular signalling during fruiting body formation in *M. xanthus*. *Dev Cell***15**: 627-634.

Konovalova, A., Y. Kobozyev, N. Grabchenko & I. Ganova, (2008) Synthesis of recombinant analogue of p24 protein of BLV.*Biotechnology***1** (in Ukranian)

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Konovalova, A., S. O. Shylin & P. V. Rokytko, (2006) Isolation and preliminary characterization of carotenoids from pink-pigmented methylotrophs.*Ukr Biokhim Zh***78**: 146-150 (in Ukranian)

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Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel „Regulation of secretion of the signalling protease PopC in *Myxococcus xanthus*“ selbstständig verfasst, keine anderen als die im Text angegebenen Hilfsmittel verwendet und sämtliche Stellen, die im Wortlaut oder dem Sinn nach anderen Werken entnommen sind, mit Quellenangaben kenntlich gemacht habe.

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Anna Konovalova Ort, Datum

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