Thermoresponsive secretion of the extracellular enzyme levansucrase from *Pseudomonas syringae*

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Hongqiao Li

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Erstgutachter: Dr. habil. M. Ullrich

Zweitgutachter: Prof. Dr. R. K. Thauer

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Li, H., Schenk, A., Hettwer, U., Rudolph, K. and Ullrich, M.S. Temperature-dependent secretion of the extracellular enzyme levansucrase in *Pseudomonas syringae* pv. glycinea PG4180. In preparation (to be submitted to Appl. Environ. Microbiol.).

Li, H., Schenk, A., Weingart, H. and Ullrich, M.S. Role of exopolysaccharide synthesis and the global regulator GacS on virulence in *Pseudomonous syringae* pv. glycinea. In preparation (to be submitted to Mol. Plant-Microbe Interact.)

Abbreviations

A_{xxx}	absorbance at xxx nm
AP	alkaline phosphatase
APS	ammonium peroxodisulfate
ATP	adenosine-5'-triphosphate
bp	base pair
COR	coronatine
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiotreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Fig	figure
g	gram
GUS	β-glucuronidase
h	hour
HSC	Hoitink-Sinden medium optimized for coronatine production
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo bases
KB	King's B
kDa	kilo Dalton
LacZ	β-galactosidase
LB	Luria-Bertani
lsc	gene encoding for levansucrase
Lsc	levansucrase
MBP	maltose-binding protein
MG	Mannitol-Glutamat
mg	milligram
min	minute
ml	milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
nm	nanometer
OD_{xxx}	optical density at xxx nm

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
phoA	gene encoding for alkaline phosphatase
pv.	pathovar
P. syringae	*Pseudomonas syringae*
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
SDS	sodium dodecyl sulfate
sec/s	seconds
SSC	saline sodium citrate
TAE	Tris-acetate-EDTA
TCA	trichloroacetic acid
TE	Tris/EDTA
TEMED	N, N, N',N'-tetramethylendiamine
Tris	tris-(hydroxymethyl)-aminomethane
U	unit
uidA	gene encoding β-glucuronidase
UV	ultraviolet
v/v	volume to volume
WT	wild type
w /v	weight to volume
X-Gal	5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indoyl-β-glucuronic acid
X-PhoA	5-bromo-4-chloro-3-indoyl-β-phosphate-p-toluidine salt
µg	microgram
µl	micro liter

1 SUMMARY

In the plant pathogen *Pseudomonas syringae* pv. glycinea PG4180 and other bacterial species, synthesis of the exopolysaccharide levan is catalyzed by the extracellular enzyme levansucrase. Southern blot and PCR analysis indicated the presence of three levansucrase-encoding genes in PG4180: *lscA*, *lscB*, and *lscC*. In this study, *lscB* and *lscC* were cloned from a genomic library of PG4180. Sequence analysis of the two *lsc* genes showed that they were virtually identical to each other and highly similar to the previously characterized *lscA* gene. *lscA* and *lscC* had a chromosomal location whereas *lscB* resided on an indigenous plasmid of PG4180. PCR screening in various *P. syringae* strains with primers derived from the three characterized *lsc* genes demonstrated the presence of multiple *lsc* genes in other *P. syringae* pathovars.

Comparison of extracellular protein profiles of PG4180 cultures grown in minimal medium at 18°C and 28°C revealed a protein band of approximately 50 kDa which was predominantly found in the supernatant at 18°C and which represented levansucrase. Mutants impaired in expression of individual *lsc* genes as well as double mutants were generated by marker exchange mutagenesis. Determination of levansucrase activities in these mutants revealed that the *lscB* gene product but not that of *lscA* or *lscC* was secreted at the lower temperature. Our results indicated that *lscB* and *lscC* but not *lscA* contributed to periplasmic levan synthesis of PG4180. The *lscB lscC* double mutant was completely defective in levan formation and could be complemented by either *lscB* or *lscC.* Data of this study suggested a compartment-specific localization of two *lsc* gene products with LscB being the secreted and LscC being the predominantly periplasmic levansucrase. Results of Western blot analyses indicated that *lscA* was not expressed. LscA could only be detected in PG4180 when transcribed from the vector-borne P_{lac} promoter. Northern blot analysis indicated that transcription of *lscB* and *lscC* was temperature-dependent. Quantitative immunological detection of levansucrase in extracellular protein pools and total cellular protein samples confirmed that LscB secretion at low temperature was due to the combination of a temperature-regulated transcription and thermoresponsive secretion.

LscB and LscC may differ in the number of potential disulfide bridges and the herein reported successful identification and cloning of *dsbA* and *dsbC,* encoding periplasmic disulfide-bond forming enzymes, in the genome of PG4180 will help to further investigate a potential linkage between protein folding and secretion. Results of temperature shift experiments suggested that the factor(s) responsible for LscB secretion depended on *de*

novo protein synthesis, were only present at 18°C, and were relatively stable once the bacterial cultures were shifted from inducing to non-inducing temperature conditions. Additionally, preliminary studies on the effects of levan formation on *in planta* survival of PG4180 on soybean plants indicating that fewer symptoms developed and less bacterial multiplication occurred when a levan-deficient mutant was used as the inoculum as compared to its wild type. Consequently, levan formation might contribute to bacterial fitness and potentially virulence. Mutation of *gacS*, encoding a global kinase implicated in regulation of virulence in *P. syringae*, demonstrated that alginate formation and levan production were not coordinately controlled and that neither the transcription of *lsc* genes nor the secretion of LscB depended on GacS.

1 ZUSAMMENFASSUNG

Im Pflanzenpathogen *Pseudomonas syringae* pv. glycinea PG4180 und anderen Bakterien bildet das extrazelluläre Enzym Levansucrase (Lsc) das Exopolysaccharid Levan. Southern Blot und PCR Analyse ermöglichten die Identifizierung von drei Lsc-kodierenden Genen: *lscA*, *lscB* und *lscC*. In der vorliegenden Studie wurden *lscB* und *lscC* kloniert und sequenziert. Die Sequenzanalyse zeigte, daß die beiden Gene nahezu identisch zu einander und sehr ähnlich zu dem zuvor charakterisierten *lscA*-Gen sind. Während *lscB* plasmid-kodiert ist, liegen *lscA* und *lscC* chromosomal vor. Eine PCR-Analyse erbrachte, daß das multiple Auftreten von *lsc*-Genen in diversen Pathovars von *P. syringae* weit verbreitet ist.

Der Vergleich von extrazellulären Proteinmustern von bei 18 bzw. 28°C gewachsenen PG4180-Kulturen zeigte ein 50-kDa Protein, das nur bei 18°C im Kulturüberstand auftrat und Lsc-Aktivität besaß. Mittels Markeraustausch-Mutagenese wurden PG4180-Mutanten bzw. –Doppelmutanten erzeugt, die in individuellen *lsc*-Genen defekt waren. Die Ermittlung der zellkompartiment-spezifischen Levansucrase-Aktivitäten in diesen Mutanten erbrachte, daß das Genprodukt von *lscB*, nicht jedoch die von *lscA* oder *lscC*, bei 18°C sekretiert wurde. Unsere Ergebnisse wiesen weiterhin daraufhin, daß LscB und LscC, nicht jedoch LscA, periplasmatisch auftraten. Eine *lscB lscC* Doppelmutante bildete kein Levan und konnte sowohl durch *lscB* als auch durch *lscC* komplementiert werden. Die Ergebnisse der vorliegenden Arbeit deuten daraufhin, daß die *lsc*-Genprodukte zellkompartiment-spezifisch akkumulieren, wobei LscB das sekretierte Enzym und LscC das vorrangig periplasmatische Enzym darstellt. Ergebnisse von Western Blot Analysen zeigten, daß *lscA* nicht exprimiert wird und daß dessen Genprodukt erst nach Transkription von einem vektorstämmigen P_{lac} Promoter in PG4180 nachgewiesen werden kann. Northern Blot Analysen ergaben, daß sowohl *lscB* als auch *lscC* in einer temperaturabhängigen Weise transkribiert werden. Der quantitative immunologische Nachweis von Lsc im Kulturüberstand sowie im Zellinneren machte deutlich, daß die beobachtete differentielle Sekretion von LscB das kombinierte Ergebnis von temperaturabhängiger Genexpression und thermoregulierter Proteinsekretion ist.

LscB und LscC könnten sich in der Anzahl der jeweiligen Disulfidbrückenbindungen unterscheiden. Die in der vorliegenden Arbeit erreichte Klonierung von zwei Genen, *dsbA* und *dsbC*, deren periplasmatische Genprodukte an der Disulfidbrückenbildung beteiligt sind, könnten in zukünftigen Arbeiten einen eventuellen Zusammenhang zwischen

Proteinfaltung und Sekretion nachweisen helfen. Temperatur-Shift-Experimente mit PG4180-Kulturen machten deutlich, daß die für die LscB-Sekretion notwendigen Faktoren durch *de novo* Proteinsynthese bei 18°C gebildet werden und nachfolgend bei 28°C relativ stabil sind. Vorläufige Pflanzeninokulations-Experimente deuten daraufhin, daß die Levanbildung einen wichtigen Fitness- und Virulenzfaktor für *P. syringae* darstellt, da nach Sprühinokulation einer Levan-defekten Mutante deutlich weniger Symptome und eine verringerte bakterielle Vermehrung *in planta* beobachtet wurde. Die Mutation von *gacS*, dem Gen für eine Histidinkinase, die an der globalen Regulation von Virulenzfaktoren beteiligt ist, führte weder zu einer Veränderung der Expression von *lsc* noch zu einer Beeinträchtigung der Lsc-Sekretion. Jedoch konnte nachgewiesen werden, daß GacS eine wichtige Rolle bei der Bildung von Alginat spielt, welches das zweite von *P. syringae* produzierte Exopolysaccharid darstellt.

2 INTRODUCTION

Phytopathogenic bacteria are capable of causing disease in a myriad of plant hosts. Plant pathogens damage and destroy billions of dollars worth of crops worldwide each year. The economy of developing countries is much more dependent on agriculture than that of industrialized countries. The heavy reliance on the agricultural sector makes developing countries particularly sensitive to this economic damage. Because of this, plant pathogenic bacteria are indirectly responsible for much morbidity and mortality worldwide. The fight against plant disease remains one of the biggest challenges in agriculture as we stepped into the 21st Century.

Plant pathogens can grow and multiply rapidly on the diseased plants, spread from the diseased plants to healthy ones, and thereby cause additional plants to become diseased. Such diseases decrease both the quantity and quality of the crops, and damage the stored product as well. In some cases a crop which could have been profitable can not be grown because a bacterial pathogen is extremely destructive over seasons. The ever-increasing incidence of antibiotic resistance, combined with new and reemerging bacterial pathogens, has heightened our awareness of bacterial infectious diseases in agriculture.

2.1 *Pseudomonas syringae* pv. glycinea

The genus *Pseudomonas* represents a large group of medically and environmentally important bacteria that inhabit a great diversity of habitats. For example, pseudomonads occur in and on plants and in water and soil. *Pseudomonas syringae* might be the most significant, and best-studied plant pathogenic bacterium. *P. syringae* is divided into pathogenic variants (pathovars), which vary in host range (Huynh *et al*, 1989) and which remain the subject of intensive scientific investigation.

The leaves and flowers of healthy plants are normally colonized by a large number of bacteria, including plant pathogenic bacteria. *P. syringae* grows asymptomatically on the surface of a wide variety of plants. Many strains of *P. syringae* have the capacity to cause disease in several economically important plant species (Agrios, 1997). In fact, initiation of disease by epiphytically grown *P. syringae* is unlikely unless relatively large epiphytic population size of these bacteria develop (Rouse *et al.* 1985). Disease occurs when bacteria are introduced into the tissue of a susceptible plant species and begin to multiply in the

intercellular spaces. Tissue chlorosis and necroses, called leaf blights, commonly develop following colonization of tissue. The development of bacterial blight symptoms is most severe during periods of cold and humid weather conditions (Dunleavy, 1988).

The bacterial blight pathogen of soybean, *Pseudomonas syringae* pv. glycinea PG4180, causes formation of water-soaked lesions which develop into necrotic leaf spots surrounded by chlorotic halos (**Fig. 1**). The phytotoxin coronatine (COR) appears to be a major virulence factor for this model organism (Budde and Ullrich, 2000). COR production was also shown to enhance the virulence of *P. syringae* strains on tomato, *Arabidopsis thaliana*, and Chinese cabbage plants (Bender *et al.* 1987; Mittal and Davis 1995; Tamura *et al.* 1998).

Fig. 1. *Pseudomonas syringae* pv. glycinea causes bacterial blight on soybean plants.

Different factors of stain PG4180 have been genetically and biochemically studied in our lab:

- Biosynthesis of the polyketide phytotoxin COR by *P. syringae* has been shown to be thermoresponsive at the transcriptional level (Budde *et al*., 1998; Rohde *et al*., 1998).
- A modified two-component regulatory system has been identified to control the temperature-dependent transcription of biosynthetic genes involved in COR synthesis (Ullrich *et al*., 1995; Rohde *et al*., 1998; Wang *et al*., 1999).
- A number of new genes or gene products which are differentially expressed at 18 or 28°C were identified (Rohde *et al*., 1999; Ullrich *et al*., 2000; Smirnova *et al*., 2001).
- Levansucrase has been cloned from *P. syringae* (Hettwer et al., 1998).
- The ethylene forming enzyme (EFE) has genetically been characterized (Weingart *et al*., 1999)

2.2 Pathogenicity and virulence determinants of *Pseudomonas syringae*

There are two possible reactions when *P. syringae* cells dock onto the surface of plant cells. One potential outcome is the compatible, susceptible interaction, that is characterized by a symptom called water soaking, a reaction which is followed by pathogen multiplication and advanced symptom development. Phytotoxins play an important role in this pathogen-plant interaction (Bender *et al*., 1999; Feys *et al*., 1994; Mittal and Davis, 1995). Although phytotoxins are not required for pathogenicity in *P. syringae,* they generally function as virulence factors for this pathogen and enhance the disease severity. Phytopathogenic pseudomonads also encode other gene products that enhance virulence, including extracellular polysaccharides, cell wall-degrading enzymes, and phytohormones (**Fig. 2**) (Alfano and Collmer, 1996; Costacurta and Vanderleyden, 1995; Denny, 1995; Gross, 1991).

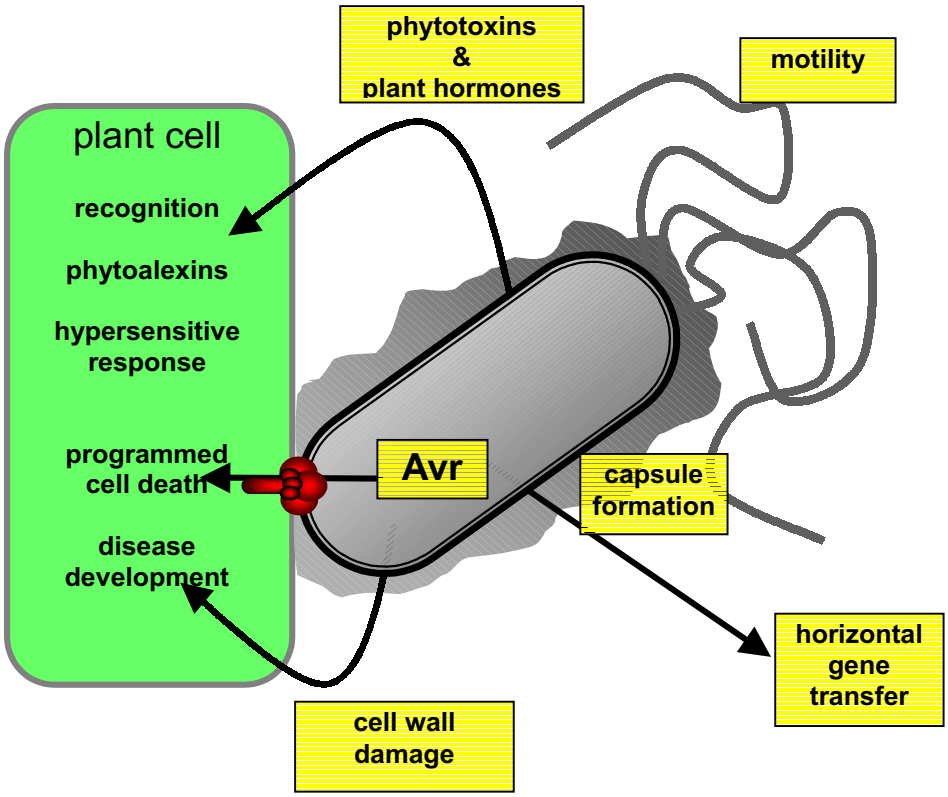

Fig. 2. Possible interactions of *Pseudomonas syringae* with host and non-host plants.

The other reaction is known as the hypersensitive reaction (incompatible reaction). This reaction leads to necrosis 12 to 24 h after bacterial inoculation. As shown in **Fig. 2**, the plant's recognition of a phytopathogenic bacterium is specified genetically by the presence

of bacterial avirulence (Avr) genes and resistance in hosts containing the corresponding disease resistance gene (Leach and White, 1996; Huynh *et al.* 1989). In susceptible plants, Avr products might function as pathogenicity factors. By evolving precise defense pathways, plants actively recognize invading bacteria to impede their *in planta* multiplication. The phenotype of plant resistance is the rapid induction of a programmed cell death. Major groups of gram-negative plant pathogenic bacteria contain hypersensitive reaction and pathogenicity (*hrp*) genes. In plant pathogenic bacteria the type III secretion system encoded by *hrp* genes is called the Hrp system (He *et al.*, 1993; Bogdanove *et al*., 1996). Phytopathogenic bacteria require the gene products of the *hrp* cluster to elicit an HR, characterized by the rapid collapse of the leaf tissue followed by necrosis (Bonas, 1994; He, 1996). HR is associated with the production of reactive oxygen intermediates, the alteration of ion fluxes, the oxidative cross-linking of cell wall structural proteins, and the synthesis of antimicrobial compounds, including phytoalexins and pathogenesis-related proteins (Greenberg, 1997; Lamb and Dixon, 1997). The detailed mechanism(s) for pathogen inhibition remains unknown.

2.3 Thermoregulated expression of virulence factors in plant-associated bacteria

Plants as well as plant pathogens require certain minimum temperatures to grow and maintain metabolic activities. Very low temperatures of late fall, winter, and early spring are below the minimum temperature required by most pathogens. With the advent of moderate temperatures, however, pathogens become active and, when other conditions are favorable, they can infect plants and cause diseases. For our model organism, *P. syringae* PG4180, infection begins and develops on young leaves of soybean plants primary in mid-spring. During these periods temperature is high enough for this pathogen to grow but are yet low enough to permit optimal host defense. More importantly, during these climatic conditions humidity is high and the pathogen require water film and aerosols to infect the plant tissue.

Temperature as one of the important environmental factors has a significant impact on many cellular processes, and therefore, bacteria must possess molecular thermoresponsive devices in order to adjust to changes in temperature. Moreover, bacterial virulence functions are often temperature-regulated and thus temperature sensing and thermoresponsive gene regulation mostly occur in pathogenic organisms. In general,

temperature-mediated virulence factor regulation can occur at the levels of transcription, protein conformation and protein stability. DNA supercoiling, changes in mRNA conformation and protein conformation are all implicated in thermosensing by animal pathogens as reviewed by Hurme and Rhen (1998) (**Fig. 3**).

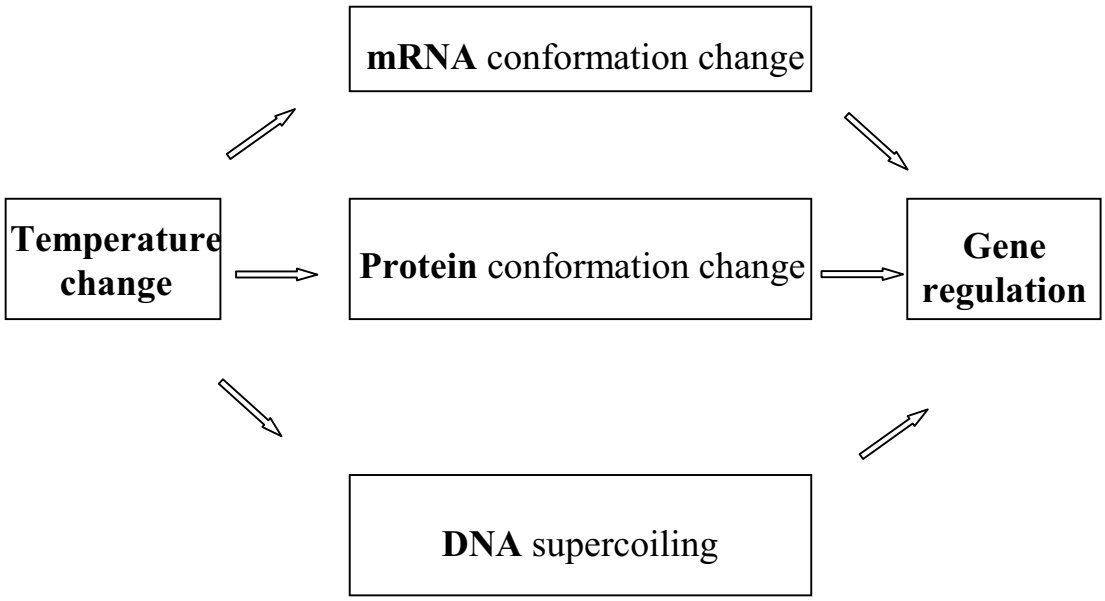

Fig. 3. Schematic representation of thermosensing mechanisms affecting gene regulation. To be converted into gene regulatory signals, temperature changes have to be sensed by cellular components. This may occur via conformational changes in mRNA or protein, or through DNA supercoiling.

Transcription of virulence genes in pathogens of warm-blooded hosts is induced at higher temperatures, which are typical for body cavities and host tissues. This leads to synthesis of virulence factors preferably at the time point and location when and where they are actually needed: in the interior of the warm-blooded host. Thermoregulation of virulence factors in plant pathogens is not as obvious. However, it is interesting to note that many plant pathogenic bacteria exhibit a stronger virulence at lower temperatures although their optimal growth temperatures usually range from 25-30°C.

In analogy to the temperature-dependent expression and secretion of virulence factors in human and animal pathogens (Hurme and Rhen, 1998), those of phytopathogens may also be subjected to temperature fluctuations. Biosyntheses of virulence factors in plant pathogenic bacteria, for instance, phytotoxins, exopolysaccharides, plant cell wall

degrading enzymes, and horizontal gene transfer, represent very energy-expensive processes. Thus, a differential expression of these secondary metabolites appears to be highly beneficial to the over-all cellular energy pool of the pathogens. The type III protein secretion apparatus and their regulatory components encoded by *hrp* genes are strongly influenced in a temperature-dependent manner in *Erwinia amylovora* (Wei *et al*., 1992; 2000). Likewise, secretion of *avr* gene products via the Hrp system of *P. syringae* was demonstrated to be affected by temperature (Van Dijk *et al*., 1999). Phytotoxin biosynthesis in *P. syringae* pvs. glycinea and phaseolicola was shown to be thermoresponsive with optimal synthesis levels at 18°C (Palmer and Bender, 1993; Rowley *et al*., 1993; Budde *et al*., 1998). The transfer of tumor-inducing Ti-plasmid DNA from *Agrobacterium tumefaciens* into plant cells is favored at lower temperature such as 18-22°C (Fullner *et al*., 1996). Frost injury on citrus plants caused by *P. syringae* is mediated by the activity of a bacterial ice nucleation protein. As expected, the respective *inaZ* gene coding for this protein is expressed at maximal rate at 16°C and is only minimally transcribed at 24°C (Nemecek-Marshall *et al*., 1993). Interestingly, further lowering the incubation temperature did not further increase *inaZ* expression. Recently a mini-Tn*5* transposon mutagenesis revealed new thermoresponsive loci in *P. syringae* pv. glycinea (Ullrich *et al*., 2000). Temperature is also a primary environmental factor affecting the synthesis of extracellular polysaccharides amylovoran and levan due to thermoregulation at the level of gene expression in *E. amylovora* (Kelm *et al*., 1997; Bereswill *et al*., 1997). Transcriptional activation of *pel* genes encoding pectate lyases is favored at lower temperatures in *Erwinia chrysanthemi*. Pectinases, secreted isoenzymes encoded by *pelD* and *pelE*, showed the most pronounced thermoregulation and played a major role during the infection process (Hugovieux-Cotte-Pattet *et al*., 1992; 1996). Extracellular cellulases in *Erwinia carotovora* were shown to be regulated in a similar temperature-dependent manner (Lanham *et al*., 1991). Expression of *algD*, the first gene of the alginate biosynthetic operon, was shown to be induced at 28°C and was significantly lower at 18°C (Peñaloza-Vázquez et al., 1997). Alginate biosynthesis in *P. syringae* is controlled by the alternate sigma factor AlgT whose expression is heat-shock inducible (Keith and Bender, 1999) suggesting that alginate production might function in protecting the cell from heat stress.

2.4 Extracellular polysaccharides (EPS)

Bacterial cells living in its natural habitat are enveloped by slimy surface layers conditioning a close-by favorable environment for them (Dudman, 1977; Costerton *et al.,* 1981). Thus, the "actual" bacterial cell wall (peptidoglycan, outer membrane, lipopolysaccharides etc.) is covered by additional slime layers. Such surface layers are thought to be important virulence factors of plant pathogenic bacteria (Mansfield and Brown, 1986). Bacterial polysaccharides form an amorphous layer of extracellular polysaccharide (EPS) surrounding the bacterial cell. This layer may be further organized into a distinct structure termed a capsule (Cross, 1990). Capsular polysaccharides are linked to the bacterial cell surface via covalent attachments to either phospholipid or lipid-A molecules (Whitfield *et al*., 1993). In contrast, extracellular polysaccharide molecules appear to be released from the cell surface with no visible means of attachment and are often sloughed off to form slime. In spite of the difference in their association to the cell, both types of polysaccharide molecules have been implicated as important pathogenic factors.

The virulence of phytopathogenic bacteria, including *Ralstonia solanacearum*, *Erwinia amylovora*, *E. stewartii* and *Xanthomonas campestris*, has been correlated with their ability to produce EPS *in planta* (Dolph *et al*., 1988; Kao *et al*., 1992; Geier and Geider, 1993; Saile *et al*., 1997; Katzen *et al*., 1998). *P. syringae* pathovars generally produce two EPS molecules: levan (a β-2, 6-linked polyfructan) and alginate, a co-polymer of *O*-acetylated β-1,4-linked d-mannuronic acid and its C-5 epimer, l-guluronic acid (Fett *et al*., 1986; Gross and Rudolph, 1987). Alginate in *P. syringae* has been demonstrated to be a significant contributing factor to the pathogenic interaction on bean plants (Yu *et al*., 1999). Furthermore, a positive correlation between the virulence of *P. syringae* and the quantity of alginate produced *in planta* has been demonstrated (Osman *et al*., 1986; Gross and Rudolph, 1987). However, due to lack of respective mutants the role of levan in the virulence of *P. syringae* has not been assessed so far.

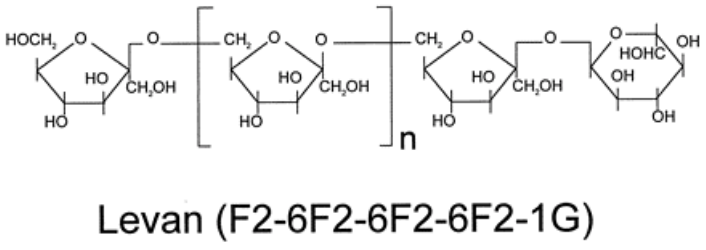

Fig. 4. Structure of the EPS levan as a β-(2,6) polyfructan.

The synthesis of EPS is a common characteristic of plant pathogenic bacteria and its role in virulence has been previously reviewed in detail (Alfano and Collmer, 1996; Leigh and Coplin, 1992; Saile *et al*., 1997; Denny, 1995). The EPS levan is a β-(2,6) polyfructan with extensive branching through β-(2,1) linkages (**Fig. 4**). Polyfructans are a group of storage carbohydrates that are widely distributed in nature, and rival in abundance the better known glucose polymers, starch and glycogen. Like other EPS, levan could have particular functions prior to or during the infection process (Leigh and Coplin, 1992; Denny, 1995). Levan might be particularly important during early stages of infection by masking and protecting the cell and by supporting the proliferation of the pathogen in the host tissue (Kasapis *et al*., 1994; Lindow, 1991). EPS provide a selective advantage to bacteria, have multiple functions, and are thought to enhance bacterial survival by generating a hydrogenated matrix, minimizing direct contacts with plant surfaces, preventing host recognition, or functioning as a detoxifying barrier versus plant defense compounds. Additionally, EPS formation may protect bacterial cells from desiccation, concentrate minerals and nutrients, and improve attachment to surfaces during epiphytic growth.

2.5 The extracellular enzyme levansucrase

Carbon compounds alone or both carbon and nitrogen compounds were shown to be limiting factors for bacterial populations on plant leaves (Wilson and Lindow, 1994). Leaves of different plant species support different numbers of epiphytic bacteria (O'Brien and Lindow, 1989) and the epiphytic bacterial population size on leaves is due to the amount of sugars initially present on the leaves (Mercier and Lindow, 2000). Two types of sucrose-utilizing systems were identified in bacteria: intracellular and extracellular. Within the cell, sucrose is transported to the cytoplasm or periplasm by a phosphoenolpyruvate-dependent carbohydrate phosphotransferase system, and is subsequently cleaved by the intracellular sucrose-phosphate hydrolase (Klier and Rapoport, 1988). In case of the other sucrose-utilizing system sucrose is first hydrolyzed extracellularly to monomeric sugars and then transported into the cell. Glucose and fructose generated from sucrose by extracellular saccharolytic enzymes enter into the cell by a facilitated diffusion system and are utilized by the central Entner-Doudoroff glycolytic pathway. Extracellular saccharolytic enzymes such as levansucrase or sucrase constitute a primary metabolic

pathway that allows these bacteria to utilize sucrose as an energy source (Jones *et al*., 1991).

The extracellular enzyme levansucrase (Lsc) (EC 2.4.1.10) catalyzes the following three reactions: (i) synthesis of levan from sucrose by transfructosylation while releasing glucose, (ii) hydrolysis of levan to monosaccharides of fructose, and (iii) exchange of [^{14}C] glucose in the reaction fructose-2,1-glucose + [^{14}C] glucose to fructose-2,1-[^{14}C]glucose + glucose (Gross and Rudolph, 1987).

Levansucrases have been described in various bacteria such as *Bacillus subtilis*, *Streptococcus mutans*, *Zymomonas mobilis*, *Acetobacter diazotrophicus*, *Erwinia amylovora* and *Pseudomonas syringae* (Arrieta *et al*., 1996; Dedonder, 1966; Gross *et al*., 1992; Hettwer *et al*., 1995; Lyness and Doelle, 1983; Sato *et al*., 1984). In contrast to levansucrases from gram-positive bacteria, which differ widely in their biochemical characteristics, the respective enzymes of gram-negative bacteria are similar in their molecular mass and substrate-independent expression (Geier and Geider, 1993; Hettwer *et al*., 1995; Hettwer *et al*., 1998; Song *et al*., 1993).

2.6 Protein secretion in gram-negative bacteria

Many bacterial proteins function outside the bacterial cell. Extracellular proteins often play important roles in bacterial virulence because they can interact directly with host cells. Proteins secreted into the extracellular space by gram-negative bacteria have to traverse a cell envelope consisting of two membranes, separated by the periplasmic compartment. Although protein secretion is required for numerous aspects of the bacterial life cycle only a few pathways exist by which these proteins are transported from the bacterial cytoplasm to the extracellular space. Four pathways of protein secretion (type I to IV) have been described for gram-negative bacteria (**Fig. 5**). (Fath and Kolter, 1993; Finlay and Falkow, 1997; Hueck, 1998; Salmond and Reeves, 1993; Van Gijsegem *et al*., 1993). The term "secretion" is used to describe the active transport of proteins from the cytoplasm across the inner and outer membranes into the extracellular medium. Secretion is distinguished from export, which refers to the transport of proteins from the cytoplasm to the periplasmic space (Pugsley, 1993; Salmond and Reeves, 1993).

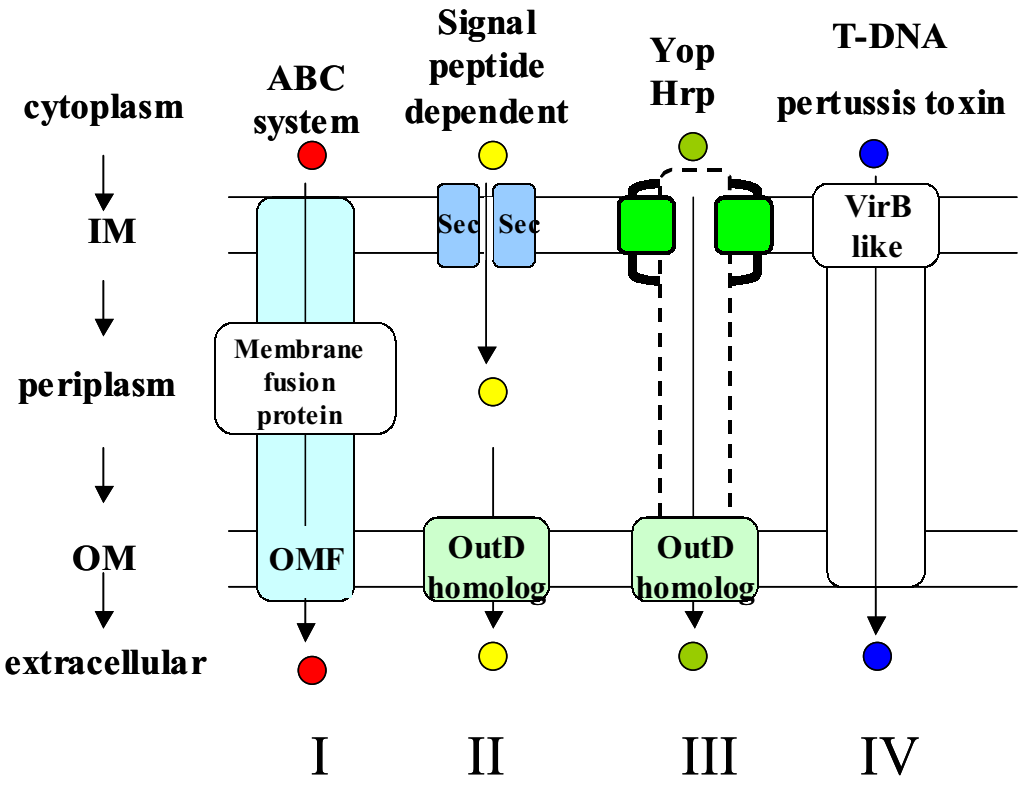

Fig. 5. Protein secretion mechanisms in gram-negative bacteria. Proteins (filled circles) are secreted across the inner membrane (IM) and outer membrane (OM) of gram-negative bacteria via Sec-dependent and Sec-independent mechanisms. (I) During the type I or ATP-binding cassette (ABC) secretion, the periplasmic membrane fusion protein (MFP) interacts with both the IM ABC exporter and the OM channel-forming protein (OMP) to allow secretion to the extracellular medium without a periplasmic intermediate. (II) Type II substrates cross the IM via the Sec system accompanied by signal-sequence cleavage and protein folding in the periplasm. (III) Type III secretion involves cytoplasmic chaperones which bind to presecretory proteins. Type III and type II secretion share a homologous multimeric outer membrane component. (IV) Protein secretion by the type IV pathway may take place via a periplasmic intermediate, with substrates first traversing the IM via the Sec pathway. T-DNA secretion probably takes place from the cytoplasm in a single step, without a periplasm intermediate.

Proteins secreted by the type I pathway are normally high-molecular-weight toxins and extracellular enzymes. In general, type I secretion systems are a group of structurally and functionally related protein secretion complexes, whose substrates do not possess an N-terminal signal peptide. Instead, they are recognized through their C-terminal domain (Blight and Holland, 1994; Lory, 1998). Proteins secreted through the type I pathway are translocated from the cytoplasm to the extracellular medium across the inner and the outer membrane without a periplasmic intermediate (Koronakis *et al.,* 1989; Blight and Holland,

1994; Binet *et al.*, 1997). The type I secretion machineries consist of three polypeptides: one is an integral outer membrane protein forming a trimer with a single hydrophilic pore such as TolC (Paulsen *et al*., 1997); the other two are an inner membrane protein belonging to the ATP binding cassette (ABC) superfamily of transporters, for example, HlyB, (Binet *et al*., 1997; Jones and George, 1999) and a membrane fusion protein (MFP) such as HlyD (Dinh *et al*., 1994). Structural and/or functional homologous of TolC, HlyB and HlyD exist in all bacterial type I systems characterized so far.

The type II secretion pathway is responsible for the secretion of toxins and hydrolytic enzymes in many gram-negative bacteria. This pathway is referred to as the general secretory pathway (GSP). Secretion via type II pathway occurs in two steps. First, proteins to be secreted are produced with a N-terminal signal peptide, which allows for a Sec-dependent translocation across the inner membrane. During this process the signal peptide is cleaved off by Sec-protease. Following the removal of the signal peptide, folding, and release of the mature proteins into the periplasm, these proteins may undergo further modifications, such as disulfide bond formation or subunit assembly, before they are translocated across the outer membrane via the actual type II secretion apparatus also termed the secreton. Genes encoding the secretion apparatus and the secreted proteins themselves are usually clustered. The type II secreton includes two outer membrane components: GspD and GspS (Russel, 1998; Pugsley *et al*., 1997). GspD is an integral outer membrane protein and GspS is a small lipoprotein required in at least some type II systems for proper targeting and insertion of GspD in the outer membrane (Nouwen *et al*., 1999).

Most of the other secreton components are associated with the inner membrane (Russel, 1998): GspG, H, and J with limited homology to the type IV pilus structural subunit, pilin (Strom *et al*., 1991), and GspC, F, K, L, M, and N with extensive periplasmic domains. GspE is a cytoplasmic protein that localizes to the inner membrane via interaction with GspL (Sandkvist *et al*., 1995; Py *et al*. 1999). GspE contains a conserved ATP-binding motif and has autokinase activity (Sandkvist *et al*., 1995; Pssot and Pugsley, 1997). GspE may regulate secretion or energize the secretion process and/or assembly of the secreton. This leads to a model in which GspE uses ATP to effect conformational changes in the inner membrane proteins that are transmitted to the periplasmic domains and then to the outer membrane. GspC, which fractionates with both the inner and outer membranes, may be responsible for energy transduction to GspD in the outer membrane (Thanassi *et al*., 2000).

Type III secretion pathways capable of translocating anti-host factors into targeted eukaryotic cells have been identified in a number of animal and plant pathogens (Hueck, 1998). The injected proteins often resemble eukaryotic factors with signal transduction functions and are capable of interfering with eukaryotic signaling pathways. Like the type I secretion pathway, type III secretion is Sec-independent and does not exhibit a periplasmic intermediate. The type III secretion apparatus is composed of approximately 20 proteins that assemble into a large structure that spans both, inner and outer membrane, and possibly the host cell membrane as well. Type III secretion requires a cytoplasmic, probably membrane-associated ATPase. The majority of type III components is thought to localize to the inner membrane and are closely related to components of the flagellar basal body (Hueck, 1998). Secretion of *Yersinia* outer proteins (Yops) by *Yersinia* spp. represents the prototypical type III secretion pathway, allowing the bacteria to disturb host defense mechanisms (Galan and Bliska, 1996). A variety of gram-negative phytopathogenic bacteria use type III secretion for pathogenesis, too. The *hrp*–encoded type III secretion pathway has revealed the mechanisms by which phytopathogenic bacteria infect plants (Lindgren *et al*., 1986). Furthermore, several proteins have been observed to be secreted via the type III pathway to the supernatant of in vitro-grown cultures of *P. syringae* (Yuan and He, 1996).

The type IV secretion pathway is prototyped by the IncP and IncN bacterial conjugation systems and the VirB system of *Agrobacterium tumefaciens* that facilitates translocation of oncogenic T-DNA into plant cells (Burns, 1999). A type IV system is also required for pertussis toxin (PT) secretion by *Bordetella pertussis*, the causative agent of whooping cough (Segal *et al*., 1998; Weiss *et al*., 1993; Vogel *et al*., 1998; Covacci *et al*., 1997). Most information regarding type IV secretion comes from studies focused on the VirB system. VirB proteins are membrane-associated, interact with each other, and are required in multiple copies for proper assembly. A subset of VirB proteins consisting of VirB3, 4 and 7-10 facilitates transfer of conjugal plasmid DNA and may represent a core type IV secretion system (Bohne *et al*., 1998). VirB4 oligomerization, but not its nucleotide-binding activity is required for function of this minimal apparatus (Dang *et al*., 1999). Pertussis toxin secretion requires nine gene products of the *ptl* locus, all with homologous in the *virB* locus of *A. tumefaciens*, which contains eleven genes. DNA secretion by the VirB and conjugal systems is thought to occur in a single step from the cytoplasm to the outside of the cell. However, pertussis toxin secretion might occur in two steps, with toxin subunits first crossing the inner membrane via the Sec system (Burns, 1999).

2.7 The Dsb system

Most of the proteins exported from the cytosol of prokaryotes and eukaryotes contain disulfide bonds, which are involved in stabilizing their tertiary structures. For several bacterial proteins which are secreted via the type II mechanism, folding in the periplasm is essential for the subsequent translocation across the outer membrane (Pugsley, 1992; Bortoli-German *et al*., 1994; Hardie *et al.,* 1995). The formation or isomerization of disulfide bonds is a slow process requiring catalysis. Protein disulfide bonds are formed in gram-negative bacteria through the action of so called Dsb (disulfide bond forming) enzymes that are located or have their catalytic sites directed towards the periplasmic space (Raina and Missiakas, 1997; Rietsh and Beckwith, 1998).

The Dsb system consists of at least five redox proteins belonging to the thioredoxin superfamily. In *E. coli* formation of disulfide bonds is catalyzed by the thiol-disulfide oxidoreductase DsbA (Bardwell *et al*., 1991; Kamitani *et al*., 1992). DsbA acts as a donor of disulfides to newly synthesized periplasmic proteins. Studies with purified DsbA in vitro showed that it is an extremely efficient catalyst of disulfide bond formation (Akiyama *et al*., 1992; Wunderlich *et al*., 1993; Zapun *et al*., 1993). DsbC is another protein, which acts as a disulfide isomerase (Sone *et al*., 1997; Zapun *et al*. 1995). Because of DsbA and DsbC as well as the oxidative environment, the periplasm provides an adequate compartment for generation of proteins with multiple disulfide bonds (Baneyx, 1999; Georgiou and Valax, 1996). However, periplasmic proteins with multiple disulfide bonds often occur in low yield or are inactive (Baneyx, 1999; Wulfing and Pluckthun, 1994). This may result from limited or incorrect formation of disulfide bonds in the target proteins because of low activity of the disulfide isomerase DsbC (Joly and Swartz, 1994) and/or incorrect formation of disulfide bonds (Joly and Swartz, 1997; Rietsch *et al*., 1996). Additionally, DsbB and DsbD modulate DsbA and DsbC activities, respectively (Bardwell *et al*., 1993; Missiakas *et al*., 1993; Missiakas *et al*., 1995). More recently, DsbG of *E. coli* was described as a novel member of the Dsb family, which oxidizes so far unknown substrate(s) (Andersen *et al*., 1997; Besette *et al*., 1999). All of these Dsb enzymes have the conserved active site motif Cys-X-X-Cys and seem to be common to all investigated gram-negative bacteria (Raina and Missiakas, 1997).

Doubtlessly, the process of protein secretion in gram-negative bacteria is related to the function of the Dsb system. Because the laboratory strain *E. coli* K12 does not secrete protein into the extracellular medium via the GSP under standard laboratory growth conditions (Francetic and Pugsley, 1996), most studies focused thus far on other gram-

negative bacterial species. The DsbA-dependent disulfide bond formation has been described to be essential for pectate lyases and cellulase EGZ in *Erwinia chrysanthemi* (Bortoli-German *et al*., 1994; Shevchik *et al*., 1995), extracellular elastase and lipase in *Pseudomonas aeruginosa* (Braun *et al*., 2001; Urban *et al*., 2001), and cholera toxin and hemagglutinin-protease in *Vibrio cholerae* (Peek and Taylor, 1992).

2.8 The GacS sensor kinase

The GacA-GacS (global regulator for antibiotics and cyanide production) two-component global regulatory system is widely distributed in gram-negative bacteria. This regulatory system is composed of GacS (previously also called ApdA or LemA), which is a histidine protein kinase (Barta *et al*., 1992), and the cognate response regulatory GacA, which has significant homology to members of the FixJ family of regulatory proteins (Rich *et al*., 1994).

The histidine kinase GacS is an important positive regulator of gene expression for virulence factor syntheses involved in regulating virulence factors in both plant and animal pathogens. In *P. syringae* pv. syringae, the causal agent of bacterial brown spot of bean, GacS has been demonstrated to regulate bacterial swarming, the production of the phytotoxin syringomycin, extracellular protease, and *N*-acyl-L-homoserine lactone in addition to other undefined gene products that are required for pathogenicity (Barta *et al*., 1992; Rich *et al*., 1992; Kinscherf and Willis; 1999; Kitten *et al*., 1999). Moreover, GacS is required for the production of pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, HCN, extracellular protease(s), and tryptophan side chain oxidase (TSO) in *P. fluorescens* (Corbell and Loper 1995; Gaffney *et al*., 1994; Laville *et al*., 1992).

GacS also plays a role as a regulator of synthesis of pectate lyase (Liao *et al*., 1994), cellulase (Frederick *et al*., 1997), and the extracellular polysaccharide alginate (Castaneda *et al*., 2000; Willis *et al*., 2001; Parkins *et al*., 2001) in *Azotobacter vinelandii, P. syringae, P. aeruginosa*. As a major virulence factor, alginate biosynthesis has been extensively studied in both *P. aeruginosa* and *P. syringae.* Recent reports provided evidence that mutation of *gacS* significantly reduced alginate production and transcription of *algD*, the gene coding for the key enzyme GDP-mannose dehydrogenase of the alginate biosynthetic pathway in *Azotobacter vinelandii* (Castaneda *et al*., 2000). Additionally, mutation of *gacS* has a general effect on microbe-host interactions (Grewal *et al*., 1995; Liao *et al*., 1994; Willis *et al*., 1990). The ability of a *P. syringae gacS* mutant to persist in the field setting

was greatly reduced although the bacteria appeared to grow *in planta* nearly as well as the wild type (Hirano *et al*., 1997).

2.9 Aim of this study

Previously, the biochemical characteristics of levansucrase from *P. syringae* pv. phaseolicola were investigated (Hettwer *et al*., 1995) and two genetic loci coding for this enzyme in *P. syringae* pv. glycinea and *P. syringae* pv. phaseolicola were identified (Hettwer *et al*., 1998). In the latter study, levansucrase of *P. syringae* could be expressed in *Escherichia coli*. Furthermore, it was shown that both enzymes were transported into the periplasm of *E. coli* without lethal effects. The optimal temperature for levan formation by purified levansucrase of *P. syringae* pv. phaseolicola was 18°C (Hettwer *et al*., 1995).

In contrast to levan synthesis in *Erwinia amylovora* (Geier and Geider, 1993), little is known about the regulation of levan formation in *P. syringae*. A report on the in *vitro* variability of levan formation in various *P. syringae* isolates (Fett *et al*., 1989) showed that environmental factors, such as temperature, influence the composition of total bacterial EPS.

Aims of the current study were the cloning and expression of two new *lsc* genes from PG4180 in *Escherichia coli*, determination of their nucleotide sequences, analysis of cell compartment-specific levansucrase activities in PG4180 mutants impaired in individual *lsc* genes, and analysis of their distribution in other pathovars of *P. syringae.* Moreover, transcription of levansucrase genes and secretion of the respective gene products in dependence of temperature were to be analyzed. In addition, studies on the virulence phenotype and the *in planta* survival of a levan-deficient mutant of PG4180 on soybean plants needed to be carried out.

Secreted proteins often form disulfide bonds in the oxdizing environment of periplasm via the assistance of the Dsb system. LscB contains three cysteines whereas LscC contains four cysteines. In the present study, the identification and cloning of *dsbA* and *dscC* in *P. sringae* PG4180 for further mutational analysis was another aim.

Of additional interest to our laboratory was the global sensor kinase GacS, which could potentially influence expression of virulence factors. Therefore, the *gacS* gene of PG4180 was to be cloned and knocked-out, and a *gacS* mutant was to be analyzed in terms of temperature-dependent secretion of levansucrase.

3 MATERIALS

3.1 Apparatus used in this study

Tab. 1. **Equipment used in this study**

Equipments	Name	Manufacturer
Centrifuge with rotors	RC 5B + SS34 20,500 rpm max SLA-3000 12,000 rpm max	SORVALL, Bad Homburg
Dryer	Slab Gel Dryer (SGD 2000)	Savant, Frankfurt
Electrotransfer apparatus	Mini Trans-blot Electrophoretic Transfer Cell	BioRad, München
Electrophoreses-Chambers	Agagel Mini Agagel Midi-Wide	Biometra, Göttingen
Electroporation apparatus	GenePulser II	BioRad, München
Fluorometer	Fluorolite 1000	Dynatech Laboratories, Denkendorf
Microcentrifuge	Centrifuge 5415 C	Eppendorf, Hamburg
Microplate reader	MRX	Dynatech Laboratories, Denkendorf
PCR machines	Hybaid Touchdown	Hybaid, Middlesex; England
	GeneAmp PCR System 2400	Perkin Elmer, Norwalk, USA
pH meter	CG840B	Schott-Geräte, Hoheim
Polaroid camera	MP 4+	AGS, Heidelberg
Power supply	Power Pack P200/Pack P3000	BioRad, München
RNA/DNA calculator	Gene Quant	Pharmacia Biotech, Freiburg
SDS-PAGE apparatus	Mini Protean II; Protean II xi	BioRad, München
Sequence apparatus	LICOR Model 4000	MWG Biotech, Ebersberg
	ALF *express*	Amersham Pharmacia Biotech, Freiburg
Thermomixer	Eppendorf Thermomixer 5436	Eppendorf, Hamburg
Ultrasonic Appartus	Type UW 70	BANDELINelectronic, Berlin
UV/Visible spectrophotometer	Ultrospec 2100 *pro*	Amersham Pharmacia Biotech, Freiburg

UV-Cross-Linker	UV Stratalinker 2400	Stratagene, Heidelberg
Vacu blot apparatus	Vacu-Blot VB11	Biometra, Göttingen
Freeze dryer	Freeze dryer MicroModulyo	Edwards, England

3.2 Chemicals and Enzymes

Chemicals were purchased from Bio-Rad (München), Biomol (Hamburg), Boehringer Mannheim (Mannheim), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen). Enzymes were purchased from Amersham-Pharmacia Biotech (Freiburg) and Boehringer-Mannheim (Mannheim).

3.3 Kits

Tab. 2. **Kits used in the study.**

Kit	Manufacturer
DIG DNA Labeling and Detection Kit	Roche, (Mannheim)
Gluco-quant Glucose/HK assay kit	Roche, (Mannheim)
PCR purification kit	Qiagen (Hilden)
Taq PCR Core	Qiagen (Hilden)
Thermo Sequenase fluorescent labelled primer cycle sequencing kit	Amersham-Pharmacia Biotech (Freiburg)
QIAEX II	Qiagen (Hilden)

3.4 Antibodies

Polyclonal antibodies raised against levansucrase derived from *P. syringae* pv. phaseolicola were provided by U. Hettwer and K. Rudolph (Univ. Göttingen, Germany). The specificity of the levansucrase antiserum at a dilution of 1:3,000 was evaluated with recombinant levansucrase protein from *E. coli* and crude protein extracts of PG4180. For signal detection, secondary anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Sigma, Darmstadt, Germany) were used at a concentration of 1:7,500 and the reaction was visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt.

3.5 Media

Ingredients were added to water by stirring until dissolved, poured into bottles with loosen caps and autoclaved 20 min at 15 lb/in^2. For solid media, 1.5% (w/v) agar was added to medium.

3.5.1 Media for *Escherichia coli*

LB-Medium pH 7.0

Luria-Bertani-Medium, (Sambrook *et al.*, 1989)

	10	g	Bacto-Trypton
	10	g	NaCl
	5	g	Bacto-yeast extract
add	1	liter	H_2O

SOC-Medium (Sambrook *et al.*, 1989)

20	g	Bacto-Trypton
1	g	Bacto-yeast extract
5	g	NaCl
1	ml	1 M KOH stock solution

Sterilize, then add

1	ml	1 M $MgCl_2$ stock solution

3.5.2 Complex media for *Pseudomonas syringae*

KB-Medium pH 7.2

King´s B Medium, (King *et al.*, 1954)

	20	g	Pepton
	1.5	g	K_2HPO_4
	1.5	g	$MgSO_4$ x 7 H_2O
	10	ml	Glycerin
add	1	liter	H_2O

3.5.3 Minimal media for *Pseudomonas syringae*

HSC-Medium pH 6.8

Hoitink Sinden medium (optimized for coronatine synthesis, Palmer & Bender, 1993)

Part A:		1	g	NH_4Cl
		0.2	g	$MgSO_4$ x 7 H_2O
		4.1	g	KH_2PO_4
		3.6	g	K_2HPO_4
		0.3	g	KNO_3
		10	ml	$FeCl_3$ (2 mM)
	add	0.9	liter	H_2O
Part B:		20	g	Glucose
	add	0.1	liter	H_2O

Sterilize separately, then mix both parts 9:1 after the solutions cooled to 60°C or less.

MG-Medium pH 7.0

Mannitol Glutamat Medium, (Keane *et al.*, 1970)

10	g		Mannitol
2	g		L-Glutamic acid
0.5	g		KH_2PO_4
0.2	g		NaCl
0.2	g		$MgSO_4$ x 7 H_2O
add 1	liter		H_2O

3.6 Antibiotics

Tab. 3. **Antibiotics used in the study**

Antibiotics	Concentration	End concentration in medium
Ampicillin	50 mg/ml	50 mg/l
Chloramphenicol	25 mg/ml	25 mg/l
Gentamycin	25 mg/ml	2,5 mg/l
Kanamycin	25 mg/ml	25 mg/l
Spectinomycin	25 mg/ml	25 mg/l
Streptomycin	25 mg/ml	25 mg/l
Tetracycline	25 mg/ml	25 mg/l

3.7 Computer software

Tab. 4. **Computer softwares used in this study.**

Name	Manufacturer, provider
DNA-STAR	Lasergene, USA
BLASTN, BLASTP, TBLASTN, BLASTX, FASTEMBL, BESTFIT	University of Wisconsin, Genetic Computer Group
SignalP version 1.1 program	Center for Biological Sequence Analysis, Dept. Biotechnology, Technical University of Denmark
Vector NTI	Informax Inc., USA
MS Office	Microsoft, USA

3.8 Microorganisms

Tab. 5. **Bacterial strains used in this study.**

Bacterial strain	Relevant characteristics	Reference or source
Escherichia coli DH5α	F^-/ *endA1*, *deoR,* (ϕ80*dlacΔ(lacZ)M15*), *recA1, gyrA* (Nal^r), *thi-1, hsdR17* (r_K^-, m_K^+), *supE44, relA1, Δ(lacZYA-argF)U169*	(Ausubel *et al.*, 1987)
Pseudomonas syringae pv. glycinea		
PG4180	wild type, $levan^+$	(R.E. Mitchell, 1975)
PG4180.muc	wild type, $Levan^+$, $Alginate^+$, mucoid	(Bender, 2001)
PG4180.M1	*lscA* mutant, Sm/Sp^r	(Jaeckel, 1999)
PG4180.M2	*lscB* mutant, Gm^r	This study
PG4180.M3	*lscA lscB* mutant, Sm/Sp^r, Gm^r	This study
PG4180.M4	*lscC* mutant, Gm^r	This study
PG4180.M5	*lscA lscC* mutant, Sm/Sp^r, Gm^r	This study
PG4180.M6	*lscB lscC* mutant, Gm^r, Sm/Sp^r	This study
PG4180.ML	*gacS* mutant of PG4180, Sm/Sp^r	This study
PG4180.ML2	*gacS* mutant of PG4180.muc, Sm/Sp^r	This study
Psg 7a/90, 16/83,	$Levan^+$	(Ullrich.*et al.,*1993)
P. syringae pv. savastanoi		
GSPB 2264	$Levan^+$	GSPB[a]
GSPB 2259	$Levan^+$	GSPB
P. syringae pv. phaseolicola		
NCPPB1321	$Levan^+$	Hettwer, 1998

GSPB 796	Levan$^+$	GSPB
Psph 6/0	Levan$^+$	B. Völksch
P. syringae pv. morsprunorum		
D5	Levan$^+$	K. Naumann
GSPB886, 1013	Levan$^+$	GSPB
Pm 7	Levan$^+$	A. Jones
P. syringae pv. atropurpurea		
MAFF 301309	Levan$^+$	B. Völksch
P. syringae pv. syringae		
FF5	Levan$^+$	G.W. Sundin
B301D	Levan$^+$	D. C. Gross
Pss B48	Levan$^+$	G.W. Sundin
Pss 3525	Levan$^+$	G.W. Sundin
P. syringae pv. tomato		
DC3000	Levan$^+$	D. Cuppels
DSM 50315	Levan$^+$	DSM[b]
GSPB 119	Levan$^+$	GSPB
P. syringae pv. maculicola GSPB 2145	Levan$^+$	GSPB
P. syringae pv. pisi GSPB 104	Levan$^+$	GSPB
P. syringae pv. lachrymans GSPB 77	Levan$^+$	GSPB
P. syringae pv. coriandricola GSPB 1784	Levan$^+$	GSPB
P. syringae pv. photiniae CFBP 11034	Levan$^+$	CFBP[c]
P. syringae pv. myricae CFBP 11005	Levan$^+$	CFBP
P. syringae pv. persicae GSPB 1025	Levan$^+$	GSPB
P. syringae pv. hibisci CFBP 11294	Levan$^+$	CFBP
P. syringae pv. mellea CFBP 2344	Levan$^+$	CFBP
P. syringae pv. striafaciens GSPB 1850	Levan$^+$	GSPB
P. syringae pv. helianthi GSPB 2688	Levan$^+$	GSPB
P. syringae pv. zizaniae CFBP 11040	Levan$^+$	CFBP
P. syringae pv. pisi GSPB 104	Levan$^+$	GSPB
P. syringae pv. apii GSPB 2153	Levan$^+$	GSPB

[a]GSPB, Göttinger Sammlung phytopathogener Bakterien, Germany
[b]DSM, Deutsche Sammlung für Mikroorganismen, Germany
[c]CFBP, Collection Francaise des Bacteries Phytopathogenes, France

3.9 Plasmids

Tab. 6. **Plasmids used in this study**

Plasmid	Relevant characteristics	Reference/source
pBlueskript II SK$^+$	Amp^r, cloning vector	Stratagene, Heidelberg
pBBR1MCS	Cm^r, broad host-range cloning vector	(Kovach *et al.*, 1994)
pRK415	Tc^r, RK2-derived broad host-range cloning vector	(Keen, 1988)
pMAL-c2	Amp^r, ColE1 origin, *tac* promoter, encodes *malE*	New England Biolabs, Schwalbach

pTYB1	Amp^r, cloning vector	New England Biolabs, Schwalbach
pRK2013	Km^r; mob^+, tra^+; helper plasmid for conjugation	(Figurski and Helsinki, 1979)
pCAM140	Amp^r, Sm/Sp^r, contains 2.0-kb *Eco*RI fragment with Sm/Sp^r cassette and 2.0-kb *Not*I fragment with promoterless *uidA* gene	(Wilson *et al.*, 1995)
pK18mobGII	Km^r, mobilizable suicide vector	(Katzen *et al.*, 1999)
pRG960sd	Sm/Sp^r, contains promotorless *uidA* with start codon and Shine-Dalgarno sequence	(Van den Eede *et al.*, 1992)
pPHO7	Ap^r; contains promoterless *phoA* without signal peptide and a ribosome-binding site	(Gutierrez and Devedjian, 1989)
pMGm	Gm^r, contains 1.9-kb Gm^r-cassette	(Murillo *et al*, 1994)
pSKL3	Amp^r, contains *lscA* on 3.0-kb *Pst*I fragment (P_{lac}>*lscA*), $levan^+$	(Hettwer *et al*, 1998)
pRA3.1	Tc^r, contains *lscA* under control of P_{lac} on 3.0-kb *Pst*I fragment from pSKL3 in pRK415, $levan^+$	(Jaeckel, 1999)
p7C7	Tc^r, genomic library clone of PG4180 with 25-30 kb insert in pRK7813, contains *lscB*, $levan^-$	This study
pLB7.2	Amp^r, contains 7.2-kb *Eco*RV fragment from p7C7 in pBluescript (*lscB*>P_{lac}), $levan^-$	This study
pLB7.2R	Amp^r, contains 7.2-kb *Eco*RV fragment from p7C7 in pBluescript (P_{lac}>*lscB*), $levan^-$	This study
pRB7.2	Cm^r, carries *lscB* on 7.2-kb *Eco*RV fragment from pLB7.2 in pBBR1MCS, $levan^-$	This study
pLB2.4	Amp^r, contains 2.4-kb *Pst*I/*Sal*I fragment from pLB7.2 derived by PCR in pBluescript (P_{lac}>*lscB*), $levan^+$	This study
pLB7.2-Gm	Amp^r, Gm^r, contains *lscB* mutagenized by insertion of Gm^r cassette inserted in *Xho*I site of pLB7.2, $levan^-$	This study
pKB7.2-Gm	Km^r, Gm^r, contains *lscB* mutagenized by insertion of Gm^r cassette on 9.2-kb *Sal*I/*Pst*I fragment from pLB7.2-Gm in pKmobGII, $levan^-$	This study
p5C10	Tc^r, genomic library clone of PG4180 with 25-30 kb insert in pRK7813, contains *lscC*, $levan^-$	This study
pLC5.5	Amp^r, contains 5.5-kb *Pst*I fragment from p5C10 in pBluescript (*lscC*>P_{lac}), $levan^-$	This study
pLC5.5-Gm	Km^r, Gm^r, contains *lscC* mutagenized by insertion of Gm^r cassette in *Xho*I site of pLC5.5, $levan^-$	This study
pKC5.5-Gm	Km^r, Gm^r, contains *lscC*:Gm^r on 7.5-kb *Sal*I fragment from pLC5.5-Gm in pKmobGII, $levan^-$	This study
pLC5.5-Sm	Amp^r, Sm/Sp^r, contains *lscC* mutagenized by insertion of Sm/Sp^r cassette in *Xho*I site of pLC5.5, $levan^-$	This study

pKC5.5-Sm	Km^r, Sm/Sp^r, contains *lscC*:Sm/Sp^r on 7.5-kb *Sal*I fragment from pLC5.5-Sm in pKmobGII, $levan^-$	This study
pLB-uidA	Amp^r, carries the *uidA* gene from pCAM140 inserted in *Xho*I sites on pSKLB, $levan^-$	This study
pRB-BG	Tc^r, contains a *lscB::uidA* reportergene fusion on pRK415	This study
pTYB-uidA	Amp^r, carries the *uidA* gene from pCAM140 inserted in *Not*I sites on pTYB1	This study
pLC-uidA	Amp^r, carries the *uidA* gene from pCAM140 inserted in *Xho*I sites on pLC5.5, $levan^-$	This study
pRK-CG	Tc^r, contains a *lscC::uidA* reportergene fusion on pRK415	This study
pMal-lscB	Amp^r, contains *lscB* on a 1.3-kb *Bam*HI fragment; derived from pLB7.2 by PCR cloning in pMAL-c2	This study
pMal-lscC	Amp^r, contains *lscC* on a 1.3-kb *Bam*HI fragment; derived from pLC5.5 by PCR cloning in pMAL-c2	This study
pAS-lacZ	Cm^r, contains translational *corR*::*lacZ* fusion in pBBR1MCS, $LacZ^+$	(Smirnova, 2001)
pLC-PhoA	Amp^r, contains translational *lsc*::*phoA* fusion in pBluescript SK+	This study
pHL-PhoA	Tc^r, contains translational *lsc*::*phoA* fusion in pRK415, $PhoA^+$	This study
p2D7	Tc^r, genomic library clone of PG4180 with 25-30 kb insert in pRK7813, contains *gacS*	This study
pBluelemA6.3	Amp^r, contains *gacS* on a 6.3-kb *Ava*I fragment in pBluescript SKII	This study
pKLemA6.3	Km^r, contains *gacS* on a 6.3-kb *Xho*I/*Bam*HI fragment in pKmobGII	This study
pKLemA6.3-Sp	Km^r, Sm/Sp^r, contains *gacS*:: Sm/Sp^r on a 6.3-kb *Xho*I/*Bam*HI fragment in pKmobGII	This study
pEMH97	Tc^r, contains *gacS* gene on 9.7 kb *Hind*III fragment from *Pseudomonas syringae* pv. syringae	(Hrabak and Willis, 1992)
p7H1	Tc^r, genomic library clone of PG4180 with 25-30 kb insert in pRK7813, contains *dsbA*	This study
p8G3	Tc^r, genomic library clone of PG4180 with 25-30 kb insert in pRK7813, contains *dsbC*	This study

3.10 Oligonucleotides

Oligonucleotides were synthesized by MWG Biotech (Ebersberg).

Tab. 7. **Oligonucleotides used for PCR with their restriction enzyme recognition sites.**

Name)	**Strand)[1]**	**Nucleotide sequences)[2]**	**Recognition site**
lscA-F	s	5'-ATG AGT AAC ATC AAT TAC-3′	-
lscA-R	as	5'-TCA GCT CAG CAC CAC GTT CT-3′	-
Lsc-F5	s	5′-ATG TCC ACT AGC AGC TCT-3′	-
Lsc-R	as	5′- TCA GCT TAG CGT CAC GTC -3′	-
Lsc-F5b	s	5′- TCG CTG CAG ATG TCC ACT AGC AGC TCT -3′	*Pst*I
LscB-FC	s	5′- TCA CTG CAG GCC CTA GCG CTG ACC AAA -3′	*Sal*I
Lsc-R5	as	5′-CGA CTG CAG TCA GCT TAG CGT CAC GTC -3′	*Pst*I
LscF8	s	5'-TCA CTG CAG GCC CTA GCG CTG ACC AAA -3′	*Pst*I
LscF9	s	5′-TCG CTG CAG GTT TTC CAT GTA CAC CTC-3′	*Pst*I
LscR9	as	5′-CGA GTC GAC TCA GCT CAG TTG CAC GTC -3′	*Sal*I
LscBFBam	s	5′-TCG GGA TCC ATG TCC ACT AGC AGC TCT-3′	*Bam*HI
LscBRBam	as	5′-CGA GGA TCC TCAGCTCAGTTGCACGTC -3′	*Bam*HI
LscCRBam	as	5′-CGA GGA TCC TCA GCT CAG TTG CAC GTC-3′	*Bam*HI
DsbAF	s	5′-CCC ATC GAG TCC GGC AAA CAA TA -3′	-
DsbAR	as	5′-TGA TAC CAG AAC GGG AAA -3′	-
DsbAF1	s	5′-CGC CAG TCT GTT CGG TAT GTC -3′	-
DsbAR1	as	5′-AGG AGT CGA ACG TCT TGA GGA -3′	-
DsbCF	s	5′-GTA TCA CGC ACG CTC CTG T -3′	-
DsbCR	as	5′-CAA CCT GAC CGA AAA GAC C -3′	-
LemAF1	s	5′-AAG TTC ACC CGC GAA GGC AC -3′	-
LemAR1	as	5′-GTC GGG CAC AGT ACC ATC ACC T -3′	-
T7	as	5'-TAATACGACTCACTATAGGGAGGAGGATTACCCCTCGA-3′	-
Lsc-fwd	a	5'- GTC AGT GCG GAC TTT CCG GTC ATG –3'	-
LscR-T7	as	5'- TAATACGACTCACTATAGGGAGGGATCGCGAAAGTT CCAGCT -3'	-

gusF	s	5′-TGAATCCGCACCTCTGGCAA-3’	-
gusR-T7	as	5’- TAATACGACTCACTATAGGGAGGCAATACTCCACAT CACCAC -3’	-

)[1] „s“ *sense*-strand, „as“ *antisense*-Strand.

)[2] restriction enzyme sites underlined.

Tab. 8. **Cy5-labeled oligonucleotides for sequencing.**

Name)	**Strand)[1]**	**Nucleotide sequences**
T3	s	5’-AAT TAA CCC TCA CTA AAG GG -3′
T7	as	5’-TAA TAC GAC TCA CTA TAG GG -3′
LscBFL-1	s	5′-ATG TCCACT AGC AGC TCT -3′
LscBRL-1	as	5′-TCA GCT TAG CGT CAC GTC -3′
LscBFL-2	s	5′-ATC GTA TCG GCG GGA CC -3′
LscBRL-2	as	5′-CAG ACA GTG GGT TCG TAG TTG ATG -3′

)[1] „s“ *sense*-strand, „as“ *antisense*-Strand.

4 METHODS

4.1 Bacterial growth conditions

4.1.1 Growth conditions for *Escherichia coli*

Escherichia coli were routinely cultivated in LB medium plates at 37°C. Single colonies were streaked out and liquid cultures were carried out in LB medium containing the appropriate antibiotics at 37°C with 250 rpm shaking for overnight.

4.1.2 Growth conditions for *Pseudomonas syringae*

Pseudomonas strains were maintained on solid mannitol-glutamate medium (MG) at 28°C. Single colonies of *P. syringae* grown on MG agar for 96 h were resuspended in 5 ml of King's B (KB) medium and incubated overnight on a rotary shaker at 280 rpm and 28°C. Subsequently, this overnight culture was used to inoculate HSC medium or KB medium, which was incubated on a rotary shaker at 280 rpm and at 18°C for 24 to 48 h or at 28°C for 12 to 24h.

4.1.3 Storage of bacterial strains

Freshly grown bacteria were suspended in 1 ml of 15% sterile glycerol, mixed and stored at –80°C.

4.2 DNA manipulations

4.2.1 Isolation of plasmid DNA from *E. coli* cells

The so-called 1-2-3-preparation is a rapid method for isolation of plasmid DNA (Birnboim and Doly, 1979). 1.5 ml *E. coli* overnight-culture was centrifuge at 13,000 g for 1 min, the cell pellet was resuspended in 150 µl buffer P1. After 150 µl buffer P2 was added, the sample was shaken carefully and incubated at room temperature for 5 min, then 150 µl buffer P3 was added and the sample was incubated on ice for 10 min. After centrifugation at 13,000 g for 15 min, the supernatant was pipetted into a new tube and the DNA was precipitated with 0.7 vol. isopropanol and 0.1 vol. 3 M NaAcetate. At last, the pellet was washed with 70% ethanol, dried, and resuspended in 25 µl TE buffer.

Relatively large quantities of plasmid DNA from *E. coli* clones were prepared according to Birnboim and Doly (1979) using Qiagen Midi plasmid purification kits. Bacteria were grown in 25 ml (for high-copy plasmids) or 100 ml (for low-copy plasmids) of LB medium at 37°C and 250 rpm overnight. After centrifugation at 6,000 g at 4°C for 15 min, the cell pellet was resuspended in 4 ml buffer P1. 4 ml buffer P2 was added. The sample was then mixed gently and incubated at room temperature for 5 min. 4 ml buffer P3 was added and the sample was mixed and incubated on ice for 15 min. Centrifugations at 13,000 g, 4°C for 30 min and 15 min were carried out and the supernatant was secured. The supernatant was applied to an equilibrated QIAGEN-tip (equilibrated with 4 ml buffer QBT). The QIAGEN-tip was then washed with 2 x 10 ml of buffer QC and the DNA was eluted with 5 ml buffer QF. Then the DNA was precipitated with 3.5 ml isopropanol (room temperature) and washed with 2 ml 70 % ethanol. At last, the DNA was dried, redissolved in 250 µl TE Buffer and stored at –20°C.

P1-Buffer:		**P2-Buffer:**		**P3-Buffer:**
50 mM	Tris/HCl, pH 8.0	200 mM	NaOH	3.0 M potassium acetate, pH 5.5
10 mM	EDTA	1 %	SDS	
100 µg / ml	RNase A			

QBT-Buffer:		**QC-Buffer:**		**QF-Buffer:**	
750 mM	NaCl	1 M	NaCl	1.25 M	NaCl
50 mM	MOPS, pH 7.0	50 mM	MOPS, pH 7.0	50 mM	Tris/HCl, pH8.5
15 %	isopropanol	15 %	isopropanol	15 %	isopropanol
0.15 %	Triton X-100				

4.2.2 Isolation of plasmid DNA from *P. syringae* cells

A single colony of *P. syringae* was inoculated to 5 ml KB medium. 1.5 ml overnight-culture was centrifuged at 13,000 g for 1 min, the cell pellet was resuspended in 166 µl E-buffer. 333µl lysis buffer was added, and then the sample was gently mixed for 1 min and incubated at 65°C for 40 min. After that, 500µl phenol and 500 µl chloroform was added followed by gentle mixing for 1 min. Subsequently, centrifugation was carried out at 13,000 g for 10 min. The supernatant containing the plasmid DNA was carefully taken to a

fresh tube. 2 µl of 6× loading buffer was added to 25 µl of supernatant sample and loaded on a 0.8 % agarose gel. The electrophoresis was done at 45-55 V for 4-5 h.

E-buffer		**Lysis buffer**	
40 mM	TrisHCl pH 7.9	50 mM	Tris/HCl pH 12.6
2 mM	EDTA	3 %	SDS

4.2.3 Isolation of total genomic DNA from *P. syringae*

4.2.3.1 Chloroform extraction

Isolation of total genomic DNA from *P. syringae* according to a standard preparation protocol (Ausubel *et al.*, 1987) resulted in high yields of DNA. Cells of a 1.5-ml bacterial culture (OD_{600} = 2.5) were centrifuged and the pellet was resuspended in 567 µl TE buffer (10mM Tris-Cl, 1 mM EDTA, pH8.0). After addition and mixture of 30 µl of 10 % SDS and 3 µl of 20 mg /ml proteinase K, the suspension was kept at 37°C for 1 h. To this suspension 100 µl of 5 M NaCl and 80 µl CTAB/NaCl solution (10 % CTAB, 0.7 M NaCl) were added and incubated for further 10 min at 65°C. 700 µl chloroform/isoamyl alcohol (24:1) was added and mixed, followed by centrifugation. The aqueous upper layer was transferred to a fresh tube and mixed with 450 µl isopropanol. After centrifugation, the precipitate was washed with 70 % ethanol. The DNA pellet was collected by centrifugation and resuspended in 20 µl TE buffer.

4.2.3.2 QIAamp procedure

The QIAamp tissue kit (Qiagen, Hilden) provides a fast and easy way to purify total DNA from *P. syringae*. Bacterial pellets from 1.5 ml overnight culture were resuspended in 180 µl buffer ATL. Following the addition of 20 µl of proteinase K stock solution, the bacterial cells were lysed by incubation at 55°C for 1 h. 200 µl buffer AL was added to this sample. After incubation at 70°C for 10 min, 210 µl of 95% ethanol was added. The mixture was applied to a QIAamp spin column and centrifuged down at 8000 rpm for 3 min. The QIAamp spin column was washed twice with 500 µl of Buffer AW. The total DNA was eluted in buffer AE. (Buffers ATL, buffer AL, AW, and AE and as well as proteinase K stock solution were included in the QIAamp tissue kit).

4.2.4 Ethanol precipitation of DNA

Contamination by small nucleic acids fragments and proteins can also be reduced to an acceptable level by precipitating the DNA with 0.1 M sodium chloride. To do so, 1/10 volume of 1 M NaCl and 10 volumes of ice-cold ethanol were added to the DNA solution. The sample was incubated at –20°C for 20 min and then centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the DNA pellet was washed in cold 70 % ethanol solution with subsequent centrifugation at 13,000 rpm for 15 min. The DNA was dried in a speed-vacuum centrifuge and dissolved in a proportional volume of sterile water.

4.2.5 DNA electrophoresis through agarose gels

DNA electrophoresis through agarose gel is the standard method to separate, identify, and purify DNA fragments. An agarose gel of 0.8-2 % (*w/v*), was prepared by boiling a weighed amount of agarose in 1× TAE buffer and pouring it into a flat electrophoretic tank. DNA samples were mixed with 1/6 vol. 6× loading buffer, loaded onto the gel and DNA fragments were separated at 10 V/cm for 90 to 300 min in 1× TAE buffer. For UV visualization of DNA, the gel was stained in a solution of ethidium bromide (2 µl/ml in 1× TAE buffer). Photographs were taken at UV light of a wavelength of 312 nm.

1×TAE-Buffer (pH 8.0)

40	mM	TrisHCl
1.3	mM	EDTA
0.47	mM	glacial acetic acid

Loading-Buffer

0.25	%	bromophenol blue
0.25	%	xylene cyanol FF
40	% (w/v)	sucrose

4.2.6 Digestion of DNA with restriction endonucleases

Samples of DNA (200 ng) were incubated with the restriction endonuclease(s) accompanied by appropriate reaction buffers. The amount of enzyme and DNA, the buffer composition and ionic concentration, and the duration of the reaction varied depending upon the specific requirements of the enzyme (in general: 37°C for 2 h to overnight). In case where it was necessary to treat the same DNA sample with different restriction enzymes and buffers, the reaction was first carried out for the restriction enzyme with the

lower salt concentration buffer and then the salt concentration was increased to proceed with the treatment with the second restriction enzyme.

4.2.7 DNA extraction from agarose gels by the QIAEX II kit method

To elute DNA from agarose gels, the QIAEX II extraction system (Qiagen, Hilden) was used as recommended by the manufacturer. The DNA band was excised from an ethidium bromide stained agarose gel (less than 250 mg of gel) and transferred to an eppendorf cup. Buffer QX1 was added in a ratio of 3 µl/mg of gel (for fragments 100 bp – 4 kb). Alternatively, 3 µl buffer QX1 and 2 µl H_2O were added per mg gel (for DNA fragments of more than 4 kb size). Incubation at 50°C for 10 min with occasional mixing was followed by centrifugation at 13,000 rpm for 30 s. Samples were then washed once with 500 µl of QX 1 and two times with 500 µl of PE solution. The final precipitate was air-dried and eluted twice with 20 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8,0) or H_2O by gentle mixing at 37°C. Buffers QX1 and PE were provided within the QIAEX II kit.

4.2.8 Dephosphorylation of digested DNA

Following a restriction digestion, the vector DNA was treated with shrimp alkaline phosphatase (SAP) to remove the phosphate groups from the 5'-ends and to facilitate more efficient cloning of insert DNAs. This prevents self-ligation of the vector DNA. SAP can be added directly to a digestion mix because the SAP buffer is usually compatible with the buffers for restriction endonucleases. 1/10 volume of the SAP buffer and 0.1 units/pmole 5'-ends (final concentration) of SAP were added to a sample. This mix was incubated for 30 min at 37°C to accomplish desphosphorylation. Afterwards, SAP and restriction enzymes were inactivated by heating at 65°C for 15 min. For subsequent cloning steps the DNA was purified.

4.2.9 Klenow filling

If an insert and vector were digested with different restriction enzymes, it was necessary to convert the sticky ends to blunt-ends for the further ligation process. An enzyme suitable for the filling of gaps and for the repair of the termini of double-stranded DNA is Klenow

(DNA Polymerase I Large Fragment) polymerase. Following the digestion with a restriction enzyme, the enzyme was inactivated by heating. 1/20 volume of dNTPs, 1/10 volume of Klenow buffer and 3 to 5 U units of Klenow polymerase were added to the sample which was then incubated at 30°C for 20 min. The reaction was stopped by heating at 75°C for 10 min. For subsequent cloning steps the DNA was purified.

Klenow-Buffer:	**Klenow enzyme**	**dNTP-Mix:**
20 mM Tris/HCl, pH 8.0	3-5 U Klenow fragment	0.5 mM dATP,
100 mM $MgCl_2$		dCTP, dGTP, dTTP

Some other enzymes, for instance *Kpn*I or *Sac*II, generate 3'-overhanging sticky ends. Although the Klenow fragment has a 3'-5' exonuclease activity, T4 DNA polymerase is preferred over Klenow for converting 3'-overhanging ends to blunt ends, because this enzyme exhibits a 250-fold stronger 3'-5' exonuclease activity than Klenow fragment. T4 DNA polymerase catalyzes the synthesis of DNA in the 5'-3' direction and lacks 5'-3' exonuclease activity. 1/20 volume of 0.5 mM dNTPs, 1/10 volume of 10×buffer, 1/10 volume of 0.1 % BSA and 3 to 5 U of T4 DNA polymerase were added to a sample which was then incubated at 16°C in a water bath for 30 min. The reaction was stopped by heating at 75°C for 10 min.

4.2.10 DNA ligation

For sticky end ligation reactions, restriction enzyme-digested insert dsDNA and a proportional amount of linearized plasmid DNA were mixed with one unit of T4 DNA ligase and 1 µl of 10 × T4 DNA ligation buffer in a final volume of 10 µl and incubated at 16°C for at least 12 h. The ligation mixture was then used for transformation of competent *E. coli* cells. For blunt-ended restriction DNA fragments, 10-20 µl of 10 × ligation buffer, 2-3 µl of low (1U/µl) or high concentrated T4 DNA ligase (10 U/µl), and 50-100 µl of sterile water were added to 10 µl of over-night ligation mix. Following an additional incubation for 20 hours, the ligation mix was transformed to competent *E. coli* cells.

4.2.11 Preparation of competent *E. coli* cells using calcium chloride

A single colony of fresh *E. coli* DH5α cells was inoculated in 5 ml of LB-medium and cultured overnight at 37°C. The overnight pre-culture was added to 500 ml of LB medium and cultured at 37°C until bacteria reached the early exponential phase (OD_{600} 0.4-0.5) at which competence of the cells can be efficiently induced for transformation. Following incubation on ice for 10 min, 300 ml of the cells were harvested at 5,500 rpm for 5 min at 4°C and washed with 240 ml of cold sterile 0.1 M $MgCl_2$ solution and 240 ml of cold sterile 0.1 M $CaCl_2$ solution, individually. Finally, cells were suspended in 15 ml of cold sterile 0.1 M $CaCl_2$ solution containing 15% glycerol. Aliquots of 200 µl cells were frozen in liquid nitrogen and stored at –80°C.

4.2.12 Transformation of *E. coli* by heat shock

Bacteria treated with ice-cold $CaCl_2$ solution and then briefly heated at 37°C can be transformed with plasmid DNA (Cohen *et al*., 1972). An aliquot of 100 µl of Ca^{2+}-competent cells was mixed with 10 to 100 µl of ligation mix and incubated on ice for 30 min. Uptake of DNA was induced by heat shock (5 min at 37°C). Following the heat shock, the cells were diluted in 800 µl of pre-warmed LB medium (37°C) and incubated at 37°C for 45 min by shaking at 250 rpm. 50-500 µl of the cell suspensions were plated on LB agar plates containing the appropriate antibiotic(s). Plates were incubated overnight at 37°C until single *E. coli* colonies were visible.

4.2.13 Preparation and transformation of competent *P. syringae* cells by electroporation

A single colony of fresh *P. syringae* cells was inoculated in 5 ml of KB medium and cultured at 28°C until cells reached the early exponential phase (OD_{600} 0.5-0.7). After a short incubation on ice bacteria were harvested at 7,000 rpm for 5 min. The pellet was resuspended in 5 ml of cold sterile 0.5 M sucrose solution and centrifuged again at 5,000 rpm for 3 min. The washing step with sucrose was repeated twice. Finally, cells were resuspended in 0.5 ml of 0.5 M sucrose solution and directly used for electroporation.

100 µl of electro-competent cells were mixed with 0.1-1 µg of plasmid DNA, and exposed to electric shock in a pre-chilled cuvette with a GenePulser-Apparatus (BioRad, München) set to 2.5 kV and 200 Ω. 1 ml of KB medium was added to the cuvette

immediately after the pulse. The cells were then incubated at 28°C for 60 min by shaking at 280 rpm, and plated on MG agar containing the appropriate antibiotic(s). Subsequently, plates were incubated at 28°C for 3-5 days until single *P. syringae* colonies were visible.

4.2.14 Conjugation of plasmid DNA into *P. syringae* by triparental mating

Most of the plasmids used for recombinant DNA research lack conjugative functions. The helper strain *E. coli* HB101 (pRK2013) carries transfer functions (*tra*) necessary for conjugation. Donor plasmids with *mob* function, so called broad-host-range plasmids, were introduced into *P. syringae* in the presence of the helper strain (triparental mating). Recipient *P. syringae* strains were grown for 2-3 days on MG agar plates prior to the conjugation. Approximately one loop full of recipient bacteria was resuspended in 1 ml of sterile water. Subsequently, single colonies of overnight grown donor and helper *E. coli* strains were resuspended in the same suspension. The suspension was mixed well and spotted (10 µl) on a KB agar plate without antibiotics. The conjugation plate was then incubated for 8-16 hours at 28°C. Mating spots were scraped off the plate and resuspended in 1 ml sterile water. Cell suspensions were then diluted from $1:10^{-1}$ to $1:10^{-3}$ in sterile water. 100 µl of this dilution series were plated on MG plates with the appropriate antibiotic(s) to select for transconjugants. Subsequently, plates were incubated at 28° for 3-5 days until single *P. syringae* colonies were visible. The single colonies were re-streaked at least three times on MG plates containing the appropriate antibiotic(s) and the presence of plasmids was determined by DNA plasmid isolation or PCR detection.

4.2.15 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) (Mullis & Faloona, 1987) is a widespread technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. This method is based on the annealing and extension of two specific oligonucleotides (generally in the range of 15 –30 bases) that flank the target region in duplex DNA. After denaturation of the DNA, achieved by heating to 95°C, each oligonucleotide hybridizes to one of the two separated strands so that extension from each 3'-hydroxyl end is directed toward to the other. The annealed oligonucleotides are then extended on the template strand using thermostable DNA polymerase such as *Taq* and a dNTP mixture. The three steps (denaturation, primer

binding, and DNA synthesis by primer extension) constitute a single PCR cycle. Consequently, repeated cycles of these three steps result in the exponential accumulation of a discrete fragment whose termini are defined by the 5′ ends of the two oligonucleotides.

Components for standard PCR reactions (25 µl):

2.5	µl	10 x PCR buffer
4.0	µl	$MgCl_2$ (25 mM)
1.0	µl	dNTP mix (25 mM each)
1.0	µl each	primers A and B (50pM)
0.5	µl	template DNA
0.4	µl	*Taq* DNA polymerase
14.6	µl	distilled H_2O

A typical PCR program was used as following:

Initial denaturation:	94 °C	5 Min	
Denaturation:	94 °C	30 s	25 cycles
Annealing:	50 - 55 °C	30 s	
Extension:	72 °C	1 - 2 Min	
Final extension:	72 °C	7 Min	
	4 °C	∞	

Formula for estimating the melting temperature (T_m):

T_m [°C] = 4°C × (G+C) + 2°C × (A+T).

The annealing temperature used for DNA amplification was 4-5°C below T_m.

4.2.16 QIAquick PCR purification kit

The QIAquick-kit (Qiagen, Hilden) was used to purify PCR products from the reaction mixture which contaminated primers, nucleotides, polymerases, and salts. 5 volumes of buffer PB were added to 1 volume of the PCR reaction and mixed. The sample was applied to a QIAquick spin column in a 2-ml collection tube, and shortly centrifuged to accomplish binding of PCR products to the column. Then the column was washed with 0.75 ml of buffer PE and placed into a 1.5 ml Eppendorf tube. To elute, 30 µl of sterile water was

added to the center of the column surface and after 1-3 min the sample was shortly centrifuged (1 min, 13,000 rpm). DNA samples were stored at –20°C. Buffer PB and PE were supplied in the kit.

4.2.17 DNA sequencing

DNA fragments cloned in a vector (pBluescript II SK) or obtained by PCR without cloning were sequenced manually by the dideoxy-chain termination method (Sanger *et al*., 1977) with the Thermo Sequenase™ fluorescent labelled primer cycle sequencing kit (Amersham-Buchler, Braunschweig). Sequencing primers were Cy5-labelled. The sequencing reactions were carried out by PCR.

1	µl	Cy5 labelled oligonucleotide primer (0.2 pmol)
2	µl	dNTPs and ddNTPs
5	µl	template DNA 0.5-5 µg
1	µl	Sequenase (T7 DNA polymerase)

The thermocycling conditions for the sequencing reactions were:

Initial denaturation	95 °C	5 Min	
Denaturation	95 °C	30 Sec	} 25 cycles
Extensions	60 °C	30 Sec	
	4 °C	∞	

The sequencing reaction mixtures were ethanol-precipitated. Then 3.5 µl of formamide loading dye was added. Automated DNA sequencing was accomplished with an ALF Express sequencing apparatus. The conditions for the sequencing electrophoresis were: 1600 V; 38 mA; 34 W; 2 seconds sample interval; 1000 V running; 55 °C.

4.2.18 Southern blot hybridization

4.2.18.1 DNA labeling with digoxigenin (DIG)

DNA labeling with digoxigenin was carried out following the protocol from the "DIG DNA Labeling and Detection Kit" (Boehringer Mannheim, Mannheim). DNA fragments (400 ng) generated by restriction digestion or PCR were gel purified as described above. After DNA fragments were denatured at 95°C for 10 min, the labeling reaction was prepared as follow:

2	µl	Hexanucleotide mixture
2	µl	dNTP mixture
1	µl	2 units/µl of Klenow enzyme
15	µl	The denatured DNA fragment

The reaction mixture was incubated at 37°C overnight. The enzyme was inactivated with 1 µl of 0.5 M EDTA. Subsequently, the reaction mixture was precipitated with 2.5 µl of 4 M $LiCl_4$ and 75 µl of chilled 95 % ethanol. Following incubation at –80°C for 20 min, the mixture was centrifuged at 13,000 rpm for 15 min. The pellet was washed twice with 180 µl of 70 % chilled ethanol. The pellet was dried under vacuum, dissolved in 20 µl sterile water. The labeled DNA fragment was denatured at 95°C for 10 min and then used as the probe.

4.2.18.2 Detection of DNA labeling efficiency

The DNA labeling efficiency was verified by DIG quantification and DIG control test stripes (Roche, Mannheim) which were subjected to immunological detection with anti-digoxigenin-AP conjugate and a pre-mixed stock solution of NBT/BCIP (75 mg/ml nitroblue tetrazolium salt in 70 % (v/v) dimethylformamide/ 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 % dimethylformamide). A series of dilutions of DIG-labeled DNA was applied as 1 µl aliquots to the marked squares on the DIG quantification test stripes. The test stripe was then air-dried for 2 min. The test stripe was dipped into the following solutions in the following order: blocking solution for 2 min, antibody binding solution for 3 min, blocking solution for 1 min, maleic acid buffer for 1

min, detection buffer for 1 min, and color reaction buffer for 5 – 30 min. The reaction was stopped by addition of tap water.

Maleic acid buffer:

0.1	M	maleic acid
0.15	M	NaCl

adjusted to pH 7.5 with solid NaOH

Blocking solution:

Diluted 10 × blocking buffer provided in the kit in maleic acid buffer

Antibody binding solution:

Dilute anti-digoxigenin-AP 1:2,000 in blocking buffer

Detection buffer:

0.1	M	Tris-HCl	pH 9.5
0.1	M	NaCl	
50	mM	$MgCl_2$	

Color reaction buffer:

40 µl of the NBT/BCIP stock solution in 2 ml of detection buffer

4.2.18.3 Southern Blot hybridization

Genomic DNA or plasmid DNA was digested with restriction enzyme(s). After DNA fragments were separated by gel electrophoresis, the gel was stained with ethidium bromide solution and photographed. The gel was then briefly washed in water. Subsequently, the gel was incubated in depurination solution (0.25 M HCl) for 10 min and in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 × 15 min, respectively. The DNA in the gel was transferred by capillarity or vacuum blot to Nylon membranes (Hybond- N^+, Amersham, Freiburg). The DNA was UV-cross linked onto the membrane for 3 min.

Subsequently, the filter was incubated for at least 1 h at 68°C (for high stringency) or 55°C (for low stringency) in standard hybridization buffer and incubated with the labeled DNA probe in 25 ml hybridization buffer overnight at the same temperature. The filter was then washed twice for 5 min in 2 × SSC, 0.1 % SDS at room temperature and 2 × 15 min in 0.1 × SSC, 0.1 % SDS at 65°C. Subsequently, the filter was incubated in a plastic bag with 50 ml of 0.5% blocking reagent solution for 30 min. Then, in a plastic bag the filter was incubated with 20 ml of buffer 1 containing the anti-digoxigenin-AP at 1:2,000 dilution.

Excess of conjugates was removed by washing the filter with buffer I for 2×15 min. The filter was equilibrated with buffer 3 for 3 min before detection of DIG-labeled hybrids.

With the DIG system, detection can be performed with the colorimetric detection or using chemiluminescent detection in which a light signal is produced at the site where the hybridizing probe was located. For colorimetric detection of anti-digoxigenin-AP, 10 ml of NBT/BCIP color substrate solution was added to the filter and the filter was incubated in a sealed plastic bag in the dark. Once signals were visible, the filter was washed with tap water. For chemiluminescent detection, CSPD® substrate was used at 1:10 dilution. For this, the membrane was incubated with 0.5 ml of CSPD® substrate solution at 37°C for 15 min. Then the membrane was exposed to an X-ray film at room temperature for 5-60 min. The film was developed and signals were visually monitored.

20 x SSC: pH 7.0

3 M	NaCl
0.3 M	sodium citrate
1000 ml	distilled water

Hybridization buffer:

5 x SSC	
0.1%	N-Lauroylsarcosine
0.02 %	SDS
1 %	blocking solution
add 100 ml	distilled water

2 x SSC/0,1 % SDS:

20 ml	20 × SSC
2 ml	10 % SDS
178 ml	distilled water

0,1 x SSC/0,1 % SDS:

1 ml	20 × SSC
1 ml	10 % SDS
198 ml	distilled water

Buffer 1:

8.77 g	Tris/HCl, pH 7.5
12.11 g	NaCl

Adjust to 1000 ml distilled water

Buffer 2:

0.5 % Blocking reagent in buffer 1

Buffer 3: pH 9.5

12.11 g	Tris/HCl
5.84 g	NaCl
10.17 g	$MgCl_2 \times 6\ H_2O$

Color substrate solution:

10 ml	buffer 3
200 µl	of reagent of NBT and BCIP (X-Phosphate)

4.3 RNA manipulations

4.3.1 Isolation of total RNA from *P. syringae*

Prior to RNA isolation, all glassware was thoroughly cleaned. Subsequently, glassware was heated for 12 hours at 200°C. Aqueous solutions were prepared using water supplemented with 0.1 % (v/v) DEPC overnight treatment and autoclaved. Plastic materials were treated with 3 % H_2O_2 aqueous solution. Total RNA was isolated from bacterial cells by use of the RNeasy™ kit (Qiagen, Hilden).

4.3.2 RNA electrophoresis

Size separation of RNA molecules is the first step of Northern blot hybridization. An agarose gel (1.2 % agarose, 1 × MOPS, 6 % formaldehyde) was prepared for RNA electrophoresis. 1 × MOPS (200 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 7.0) electrophoresis buffer was added to cover the gel. RNA retains much of its secondary structure during electrophoresis unless it is first denatured. To do so, 12 µl RNA sample was mixed with 12 µl 2 × RNA loading buffer. The mixture was heated at 85°C for 10 min before loading to the wells of the gel. The gel was run at 4-5 V/cm for 2-3 h 1 × MOPS.

2 × RNA loading buffer:

24 % (v/v)	formaldehyde
66.4 % (v/v)	deionized formamide
0.5 mg/ml	bromophenol blue
0.5 mg/ml	xylene cyanol FF
2 %	sucrose
2 ×	MOPS
30 µg/ml	ethidium bromide

4.3.3 Probe labeling

The RNA probes used for Northern blots were synthesized by the Strip-EZ™ RNA Probe Synthesis & Removal kit (Ambion, Wiesbaden). RNA probes were produced by in vitro transcription using PCR product of DNA templates. PCR was used to add the T7 promoter by including its sequence at the 5' end of the PCR primers. The primers used

were Lsc-fwd/LscR-T7 and gusF/gusR-T7. 1-2 µl of the PCR reactions (about 0.1 to 0.2 ηg of DNA) was used as the template for the Strip-EZ RNA reaction. The RNA probe was labeled using enzymatic incorporation of digoxigenin-labeled nucleotides during RNA synthesis. After transcription proceeded, 1 µl of DNase I was added, mixed by flicking, and incubated at 37°C for 15 min to remove the DNA template. The DNase reaction was stopped by adding 1µl of 500mM EDTA and incubating at 75°C for 5min.

Components for standard transcription reactions (20 µl):

2.0 µl	10 × transcription buffer
1.0 µl	10 mM ATP
1.0 µl	2 mM modified CTP
1.0 µl	10 mM GTP
0.33 µl	10 mM UTP
0.67 µl	10 mM labeled UTP
1.0 µl	DNA template
2.0 µl	T7 RNA polymerase + Ribonuclease inhibitor
11.0 µl	Nuclease-free H_2O

4.3.4 Northern blot hybridization

The electrophoresis gel was placed in an RNase-free dish and rinsed repeatedly with deionized water for 4 x 20 min. The RNA was transferred to nitrocellulose membrane (Schleicher & Schull, Dassel) by capillarity or vacuum blot. Subsequently, the membrane was placed on a UV transilluminator (254 nm) and exposed for 3 min. The membrane was then rinsed in 5 × SSC and placed in a hybridization tube. 25 ml of hybridization solution was added the tube which was then placed in the hybridization oven and incubated with rotation for 2 hr at 68 °C. Subsequently, 25 µl of the labeled RNA probe was added followed by an incubation for 12 hr at 68°C. The membrane was then washed twice for 5-10 min with 2 × SSC/ 0.01% SDS solution at room temperature and twice for 15 min at 65 °C with 0.1 × SSC/ 0.1 % SDS. Subsequently, the filter was incubated in a plastic bag with 50 ml of 0.5 % blocking reagent solution for 30 min. Then, in a plastic bag the filter was incubated with 20 ml of buffer1 containing the anti-digoxigenin-AP at 1:2,000 dilution. Excess of conjugates was removed by washing the filter with buffer I for 2×15 min. The filter was equilibrated with buffer 3 for 3 min prior to detection of DIG-labeled hybrids. Detection was performed with the colorimetric detection method. 10 ml of NBT/BCIP

color substrate solution was added to the filter and the filter was incubated in a sealed plastic bag in the dark. Once signals were visible, the filter was washed with tap water. (The components of the buffers are listed in **4.2.18.3**).

4.4 Protein manipulations

4.4.1 Absorption spectrophotometry

The technique of absorption measurement was used for quantitative analysis of proteins or enzymatic reaction products. For quantitative measurement, a wavelength where the analyte absorbs maximally was used to determine its concentration applying the Beer-Lambert´s relation:

$A = \varepsilon \cdot c_m \cdot l = a \cdot c \cdot l$

A, absorbance; ε, molar absorption coefficient (1 mol^{-1} cm^{-1}); c_m, concentration (mol l^{-1}); *l*, light path length (cm); a, specific absorption coefficient (l g^{-1} cm^{-1}); and *c*, concentration (g l^{-1}).

4.4.2 Determination of protein concentration (Bradford assay)

Protein concentrations were determined using the protein dye reagent, Coomassie Brilliant Blue (Bio-Rad, München), according to Bradford (1976). The determination of protein concentration for cell extracts was carried out in microtiter plates. 180 µl of the cell extract was transferred to fresh Eppendorf tubes and precipitated with an equal volume of cold 10 % trichloroacetic acid (TCA) solution. After precipitation with TCA, proteins were denatured by boiling for 5 min. After centrifugation for 5 min, the protein pellet and 1-2 mg of a protein standard (γ-globulin) were separately dissolved in 100 µl of 1 M NaOH and diluted to 1 ml with 900 µl distilled water. 150 µl of the protein solutions were transferred to wells A1-11 of a microtiter plate. Well A12 and all wells in rows B, C, D, E, F, G, and H were filled with 100 µl of distilled water. Subsequently, 50 µl of the protein solutions were transferred from row A to rows B, C, D, E, F, G, and H resulting in a 1:3 dilution for each row. 200 µl of Bradford reagent (8.5 % (v/v) H_3PO_4; 4.75 % (v/v) C_2H_5OH; 0.01 % (w/v) Coomassie Brilliant Blue G250) was added to the wells. The A_{595} was measured and the protein concentration of the sample was determined from plotting it to the γ-globulin standard curve.

4.4.3 Subcellular fractionation of *P. syringae*

Subcellular fractionation was done according to the method described by Boyd *et al*. (1987) with some modifications. 1.5 ml of an exponentially grown bacterial culture was centrifuged down at 5,000 rpm and 4°C. The supernatant was filtered (0.2 µm pore size) and used as the extracellular fraction. Cells were permeabilized by resuspending the pellet in 150 µl of cold SP buffer (0.1 M Tris/HCl, pH 7.5, 0.5 mM EDTA-Na, 0.5 M sucrose) and subsequent incubation on ice for 5 min. Permeabilized cells were centrifuged down at 5,000 rpm and 4°C and carefully resuspended in 100 µl of ddH_2O. Subsequently, the permeabilized cells were osmotically shocked by addition of 5 µl of 20 mM $MgCl_2$. After centrifugation of the cells for 5 min at 13,000 rpm at 4°C, the supernatant was further used as the periplasmic fraction. The pellet (spheroplasts) was resuspended in 500 µl of a 50 mM Tris/HCl (pH 8.0) buffer. Spheroplasts were lysed by 5 × 10 s of ultrasonic treatment. Subsequent centrifugation for 30 min at 13,000 rpm and 4°C separated the cytoplasmic fraction (supernatant) from the membrane fraction (pellet). The membrane pellet was resuspended in 100 µl of 50 mM Tris/HCl (pH 8.0).

4.4.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE according to Laemmli (1970) was used to separate proteins based on their molecular masses. The polyacrylamide gel was casted as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus (Mini Protean II). The gel (8.2 x 7.3 x 0.075 cm^3) was prepared according as follows:

	Stacking gel	Separating gel		
	4 %	7.5 %	10 %	12.5 %
Acrylamid/Bisacrylamid	0.94 ml	3 ml	4 ml	5 ml
0,5 M Tris/HCl (pH 6,8)	1.76 ml			
1 M Tris/HCl (pH 8,8)		4.5 ml	4.5 ml	4.5 ml
H_2O	3.84 ml	3.84 ml	2.86 ml	1.86 ml
10 % (w/v) SDS	70 µl	120 µl	120 µl	120 µl
TEMED	10 µl	12 µl	12 µl	12 µl
1 % (w/v) APS	0.4 ml	0.5 ml	0.5 ml	0.5 ml

Protein-loading buffer was added to the protein samples which were then incubated at 95° for 5 min. The samples were loaded to the gel and the electrophoresis was started at

20 mA until the line of blue tracking dye reached the top of the separating gel, then followed by 40 mA until the tracking dye reached the bottom of the separating gel. After that, the gel cast was disassembled and the gel were stained by 0.04 % Coomassie blue R 250 in a mixture of methanol / H_2O / acetic acid (4:5:1). Destaining was carried out in 250 ml of a methanol / H_2O / acetic acid mixture (4:5:1) until the Coomassie blue-stained protein bands became visible; then the gel was dried at 60°C for 2 h.

Protein Loading-buffer:			Electrophoresis running buffer:		
125 mM	TrisHCl	pH 6,8	25.0 mM	TrisHCl	pH 8,3
4 % (w/v)	SDS		192.0 mM	glycine	
20 % (v/v)	glycerin		0.1 % (w/V)	SDS	
10 % (v/v)	2-β mercaptoethanol				
20 µg/ml	bromphenol blue				

For detection of very small amounts of protein, the silver staining method (Switzer *et al*., 1979) was carried out. After the gel was incubated in fixation solution for overnight, the gel was incubated in 50 % ethanol by gentle shaking for 3 x 20 min. After that, the gel was incubated in solution 1 for 1 min, washed in H_2O for 3 x 20 sec, incubated in solution 2 for 20 min, washed in H_2O for 3 x 20 sec again, then incubated in solution 3 until the desired band intensity was visible. The reaction was stopped by replacing solution 3 with concentrated acetic acid.

Fixation Solution		Solution 1	
50 %	methanol	0.8 mM	$Na_2S_2O_3$
12 %	acetic acid		
500 µl	formaldehyde		
380 ml	ddH_2O		

Solution 2		Solution 3	
0.5 g	$AgNO_3$	15 g	Na_2CO_3
187.5 µl	formaldehyde	125 µl	formaldehyde
to 250 ml	ddH_2O	5 ml	Solution 1

4.4.5 Nondenaturing native polyacrylamide gel electrophoresis

To perform PAGE under nondenaturing native conditions, the same gel preparation scheme indicated above was applied but additional SDS and the stacking gel were omitted. The electrophoresis was done with Tris-glycine buffer (pH8.3) in the absence of SDS. Samples were applied in loading buffer that did not contain SDS and 2-β mercaptoethanol (2-ME), and without prior boiling.

4.4.6 Protein overexpression with the pMal™-system

The pMal™-c2 vectors provide a method for overexpressing and purifying a recombinant protein produced from a cloned gene. The cloned gene is inserted into the pMal-c2 vector downstream of and in frame with the *malE* gene, which encodes maltose-binding protein (MBP). The technique uses the strong IPTG inducible P_{tac} promoter and the translation initiation signals of MBP to express large amounts of the fusion protein. The fusion protein is then isolated from crude protein by one-step affinity purification for MBP.

This system also takes advantage of the fact the cloned gene is inserted within the *lacZα* gene allowing a blue/white screen for inserts on X-gal containing agar plates. In pMal-c2, the *malE* signal sequence is deleted resulting in cytoplasmic expression of the fusion protein. Between the *malE* sequence and the polylinker there is a spacer sequence coding for 10 asparagine residues. This spacer insulates MBP from the protein of interest, increasing the chances that a particular fusion protein will bind tightly to the amylose resin applied for affinity chromatography. The vector also includes a sequence coding for the recognition site of protease factor Xa. This allows the protein of interest to be cleaved from MPB after purification.

4.4.6.1 Protein overexpression in *E. coli*

1.5 ml of an overnight culture of *E. coli* DH5α containing the fusion plasmid was inoculated to 100 ml LB medium and grown at 37°C with 250 rpm to an OD_{600} of 0.45 – 0.5. An aliquot was removed (uninduced sample) and 0.3 mM IPTG was added; the sample was incubated continuously at 37°C with 250 rpm for 4 h. The cells were harvested by centrifugation at 10,000 g for 10 min and the cell pellet was stored at -20°C.

4.4.6.2 Sonication

About 600-700 µl of cells pelleted by centrifugation and resuspended in protein extraction buffer was sonicated 4 × 15 sec at medium power (output control should be 4 - 5). After centrifugation at 4°C for 20 min, the supernatants were collected in fresh pre-chilled test tubes. Then the pellets of cell debris were resuspended in 600 µl of fresh protein extraction buffer. The protein suspensions were stored at -20°C.

4.4.6.3 Bacterial lysis by the freeze-thawing method

This extraction method was used for small-scale extraction of total cellular protein. Resuspended in 50 mM Tris-HCl buffer after centrifugation, the cells were frozen at -80°C for 30 min, followed by immediate incubation at 37°C for 10 min. Subsequently, this step was repeated three times. Then the sample were centrifuged, the supernatants were collected and stored at -20°C.

4.4.7 Protein purification

For this, a simplified version of the method of affinity chromatography was used based on the pMal™ overexpression system. 300µl amylose resin was washed with column buffer in 1.5-ml eppendorf tubes, 1 ml protein extracts was added and vortexed. The sample was incubated at 4°C for 10 min. After removal of the supernatant by centrifugation, the amylose resin was washed 3 times with 0.5 ml column buffer, and then 100 µl elution buffer was added to the resin and vortexed. The sample was put on ice for 10 min and centrifuged, subsequently the supernatant containing the purified protein was collected. Purified products were qualitatively visualized by SDS-PAGE and the concentration of the proteins was determined by Bradford Assay or absorption spectrophotometry.

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4.4.8 Precipitation of proteins

4.4.8.1 Precipitation by TCA

10 % TCA was added to the protein sample, mixed and the mixture was left on ice for 20 min. After centrifugation, the protein pellet was washed with chilled 95 % ethanol. The pellet was dried and redissolved in 50 mM Tris-HCl (pH 8.0).

4.4.8.2 Concentration of proteins by lyophilization

To obtain the extracellular protein samples of bacterial cultures, lyophilization was used to concentrate the cell-free supernatants of the cultures. After the lyophilizator apparatus cooled down and the protein suspension was frozen, the protein was dried at -20°C in vacuum overnight.

4.4.9 Immunoblotting

Proteins from crude cell extracts, concentrated supernatant extracts or subcellular fractions were diluted and equal amounts (2 µg/lane) were separated by electrophoresis using 10 % SDS-PAGE. Subsequently, proteins in the gel were electrotransferred to Hybond-C nitrocellulose membranes (Amersham-Pharmacia Biotech, Freiburg). Electroblotting was performed in electrotransfer buffer overnight at 20 mA per gel using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, München). Following the electrotransfer, the membrane was blocked with 10 ml of blocking buffer at 37°C for 1 h. Subsequently, the membrane was incubated in a plastic bag with 5 ml of antibody incubation buffer containing the levansucrase antiserum at a dilution of 1:3,000 at 37°C for 1.5 h. The unbound primary antibody was then removed by washing the membrane 3 × 15 min with washing buffer I at 37°C. For antibody detection, secondary anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used at a concentration of 1:7,500. Excess of conjugates was removed by washing the membrane with washing buffer I for 3×15 min at 37°C. To remove TritonX-100, the membrane was then washed in washing buffer II for 2 × 5 min at room temperature. The reaction was visualized by adding 20 ml of color reaction buffer containing 35 µl of 5 % (w/v) 5-bromo-4-chloro-3-indolyl phosphate dissolved in dimethylformamide and 45 µl of 7.5 % (w/v) nitroblue tetrazolium salt dissolved in 70 % (v/v) dimethylformamide. The reaction was stopped by washing the membrane several times with tap water.

Electrotransfer buffer (1 liter):

14.41 g	Glycine
3.03g	Tris/HCl, pH 8,3
100 ml	Methanol

Blocking buffer (1 liter):

3.12 g	Na_2PO_4, pH 7,4
11.69 g	NaCl
5%	dried skim milk (freshly added)

Washing buffer I (1 liter):

Antibody incubation buffer:

3.12 g	NaH_2PO_4, pH 7,4	Washing buffer I
11.69 g	NaCl	1 % dried skim milk (freshly added)
372 mg	EDTA	
3 ml	Triton X-100	

Washing buffer II (1 liter):

2.42 g	Tris/HCl, pH 7,4
11.69 g	NaCl

Color reaction buffer (1 liter):

12.11 g	Tris/HCl, pH 9.5
5.84 g	NaCl
10.17 g	$MgCl_2$

4.5 Enzymatic assays

4.5.1 Qualitative assays for levansucrase activity

Qualitative estimation of levansucrase activity in sterile bacterial supernatants and subcellular fractions was carried out by spotting 5-10 µl of samples on water-agar plates containing 5 % sucrose. Enzyme activity was visualized by the formation of opalescent slime plugs following an incubation of 24-48 h at 18°C. Zymograms with proteins from cell-free supernatants and subcellular fractions were prepared by PAGE under non-denaturing conditions as described above. For this, 15 µl aliquots of native protein samples derived from concentrated supernatants or subcellular fractions of exponentially growing or stationary phase cultures were loaded to 10 % polyacrylamide gels. Following electrophoresis, gels were incubated in sterile water containing 10 % sucrose for 24-48 h at 18°C. Protein bands representing levansucrase were detected by a whitish swelling of the gel matrix that corresponded to levan formation.

4.5.2 Quantitation of levansucrase activity

Levansucrase activity was quantified by measuring the amount of glucose liberated during incubation of crude protein extracts with sucrose using the Gluco-quant Glucose/HK assay kit (Roche, Mannheim). Non-concentrated 20 µl-samples of glucose-free supernatant or 20 µl of subcellular fractions were mixed with 20 µl of assay buffer (10 % sucrose + 0.09 % NaCl) and 2 ml test reagent (83 mmol/l Tris and 5 mmol/l HEPES [pH 7.7] containing 4 mmol/l Mg^{2+}, 1.4 mmol/l ATP, 0.83 mmol/l NADP, 1.4 U/ml hexokinase and 2.5 U/ml Glucose-6-phosphate dehydrogenase). Subsequently, the reaction

was incubated at 25°C and the absorbance was measured at A_{365} in 15-min intervals for 1 hour. One unit of Levansucrase activity represented the amount of enzyme in 1 ml of bacterial culture that liberated 1 μmol of glucose per minute.

4.5.3 Determination of K_m

The apparent value of K_m of levansucrase was estimated using sucrose as substrate. Different concentrations of sucrose (40, 80, 120,160, and 200 mM) were incubated with an appropriate amount of MBP-Lsc in 0.09 % NaCl solution in a final volume of 2 ml. The absorbance of the reaction product (glucose) at A_{365} was continuously recorded. The difference in molar absorbance, 10,000 $M^{-1}cm^{-1}$, was used to express the product increase in nmol. The values for K_m and V_{max} were extrapolated from the Eadie-Hofstee plot of the rearranged Michaelis-Menten equation,

$$V = V_{max} - K_m V/ [S]$$

V, velocity; V_{max}, maximum velocity; K_m , Michaelis-Menten constant; and [S], concentration of substrate.

The plot of line $V/$ [S] *versus* V provided $-1/ K_m$ as a slope and V_{max} as its X-intercept.

4.5.4 Assay of extracellular lipase

Lipase activity in cell-free *P. syringae* culture supernatants was assayed using *p*-nitrophenyl-palmitate (sigma) as the substrate (Winkler and Stuckmann, 1979). 10 ml of isopropanol containing 30 mg of *p*-nitrophenyl-palmitate was mixed with 90 ml of 0.05 M Sörensen phosphate buffer, pH 8.0, containing 207 mg of sodium deoxycholate and 100 mg of gum arabi. A 2.4 ml amount of this freshly prepared substrate solution was pre-warmed at 37°C and then mixed with 0.1 ml of cell-free supernatant. After 15 min incubation at 37°C, the OD_{410} was measured against an enzyme-free control. One enzyme unit is defined as 1 nmol of *p*-nitrophenol enzymatically released from the substrate per milliliter per minute.

4.5.5 β-Glucuronidase (GUS) assay

An approach to investigate changes in gene transcription is to link the presumed cis-acting promoter sequence from the gene of interest to the coding sequence for an unrelated

reporter gene without its own promoter sequence. The *E. coli* β-glucuronidase gene (*uidA* or *gusA*) (Jefferson *et al*., 1986) is predominantly used in plant pathogenic bacteria as a reporter gene to demonstrate environmentally controlled gene expression since plants do not contain β-glucuronidase. β-glucuronidase activity (GUS) can be quantified by spectrophotometric or fluorescence assays (Xiao *et al*., 1992).

Samples of 1.5 ml were taken from *P. syringae* cultures carrying recombinant plasmids, which harbor transcriptional fusions, grown at 18 and 28°C when the OD_{600} reached values of 1.5-2.0. After discarding supernatants, cells were resuspended in 500 µl GUS extraction buffer and kept on ice for 30 min. Subsequently, cells were disrupted by 3 × 15 sec ultrasonic treatment. The wells of rows C, D, E, F, G, H from 1 to 12 were filled with 180 µl carbonate stop-buffer. Additionally, the well B12 was filled with 180 µl carbonate stop-buffer. 200 µl of cell extract were filled into wells A1-10, whereas A11 was filled with 200 µl of water, and A12 was filled with 200 µl of MU-standard (1 mM 7-hydroxy-4-methylcoumarin, sodium salt). Finally, wells B1-11 were filled with 180 µl of assay buffer. The reaction was initiated by transferring 20 µl of cell extract to the substrate buffer wells from row A to row B (1:10 dilution) by use of a multi-channel pipetman. Immediate transfer of 20 µl from row B to rows C and D stopped the reaction. Therefore, the fluorescence values in wells C1-10 represented the F (t_0) values. The microtiter plate was immediately placed into a water bath at 37°C. After 10 min the reaction was stopped by transfer of 20 µl from row B to row E, and subsequently to rows F, G and H. The plate was immediately read in a Fluorolite fluorometer (Dynatech Laboratories, Denkendorf) with the following set-up: extinction filter at 390 nm, emission filter at 450 nm, and lamp voltage at 3.0 V. The fluorescence values in wells E1-10 represented the F (t_{10}) values. The fluorescence in the well B12 represented the standard fluorescence (F_{st}) of 100 µM MU-solution. The GUS activity was calculated according to the following equations:

$\Delta F_{450} = F(t_{10}) - F(t_0)$

GUS activity (U) = $\Delta F_{450} \times 100 / F_{st}$

Specific activity (U/mg protein) = UGUS / mg protein, where

F_0 , fluorescence of time point ‘0’;

F_{10}, fluorescence of time point ‘0’;

F_{st}, fluorescence of 100µM MU-standard.

GUS extraction buffer (for 100 ml) **Carbonate-buffer:**

5 ml	1M $NaHPO_4$ pH 7.0
70.0 µl	β-mercaptoethanol
2 ml	0.5M EDTA pH 8,0
330.0 µl	30% N-laurolylsarcosyl sodium salt
1 ml	10 % Triton X-100
91.6 ml	distilled water.

21.2 g	Na_2CO_3
1000 ml	distilled water

MU-Standard solution:

19,8 mg	7-hydroxy-4-methylcoumarin
100 ml	distilled water

Assay-buffer:

4 mg 4-methylumbelliferyl β-D-glucuronide

5 ml GUS extractions buffer

4.6 Plant experiments

Soybean plants were grown in the greenhouse at 20-25°C, 60 % humidity and 15,000 lux. *P. syringae* cells were grown in 100 ml HSC medium at 18°C until they reached OD_{600} of 1.0. Cells were washed with 0.85 % NaCl buffer and resuspended in 15 ml of the same buffer to prepare the inoculum for 7 pods of 4-week-old soybean plants. Plants were sprayed with the bacterial suspension at a concentration of approximately 1×10^9 cfu/ml. Following inoculation, the soybean plants were incubated in growth chambers (Controlled Environments Inc., North Carolina, USA) at 18°C for 21 days. Bacterial populations in leaves were monitored by homogenizing the inoculated leaves in isotonic NaCl solution and serially diluting the cells on MG agar plates.

5 RESULTS

5.1 Cloning and characterization of the second *lsc* gene of *P. syringae* pv. glycinea PG4180

Previously, a *lsc* gene coding for levansucrase in *P. syringae* pv. glycinea PG4180 was identified and characterized to be functional in *E. coli* (Hettwer *et al*.,1998). However, Jaeckel (1999) reported the experimentally determined N-terminal amino acid sequence of a secreted 50-kDa levansucrase which was identical to the predicted N-terminal amino acid sequence of levansucrase from *P. syringae* pv. phaseolicola NCPPB1321 (Hettwer *et al*., 1995 and 1998) but not to the previously predicted N-terminus of levansucrase from strain PG4180 (**Fig. 6.**). Moreover, when this gene had been knocked-out by marker exchange mutagenesis (Jaeckel, 1999), the mutant did not exhibit a levan-deficient phenotype, suggesting the existence of at least one additional allele of this gene in PG4180. According to the previously published nucleotide sequence coding for levansucrase from *P. syringae* pv. phaseolicola NCPPB1321 (Hettwer *et al*., 1998), oligonucleotide primers lsc-F5 and lsc-R were designed to amplify an approximately 1.3-kb PCR product from genomic DNA of PG4180 (**Fig.7.**). These results indicated the presence of an additional *lsc* gene in PG4180. Consequently, the original levansucrase gene was renamed *lscA* and the second one was designated *lscB*.

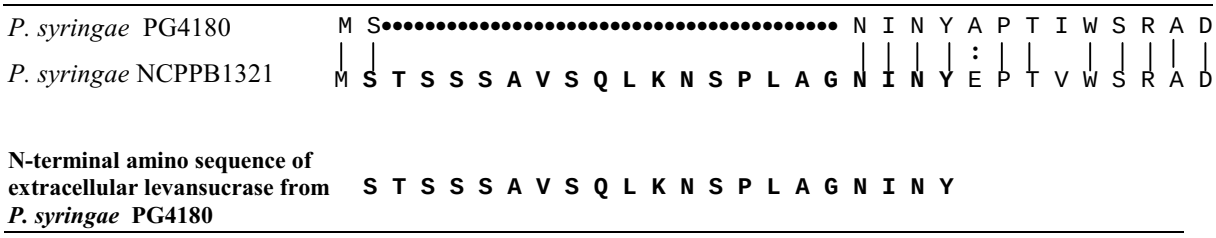


Fig. 6. Comparison of N-terminal sequence data for levansucrases. The predicted N-terminal amino acid sequences of *lsc* gene products from *P. syringae* pv. glycinea PG4180 and *P. syringae* pv. phaseolicola NCPPB1321 (Hettwer *et al*.,1998) and N-terminal amino acid sequence of extracellular levansucrase from *P. syringae* pv. glycinea PG4180.

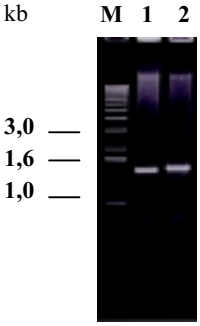


Fig. 7. PCR-analysis using genomic DNA of PG4180 and the specific primer pairs LscA-F/A-R and LscF5/R5 for detection of two different *lsc* genes. Lanes: 1, PCR product with primers lscA-F/A-R for amplifying the previous described *lsc* gene in PG4180; 2, PCR product with primers lscF5/R5 designed from the previously published nucleotide sequence of *lsc* from *P. syringae* pv. phaseolicola NCPPB1321.

5.1.1 Screening for the second levansucrase gene in the PG4180 cosmid library

A genomic library of *P. syringae* PG4180 was previously constructed by cloning partially *Sau*3A-digested genomic DNA of PG4180 into *Bam*HI-treated cosmid vector pRK7813 in our laboratory (Hettwer *et al*., 1998). With this library, a total of 960 *E. coli* recombinants, each containing a cosmid with approximately 25- to 35-kb of insert DNA, were screened for the presence of DNA homologous of *lsc* of strain NCPPB1321 by PCR with oligonucleotide primers lsc-F5 and lsc-R. This primer pair does not allow amplification of the *lscA* gene from PG4180 (data not shown). One positive cosmid designated p7C7 was identified. Restriction analysis of the insert DNA of p7C7 did not give the 3.0-kb *Pst*I fragment which was common to the four cosmid clones containing the previously described *lsc* gene in PG4180 (Hettwer *et al*., 1998). Southern blot analysis was carried out with cosmid p7C7 digested with different restriction enzymes and a DNA probe containing the previously described *lsc* gene from PG4180 (**Fig. 8.**). These results confirmed that cosmid clone p7C7 contained the second levansucrase gene from PG4180.

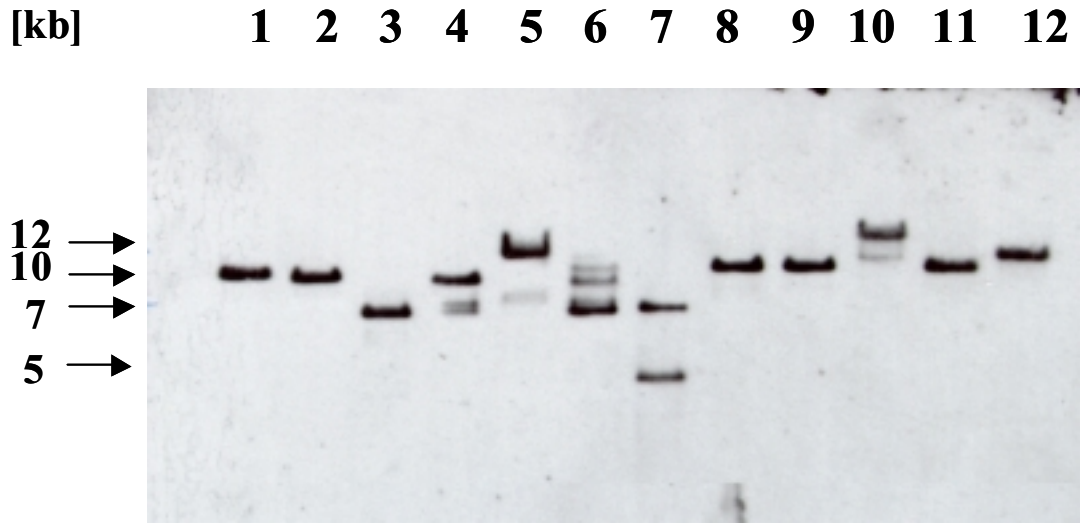


Fig. 8. Southern blot hybridization analysis of genomic library clone p7C7 with various restriction enzyme-digestions and a digoxigenin-labeled probe specific to the previously described PCR-amplified *lsc* gene of PG4180. In *Xho*I-digested cosmid p7C7 (lane 7) there are two signals due to the presence of an *Xho*I site in this *lsc* gene. Lane: 1, *Sal*I; 2, *Eco*RI; 3, *Eco*RV; 4, *Pst*I; 5, *Cla*I; 6, *Hind*III; 7, *Xho*I; 8, *Xba*I; 9, *Xba*I / *Pst*I; 10, *Kpn*I / *Sac*I; 11, *Xba*I / *Eco*RI; 12, *Bam*HI.

5.1.2 Subcloning of *lscB* from the PG4180 genomic library clone p7C7

In cosmid clone p7C7, a 7.2-kb *Eco*RV hybridized to the DNA probe amplified with primers lsc-F5/R5 from PG4180 in a Southern blot analysis (**Fig. 8**). Subsequently, this fragment was subcloned into pBluescript II SK(+) in both orientations to obtain plasmids pLB7.2 and pLB7.2R. The partial physical map of this 7.2-kb insert is showed in **Fig. 9**. When plated on LB agar plates containing 5 % of sucrose, neither plasmid conferred levan formation to *E. coli*.

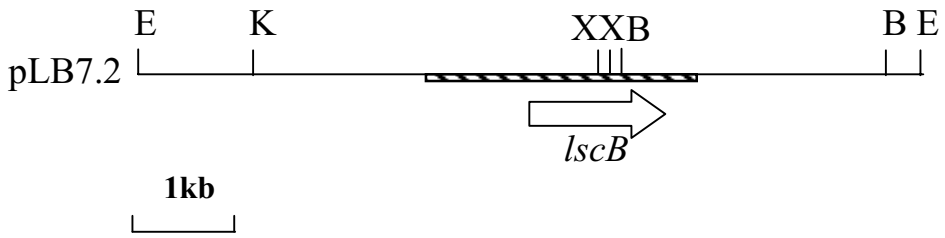
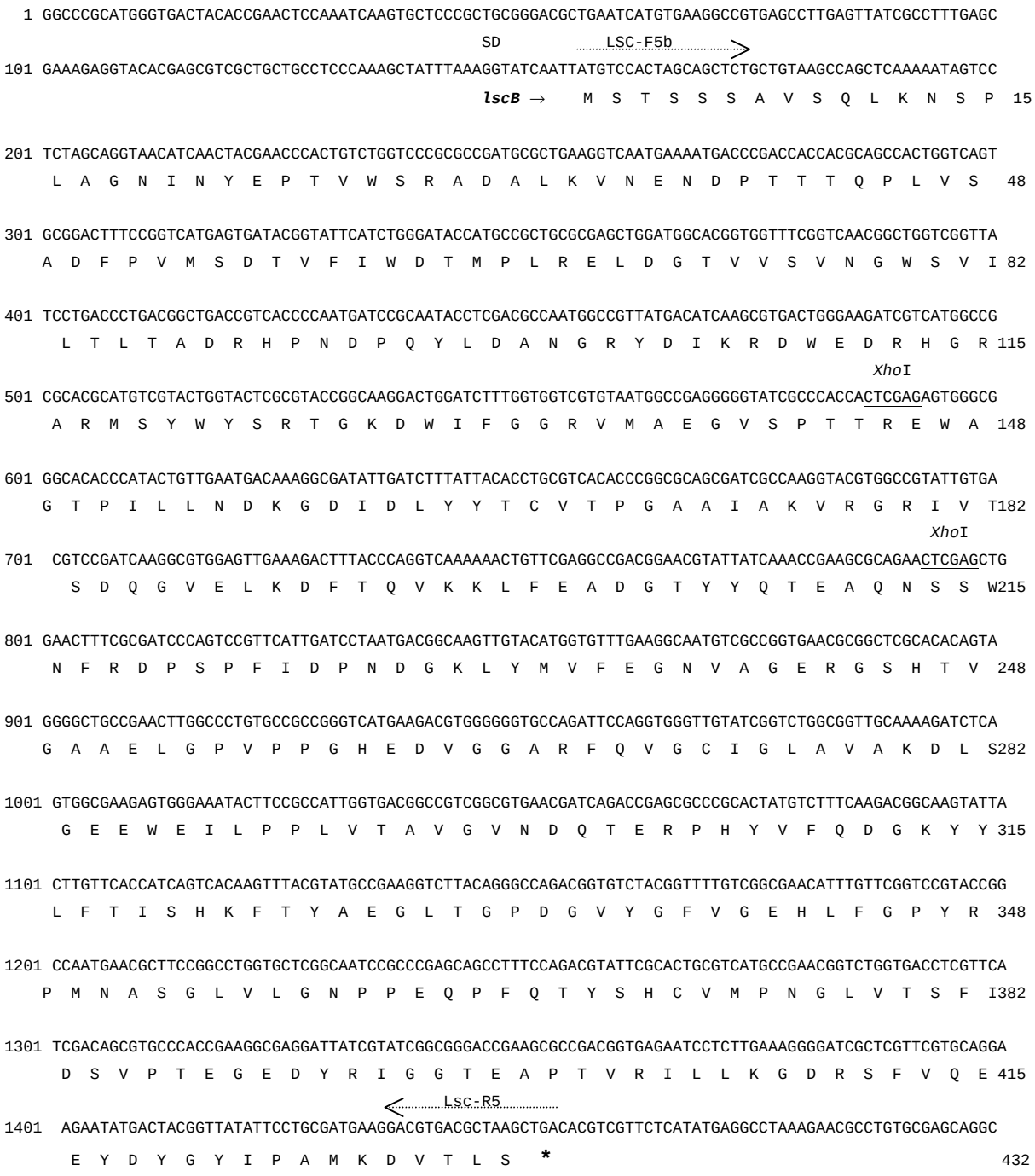


Fig. 9. Partial restriction map of the 7.2-kb insert of pLB7.2. The striped line indicates the DNA for which the nucleotide sequence has been determined. The open arrow symbolizes the identified ORF of *lscB* with its direction of transcription. Restriction enzyme sites: E, *Eco*RV; K, *Kpn*I; X, *Xho*I; B, *Bgl*II; and S, *Sal*I.

5.1.3 Determination, analysis, and comparison of the *lscB* nucleotide sequence

Part of the 7.2-kb insert of pLB7.2 was sequenced based on the four oligonucleotide primers LscBFL-1, LscBRL-1, LscBFL-2, and LscBRL-2 derived from the previously described *lsc* genes and additional primer walking (Hettwer *et al*., 1998). The nucleotide sequence of a 1296-bp ORF designated *lscB* was determined and found to be identical to the previously described *lsc* gene from *P. syringae* NCPPB1321 (Hettwer *et al*., 1998). The nucleotide sequence of *lscB* gene and its deduced amino acid sequence are shown in **Fig. 10**. The sequence of *lscB* showed a very high degree of similarity to *lscA* at the nucleotide level (86 %) and at the deduced amino acid sequence level (95 %). The N-terminus of the deduced *lscB* gene product was identical to the experimentally determined N-terminus of the extracellular 50-kDa protein identified as levansucrase (Jaeckel, 1999). This indicated that secretion of the *lscB* gene product might occur in a sec-independent manner.

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  1 GGCCCGCATGGGTGACTACACCGAACTCCAAATCAAGTGCTCCCGCTGCGGGACGCTGAATCATGTGAAGGCCGTGAGCCTTGAGTTATCGCCTTTGAGC
                                                SD          LSC-F5b
101 GAAAGAGGTACACGAGCGTCGCTGCTGCCTCCCAAAGCTATTTAAAGGTATCAATTATGTCCACTAGCAGCTCTGCTGTAAGCCAGCTCAAAAATAGTCC
                                              lscB →    M  S  T  S  S  S  A  V  S  Q  L  K  N  S  P  15

201 TCTAGCAGGTAACATCAACTACGAACCCACTGTCTGGTCCCGCGCCGATGCGCTGAAGGTCAATGAAAATGACCCGACCACCACGCAGCCACTGGTCAGT
     L  A  G  N  I  N  Y  E  P  T  V  W  S  R  A  D  A  L  K  V  N  E  N  D  P  T  T  T  Q  P  L  V  S  48

301 GCGGACTTTCCGGTCATGAGTGATACGGTATTCATCTGGGATACCATGCCGCTGCGCGAGCTGGATGGCACGGTGGTTTCGGTCAACGGCTGGTCGGTTA
    A  D  F  P  V  M  S  D  T  V  F  I  W  D  T  M  P  L  R  E  L  D  G  T  V  V  S  V  N  G  W  S  V  I 82

401 TCCTGACCCTGACGGCTGACCGTCACCCCAATGATCCGCAATACCTCGACGCCAATGGCCGTTATGACATCAAGCGTGACTGGGAAGATCGTCATGGCCG
      L  T  L  T  A  D  R  H  P  N  D  P  Q  Y  L  D  A  N  G  R  Y  D  I  K  R  D  W  E  D  R  H  G  R 115
                                                                                          XhoI
501 CGCACGCATGTCGTACTGGTACTCGCGTACCGGCAAGGACTGGATCTTTGGTGGTCGTGTAATGGCCGAGGGGGTATCGCCCACCACTCGAGAGTGGGCG
     A  R  M  S  Y  W  Y  S  R  T  G  K  D  W  I  F  G  G  R  V  M  A  E  G  V  S  P  T  T  R  E  W  A  148

601 GGCACACCCATACTGTTGAATGACAAAGGCGATATTGATCTTTATTACACCTGCGTCACACCCGGCGCAGCGATCGCCAAGGTACGTGGCCGTATTGTGA
    G  T  P  I  L  L  N  D  K  G  D  I  D  L  Y  Y  T  C  V  T  P  G  A  A  I  A  K  V  R  G  R  I  V  T182
                                                                                          XhoI
701  CGTCCGATCAAGGCGTGGAGTTGAAAGACTTTACCCAGGTCAAAAAACTGTTCGAGGCCGACGGAACGTATTATCAAACCGAAGCGCAGAACTCGAGCTG
       S  D  Q  G  V  E  L  K  D  F  T  Q  V  K  K  L  F  E  A  D  G  T  Y  Y  Q  T  E  A  Q  N  S  S  W215

801 GAACTTTCGCGATCCCAGTCCGTTCATTGATCCTAATGACGGCAAGTTGTACATGGTGTTTGAAGGCAATGTCGCCGGTGAACGCGGCTCGCACACAGTA
     N  F  R  D  P  S  P  F  I  D  P  N  D  G  K  L  Y  M  V  F  E  G  N  V  A  G  E  R  G  S  H  T  V  248

901 GGGGCTGCCGAACTTGGCCCTGTGCCGCCGGGTCATGAAGACGTGGGGGGTGCCAGATTCCAGGTGGGTTGTATCGGTCTGGCGGTTGCAAAAGATCTCA
    G  A  A  E  L  G  P  V  P  P  G  H  E  D  V  G  G  A  R  F  Q  V  G  C  I  G  L  A  V  A  K  D  L  S282

1001 GTGGCGAAGAGTGGGAAATACTTCCGCCATTGGTGACGGCCGTCGGCGTGAACGATCAGACCGAGCGCCCGCACTATGTCTTTCAAGACGGCAAGTATTA
      G  E  E  W  E  I  L  P  P  L  V  T  A  V  G  V  N  D  Q  T  E  R  P  H  Y  V  F  Q  D  G  K  Y  Y 315

1101 CTTGTTCACCATCAGTCACAAGTTTACGTATGCCGAAGGTCTTACAGGGCCAGACGGTGTCTACGGTTTTGTCGGCGAACATTTGTTCGGTCCGTACCGG
     L  F  T  I  S  H  K  F  T  Y  A  E  G  L  T  G  P  D  G  V  Y  G  F  V  G  E  H  L  F  G  P  Y  R  348

1201 CCAATGAACGCTTCCGGCCTGGTGCTCGGCAATCCGCCCGAGCAGCCTTTCCAGACGTATTCGCACTGCGTCATGCCGAACGGTCTGGTGACCTCGTTCA
    P  M  N  A  S  G  L  V  L  G  N  P  P  E  Q  P  F  Q  T  Y  S  H  C  V  M  P  N  G  L  V  T  S  F  I382

1301 TCGACAGCGTGCCCACCGAAGGCGAGGATTATCGTATCGGCGGGACCGAAGCGCCGACGGTGAGAATCCTCTTGAAAGGGGATCGCTCGTTCGTGCAGGA
      D  S  V  P  T  E  G  E  D  Y  R  I  G  G  T  E  A  P  T  V  R  I  L  L  K  G  D  R  S  F  V  Q  E 415
                                                Lsc-R5
1401  AGAATATGACTACGGTTATATTCCTGCGATGAAGGACGTGACGCTAAGCTGACACGTCGTTCTCATATGAGGCCTAAAGAACGCCTGTGCGAGCAGGC
       E  Y  D  Y  G  Y  I  P  A  M  K  D  V  T  L  S  *                                                 432
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Fig. 10. Nucleotide sequence and deduced amino acid sequence of the *lscB* gene from PG4180. Nucleotides and amino acid residues are numbered on the left and right, respectively. The putative ribosome binding site (SD), the *Xho*I sites (underlined) used for insertion of a Sm^r-Sp^r cassettes (5.1.7) and the stop codon (*) are indicated. Primer binding sites used for PCR screening of the *lscB* are indicated by dotted arrows. SD, Shine-Dalgarno sequence.

5.1.4 Expression and characterization of LscB from *P. syringae* in *E. coli*

Cloning of *lscB* in plasmid pLB7.2 or pLB7.2R resulted in no detectable levansucrase expression in *E. coli*. Therefore, we subcloned *lscB* with primers lscB-FC and Lsc-R5 into pBluescript II SK(+) as a 2.4-kb PCR product amplified from pLB7.2 yielding plasmid pLB2.4. Since in plasmid pLB2.4 the vector-based P_{lac} promoter was situated in a much closer proximity to the translational start codon of *lscB* than it was in plasmid pLB7.2, this gene could now be expressed leading to levan formation in *E. coli*. This indicated that *lscB* was not transcribed from its native promoter in *E. coli*. The relative distribution of levansucrase activities was analyzed in the extracellular, periplasmic and cytoplasmic fractions of *E. coli* (pLB2.4). No levansucrase activity was detected in the culture supernatant. The majority of levansucrase activity was found in the periplasmic fraction. Approximately 10 % of levansucrase activity was detected in the cytoplasmic fraction. However, we could not rule out the possibility of contamination of levansucrase from the periplasm in the cytoplasmic fraction by generating fractions with the method of Boyed *et al.* (1987). The levels of LscB activities assayed in different cell fractions of *E. coli* (pLB2.4) thereby confirmed earlier data for *lscA* (Hettwer *et al*., 1998). This result is in stark contrast to the situation in *P. syringae*, where levansucrase is secreted to the supernatant (see below). These results suggested that the secretory pathway responsible for levansucrase translocation in *P. syringae* is not present in *E. coli*.

5.1.5 Overexpression and purification of the fusion protein MalE::LscB in *E. coli*

Overexpression of the *lscB* gene as translational fusion to the *malE* gene coding for maltose binding protein (MBP) was performed with the expression vector pMal-c2 in *E. coli* DH5α. The *lscB* gene was amplified by PCR, with plasmid pLB7.2 serving as the template DNA and oligonucleotides LscBFBam and LscBRBam with *Bam*HI restriction site as primers, respectively. The *Bam*HI restriction sites in flanking oligonucleotides were used to clone the amplification product into the expression vector pMal-c2 (**Fig. 11A**). Plasmid pMal-lscB was obtained this way and used for overexpression and small-scale protein purification of the *lscB* gene product. After induction of the MBP-LscB fusion expression with IPTG, the fusion protein could be detected in the soluble protein fraction of *E. coli* DH5α with a molecular mass of approximately 90 kDa. The purified MBP-LscB could be obtained with the aid of the affinity of MBP to amylose resin. Eluted protein

together with crude proteins were loaded to an SDS-PAGE gel and stained with Coomassie blue (**Fig. 11B**).

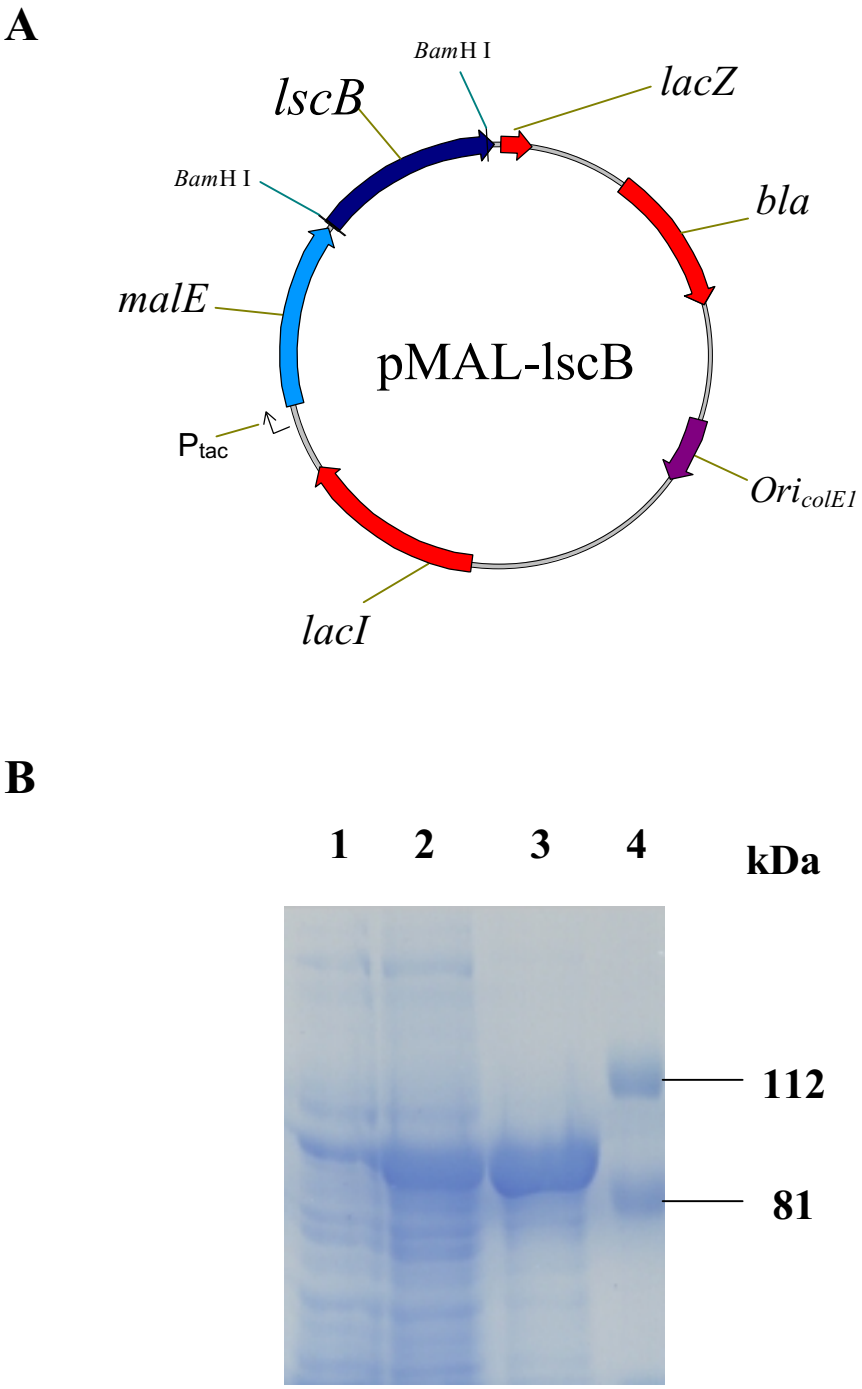

Fig. 11. (A) Scheme for the construction of translational fusion *malE*::*lscB*. (B) Overexpression and purification of MBP-LscB in *E. coli*. Proteins were separated by 10 % SDS-PAGE. 1, crude proteins from *E. coli* (pMal-lscB), uninduced; 2, crude proteins from *E. coli* (pMal-lscB), induced with IPTG; 3, purified MBP-LscB; 4, molecular weight marker.

5.1.6 Enzymatic characterization of LscB

The purified protein MBP-LscB was tested for its ability to release glucose with sucrose as the substrate. The reaction velocity produced by the 30 μg of purified MBP-LscB with varying concentrations of sucrose (20, 40, 80, 160, 240, 320 mM) was determined and the Eadie-Hofstee plot of the velocity at the specific sucrose concentration against the value V/[S] resulted in an apparent K_m = 11 mM (**Fig. 12)**.

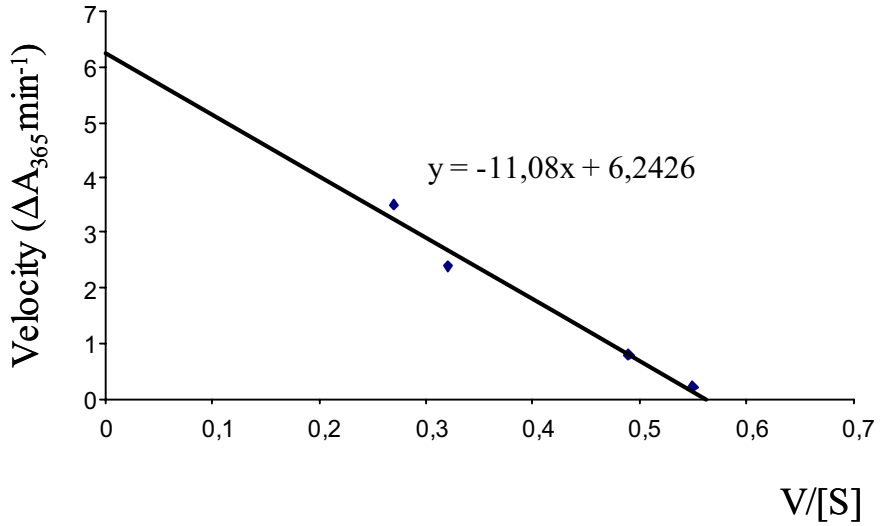

Fig. 12. Eadie-Hofstee plot of the activity of MBP-LscB on sucrose. *V* velocity (nmol/min) and S substrate concentration (mM).

5.1.7 Generation of *lscB*-deficient mutants of PG4180 and PG4180.M1 by marker exchange mutagenesis

Previously, the initial *lsc* gene (*lscA*) of PG4180 was knocked-out by insertion of a streptomycin-spectinomycin resistance (Sp^r-Sm^r) gene cassette to obtain mutant PG4180.M1 (Jaeckel, 1999). When growing on sucrose-containing MG agar plates, there was no obvious phenotype for PG4180.M1. The *lscB*-deficient mutants of PG4180 and PG4180.M1 were generated by marker exchange mutagenesis as follows. The 9.1-kb *Eco*RV insert of plasmid pLB7.2-Gm, which contained *lscB* mutagenized by insertion of a 1.9-kb gentamycin resistance (Gm^r) cassette derived from pMGm with the restriction enzyme *Sal*I and ligated to *Xho*I-treated plasmid pLB7.2, was subcloned into the mobilizable suicide vector pKmobGII yielding plasmid pKB7.2-Gm. This plasmid was mobilized into PG4180 and PG4180.M1, respectively, by triparental mating thereby obtaining mutants PG4180.M2 (*lscB*$^-$) and PG4180.M3 (*lscA*$^-$ *lscB*$^-$) via homologous marker exchange mutagenesis. The genotype of both mutants was confirmed by PCR and

Southern blot analysis (**Figs. 13 and 20**). However, neither mutant exhibited a levan-deficient phenotype when streaked on MG agar plates containing 5 % sucrose.

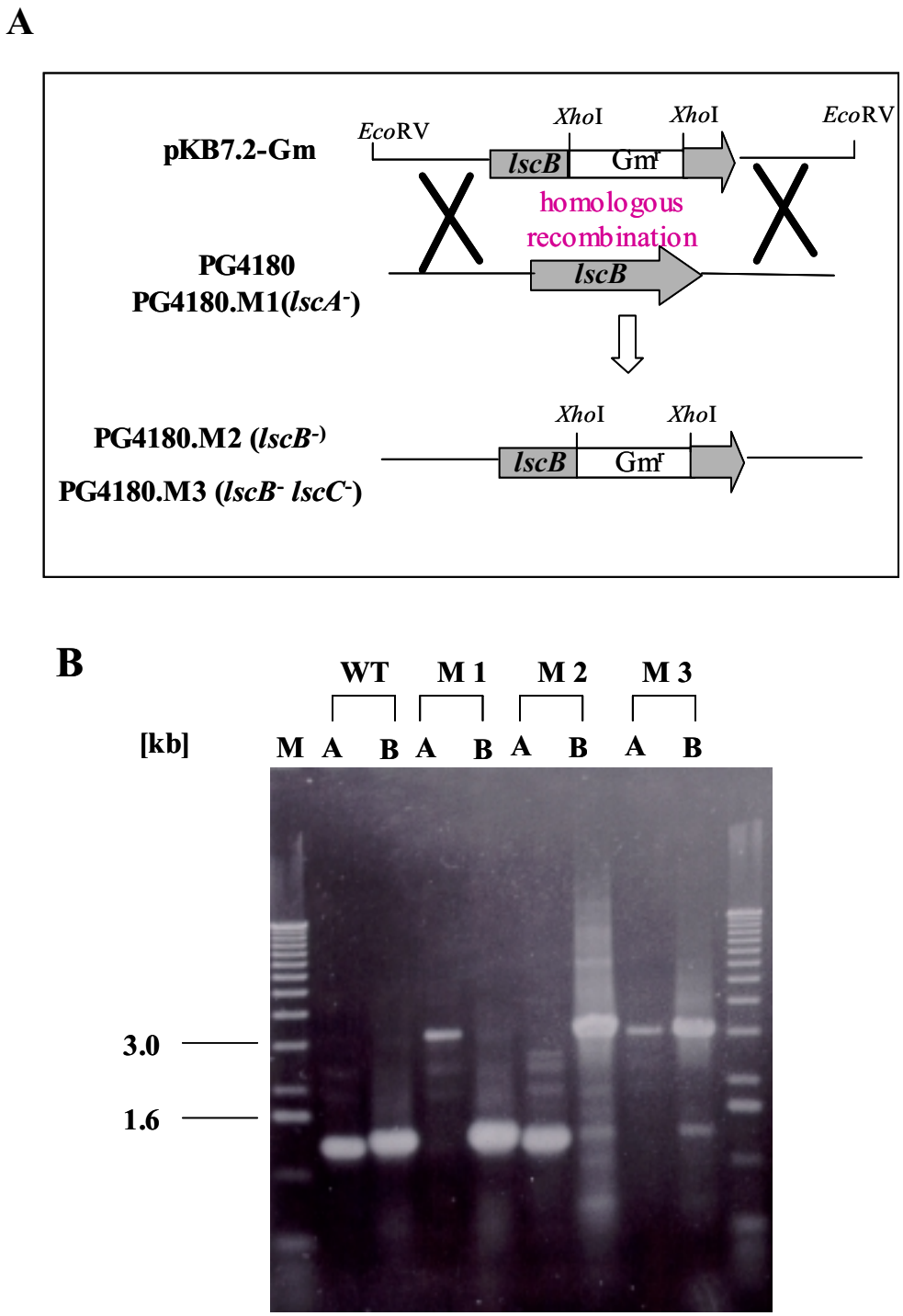

Fig. 13. Mutagenesis of *lscB* by insertion of a Gm^r resistance cassette. (A) The 1.9-kb *Sal*I-digested Gm resistance cassette was inserted into *Xho*I sites of *lscB*. The resultant plasmid, pKB7.2-Gm, is unable to replicate in *P. syringae* and exchanges its homologous insert DNA with the genome to obtain Gm-resistant and Km-sensitive clones. (B) PCR analysis of genomic DNA of PG4180 and its mutants PG4180.M1, PG4180.M2, and PG4180.M3 and the primer pairs lscA-F/R and lsc-F5/R for *lscA* and *lscB*, respectively. Lanes: A, *lscA* primers; B, *lscB* primers .

5.2 Cloning and characterization of the third *lsc* gene of *P. syringae* PG4180

5.2.1 Detection of *lsc* genes of PG4180

After both, *lscA* and *lscB*, had been inactivated by marker exchange mutagenesis, the double mutant did not exhibit a levan-deficient phenotype, indicating the existence of more than two alleles of this gene in PG4180. Therefore, we analyzed the PG4180 genome for additional *lsc* gene(s). Southern blot hybridizations under conditions of low stringency (hybridization temperature of 55°C) with genomic DNA of PG4180 digested with the restriction enzyme *Sal*I and a DNA probe containing the previously described *lscB* were carried out. The probe hybridized to three fragments of 5.5, 7.0, and 10.5 kb (**Fig. 14**). This result and Southern blot analysis with further restriction enzymes indicated the presence of one additional *lsc* gene in PG4180. The 5.5-kb *Sal*I fragment did not occur in the previously identified cosmid clones containing *lscA* and *lscB*, respectively. Consequently, the potential *lsc* gene in the 5.5-kb *Sal*I fragment was designated *lscC* and was to be cloned.

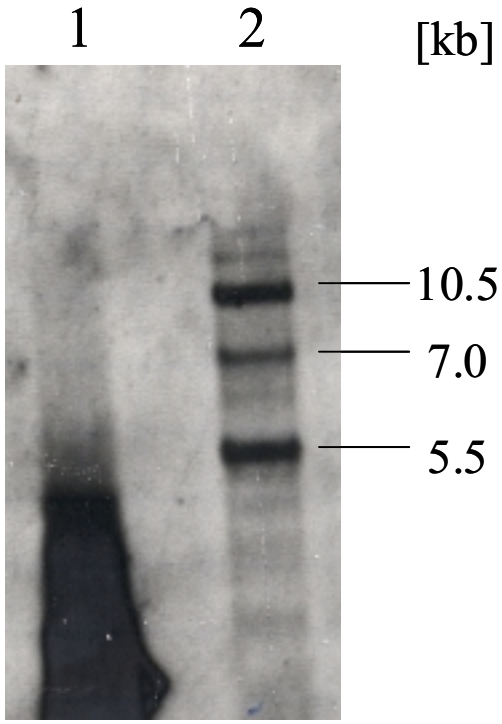

Fig. 14. Detection of three *lsc* genes in genomic DNA from PG4180 by Southern blot hybridization. A DIG-labeled PCR product of the previously characterized *lscB* was used as a DNA probe under conditions of low stringency (hybridization temperature 55°C). Lanes: 1, 1.3-kb PCR product with *lsc* from NCPPB1321; 2, *Sal*I treated genomic DNA of PG4180.

5.2.2 Cloning of *lscC* from *P. syringae* pv. glycinea

In order to find the third *lsc* gene, *lscC*, in the genomic library of PG4180 in *E. coli,* Southern blot hybridizations were performed to screen for a 5.5-kb fragment which hybridized with the probe derived from *lscA* or *lscB* (data not shown). Three individual cosmid clones contained a common 5.5-kb *Sal*I fragment that hybridized with the DNA probe. The 5.5-kb fragment of one of those cosmids, p5C10, was subcloned to pBluescript II SK(+) to yield plasmid pLC5.5. Just as the subclone containing *lscB*, this plasmid did not mediate levan synthesis in the respective *E. coli* transformants. All attempts to insert the 5.5-kb *Sal*I fragment containing *lscC* into pBluescript SK in a manner that allowed P_{lac} control of *lscC* failed. This suggested that the lack of immediate transport of its gene product to the periplasm or intrinsic characteristics of LscC might be toxic to *E. coli*.

5.2.3 Nucleotide sequence analysis of *lscC* from PG4180

The 5561-bp insert of plasmid pLC5.5 was completely sequenced (**Fig. 15**). Three complete ORFs designated *orf2*, *lscC*, and *orf4*, and an N-terminally truncated ORF named *orf1* were identified following a comparison with database entries. The deduced amino acid sequences of *orf2* and *orf4* showed 72 and 71 % similarity to an autolytic lysozyme from *Xylella fastidiosa* and a hypothetical protein from *E. coli*, respectively (accession numbers G82563 and F64902). The deduced amino acid sequence of the ORF fragment *orf1* showed 89 % similarity to a putative transposase from *Pseudomonas sp.* JR1 (accession number AF155505). The 1296-bp ORF designated *lscC* (**Fig. 16**) showed 98 % identity to *lscB* at the nucleotide sequence level. Nucleotide sequences of about 450 bp upstream of *lscB* and *lscC* were 97 % identical to each other. Both *lscB* and *lscC* showed almost 99 % identity at the deduced amino acid sequence level to each other. In their respective amino acid sequences, the gene products of *lscB* and *lscC* differed in only five residues distributed throughout the central and C-terminal regions (N92D, S119C, E327D, L329I, and T429Q). Both genes also showed 86 % identity to *lscA* at the nucleotide level and high similarity at the deduced amino acid sequence level (95 %). The gene products of *lscA*, *lscB*, and *lscC* showed amino acid sequence similarities to various levansucrases of gram-negative and gram-positive bacteria comparable to those observed for *lsc* of *P. syringae* pv. phaseolicola (Hettwer *et al*., 1998).

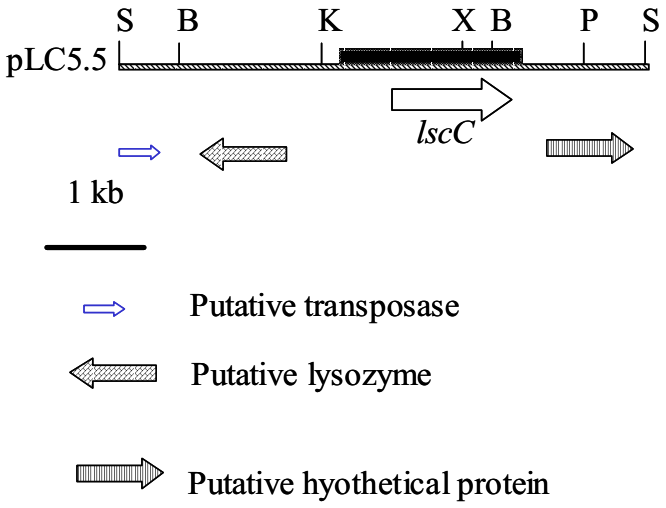

Fig. 15. Physical map of the 5.5-kb insert of pLC5.5. The striped line indicate the DNA for which the nucleotide sequence has been determined. Open arrows symbolize identified ORFs with their directions of transcriptions. Restriction enzyme sites: K, *Kpn*I; X, *Xho*I; B, *Bgl*II; S, *Sal*I; and P, *Pst*I.

5.2.4 Search for putative N-terminal signal peptide sequences

The deduced amino acid sequences of all three *lsc* genes were analyzed for putative signal peptide sequences using the SignalP version 1.1 program. No putative signal peptidase cleavage site was found in the predicted amino acid sequence of *lscA*. Although a putative signal peptidase recognition site was observed in each of the deduced amino acid sequences of *lscB* and *lscC*, the SignalP program predicted them to be too close to the N-terminus (8 amino acid residues downstream from the start codon) to be meaningful cleavage sites. Previously it was proven that no peptide cleavage occurred during the translocation of levansucrase through the two membranes by determining the N-terminal amino acid sequence of a potential Lsc isoenzyme mixture from the supernatant of PG4180 cultures (Jaeckel, 1999). It was identical to the predicted N-terminus derived from the nucleotide sequences of *lscB* and *lscC* but not to that of *lscA* (**Fig. 17**). This result together with the computer prediction clearly indicated that these proteins are not proteolytically processed during translocation across the inner and outer membrane and that the transport might therefore occur via a *sec*-independent mechanism.

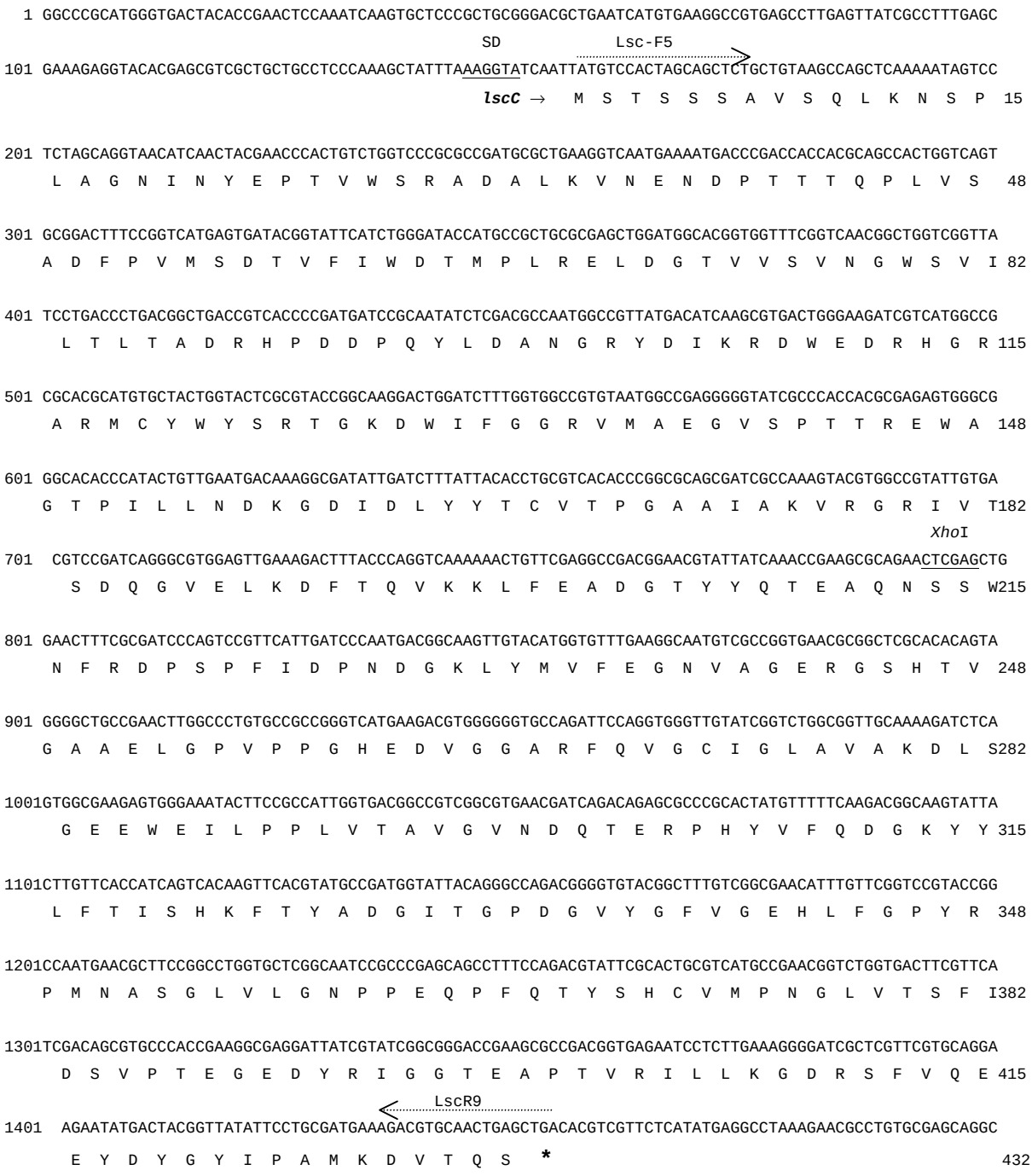

Fig. 16. Nucleotide sequence and deduced amino acid sequence of the *lscC* gene from PG4180. Nucleotides and amino acid residues are numbered on the left and right, respectively. The putative ribosome binding site (SD), the *Xho*I site (underlined) used for insertion of the Gm^r or Sm^r-Sp^r cassettes and the stop codon (*) are indicated. Primer binding sites used for PCR screening of *lscC* are indicated by dotted arrows. SD, Shine-Dalgarno sequence.

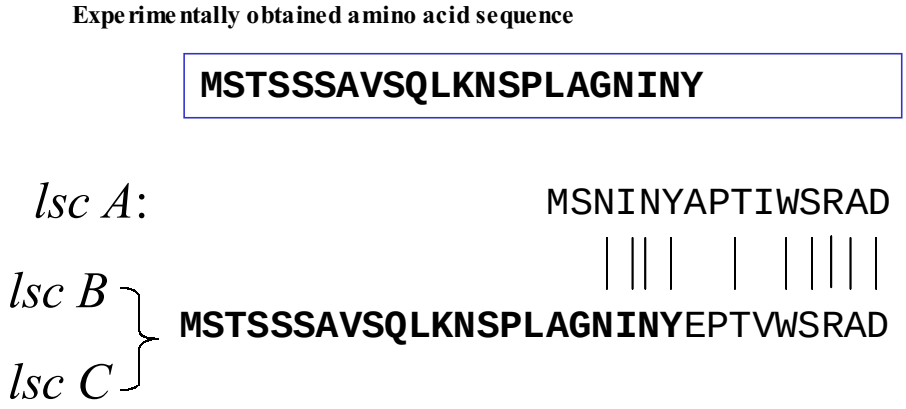

Fig. 17. Comparison of the N-terminal sequences of *lsc* gene products. The amino acid sequence in the box represents that of the ca 50-kDa protein which occurred in the supernatant of PG4180 predominantly at 18°C. The predicted N-terminal amino acid sequences of the three *lsc* gene products are given below.

5.2.5 Generation of *lscC*-deficient mutants of PG4180, PG4180.M1, and PG4180.M2 by marker exchange mutagenesis

To obtain *lscC*-deficient mutants of PG4180 and PG4180.M1 respectively, a 1.9-kb gentamycin resistance (Gm^r) cassette, which was derived by pMGm with digestion with restriction enzyme *Sal*I, was ligated to *Xho*I-treated plasmid pLC5.5. The resulting 7.5-kb *Sal*I-insert of plasmid pLC5.5-Gm was subcloned into the mobilizable suicide vector pKmobGII yielding plasmid pKC5.5-Gm. This plasmid was mobilized to PG4180 by triparental mating thereby obtaining mutant PG4180.M4 (*lscC*$^-$) via homologous marker exchange mutagenesis (**Fig. 18A**). The double mutant PG4180.M5 (*lscA*$^-$ *lscC*$^-$) was obtained by mobilization of plasmid pKC5.5-Gm into mutant PG4180.M1. The genotype of both new mutants was confirmed by PCR analysis of genomic DNA (**Fig. 18B**) and Southern blot hybridization (data not shown).

To generate an *lscB lscC* double mutant, a 2.0-kb *Sal*I-*Xho*I fragment containing a streptomycin-spectinomycin resistance cassette (Sm^r-Sp^r) from plasmid pCAM140 was ligated to plasmid pLC5.5 that had been linearized with *Xho*I. The 7.5-kb *Sal*I insert of the resulting plasmid pLC5.5-Sm was then subcloned into pKmobGII to generate plasmid pKC5.5-Sm. This plasmid was subsequently mobilized to mutant PG4180.M2 by triparental mating to obtain mutant PG4180.M6 (*lscB*$^-$ *lscC*$^-$) via homologous recombination (**Fig. 19A**). The genotype of this mutant was also confirmed by PCR (**Fig. 19B**) and Southern blot analysis (**Fig. 20**).

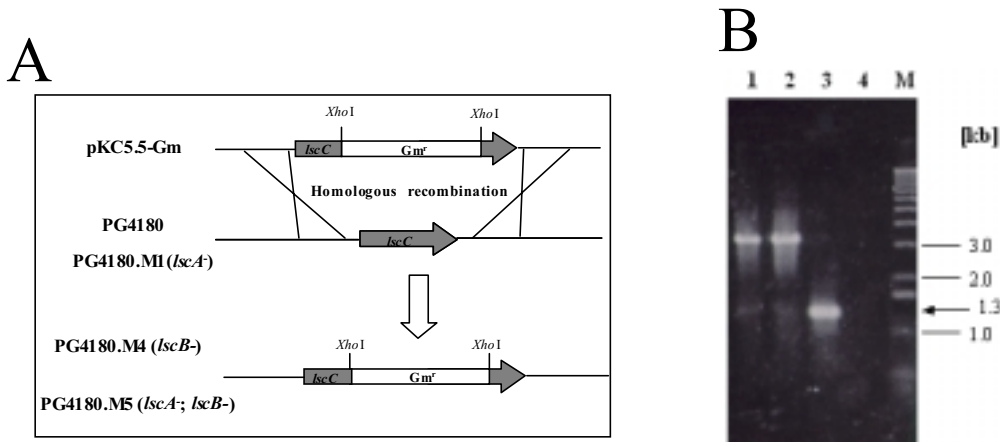

Fig. 18. Mutagenesis of *lscC* by insertion of a Gm^r resistance cassette. (A) The 1.9-kb *Sal*I-digested Gm resistance cassette was inserted into the *Xho*I site of *lscC* gene. The resultant plasmid, pKC5.5-Gm, is unable to replicate in *P. syringae* and facilitates exchange with the chromosome to obtain Gm-resistant and Km-sensitive clones. (B) PCR analysis of genomic DNA of PG4180.WT and its mutants PG4180.M4 and PG4180.M5 and the primer pairs lscC-F5/R9 specific for *lscC*. Lanes: 1, PG4180.M4; 2, PG4180.M5; 3, PG4180; 4, negative control; and M, molecular marker.

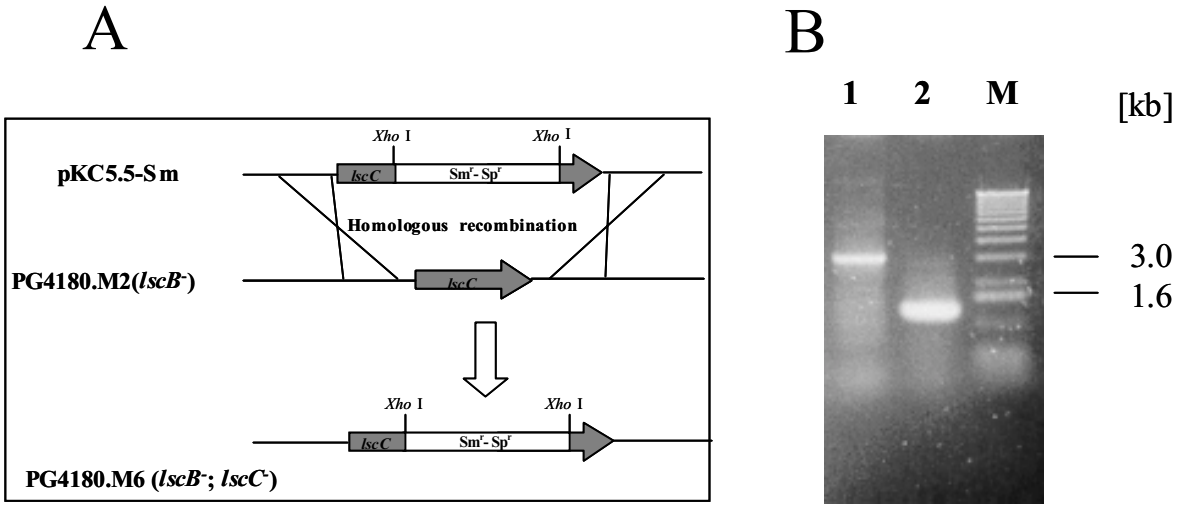

Fig. 19. Mutagenesis of *lscC* by insertion of a Sp^r-Sm^r resistance cassette. (A) The 2.0-kb *Sal*I/*Xho*I-digested Sp^r-Sm^r resistance cassette was inserted into the *Xho*I site of *lscC*. The resultant plasmid, pKC5.5-Sm, is unable to replicate in *P. syringae* and facilitates exchange with chromosome to obtain Gm-resistant and Km-sensitive clones. (B) PCR analysis of genomic DNA of PG4180 and its mutants PG4180.M6 and the primer pairs lscC-F5/R9 specific for *lscC*. Lanes: 1, PG4180.M6; 2, PG4180; and M, molecular marker.

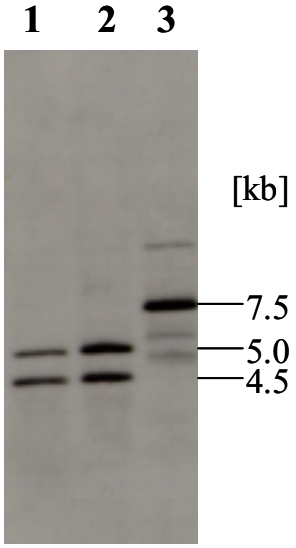

Fig. 20. Southern blot hybridization confirming the disruption of *lscB* in *P. syringae* PG4180 mutants. Southern blot analysis was performed on *Eco*RV digests of total plasmid DNA from PG4180, PG4180.M3 and PG4180.M6 using a digoxigenin-labeled PCR product of *lscB* . Since *lscB* is plasmid borne and *Eco*RV cuts within the Gm resistance cassette, the probe hybridizes to a 7.5 kb *Eco*RV fragment from PG4180 and two *Eco*RV fragment of 4 kb and 5.5 kb, respectively, from PG4180.M3 and PG4180.M6. Lanes: 1, PG4180.M3; 2, PG4180.M6; 3, PG4180.

5.3 Genomic localization of *lscA*, *lscB*, and *lscC*

PG4180 harbors five indigenous plasmids with molecular sizes between 45-100 kb which encode various virulence and fitness determinants (Bender *et al.*, 1991; Watanabe *et al.*, 1998). In order to test whether any of the *lsc* genes might be plasmid-borne, a Southern blot experiment was carried out with undigested plasmid DNA of PG4180 and a probe containing *lscA* (**Fig. 21**). A clear signal was detected with a band representing the approximately 60-kb plasmid p4180D (Bender *et al*., 1991; Ullrich *et al*., 1993). When plasmid DNA of PG4180 was digested with the restriction enzyme *Sal*I, a 10.5-kb fragment hybridized to the probe (**Fig. 21**). To find out which *lsc* gene was plasmid-encoded, Southern blot analyses with DNA probes derived from upstream DNA of *lscB* and *lscC*, respectively, were carried out (**Fig. 22**). The probe from the upstream region of *lscB* but not that associated with *lscC* hybridized to plasmid p4180D and to the 10.5-kb *Sal*I fragment from total plasmid DNA of PG4180. Furthermore, a 10.5-kb *Sal*I fragment was identified in cosmid p7C7, from which *lscB* was subcloned. These results suggested that *lscA* and *lscC* were chromosomally located whereas *lscB* was plasmid-borne.

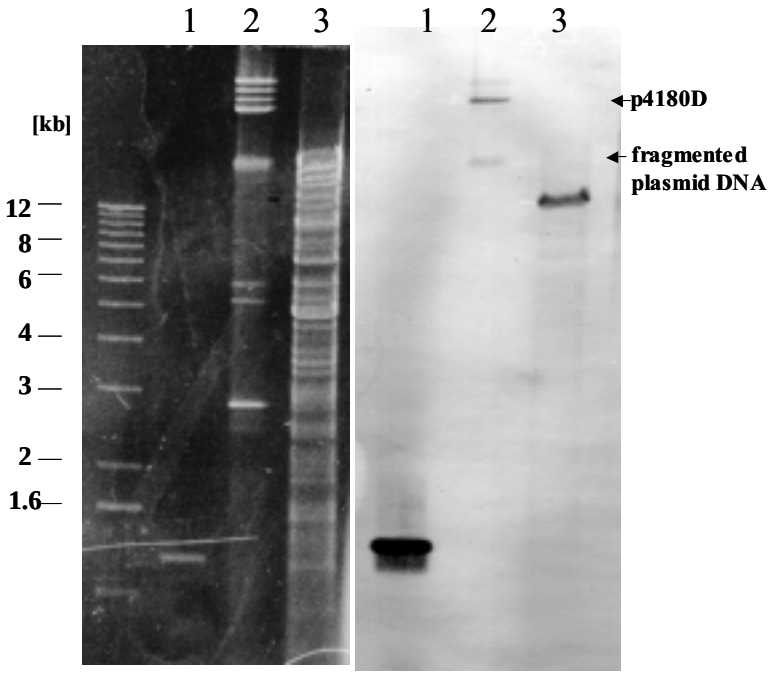

Fig. 21. Analysis of the plasmid-borne location of an *lsc* gene from PG4180 by Southern blot hybridization (hybridization temperature 68°C) with *lscA* as a DNA probe. The approximately 60-kb plasmid p4180D, the fragmented plasmid and linearized chromosomal DNA are marked by arrows. Lanes: M, molecular size marker; 1, PCR product of *lscA*; 2, total undigested plasmid DNA of PG4180; 3, *Sal*I-digested total plasmid DNA of PG4180.

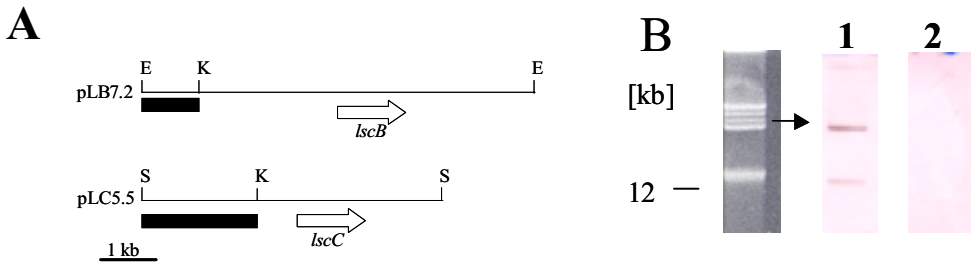

Fig. 22. Localization of *lsc* genes from PG4180 by Southern blot analysis with undigested plasmid DNA of PG4180. (A). Scheme of probes from the upstream DNA regions of 7.2-kb and 5.5-kb inserts of pLB7.2 and pLC5.5, respectively. Black boxes indicate DNA regions upstream of *lscB* and *lscC* used as probes for the Southern blot analyses. E, *Eco*RV; K, *Kpn*I; and S, *Sal*I. (B). Southern blot analyses using the upstream DNA of *lscB* as a probe (panel 1) and the upstream DNA of *lscC* as a probe (panel 2).

5.4 Phenotypic analysis of *lsc*-deficient mutants of PG4180

Mutants of PG4180 disrupted in all three *lsc* genes were generated. Genotypes of all mutants were verified by PCR and Southern blot analyses indicating that *lscA*:Sm^r, *lscB*:Gm^r, *lscC*:Gm^r, and *lscC*:Sm^r had replaced their native alleles in the respective mutants. Subsequently, all mutants were grown on MG agar plates supplemented with 5 %

sucrose. Mutants PG4180.M1 (*lscA*$^-$), PG4180.M2 (*lscB*$^-$), PG4180.M3 (*lscA*$^-$ *lscB*$^-$), PG4180.M4 (*lscC*$^-$), and PG4180.M5 (*lscA*$^-$ *lscC*$^-$) did not exhibit a levan-deficient phenotype (data not shown), suggesting that at least two *lsc* gene products contribute to levan formation in PG4180. When plated on MG agar containing 5 % sucrose, mutant PG4180.M6 (*lscB*$^-$ *lscC*$^-$) did not produce levan (**Fig. 23**), indicating that *lscB* and *lscC* are functional whereas *lscA* might not be transcribed or its gene product might exhibit an undetectable enzymatic activity.

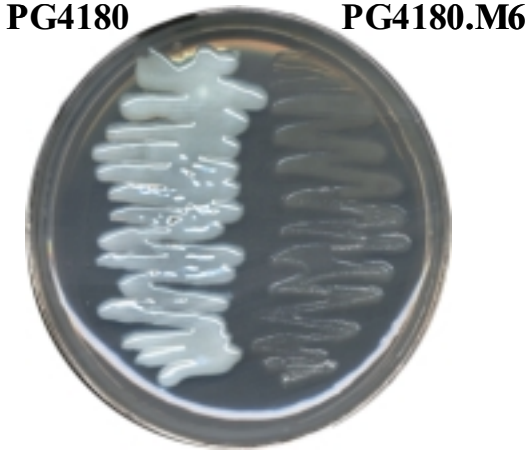

Fig. 23. Levan formation by PG4180 and mutant PG4180.M6 (*lscB*$^-$ *lscC*$^-$). Bacteria were streaked on MG agar plates containing 5 % sucrose and incubated at 18°C for 7 days.

5.4.1 Compartment-specific analysis of levansucrase activities in PG4180

In order to quantitatively determine the contribution of each *lsc* gene product to the total levansucrase activity inside and outside the cell, cultures of PG4180 and its *lsc* mutants were grown in HSC medium at 18 °C until they reached an OD_{600} of 1.5 – 2.0. Subsequently, levansucrase activities in the total cell lysate, periplasmic fraction, cytosolic fraction, and the cell-free supernatant were quantified photometrically (**Fig. 24**). Although visually not distinguishable from the wild type's levan formation, all mutants except PG4180.M6 (*lscB*$^-$ *lscC*$^-$) consistently showed a slight to moderate decrease of total levansucrase activities ranging from 46 to 78 % of the wild type level. No levansucrase activity could be measured for the *lscB*$^-$ *lscC*$^-$ double mutant PG4180.M6. The periplasmic portions of levansucrase activities were relatively unaffected in all levan-producing mutants and represented the largest part of the total levansucrase activity (**Fig. 24**). In contrast, cytosolic fractions contained only minor amounts of levansucrase suggesting that the *lsc* gene products were efficiently exported in PG4180. Levansucrase activities in the cytosolic and extracellular fractions were the lowest in mutants disrupted in *lscB*

(PG4180.M2, PG4180.M3, and PG4180.M6) indicating that *lscB* expression might represent the major source of total levansucrase activity. When comparing supernatant samples of the three single mutants, PG4180.M1 (*lscA*$^-$), PG4180.M2 (*lscB*$^-$), and PG4180.M4 (*lscC*$^-$), it appears that the *lscB* gene product contributes most to the extracellular levansucrase activity (**Fig. 24**). This result could be confirmed when the extracellular levansucrase levels of the double mutants were compared. LscB and LscC contributed equally to the levansucrase activity in the periplasm. Mutations in *lscA* had the least significant impact on levansucrase activity regardless of the fraction studied. Additionally, we analyzed the membrane fractions of various mutants and the wild type of PG4180 for levansucrase activities. Levansucrase activity was negligible in those fractions regardless of the mutant background suggesting that levansucrase is not membrane-bound.

To ensure that the subcellular fractions were not significantly contaminated with proteins from other fractions, cells of PG4180 (pAS-LacZ) and PG4180 (pHL-PhoA) were incubated under the same conditions as described above and subjected to subcellular fractionation. The two recombinant plasmids harbor translational fusions of β-galactosidase (LacZ) to CorS (Smirnova, 2000) and alkaline phosphatase (PhoA) to LscB, respectively. Subsequently, LacZ and PhoA activities were quantified photometrically in three individual experiments with each three replicates. The cytosolic fraction of PG4180 (pAS-LacZ) showed 198 U β-galactosidase activity while the cognate periplasmic fraction exhibited 54 U LacZ activity indicating that the periplasmic fraction could have been contaminated with cytosolic LacZ by approximately 27 %. When fractions of PG4180 (pHL-PhoA) were analyzed for PhoA activities, 22.5 U PhoA activity were measured in the periplasm as compared to 3.3 U PhoA activity in the cytosolic fraction suggesting that the latter fraction was contaminated with periplasmic PhoA by 15 %. Neither LacZ nor PhoA activities were detected in the extracellular fractions.

To confirm our quantitative data, we subsequently analyzed the subcellular fractions of the three double mutants (PG4180.M3, PG4180.M5, and PG4180.M6) by zymographic detection of levansucrase activities. As shown in **Fig. 25**, levansucrase accumulated in the periplasm of the *lscA*$^-$ *lscB*$^-$ mutant whereas only minor traces of levansucrase activity could be observed in the supernatant of this mutant. In contrast, the *lscA*$^-$ *lscC*$^-$ mutant showed a remarkable accumulation of levansucrase in the supernatant furthermore suggesting that LscB was the major source of extracellular levansucrase activity. As expected, mutant PG4180.M6 did not show any periplasmic or extracellular levan formation (**Fig.25**). In summary, our compartment-specific analyses suggested that LscB

and LscC but not LscA contribute to levansucrase activity in the periplasm in significant amounts. In contrast to LscC, LscB is further required for detectable levan formation in the cell's exterior.

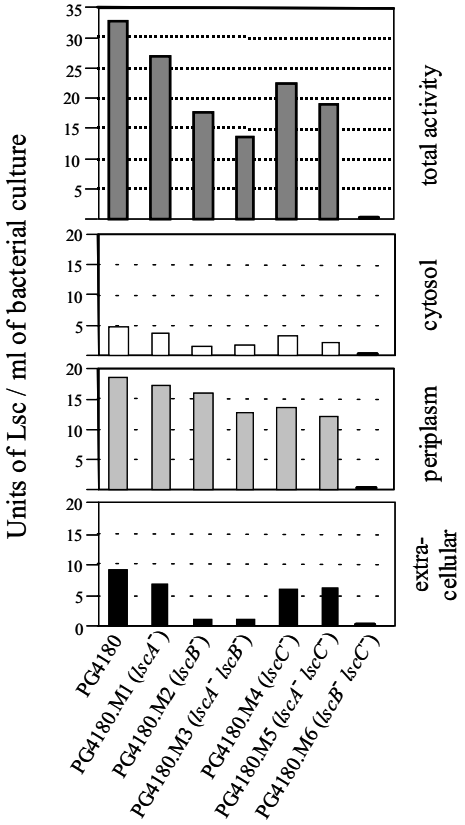

Fig. 24. Quantitative analysis of the compartment-specific levansucrase activities in PG4180 and its *lsc* mutants. Bacterial cultures were incubated in HSC medium at 18°C until they reached an OD_{600} of 1.5 – 2.0. Levansucrase activities normalized to 1 ml of culture were photometrically determined in the total cell culture and in cytosolic, periplasmic, and extracellular fractions. Data represent average values from three independent experiments with each three replicates.

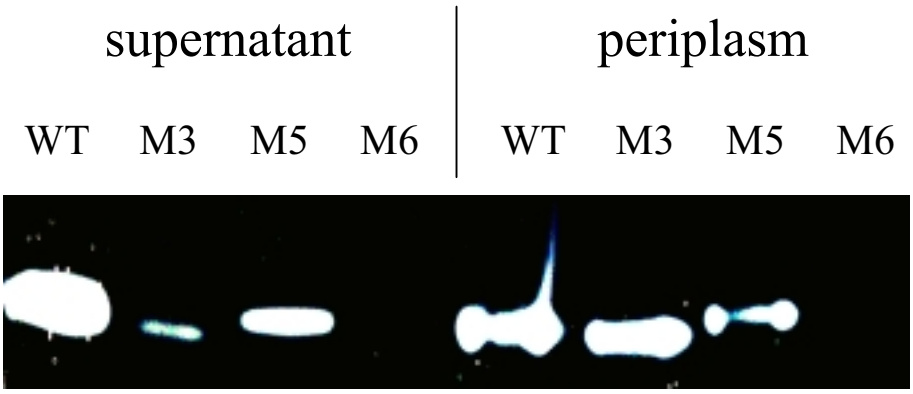

Fig. 25. Zymographic detection of levansucrase activities from periplasmic and extracellular fractions of PG4180 and the *lsc* double mutants PG4180.M3 (*lscA*⁻ *lscB*⁻), PG4180.M5 (*lscA*⁻ *lscC*⁻), and PG4180.M6 (*lscB*⁻ *lscC*⁻). Protein samples were concentrated 30-fold, loaded to a polyacrylamide gel, and separated under non-denaturing conditions. The gel was subsequently soaked in water containing 10 % sucrose. The whitish swelling of the gel matrix corresponds to levan formation.

5.4.2 Immunological detection of Lsc in different cell compartments

To distinguish between protein secretion and enzymatic activity of levansucrase, Western blot experiments were carried out with total cellular protein samples and extracellular protein fractions of PG4180 and its *lsc* mutants using polyclonal antibodies raised against levansucrase of *P. syringae* pv. phaseolicola (Hettwer *et al*.,1995). Results given in **Fig. 26** demonstrate that levansucrase could be detected in crude protein extracts and in the supernatant of PG4180 but not in any protein fractions of PG4180.M6 (*lscB*$^-$ *lscC*$^-$) suggesting that *lscA* is not expressed. Signals for levansucrase were detected in all levan-producing mutants of PG4180 (**Fig. 26A**). Moreover, signals were strongly decreased or absent from the extracellular fractions of mutants disrupted in *lscB* (PG4180.M3 and PG4180.M6) but were present in the *lscA*$^-$ *lscC*$^-$ mutant PG4180.M5 (**Fig. 26B**) suggesting that LscB was the secreted enzyme. These results show that the compartment-specific occurrence of levansucrase activities strictly correlates with the presence of the enzyme(s), allowing the possibility of a catalytic inactivation of LscC in the culture supernatant of PG4180 to be ruled out.

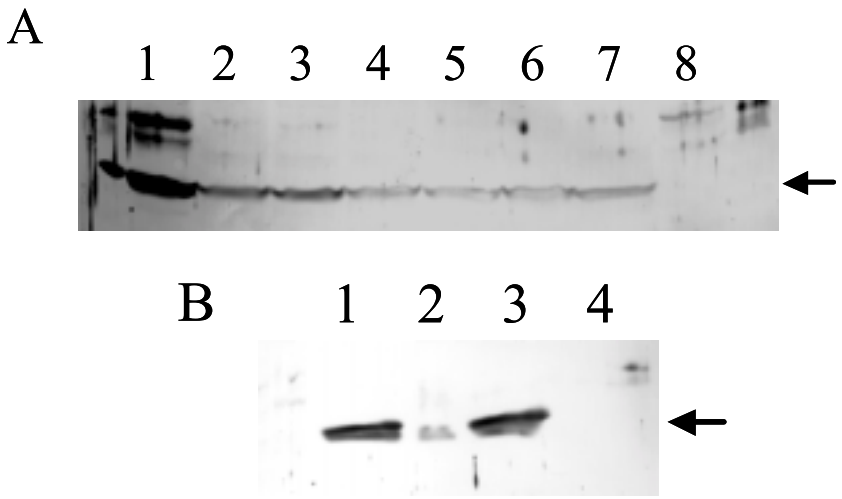

Fig. 26. Western blot analysis of PG4180 and its *lsc* mutants using a polyclonal antiserum raised against levansucrase from *P. syringae* pv. phaseolicola NCPPB1321. (A) 10 % SDS-PAGE with crude protein extracts. Cells were grown to an OD_{600} of 1.5 – 2.0 at 18°C and then subjected to total protein isolation. Lanes: 1, *E. coli* (pLB2.4); 2, PG4180; 3, PG4180.M1 (*lscA*$^-$); 4, PG4180.M2 (*lscB*$^-$); 5, PG4180.M3 (*lscA*$^-$ *lscB*$^-$); 6, PG4180.M4 (*lscC*$^-$); 7, PG4180.M5 (*lscA*$^-$ *lscC*$^-$); and 8, PG4180.M6 (*lscB*$^-$ *lscC*$^-$). (B) 10 % SDS-PAGE with cell-free extracellular fractions of PG4180 and its *lsc* double mutants. Cells were grown to an OD_{600} of 1.5 – 2.0 at 18°C and then centrifuged. The supernatants were filter-sterilized, concentrated 30-fold, and applied to the gel. Lanes: 1, PG4180; 2, PG4180.M3 (*lscA*$^-$ *lscB*$^-$); 3, PG4180.M5 (*lscA*$^-$ *lscC*$^-$); and 4, PG4180.M6 (*lscB*$^-$*lscC*$^-$). Arrows mark the specific signals representing levansucrase.

5.4.3 Complementation of the levan-deficient mutant PG4180.M6

To provide evidence that the gene product of *lscB* is indeed secreted by PG4180, the 7.2-kb insert of pLB7.2 was subcloned in the broad-host range vector pBBR1MCS to yield pRB7.2. This plasmid and cosmid p5C10 carrying copies of *lscB* and *lscC*, respectively, were separately introduced to the *lscB*$^-$ *lscC*$^-$ mutant PG4180.M6. The accumulation of levansucrase in subcellular fractions of the respective transconjugants was assayed by zymographic levansucrase detection and Western blot analysis (data not shown). As expected, levansucrase was secreted to the exterior of transconjugant PG4180.M6 (pRB7.2) but not to that of PG4180.M6 (p5C10). However, both transconjugants were visibly mucoid when streaked on MG plates containing 5 % sucrose. These results supported our previous findings and suggested that even though functional *lscC* restored levan formation to PG4180.M6, its gene product was not secreted to the supernatant but instead must have accumulated in the periplasm.

5.4.4 Effects of *lscB lscC* mutation in PG4180 on virulence and saprophytic survival *in planta*

To investigate the role of levan formation of *P. syringae* PG4180 during infection of soybean plants, the *in planta* survival of the wild type strain and its mutant PG4180.M6 was tested by infecting soybean plants (4-6 weeks old) and measuring bacterial growth in the infected leaves. When leaves were infected with almost equal amounts of the wild type and the mutant by spray inoculation, the initial *in planta* growth rate was almost identical between the two strains (**Fig. 27**). However, 11 days after infection a clear difference occurred between both treatments. While the wild type continued to multiply inside the plant tissue, colony forming unit numbers declined in the case of infection with the levan-deficient mutant. 14 and 21 days post infection, mutant PG4180.M6 did not multiply inside the plant anymore (**Fig. 27**).

Additionally, virulence of the wild-type strain PG4180 and its levan-deficient mutant was determined by an alternative infiltration assay on soybean leaf tissue where bacteria were directly injected into the leaf tissue (data not shown). Interestingly, under those conditions no obvious difference between the wild type and mutant PG4180.M6 could be observed. These results suggested that levan formation contributes to the bacterial survival on the plant surface and possibly to the natural infection process but does not contribute

significantly to the overall pathogenicity of PG4180 once the bacteria are inside the plant's apoplast.

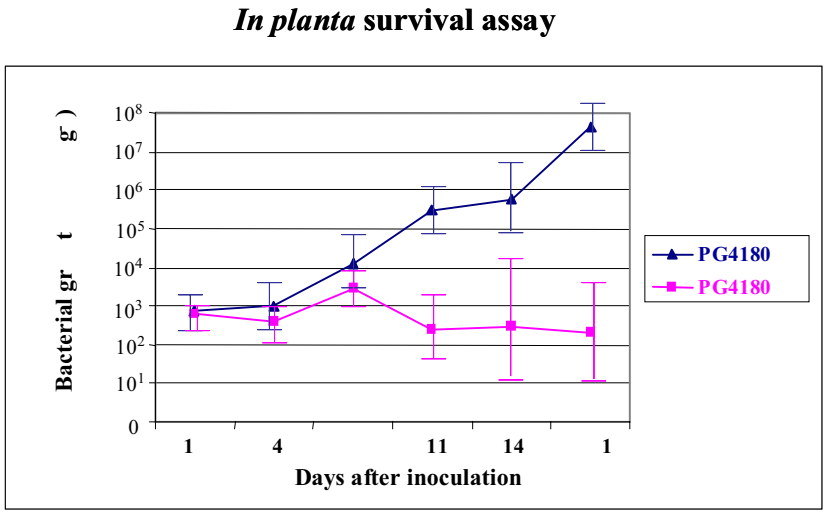

Fig. 27. *In planta* bacterial multiplication of PG4180 (▲) and PG4180.M6 (■) after spray-inoculation with bacteria derived from 18°C. Results represent data from three independent experiments with each two replicates.

5.5 Screening for multiple levansucrase alleles in various pathovars of *P. syringae*

Thirty-six strains representing 21 different pathovars of *P. syringae* were screened for multiple levansucrase genes by PCR (**Table 9**). For strains of the pathovars glycinea, tomato, phaseolicola, lachrymans, and morsprunorum, Hettwer *et al.* (1998) had previously demonstrated the presence of *lscA* homologues by PCR analysis. In this study, the primer sets lscB-F/lscB-R and lscC-F/lscC-R were used to amplify 1.3-kb PCR products representing *lscB* and *lscC*, respectively (**Fig. 28**). Both signals were amplified from genomic DNA of all tested strains of the pathovars glycinea, tomato, phaseolicola, tabci, lachrymans, coriandricola, and photiniae. In contrast, the PCR product of *lscC* but not that of *lscB* was amplified from strains representing the pathovars savastanoi, syringae, myricae, garcae, morsprunorum, and persicae. Samples of representative strains from nine additional pathovars did not yield any detectable PCR products. These results were in part confirmed by Southern blot analysis of the 1.3-kb PCR products using *lscB* of PG4180 as the DNA probe (data not shown). When the respective PCR products were treated with

*Xho*I, a restriction fragment length polymorphism was observed among samples from *P. syringae* pvs. glycinea, phaseolicola, syringae, tomato, tabci, lachrymans, coriandriocola, photiniae, respectively (**Fig. 28**). These data revealed that the occurrence of multiple copies of *lsc* is widespread in various pathovars of *P. syringae*.

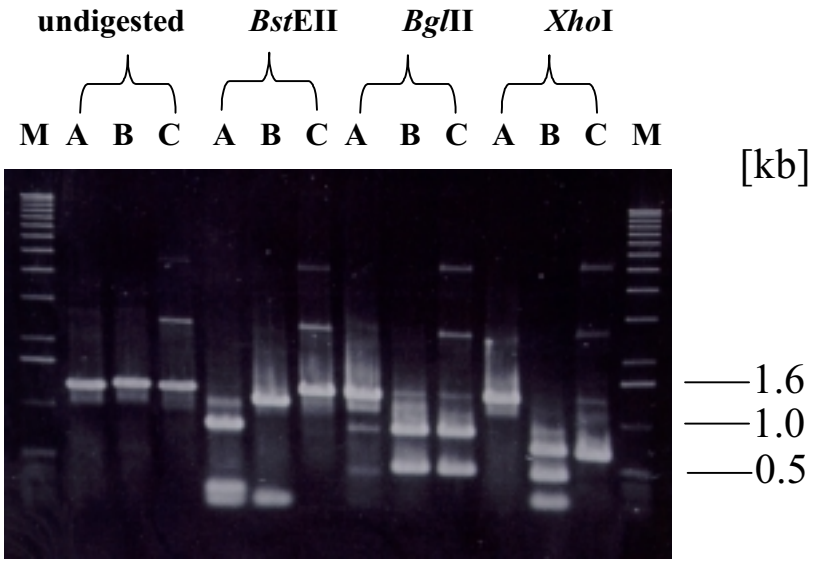

Fig. 28. PCR detection of individual *lsc* genes using genomic DNA of PG4180 and the specific primer pairs lscA-F/R, lsc-F5/R, and lsc-F5/R9 for *lscA*, *lscB*, and *lscC*, respectively and restriction analysis of the three gene products with *Bst*EII, *Bgl*II, and *Xho*I. Lanes: A, *lscA* primers; B, *lscB* primers; C, *lscC* primers; and M, molecular marker.

Tab. 9. **PCR screening for levansucrase alleles in *P. syringae* pathovars**

Strain		Result of PCR analyses for:		Reference or source [a]
		lscB	***lscC***	
P. syringae pv. phaseolicola	NCPPB1321	+	+	(Hettwer *et al*., 1995)
	Psph 6/0	-	+	B. Völksch
	GSPB 796	+	+	GSPB[a]
P. syringae pv. glycinea	PG4180	+	+	R.E. Mitchell
	Psg 16/83	+	+	(Ullrich *et al*., 1993)
	Psg 7a/90	+	+	(Ullrich *et al*., 1993)
P. syringae pv. tomato	DC3000	+	+	D. Cuppels
	DSM 50315	+	+	DSM[b]
	GSPB 119	+	+	GSPB

P. syringae pv. tabaci	GSPB 113	+	+	GSPB
	GSPB 117	+	+	GSPB
P. syringae pv. lachrymans	GSPB 77	+	+	GSPB
P. syringae pv. coriandricola	GSPB 1784	+	+	GSPB
P. syringae pv. photiniae	CFBP 11034	+	+	CFBP[c]
P. syringae pv. savastanoi	GSPB 2264	-	+	GSPB
	GSPB 2259	-	+	GSPB
P. syringae pv. syringae	FF5	-	+	G.W. Sundin
	B301D	-	+	D.C. Gross
	Pss B48	-	+	G.W. Sundin
	Pss 3525	-	+	G.W. Sundin
P. syringae pv. myricae	CFBP 11005	-	+	CFBP
P. syringae pv. garcae	CFBP 1634	-	+	CFBP
P. syringae pv. morsprunorum	D5	-	+	K. Naumann
	GSPB 886	-	+	GSPB
	GSPB 1013	-	+	GSPB
	Pm 7	-	+	A. Jones
P. syringae pv. persicae	GSPB 1025	-	+	GSPB
P. syringae pv. atropurpurea	MAFF 01309	-	+	K. Nishiyama
P. syringae pv. hibisci	CFBP 11294	-	-	CFBP
P. syringae pv. mellea	CFBP 2344	-	-	CFBP
P. syringae pv. striafaciens	GSPB 1850	-	-	GSPB
P. syringae pv. helianthi	GSPB 2688	-	-	GSPB
P. syringae pv. zizaniae	CFBP 11040	-	-	CFBP
P. syringae pv. pisi	GSPB 104	-	-	GSPB
P. syringae pv. apii	GSPB 2153	-	-	GSPB

[a]GSPB, Göttinger Sammlung phytopathogener Bakterien, Göttingen, Germany
[b]DSM, Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany
[c]CFBP, Collection Francaise des Bacteries Phytopathogenes, France

5.6 Analysis of the gene product of *lscA*

Although *lscA* was previously expressed in *E. coli* giving rise to levan formation by this organism (Hettwer *et al*., 1998), our current data for the levan-deficient mutant PG4180.M6 (*lscB*$^-$ *lscC*$^-$) suggested that *lscA* was not expressed in PG4180 under the tested

in *vitro* conditions. In order to confirm this, three strategies were used to investigate this as outlined below.

5.6.1 Analysis of the 3.1-kb *Pst*I fragment containing *lscA*

As described previously, a 3.1-kb *Pst*I fragment containing *lscA* was subcloned from cosmid clone pCK2, which led *E. coli* harboring it to exhibit a clearly mucoid phenotype when incubated on LB agar plate containing 5% sucrose (Hettwer *et al*., 1998). When we subcloned this 3.1-kb *Pst*I fragment into pBluescript SK with *lscA* in both orientation to P_{lac}, both clones conferred a mucoid phenotype when the transformants were incubated on LB agar plates with 5 % sucrose. In order to elucidate the promoter region upstream of *lscA*, we subcloned a 7.5-kb *Sal*I fragment from cosmid pCK2 which harbors the 3.1-kb *Pst*I fragment. Interestingly, the upstream region of *lscA* in this 7.5-kb *Sal*I fragment showed the same restriction sites as the cosmid vector, pRK7813, in which the genomic library of PG4180 had been constructed. Furthermore, Southern blot hybridization with a DNA probe derived from DNA upstream of *lscA* (**Fig. 29A**) was carried out, in which a signal was detected for the empty cosmid pRK7813 (**Fig. 29B**). Our data indicated that the DNA upstream of *lscA* might have been derived from vector pRK7813 and might facilitate the transcriptional activation of *lscA* leading to levan formation in recombinant *E. coli* harboring *lscA*.

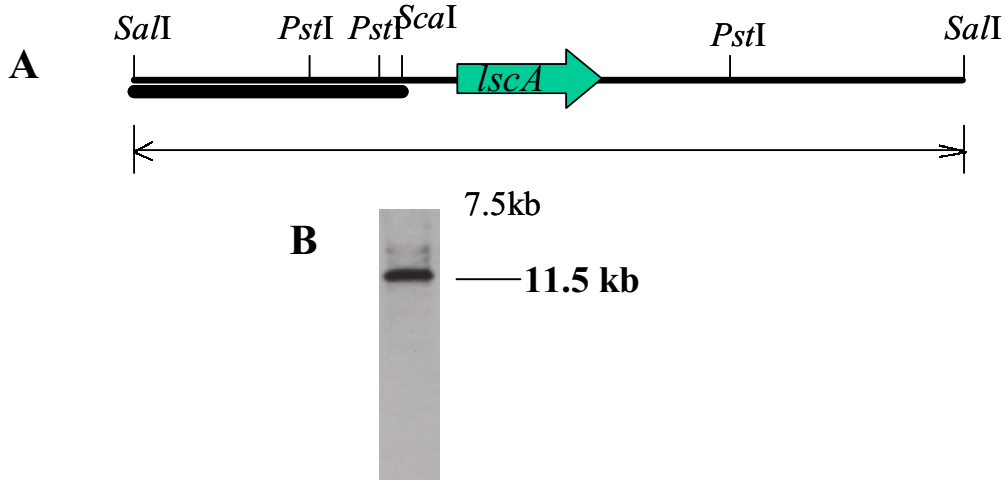

Fig. 29. Analysis of the upstream region of *lscA* in cosmid pCK2. (A) Restriction map of the 7.5-kb *Sal*I fragment from pCK2. The black box indicates a DNA region upstream of *lscA* used as a probe for Southern blot hybridization. (B) Analysis of vector pRK7813 by Southern blot hybridization with the *Sal*I-*Sca*I region from the 7.5-kb fragment of pCK2 used as probe.

5.6.2 Northern blot analyis

The following experiment was intended to determine whether or not transcription of *lscA* occurred in PG4180.

Total RNA extracts were prepared from *P. syringae* PG4180 and PG4180.M6 cells grown at 18°C. Total RNA preparations contain primarily ribosomal RNA and transfer RNA (up to 80% - 90%) and only minor amounts of mRNA. Northern blot hybridizations were carried out with a labeled RNA probe derived from the coding region of *lsc* genes by PCR using primers Lsc-fwd and LscR-T7. Based on nucleotide sequence analysis, the size of the mRNA transcript derived from any of the *lsc* loci could be expected to range from ca 1.3- to 1.5-kb. A signal indicating a transcript size of 1.5-kb was detected in RNA samples of PG4180 and corresponded well to the predicted transcript sizes of *lscA*, *lscB*, or *lscC* (**Fig. 30**). No such signal was detected in RNA samples derived from mutant PG4180.M6, which is disrupted in *lscB* and *lscC*. In this mutant a hybridizing signal was much smaller. These results indicated that *lscA* was not transcribed in the mutant PG4180.M6 under the tested conditions and suggested that this gene might not be transcribed in PG4180 at all.

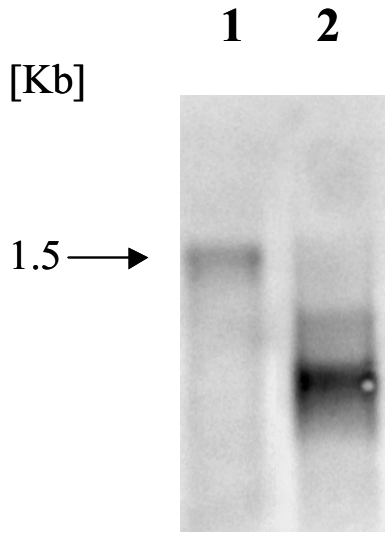

Fig. 30. Northern blot analysis of total RNA of *P. syringae* PG4180 and its mutant PG4180.M6 grown at 18°C. Cells were grown to an OD_{600} of 1.5 and then subjected to total RNA extraction. Following electrophoresis RNA samples were transferred to nitrocellulose filters and hybridized with a digoxigenin-II-UTP labeled RNA probe derived from *lsc*. The *lsc* signal is marked by an arrow. Lanes: 1, PG4180; 2, PG4180.M6.

5.6.3 Immunological analysis of LscA

Next, it was to be tested whether a gene product of *lscA* exists in PG4180 under the tested laboratory condition.

The 3.1-kb *Pst*I fragment from plasmid pSKL3 harboring a functional *lscA* gene was subcloned into the broad-host-range vector pRK415 yielding plasmid pRA3.1 (**Fig. 31A**), in which *lscA* was transcriptionally linked to the vector-borne P_{lac} promoter. This plasmid

was then introduced to PG4180 and its mutant PG4180.M6. Transconjugant PG4180.M6 (pRA3.1) showed a mucoid phenotype when streaked on MG plates containing 5 % sucrose indicating that the *lscA* gene product is functional but its gene is not expressed from its own promoter under the tested in *vitro* conditions in PG4180. Subsequently, Western blot experiments were carried out with PG4180, PG4180.M6 and their respective transconjugants harboring plasmid pRA3.1 (**Fig. 31B**). The immunologically detectable gene product of *lscA* can clearly be distinguished from those of *lscB* or *lscC* due to its smaller size. LscA could be detected when its gene was transcribed from the P_{lac} promoter in PG4180 (pRA3.1) and PG4180.M6 (pRA3.1) but not in PG4180 and its mutant PG4180.M6 (**Fig. 31B**) confirming previous observations.

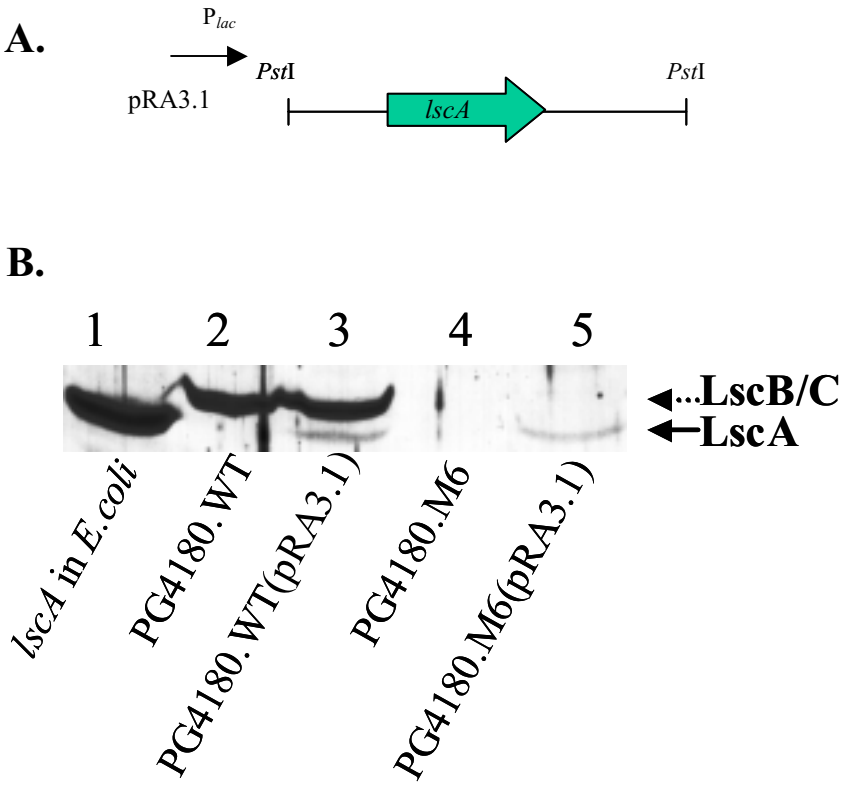

Fig. 31. Detection of the gene product of *lscA* in *P. syringae* by Western blot analysis following expression of *lscA* under control of P_{lac} on plasmid pRA3.1. (A) Scheme for the 3.1-kb *Pst*I fragment in plasmid pRA3.1. (B) Western blot analysis. The antiserum used was raised against levansucrase from *P. syringae* pv. phaseolicola NCPPB1321. 10 % SDS-PAGE with crude protein extracts. Cells were grown to an OD_{600} of 1.5 – 2.0 at 18°C and then subjected to total protein extraction. Lanes: 1, *E. coli* (pSKL3); 2, PG4180; 3, PG4180 (pRA3.1); 4, M6; and 5, M6 (pRA3.1). LscA (solid arrow) and LscB or LscC (dashed arrow) are distinguishable by their different molecular weights.

5.7 Temperature-dependent secretion of levansucrase in PG4180

5.7.1 Detection of levansucrase in extracellular protein fractions of PG4180 at 18°C and 28°C

P. syringae PG4180 cells were grown in HSC medium at 18°C and 28°C and extracellular protein fractions were collected in the exponential (OD_{600} = 1.5) growth phase. Analysis of the proteins secreted into the culture medium was subsequently carried out by electrophoretic separation of concentrated cell-free samples of the supernatant on a 10 % SDS polyacylamide gel. In samples of 18°C grown PG4180 cultures a distinct protein band of approximately 50 kDa was detected which was hardly visible in the samples of 28°C grown cells (**Fig. 32A**). Zymograms with 10 % sucrose and electrophoretically separated native extracellular protein samples from 18 and 28°C incubations of PG4180 demonstrated that the protein corresponding to that band exhibited levan formation activity (**Fig. 32B**). This result confirmed that levansucrase activities of PG4180 predominantly accumulated in the bacterial supernatants when the cell grew at 18°C. The experiment was repeated with complex KB medium; however, a clear difference in protein profiles of samples derived from 18 and 28°C cells could not be observed suggesting that this effect was nutrient-dependent (data not shown).

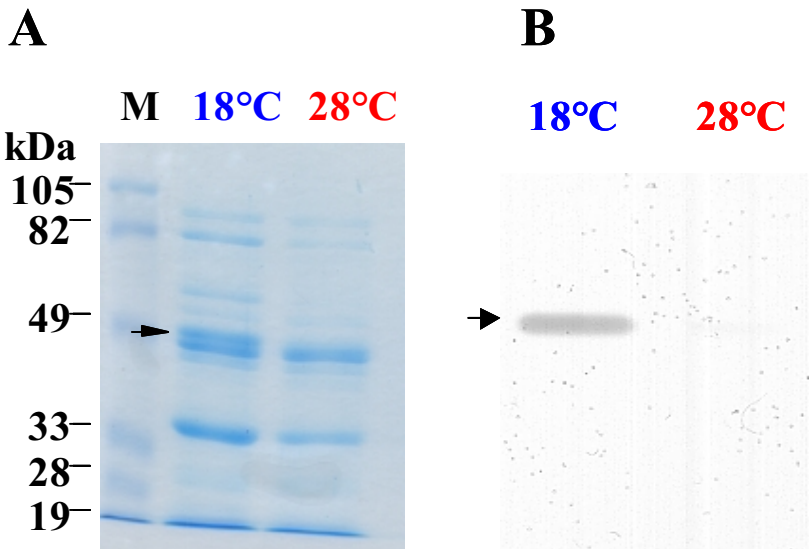

Fig. 32. Thermoresponsive secretion of levansucrase in *P. syringae* PG4180. (A) SDS-PAGE analysis of extracellular proteins of supernatants of PG4180 cultures. Extracellular proteins from 18°C- and 28°C- cultures were precipitated with TCA and separated on a 10 % SDS-PAGE. The black arrow marks an approximately 50 kDa protein band, predominately visible in samples derived from 18°C- cultures. (B) Zymogram with extracellular proteins of PG4180. Extracellular protein samples from 18°C- and 28°C- grown cultures were loaded on a native polyacrylamid gel and separated under non-denaturing conditions. The whitish swelling of the gel corresponds to levan formation.

5.7.2 Immunological detection of levansucrase in extracellular protein fractions at 18°C and 28°C

To rule out the possibility that differences in the extracellular levansucrase activity between 18°C- and 28°C- incubated PG4180 cultures were due to malfunction of the enzyme rather than differential secretion, Western blot experiments were carried out with concentrated supernatant samples and cell lysates of PG4180 and its *lsc* mutants grown at 18°C and 28°C, respectively (**Fig. 33**). Levansucrase could be detected in the cell lysates of both, 18°C- and 28°C- incubated PG4180 cultures, whereas levansucrase was only detected in the supernatant sample of 18°C- incubated PG4180 but not in that of 28°C-grown cells (**Fig. 33A**). As shown in **Fig. 33B**, signals for levansucrase were only found in the supernatant samples from PG4180 and PG4180.M5 (*lscA*$^-$ *lscC*$^-$) incubated at 18°C but not in those derived from the respective cultures grown at 28°C. No signal was detected in supernatant samples from PG4180.M3 (*lscA*$^-$ *lscB*$^-$) and PG4180.M6 (*lscB*$^-$ *lscC*$^-$) incubated at 18 and 28°C, furthermore supporting that LscB might be secreted in temperature-dependent manner.

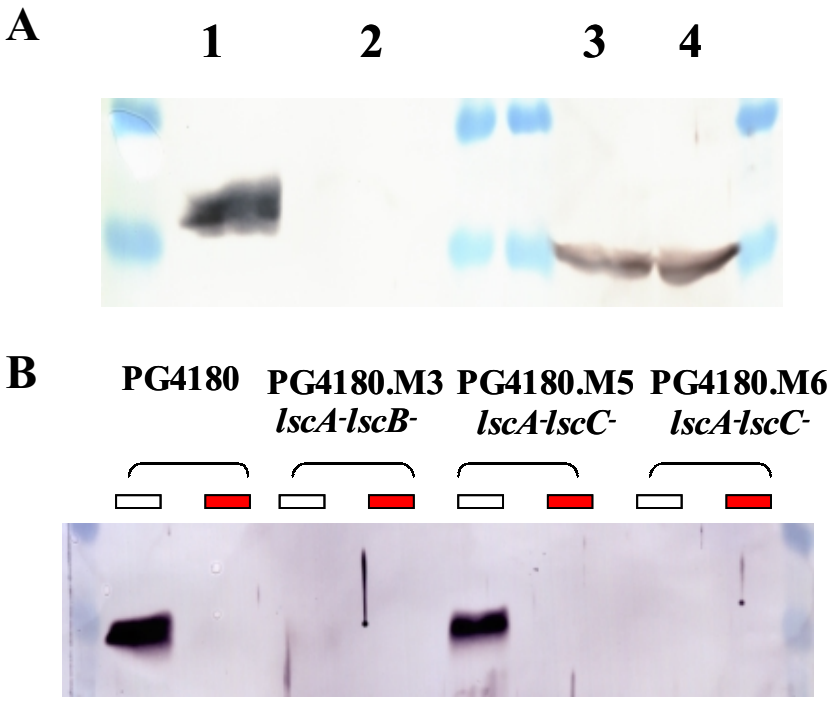

Fig. 33. Western blot analysis of (A) extracellular proteins in the cell-free supernatant and of total cellular protein samples of *P. syringae* PG4180 grown at 18 and 28°C. Lanes: 1, extracellular protein of PG4180 at 18°C; 2, extracellular protein of PG4180 at 28°C; 3, cell lysates of PG4180 at 18°C; and 4, cell lysates of PG4180 at 28°C. (B) Extracellular proteins in cell-free supernatants of *P. syringae* PG4180 and its *lsc* double mutants grown at 18 (▭) and 28°C (▬).

5.7.3 Analysis of promoter activities for *lsc* genes

5.7.3.1 *In trans* analysis of *lscB* and *lscC* transcription

A transcriptional *lscB*::*uidA* fusion was constructed by blunt end insertion of the promoterless *uidA* reporter gene located on a 2-kb *Not*I-fragment from plasmid pCAM140 into *Bgl*I-cleaved *lscB* on plasmid pSKL7.2 yielding plasmid pSKL7.2-uidA. Subcloning of a 4.6-kb *Pst*I fragment of pSKL7.2-uidA carrying the *lscB*::*uidA* fusion into pRK415 in opposite orientation to the vector-borne P_{lac} promoter generated plasmid pRK-BG (**Fig. 34A**). This plasmid was subsequently introduced to PG4180 by triparental mating generating transconjugant PG4180 (pRK-BG). In order to generate a *lscC*::*uidA* transcriptional fusion, the 2-kb *Not*I fragment carrying *uidA* was first inserted into vector pTYB-1 to generate plasmid pTYB-uidA. A 2-kb *Sal*I/*Xho*I fragment containing *uidA* from pTYB-uidA was then inserted into *Xho*I-digested plasmid pLC5.5 to generate pLC-uidA. Subsequently, a 4.5-kb *Xba*I/*Hind*III fragment from pLC-uidA was ligated to pRK415 in opposite direction to P_{lac} to generate plasmid pRK-CG (**Fig. 34A**). Plasmid pRK-CG was introduced to PG4180 to generate transconjugant PG4180 (pRK-CG). Both reporter gene fusions were flanked by at least 1 kb of DNA upstream of the translational start sites of *lscB* and *lscC*, respectively, to ensure the presence of putative promoter regions in the genetic constructs. Transconjugants PG4180 (pRK-BG) and PG4180 (pRK-CG) were cultured in HSC medium at 18°C and 28°C until they reached an OD_{600} of 1.5 - 2.0. Subsequently, cells were harvested and analyzed with respect to glucuronidase activities. As depicted in **Fig. 34B**, reporter gene activities for the *lscB*::*uidA* and *lscC*::*uidA* fusions were of similar strength regardless of the applied incubation temperature. This result suggested that transcription of neither *lscB* nor *lscC* was temperature-dependent.

Additionally, we tested whether the transcription of *lscB* or *lscC* was substrate inducible. To do so, HSC medium was supplemented with 10-120 mM of sucrose and parallel cultures of PG4180 transconjugants harboring pRK-BG and pRK-CG were incubated at 18 and 28°C and evaluated for glucuronidase activity in the exponential growth phase (OD_{600} = 1.5 – 2.0). The addition of substrate had no impact on the activities of the *lscB*::*uidA* and *lscC*::*uidA* transcriptional fusions. These results indicated a constitutive expression of the *lsc* genes and led the preliminary conclusion, that the increased levansucrase activity in culture supernatants of 18°C incubated PG4180 cells might be mainly due to a temperature-regulated secretion of levansucrase.

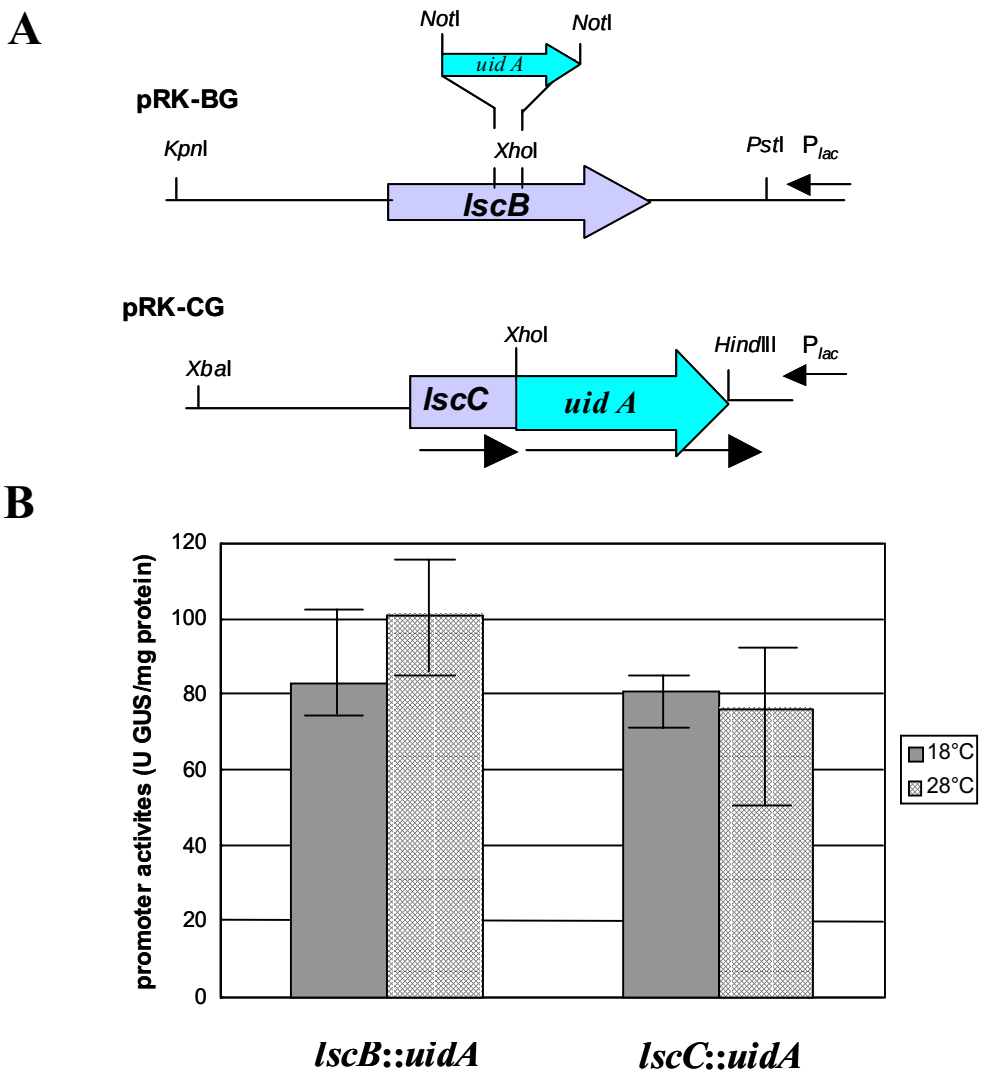

Fig. 34. Transcriptional analysis of *lscB* and *lscC in trans*. (A) Scheme for the transcriptional fusions *lscB::uidA* and *lscC::uidA* generated to analyze the expression of the reporter gene product β-glucuronidase. (B) Measurement of the β-glucuronidase activities of PG4180 harboring *lscB::uidA* or *lscC::uidA* transcriptional fusions at 18°C and 28°C. Glucuronidase activities represent values derived from three individual experiments with each three replicates.

5.7.3.2 Transcriptional analysis of *lsc* genes in temperature-dependent manner by Northern blot hybridization

Data obtained from the transcriptional fusions *lscB*::*uidA* and *lscC*::*uidA* seemed to be partially contradictory towards our results of quantitative levansucrase measurements in the different cell compartments of PG4180 (**5.4.1**). Those experiments had shown that export of levansucrase(s) to the periplasm yielded in temperature-independent accumulation of levansucrase in this compartment while levansucrase occurred in the supernatant in a strongly temperature-dependent manner. In order to obtain accurate *lsc*

gene expression profiles under different temperature conditions, total RNA samples were isolated from *P. syringae* PG4180 and its double-mutants PG4180.M3 and PG4180.M5 incubated at 18 and 28°C. The total RNA was analyzed by agarose gel electrophoresis (**Fig. 35A**) and expression of levansucrase at 18 and 28°C was examined by Northern blot analysis (**Fig. 35B**) with a RNA probe derived from *lsc*. As shown in **Fig. 35A**, comparable amounts of RNA (2.5 µg) were loaded per lane. The 1.5-kb signals corresponding to the *lsc* transcriptional units were clearly detected in RNA samples from PG4180 and mutants PG4180.M3 and PG4180.M5 incubated at 18°C. In contrast, approximately 10-fold decreased signals were observed in samples from cultures grown at 28°C regardless of the genotype (**Fig. 35B**). This result indicated that mRNA abundance for *lsc* transcripts varies strongly in a temperature-dependent manner.

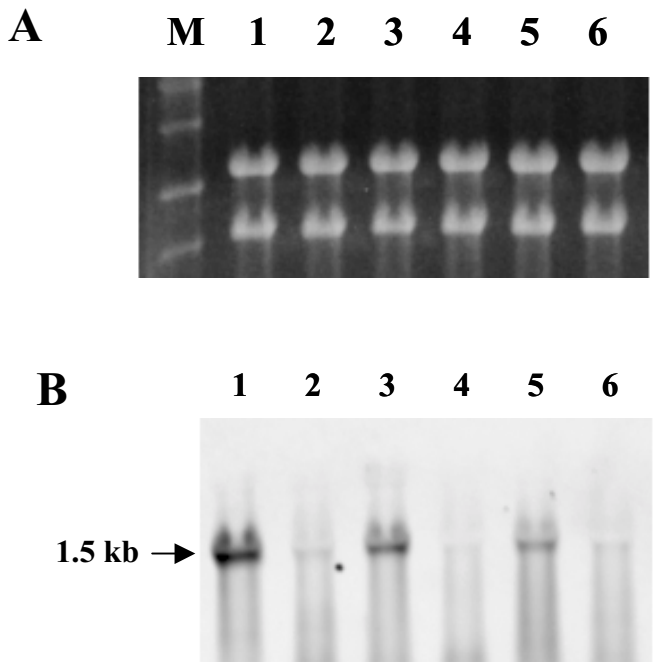

Fig. 35. Northern bolt analysis of *lsc* transcription. (A) Total RNA samples were electrophoretically separated on a 1.2 % agarose gel. Total RNA was isolated from *P. syringae* PG4180 and its mutants PG4180.M3 and PG4180.M5 grown at 18 and 28°C. Cells were grown to an OD_{600} of 1.5 and then subjected to total RNA extraction. 2.5 µg of total RNA was loaded per lane. Lanes: M, marker; 1, PG4180, 18°C; 2, PG4180, 28°C; 3, PG4180.M3, 18°C; 4, PG4180.M3, 28°C; 5, PG4180.M5, 18°C; 6, PG4180.M5, 28°C. (B) Northern blot analysis of total mRNA of *P. syringae* PG4180 and its mutants PG4180.M3 and PG4180.M5 grown at 18 and 28°C. After electrophoresis of the total RNA samples, RNA was transferred to nitrocellulose filters, and hybridized with a digoxigenin-II-UTP labeled RNA probe derived from *lsc*. The transcript of *lsc* is marked by an arrow. Lane numbering corresponds to part A of this figure.

This result was in stark contrast to the data obtained with transcriptional fusions of *lscB* and *lscC* to the β-glucuronidase gene *uidA* (**5.7.3.1**). To again test the transcriptional fusions *lscB*::*uidA* and *lscC*::*uidA* in their respective transconjugants, Northern blot analysis was carried out with an RNA probe derived from *uidA*. For this, the two promoter probe plasmids pRK-BG and pRK-CG were additionally conjugated into the double-mutants PG4180.M3 and PG4180.M5. RNA samples were prepared from the six transconjugants PG4180 (pRK-BG), PG4180 (pRK-CG), PG4180.M3 (pRK-BG), PG4180.M3 (pRK-CG), PG4180.M5 (pRK-BG) and PG4180.M5 (pRK-CG) after incubation in HSC medium at 18 and 28°C, respectively, and subjected to Northern blot analysis (**Fig. 36A**). As expected, similar signal strength was detected among RNA samples from all cultures regardless of the incubation temperature (**Fig. 36B**). From this result together with previous data, it was concluded that the controversial results of the transcriptional fusions and the *in-cis* measurement of *lscB* and *lscC* transcription by Northern blot analysis might be due to modified mRNA secondary structure in the transcriptional fusions *lscB*::*uidA* and *lscC*::*uidA*.

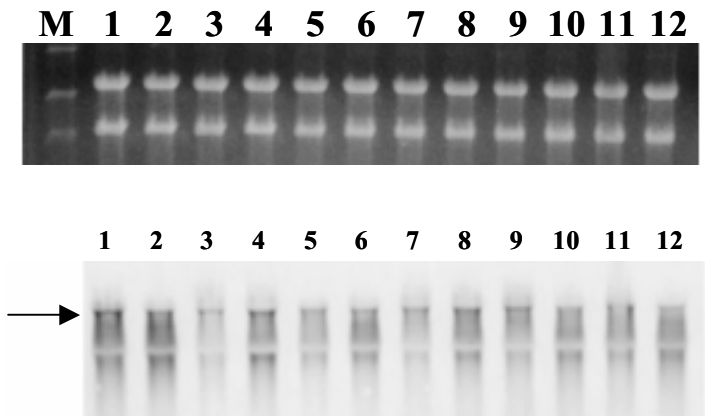

Fig. 36. Northern blot analysis of the expression of transcriptional fusions *lscB::uidA* and *lscC::uidA*. (A) Total RNA samples were electrophoretically separated by 1% agarose gel. Total RNA was isolated from transconjugants PG4180, PG4180.M3, PG4180.M5 harboring pRK-BG or pRK-CG, respectively, grown at 18 and 28°C. Cells were grown to an OD_{600} of 1.5 and then subject to total RNA extraction. 2.5 µg of total RNA was loaded per lane. Lanes: M, marker; 1, PG4180 (pRK-BG), 18°C; 2, PG4180 (pRK-BG), 28°C; 3, PG4180 (pRK-CG), 18°C; 4, PG4180 (pRK-CG), 28°C; 5, PG4180.M3 (pRK-BG), 18°C; 6, PG4180.M3 (pRK-BG), 28°C; 7, PG4180.M3 (pRK-CG), 18°C; 8, PG4180.M3 (pRK-CG), 28°C; 9, PG4180.M5 (pRK-BG), 18°C; 10, PG4180.M5 (pRK-BG), 28°C; 11, PG4180.M5 (pRK-CG), 18°C; 12, PG4180.M5 (pRK-CG), 28°C. (B) Northern blot analysis. After electrophoresis of the total RNA samples, RNA was transferred to nitrocellulose filters, and hybridized with a digoxigenin-II-UTP labeled RNA probe derived from *uidA*. The transcript of *lscB*::*uidA* or *lscC*::*uidA* is marked by an arrow. Lane numbering corresponds to part A of this figure.

5.7.4 Western blot analysis of levansucrase in cell lysates of mutants producing only one *lsc* gene product

In order to determine whether the observed temperature effect on transcription of *lscB* and *lscC* (**5.7.3.2**) is directly responsible for the likewise observed thermoresponsive levansucrase secretion, Western blot analyses were carried out with cell lysates of mutants impaired in either *lscB* or *lscC*. For this, equal amounts (5 µg) of cell lysates from mutants PG4180.M3 (*lscA*$^-$ *lscB*$^-$), PG4180.M5 (*lscA*$^-$ *lscC*$^-$), and the wild type grown at 18 and 28°C were loaded on 10 % SDS-PAGE and blotted with antiserum raised against levansucrase (**Fig. 37**). Due to their genotypes, the two mutants each produce only one isoform of levansucrase, PG4180.M3 produces only LscC whereas PG4180.M5 produces only LscB (**5.1.7** and **5.2.5**). Almost identical amounts of LscB were detected in the lysates of PG4180 and mutant PG4180.M5 regardless of the applied incubation temperature. In contrast, cell lysates of PG4180.M3 showed substantially less LscC when grown at 18°C as compared to 28°C. These results indicated that in the periplasmic compartment, where most of the cellular levansucrase was observed, there is no clear thermoresponsiveness of levansucrase accumulation. Our results furthermore suggested that LscC but not LscB might undergo a yet unknown type of modification which leads to its increased abundance at 28°C or its accelerated degradation at 18°C.

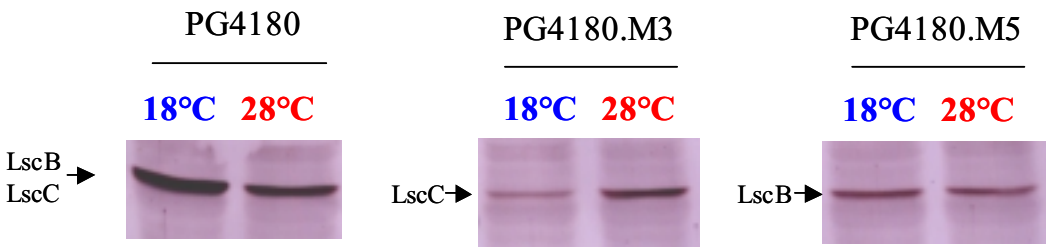

Fig. 37. Western blot analysis of total cellular proteins of *P. syringae* PG4180, PG4180.M3 and PG4180.M5 grown at 18 and 28°C. Cells were grown to an OD_{600} of 1.5 at 18 and 28°C and then subjected to total protein extraction. Protein samples (5 µg per lane) were electrophoretically separated, transferred to nitrocellulose filters, and then blotted using antiserum raised against levansucrase.

5.7.5 Analysis of levansucrase secretion after temperature shifts

The notable feature that levansucrase secretion was more pronounced at 18°C as compared to 28°C raised the question which pathway PG4180 utilizes for this secretion. Therefore, the influence of temperature shifts on levansucrase secretion was investigated in

two sets of experiments. In the first experiment, cultures of PG4180 were initially grown at 28°C until they reached an OD_{600} of 1.0. Cultures were divided into four aliquots. Two aliquots were treated with chloramphenicol (400 µl/ml) and furthermore incubated at 18 and 28°C, respectively. The other two aliquots were incubated at 18 and 28°C, respectively, but without antibiotics. After various time points, samples of supernatants were collected and applied to native PAGE and subsequent zymographic levansucrase detection. As shown **Fig. 38**, levansucrase activities could be detected in samples without chloramphenicol treatment at 6-10 hours after the temperature shift. In contrast, no levansucrase activities were found in the supernatant at 28°C or when cultures were treated with chloramphenicol regardless of the temperature to which the cultures were shifted to. These results suggested that *de novo* protein synthesis is required for levansucrase secretion at 18°C and that this secretion process is not occurring rapidly after a temperature down-shift.

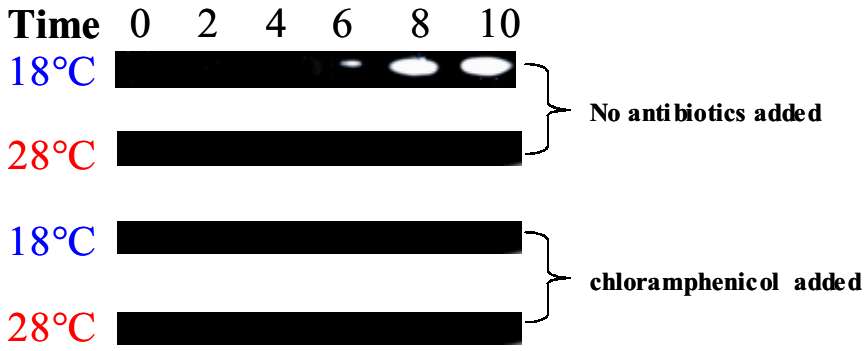

Fig. 38. Zymographic analysis of extracellular levansucrase activities following temperature shift from 28 to 18°C in PG4180. PG4180 cells were grown to an OD_{600} of 1.0 at 28°C, aliquoted four-fold and then two parallels were treated with chloramphenicol. Each one of the subcultures was incubated at 18°C, and the other remained at 28°C. The cell-free supernatant samples were collected at the indicated time points, and the supernatant samples were subjected to zymographic levansucrase analysis. Time point 0 indicates the temperature shift from 28 to 18°C.

Next, cultures of PG4180 were grown at 18°C until they reached an OD_{600} of 1.0 and cells were centrifuged down at 4,000 × g for 5 min. Pellets were resuspended in fresh HSC medium, aliquoted two-fold, and subsequently cultured at 18 and 28°C, respectively. At various time points, supernatant samples were collected and subjected to native PAGE and zymographic analysis of levansucrase activities. This experiment allowed the stability of protein(s) responsible for levansucrase secretion to be estimated at the higher temperature. As shown in **Fig. 39**, levansucrase activities were detected in cell-free supernatant samples

over an extended period of time. This suggested that the protein(s) required for levansucrase secretion might be rather stable at 28°C once synthesized at 18°C.

In summary, synthesis of protein(s) essential for levansucrase secretion might only occur at lower temperature 18°C and might remain stable regardless of the temperature to which the culture is shifted to.

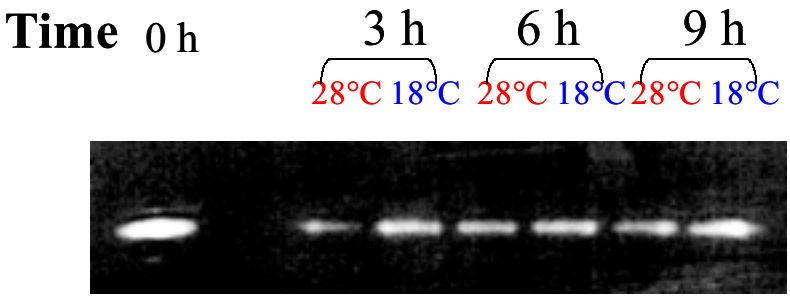

Fig. 39. Zymographic analysis of extracellular levansucrase activities following a temperature shift from 18 to 28°C in *P. syringae* PG4180. Cells of PG4180 were grown to an OD_{600} of 1.0 at 18°C and then centrifuged. Subsequently, the pellets were resuspended in fresh HSC medium and divided into two aliquots. One of the subcultures was incubated at 28°C and the other remained at 18°C. The cell-free supernatant samples were collected at the indicated time points, and the supernatant samples were subjected to native PAGE and zymographic analysis of levansucrase activities. Time point 0 indicated the temperature shift from 18°C to 28°C.

5.8 Identification and cloning of *dsbA* and *dsbC* genes from PG4180

Since LscB and LscC potentially differ in the number of disulfide bonds, two genes encoding enzymes involved in their formation, *dsbA* and *dsbC* were to be cloned for future studies. *dsbA* and *dsbC* nucleotide sequences of diverse origins were collected from databases and aligned to identify perspective conserved genes *P. syringae* DC3000, for which the genome sequence has almost been completely determined (http://www.tigr.org/). Based on both potential sequences from DC3000, we designed two pairs of primers (DsbAF/DsbAR and DsbCF/DsbCR) to PCR amplify two fragments of 600 bp for *dsbA* and of 650 bp for *dsbC* from DC3000 (data not shown). The same primer pairs were then used to amplify potential *dsbA* and *dsbC* fragments from genomic DNA of *P. syringae* PG4180. Only a *dsbA* fragment could be amplified from PG4180 with primers DsbAF/DsbAR (data not shown). Subsequently, this pair of primers was used to screen for *dsbA* in the genomic library of *P. syringae* PG4180. A cosmid clone, designated p7H1 was found to contain a potential *dsbA* gene on a 6-kb *Eco*RI fragment by Southern blot analysis with a probe derived from the *dsbA* PCR (data not shown). The procedure was repeated

with the PCR product of the *dsbC* fragment derived from DC3000 and an additional cosmid clone, designated p8G3, was identified, which harbored a 5-kb *Hind*III fragment containing *dsbC* of PG4180. Both fragments harboring *dsbA* and *dsbC*, respectively, were subcloned in pBluescript II SK(+) before further subcloning was carried out to provide templates for the determination of the complete nucleotide sequences of *dsbA* and *dsbC*. When the predicted amino acid sequences were derived for both gene products, they showed 65 % and 49 % similarity to the respective protein sequences of *P. aeruginosa.* The active sites for both enzymes were highly conserved (**Fig. 40**).

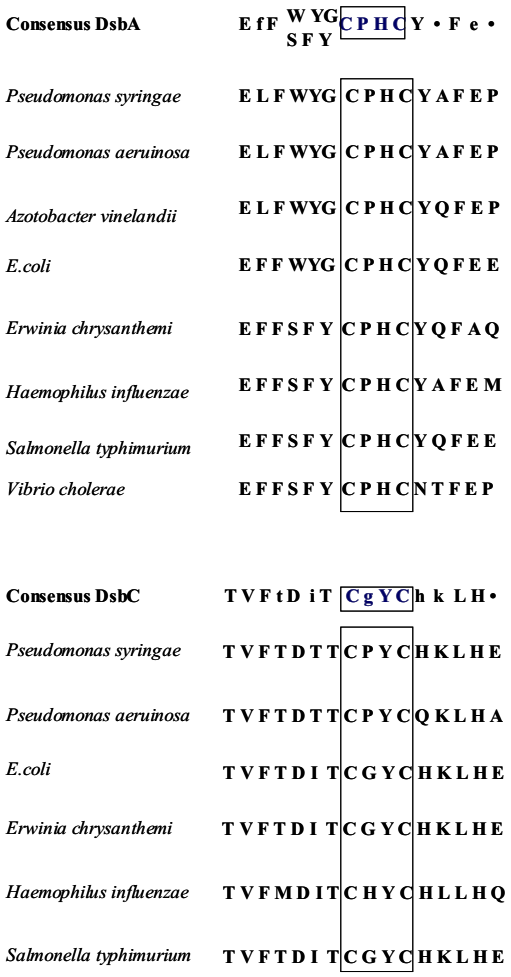

Fig. 40. Alignment of the active site amino acid residues of DsbA and DsbC from *P. syringae* PG4180 and other bacterial species. The C-X-X-C consensus motive characteristic for the thioredoxin superfamily is boxed.

5.9 Influence of the sensor kinase GacS on secretion of extracellular enzymes in PG4180

5.9.1 Identification and cloning of *gacS* gene from PG4180

The histidine protein kinase GacS had been implicated in secretion of various extracellular virulence factors in *P. syringae* (Kitten *et al*., 1998; Kinscherf and Willis, 1999; Hirano *et al*., 1999; Willis *et al*., 2001). Sensor kinases of the two-component regulatory systems usually act as regulators by receiving an environmental signal and subsequently phosphorylating their cognate response regulator. In case of GacS, this response regulator is termed GacA (Rich *et al*., 1994). Previously, in our laboratory a cosmid clone designated p2D7 and containing the complete *gacS* coding region was identified in the genomic library of PG4180 by Southern blot hybridization using a *gacS* probe derived from *P. syringae* pv. syringae B728a. This cosmid contained a 6.3-kb *Ava*I fragment which hybridized with the probe. The fragment was subcloned in pBluescript II SK(+), resulting in pBluelemA6.3 and a partial physical map of its insert was generated and is depicted in **Fig. 41**.

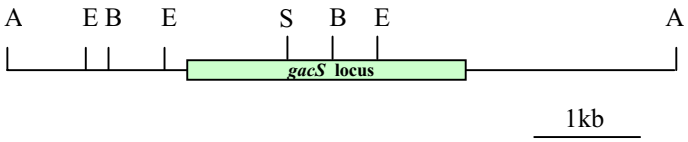

Fig. 41. Partial physical map of the 6.3-kb *Ava*I insert of pBluelemA6.3. Abbreviations: A, *Ava*I; B, *Bst*EII; E, *Eco*RI; S, *Stu*I.

5.9.2 Construction and characterization of *gasS* mutants of PG4180 and PG4180.muc

Beside levan, PG4180 produces a second EPS, termed alginate. In our laboratory, two versions of strains PG4180 exist: the alginate-minus strain PG4180 and its alginate-producing derivative, PG4180.muc. In contrast to PG4180.muc, PG4180 harbors a point mutation in *algT*, the alternative sigma factor required for alginate synthesis (Schenk, personal communication). Mucoidy could be restored to PG4180 by the introduction of a plasmid containing the *algT* from PG4180.muc. To investigate a potential role of *gacS* on

levan and alginate biosynthesis, both PG4180 and PG4180.muc were used to construct *gacS* mutants.

The streptomycin-spectinomycin resistance (Sp^r-Sm^r) gene from plasmid pCAM140 was used to construct a mutation in *gacS*. pBluelemA6.3 was digested with restriction enzymes *Xho*I and *Bam*HI and the resulting 6.3-kb fragment containing *gacS* was subcloned into the mobilizable suicide vector pKmobGII, yielding plasmid pKLemA6.3. This plasmid was linearized with *Stu*I, which recognizes a unique site within *gacS* (**Fig. 41**). The Sp^r-Sm^r cassette was cut as a 2.0-kb *Sma*I fragment from pCAM140 and ligated into linearized pKLemA6.3, resulting in pKLemA6.3-Sp. This plasmid was then introduced to *P. syringae* PG4180 and PG4180.muc, respectively, by triparental mating to facilitate homologous recombination (**Fig. 42**). The genotype of *gacS* mutants, PG4180.ML (derived from PG4180.WT) and PG4180.ML2 (derived from PG4180.muc), were confirmed by PCR.

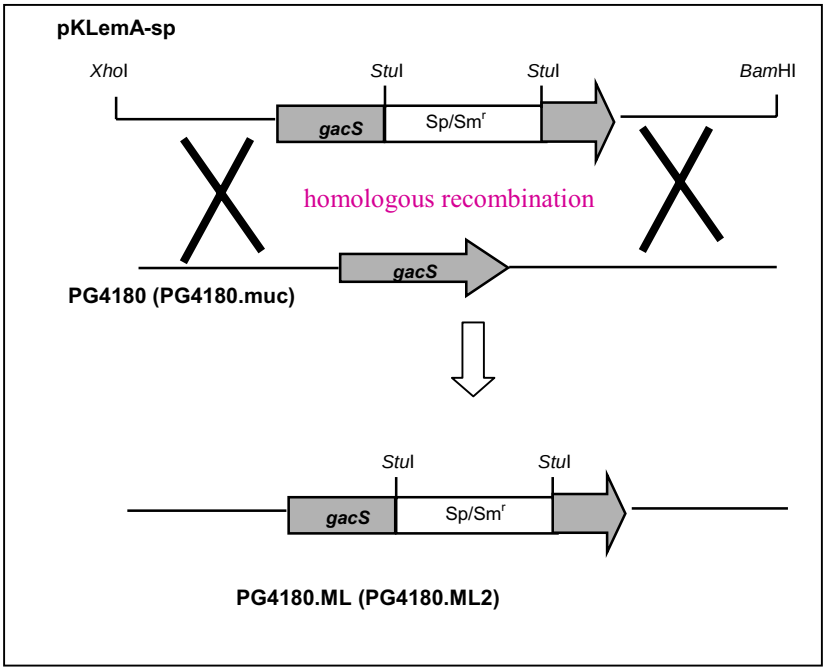

Fig. 42. Mutagenesis of g*acS* by insertion of a Sp-Sm^r resistance cassette. The 2.0-kb *Sma*I-digested Sp/Sm^r resistance cassette was inserted into *Stu*I site of *gacS*. Plasmid pKLemA--Sp is unable to replicate in *P. syringae* and facilitates exchange with the chromosome by homologous recombination to obtain Sp-resistant and Km-sensitive clones.

5.9.3 Characterization of *gacS* mutants

Mutant PG4180.ML was tested with respect to levan formation and levansucrase secretion using phenotypic observation and Lsc-specific quantitative assays. However, no differences was observed for PG4180 and PG4180.ML indicating that GacS does neither play a role for transcription of *lsc* genes nor for the secretion of LscB. Furthermore, extracellular protein profiles, extracellular lipase activities and coronatine production were evaluated for PG4180 and its *gacS* mutant PG4180.ML. However, none of these features was affected when *gacS* was knocked-out. Additionally, motility of the *gacS* mutant was compared to that of the wild type. It was also not influenced by a *gacS* mutation. These results suggested that extracellular traits for which an influence of *gacS* had been shown in other pathovar of *P. syringae* (Kinscherf and Willis, 1999; Rich, *et al*., 1994; Kitten and Willis et al., 1996) were not influenced by *gacS* in PG4180 at all.

Fig. 43. Alginate production of PG4180.WT, PG4180.muc and their mutant derivatives. Four strains were streaked on MG agar plate. Picture was taken after 5 days of streaking out and incubation at 28°C.

In order to test whether *gacS* plays a regulatory role in alginate biosynthesis, PG4180, PG4180.ML, PG4180.muc, and its mutant PG4180.ML2 were grown on MG agar plates.

Mucoidy indicative for alginate production was clearly visible for strain PG4180.muc but not for mutant PG4180.ML2 (**Fig. 43**). As expected, neither PG4180 nor its *gacS* mutant PG4180.ML produced visible amounts of the EPS alginate. Mucoidy could be restored to PG4180.ML2 by introducing plasmid pEMH97 containing the *gacS* gene from *P. syringae* pv. syringae strain B728a (data not shown), indicating that the disruption of *gacS* was responsible for the nonmucoid phenotype of PG4180.ML2. In summary, it can be concluded that regulation of levan biosynthesis and control of alginate synthesis are not coordinated by common regulatory principles such as *gacS*.

6 DISCUSSION

Despite the use of levan formation for taxonomic identification of *P. syringae* (Bradbury, 1986; Schaad, 1988), the genes encoding levansucrase and regulation of levan formation had not been characterized in detail prior to this work. Knowledge of the genetics of levansucrase is essential for further analysis of the role of levan formation in plant-microbe interaction. Mutants lacking levan synthesis were reduced in virulence even though levan formation did not appear to be required for pathogenicity in *Erwinia amylovora* (Geier and Geider, 1993). However, little was known about the role of levan formation in host-pathogen interactions due to the previous lack of levan-deficient mutants of *P. syringae*. Previously, Hettwer *et al*. (1995) characterized purified levansucrase from the supernatant of *P. syringae* pv. phaseolicola. When an *lsc* gene from *P. syringae* PG4180 was successfully expressed in *E. coli,* its gene product was not secreted to the supernatant but accumulated in the periplasm. Thus, *E. coli* cells obviously lack additional secretory factor(s), which might only be present in *P. syringae*. In contrast to the well-understood process of levansucrase export in *B. subtilis* (Chambert and Petit-Glatron, 1988; Petit-Glatron *et al*. 1987), no information was available about the secretion of this enzyme in *P. syringae*. This prompted us to investigate how levansucrase from PG4180 is exported to the periplasm and how its secretion to the extracellular medium occurs. Furthermore, in this study we investigated how gene expression and/or secretion(s) mechanism for levansucrase are regulated by environmental factor, such as temperature.

The present study showed that there are three *lsc* genes coding for levansucrase in *P. syringae* pv. glycinea PG4180. Gene disruption experiments revealed that only *lscB* and *lscC* are functional and responsible for levan formation in PG4180. As a result of compartment-specific analysis of levansucrase activities in PG4180, we conclude that *lscB*-deficient mutants accumulated the periplasmic isoform of levansucrase but failed to secrete the enzyme at both, 18 and 28°C. Additionally, *lscC*-deficient PG4180 mutants could secrete LscB at 18°C but not at 28°C. Furthermore, secretion of levansucrase proceeded in a signal-independent manner. These findings indicated that LscB secretion might occur by a two-step process which requires additional periplasmic factor(s) to transport the periplasmic intermediate LscB across the outer membrane. Results of temperature shift experiments suggested that the secretory factor(s) might be synthesized *de novo* at the lower temperature, 18°C. Compared to widely and well-established protein secretion mechanisms in many gram-negative bacteria, levansucrase secretion does not

perfectly fit into any of the four major secretion pathway principles for this group of microbial organisms. Therefore, an in-depth analysis of levansucrase secretion has to succeed the present study in the future.

6.1 Gene duplication of *lsc*

Herein, we provide unprecedented evidence for the presence of multiple levansucrase genes in the plant pathogen *P. syringae*. Southern blot hybridizations, PCR detection, and mutational analysis revealed the presence of three individual *lsc* genes in PG4180 that could be cloned and sequenced suggesting a high degree of similarity of the primary sequence data. While the nucleotide sequences of *lscB* and *lscC* were almost identical to each other and to *lsc* from *P. syringae* pv. phaseolicola (Hettwer *et al*., 1998), they differed from that of *lscA* by approximately 14 %.

A screening for multiple *lsc* copies among various pathovars of *P. syringae* revealed that the multiple occurrence of *lsc* genes seems to be conserved among *P. syringae* pathovars other than pv. glycinea despite of quantitative differences in levan formation. Gene duplication, which might be the cause for this, is believed to have a major role in evolution: one gene copy maintaining its original function in response to selective constraints, thereby freeing the other to generate possibly advantageous mutations and new functions (Force *et al*., 1999). These processes might facilitate the formation of variant families of proteins and of proteins with novel functions, thus providing pathogenic organisms such as *P. syringae* with the ability to evade the host immune response, to facilitate transmission to the next host, or to adapt to different microenvironments. Failure of a duplicated gene to facilitate a beneficial function for the organism may in turn result in loss of this allele from the organism's genome (Force *et al*., 1999).

Our data indicated that *lscB* was located on an indigenous plasmid and was found to be identical to a levansucrase gene from *P. syringae* pv. phaseolicola NCPPB1321 (Hettwer *et al*., 1998). This result confirmed reports on the conservation of *lsc* genes in plant-associated gram-negative bacteria (Arrieta *et al*., 1996; Geier and Geider, 1993; Hettwer et al., 1998) and implied that horizontal gene transfer might have been involved in its distribution. In a subsequent experiment, plasmid DNA from *P. syringae* pv. phaseolicola NCPPB1321 was analyzed by Southern blot hybridization for the presence of *lsc* (data not shown). Indeed, a signal representing a plasmid of yet unidentified size could be obtained indicating that at least one *lsc* gene copy of NCPPB1321 is plasmid-borne. The

location of *lscB* on an indigenous plasmid allows us to speculate that this gene might occur in a multiple copy number therefore giving rise to higher abundance of its gene product as compared to LscC.

So far, all bacterial organisms tested contained a single copy of levansucrase gene in their genomes (Gay *et al*., 1983; Gier and Geider, 1993; Song *et al*., 1993; Arrieta *et al*., 1996; Song *et al*., 1998; Tajima *et al*., 2000; Hernandez *et al*., 2000). Redundance of *lsc* in the genome of *P. syringae* might either highlight the ecological importance of this enzyme or might hint to different functions for each of the gene products.

The role of *lscA* in levan formation of *P. syringae* remains obscure. As shown by heterologous expression under P_{lac} control in *E. coli* (Hettwer *et al*., 1998), *lscA* encodes a functional enzyme synthesizing levan. However, the *lscB*$^-$ *lscC*$^-$ mutant PG4180.M6 exhibited a levan-deficient phenotype in *vitro* and did not show a signal for levansucrase in Western blot analyses suggesting that the gene product of *lscA* is not translated and is dispensable for levan formation in *P. syringae* under laboratory conditions. When expressed under control of P_{lac}, *lscA* restored levan production to the *lscB*$^-$ *lscC*$^-$ mutant and LscA could be immunologically detected in the respective transconjugants. Therefore, we conclude that transcription of *lscA* under the tested conditions either does not occur or is too low to be detected. However, it remains to be tested whether *lscA* is transcribed under natural conditions *in planta*.

The analysis of bacterial genomes clearly indicated that evolution of biochemical diversity has involved substantial levels of gene duplication. It generally accepted that enzyme evolution is more efficient if one duplicate is rendered non-functional at the level of transcription or translation, so that later it may revert to an active allele under favorable conditions (Koch, 1972). We assume that *lscA* might have become inactive after its functional duplicate *lscB* had been acquired in *P. syringae* possibly by natural plasmid transfer. The mechanism may have started with gene duplication followed by silencing of *lscA* at the transcriptional or translational level. Further evolution of this ‘silenced’ gene, initially generated by a mutation causing reduced transcription, maintained it in the bacterial population for a finite period of time, then modified it via accumulation of a variety of additional mutations. Eventually such a ‘silenced’ gene may be lost by deletion but, prior to this, it may acquire a modified enzymatic function that will persist if of selective value to the cell (Koch, 1972; Rigby *et al*., 1974; Beacham, 1987). In one allele of the duplicates, lack of a functional gene product may also result from a missense mutation(s) causing, for instance, aberrant folding of the encoded protein (Rigby *et al*.,

1974). In spite of lack of evidence for gene duplication in case of *lscA*, we suggest that the existence of *lscA* might be a 'silent gene intermediate' in the evolution to new enzyme activities.

6.2 Expression of *lsc* genes from PG4180 in *E. coli*

Even though all three *lsc* genes could be cloned in *E. coli*, only transcription of *lscA* and *lscB* under control of the vector-based P_{lac} promoter gave rise to recombinant levan formation. This result is consistent with various reports showing that many *P. syringae* promoters are non-functional in enterobacteriaceae and suggests that *E. coli* might lack the native regulatory system(s) for transcription of *lsc* genes from *P. syringae*. With restriction analysis and Southern blot analysis, we have demonstrated that initial screening for levan synthesis within the genomic library of PG4180 had selected transcriptional fusion between the vector-based P_{lac} and the *lscA* gene, which is clearly an experimental artifact.

Possibly, lack of export of LscC in enterobacteria could be the primary reason for our failure to express LscC in *E. coli*. Intracellular accumulation of LscC might be toxic to this organism as described for *sacB*, the *Bacillus subtilis* gene coding for levansucrase (Gay *et al*., 1985). Furthermore, the gene products of *lscA* and *lscB* were detected in the periplasm but not in the supernatant of *E. coli* suggesting a lack of the appropriate secretion machinery for Lsc in this organism.

6.3 The role of levan formation for *P. syringae in planta*

Redundance of *lsc* in the genome of *P. syringae* could signal its ecological importance or might hint at different functions for each of the gene products. In the fire blight pathogen, *E. amylovora*, levan formation was shown to significantly contribute to virulence and *in planta* bacterial multiplication (Geier and Geider, 1993). Whether or not levan formation plays a critical role during the infection of soybean plants by PG4180 has been investigated in this study. Our preliminary results for virulence and *in planta* survival assays with the levan-deficient mutant PG4180.M6 and the wild type of PG4180 suggest that this mutant is not impaired in pathogenicity when infiltrated into soybean leaf tissue. However, when spray-inoculated, PG4180.M6 showed a significantly decreased *in planta* survival indicating that levan formation might be required for the general fitness of *P. syringae*. Moreover, results of the spray-inoculation experiments, which resemble the

natural infection process much more than the infiltration technique, also suggested that symptom development was decreased in the levan-deficient mutant as compared to the wild type. A detailed analysis of *in planta* levan formation and *lsc* expression will be conducted in the future in our laboratory. Nevertheless, the presence of two functional *lsc* genes and their constitutive expression suggests a high importance of levan formation during plant-pathogen interaction. Levansucrase activities might be particularly required during early stages of infection, masking, and supporting the proliferation of the pathogen in the host tissue (Kasapis *et al*., 1994; Kiraly *et al*., 1996; Lindow, 1991). However, as shown for an *Erwinia amylovora* mutant with abolished levansucrase activity that was tested for fireblight symptoms, levan synthesis is not strictly necessary for symptom development (Bereswill and Geider, 1997). Other EPS such as alginate and amylovoran were also demonstrated to contribute to the virulence *in planta* and strongly affect the formation of disease symptoms of a plant pathogen (Yu *et al*., 1999; Bereswill and Geider, 1997).

6.4 Temperature-dependent expression and secretion of levansucrase in PG4180

For the first time, we herein provide evidence for a thermoresponsive and sec-independent secretion of levansucrase in *P. syringae*. It was demonstrated that accumulation of the gene product of *lscB* was responsible for this phenotype. This was shown by extracellular protein profiling, determination of the N-terminal protein sequence, compartment-specific enzymatic detection of levansucrase, and was ultimately confirmed by mutational analysis. Western blot analyses with levansucrase-specific polyclonal antibodies added evidence to strengthen this result.

P. syringae pv. glycinea, the causal agent of bacterial blight on soybean plants, is also termed a “cold-weather” pathogen because bacterial blight symptoms preferential occur during and past periods of cold and humid weather (Dunleavy, 1988). Therefore, ecologically it makes perfect sense that a factor like levan formation which contributes to the overall fitness of the pathogen *in planta* is preferentially synthesize at low temperatures.

Effects of temperature on protein secretion in pathogenic bacteria have been reported in numerous studies. Human and animal pathogens like *Yersinia* species secrete proteogenous virulence factors preferentially at 37-40°C via type III secretion systems whose assembly depends on the temperature-regulated transcription of so-called *yop* genes (Cornelis *et al*.,

1998). As shown by Amoako *et al*. (1996), hemolysin secretion by the animal pathogen *Fusobacterium necrophorum* was repressed at low temperature. Furthermore, virulence-associated protein secretion in *Escherichia coli* was induced at 37°C, a temperature simulating the environment of the warm-blooded host (Ebel *et al*., 1996; Kenny *et al*., 1997). In the plant pathogen *Erwinia chrysanthemi*, secretion of extracellular pectolytic enzymes which function as major virulence factors occurs in a temperature-dependent manner with maximal secretion at 25°C (Hugouvieux-Cotte-Pattat *et al*., 1996). Exoenzyme secretion in *Erwinia carotovora*, another soft rot plant pathogen, is also controlled by temperature (Housby *et al*., 1998). Although not experimentally shown, it may be speculated that protein secretion via the type III *hrp* secretion apparatus of the fire blight pathogen, *Erwinia amylovora*, (Bogdanove *et al*., 1996) might be thermoresponsive since the *hrp* gene expression of this organism was induced at 18°C and repressed at 28°C (Wei *et al*., 1992). *P. syringae* pathovars, which preferentially infect their hosts under conditions of cold and humid weather, secrete Avr proteins via the Hrp system in a temperature-dependent manner (Van Dijk *et al*., 1999). In most of these cases gene expression was influenced by temperature therefore complicating a clear differentiation between temperature-regulated protein synthesis and protein transport. In this study, we provide direct evidence that gene expression of *lscB* and *lscC* as well as secretion of the *lscB* gene product could be influenced by the incubation temperature and proceeds maximally at 18°C, a temperature under which *P. syringae* is most virulent.

In order to study gene transcription in *P. syringae*, the *uidA* gene encoding β-glucuronidase is heavily used as a promoterless reporter gene since plants lack β-glucuronidase making *in planta* detection of gene expression in *P. syringae* possible. It is widely assumed that the specific β-glucuronidase activities derived from transcriptional fusions of *uidA* with native promoters reflect the level of transcription of the genes of interest. Respective experiments with transcriptional fusions expressed *in trans* have successfully been carried out with our model organism (Ullrich and Bender, 1994; Budde *et al*. 1998). However, accurate measurement of the *lsc* promoter strength by the transcriptional fusions *lscB*::*uidA* and *lscC*::*uidA* failed in this study and did not confirm our Northern blot analysis results. Transcriptional fusions appeared to be expressed in a temperature-independent manner while mRNA abundance strongly depends on temperature. It could be speculated that mRNA secondary structures or *in trans* effects might have obscured these results. Similar technical problems were recently described and discussed by Pessi *et al.* (2001).

Interestingly, the thermoresponsive levansucrase secretion was more pronounced when PG4180 cells were incubated in minimal medium as compared to complex medium suggesting that additional environmental factors might influence the extracellular accumulation of levansucrase. Biosynthesis of various secondary metabolites, fitness determinants, and virulence factors of *P. syringae* were previously shown to be dictated by nutritional factors (Lindow, 1991; Palmer and Bender, 1993; Rahme *et al*., 1992; Xiao *et al*., 1992).

6.4.1 Export of levansucrases from the cytoplasm to the periplasm in PG4180

Levansucrase activities were negligible in cytoplasmic and membrane fractions of PG4180. In contrast, high enzymatic activities were found in periplasmic fractions and in the supernatants. This result suggested a putative two-step transport mechanism for levansucrase via the periplasm as described for levansucrases of *Bacillus subtilis* and *Acetobacter diazotrophicus* (Arrieta *et al*., 1996; Chambert and Petit-Glatron, 1988; Petit-Glatron et al., 1987; Leloup et al., 1999). However, nucleotides encoding a signal peptide sequence typical for the sec-dependent type II general secretory pathway (Fekkes and Driessen, 1999) were not found in either *lsc* gene. More importantly, the determined N-terminal protein sequence of the secreted *lsc* gene product was identical to its predicted N-terminal sequence. These data indicated that the enzyme may not be proteolytically processed during its translocation across the inner and outer membrane and that its transport might therefore occur via a sec-independent mechanism. The presence of levansucrase activity in the periplasm contradicts the possibility that protein transport might have occurred by a classical type I or III secretion mechanism. In these secretion pathways, proteins bypass the periplasm and are directly secreted from the cytoplasm (Binet *et al*., 1997; Lory, 1998; Hueck, 1998). Charkowski *et al*. (1997; 1998) recently demonstrated that mutations within genes for the type III *hrp* secretion pathway in *P. syringae* caused a significant accumulation of transported proteins in the periplasm. However, this particular secretion pathway functions perfectly in *P. syringae* PG4180 (Budde and Ullrich, 2000) and therefore could not have been affected by mutations. Additionally, the environment of the periplasmic space is more oxidizing than the cytoplasm and can favor the correct folding of levansucrases containing disulfide bonds. This folding might ultimately lead to the secretion of LscB to the extracellular space. In *E. amylovora*, export of levansucrase across the inner membrane was suppressed when the

C-terminus of this enzyme was mutated (Geier and Geider, 1993). The detailed mechanism by which *P. syringae* levansucrase is secreted from the cytoplasm and which particular signals are necessary for this transport remain to be investigated in the future.

6.4.2 Secretion of levansucrase at 18°C in PG4180

Determination of levansucrase activity in cell-free culture supernatants from 18°C grown PG4180 cultures revealed a 10- to 15-fold higher enzymatic activity as compared to samples derived from 28°C. In contrast, equal levels of levansucrase activity were measured in cell lysates derived from 18 or 28°C incubations. In this study, knock-out mutations were generated for three individual *lsc* genes and double mutants were obtained as well. This allowed us to separately investigate the secretion of either gene product. The *lscB* gene product was demonstrated to be secreted in a temperature-dependent manner. We concluded that the gene product of *lscC* predominantly has a periplasmic function whereas LscB might be specifically required for levan formation outside the cell at the lower temperature.

Furthermore, we determined whether lack of levansucrase activity in a particular compartment and at 28°C was due to catalytic dysfunction or to lack of protein transport. Western blot analysis demonstrated that the presence of a given *lsc* gene product was strictly linked to its enzymatic activity. These results essentially ruled out the possibility that LscC might be secreted to the supernatant but might be non-functional in this particular environment. They also ruled out the possibility that LscB might be secreted to the extracellular medium but might be inactive at 28°C.

As experimentally demonstrated, both *lscB* and *lscC* gene products but not that of *lscA* are synthesized and efficiently exported to the periplasm at both, 18 and 28°C. Both gene products accumulated in the periplasm when PG4180 cultures grew at 28°C. However, *lscB* is not only strongly expressed at 18°C but also its gene product is efficiently secreted to the extracellular medium. These results were obtained by Northern blot analysis of transcription of *lsc* genes and Western blot analysis of levansucrase indicating a temperature-dependent gene expression for both genes and a temperature-dependent secretion for LscB. Even though transcription of neither *lscB* nor *lscC* was substrate-dependent, additional experiments will need to be carried out in the future to analyze the precise mode of transcriptional regulation of these genes.

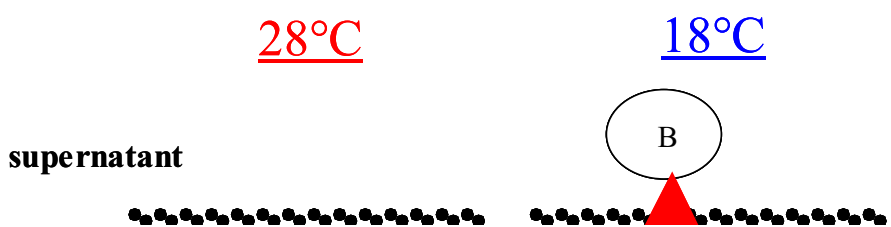

Fig. 44. **Hypothetical model of levansucrase expression, export, and secretion.** *lscA* is not transcribed while both *lscB* and *lscC* show a temperature-dependent transcriptions. LscB is the dominantly secreted dominant form of levansucrase when cells grown at 18°C while LscC remains in periplasm regardless of the applied temperature. Periplasmic proteolysis might occur on both, LscB and LscC, at 18°C.

Our hypothetical model for export and secretion of *lsc* gene products presented in **Fig. 44** might help to explain the phenomena described above: The observed temperature-independent accumulation of levansucrase in the periplasm suggested that at 18°C proteolysis might contribute to decay of LscB and LscC in this compartment and that this proteolysis might compensate for the strong transcription of *lscB* and *lscC* at this temperature. This assumption was supported by Western blot analysis results obtained from the double mutants PG4180.M3 and PG4180.M5 which solely produced LscC or LscB, respectively. To test this assumption in the future, purified MBP-coupled levansucrase will be mixed with PG4180 lysates derived from cells grown at either 18 or 28°C and incubated at different temperatures. Subsequently, MBP-Lsc will be recovered by affinity chromatography and quantified to determine whether proteolytic factors contribute to levansucrase degradation specific at 18°C.

A screening for thermoresponsive levansucrase secretion in different pathovars of *P. syringae* revealed that a more pronounced levansucrase secretion at 18°C was common to many *P. syringae* strains of different origins and indicated that this phenomenon was widespread among representatives of this plant pathogenic bacterium (Hettwer and Ullrich, personal communication). Temperature regulated levansucrase secretion in pathovars of

P. syringae could not be strictly correlated to the type of symptoms caused by or the phylogenetic relatedness among the tested pathovars.

6.4.3 The potential effect of disulfide bond formation for levansucrase secretion

It remains to be elucidated what determines the herein observed compartment-specific accumulation of the two levansucrase isoforms. LscB and LscC differ in only five amino-acyl residues from which the conservative changes in amino acid residues 92, 327, 329, and 429 might not be important for the structure and physicochemical characteristics of Lsc. However, the alteration in position 119, a serine residue in LscB changed to a cysteine residue in LscC, could impact the putative number of disulfide bridges the proteins might form. Accordingly, LscC might contain two disulfide bridges whereas LscB might only possess one disulfide bond and a free cysteine in its structure. Such an alteration could significantly influence the overall structure of the enzyme, possibly leading to a selective transport across the outer membrane. Two gene products, DsbA and DsbC, which represent disulfide bridge forming and modulating periplasmic enzymes, respectively, might be involved in folding of LscB and LscC. The genes encoding both proteins were identified in the genome of PG4180 suggesting that this organism might indeed express both gene products. It is very likely that DsbA and DsbC of PG4180 possess similar functions as their counterparts from other gram-negative bacteria.

In future studies, both, *dsbA* and *dsbC*, should be knocked-out in order to determine the potential role of their gene products in folding of LscB and LscC. Moreover, experimental exchange of the particular amino acid residues in which LscB and LscC differ could reveal their potential to target the respective gene product to the extracellular space. Another open question is that of the particular function of LscB versus LscC. Possibly, the levans produced by either levansucrase might differ and might have distinct functions inside or outside the periplasm. Structural analyses of the polymeric levan products of LscB and LscC derived from respective double mutants will be helpful to determine the particular role of periplasmic and extracellular levan formation for *P. syringae.*

6.5 The influence of *gacS* mutants on virulence factors in PG4180

One aim of the present study was to clarify the role of the GacS/GacA two-component regulatory system on levansucrase expression and secretion. This regulatory system had

been implicated in synthesis of various extracellular virulence factors in *P. syringae* (Hrabak and Willis, 1992; Rich *et al*., 1994). Therefore a *gacS* mutant of PG4180 was generated. Its mutation did neither influence levan synthesis nor levansucrase secretion, indicating that the signal transduction initiated and governed by GacS does not exert on the regulation of *lsc* genes or any of its yet to be identified secretory components. Subsequently, other cellular features important for virulence (coronatine) or simply secreted to the environment (lipase; extracellular protein profiles) were compared for PG4180 and its *gacS* mutant. However, in no case there was a significant effect suggesting that function of the GacS/GacA system as described for *P. syringae* pv. syringae (Hrabak and Willis, 1992; Barta *et al.*, 1992; Rich *et al*., 1994) do not apply for *P. syringae* pv. glycinea.

Levan and alginate are the two main EPS produced by *P. syringae* (Fett *et al*., 1989; Gross and Rudolph, 1987). The regulation of alginate synthesis in *P. syringae* has been investigated in detail (Fakhr *et al*., 1999; Keith and Bender, 1999). In contrast, virtually no information exists how the temperature-responsive transcription of *lsc* genes is influenced by regulatory protein(s). A complex regulatory network for the biosynthesis of the highly complex EPS in the plant pathogen *Ralstonia solanacearum* has been uncovered (Huang *et al*., 1995). Three separate signal transduction systems, PhcA, a LysR-type transcriptional regulator and the dual two-component regulatory systems, VsrA/VsrD and VsrB/VsrC, have been uncovered.

In this study, a PG4180.muc mutant with disruption in *gacS* showed a clear reduced ability to form alginate. The profound effects of mutations in the GacS/GacA system on alginate synthesis observed herein and reported elsewhere (Willis *et al*., 2001) hint to a requirement of functional GacS for alginate biosynthetic gene expression via GacA. One potential target for GacA in these terms might be the promoter region of a*lgD,* the gene encoding GDP-mannose dehydrogenase, which is the key enzyme in the alginate biosynthetic pathway. Transcriptional regulation of *algD* seems to be highly regulated, because its transcription can initiate from three different sites: p1, a σ^{70} type promoter (Campos *et al*., 1996); p2, controlled by σ^{E}; and p3 (Moreno *et al*., 1998). As evidenced by measuring reporter gene activities of *algD*::*lacZ* and primer extension analysis of *algD* transcription in *Azotobacter vinelandii* wild-tpye and its *gacS* mutants, GacA must mediate signal transduction between GacS and *algD* transcription (Castaneda *et al*., 2000). This could also apply to the GacS/GacA system in *P. syringae.* Whether GacA directly interacts with the *algD* promoter region remains to be determined in future studies. In summary, our

results indicated that regulation of alginate and levan formation does not proceed in a concerted manner and that levan production is not controlled by the GacS/GacA system.

6.6 Outlook

Experimental evidence of this study indicated that LscB was secreted only when the producing PG4180 culture grew at 18°C. However, its isoform LscC accumulated in the periplasm regardless of the applied incubation temperature. The predicted primary sequence of both enzymes suggested that disulfide bond formation might be of importance for this differential secretion. DsbA and DsbC play an important role for generating the disulfide bridges of periplasmic proteins. Therefore, mutation of DsbA and/or DsbC should potentially provide new insights into the temperature-dependent secretion of LscB in *P. syringae*. Another important approach will be to use site-directed mutagenesis of LscB and LscC to investigate the role of the cysteine residues in secretion of LscB. Ultimately, we need to understand what determines such a differential secretion mechanism. In contrast to the transcriptional regulation of alginate biosynthesis in *P. syringae*, little is known about the regulation of transcription of *lsc* genes in this organism. Characterization of the alginate biosynthetic gene cluster in *P. syringae* pv. syringae has shown that its central promoter depend on temperature, osmolarity, and GacS (Penaloza-Vazquez *et al*., 1997; Willis *et al*., 2001). Our preliminary data indicated that the two major EPS of *P. syringae*, levan and alginate, are not coordinately regulated in *P. syringae* PG4180. Therefore, further investigation is needed to understand the temperature-dependent transcription of *lsc* genes and its transcriptional activator(s) need to be determined. One possible scenario to explain thermoresponsiveness of LscB secretion is the presence of a secretory complex which is synthesized and assembled *de novo* at low temperatures. Molecular tools (e.g., mutants and translational fusions) to dissect this secretory pathway are now available and are being used in ongoing studies.

7 LITERATURE

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