Expression and regulation of levansucrase in *Pseudomonas syringae*

by

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Summary

*Pseudomonas syringae* pv. glycinea PG4180 is an opportunistic plant pathogen which causes bacterial blight disease of soybean (*Glycine max*). This organism can utilize sucrose, the most abundant plant sugar, with the help of enzyme levansucrase (Lsc). Sucrose is eventually cleaved into glucose, which is further metabolised by the cell, and fructosyl residues, which are polymerized by the same enzyme to form the exopolysaccharide levan. PG4180 has three genes for Lsc, of which two, *lscB* and *lscC* are expressed while *lscA* is not expressed.

The first part of this study dealt with determining the presence of multiple *lsc* genes in *P. syringae*. Nucleotide sequence alignments between *lscB/C* and *lscA* helped identify a prophage associated promoter element present in the upstream of *lscB/C* ORF but not associated with *lscA*. We could, for the first time, express *lscA* in PG4180 under the *lscB* promoter. However, *lscB* could not be expressed under the *lscA* promoter indicating that *lscA* upstream region does not contain a promoter region. We speculate that *lscA* might be an ancestral Lsc variant in front of which an active promoter, possibly derived from a bacteriophage, got inserted leading to expression of *lscB/C*.

The second part of this study dealt with identifying transcription factors involved in regulation of Lsc expression in *P. syringae*. The binding site for a hexose metabolism regulator, HexR, was identified in the promoter region of *lscB*. As compared to the wild-type, the ∆*hexR* mutant had severely hampered growth characteristics in medium containing glucose or sucrose as sole source of carbon as well as *in planta*. DNA affinity chromatography was done to identify two H-NS-like regulatory proteins MvaT-MvaU binding to the promoter region of *lscB*. Protein-DNA binding assay and overexpression of MvaU in PG4180 suggested that these two proteins could act as repressors of *lsc* expression. The data suggest a co-regulation of genes involved in extra-cellular sugar acquisition with those involved in intra-cellular energy-providing metabolic pathways as well as virulence and biofilm expression in *P. syringae*.
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I dedicate this thesis to my father, who passed away in 2010.
1

Introduction

1.1 Plant-pathogen interactions

Molecular-clock estimate studies suggest that land plants evolved more than 700 million years ago [40]. Plants are constantly exposed to microbes during their lifetime, be it bacteria, fungi or viruses. In this respect, bacteria are omnipresent on plant surfaces. Interactions between any two organisms can be termed as mutualism, commensalism or parasitism, depending on the outcome for one or both the organisms involved. Mutualism, also termed as symbiosis, is the interaction where both organisms benefit from each others’ presence. If only one of the two organisms is benefited while the other is unharmed, the association is called commensalism. Parasitism has the most drastic effects since one organism harms the other organism in order to survive.

1.1.1 Entry into the host

To be pathogenic, a microbe has to access the plant interior in some way like through stomatal openings, wounds and lesions or directly through the epidermis of the leaf, stem or root. However, adhering to the plant surface or entering it does not necessarily lead to a disease. Pathogenesis is strongly dependent on environmental factors especially temperature and humidity. Both these factors affect the epiphytic life of the pathogen such as its growth rate, chemotaxis, or exopolysaccharide (EPS) production. Gram-negative bacteria belonging to the family *Pseudomonadaceae
colonize the apoplast, the extracellular space surrounding the plant cells [1]. Such pathogens are called hemibiotrophs because they are able to obtain nutrients from living host cells in order to multiply in the apoplast and infect neighbouring tissues [75].

1.1.2 Defense response: Disease or No disease?

Once inside the apoplast, plants have the ability to recognize foreign bacteria and potential pathogens by the bacterial surface receptors, the so-called pathogen-associated molecular patterns (PAMPs) and trigger PAMP-triggered immunity (PTI) [70, 105]. The bacteria counter this PTI by blocking this immune response and by secreting effector proteins. Most bacterial plant pathogens possess type III secretion system (T3SS), by which they can inject the T3SS effector proteins like Avr and Hop proteins into the plant cell using a syringe like ‘injectisome’ apparatus [17].

In-turn, the plants have developed a second response to these effector proteins which is known as effector-triggered immunity (ETI) [48]. The phenomenon was first proposed by H. Flor and called ‘gene-for-gene’ resistance [30]. Resistant plants possess cognate R effector proteins which are complementary to the bacterial Avr proteins. A complementary Avr-R interaction leads to incompatible host-microbe interaction leading to a hypersensitive reaction (HR), i.e. a programmed cell death at the site of infection. Such an HR results in suppression of the disease. If Avr-R reaction is not complementary, the result is invasion of the host by the bacterium and onset of disease [19]. Indeed, HR represents an important criterion for differentiation of bacterial species into pathovars depending on HR elucidated by host and non-host plants.

Interestingly, the pathogens have also adapted themselves to secrete effectors which interfere with ETI [14]. Thus, resistant hosts lead to more potent pathogens and vice versa. This viscous circle predominates the co-evolution of plants and pathogens associated with them in order to survive.
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1.2 *Pseudomonas syringae*

*Pseudomonas syringae* is a Gram-negative, rod-shaped, aerobic and motile γ-proteobacterium possessing several polar flagella [33]. The organism was first isolated in 1902 by van Hall from common liliac plant *Syringa vulgaris*, from which it has got its name [102]. The optimum temperature for growth its is 25-30°C [83]. It is catalase and oxidase negative. Production of siderophores like pyoverdin under iron-limiting conditions is a property shared by many *Pseudomonas* species including *P. syringae*. These siderophores make these so-called fluorescent pseudomonads easy to detect on solid media like King’s B medium [50]. Pseudomonads, in general, are nutritionally versatile organisms and can utilize a variety of carbon sources. Although primarily a phytopathogen, epiphytic growth is ecologically important for *P. syringae’s* survival and spread in the field, and is a topic of intensive study. Existence of non-pathogenic strains of *P. syringae* have also been reported [97, 62].

Taxonomic classification of *Pseudomonas syringae*

Kingdom: Bacteria  
Phylum: Proteobacteria  
Class: Gamma Proteobacteria  
Order: Pseudomonadales  
Family: *Pseudomonadaceae*  
Genus: *Pseudomonas*  
Species: *syringae*

The isolates are divided into more than 50 pathovars depending on their host specificity and the diseases they cause [46]. Apart from T3SS and Avr proteins, the pathovars also secrete different phytotoxins like phaseolotoxin, tabtoxin, coronatine, syringolin, syringopeptin and syringomycin [4]. These act as additional virulence factors although they are not necessary for pathogenicity. A pathovar may secrete one or more of these toxins but most lack host-specificity and have wider range of activity.

Dissemination of bacteria usually occurs via aerosols and is dependent on the
water cycle [64, 65]. *P. syringae* causes a variety of diseases on a wide range of plants like bacterial speck of tomato, brown spot of bean, halo blight of bean, bleeding canker of European horse chestnut, bacterial blight of soybean, leaf spot of Indian horse chestnut and many others. Not surprisingly, it is the most studied phytopathogenic bacterium in the world [57]. Three pathovars namely, pv. tomato DC3000 [7], pv. phaseolicola 1448A [47] and pv. syringae B728a [28] have been fully sequenced while more than additional 30 have been partially sequenced (www.pseudomonas-syringae.org). Not surprisingly, the strains DC3000, 1448A and B728a have become excellent model systems to study plant-bacterial interactions.

### 1.2.1 *Pseudomonas syringae* pv. glycinea PG4180

*P. syringae* pv. glycinea PG4180 causes bacterial blight of soybean plants. Typical symptoms include water-soaked lesions which eventually turn into necrotic regions surrounded by chlorotic halos in the leaves.

### 1.3 Bacterial blight of soybean

Soybean (*Glycine max*) is a legume native to East Asia although it is now grown worldwide (Figure 1.1A). The bean has various uses like protein for animal feed, soymilk, tofu, as well as substitute for meat analogues. Being widely cultivated, it also susceptible to diseases, most common being the bacterial blight of soybean [49]. Although it was reported much earlier, the first detailed description of the disease was given by Coerper in 1919 [16].

The disease initially shows water-soaked lesions, most conspicuously on the leaves. The water-soaking is due to filling of the intra-cellular spaces with EPSs [78]. The phytotoxin coronatine induces chlorosis (Figure 1.1B) and its production is temperature-dependent. Highest coronatine synthesis is found at 18°C which is also the ambient temperature for pathogenesis for the organism [3]. The chlorotic regions eventually become necrotic with the leaf finally assuming a very ragged appearance. The disease is not limited to leaves but is also seen in infected stems, petioles and pods [16].
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Sucrose is the most abundant plant storage sugar and is thought to also simply leach from the interior of the plant [92, 61]. Glucose and fructose are linked through a glycosidic bond to form the disaccharide sucrose. Grain legumes such as soybeans contain 2-10% of α-galactosides of sucrose, namely raffinose, stachyose and verbascose [38]. Ability to utilize this sucrose forms a distinct advantage for the bacteria in order to survive and replicate in the otherwise nutrient-deprived interior of plant cells. Some bacteria have the ability to utilize sucrose by importing the sugar through a sucrose permease porin. The sucrose is then metabolised by sucrose phosphorylase or first phosphorylated and then metabolised by sucrose-6-phosphate hydrolase (ScrB) [72]. Other bacteria like *P. syringae* utilize the sucrose via extra-cellular enzymes like levansucrase or sucrose-6-phosphate hydrolase. Although *P. syringae* also possesses ScrB, its exact role in relation to Lsc is still under investigation.

1.4 Exopolysaccharides

An important virulence mechanism conducted by *P. syringae* is the production of exopolysaccharides (EPSs). The composition of EPS varies greatly but they are
usually high molecular weight compounds in the range of $0.5-2.0 \times 10^6$ Da [86]. EPSs consist of 97% water thereby playing a major role in preventing desiccation of the bacterium. Apart from this, they are thought to play other roles to give the bacterium competitive advantage while invading the host. EPSs chelate ions which activate T3SS [45] or they can play protective roles like adsorbing copper thereby preventing the metal from entering the bacterial cell [35]. EPSs have also been shown to enhance attachment to biotic or abiotic surfaces [9, 34, 22] and contribute to bacterial movement in-planta [5].

The two major EPSs of *P. syringae* pv. glycinea are alginate and levan. However, *P. syringae* pv. glycinea PG4180 produces very little alginate due to a point mutation leading to a truncation of the *algT* gene product, an alternative sigma factor involved in the synthesis of alginate. A natural revertant PG4180.muc produces both levan and alginate [80]. Since wild-type PG4180 produces levan as its major exopolysaccharide, it is a good model organism to study exopolysaccharide production and biofilms.

1.4.1 Levan

Bacterial levan is an EPS formed when bacteria encounter moderate to high sucrose concentrations in their environment. Levan is produced by many genera including commercially important bacteria like *Azotobacter, Lactobacillus, Pseudomonas, Streptococcus* and *Zymomonas*. Levan is produced by many if not all pathovars and hence serves as a taxonomic marker of *P. syringae* [42]. Levan appears as mucoid transluscent slime around the colonies when bacteria are grown on medium supplemented with sucrose (Figure 1.2A). Structurally, it is a $\beta$-(2,6) polyfructan with extensive branching through $\beta$-(1,4) linkages as seen in Figure 1.2B [71, 36, 42]. Levan is formed by the enzyme levansucrase (Lsc). Lsc cleaves sucrose into glucose and fructosyl residues. The glucose is utilized by the organism as a source of energy. The fructosyl residues are polymerized to from levan by the same enzyme. Levan has been shown to enhance tolerance to salinity, dessication, and the ability to form cell aggregates on abiotic surfaces [95].
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Figure 1.2: (A) *Pseudomonas syringae* pv. glycinea PG4180 colonies showing the characteristic mucoid slime of levan after growing on King’s B agar containing 5% sucrose (B) Structure of levan showing branched fructosyl residues with a terminal glucose moiety at the right. Polymer length changes according to the number of chained fructosyl residues (n).

1.5 Levansucrase

Levansucrase (E.C. 2.4.1.10) belongs to family 68 of glycoside hydrolases [8]. It is an extracellular enzyme produced by many Gram-positive as well as Gram-negative bacteria. This enzyme cleaves sucrose and use the bond energy between glucose and fructose to couple a fructosyl unit to other fructosyl units to form the polymer of levan. Lscs can also catalyze the fructosyl transfer to a number of acceptors (other than the fructan polymer) such as water, sucrose, raffinose, and various mono- and disaccharides [81].

Lsc has been studied in detail in Gram-positive bacteria like *Bacillus subtilis*, different food-grade lactobacilli, oral *Streptococcus* sp., and *Leuconostoc mesenteroides* [89, 43, 63, 76], and in Gram-negative bacteria like *P. syringae*, *Erwinia amylovora*, *Zymomonas mobilis*, *Glucanacetobacter diazotrophicus*, *Rahnella*
1. INTRODUCTION

*aquatilis* and *Acetobacter xylinum* [84, 87, 96, 82, 58].

Experimental work with Lsc indicated three major features, viz. (i) intrinsically high protein stability as an extra-cellular enzyme, (ii) no need for co-factors, and (iii) ease of enzymatic detection due to its glucose releasing activity. Crystal structures of few Lscs, namely from *B. subtilis*, *B. megaterium*, *Arthrobacter* sp. K-1 and *G. diazotrophicus* are available. Peptide sequences, enzymatic properties, protein location, and transcriptional regulation of Lsc differ most noticeably between Gram-positive and Gram-negative bacteria. However, they do share some similarities as well. In general, Lsc has a five bladed β-propeller fold with central acidic pocket for substrate binding [60, 58, 85, 90].

Lsc synthesis has been studied previously with different aims and extents by many researchers. For e.g.: Lsc-encoding *lsRN* from *B. licheniformis* RN-01 is constitutively expressed independently of the presence extracellular sucrose [66], while Lsc from a thermophilic *Bacillus* sp. is sucrose inducible [2]. *levU* from *Z. mobilis* is encoded in a bicistronic manner, a feature quite different from other studied Gram-negative bacteria [84]. *lsrA* from *R. aquatilis* has been shown to be stationary phase-dependent but independent from RpoS, a sigma factor needed for transcription under starvation conditions in stationary phase [82].

*P. syringae* pv. glycinea PG4180 possesses three *lsc* genes, *lscA*, *lscB* and *lscC* [54]. *lscA* and *lscC* are chromosomally encoded while *lscB* is encoded on a native 60-kb plasmid. However, not all the three genes synthesize Lsc. Only *lscB* and *lscC* are active while *lscA* is found to be inactive under the tested lab conditions [54]. Single mutants of *lscB* and *lscC* in PG4180 produce levan while the *lscBlscC* double mutant is levan negative. Analysis of the single *lsc* mutants also shows that LscB is predominantly secreted from the cell while LscC is retained in the periplasm. *lscA* can be expressed under an artificial promoter but not under its native promoter. Synthesis of *lsc* is highest at OD_{600} of 0.5 i.e. in early logarithmic phase and decreases as the bacterial cultures age [53]. LscB and LscC are 98% identical, differing in only five amino acid residues at the protein level. Interestingly, four of the five divergent amino acyl residues from the predicted protein sequences are conservative replacements while the fifth is a cysteine-to-serine exchange allowing speculations about different secondary structure formation with respect to disulfide bridges. Since
Lsc is involved in degradation of sucrose to obtain glucose, it is assumed to be an integral enzyme of the sugar metabolism pathway in this organism.

1.6 Regulation of sugar metabolism in Pseudomonads

The regulation of sugar metabolism is a global, multi-factorial, and essential feature of heterotrophic bacterial species. Pathways for utilization of sugars are greatly adapted in bacteria. As compared to E. coli which utilizes glucose via the Emden-Meyerhof-Parnas (EMP) pathway and the tricarboxylic acid (TCA) cycle, Pseudomonas sp. utilize glucose via the Entner-Douderoff (ED) pathway and the glyoxylate-shunt of the TCA cycle [26, 32]. It has recently been shown that the ED pathway is necessary for generating redox currency in from of NADPH, which is required for counteracting oxidative stress [13]. Since generation of oxidative stress by production of reactive oxygen species is a very common plant defense reaction to pathogens [91], organisms like P. syringae could favour the ED pathway over the EMP pathway to tolerate and overcome this oxidative stress.

In Pseudomonas putida and Pseudomonas fluorescens, extracellular glucose is first converted to gluconate and 2-keto-gluconate in the periplasm and then channeled into the ED pathway [25, 56, 88, 21]. del Castillo et al. have also shown that the peripheral glucose pathways converge to give a common intermediate of 6-phosphogluconate in P. putida KT2440 [12].
Aims of the study

Utilization of sucrose by Lsc comprises of an energetically important process of the plant pathogen *P. syringae* pv. glycinea PG4180. Like any central metabolic process, this has to be correctly regulated to save energy. Even though some work on Lsc and levan from PG4180 has been done, not much is known about the regulation of transcription of *lsc* in PG4180. Redundancy in the *lsc* gene copies in *P. syringae* is also an area not investigated in detail. In this study, two important aspects of levansucrase, namely its genomic distribution and the activity of *lscA*, and the regulation of *lsc* by transcription factors, were investigated.

In the first part of this thesis work, the analysis of the genomic distribution of the three *lsc* genes of *P. syringae* was used to establish an evolutionary model on how this type of gene was acquired and how its expression got controlled by a potentially phage-borne upstream region and associated regulators. Further, experiments were done to understand the cryptic expression of *lscA*. Protein chimeras were constructed by fusing the promoter regions of *lscB* with the ORF of *lscA* and vice versa. This particular scope of the study was broadened by analyzing the *lscA* expression patterns in other *P. syringae* pathovars.

In the second part, the hexose metabolism regulator HexR was found to be a repressor of *lsc* due to presence of a conserved binding site in the promoter region of *lsc*. DNA affinity chromatography experiments had shown two H-NS-like proteins, MvaT and MvaU, binding to the upstream region of *lscB*. Experiments like Western blot, growth curve analyses, DNA-protein binding assay and over-expression studies were done to understand the role of these transcription factors on regulation of *lsc*. 
Results

The results are represented by publications and manuscripts originated during the course of this PhD thesis.


AbS conducted the bioinformatics analysis; NAK conducted the E. amylovora-related experiments; ArS conducted the glycosyl hydrolase-related experiments; HW conducted the com gene analysis; AbS, SK and MU prepared and revised the manuscript.


SK conducted all the experiments; DP generated a fusion construct; AnS conducted the MALDI-TOF analysis; SK and MU prepared the manuscript.

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SK conducted the growth curve, Western blot and qRT-PCR experiments; DZ and GAE generated the hexR deletion mutant; DZ performed the in-planta assay; AbS, DP and AM conducted the bioinformatics analysis; NAK conducted the HR and protein secretion experiments; SK, DZ, AbS and MU prepared the manuscript.

Shaunak Khandekar, Daria Zhurina, Annette Arndt, Melanie Brocker, Michael Bott, Bernhard Eikmanns and Matthias S. Ullrich, “H-NS-like proteins MvaT and MvaU regulate expression of levansucrase in Pseudomonas syringae”, (In preparation)

SK conducted the EMSA, overexpression and mvaT mutational analysis; DZ conducted the DNA affinity chromatography, transcriptional analysis and generated the mvaU mutant; MeB and MiB conducted and helped with the MALDI-TOF analysis; AA and BE helped with the DNA affinity chromatography; SK, DZ and MU prepared the manuscript.
3. RESULTS

3.1 Genomic distribution and divergence of levansucrase-coding genes in \textit{Pseudomonas syringae}
Article

Genomic Distribution and Divergence of Levansucrase-Coding Genes in Pseudomonas syringae

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Abstract: In the plant pathogenic bacterium, Pseudomonas syringae, the exopolysaccharide levan is synthesized by extracellular levansucrase (Lsc), which is encoded by two conserved 1,296-bp genes termed lscB and lscC in P. syringae strain PG4180. A third gene, lscA, is homologous to the 1,248-bp lsc gene of the bacterium Erwinia amylovora, causing fire blight. However, lscA is not expressed in P. syringae strain PG4180. Herein, PG4180 lscA was shown to be expressed from its native promoter in the Lsc-deficient E. amylovora mutant, Ea7/74-LS6, suggesting that lscA might be closely related to the E. amylovora lsc gene. Nucleotide sequence analysis revealed that lscB and lscC homologs in several P. syringae strains are part of a highly conserved 1.8-kb region containing the ORF, flanked by 450-452-bp and 49-51-bp up- and downstream sequences, respectively. Interestingly, the 450-452-bp upstream sequence, along with the initial 48-bp ORF sequence encoding for the N-terminal 16 amino acid residues of Lsc, were found to be highly similar to the respective sequence of a putatively prophage-borne glycosyl hydrolase-encoding gene in several P. syringae genomes. Minimal promoter regions of lscB and lscC were mapped in PG4180 by deletion analysis and were found to be located in similar positions upstream of lsc genes in three P. syringae genomes. Thus, a putative 498-500-bp promoter element was identified, which possesses the prophage-associated com gene and DNA encoding common N-terminal sequences of all 1,296-bp Lsc and two glycosyl hydrolases.
Since the gene product of the non-expressed 1,248-bp lscA is lacking this conserved N-terminal region but is otherwise highly homologous to those of lscB and lscC, it was concluded that lscA might have been the ancestral lsc gene in E. amylovora and P. syringae. Our data indicated that its highly expressed paralogs in P. syringae are probably derived from subsequent recombination events initiated by insertion of the 498-500-bp promoter element, described herein, containing a translational start site.

**Keywords:** levansucrase; phage-associated promoter element; Pseudomonas syringae; Erwinia amylovora; pro-phage

---

### 1. Introduction

*Pseudomonas syringae* is a Gram-negative phytopathogenic bacterium classified into 51 pathovars based on distinct host specificities [1]. These bacteria produce various extracellular polysaccharides (EPSs). In *P. syringae*, *Erwinia amylovora*, and several other bacterial species, the EPS levan is synthesized from sucrose by the extracellular enzyme levansucrase (Lsc; EC 2.4.1.10), which is a member of the glycosyl hydrolase 68 family. Levan is a high-molecular β-(2,6)-polyfructan with extensive branching through β-(2,1)-linkages [2].

*P. syringae* pv. glycinea PG4180 causes bacterial blight on soybean plants and has been used as a model strain for levan formation [2–4]. Full genome sequences of three other *P. syringae* strains are available: pv. phaseolicola 1448A, pv. syringae B728a, and pv. tomato DC3000. Partial shot-gun genome sequences of several other *P. syringae* strains are available on the NCBI’s Genbank website. Comparison of genome sequences in *P. syringae* strains revealed the presence of 2–3 copies of the *lsc* gene in all of the analyzed strains [3–9]. In previous years extensive progress has been made in terms of the heterologous expression and the protein and polymer characterization of *lsc* genes from *P. syringae* [10–12].

Bacterial genomes are comprised of core and flexible components. Core genomes include genes essential for the survival of the organism, such as e.g., 365 housekeeping genes in *P. syringae* [6]. Phylogenetic comparison of several housekeeping genes allowed classification of *P. syringae* strains into four monophyletic groups, where pv. tomato DC3000 and T1 belonged to group 1, pv. syringae B728a to group 2, and pv. phaseolicola 1448A as well as pv. glycinea PG4180 to group 3 [13]. Flexible genomic components comprise genes important for adaptation to specific ecological niches or specific growth conditions such as e.g., virulence-associated genes, resistance genes, or mobile genetic elements like phage-borne genes, plasmids, conjugative transposons, or insertion sequence (IS) elements [13].

In contrast to any other investigated levan-forming bacteria, multiple copies of *lsc* have been reported for *P. syringae* strains. However, a reasonable explanation for the occurrence of multiple *lsc* copies in *P. syringae* genomes is missing. Recently, genomic data of plant-pathogenic and plant-associated bacteria has emerged rapidly and a close observation indicates the presence of more than one copy of *lsc* genes present in *P. syringae* strains [3–9]. The *lsc* alleles in PG4180 were termed lscA, lscB, and lscC [3] while the corresponding genes in DC3000 and T1 were termed lsc2, lsc3, and lsc1 [5,8]. Two distinguishable variants of *lsc* were observed: lscA and lsc2 are 1,248 bp in length
while lscB, lsc3, lscC, and lsc1 comprise 1,296 bp. Strain B728a possesses only one 1,296-bp lsc allele [7]. For easier understanding, herein the 1,248-bp lsc gene variants were designated as ‘variant A’ while the 1,296-bp lsc alleles were termed ‘variant BC’.

*E. amylovora* Ea7/74, which causes fire blight on rosaceous plants, possesses a single variant A homolog [2,14]. Mutation of lsc in Ea7/74 led to a levan-negative phenotype [15]. Previously, variant A from PG4180 was shown to be not expressed under various conditions tested in its native host. Mutation of variant A in PG4180 still rendered the mutant levan-positive [3]. Only simultaneous mutation of lscB and lscC yielded the levan-negative mutant, PG4180.M6 [3]. Furthermore, lscA of PG4180 is not expressed from its native promoter in *Escherichia coli*, a close enterobacterial relative of *E. amylovora* [2,3].

In the current study, the cryptic PG4180 variant A lsc gene was expressed from its native promoter in the *E. amylovora* lsc-negative mutant, Ea7/74-LS6, demonstrating that the *E. amylovora* genetic background was sufficient for its expression. A comparative bioinformatics approach was used to analyze the upstream and downstream sequences of variant BC alleles in five *P. syringae* strains, giving rise to an interesting model on how lsc genes might have evolved and been distributed among *P. syringae* pathovars. Furthermore, it was determined that variant BC alleles might be expressed from a newly defined phage-associated promoter element (PAPE).

2. Results and Discussion

2.1. Heterologous Expression of lscA in *E. amylovora*

Previously, it had been shown that lscA was not expressed in PG4180 while variant BC alleles were functional [3]. When lscA of PG4180 along with its 940-bps upstream sequence was introduced to the lsc-negative *E. amylovora* mutant Ea7/74-LS6 [15], the resulting transconjugant showed levan formation on agar plates supplemented with 5% sucrose, in contrast to mutant Ea7/74-LS6 (Data not shown). Since lscA was placed in the opposite direction to vector-borne promoters of the plasmid, this result indicated lscA expression from its native promoter. To substantiate this, Western blot analysis with Lsc-specific antiserum and protein extracts of Ea7/74 wild type, its lsc-deficient mutant, and the Ea7/74-LS6 transconjugant carrying lscA was conducted, revealing a clear signal for Lsc in the transconjugant (Figure 1A). Concentrated cell-free supernatants of the Ea7/74 derivatives were spotted on water agar containing 5% sucrose revealing levan formation in the transconjugant thus confirming heterologous lscA expression (Figure 1A).

2.2. Nucleotide Sequence Comparison of Variant A lsc Genes

PG4180 LscA shows 87.5% identity at the amino acid level with variant BC enzymes and shares 75.9% identity to Lsc of Ea7/74. It was speculated that the common ancestor of PG4180 genes lscA, lscB, and lscC might be related to the lsc gene of *E. amylovora*, and that all three genes present in *P. syringae* might have diverged from this common ancestor. This hypothesis was supported by a phylogenetic analysis of all available protein sequences of Lsc’s built by the Neighbor-Joining method (Appendix Figure A1). Variant A alleles of all *P. syringae* strains were found to be clustered closer to the Lsc’s from *Enterobacteriaceae* including *E. amylovora* Ea7/74 as opposed to variant BC alleles. Aside of variant A alleles being present in single copy in several *P. syringae* strains and in
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*E. amylovora*, this gene variant was also found in single copy in other enterobacterial species such as *Erwinia tasmaniensis* and *Rahnella aquatilis* ATCC33071 (Table 1). Since these bacterial species are commonly found to be associated with host plant species such as soybeans [16,17], *P. syringae*, *E. amylovora*, and *R. aquatilis* might have exchanged genetic information during cohabitation and *lscA* can be considered as an example of such horizontal gene transfer.

**Figure 1.** (A) Western blot detection of extra-cellular levansucrase (*Lsc*) of *E. amylovora* Ea7/74 and *LscA* of *P. syringae* pv. glycinea PG4180 expressed in Ea7/74-LS6 (*lsc* deficient mutant). LS6 (*lscA*) harbored PG4180 *lscA* in opposite orientation to the vector-borne *P*<sub>lac</sub> promoter. The arrow represents the signal for the 55-kDa *Lsc* proteins. The lower panel shows the levan formation assay for cell-free concentrated culture supernatants on water agar plates supplemented with 5% sucrose. The sample was incubated at 18 °C for one week on a water agar plate with 1.5% agar content (B) Comparison of the predicted catalytic blocks present in the active sites of PG4180 variant A and BC with that of *E. amylovora* Ea7/74 *Lsc*. Residues Asp, Asp, Glu in blocks I, II, and III are the predicted key residues involved in activity of the enzyme. *Lsc* of Ea7/74 and PG4180 variant A share the same codon usage for Block II Asp and Block III Glu.
Table 1. Distribution of variant A and variant BC alleles in *Pseudomonadaceae* and *Enterobacteriaceae*.

<table>
<thead>
<tr>
<th>Pathovar, strain</th>
<th>Gene name</th>
<th>Locus tag</th>
<th>Genomic location</th>
<th>Gene name</th>
<th>Locus tag</th>
<th>Genomic location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycinea PG4180</td>
<td><em>lscA</em></td>
<td>-</td>
<td>Chr.</td>
<td><em>lscB</em></td>
<td>-</td>
<td>Plasmid</td>
<td>[3]</td>
</tr>
<tr>
<td>phaseolicola 1448A</td>
<td>levansucrase</td>
<td>PSPPH_2074</td>
<td>Chr.</td>
<td>levansucrase</td>
<td>PSPPH_A0027</td>
<td>Plasmid</td>
<td>[6]</td>
</tr>
<tr>
<td>syringae B728a</td>
<td>levansucrase</td>
<td>Psyr_2103</td>
<td>Chr.</td>
<td>levansucrase</td>
<td>Psyr_0754</td>
<td>Chr.</td>
<td>[7]</td>
</tr>
<tr>
<td>acinitidiae M302091</td>
<td>-</td>
<td>-</td>
<td>plasmid</td>
<td><em>lscC</em></td>
<td>PSYAC_19498</td>
<td>?</td>
<td>[42]</td>
</tr>
<tr>
<td>aesculi NCPPB3681</td>
<td>levansucrase</td>
<td>PsyrPaN_010100019209</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>plasmid</td>
<td>[43]</td>
</tr>
<tr>
<td>lachrymans M302278PT</td>
<td>levansucrase</td>
<td>PLA107_25445</td>
<td>?</td>
<td>levansucrase</td>
<td>PSYMP_24576</td>
<td>?</td>
<td>[43]</td>
</tr>
<tr>
<td>morsprunorum M302280PT</td>
<td>-</td>
<td>-</td>
<td>plasmid</td>
<td><em>lscC</em></td>
<td>PsyrptA_020100005135</td>
<td>?</td>
<td>[42,43]</td>
</tr>
<tr>
<td>tomato DC3000</td>
<td><em>lsc</em>-2</td>
<td>PSPTO_2305</td>
<td>Chr.</td>
<td><em>lsc</em>-3</td>
<td>PSPTO_A0032</td>
<td>Plasmid</td>
<td>[5]</td>
</tr>
<tr>
<td>tomato T1</td>
<td><em>lsc</em>-2</td>
<td>PSPTOT1_4965</td>
<td>Chr.</td>
<td><em>lsc</em>-3</td>
<td>PSPTOT1_4913</td>
<td>?</td>
<td>[8]</td>
</tr>
<tr>
<td>tomato K40</td>
<td>-</td>
<td>levansucrase</td>
<td>PsyrptK_010100027584</td>
<td>?</td>
<td>-</td>
<td>Vinatzer et al. (unpublished) Genbank</td>
<td></td>
</tr>
<tr>
<td>tomato NCPPB 1108</td>
<td>levansucrase</td>
<td>PsyrptN_010100027628</td>
<td>?</td>
<td>-</td>
<td>-</td>
<td>Vinatzer et al. (unpublished) Genbank</td>
<td></td>
</tr>
<tr>
<td><em>Erwinia amylovora</em></td>
<td><em>Lsc</em></td>
<td>-</td>
<td>Chr.</td>
<td>-</td>
<td>-</td>
<td>[15]</td>
<td></td>
</tr>
<tr>
<td>CFPB 1430</td>
<td><em>Lsc</em></td>
<td>-</td>
<td>Chr.</td>
<td>-</td>
<td>-</td>
<td>[44]</td>
<td></td>
</tr>
<tr>
<td><em>Erwinia tasmaniensis</em></td>
<td><em>Lsc</em></td>
<td>-</td>
<td>Chr.</td>
<td>-</td>
<td>-</td>
<td>[45]</td>
<td></td>
</tr>
<tr>
<td>Rahnella aquaticis</td>
<td><em>lsrA</em></td>
<td>-</td>
<td>Chr.</td>
<td>-</td>
<td>-</td>
<td>[46]</td>
<td></td>
</tr>
</tbody>
</table>

*lsc*: levansucrase, Chr.: Chromosomal location.
It had been reported that three acidic residues are highly conserved among members of the Glycosyl hydrolase families 32, 43, 62, and 68 at the catalytic active sites termed as block I (Asp/Glu), block II (Asp) and block III (Glu) [12,18,19]. Therefore, conserved regions within the catalytic blocks of variant A and variant BC were compared for Ea7/74 and PG4180 (Figure 1B). The catalytic centers Asp (block II) and Glu (block III) shared a common codon usage for these nucleotide sequences with both organisms (www.kazusa.or.jp/codon/). This further suggested that lscA in PG4180 and lsc of Ea7/74 might share similar regulatory features particularly present in E. amylovora.

2.3. Nucleotide Sequence Comparison of Variant BC lsc Genes

In contrast to variant A genes, variant BC lsc alleles are found only in P. syringae pathovars (Table 1, Appendix Figure A1). The coding sequences of all variant BC genes exhibited 94.0% identity at the nucleotide sequence level and 98.1 to 99.8% similarity at the protein sequence level, demonstrating a high degree of conservation among otherwise variable P. syringae pathovars [13,20].

Comparison of the up- and downstream sequences of known variant BC alleles interestingly revealed a common ~1.8-kb highly conserved nucleotide sequence with an average of 87.3% identity (Figure 2, Appendix Figure A2). This conserved region comprises 450 to 452 bp of upstream sequence, 1,296 bp of the lsc ORF and 49–51 bp of downstream sequence, possibly involved in the formation of stem-loop structure for ρ-independent transcriptional termination (Appendix Figure A3). To map the promoter element of variant BC genes, a nested deletion analysis of the lscB upstream sequences ranging from -666 to -50 bp of the respective lsc translational start (TS) was conducted (Figure 3). For this, the lscB- and lscC-deficient mutant PG4180.M6 was complemented with various plasmid-borne deletion constructs and the phenotypes of the transconjugants were analyzed with respect to levan production (Appendix Figure A4). Deletion constructs ending 5’ at position −440-bp upstream of the TS and any larger upstream sequence fully complemented the mutant PG4180.M6 with respect to levan formation. In contrast, the deletion construct ending at position −332-bp did not complement this mutant, thereby identifying the minimal promoter required for lscB expression (Figure 3). The experiment was repeated with respective deletion constructs for lscC giving identical results (Data not shown). Since nucleotides 450–452 bp upstream of variant BC ORFs are highly conserved in all four P. syringae strains analyzed (Figure 2, Appendix Figure A2), it may be speculated that the minimal promoter sequences of the variant BC alleles of the other P. syringae pathovars not experimentally studied are similar to those of strain PG4180. Nucleotide sequences flanking the conserved ~1.8 kb region varied considerably among the strains investigated, with the exception of two plasmid-borne variant BC alleles in strains DC3000 and 1448A, for which 93% of the nucleotides up to ~2,800 bp with respect to the translational start sites were identical (Figure 2). This 2,800-bp upstream sequence of plasmid-borne variant BC genes is bordered by a truncated transposase gene resembling that of transposon ISPsy16 [6] (Data not shown).
Figure 2. Genetic map showing variant BC alleles and their surrounding sequences in *P. syringae* pathovars represented by PG4180, 1448A, DC3000 and B728a. The 1.8-kb conserved nucleotide sequence contains 1.296-bp *lsc* coding sequence, 450 bp up-, and 49–51 bp downstream sequences of variant BC. The 500-bp conserved sequence represents the phage-associated promoter element (PAPE) linked with variant BC and the putative pro-phage-borne glycosyl hydrolase genes with 48 bp conserved N-termini of the coding sequences. PSPPH_0655 and Psyr_4600 are putative glycosyl hydrolase genes in 1448A and B728a, respectively. The PAPE contains the promoter of *lsc* and *com* genes. Variant BC comparison in their downstream sequences showed a different length of nucleotide conservation. The 25–100 bp sequences downstream of *lscB/C* stop codon show conservation with a 75-bp *lscA* downstream sequence and the 3’ end of the *nasT* gene. Variant A is depicted devoid of the 48-bp conserved sequence which is always associated with variant BC. Minus (‘−’) values depict upstream sequences to the translational start codon of *lsc* and positive values represent the nucleotide sequences downstream of *lsc* translational stop codon.
Figure 3. Nested deletion analysis of the lscB upstream sequence. (A) Schematic presentation of lscB and its upstream sequence. (■) represents the promoter region of lscB. Levan phenotypes after complementation of mutant PG4180.M6 with different deletion constructs are provided below. (B) Western blot analysis of 30-fold concentrated cell-free supernatants of PG4180.M6 complemented with deletion constructs using Lsc-specific antiserum. (C) Lsc activities in 1 mL of cell-free supernatants. Cells were grown at 18 °C and harvested at OD$_{600}$ of 1.5 to 2.0. Data represent average values with standard deviation from three independent experiments each with three replicates.

2.4. Downstream Sequence Comparison of Variant BC lsc Alleles

Aside from the 49–51-bp highly conserved DNA sequences, downstream of the translational stop of variant BC genes, the nucleotide sequences of those alleles further downstream exhibited ~87% identity until +1,530 bp with respect to lsc translational stop codon, except for lscC of B728a and lsc-1 of DC3000, which both had an additional 130-bp 85% similar downstream sequence (Figure 2, Appendix Figure A2). Since strain B728a lacks any native plasmid [7] but possesses conserved sequences surrounding lscC (~478 bp with respect to translational start and +1,420 bp with respect to the translational stop of the lsc gene) to that of DC3000 lsc-1 (Figure 2), it was speculated that lsc-1 of the pv. tomato strain might have been associated with the phylogenetic source of lscC in B728a.
**Figure 4.** Amino acid sequence alignment of N-termini of predicted gene products (variant A, variant BC, putative pro-phage-borne glycosyl hydrolase). A 16 residue-spanning conserved sequence in variant BC and the glycosyl hydrolase is missing in variant A. Color coding: blue—conservative residue, green—block of similar residues, yellow/orange—identical residues, white/green—weakly similar residues, black—non-similar residues.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ntermini</td>
<td>[Alignment Data]</td>
</tr>
</tbody>
</table>

Nucleotide sequence alignments revealed sequences downstream of variant BC alleles with significant similarities to sequences of transposases (www.pseudomonas.com). These could be considered as presumable evolutionary scars of transposable elements involved in the course of variant BC allele distribution in *P. syringae* (Figure 2). In the genome of strain B728a a sequence showing 92% identity with transposase orfA of ISPsy24 [6] was located 1,450–1,488 bp downstream of the lscC stop codon. Interestingly, the sequence of 1,420 bp downstream of B728a lscC coincided with the 1,290-bp downstream sequences of lscC in strains 1448A and PG4180 as well as of lsc3 in strains DC3000 and T1 (Appendix Figure A2). This finding further supported the hypothesis that lscC of strain B728a might be derived from a similar source to that of lsc-1 from strain DC3000. Previously, studies on *E. coli* transposon IS911 showed that production of OrfA either in cis or in trans stimulated production of excised circular transposon copies, suitable for intermolecular transposition into a plasmid target [21]. Previously, IS elements had been predicted to be involved in the horizontal transmission of avirulence genes and the coronatine gene cluster in *P. syringae* [22,23]. In a comparative study, lsc-1 (chromosomal) was located on the variable region (VR) 38 and supposed to be in a hotspot zone [24]. It has been reported that the same location is functionally classified as a lineage-specific region (LSR) i.e., virulence type LSR no. 14, in the DC3000 chromosome. The majority of DC3000-specific genes were suggested to be linked with lateral gene transfer events and responsible
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for the fitness as well as adaptation to the environment. Such LSRs were also predicted to help in utilizing plant-derived energy sources, e.g., sucrose [6].

Interestingly, the 25-100-bp downstream sequences of \(lscC\) in strain B728a, of \(lscB\) in strains 1448A and PG4180, and of \(lsc-I\) in strain DC3000, showed an overall 72% sequence identity with the 75-bp downstream sequences of variant A \(lsc\) alleles. Forty nucleotides of this 75-bp sequence were found to be conserved in the 3'-end of the \(nasT\) gene coding for a response regulator protein, required for expression of nitrite-nitrate reductase genes (Figure 2) suggesting but not proving a phylogenetic link between a putative initial insertion of an ancestral variant A allele to \(P. syringae\), which later diverted into several chromosomal or plasmid-borne variant BC alleles.

2.5. Upstream Sequence Comparison of Variant BC lsc Alleles and a Putative Prophage-Borne Glycosyl Hydrolase Gene

A genome-wide comparison of the 450–452-bp upstream sequences of variant BC \(lsc\) alleles with the genomic sequences of strains 1448A, DC3000, and B728a, respectively, revealed 452-bp and 70% conserved sequences upstream of two putative family 2 glycosyl hydrolase genes located within the sequences of pro-phage PSPPH01 of strain 1448A and pro-phage GH5 of strain B728A (Table 2). The sequence focused-on herein was also found to be associated with a putative bacteriocin gene in DC3000 although to a lesser extent (Table 2). In \(Klebsiella\) sp., a gene cluster, required for production of the bacteriocin klebcin, was previously reported to be associated with a phage sequence suggesting its lateral gene transfer and diversification [25].

**Table 2.** Genomic location of PAPE associated with variant BC \(lsc\) alleles.

<table>
<thead>
<tr>
<th>PAPE association with</th>
<th>Genomic location</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophage PSPPH01, putative cellulase (PSPPH 0655), 1448A chromosome *§</td>
<td>773003–773504</td>
<td>502</td>
</tr>
<tr>
<td>putative GH5 Cellulase, (Psyr_4600), B728a chromosome #§</td>
<td>5460116–5459615</td>
<td>502</td>
</tr>
<tr>
<td>putative bacteriocin, (PSPTO_0572), DC3000 chromosome *§</td>
<td>629397–629790 *</td>
<td>394</td>
</tr>
<tr>
<td>(lsc-I/C), DC3000 chromosome #</td>
<td>1595373–1594873</td>
<td>501</td>
</tr>
<tr>
<td>(lsc-3/B), DC3000 plasmid pDC3000A *</td>
<td>34651–34152</td>
<td>500</td>
</tr>
<tr>
<td>(lscC) (Psyr_0754), B728a chromosome *</td>
<td>859840–859339</td>
<td>502</td>
</tr>
<tr>
<td>(lscB) (PSPPH_A0027), 1448A large plasmid #</td>
<td>22669–22170</td>
<td>500</td>
</tr>
<tr>
<td>(lscC) (PSPPH_4994), 1448A chromosome #</td>
<td>5662790–5663289</td>
<td>500</td>
</tr>
<tr>
<td>(lscB), PG4180, plasmid #</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>(lscC), PG4180, chromosome #</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>glycosyl hydrolase, PG4180 *#</td>
<td>-</td>
<td>502</td>
</tr>
</tbody>
</table>

PAPE: Phage-associated promoter element; *#(57bp upstream to PSPTO_0572). Reference/tool: BLAST-N (#), www.pseudomonas.com (§), This study (≤).

Interestingly, the 48-bp 5’-coding sequences of variant BC \(lsc\) alleles and the glycosyl hydrolase genes were found to be 80% identical. These 48 nucleotides encode with almost identical N-termini of variant BC \(lsc\) gene products as well as the putatively pro-phage-borne glycosyl hydrolases (Figure 4). Furthermore, the conserved upstream sequences included the experimentally determined promoter region of variant BC alleles and a phage-associated \(com\) gene encoding a putative translational regulator [26] (Figure 3).
The distribution of the ~500-bp conserved upstream and N-terminus-encoding sequences at various genomic positions in diverse *P. syringae* strains suggested their mobile nature (Table 2). Mobile DNA sequences possessing potential promoter regions are generally termed mobile promoter elements [27,28]. These DNA elements, often phage-associated, allow for expression of adjacent genes or re-activation of silent genes such as shown for the IS3-mediated activation of *argE* in *E. coli* [27], and are therefore termed phage-associated promoter elements (PAPE). Interestingly, the herein observed PAPE of *P. syringae* seems to be associated with genes encoding for extra-cellular levansucrases and putative glycosyl hydrolases, both of which might play an important role for nutrient acquisition and *in planta* fitness of the pathogen.

2.6. Investigating the Role of com Gene and Glycosyl Hydrolase Gene in PG4180

The location of the mapped minimal promoter required for lsc expression appeared to be located ≥332-bp upstream from the translational start of the *lscB/C* ORF. This stimulated some interest to scan this intergenic region for additional genes by comparing this sequence with entries of the GenBank nucleotide sequence database using the method BLAST-N. The search for potential additional coding sequences revealed the presence of a 192-bp ORF starting at position -204 and ending -12 bp upstream of the translational start sites of *lscB* and *lscC*, respectively. This ORF was homologous to the *com* gene [29,30] (Figure 3). Its predicted amino acid sequence exhibited a high degree of similarity to Com translational regulators found in pseudomonad bacteriophages, including Mu-like phage of *P. entomophila* L48 (85.4% similarity), phage B3 of *P. aeruginosa* (78.3% similarity), and phage DVM 2008 of *P. fluorescens* Pf-5 (65% similarity). *In-silico* structural prediction at the ‘SUPERFAMILY’ database of the structural and functional protein website (supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/index.html) suggested that the predicted *com* gene products possess classic C2-H2 zinc-finger domain as reported for Com earlier [31]. Homologs of the putative *com* gene in PG4180 were also found upstream of the *lsc* and glycosyl hydrolase genes in 1448A, DC3000 and B728a (Figure 2). Moreover, data derived from the www.pseudomonas.com website suggested that the putative phage-borne *cellulase* gene (PSPPH0655) in 1448A and its homolog in B728a (Psyr_4600) are surrounded by phage-associated genes, which with caution suggested potential ancestral pro-phage insertions.

A potential impact of *com* on levan production was investigated by inserting a premature stop codon to *com* using site-directed mutagenesis of plasmid –666-*lscB*. The mutated plasmid was introduced to the levan-deficient mutant PG4180.M6 [3] and compared to a transformant of PG4180.M6 carrying a non-mutated plasmid. Both transformants exhibited similar levels of levan formation and Lsc secretion (Data not shown) demonstrating that the putative translational regulator, Com, was not involved in expression of *lscB*. Its potential role in controlling translation of the glycosyl hydrolase gene needs to be tested in future studies.
Figure 5. Quantitative Reverse Transcriptase PCR analysis of growth phase-dependent lsc and glycosyl hydrolase gene expression in P. syringae PG4180. Cells were grown at 18 °C in HS + glutamate as sole carbon source. Relative mRNA levels were related to the mean value determined for the signals of lsc gene of PG4180 at an OD$_{600}$ of 0.5, which was defined as 100%. Data show the means and standard errors of two experiments with three replicates. Grey bars depicts the expression of lsc gene and white bars represent the glycosyl hydrolase gene expression in PG4180.

Over-expression of the glycosyl hydrolase gene associated with the herein discovered PAPE in PG4180 and the subsequent protein purification yielded no detectable cellulose-degrading activity [32,33] (Data not shown), suggesting that the encoded enzyme is not a cellulase. A growth-phase dependent transcriptional analysis of the glycosyl hydrolase gene of PG4180 was conducted using qRT-PCR to compare if its expression resembles that of lsc as published earlier [4]. Cells of PG4180 wild type were grown in minimal medium containing glutamate at 18 °C. Results indicated that lsc and the glycosyl hydrolase gene associated with the PAPE showed maximal expression at the early exponential growth stage (Figure 5). A very similar pattern of expression had previously been observed for the lscBC genes [4]. Thus, the result indicated that the PAPE might be the site of transcriptional regulation where common, regulator(s), yet-to-be-identified, might bind and lead to gene expression.

2.7. Putative Scenario for lsc Gene Distribution in P. syringae

Due to its sequence similarity, its size, and the heterologous expression of the variant A lsc gene of PG4180 observed herein, it is tempting to speculate that variant A alleles were initially obtained by horizontal gene transfer from enterobacterial species such as E. amylovora. This assumption is fueled by the fact that P. syringae is the only known organism, in which multiple copies of lsc exist. Consequently, variant A might represent the most ancestral lsc variant in P. syringae. However, due to
its potentially inactive promoter sequence, this variant remained cryptic in \textit{P. syringae}. In an unknown sequence of events initially involving a gene duplication of \textit{lscA}, a pro-phage insertion bearing an active promoter, a potential translational regulator, and the pro-phage-borne N-terminal sequence might have inserted upstream of one of the two variant A gene copies yielding an ancestral variant BC \textit{lsc} copy. Subsequently, transposon-mediated transposition events might have led to a spreading of variant BC copies, now functional, in various \textit{P. syringae} genomes. The latter assumption is indirectly supported by the fact that \textit{P. syringae} pv. tomato PT23 and pv. glycinea race 4 contain multiple copies of IS1240 with the \textit{tnpA} gene coding for a transposase [20,22,34].

Since there are several plasmid-borne variant BC alleles, it is tempting to speculate furthermore that conjugative transfer of \textit{lsc}-bearing plasmids might have led to an accelerated distribution of variant BC alleles among \textit{P. syringae} pathovars. Interestingly, the plasmid-borne \textit{lsc-3} of DC3000 shows an upstream sequence very similar to that of plasmid-borne variant BC alleles of strains PG4180 and 1448A. However, its downstream sequence compares better to the downstream sequences of chromosomal orthologs in strains PG4180 and 1448A, respectively, (Figure 2 and Appendix A2) thus complicating the clarity of further definition of the phylogenetic pathway. However, the presence of two copies of variant BC, of which one is located on a plasmid, might indicate the importance of Lsc for the evolutionary fitness of the leaf pathogen, \textit{P. syringae}.

3. Materials and Methods

3.1. Bacterial Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 3. \textit{Escherichia coli} and \textit{Erwinia amylovora} strains were maintained and grown on Luria-Bertani (LB) medium at 37 °C and 28 °C, respectively [14,35]. \textit{P. syringae} cultures were grown in a modified Hoitink Sinden (HS) medium [36] supplemented with glutamate as sole carbon and nitrogen source at 18 °C. Bacterial growth in liquid LB media was continuously monitored by measuring the optical density at 600 nm (OD$_{600}$) and harvested for protein sampling at an OD$_{600}$ of 1.0. Antibiotics were added to the media at the following concentrations (µg/mL), respectively: ampicillin, 50; kanamycin, 25; tetracycline, 25. Cellulolytic activity was assessed according to Kasana \textit{et al.}, 2008 [32].

3.2. Molecular Genetic Techniques

Small scale isolation of plasmid DNA, restriction enzyme digests, agarose gel electrophoresis, purification of DNA fragments from agarose gels, electroporation, ligation of DNA fragments and other routine molecular methods were performed using standard protocols [35]. Nucleotide sequencing was carried out commercially (Eurofins MWG Operon, Ebersberg, Germany). The 3.1-kb \textit{PstI} fragment containing PG4180 \textit{lscA} was obtained from pSKL3 [3] and re-cloned in pBBR1MCS-3 [37] in the opposite direction to the vector-borne \textit{lac} promoter yielding pBBR3(\textit{lscA}). This construct was subsequently electroporated into competent cells of Ea7/74-LS6 yielding the transformant LS6(\textit{lscA}), which was then grown on LB agar plate containing Km\textsuperscript{r} and Tc\textsuperscript{r}. Later, the transconjunct was streaked on 5% sucrose-containing LB agar media.
Table 3. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Strain</th>
<th>Relevant characteristics</th>
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<td><em>Pseudomonas syringae</em> pv. <em>Glycinea</em></td>
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<tr>
<td>PG4180</td>
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<td>Sp', Gm', lscB lscC mutant of PG4180, levan-</td>
<td>[3]</td>
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<tr>
<td>PG4180.M6</td>
<td></td>
<td>PG4180.M6</td>
<td>Sp', Gm', Te', lscB lscC mutant of PG4180 bearing pBBR1MCS</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pBBR1MCS-3)</td>
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<td><em>Erwinia amylovora</em></td>
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<td><em>Escherichia coli</em></td>
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<td>Ea7/74</td>
<td>DH5α</td>
<td><em>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1</em></td>
<td>[35]</td>
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<td></td>
<td></td>
<td>pSKL3</td>
<td><em>gyrA96 thi-1 relA1</em></td>
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<td></td>
<td></td>
<td>pBBR3(lscA)</td>
<td><em>Ap</em>, contains lscA on 3.0-kb PstI insert, (Plac &gt; lscA)</td>
<td>[3]</td>
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<tr>
<td></td>
<td></td>
<td>pBBR1MCS</td>
<td>*Te', contains lscA on 3.0-kb PstI insert, (lscA &gt; Pm)</td>
<td>This study</td>
</tr>
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<td></td>
<td></td>
<td>pBBR1MCS-3</td>
<td><em>Te', broad-host-range cloning vector</em></td>
<td>[37]</td>
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<td></td>
<td></td>
<td>-666-lscB</td>
<td><em>Te', lscB gene with -666bp upstream sequence in pBBR1MCS-3</em></td>
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<td>This study</td>
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<td>-440-lscC</td>
<td><em>Te', lscC gene with -101bp upstream sequence in pBBR1MCS-3</em></td>
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<td></td>
<td>-332-lscC</td>
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<td></td>
<td>-666-lscB.0m1</td>
<td><em>Cm', lscB gene with -666 upstream sequence in pBBR1MCS, com gene containing a premature stop codon</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

*Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

Nested deletion analysis of the upstream region of *lscB* in plasmid pRB7.2 [3] was conducted using the Erase-a-Base® kit (Promega, Madison, USA). For analysis of the *lscC* upstream region, PCR was used to generate products covering the same region as in the deletion constructs of *lscB* (Table 3). PCR products of the *lscC* upstream region were cloned in vector pBBR1MCS-3 (Table 4). All constructs were introduced to *E. coli* DH5α via electroporation and then transferred by tri-parental mating [38] with helper plasmid pRK2013 [39] to the *lscB lscC* mutant PG4180.M6 [3]. Transconjugants were streaked on 5% sucrose-containing MG agar medium for assessment of levan production.
Table 4. Oligonucleotide primers used in this study.

<table>
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<th>Oligonucleotides</th>
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<td>lscB_PG-500_fwd</td>
<td>GATGAGCTCAGTCGCAAATATGCGAG</td>
</tr>
<tr>
<td>lscB_PG-440_fwd</td>
<td>GATGAGCTCCAGTCGCAAATGCGAG</td>
</tr>
<tr>
<td>lscB_PG-332_fwd</td>
<td>GATGAGCTCCAGTCGCAAATGCGAG</td>
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<td>lscB_PG-300_fwd</td>
<td>GATGAGCTCCAGTCGCAAATGCGAG</td>
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<td>lscB_PG-250_fwd</td>
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<td>lsc_C_rev</td>
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<tr>
<td>lscC_PG-666_fwd</td>
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<td>lscC_PG-332_fwd</td>
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<td>lscC_PG_rev</td>
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<td>comI</td>
<td>GCAAAATGTGAAAGACTACCAGTCCGAGTGC</td>
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<td>lscBC_RT_rev</td>
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<td>Cel_RT_fwd</td>
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</tr>
<tr>
<td>Cel_RT_rev</td>
<td>TCGCTTTATCGAGAGTGCTTTATTAC</td>
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</table>

a Restriction sites incorporated in primers are underlined; GAGCTC—SacI, TCTAGA—XbaI.

3.3. Extra-Cellular Lsc Detection

Extra-cellular fractions obtained from Ea7/74, Ea7/74-LS6, LS6 (lscA), PG4180, and lsc deletion constructs-harbor PG4180.M6 transformants and the use of polyclonal antibodies were carried out as described previously [4]. For immunological detection of Lsc enzyme, Western blot experiments were performed with total extra-cellular fractions using polyclonal antibodies raised against purified Lsc of *P. syringae* pv. phaseolicola as described earlier [3]. Water agar plates with 1.5% agar and 5% sucrose were used for the qualitative visualization of extra-cellular Lsc. Lsc activity was quantified by measuring the amount of glucose liberated during incubation with sucrose using the Gluco-quant Glucose/HK assay kit (Roche Diagnostics, Mannheim, Germany) at an absorbance of 340 nm. One unit of Lsc activity corresponded to the amount of enzyme which liberates 1 μmol glucose per minute from sucrose. The experiments were repeated three-fold and mean values were expressed as the quantity of glucose release.

3.4. Analysis of Glycosyl Hydrolase gene Expression by Quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

Bacterial cells were grown in HS + glutamate medium at 18 °C. When cultures reached distinct OD<sub>600</sub> values, total RNA was isolated by acid phenol/chloroform extraction as described previously [40]. The yield and the purity of RNA were determined by measuring absorption at 260 and 280 nm. Total mRNA samples were treated with TURBO DNA-free (Applied Biosystems,
Darmstadt, Germany) to remove remaining traces of genomic DNA as described by the manufacturer’s recommendation.

SYBR green-based qRT-PCR was performed with 1 ng RNA template and 200 nM primers (cel_RT_fwd and cel_RT_rev) (Table 4) using the QuantiTect SYBR Green one-step RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The thermocycler program comprised an initial step of 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. Reactions were performed in technical duplicates and biological triplicates with a Mastercycler® ep realplex2 real-time PCR system (Eppendorf, Hamburg, Germany) as described by the manufacturer using their universal program. Reactions with no addition of reverse transcriptase served as negative controls and proved lack of DNA contamination. Specificity of amplification was assessed by analyzing the melting curve of the amplification product. Due to very high sequence identity between lscB and lscC, it was not possible to design primers discriminating between these two mRNAs, thereby the expression profile of lsc is always referred to as a combination of both genes.

3.5. Bioinformatics Analyses

Vector NTI Advance 10.1.1 (Invitrogen Corporation, USA) was used for the nucleotide and amino acid sequence alignments and for dendrogram generation. BLAST-N and BLAST-P programs were used for online sequence analyses and for identifying transposase-like sequences and mobile promoter elements [41]. The website www.pseudomonas.com was consulted for the determination of P. syringae gene orthologs and paralogs.

4. Conclusions and Future Scope

Due to the high degree of conservation we can hypothesize that the conserved PAPE, identified herein, appears to be involved in the expression of variant BC lsc alleles and the glycosyl hydrolase gene in a coordinated manner. The sequence of the PAPE might harbor binding sites for regulatory proteins possibly controlling sugar utilization in P. syringae. This exciting hypothesis is fueled by the idea that P. syringae might need to produce both sucrose-utilizing Lsc and plant cell wall-degrading glycosyl hydrolase, in order to obtain glucose for central metabolism. Phage-mediated genetic rearrangements might have made possible such a coordinated control of the gene products of sugar metabolism. It is tempting to speculate that this type of regulation might be linked to that of central cellular sugar utilization. Thus, future experiments will focus on analyzing the potentially coordinated expression of variant BC lsc genes, the glycosyl hydrolase gene, and other genes required for central glucose metabolism. Likewise, identification of the enzymatic function of the gene product encoded by the glycosyl hydrolase gene, discovered herein, will be part of our future research focus. Occurrence of three isoforms of Lsc in P. syringae indicates their importance in this plant pathogenic bacterium. The precise additional roles of levan formation aside from nutrient acquisition, adherence to plant surfaces, or as protective functions remain to be determined.
Acknowledgments

The authors thank Georgi Muskhelishvili and Frank Oliver Glöckner for their valuable suggestions and Anna Behrendt for excellent technical support. This study was financed by the Deutsche Forschungsgemeinschaft.

References


Appendix

**Figure A1.** Neighbor-Joining phylogenetic tree generated by Mega 5.05 for known Lsc amino acid sequences of *P. syringae* strains, *Erwinia amylovora*, *Rahnella aquatilis* and *Zymomonas mobilis*. The blue arrows represent Lsc in PG4180 while the dashed arrow represents Lsc of Ea7/74.
Figure A2. Phylogenetic dendrograms of variant BC lsc genes and their up- and downstream sequences.

A

*iscA* upstream sequence

B

*iscB/C* upstream sequence

*iscB/C* coding sequence

*iscB/C* downstream sequence
**Figure A3.** Schematic depiction of predicted stem loop structures at the mRNA level for *lscA*, *lscB*, and *lscC* transcripts. The poly U runs suggest ρ-independent transcriptional termination. Blue nucleotide letters were found identical to those of the *lscA* downstream sequence. Red letters symbolize the position of the stop codon of *nasT* gene in case of *lscA*.

**Figure A4.** Phenotype of the *lscBC*-deficient mutant PG4180.M6 complemented with various *lscB* deletion constructs (ranging from -50 to -666-bp upstream of the translational start of *lscB* gene) and grown on MG agar supplemented with 5% sucrose. PG4180.M6 (pBBR1MCS) and PG4180.M6 served as negative controls. WT represents PG4180.WT serving as the positive control.
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3. RESULTS

3.2 Expression of levansucrases in *Pseudomonas syringae* pathovars
Expression of levansucrases in *Pseudomonas syringae* pathovars

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Abstract

*Pseudomonas syringae* pv. glycinea PG4180 produces the exopolysaccharide levan by enzyme levansucrase. Levansucrase has three copies in this organism, two of which, *lscB* and *lscC*, are expressed while the third, *lscA*, is not expressed. Nucleotide sequence alignments of *lscB/C* variants in *P. syringae* showed that a ~450-bp phage-associated promoter element (PAPE) including the first 48 nucleotides of the ORF are absent in *lscA*. Herein, we tested whether this upstream region is responsible for the differential expression of *lscB/C* and *lscA*. For this, we fused the PAPE including the first 48-bp of *lscB* with the ORF of *lscA* (*lscB*<sub>UpN</sub>*A*) and expressed this fusion in a levan-negative mutant of PG4180. Other fusion proteins comprising of the non-coding part of the upstream region of *lscB* with *lscA* (*lscB*<sub>Up</sub>*A*) and the upstream region of *lscA* with *lscB* (*lscA*<sub>Up</sub>*B*) were also generated. Transformants harboring the *lscB*<sub>UpN</sub>*A* or the *lscB*<sub>Up</sub>*A* fusion, respectively, showed levan formation while the transformant carrying *lscA*<sub>Up</sub>*B* did not. MALDI-TOF analysis was performed to demonstrate the synthesis of the corresponding fusion proteins. qRT-PCR and Western blot analyses showed that *lscB*<sub>UpN</sub>*A* had an expression similar to *lscB* while *lscB*<sub>Up</sub>*A* showed a lower expression suggesting that the upstream sequence of *lscB* is essential for expression of levansucrase and that the N-terminus of LscB mediates an enhanced expression. In contrast, the upstream region of *lscA* does not seem to contain a promoter region leading to an inactive gene. We propose that *lscA* might be an ancestral levansucrase variant in front of which the PAPE got inserted by transposition events leading to expression of *lscB/C*.
3. Results

Introduction

*Pseudomonas syringae* strains comprise a large and well-studied group of plant-pathogenic bacteria [1]. They infect a broad range of host plants and are subdivided into more than 50 different pathogenic variants called pathovars [2]. They possess a number of well-studied virulence and pathogenicity factors like the type III effector trafficking system, various phytotoxins, compounds and mechanisms suppressing the plant defense, or synthesis of exopolysaccharides [3]–[5]. Exopolysaccharides play a variety of roles in virulence and pathogenicity not only in *Pseudomonas* but also in other biolfilm-producing organisms [6][7]. The two major exopolysaccharides produced by *P. syringae* pv. glycinea are alginate and levan [7]. Levan is a β-(2,6) polyfructan with extensive branching through β-(1,4) linkages, while alginate is a copolymer of O-acetylated β-(1,4)-linked D-mannuronic acid and its C-5 epimer, L-guluronic acid [7]–[10].

*P. syringae* pv. glycinea PG4180 causes bacterial blight of soybean plants. Like some other *Pseudomonas* species, this organism utilizes sucrose as a carbon source with the help of the enzyme levansucrase (EC 2.4.1.10, Lsc) in a process leading to the production of glucose and the exopolysaccharide levan. PG4180 produces very little alginate due to a frameshift mutation in the *algT* gene and hence, the exopolysaccharide matrix of this strain is mainly composed levan (R. Mitchell, 1975, [11]). Apart from several draft genome sequences [12]–[18], the complete genome sequences of three *P. syringae* pathovars are available, namely pv. tomato DC3000 [19], pv. phaseolicola 1448A [20] and pv. syringae B728a [21]. All of these strains serve as excellent model organisms to study plant-microbe interactions. Like in some other *P. syringae* pathovars, the PG4180 genome contains three copies of the *lsc* gene, of which two – *lscA* and *lscC* are chromosomally encoded while *lscB* is plasmid-encoded. Of the three copies, only *lscB* and *lscC* have been shown to be expressed while no expression was observed for *lscA* under the tested growth conditions [10]. Even though LscB is predominantly extracellular and LscC is predominantly retained in the periplasm, the two enzymes are 98% identical at the amino acyl level. There are only 5 amino acid differences, four of which are conserved changes. The enzymes are highly similar in their structure as well as function and hence, the experiments in this study were done using *lscB* only.

As reported by Srivastava *et al.* [22], nucleotide sequence comparison of the *lscA* variants with those of *lscB/C* variants of *P. syringae* pathovars showed that the first
48-bp of the N-terminus of the ORF lscB/C were absent in lscA. In silico deletion of this N-terminal region increased the identity from 87.5% to 93% at the amino acyl residue sequence level between LscA and B/C variants. The comparison also showed that a ~450-bp upstream region, which is highly conserved in all lscB/C variants, is missing in lscA. This region spanning from -450-bp to +48-bp with respect to the translational start site of lscB/C was predicted to be a pro-phage borne DNA based on sequence similarities and hence was termed phage-associated promoter element (PAPE).

P. syringae is the only Lsc-synthesizing organism having multiple copies of the same enzyme. The reason for the occurrence of multiple lsc copies some of which carry upstream PAPEs remained obscure and prompted the current study, during which the PAPE with or without the N-terminal coding sequence was fused to lscA. Additionally, the upstream region of lscA was fused with the coding sequence of lscB while lscB and lscA with their native upstream sequences served as controls. All fusion constructs were expressed in the levan-negative mutant PG4180.M6, and tested for their levan formation ability by zymographic detection linked to MALDI-TOF analysis and Western blotting. Furthermore, the expression of fusions at the mRNA level was checked by qRT-PCR analysis. In addition, a PCR approach with cDNA was undertaken to show that the expression of lscA is cryptic in other P. syringae pathovars.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains, plasmids and oligonucleotide primers used in this study are listed in Tables 1 and 2. Escherichia coli DH5α was used as the cloning host [23] and grown in Lysogeny Broth (LB) medium at 37°C. P. syringae cultures were grown in HSC medium [24] at 18°C. Bacterial growth in liquid media was monitored by measuring the optical density at 600 nm (OD$_{600}$) and harvested for (i) protein sampling at an OD$_{600}$ of 2.0 or (ii) RNA extraction and cDNA synthesis at an OD$_{600}$ of 0.5 and 2.0. Antibiotics were added to the media at the following concentrations (µg/ml), respectively: ampicillin 50; tetracycline 25, and chloramphenicol 25.
**Molecular genetic techniques**

Plasmid isolation, restriction enzyme digests, agarose and polyacrylamide gel electrophoreses, electroporation, PCR, and other routine molecular methods were performed using standard protocols [23].

**Table 1: Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or Source</th>
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<td>pv. glycinea PG4180</td>
<td>wild type, levan+</td>
<td>R. Mitchell</td>
</tr>
<tr>
<td>pv. phaseolicola 1448A</td>
<td>wild type, levan+</td>
<td>[38]</td>
</tr>
<tr>
<td>pv. syringae B728a</td>
<td>wild type, levan+</td>
<td>[39]</td>
</tr>
<tr>
<td>pv. tomato DC3000</td>
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<td>D. Cuppels</td>
</tr>
<tr>
<td><strong>Pseudomonas syringae</strong> pv. glycinea</td>
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<tr>
<td>PG4180.M6</td>
<td>$Sp^r$, $Gm^r$, $lscB$ $lscC$ mutant of PG4180, levan-</td>
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<tr>
<td>PG4180.M6(pRA3.1)</td>
<td>$Sp^r$, $Gm^r$, $Tc^r$, $lscB$ $lscC$ mutant of PG4180, containing $lscA$ under control of $P_{lac}$ on 3.1-kb PstI fragment in pRK415,</td>
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</tr>
<tr>
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<td>[23]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBBR1MCS-3</td>
<td>$Te^c$, broad-host-range cloning vector</td>
<td>[40]</td>
</tr>
<tr>
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<tr>
<td>pBBR3(IscA)</td>
<td>$Te^c$, $IscA$ gene containing insert from pRA3.1 in pBBR1MCS-3 not under control of $P_{lac}$</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td>$Te^c$, fusion of 518-bp upstream region of $lscB$ (including first 48-bp of coding region) and $lscA$ (including start codon and downstream region) in pBBR1MCS-3</td>
<td>This Study</td>
</tr>
<tr>
<td>pBBR3(IscB&lt;sub&gt;UpN&lt;/sub&gt;A)</td>
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<tr>
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<tr>
<td>pBBR3(IscA&lt;sub&gt;UpB&lt;/sub&gt;)</td>
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<td></td>
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</table>

*Ap - Ampicillin, Tc – Tetracycline, Sp – Spectinomycin, Gm – Gentamycin*
### Table 2: Oligonucleotide primers used in this study

<table>
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<tr>
<th>Oligonucleotides</th>
<th>Nucleotide sequence (5' - 3')*</th>
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<td>lscB_UpN_r</td>
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<td>lscA_Up_r</td>
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</tr>
<tr>
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<tr>
<td>hexR_rv</td>
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</tbody>
</table>

*Restriction sites in the primers are underlined: AAGCTT - HindIII, GCTAGC - NheI, GGATCC - BamHI, TCTAGA - XbaI,

### Generation of fusion constructs

All genes or DNA fragments were obtained by PCR amplification unless otherwise stated. All restriction enzymes used were obtained from Fermentas (St. Leon Rot, Germany). The nucleotide sequencing was done by Eurofins MWG Operon (Ebersberg, Germany).

**Generation of lscB_UpN** and **lscB_UpA**: The sequences of the PAPE (518-bp) and the lscB upstream region without the 48-bp coding sequence (470-bp), respectively, were ligated to the N-terminus of lscA (1,748-bp) using T4 DNA Ligase (Fermentas) after treating the DNA with restriction enzyme NheI. The ligation products were then treated with HindIII, analysed by agarose gel electrophoresis, and the bands corresponding to the
fusion products (2,284 and 2,224-bp, respectively) were purified from the gel using GeneJET Gel Extraction kit (Fermentas). The purified fusion products were ligated into pBluescript-KS(II) using HindIII in such a way that the fusion products were under control of the vector-borne lac promoter (P_{lac}). Formation of levan on LB agar containing 5% sucrose indicated a functional lscA gene driven by the P_{lac}. The PAPE and lscB upstream regions were sequenced to exclude any possibility of mutations. The fusion products were then cloned into the broad host-range vector pBBR1MCS using HindII in order to ligate them in opposite orientation to the P_{lac} and then cloned into pBBR1MCS-3 using restriction enzymes PstI and XhoI to keep the same orientation with respect to P_{lac} as in case of pBBR1MCS. The constructs were introduced into mutant PG4180.M6 via electroporation.

Generation of lscA_{Up}B: A similar cloning strategy was used to generate the lscA_{Up}B construct. The C-terminus of the PCR-amplified lscA upstream region (550-bp) and the N-terminus of the PCR-amplified ORF lscB (1,704-bp) were ligated using a combination of restriction enzymes XbaI and NheI which have compatible ends. This ligation product was treated with endonucleases BamHI and HindIII and subsequently ligated into pBluescript-SK(-). The constructs were cloned into pBBR1MCS using restriction enzymes BamHI and HindII in order to ligate them in opposite orientation to the P_{lac} and then into pBBR1MCS-3 using restriction enzymes using XbaI and ApaI to keep the same orientation with respect to P_{lac} as in case of pBBR1MCS.

**Lsc detection**

Total proteins from PG4180.M6 and PG4180.M6 transformants harboring the lsc fusion constructs were obtained as described previously [25]. For immunological detection of the Lsc enzyme, total proteins were separated by 10% SDS-PAGE and Western blot experiments were performed with total extra-cellular fractions using polyclonal antibodies raised against purified Lsc of _P. syringae_ pv. phaseolicola as described [10]. Zymographic detection of Lsc was done as described previously by separating the total proteins by 10% native-PAGE and incubating the gels in 5% sucrose solution [10]. Bacterial cells grown on mannitol-glutamate agar plates with 1.5% agar and 5% sucrose were used for the qualitative visualization of Lsc activity, which led to levan formation in form of a mucoid, dome-shaped colony morphology.
**MALDI-TOF mass spectrometric analysis**

Total proteins were separated using 10% native-PAGE and incubated in 5% sucrose solution overnight [10]. As soon as in-gel levan formation became apparent, the corresponding bands were cut out from the gel and subjected to an overnight in-gel trypsin digestion adapted from Shevchenko et al. [26], Speicher et al. [27], and Granvogl et al. [28]. The used protease was modified porcine trypsin (Promega, Madison WI, USA), and nitrile gloves were worn at all times. Native gels were washed with double-distilled water for 2 h under slight agitation. For in-gel digestion, reaction cups (Nerbe plus, Winsen/Luhe, Germany) were rinsed by shaking for 12-16 h at 37°C with 150 µL acetonitrile (ACN):H₂O (1:1, v/v) containing 0.1% trifluoracetic acid. Protein bands were cut into cubes of 0.5-1 mm³ and transferred to rinsed reaction cups. 100 µL of 100 mM ammonium bicarbonate (NH₄HCO₃) buffer:ACN (1:1; v/v) were added, followed by an incubation of 30 min at room temperature (RT) under frequent mixing. 500 µL of ACN were added and cups were incubated at RT for another 10 min. The liquid was removed and the following steps were carried out on ice. Samples were covered with 20-30 µL of digestion buffer (13 ng/µL trypsin in 10 mM NH₄HCO₃ solution, containing 10% ACN (v/v)). Additional digestion buffer was added in case of complete absorption by the gel pieces. For complete trypsin saturation, samples were left on ice for additional 90 min and were afterwards covered with 5-20 µL of 100 mM NH₄HCO₃ buffer. Trypsin digestion was carried out for 12-16 h at 37°C. Supernatants (peptide samples) were directly used for MALDI-TOF MS exposure using an Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser and operated with FlexControl 3.0 software. The matrix used was a-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics). Peptide samples were acidified with 0.5% TFA in a ratio of 1:1 (v/v). HCCA stock solution was freshly prepared before measurement (1 mg/mL, dissolved in acetone). 1 volume of HCCA stock solution was mixed with 2 volumes of ethanol (EtOH). HCCA:EtOH solution was subsequently mixed with the acidified peptide sample in a ratio of 1:1 (v/v). Samples were spotted on a steel manufactured MTB AnchorChip™ target with an anchor diameter of 600 µm (Bruker Daltonics). 0.5 µL spots were loaded and air-dried. For desalting, dried spots were rinsed with 2 µL of 10 mM monobasic ammonium phosphate solution (NH₄H₂PO₄) for ~5 s. Rinsing was repeated once and the dried sample spots were exposed to MALDI-TOF MS. MS was operated in positive-ion reflection mode. The laser offset was set to
$lscA$ expression in *P. syringae*

67% with a range of 15%. The acquisition range was set to 800-4,000 Da. Peptide masses were assigned to monoisotopic peaks. Ions were extracted with a delay of 60 ns. Ion source I and II were set to a voltage of 19.00 and 16.90 kV. Single spectra were obtained from 50 laser shots, if necessary, spectra were accumulated. Calibration was performed using known trypsin auto-digestion peaks. For identification of mass artefacts resulting from sample preparation, a blank piece of polyacrylamide gel was processed. Bovine serum albumin fraction V (BSA; AppliChem, Darmstadt, Germany) was used as a control protein. Raw data were processed with FlexAnalysis, version 3.0. A signal-to-noise (S/N) ratio of 6 was applied for peak identification. Protein identification was carried out using the Mascot search engine [29], which was applied by the Biotools software, version 3.1. Mass lists were compared with the database of NCBI. Mascot score probability was set for $p < 0.05$. Oxidation of methionine (‘Oxidation (M)’) was selected as variable modification. No global modifications were selected.

**Tolerance for missed protease cleavages was 1. The mass error was set at 50 ppm. Peptide sequence analyses was done using ExPASy bioinformatics resource portal [30].**

**Analysis of $lsc$ gene expression by quantitative Reverse Transcriptase polymerase chain reaction (qRT-PCR)**

Total RNA was isolated by acid phenol/chloroform extraction as described previously [11]. The yield and the purity of RNA were determined by measuring absorption at 260 nm. Total mRNA samples were treated with TURBO DNA-free (Applied Biosystems, Darmstadt, Germany) to remove remaining traces of genomic DNA as described by the manufacturer’s recommendation. SYBR-green based qRT-PCR was performed with 5 ng RNA template and 100 µM primer with QuantiTect SYBR Green one-step RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The thermocycler program comprised an initial step of 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s. Reactions were performed with biological triplicates in a Mastercycler® ep realplex2 real-time PCR system (Eppendorf, Hamburg, Germany) as described by the manufacturer using their universal program. Reactions with no addition of reverse transcriptase served as negative control and proved the absence of DNA contamination. Specificity of amplification was assessed by analyzing the melting curve of the amplification product. Primers to amplify $lscB$ were used for constructs $lscB$ and $lscA^{UpB}$ while primers to amplify $lscA$ were used
for constructs \textit{lscA}, \textit{lscB}_{\text{UpN}}A and \textit{lscB}_{\text{Up}}A. All the results were normalized to amplification of the cDNA of \textit{gyrA} (PSPPH3667) as described previously [31].

**Analysis of \textit{lscA} gene expression by Reverse-Transcriptase polymerase chain reaction (RT-PCR)**

Template-specific primers were designed for the respective \textit{lscA} variants of \textit{P. syringae} pv. glycinea PG4180, pv. phaseolicola 1448A, pv. syringae B718a, and pv. tomato DC3000. Bacterial cells were grown in HSC medium and harvested at an OD$_{600}$ of 0.5 as well as 2.0. RNA was extracted by acid phenol/chloroform extraction method [11]. An RT-PCR was performed on total mRNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas) as recommended by the manufacturer. The strain-specific \textit{lscA} primers were used to check for presence of an \textit{lscA} mRNA by PCR using cDNA as template. Regular PCR with the same primer-pairs and genomic DNA as template were used as controls. The thermocycler program was as follows: 1 cycle of 95°C for 90 s; 25 cycles of 95°C for 15 s, 66°C for 15 s, 72°C for 30 s; 1 cycle of 72°C for 5 min. The results were analyzed by 1% agarose gel electrophoresis.

**Bioinformatics analyses**

Vector NTI Advance 10.1.1 (Invitrogen Corporation, California, USA) was used for the nucleotide, amino acid sequence alignments, as well as for generating genetic maps. BLAST-N and BLAST-P programs were used for online sequence analyses [32]. The website www.pseudomonas.com was consulted for the determination of \textit{P. syringae} gene orthologs and paralogs [33].

**Results**

**Qualitative analysis of \textit{lsc} fusion proteins**

The fusion constructs were introduced to the levan-negative mutant PG4180.M6 and were first analyzed for their levan forming ability on sucrose supplemented nutrient agar plates. Both, PG4180.M6 mutant complemented with \textit{lscB}_{\text{UpN}}A [M6(\textit{lscB}_{\text{UpN}}A)] and \textit{lscB}_{\text{Up}}A (M6-\textit{B}_{\text{Up}}A), showed levan formation indistinguishable from that of the PG4180.M6 mutant complemented with \textit{lscB} [M6(\textit{lscB})] (Figure 1). In contrast, PG4180.M6 complemented with \textit{lscA}_{\text{Up}}B [M6(\textit{lscA}_{\text{Up}}B)] was the same as levan negative
as PG4180.M6 transformed with lscA [M6(lscA)] thus suggesting that the upstream region of lscB mediates expression of downstream located genes while that of lscA does not.

**Figure 1**: Illustration of the different fusion constructs (A) Schematic representation of the DNA fusion products. The dashed line represents lscB while the solid line represents lscA. (B) Levan formation ability of the proteins encoded by the fusion constructs. The cells were grown on medium containing 5% sucrose at 18°C to check for levan formation (indicated by the dome-shaped glossy slime) around the colony. LscB, LscB_{Up}N and LscB_{Up}A showed levan formation.

**Characterization of lsc fusion proteins**

To verify the molecular sizes of Lsc encoded by the individual fusion constructs, a Western blot analysis with Lsc-specific antibodies was performed (**Figure 2A**). Under denaturing conditions, it was interesting to observe that LscB_{Up}N migrated at an intermediate rate i.e faster than LscB but slower than LscB_{Up}A. The signal for LscB_{Up}A was weaker than those representing LscB or LscB_{Up}N suggesting that the N-terminus of LscB might contribute to the expression level or stability of Lsc. In contrast, protein samples of PG4180.M6 transformed with LscA or LscA_{Up}B did not show any signal specific for Lsc at all thus confirming that lack of levan formation was due to lack of the corresponding protein.
**Figure 2:** Detection of levansucrase. (A) Western blot analysis: 10 µg of total proteins were separated by 10% SDS-PAGE, transferred onto PVDF membrane, detected with anti-Lsc antiserum and developed using BCIP/NBT. The dark bands (arrow) correspond to Lsc and the corresponding fusion proteins. (B) Zymogram: 100 µg of total proteins were separated by 10% native-PAGE and incubated in 5% sucrose solution overnight. The white bands indicate formation of levan after utilization of sucrose by Lsc and the fusion proteins.

To check for the enzymatic function of Lscs encoded by the individual fusion constructs, zymographic detection was done with non-denatured total protein samples of transformed mutants (Figure 2B). The above reported levan forming ability of colonies of M6(lscB), M6(lscB<sub>UpA</sub>N) and M6(lscB<sub>UpA</sub>) could be attributed to enzymatically functioning proteins or fusion proteins. As expected, native protein samples derived from M6(lscA) or M6(lscA<sub>UpB</sub>) did not exhibit any in-gel levan production (Figure 2B). An interesting observation was the altered electrophoretic mobility of the enzymatically active proteins. The LscB<sub>UpNA</sub> migrated slower as compared to LscB even though the predicted molecular masses of both proteins were almost identical (~47.6 kDa) suggesting possible differences in the respective protein charges. In accordance with the Western blot results, LscB<sub>UpA</sub> seemed to be less expressed than LscB or LscB<sub>UpNA</sub> suggesting an important role of the N-terminus for transcriptional or translational processes.
MALDI-TOF analysis

The altered electrophoretic migration rate of LscB_{UpNA} as compared to LscB during the native gel protein separation suggested that the two proteins were indeed different although their predicted protein sizes were almost identical. To demonstrate that LscB_{UpNA} produced a unique and novel enzyme and to show that the other two transformants indeed also produced the intended Lsc proteins, we subjected the levan-forming fusion proteins to matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. The peptides recovered in the MALDI-TOF analysis are shown in Figure 3. The recovered peptides (bold) gave rise to an overall good coverage in the protein sequences (Table 3). Some of the peptides recovered were unique to each protein (underline). For e.g., peptides SFVQEVYDYGYIPAM from LscB_{UpNA} and SFVQEEYDYGYIPAM from LscB were located at the same position in the respective amino acyl sequences of these proteins but had different masses, 1,782 Da as compared to 1,812 Da, indicating they were from different proteins. Similar differences were observed for the other peptide sequences shown in the Figure 3 indicating that the fusion constructs indeed led to the synthesis of novel fusion proteins or of the proteins intended despite the presence of different upstream regions.

Table 3: Proteins identified by MALDI-TOF analysis

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<th>NCBI accession number/gi</th>
<th>Protein description</th>
<th>Predicted molecular weight (Da)</th>
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<th>MASCOT score</th>
<th>Peptides matched</th>
<th>Sequence coverage (%)</th>
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<td>110</td>
<td>8</td>
<td>19</td>
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</table>
LscA expression in *P. syringae*

3. Results

Figure 3: Amino acid sequence alignment of LscB_{UpA}, LscB and LscB_{UpA}. Fragments in bold indicate peptides recovered from MALDI-TOF analysis. The underlined fragments indicate recovered peptides which are unique to that protein.

**Quantitative expression of lscA fusion proteins by qRT-PCR**

The difference in the amount of levan produced by LscB_{UpA} as compared to LscB_{UpNA} and LscB in the zymogram prompted us to check if this correlated at the level of RNA level. Samples were grown in HSC medium at 18°C and harvested at OD_{600} of 0.5 since *lsc* transcription is maximum at this optical density [25]. The total RNA was extracted from the cells and the expression of *lscB* and *lscA_{UpB}* was checked by *lscB*-specific primers while that of *lscA*, *lscB_{UpNA}* and *lscB_{UpA}* was checked by *lscA*-specific primers. The results showed that, considering the standard deviation obtained for the samples, the *lscB_{UpNA}* had expression levels similar to *lscB* (Figure 4) further supporting the results of the Western blot and zymogram. As compared to *lscB*, *lscB_{UpA}* had 0.6-fold lower expression level. As was the trend seen in the Western blot and zymogram, *lscA* and *lscA_{UpB}* had no expression. This indicated that even though the upstream region of *lscB* is sufficient to promote the expression of *lsc*, the expression level is enhanced by the presence 48-bp N-terminus of *lscB*.

at 30°C and harvested at OD_{600} of 0.5 since *lsc* transcription is maximum at this optical density [25]. The total RNA was extracted from the cells and the expression of *lscB* and *lscA_{UpB}* was checked by *lscB*-specific primers while that of *lscA*, *lscB_{UpNA}* and *lscB_{UpA}* was checked by *lscA*-specific primers. The results showed that, considering the standard deviation obtained for the samples, the *lscB_{UpNA}* had expression levels similar to *lscB* (Figure 4) further supporting the results of the Western blot and zymogram. As compared to *lscB*, *lscB_{UpA}* had 0.6-fold lower expression level. As was the trend seen in the Western blot and zymogram, *lscA* and *lscA_{UpB}* had no expression. This indicated that even though the upstream region of *lscB* is sufficient to promote the expression of *lsc*, the expression level is enhanced by the presence 48-bp N-terminus of *lscB*.
Native expression of *lscA* in *P. syringae* pathovars

Lack of expression of *lscA* had been shown before in *P. syringae* pv. glycinea PG4180 [10]. However, this has not been experimentally proven for other *P. syringae* pathovars. Consequently, possible expression patterns of *lscA* variants were also analyzed in the three *P. syringae* pathovars pv. phaseolicola 1448A, pv. syringae B728a and pv. tomato DC3000 using cDNA synthesis and PCR. No amplicon was detected in any of the four strains as shown in Figure 5 indicating that none of the *lscA* variants is expressed. The specificity of the primers was demonstrated by amplifying the *lscA* genes from corresponding genomic DNA all of which gave amplicons of the expected sizes. The accuracy of reverse transcription was checked by amplifying a cDNA derived from the PG4180 *lscA*-mRNA of a PG4180.M6 transformant carrying a recombinant *lscA* gene under the control of P*lac*. The *lscA* is known to be expressed under these conditions [10]. Successful cDNA synthesis of total mRNA was demonstrated by PCR amplifying the cDNA derived from the mRNA of the *hexR* gene, a hexose metabolism regulator [34]. Gene *hexR* gave an amplicon of expected size (Figure 5) indicating correct cDNA synthesis. 

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**Figure 4**: Quantitative expression of different *Lsc* genes in *P. syringae* in dependence of *lscB*. *lscB*UpN shows similar levels of expression as *lscB* while *lscB*UpA, which does not contain the first 48-bp of *lscB* ORF, has lower expression. *lscA* and *lscA*UpN were not seen to be expressed. *lscA*, *lscB*UpN and *lscB*UpA were detected using *lscA* primers (1) while the rest using *lscB* primers (2). The data represent the mean relative expression of 3 replicates ± standard deviations. Data were normalized to *gyrA* gene expression while the expression value of *lscB* was set to 100% for comparison.
Figure 5: Expression of *lscA* in different *P. syringae* strains. The bacterial cells were harvested at OD$_{600}$ of 0.5 and 2.0. Total RNA was extracted as described in the Materials and Methods followed by generation of cDNA. PCR amplification of *lscA* fragment on the total cDNA using strain-specific primers showed no amplicon (*lscA* panel) indicating no expression of *lscA*. Quality of the primers were checked by performing PCR amplification using genomic DNA (gDNA) as template while amplification using an unrelated gene *hexR* (*hexR*) signified correct reverse transcription. Artificial expression of *lscA* (by *lac* promoter) is possible in *P. syringae* as seen in M6(pRA3.1).

**Discussion**

Genomic co-existence of three highly conserved genes coding for levansucrase is a feature unique to the plant pathogen *P. syringae* despite the fact that numerous other bacterial species harbor just a single copy of this gene in their genomes. Artificial expression of *lscA* from *P. syringae* under the control of the *P$_{lac}$* had been shown previously [10]. The same study also showed that *lscA* could not be expressed under its own promoter. Major differences between *lscA* and the natively expressed genes *lscB* and *lscC* are not found in the coding sequences but in their upstream DNA regions. The upstream regions of *lscB* and *lscC* represent a possible PAPE [22]. We previously hypothesized that this PAPE might harbor regulatory sites required for expression of...
levansucrase and general sugar metabolism in \textit{P. syringae}. Herein, the PAPE of \textit{lscB} was fused to the coding sequence of \textit{lscA} and thus proven for its transcriptional activity in \textit{P. syringae}.

The nucleotide sequence of the predicted PAPE consists of two parts, the upstream region of \textit{lscB} and the first 48-bp coding for the N-terminus of LscB. The importance of these 48-bp of the ORF for the expression was tested by generating fusion constructs of the upstream region and \textit{lscA} with or without these coding nucleotides. Transformants carrying either of the two fusion constructs produced levan similar to the PG4180.M6 mutant complemented with \textit{lscB} equipped with its native upstream region. Western blotting, zymographic detection, and qRT-PCR analyses confirmed these results but also allowed a more detailed view; native \textit{lscB} and the \textit{lscB}_{UpN}A fusion had similar mRNA expression levels while that of the fusion \textit{lscB}_{UpA}, which lacked the 48-bp of N-terminal LscB-coding region, had less. Consequently, one might speculate that although the upstream DNA region of \textit{lscB} is sufficient for expression of \textit{lscA}, the first 48-bp of the \textit{lscB} ORF increase the level of its expression. Since our respective results of Western blotting and zymographic detection of Lsc activity were indistinguishable from each other, it could be concluded that the N-terminus of LscB is not involved in altering of enzymatic activities.

A peculiar observation was the electrophoretic migration of the individual proteins or protein fusions in polyacrylamide gels. The observed faster migration of LscB_{UpN}A as compared to LscB under denaturing conditions could potentially be attributed to the apparent mass shift for two proteins with nearly identical molecular masses as described earlier [35]. Interestingly, the migration of LscB_{UpN}A was significantly slower than that of LscB under native conditions despite the fact that both have similar predicted molecular masses. This finding might demonstrate that modest changes in the protein’s surface charge might result in significant alterations of electrophoretic mobility [36], [37].

Although the different migration rates of the proteins or fusion proteins under native or denaturing conditions suggested that the synthesized proteins were indeed different from each other, a MALDI-TOF analysis of each of the proteins was conducted using protein samples from zymograms. The produced levan surrounding the proteins did not seem to impact mass spectrometric analysis. The MASCOT score for each of the identified proteins was above the significance threshold of 100. The sample from the
PG4180.M6(lscB) sample gave LscB from *P. syringae* pv. phaseolicola 1448A as the first significant match which was in line with the high homology of the respective genes in the close relatives pv. glycinea and pv. phaseolicola [22]. The sample from PG4180.M6(lscB<sub>UpA</sub>) which should synthesize only LscA gave the first significant match as LscA from *P. syringae* pv. glycinea race 4 strain. This proved that the lscB<sub>UpA</sub> fusion actually synthesized an active LscA and confirmed earlier findings that artificial expression of lscA of PG4180 leads to levan formation [Li and Ullrich, 2001]. Although the majority of obtained peptides for the sample representing LscB<sub>UpN</sub>A were LscA-borne as expected, the unique 2,122-Da peptide NSPLASMSNINYAPTIWSR could be detected. This peptide is a consequence of the presence of the NheI restriction site coding for the amino acyl residues alanine and serine. Oxidation of methionine, which was chosen as a variable modification parameter, added another 16 Da to the peptide mass which subsequently increased the mass of the NSPLASMSNINYAPTIWSR fragment to 2,138 Da. This mass was exactly the same as the mass of a recovered peptide which did not find a match during the NCBI search since the respective fusion peptide is not present in the database. Thus, the synthesis of the LscB<sub>UpNA</sub> fusion protein could also be proven.

The majority of previous LscA-related studies have been performed with *P. syringae* pv. glycinea PG4180 [9], [10], [22], [25]. However, thus far, there was no evidence for a lack of lscA expression in other pathovars of *P. syringae*. Since the genomes of *P. syringae* pv. phaseolicola 1448A, pv. syringae B718a and pv. tomato DC3000 are fully sequenced [33][31][30][29], template-specific oligonucleotide primers for cDNA-based mRNA detection could be designed. Although mRNA samples were extracted during different growth stages, namely, early-logarithmic and late-logarithmic phase, no amplicons could be detected in any of the strains suggesting that lscA variants were not expressed. PCR amplification using respective genomic DNA as template proved that the primers were binding correctly. An independent gene, hexR, coding for a conserved hexose metabolism regulator protein HexR, was chosen to see if the total mRNA had been reverse transcribed correctly [30]. This PCR amplification gave correct sized amplicon of 880-bp for all the four strains demonstrating the accuracy of the used method. PCR amplification was also performed on the cDNA obtained from mRNA samples of PG4180.M6 containing lscA under the control of lac promoter. This experiment gave the same-sized amplicon as for genomic DNA again proving the
accuracy of the method. With careful controls, herein we showed for the first time that \textit{lscA} is not expressed in other \textit{P. syringae} pathovars.

In summary, we propose that \textit{lscA} could be an ancestral Lsc variant in \textit{P. syringae} as suggested by Srivastava et al. [22]. During evolution, the inactive promoter did not allow expression of \textit{lscA} after this gene had potentially been introduced to an ancestral \textit{P. syringae}. An evolutionary gene duplication of \textit{lscA} followed by an insertion of a pro-phage-borne PAPE might have led to a new \textit{lsc} variant, i.e. \textit{lscB} which in turn got duplicated again yielding in \textit{lscC} or vice-versa. As a result of this evolutionary process, two functional and expressed \textit{lsc} genes emerged in the plant pathogen, for which utilization of sucrose, and perhaps levan formation, might be particularly important. The advantage of an additional \textit{in planta} fitness-improving, and possibly virulence-promoting, factor could have this organism to selectively establish itself as a potent plant pathogen. As a consequence of this hypothesis, one could speculate on a loss of the non-expressed \textit{lscA} during further evolutionary steps.

\textbf{Acknowledgements}

We thank Helge Weingart for his helpful comments. This study was supported by the Deutsche Forschungsgemeinschaft (UL-169/5-1).

\textbf{Reference}


3. Results


[18] S. Marcelletti, P. Ferrante, M. Petriccione, G. Firrao, and M. Scortichini, “*Pseudomonas syringae* pv. actinidiae draft genomes comparison reveal strain-
specific features involved in adaptation and virulence to Actinidia species,” *PLoS ONE*, vol. 6, no. 11, p. e27297, 2011.


expression in P. syringae

3. Results


3.3 Expression of extra-cellular levansucrase in *Pseudomonas syringae* is controlled by the *in planta* fitness-promoting metabolic repressor HexR
Expression of extra-cellular levansucrase in *Pseudomonas syringae* is controlled by the *in planta* fitness-promoting metabolic repressor HexR

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**Abstract**

*Pseudomonas syringae* pv. glycinea PG4180 causes bacterial blight on soybean plants and enters the leaf tissue through stomata or open wounds, where it encounters a sucrose-rich milieu. The sucrose is utilized by invading bacteria via the secreted enzyme, levansucrase (Lsc), liberating glucose and forming the polyfructan levan. *P. syringae* PG4180 possesses two functional *lsc* alleles transcribed at virulence-promoting low temperatures. We hypothesized that transcription of *lsc* is controlled by the hexose metabolism repressor, HexR, since potential HexR binding site was identified upstream of both *lsc* genes. A *hexR* mutant of PG4180 was significantly growth-impaired when incubated with sucrose or glucose as sole carbon source, but exhibited wild type-like growth when arabinose was provided. Analyses of *lsc* expression resulted in higher transcript and protein levels in the *hexR* mutant as compared to the wild type. The *hexR* mutant’s ability to multiply *in planta* was significantly reduced. HexR did not impact *hrp* gene expression as evidenced by the *hexR* mutant’s unaltered hypersensitive response in tobacco and its unmodified protein secretion pattern as compared to the wild type under *hrp*-inducing conditions. Our data suggested a co-regulation of genes involved in extra-cellular sugar acquisition with those involved in intra-cellular energy-providing metabolic pathways in *P. syringae.*
HexR represses lsc expression in *P. syringae*

### Introduction

Fructan or glucan polymers are formed wherever microbes encounter sucrose-rich conditions, would it be in association with plants, in the oral cavity, in food manufacturing, or during bio-fuel production processes (Srivastava et al., 2009). When plant-borne sucrose is present, the soybean-infecting bacterial blight pathogen, *Pseudomonas syringae* pv. glycinea, uses levansucrase (Lsc) to synthesize the extra-cellular high-molecular fructofuranan, levan, thereby releasing glucose for primary metabolism. Three levansucrase-encoding genes, *lscA*, *lscB*, and *lscC*, are identified in *P. syringae* pv. glycinea PG4180, from which only *lscB* and *lscC* are expressed. The mutant lacking in *lscA* is levan positive while mutant lacking *lscB* and *lscC* but possessing *lscA* is levan-deficient (Li and Ullrich, 2001). Both enzymes were synthesized maximally at 18°C *in vitro* and *in planta* and their expression was optimal in the early logarithmic growth stage (Li et al., 2006; Schenk et al., 2008).

Bacterial communities growing epiphytically on plants are primarily affected by carbon availability as supported by the finding that very low sugar concentrations were sufficient to support the growth of $10^7$ to $10^8$ cells per leaf (Lindow and Brandl, 2003). Stomatal openings and wounds provide the site of entry for *P. syringae*. Under favorable micro-environmental conditions the bacterial cells live endophytically and subsequently initiate the infection process via production of the phytotoxin coronatine (Budde and Ullrich, 2000; Melotto *et al.*, 2008) and attachment to plant cell surfaces. The infection process is fostered by low environmental temperatures such as 18-20°C as opposed to the optimal growth temperature of *P. syringae*, 28°C (Dunleavy, 1988; Smirnova *et al.*, 2001). A complex sequence of events mediated by injection of bacterial Hrp effector proteins into plant cells (Mansfield, 2009) ultimately activates plant-borne K⁺ efflux and H⁺ influx, which increases the apoplastic pH from 5.5 to 7.5 (Atkinson and Baker, 1987). Subsequently, this high extra-cellular pH induces efflux of the dominant photoassimilate, sucrose, from plant cells (Atkinson and Baker, 1987). Apoplastic sucrose ranging in concentrations from 20 µM to 1-5 mM is hydrolyzed by either plant-borne invertases or by extra-cellular microbial enzymes e.g. Lsc (Roitsch and González, 2004; Biemelt and Sonnewald, 2006).

For glucose metabolism, metabolic pathway structures vary among bacterial species with different ecological niches (Papp *et al.*, 2009). In contrast to enterobacteria
HexR represses lsc expression in *P. syringae*

(Lessie and Phibbs, 1984), pseudomonads utilize glucose via the Entner-Doudoroff (ED) pathway due to lack of 6-phosphofructokinase and hence, do not catabolize sugars via the Embden-Meyerhof-Parnas pathway (Entner and Doudoroff, 1952; Portais and Delort, 2002). The ED pathway can be linear, alternative, modified with non-phosphorylated intermediates, or cyclic (Conway, 1992) and was first described in *Pseudomonas saccharophila*, which now belongs to the order *Burkholderiales* (Entner and Doudoroff, 1952; Xie and Yokota, 2005).

In *Pseudomonas putida* KT2440, key sugar metabolism genes such as *edd, eda, glk* and *zwf-1*, involved in the ED pathway and the pentose phosphate pathway, respectively, are regulated by the hexose metabolism repressor HexR (del Castillo et al., 2008; Kim et al., 2008; Daddaoua et al., 2009). In the current study, it was hypothesized that, in *P. syringae*, not only genes involved in cellular glucose metabolism but also genes encoding extra-cellular Lsc are controlled by HexR. In turn this might have consequences for our understanding about what determines bacterial *in planta* fitness and potentially virulence.

In order to address this hypothesis, a *hexR* mutant was generated in PG4180 and tested for its growth in minimal medium containing glucose, sucrose, or arabinose as sole carbon source. Analyses of *lsc* expression by quantitative Reverse Transcriptase (qRT) PCR and Western blotting were conducted for the PG4180 wild type and its *hexR* mutant. The mutant was compared to the wild type in terms of its *in planta* fitness. Furthermore, hypersensitive response (HR) reactions and profiles of secreted proteins were compared for the wild type and the *hexR* mutant when grown under hrp-inducing conditions.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was maintained at 37°C on Lysogeny Broth medium (Sambrook *et al*., 1989). *P. syringae* was routinely maintained at 28°C on mannitol-glutamate (MG) agar medium (Keane *et al*., 1970). For liquid cultures at 18°C, bacteria were grown in 200 ml of Hoitink-Sinden (HS) medium (Palmer and Bender, 1993) supplemented with various but singular carbon sources in 1-liter Erlenmeyer flasks. 113 mM of glucose (HS + glucose)
was replaced by 57 mM of sucrose in HS + sucrose medium while HS + arabinose medium had the following constituents: 0.8 mM MgSO$_4$, 30 mM KH$_2$PO$_4$, 16 mM K$_2$HPO$_4$, 16 mM KNO$_3$, 20 µM FeCl$_3$, 133 mM L-arabinose. Bacterial growth was continuously monitored by measuring the optical density at 600 nm (OD$_{600}$). Antibiotics were added to media at the following concentrations (µg/ml): ampicillin, 50; spectinomycin, 25; kanamycin, 25; tetracycline, 25; gentamicin, 2.

### Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics$^a$</th>
<th>Reference / source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Sambrook et al., 1989</td>
</tr>
<tr>
<td><strong>Pseudomonas syringae</strong> pv. glycinea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG4180</td>
<td>wild type, levan+</td>
<td>Bender et al., 1993</td>
</tr>
<tr>
<td><em>hexR</em></td>
<td>Km', <em>hexR</em> mutant of PG4180, levan++</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRK2013</td>
<td>Km', helper plasmid</td>
<td>Figurski et al., 1979</td>
</tr>
<tr>
<td>pBBR1MCS</td>
<td>Cm', broad-host-range cloning vector</td>
<td>Kovach et al., 1994</td>
</tr>
<tr>
<td>pBBR1MCS-3</td>
<td>Tc', broad-host-range cloning vector</td>
<td>Kovach et al., 1995</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Ap', vector for cloning of PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pFKm</td>
<td>Source of Km cassette flanked with <em>FRT</em> sequences</td>
<td>Choi et al., 2008</td>
</tr>
<tr>
<td>pGEM.hexR1</td>
<td>Ap', contains 456-bp upstream region of <em>hexR</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGEM.hexR2</td>
<td>Ap', contains 360-bp downstream region of <em>hexR</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGEM.hexR1-Km</td>
<td>Ap', Km, 1230-bp KpnI fragment containing Km-<em>FRT</em> cassette of pFKm1 cloned into pGEM.hexR1</td>
<td>This study</td>
</tr>
</tbody>
</table>
HexR represses lsc expression in P. syringae

Table 2. Oligonucleotide primers used in this study.

<table>
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<tr>
<th>Oligonucleotides</th>
<th>Nucleotide sequence (5’ to 3’)</th>
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<tr>
<td>HR_1f</td>
<td>GGATCC GTTCAACTCATCGAGTC</td>
</tr>
<tr>
<td>HR_1r</td>
<td>CAGATGCGACTGTTCGTC</td>
</tr>
<tr>
<td>HR_2f</td>
<td>GACCCCCGGATCAGTGCCAG</td>
</tr>
<tr>
<td>HR_2r</td>
<td>GGATCC GGTAAC CAGCCCGCTATCCGATCGAG</td>
</tr>
<tr>
<td>lscBC_RT_fwd</td>
<td>TCGGTTATCCTGACCTGAC</td>
</tr>
<tr>
<td>lscBC_RT_rev</td>
<td>CCATGACGATCTTCCCAGTC</td>
</tr>
<tr>
<td>lscB_hex_fwd</td>
<td>CGCAATTATGCGAGCCGCAGG</td>
</tr>
<tr>
<td>lscB_hex_rev</td>
<td>TTGCATTGGTCTCCTTGTGCTTC</td>
</tr>
<tr>
<td>hex_up_fwd</td>
<td>CGAGCAAGTCGCAAC</td>
</tr>
<tr>
<td>hex_down_rev</td>
<td>GAAGTCGACATGCAAGT</td>
</tr>
</tbody>
</table>

* Restriction sites incorporated in primers are underlined.

Generation of hexR mutant in PG4180

A P. syringae PG4180 hexR mutant was generated using the broad-host-range Flp-FRT recombination system (Hoang et al., 1998). Two fragments flanking the hexR gene were amplified from PG4180 genomic DNA using two pairs of primers: HexR_1f/HexR_1r and Hex_2f/HexR_2r (Table 2). PCR products were cloned into pGEM-T Easy (Promega) yielding plasmids pGEM.HexR1 and pGEM.HexR2 (Table 1). A 1,230-bp KpnI fragment containing a KmR cassette flanked with FRT sequences was removed from plasmid pFKm (Choi et al., 2008) and ligated into KpnI-digested pGEM.HexR1, yielding pGEM.HexR1-Km. A 360-bp SpeI-BamHI fragment digested from pGEM.HexR2 was ligated into SpeI-BamHI-digested pGEM.HexR1-Km, yielding plasmid pGEM.HexR-Km. Finally, a 2,046-bp EcoRI fragment was removed from...
pGEM.HexR-Km and ligated into EcoRI-digested plasmid pEX18Ap (Hoang et al., 1998), yielding the hexR gene replacement plasmid pEX.HexR-Km. This plasmid was mobilized into P. syringae PG4180 by tri-parental mating. Putative mutants were screened on MG agar plates containing kanamycin and were subsequently confirmed for the genotype by PCR using primers hex_up_fwd and hex_down_rev (Table 2).

**Analysis of lsc expression by quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)**

Bacterial cells were grown in HS + arabinose medium at 18°C. When cultures reached the respective OD_{600} values, total RNA was isolated by acid phenol/chloroform extraction, and samples were normalized by multiplexed fluorescent Northern hybridization and 23S rRNA transcript amount comparison as described previously (Schenk et al., 2008). Yield and purity of RNA were determined by measuring absorption at 260 and 280 nm. Total RNA samples were treated with TURBO DNA-free (Applied Biosystems, Darmstadt, Germany) to remove remaining traces of genomic DNA as described by the manufacturer’s recommendation.

SYBR green-based qRT-PCR was performed with 5 ng RNA template and 200 nM primers (lscBC_RT_fwd, lscBC_RT_rev) using the QuantiTect SYBR Green one-step RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The thermocycler program comprised an initial step of 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s. Reactions were performed in technical duplicates and biological triplicates with a Mastercycler® realplex2 real-time PCR system (Eppendorf, Hamburg, Germany) as described by the manufacturer using their universal program. Reactions with no addition of reverse transcriptase served as negative controls and proved lack of DNA contamination. Specificity of amplification was assessed by analyzing the melting curve of the amplification product. Due to very high sequence identity between lscB and lscC it was not possible to design primers discriminating between these two mRNAs. Thereby, expression profile of lsc is always referred as a combination of both genes.

**Immunological detection of Lsc**

Generation and concentration of cell-free supernatants of P. syringae cells and the use of polyclonal antibodies were carried out as described previously (Li et al., 2006).
Cultures were grown in HS + arabinose medium. Equal aliquots of protein fractions were loaded (10 μg/lane) and separated by 10% SDS-PAGE. Electrophoresis, electro-blotting on polyvinylidene fluoride (PVDF) membranes, and immunodetection were conducted by standard procedures (Sambrook et al., 1989).

**Growth of PG4180 wild type and hexR mutant in planta**

*In planta* growth of PG4180 and its hexR mutant was evaluated on soybeans (*Glycine max* (L.) Merr.) cultivar Maple Arrow. Soybean seedlings were germinated and grown in the greenhouse for three to four weeks prior to the growth assays. For spray inoculation, PG4180 wild type and hexR mutant were incubated for 48 h at 28°C on MG agar. Cells were suspended in distilled water, adjusted to an OD$_{600}$ of 0.1 (corresponding to approximately $10^7$ CFU/ml) and applied to the leaves with an airbrush (~8 psi) until the leaf surfaces were uniformly wet. Subsequently, inoculated plants were grown in the greenhouse (19-21°C), and survival and growth of bacterial strains was monitored by removing random leaf samples at 1-14 days post inoculation. Leaves were weighed separately and macerated in 20 ml of isotonic NaCl solution using sterile mortar and pestle. Bacterial counts (CFU/g fresh weight) were determined by plating dilutions of leaf homogenate onto MG agar and counting of fluorescent colonies after incubation for 96 h.

**Hypersensitive Response assay on tobacco**

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR1) were grown in a light chamber at 20-25°C, 60% humidity, with a 12-h photoperiod (15,000 lux). PG4180 wild type and hexR mutant were incubated for 48 h at 28°C on MG agar. Cells were suspended in sterile 0.9 % NaCl, adjusted to an OD$_{600}$ of 0.1 (corresponding to approximately $10^7$ CFU/ml). Tobacco plants were inoculated with bacterial suspensions by syringe injection of leaf veins of the third and fourth leaf. Sterile 0.9 % NaCl was used as negative control. Infiltrated areas were monitored for development of the hypersensitive reaction in form of necrosis after 24 and 48 h.

**Extra-cellular protein pool preparation and SDS-PAGE.**

For sample preparation of secreted proteins, bacteria were grown in two consecutive overnight pre-cultures in King’s B broth (King *et al*., 1954) at 28°C. From the first pre-
HexR represses lsc expression in P. syringae

Results

In silico analysis of HexR binding site upstream of lsc genes

Analysis of the nucleotide sequences upstream of lscB and lscC revealed the presence of a sequence (nucleotides +113 to +127 with respect to the corresponding transcriptional start sites) with high similarity to the conserved motif TTGTN_{7-8}ACAA previously shown to be the DNA binding site of HexR in P. putida (Petruschka et al., 2002; del Castillo et al., 2008; Daddaoua et al., 2009) (Figure 1). This finding suggested a putative binding of HexR of P. syringae to the upstream sequence of both lsc genes.

In vitro and in planta growth of P. syringae PG4180 and its hexR mutant

PG4180 wild type and its hexR mutant were comparatively grown in minimal media containing different carbon sources at 18°C (Figure 2). In contrast to the wild type, the hexR mutant did not show significant growth in HS medium containing 20 g/mL (113 mM) of glucose. Replacement of glucose by 10 g/mL (57 mM) of sucrose, did not alter the weak growth of the hexR mutant while growth of the wild type was unaffected. When glucose was replaced by an equal amount of 20 g/mL (133 mM)
arabinose as the sole carbon source, growth of the hexR mutant was not distinguishable from that of the wild type indicating that HexR might not be involved in regulation of the

lscB:

-55 CAAACTGACCTACCCGAAAACCTGCCCCCTTCCAGCCTTTGCCGAGGAGGT  
  +1 AAAGGCAACAGATATCGCATTGCGCCTCAGGSGCAGCCGCCAGATAGG  
  GCTTTAAACAGCAGCCCAAGGTAAATTGGCGATCOCGCCCAACCCGCACCACG  
  CGGCGTTTTTATAAGCTAGGAAGCACAAGGGAGAAATACAAATGTTGAAAGA  
  CTACGATGGCGCGAGTGCAAGAAGCTGGCTGCACGGGAGATGGTGACTACC  
  GAACCTCAAATCAAGTGCTCCCGCTGCGGAGCCTGAAATCTGAGGCGCCG  
  TGAGCCTTGGTTATCGCTTGTGAGGAAAGTTACAGACGGGCGCGTCGCTGCT  
  Translational start of lscB  
  GCTCCAAAGCTATTTAAAGGTATCAAATTGTCACACTAGCGCTCGTCTG  
  +362  

Figure 1. Nucleotide sequence of the upstream region of lscB containing a putative HexR binding site (underlined) similar to that described by Daddaoua et al. (2009). +1 represents the transcriptional start of lscB (Abhishek Srivastava, personal communication).

The hexR growth phenotype seems to be restricted to glucose utilization (Figure 2). For the wild type, sucrose apparently allowed for a faster adaptation as sucrose-supplemented cultures grew faster during early logarithmic growth. No significant difference was observed for the hexR mutant grown on glucose- or sucrose-supplemented medium. Arabinose allowed for the most efficient growth, independent of the genotypes studied. These results indicated a potential HexR-mediated regulatory link of intra-cellular hexose metabolism with synthesis of Lsc since sucrose is the source of glucose via the enzymatic activity of the later enzyme.

To analyze the effect of a mutation of hexR on growth in planta, PG4180 wild type and its hexR mutant were spray-inoculated onto soybean leaves with suspensions adjusted to 1 x 10^7 CFU/ml. Subsequently, the plants were kept in a plant growth chamber with temperatures ranging between 19-21°C. Bacteria were recovered between days 0 to 14 post inoculation by macerating the infected leaf areas. Interestingly, the
population size of the hexR mutant was three orders of magnitude lower than that of the wild type two weeks after spray-inoculation (Figure 3). This result indicated an important role of HexR for the in planta fitness and survival of P. syringae. Since the hexR mutant lacked significant in vitro growth when supplemented with glucose or sucrose, the result also suggested that either sugar might represent a major nutrient source for this bacterium in planta.
HexR represses lsc expression in *P. syringae*

**Figure 2.** Growth curves of PG4180 wild type (WT) (○) and its hexR mutant (●) in HS minimal media supplemented with glucose, sucrose, or arabinose as sole carbon source at 18°C. Data represent mean OD<sub>600</sub> values taken at the respective time points ± standard deviation.

![Growth curves of PG4180 wild type (WT) and its hexR mutant in HS minimal media.](image)

**Figure 3.** Growth of PG4180 wild type (○) and its hexR mutant (●) in soybean leaves. Bacterial suspensions were spray-inoculated on leaves of soybean plants grown in a greenhouse at 19-21°C. Data represent the mean values from two experiments with each three independent leaf samples ± standard deviation.

**Transcriptional analyses of lsc genes**

To analyze the effect of the hexR mutation on lsc gene expression, a growth-phase dependent transcriptional analysis was conducted using qRT-PCR. Cells of PG4180 wild type and the hexR mutant were grown in HS + arabinose medium at 18°C since levan production was shown to be maximal at this temperature (Li et al., 2006). Transcription of lsc genes in wild type and hexR mutant was highest during the early exponential growth phase and significantly decreased during further growth (**Figure 4**). Expression of lsc in the hexR mutant showed a tendency of being higher than the wild type in the early and mid-logarithmic growth phase. Interestingly, a significantly higher expression (p < 0.01) of lsc was observed in the hexR mutant as compared to that of the wild type at an OD<sub>600</sub> of 2.0 referring to late-logarithmic to stationary phase.
HexR represses lsc expression in P. syringae

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Figure 4. qRT-PCR analysis of growth phase-dependent lsc gene expression. PG4180 and its hexR mutant were grown at 18°C in HS + arabinose medium. Relative mRNA levels were related to the mean value determined for the signals of PG4180 wild type at an OD$_{600}$ of 0.5, which was defined as 100%. Data show the means and standard deviations of three biological replicates. (*= P < 0.005)

Abundance of Lsc in PG4180 wild type and hexR mutant

To qualitatively assess levan formation, PG4180 wild type and its hexR mutant were grown on sucrose-supplemented MG agar plates, resulting in indistinguishable levels of levan formation for both (Data not shown). The accumulation of Lsc in extracellular fractions of the wild type and the hexR mutant of PG4180 was tested using immunological detection. Protein samples were obtained from cell-free supernatants of bacterial cultures grown to late exponential phase at 18°C. Comparison of wild type and hexR mutant showed a slightly higher Lsc accumulation in the mutant’s culture supernatant (Fig. 5). These results further supported the hypothesis that HexR might repress lsc gene expression, resulting in more extra-cellular accumulation of its gene product in the hexR mutant.
HexR represses lsc expression in *P. syringae*

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**Figure 5.** Qualitative Western blot analysis of extra-cellular Lsc (arrow) in cell-free supernatant of PG4180 wild type (WT) and its hexR mutant (ΔhexR) grown in HS + arabinose medium at 18°C. Ten µg of protein samples per lane were electrophoretically separated, transferred to a PVDF membrane, and detected with Lsc-specific polyclonal antibody.

**Effect of HexR on protein secretion and hypersensitive response**

To explain the drastically decreased *in planta* fitness of the hexR mutant of *P. syringae* as compared to its wild type, hypersensitive response (HR) assays were performed for both on the tobacco plant *Nicotiana tabacum* (**Figure 6**). The resulting HR, after 24 hours on leaves inoculated with the mutant, was indistinguishable from that induced by the wild type. In addition, extra-cellular protein profiles were determined for PG4180 wild type and its hexR mutant incubated in hrp gene-inducing IM medium or in HS + glucose medium (**Figure 7**). After electrophoretic separation of extra-cellular proteins, nearly identical proteins profiles were observed for PG4180 wild type and its hexR mutant. In summary, these results indicated that a mutation of hexR does not influence the ability of *P. syringae* to induce an HR or alter its hrp-associated protein secretion pattern.

**Figure 6.** Typical hypersensitive response reactions elicited on tobacco plants as non-host defense responses by PG4180 wild type and its ΔhexR mutant. Sterile 0.9% sodium chloride solution was used as a negative control.
Discussion

The current study revealed that expression of genes encoding for an extra-cellular protein appear to be co-regulated with genes required for central hexose metabolism in a Gram-negative bacterium. Complementing previous studies on the global hexose metabolism repressor, HexR, in \( P. \) \textit{putida} (Petruschka et al., 2002; del Castillo et al., 2008, Kim et al., 2008, Daddaoua et al., 2009), our results suggested that involvement of HexR in regulation of \( lsc \) expression might be a selective adaptation of the plant pathogen \( P. \) \textit{syringae}, to its well-studied infection cycle (Dulla et al., 2005; Morris et al., 2008). Once \( Lsc \) is secreted, cellular resources needed for its synthesis, such as amino acyl residues and energy spent, cannot be recycled by the cell anymore. Consequently, \( P. \) \textit{syringae} might repress \( Lsc \) synthesis in coordination with hexose utilization when sufficient levels of intra-cellular glucose are available to balance the cell’s energy demands.
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In close proximity and downstream of the TSS of *P. syringae lsc* genes, palindromic sequences were identified, which resemble HexR binding sites previously predicted for *P. putida* (Daddaoua et al., 2009). Repressors such as HexR are suggested to bind to inverted repeats that partially or fully overlap RNA-polymerase binding sites (Rojo, 1999).

Multiple sequence alignments of several HexR protein sequences derived from Lsc-producing *P. syringae* strains revealed that those proteins have a high pair-wise identity scores of >97 % (Data not shown). PFAM analysis (Finn et al., 2010) of these proteins showed that there is a DNA binding domain (PFAM01418) and a SIS binding domain (PFAM 01380) as described by Daddaoua et al. (2009) for *P. putida* KT2440 (Supplementary Figure 1) suggesting a similar way of enzymatic activity. A consensus sequence obtained from multiple alignments of HexR sequences from several *P. syringae* strains revealed 91 % identity at the amino acid sequence level to that of *P. putida* (Supplementary Figure 2).

Bacterial *in planta* and *in vitro* growth analyses indicated that the substrate of Lsc i.e. sucrose, and in consequence its enzymatic product glucose, seemed to be a major nutrient sources for *P. syringae* during *in planta* growth. This is in line with previous findings reporting high molecular abundances of sucrose in bean plant’s apoplastic fluids (Atkinson and Baker, 1987). Consequently, expression and secretion of Lsc might be a major fitness factor for the *in planta* life of *P. syringae*. One may speculate that production of the levan exopolymer would rather be a ‘shunt’ product during the release of glucose from sucrose which, in turn, could be the actual major function of Lsc. Genes encoding Lsc might be part of the HexR regulon of *P. syringae* (Figure 8) in contrast to the situation in *P. putida* (del Castillo et al., 2008), which is neither phytopathogenic nor harboring any *lsc* genes. However, other bacterial species, which possess similar enzymes for cleavage of sucrose to obtain readily usable glucose, could show a similar HexR-mediated regulation. Therefore, it is suggestive to screen the most important oral cavity inhabiting bacterial species (Bergeron and Burne, 2001) as well as bacteria, which cause mucus formation in sucrose-based food manufacturing (Bekers et al., 2003) or bio-fuel production (Rojers et al., 1980), for presence of this regulatory linkage. The use of arabinose as sole carbon source, had no effect on the growth phenotype of the *hexR* mutant. This was not surprising since the assimilatory pathways of glucose and arabinose are independent. L-arabinose is converted to α–keto-glutarate in *Pseudomonas* which can
HexR represses lsc expression in P. syringae.

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directly be utilized in the TCA cycle independent of HexR regulation (Weimberg & Doudoroff, 1955; Palleroni & Doudoroff, 1957).

Currently, there is no plausible explanation for the lack of significant growth of the hexR mutant when supplemented with glucose or sucrose as sole carbon source. However, one might speculate on an up-regulation of genes normally repressed by HexR such as glk, zwf-1, pgl, edd, eda, and gap-1 as previously shown in P. putida (del Castillo et al., 2008). The effect could be potentiated in P. syringae by an increased expression of lsc, whose gene product in turn provides even more glucose. De-repressed glucose consumption in the hexR mutant might cause a surplus production of NADPH, NADH, and ATP (Figure 8). As a consequence, this may lead to an imbalance of cellular redox homeostasis thus alleviating cellular ‘reductive stress’ or even inducing ‘energy spilling’.

Figure 8. Schematic presentation of putative sucrose utilization pathway in P. syringae PG4180. Enzymes shown in blocks are presumed to be repressed by HexR in P. putida (del Castillo et al., 2008; Daddaoua et al., 2009) or in P. syringae (present study). Lsc, levansucrase; Glk, glucose kinase; Zwf, glucose-6-phosphate dehydrogenase; Pgl, 6-phosphoglucuronolactonase; Edd, 6-phosphogluconate dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; Gap, glyceraldehyde 3-phosphate-dehydrogenase; TCA, tricarboxylic acid cycle.
In *E. coli*, the redox potential influences the synthesis of fermentation products, which are formed to recycle and reoxidize NADH (Berríos-Rivera *et al.*, 2002). When *E. coli* cells are aerobically challenged with high glucose concentrations, they undergo a so-called ‘acetate switch’, which decelerates growth (Wolfe, 2005). A metabolic flux analysis of *E. coli* predicted that excess of carbon and energy might cause over-flow metabolism, which results in less efficient carbon utilization and decreased growth (Schuetz *et al.*, 2007). In *Streptococcus bovis*, excess ATP generation can cause ‘energy spilling’ by futile cycling of protons through the membrane, which leads to lesser biomass production (Bond and Russell, 2000; Russell, 2007). Whether reductive stress or ‘energy spilling’ take place in a glucose-exposed *hexR* mutant of *P. syringae* remains to be analyzed in future studies.

Expression of *lsc* genes was higher in the *hexR* mutant as compared to the wild type during late logarithmic growth. This result is in accordance with previous results of micro-array analyses of glucose metabolic genes in *P. putida* KT2440, where genes glk and zwf-1 showed a ~two-fold increased expression while genes pgl, edd, eda, and gap-1 exhibited a four- to six-fold increased expression in a respective *hexR* mutant (del Castillo *et al.*, 2008). The reason for *lsc* genes to be only moderately up-regulated in the *hexR* mutant of *P. syringae* might be explained by the peripheric role of these genes in glucose metabolism.

The extracellular protein profiles of and the HR assay conducted with the wild type and the *hexR* mutant, suggested that HexR does not influence *P. syringae*’s ability to cause an HR on non-host plants. Furthermore, our results suggested that the secretion of *hrp*-associated proteins was not affected when *hexR* was mutated. It is therefore tempting to speculate that the significantly reduced ability of the *hexR* mutant to survive *in planta* was not due to altered *hrp* gene expression but was rather due to a distorted sugar metabolism.

Data of this study prompt the question whether HexR-controlled genes such as *edd, eda, glk, pgl, zwf-1*, or *gap-1* (del Castillo *et al.*, 2008) are indeed co-regulated with *lsc* genes in *P. syringae*. This will be addressed in future studies. The current study revealed exciting options for an in-depth analysis of intra-cellular and extra-cellular hexose metabolism in the plant pathogen *P. syringae* and may allow us to better understand the potentially complex interplay of factors and parameters contributing to epiphytic or pathogenic behavior of this organism, respectively.
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Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (UL 169/5-1). The authors are grateful to Marc Auchter, Ramesh Mavathur, Petra Dangel, Helge Weingart, and Georgi Muskheilishvili for excellent technical assistance and valuable scientific advises.

References


HexR represses lsc expression in P. syringae


HexR represses lsc expression in *P. syringae*


HexR represses lsc expression in P. syringae


HexR represses lsc expression in P. syringae

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Supplementary Figure 1. Multiple sequence alignment of HexR amino acid sequences from levan-producing *Pseudomonas syringae* strains using ClustalW (Larkin et al., 2007). PFAM comparison revealed two domains: Helix-turn-helix domain from residues 6-108 (PFAM PF01418) shown in pale grey and SIS domain from residues 128-256 (PFAM PF01380) shown in dark grey. Mismatched residues are marked in blue. Residues marked in red are the predicted DNA recognition (Q46, K49, E52, R57, R60) and effector recognition (S143, S187) residues of HexR, respectively (Daddaoua et al. 2009).
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**Supplementary Figure 2.** Amino acid sequence alignment of a *P. syringae* HexR consensus sequence with that of *P. putida* KT2440 HexR (Daddaoua et al., 2009). Mismatched residues are marked in blue. Residues marked in red are the predicted DNA recognition (Q46, K49, E52, R57, R60) and effector recognition (S143, S187) residues of HexR, respectively (Daddaoua et al. 2009).
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3.4 H-NS-like proteins MvaT and MvaU regulate expression of levansucrase in *Pseudomonas syringae*
Abstract

Pseudomonas syringae pv. glycinea PG4180, an opportunistic plant pathogen, produces the exopolysaccharide levan with the enzyme levansucrase (Lsc). Exopolysaccharides promote bacterial colonization of the host and hence, synthesis of enzymes like Lsc has to be optimally regulated. Lsc is encoded by two genes, lscB and lscC. DNA affinity chromatography was used to identify two H-NS-like proteins, MvaT and MvaU, binding to the upstream region of lscB at the growth-promoting temperature of 28°C but not at the virulence-promoting temperature of 18°C, at which expression of lsc is maximal. Transcriptional analysis for mvaT and mvaU showed higher amounts of transcript produced at 18°C suggesting that the lack of MvaT/MvaU binding to the lscB upstream sequence was not due to low synthesis levels. Protein purification and subsequent electrophoretic mobility shift assays showed DNA-protein complex formation indicating MvaT-MvaU could be transcriptional regulators of Lsc. Mutagenesis of mvaU led to no phenotypic change in synthesis of Lsc or levan. MvaU was overexpressed in PG4180 and subsequent Western blotting showed a decrease in Lsc signal as compared to wild type PG480 at 18°C but not at 28°C. This indicated that MvaU might indeed represent a transcriptional repressor of lscB in PG4180. Repeated attempts to generate ΔmvaT and ΔmvaTΔmvaU mutants failed, suggesting that loss of MvaT might be lethal to the cell. Our results showed that the synthesis of lsc and, in turn, sugar metabolism and exopolysaccharide formation in plant pathogens like P. syringae is controlled by the global regulators MvaT and MvaU.
Introduction

*Pseudomonas syringae* is a well-studied group of plant-pathogenic bacteria which infect a broad range of host plants. The species is divided into more than 50 different pathogenic variants called pathovars according to the host plant and the resulting diseases [1], [2]. They possess a number of virulence and pathogenicity factors like a type-III secretion effector trafficking system for defense suppression, phytotoxins, or exopolysaccharides etc. [3]–[5]. Of these factors, exopolysaccharides play variable roles in virulence and pathogenicity not only in *Pseudomonas* but in other biofilm-producing organisms as well [6], [7]. The two major exopolysaccharides produced by *P. syringae* pv. glycinea are alginate and levan [7].

*P. syringae* pv. glycinea PG4180 causes bacterial blight disease on soybean plants. This strain produces very little alginate due to a point mutation in the *algT* gene and hence, the exopolysaccharide matrix is mainly composed of levan (R. Mitchell, 1975, [8]). Levan is a β-(2,6) polyfructan with extensive branching through β-(1,4) linkages [7], [9]–[11]. Like many other plant- or soil-associated bacteria, this organism can utilize sucrose as a source of carbon with the help of enzyme levansucrase (EC 2.4.1.10, Lsc) to produce glucose and levan. PG4180 harbours three copies of the *lsc* gene namely *lscA*, *lscB* and *lscC*. *lscA* and *lscC* are chromosomally encoded while *lscB* is plasmid-encoded. Only *lscB* and *lscC* have been shown to be natively expressed in PG4180, while no native expression of *lscA* is seen under the tested growth conditions [10]–[12]. Both, *lscB* and *lscC*, are shown to be regulated in a temperature dependent manner. Highest expression is seen at the virulence-promoting temperature of 18°C while a basal level of expression is seen at the optimal growth temperature of 28°C [11].

MvaT and MvaU are histone-nucleoid structuring (H-NS)-like proteins. The initial study on MvaT and MvaU was done on the *mva*AB operon of *Pseudomonas mevalonii* which encodes enzymes that catalyze the initial reactions of mevalonate catabolism [13]. The two proteins are global regulators controlling virulence gene expression, biofilm formation and quorum sensing [14], [15]. Interestingly, MvaT alone was shown to control more than 150 genes [15], [16].

In this study, proteins binding to the promoter region of *lscB* were isolated by DNA affinity chromatography. Using MALDI-TOF and peptide mass fingerprinting, two of these proteins were found to be 81% and 62% identical to MvaT and MvaU from
P. aeruginosa PA01 respectively. Interestingly, these two proteins were only found to bind to the lscB promoter region when they were isolated from cell grown at 28°C but not 18°C-incubated cells indicating a temperature sensitive regulation. Transcriptional analysis of the corresponding genes using Northern blot showed that both mRNAs are synthesized in higher amounts at 18°C than 28°C. Electrophoretic mobility shift assay (EMSA) using the lscB promoter region as probe showed clear shifts suggesting that the proteins were indeed DNA binding proteins of the lscB promoter region. Repeated attempts to generate deletion mutants for mvaT or mvaTΔmvaU were unsuccessful suggesting that loss of MvaT might be lethal to the cell. The deletion mutant for mvaU was phenotypically indistinguishable from the wild type PG4180 with respect to Lsc synthesis. Overexpression of MvaU in PG4180 showed a decrease in Lsc production at 18°C but not at 28°C. These results indicated that the sugar metabolism in P. syringae might be highly regulated by global regulators like MvaT and MvaU, which apparently do not only regulate expression of intracellular proteins but also extra-cellular enzymes such as Lsc.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1 and Table 2, respectively. Escherichia coli DH5α was used as the cloning host [17] and grown in Lysogeny Broth (LB) medium at 37°C. P. syringae cultures were grown in HSC medium [18] at 18°C or 28°C. Bacterial growth in liquid media was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) and cells were harvested for – (i) protein sampling at an OD<sub>600</sub> of 2.0 or (ii) RNA extraction and cDNA synthesis at an OD<sub>600</sub> of 0.5 to 2.0. Antibiotics were added to the media at the following concentrations (µg/ml), respectively: ampicillin 50; spectinomycin 25; kanamycin 25; tetracycline 25, gentamycin 2.
Table 1: Bacterial strains and plasmids used in this study

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<td></td>
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<td>DH5α</td>
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<td></td>
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<td>wild type</td>
<td>R. Mitchell</td>
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<td>PG4180.M6</td>
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<td>PG4180(pMal-mvaU-PBBR)</td>
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MvaT-MvaU regulate lsc expression in P. syringae

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\(^{a}\)Ap–Ampicillin, Tc–Tetracycline, Sp–Spectinomycin, Gm–Gentamycin, Cm–Chloramphenicol, Km–Kanamycin

**Table 2:** Oligonucleotide primers used in this study

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<sup>a</sup>Restriction sites in the primers are underlined: GGATCC-BamHI, GGTACC-KpnI, TCTAGA-XbaI, AAGCTT-HindIII, GAATTC-EcoRI, CTGAC-PstI. T7 RNA polymerase promoter sequences incorporated in primers are indicated in bold.
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#### Molecular genetic techniques

Plasmid isolation, restriction enzyme digests, agarose and polyacrylamide gel electrophoresis, electroporation, PCR, and other routine molecular methods were performed using standard protocols [17].

#### Transcriptional analysis

Total RNA was isolated from bacterial cells by acid phenol/chloroform extraction as described previously [23]. For Northern blot analysis, aliquots of total RNA (500 ng per lane), unlabeled RNA size standard (2 μg; 0.16-1.77 kB RNA Ladder, GibcoBRL, Karsruhe, Germany) and digoxigenin-labeled DNA molecular weight marker III (20 ng; 310-1517-bp; Roche, Mannheim, Germany) were separated on 1.2% glyoxal-denaturing agarose gel. The RNA was subsequently blotted onto positively charged nitrocellulose membranes (Hybond-XL, Amersham Pharmacia Biotech, Freiburg, Germany). Transfer of the RNA was verified by reversible staining of the membrane with methylene blue prior to the hybridization [24]. The membranes were incubated in hybridization solution for 2 h and subsequently hybridized with DIG-labeled RNA probes. The hybridization probes for *mvaT* and *mvaU* were generated by *in vitro* transcription using corresponding PCR products and gene-specific primers as listed in Table 2. PCR was used to add the T7 promoter by including its sequence at the 5'-end of the reverse PCR primer. After hybridization, membranes were washed twice for 5 min at room temperature in 2x SSC + 0.1% SDS followed by two 15-min washes in 0.2x SSC + 0.1% SDS at 68°C. Finally, hybridization signals were detected by incubation with anti-digoxigenin-AP Fab fragment (Roche) and ECF substrate (Amersham) using a FLA3000 phosphoimager (Amersham, Buckinghamshire, England) and the manufacturer’s image analysis software package.

#### Detection of Lsc

Total protein extraction from bacterial cells and qualitative assays for levansucrase by Western blot analysis using polyclonal antibodies were carried out as described previously [11], [12]. For immunological detection of the Lsc enzyme, total proteins were separated by 10% or 15% SDS-PAGE and Western blot experiments were performed with total extra-cellular fractions using polyclonal antibodies raised against purified Lsc of *P. syringae* pv. phaseolicola as described [12]. Bacterial cells grown on
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Mannitol-glutamate agar plates with 1.5% agar and 5% sucrose were used for the qualitative visualization of Lsc activity, which led to levan formation in form of mucoid dome-shaped colony morphology.

**MALDI-TOF mass spectrometric analysis**

Two liters of *P. syringae* culture were grown in HSC medium at 18 and 28°C, respectively. Cells were harvested at an OD$_{600}$ of 0.5, washed with 1 volume of TN buffer (50 mM NaCl, 50 mM Tris-HCl, pH 7.6), resuspended in 6 ml of disruption buffer (50 mM Tris-HCl [pH 7.6], 1 mM DTT, 10 mM MgCl$_2$, 1 mM EDTA, 10% glycerol), and disrupted by sonication. Cell debris was removed by centrifugation at 45,000 x g at 4°C for 60 min. Total protein amounts in crude extracts were determined using a Nanodrop apparatus (Thermo Fisher Scientific, Langenselbold, Germany) and visualized by 10% SDS-PAGE. Purification of DNA-binding proteins and peptide mass fingerprinting analyses were performed as described previously [25], [26]. The region from nucleotides -341 to +339 with respect to the transcriptional start site of *lscB* was used as the DNA bait. PCR amplification was performed using plasmid pRB7.2 [11] as a template and primers lscB-fw-DAC and lscB-rev-DAC (Table 2), the latter being tagged with biotin via TEG linker (MWG-Biotech).

**Generation of mvaU mutant in PG4180**

A PG4180 ΔmvaU mutant was generated by homologous recombination using suicide vector pK18mob [22] carrying a mutagenic construct generated as follows: Two fragments flanking the *mvaU* gene were amplified from PG4180 genomic DNA using the primer pairs, mvaU_1f_B / mvaU_1r and mvaU_2f/mvaU_2r_BK (Table 2). PCR products were cloned into vector pGEM-T Easy (Promega, Mannheim, Germany) yielding plasmids pGEM.MvaU1 and pGEM.MvaU2. A 1,230-bp KpnI fragment containing a kanamycin resistance cassette was removed from plasmid pFKm1 [21] and ligated into KpnI-digested pGEM.MvaU2 yielding pGEM.mvaU2-Km. A 1,700-bp SpeI-BamHI fragment derived from pGEM.MvaU2-Km was ligated into SpeI-BamHI-digested plasmid pGEM.MvaU1 yielding plasmid pGEM.MvaU-Km. Finally, a 2,145-bp EcoRI fragment was removed from pGEM.MvaU-Km and ligated into EcoRI-digested vector pK18mob yielding the *mvaU* gene replacement plasmid pK18mob.MvaU-Km. This plasmid was mobilized into *P. syringae* PG4180 by tri-parental mating. Mutants were
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subsequently plated on MG agar containing kanamycin. Genotypes of mutants were screened by PCR using primers mvaU_check_s_f and mvaU_check_s_r (Table 2). Gene replacement was verified when a 1317-bp PCR product was amplified.

Generation of mvaT mutant in PG4180

The 1.7-kb insert containing mvaT, was mutagenized by insertion of a kanamycin resistance (Km') gene cassette that was derived from plasmid pFKm1 and is expressed from its own promoter. This full construct was subcloned into the plasmid pGEM-T-easy (Promega, Mannheim, Germany) as per the manufacturers’ instructions. The plasmid pGEM.MvaT-Km was introduced into PG4180 via electroporation or mobilized by triparental mating, and the potential electroporants and transconjugants were plated on MG medium containing kanamycin. The success of homologous recombination was determined by PCR using primers mvaT_up_f and mvaT_down_r.

Purification of MvaT and MvaU

Protein purification was done using maltose-binding protein tagging and overexpression with vector pMal–C2 (New England Biolabs, Massachusetts, USA) and amylose resin beads. Two liters of E. coli culture harbouring pMal-mvaT or pMal-mvaU constructs, respectively, were grown at 37°C in LB medium with 0.2% glucose until they reached an OD$_{600}$ of 0.5. The cultures were then induced with 0.3 mM IPTG and grown for 4 hours at 37°C followed by overnight growth at 18°C. The cells were harvested, resuspended in sonication buffer (20 mM HEPES, 500 mM NaCl, 0.2 mM DTT, 1 mM EDTA, 10% glycerol) containing Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and chilled on ice for 20 min. The cells were lysed by sonicating 15 times for 10 s, and the lysate was harvested by centrifuging at 10,000 x g for 30 min. The purification column was setup and washed with 70% ethanol followed by distilled water. 600 µL of amylose resin (New England Biolabs, Massachusetts, USA) was added to the column with 1 mL of sonication buffer and the cell lysate was passed through the column three times. The column was then washed twice with 30 mL sonication buffer and eluted using 3 mL of elution buffer (20 mM HEPES, 500 mM NaCl, 0.2 mM DTT, 1 mM EDTA, 20 mM maltose, 10% glycerol). The eluate was dialysed for 2 hours using dialysis buffer (20 mM HEPES, 200 mM NaCl, 0.2 mM DTT, 1 mM EDTA, 10%
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glycerol). The final eluate was analysed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining [27].

Electrophoretic mobility shift assay

The promoter region of lscB was amplified using primers PlscB_fw and PlscB_rv (Table 2). The PCR product was purified using GeneJET PCR Purification kit (Fermentas, St. Leon Rot, Germany). 300 ng of the probe were mixed with gradually increasing quantities of protein MBP-MvaT or MBP-MvaU, respectively, and total volume was made up to 20 µL using STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0). After incubation at room temperature for 15 min, the resulting complexes were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Overexpression of MvaU in native host cells

MvaU overexpression vector pMal-MvaU was ligated with broad-host range vector pBBR1MCS using PstI and HindIII restriction sites. The resulting ligation product was transformed in E. coli DH5α. The plasmid ligation was confirmed by performing enzymatic restriction digestion. The pMal-MvaU-pBBR construct was introduced into PG4180 cells via electroporation. The correct transformant was grown in HSC medium at 18°C and 28°C. After reaching an OD₀₆₀₀ of 0.5, the cells were induced with 5 mM IPTG and the induced cells were harvested at OD₀₆₀₀ of 2.0. The effect of the overexpression of MvaU on Lsc was analysed by Western blotting of the total proteins.

Bioinformatics analyses

Vector NTI Advance 10.1.1 (Invitrogen Corporation, USA) was used for the nucleotide, amino acid sequence alignments as well for generating genetic maps. BLAST-N and BLAST-P programs were used for online sequence analyses [28]. The website www.pseudomonas.com was consulted for the determination of P. syringae gene orthologs and paralogs [29].
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Binding of MvaT-MvaU to lscB upstream sequence

DNA affinity chromatography was done using a 680-bp DNA fragment containing the lscB upstream region to identify proteins binding to the promoter region of lscB. The PG4180 cultures were grown at 18°C and 28°C and the total protein extracts were obtained. After the protein binding step elution was done using increasing concentrations of salt solutions. No significant elutable protein signals were obtained with samples derived from 18°C-grown cells (Figure 1). When protein extracts of cultures grown at 28°C were loaded, two strong protein bands were seen corresponding to sizes of 15 and 16 kDa, respectively, in all of the elution steps. Subsequently, the two protein bands were subjected to MALDI-TOF peptide mass fingerprint analyses. The 16-kDa protein showed 82% identity to MvaT, an H-NS-like repressor of P. aeruginosa PAO1 (PA4315) [15]. The 15-kDa protein was homologous and 61% identical to MvaU, a H-NS-like repressor of P. aeruginosa PAO1 (PA2667). The corresponding homologous genes encoding for these proteins were present in three fully annotated genome sequences of different P. syringae strains. Consequently, the 16-kDa protein was designated MvaT, and 15-kDa protein was named MvaU.

Figure 1: DNA affinity chromatography experiment using upstream region of lscB as bait. Total proteins extracted from PG4180 cells at 18°C and 28°C were separated using 15% SDS-PAGE and stained with colloidal Coomassie Brilliant Blue stain. The proteins observed in the figure are after elution with 1 M NaCl. 16 kDa MvaT and 15 kDa MvaU were identified by MALDI-TOF and peptide mass fingerprint analysis at 28°C but not at 18°C.
Transcriptional analysis of mvaT and mvaU

Binding of MvaT and MvaU to the lscB upstream sequence in P. syringae was observed when protein extracts were obtained from cultures incubated at 28°C but not when proteins were derived from 18°C-grown cultures. To determine whether this was due to a potential thermo-responsive expression of the genes mvaT and/or mvaU, expression profiles with total mRNA samples derived from P. syringae cells grown at 18°C or 28°C were obtained. For this, mRNA was extracted from cells during various time points of the logarithmic growth phase (OD$_{600}$ 0.5–2.0), and gene-specific probes were hybridized with the blotted mRNA (Figure 2). The approximate sizes of the corresponding transcripts were 500-bp for mvaT and 520-bp for mvaU. Expression of both the genes was more pronounced at 18°C as compared to 28°C. While mvaT mRNA was abundant throughout the tested growth phase, expression of mvaU was significantly stronger in the early logarithmic phase and declined as bacterial growth proceeded. These results showed that lack of MvaT/MvaU binding to the lscB upstream sequence at 18°C was not due to lack of expression of the corresponding genes. Consequently, these results suggested that MvaT and MvaU are present at both temperatures and that the thermoreponsive binding to the lscB upstream region must be rather due to an increased DNA binding capacity at 28°C.

![Figure 2](image-url): Transcriptional analysis of mvaT and mvaU from PG4180 cells. The RNA was extracted from cells grown at 18°C and 28°C, as indicated, in HSC medium. The RNA was then subjected to Northern blot analysis using gene specific probes. Higher level of transcription is seen at 18°C as compared to 28°C for both proteins. The 16s rRNA was used as a reference to normalize the signals.
Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) is a binding assay used to study DNA-protein interactions. Both, MvaT and MvaU proteins were purified using the maltose-binding protein (MBP) affinity tag and amylose resin column purification. The purified proteins were used with the affinity tag for the EMSA. The EMSA was done using a 500-bp upstream region of lscB as DNA probe. The DNA amount was kept constant while the protein amount was increased gradually until retardation in the DNA migration was observed (Figure 3A and 3B). Both proteins showed prominent shifts suggesting that both are DNA binding proteins. Binding to the promoter region of lscB was a strong indication that they are regulatory proteins of lscB. The EMSA with MBP alone showed no shift (Figure 3C) which suggested that the DNA binding was due to the MvaT or MvaU itself but not affected by the affinity tag.

Generation of ΔmvaU and ΔmvaT mutants

The ΔmvaU mutant was generated by homologous recombination of mvaU thereby replacing it with a kanamycin resistance cassette. The mutant was analysed in comparison to the wild type PG4180 for any difference in phenotype. The growth rate was same as for the wild type PG4180 and the amount of Lsc produced also remained unaltered as determined by Western blotting (data not shown). In line with these results, the levan forming ability of the mutant was also indistinguishable from that of the wild type. Repeated attempts to mutate the mvaT gene failed in both, PG4180 and PG4180.ΔmvaU. This suggested that the loss of mvaT could potentially be lethal to the cell. Our results furthermore indicated that loss of MvaU can be completely compensated by the cell as this mutant remained phenotypically unchanged.

Effect of MvaU overexpression on Lsc synthesis

Since the deletion of mvaU in PG4180 did not alter the synthesis of Lsc, overexpression of MvaU was conducted in order to observe its impact on Lsc synthesis. MvaU was overexpressed under the tac promoter in PG4180 as reported previously [30]. After artificially inducing MvaU synthesis, the effect on Lsc synthesis was determined by performing a Western blot experiment with the total proteins of PG4180 and PG4180.mvaU (Figure 4).
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**Figure 3:** Electrophoretic mobility shift assay using *lscB* promoter region as probe and (A) purified MvaT, (B) purified MvaU (C) purified MBP as proteins. The DNA and protein were mixed and incubated at RT for 15 min followed by separation on 1% agarose gel electrophoresis. Both MvaT and MvaU proteins showed clean shifts after forming a complex with the DNA probe, indicating they are potential regulators of *lscB*. The MBP did not show any binding suggesting it does not interfere with the binding assay. The numbers below indicate amount of protein used for each reaction in µg.

The result showed that the wild type and the PG4180.mvaU mutant cells, prior to induction produced similar amounts of Lsc. However, the overexpression of MvaU resulted in moderate decrease in the signal intensity for Lsc, indicating that MvaU possibly regulates Lsc as a transcriptional repressor. Note that this effect was only seen at 18°C. This result, although a little surprising, further supported the role of one of the H-NS-like proteins as temperature sensitive regulators.
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**Figure 4:** Western blot analysis of total protein extracts of PG4180 and PG4180.mvaU using a polyclonal antiserum raised against Lsc from \( P. \) syringae pv. phaseolicola NCPPB1321. The cells were grown at 18°C and the extracted proteins were separated by 10% SDS-PAGE. Arrow indicates the signal representing Lsc. The wild type PG4180 showed slightly more amount of Lsc produced than PG4180.mvaU (Unind). The induced PG4180.mvaU (Ind), however, showed low signal intensity as compared to the wild type PG4180.

**Discussion**

Differential gene expression plays an important role in various host-pathogen interactions. H-NS proteins are crucial for promoting conformational changes of DNA often associated with differential accessibility of the later for regulatory proteins [31], [32]. H-NS protein from enterobacteria like \( E. \) coli, \( S. \) typhimurium and \( S. \) flexneri has a high affinity to AT-rich DNA regions, which often are of foreign nature to the cells [33]–[36]. H-NS is thought to bind a specific sequence on the DNA with high affinity and then oligomerize across adjacent AT-rich regions [37]–[39]. The H-NS-like proteins MvaT and MvaU have been studied in pseudomonads like \( P. \) aeruginosa and \( P. \) mevalonii [13], [40]. Even though they are members of the H-NS family, they share little sequence identity to classical H-NS proteins of enterobacteria. Consequently, MvaT and MvaU had been included in this protein family rather for their function and overall protein structural similarity to H-NS proteins than sequence identity [41], [42].

Synthesis of extra-cellular proteins like Lsc has to be tightly regulated, since the protein along with the energy spent on its synthesis, are both ‘lost’ by the cell once the protein is secreted [43]. In this respect, other studies have previously shown that MvaT-like proteins function as regulators of exo-product formation as well as quorum sensing-associated proteins [14], [44].

In this study, we wanted to identify potential regulatory proteins of \( lscB \) in \( P. \) syringae. With DNA affinity chromatography, two proteins, the 15-kDa MvaU and the 16-kDa MvaT, were found to bind to the \( lscB \) upstream region. Interestingly, these proteins were seen to bind their target DNA when they were obtained from 28°C-grown cultures but not when they were derived from 18°C-grown cultures indicating a potential
temperature sensitive regulation. Since both the proteins were isolated together, there
could be an MvaT-MvaU heterodimer formation involved in the regulation [13]. Binding
of these H-NS-like proteins to the lscB upstream region at 28°C might explain the lack of
lsc gene expression at this temperature reported earlier [11], [12].

To test the hypothesis that absence of MvaT/MvaU DNA binding at 18°C could
be due to low expression of these proteins, a transcriptional analysis was conducted. The
analysis showed that amount of transcripts for both mvaT and mvaU were higher at 18°C
than 28°C suggesting that the differential binding of MvaT/MvaU could indeed be due to
regulatory action of the proteins rather than their abundance.

Next, MvaT and MvaU were purified to confirm their DNA binding ability. EMSA using either of the proteins showed clear shifts in the DNA migration indicating
that the proteins were indeed potential DNA-binding proteins. The EMSA using only
MBP showed no shift proving that the DNA binding ability was due to MvaT and MvaU
and that the affinity tag did not interfere with the binding assay.

In logical consequence, generation of single and double deletion mutants for the
two proteins was attempted. The ΔmvaU mutant was obtained but this mutation had no
effect on the synthesis of Lsc or levan. This was not totally unexpected since previous
studies had shown that loss of MvaU or MvaT can be compensated by an increase in
either MvaT or MvaU, respectively, in the cell [45]. However, as also observed by
Castang and co-workers, repeated attempts to generate an ΔmvaTΔmvaU double mutant
failed [46]. Since previous studies had also shown that the loss of MvaU did not affect
expression of several target genes [15], [45] it might be tempting to conclude that MvaT
seems to play a more important regulatory function and that loss of MvaT could be lethal
to P. syringae.

Overexpression of MvaU and MvaT, respectively, was carried out based on the
hypothesis that this should alter the normal cell metabolism by impacting gene
expression. A decrease in the Lsc signal intensity as compared to that of the wild type or
that found in the uninduced control clearly suggested the role of MvaU as a repressor.
The increase of the amount of repressor might have led to a weakened expression as seen
in the Northern blot. Interestingly, this repression was only seen at 18°C, but not at 28°C
where MvaT and MvaU were originally found to bind to the DNA in the DNA affinity
chromatography experiment. One could speculate that MvaU represses the synthesis of
Lsc at 28°C and hence a drastic effect was not visible after overexpression of MvaU.
Native de-repression is seen at 18°C since higher Lsc production is necessary at the virulence-promoting temperature. Hence, the effect of repression could be more prominent after overexpression of MvaU at 18°C as compared to 28°C as seen in the Western blot signals.

An interesting feature of several H-NS proteins of enterobacteria and H-NS-like proteins of *Pseudomonas* is their specific DNA binding to AT-rich regions, which are particularly frequent in foreign DNA acquired by horizontal gene transfer. Often, so-called ‘pathogenicity islands’ have a characteristic GC-content usually different from that of the host DNA and showing an increased proportion of AT-rich DNA [33], [34], [46], [47]. Recently, we have reported that the upstream regions of *lscB/C* most likely consist of phage-borne DNA [48] with an AT-content of 46.6%, higher than the average AT-content of *P. syringae* of 39-41% [49]. It was therefore proposed that *lsc* was initially acquired by lateral transfer by *P. syringae*, later duplicated and finally provided with a phage-borne promoter element. Thus, it is tempting to speculate that repression of *lsc* by MvaT-MvaU could be associated with its potential mode of acquisition. Foreign DNA, if not optimally silenced, could have deleterious effects on bacterial fitness as observed in *Salmonella* [33], [34].

Future studies include the overexpression of MvaT to check the effect on Lsc expression. A simultaneous co-overexpression of MvaU and MvaT could also be performed to test whether the phenotype of transformants is altered. These studies will hopefully give us a better understanding of the DNA-protein interactions taking place during Lsc expression.

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Discussion

Plant pathogens like *P. syringae* are under constant stress from different environmental factors. In the molecular evolutionary ‘arms race’ with their host organisms, they possess an arsenal of means such as sucrose-degrading enzymes or the ability to form EPSs to fend-off defense reactions from the plant. But being adapted as plant pathogens also comes with a price, an increase in energy dependent processes as well as its regulation. The EPS alginate has been studied to a good extent in *P. syringae* as a ‘key player’ to improve epiphytic fitness which also acts as pathogenicity and virulence factor [29, 37, 71, 103]. However, levan, in spite of being easy to produce and detect in the laboratory, has been studied to much lesser extent. Due to its accumulation in the intra-cellular voids of biofilms, levan has been proposed to be a storage of sugar polymers, possibly for nutritional use during starvation periods [52]. A good way to know more about this EPS is to study the enzyme that produces it. Study of production and regulation of Lsc with respect to its genomic distribution and transcription factors, could indirectly throw light upon novel aspects of levan itself.

4.1 Evolution of levansucrase in *P. syringae*

An interesting genomic feature of *P. syringae* is the presence of multiple gene copies for the enzyme levansucrase. To determine the reason behind the presence of three copies including a transcriptionally inactive one, nucleotide sequence alignments of *lscB/C* and *lscA* were done which showed a striking feature concerning the
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nucleotide arrangements. The shorter lscA had different synteny i.e. neighbouring genes than lscB/C counterparts. Thus, it was tempting to speculate that the presence of a so-called phage-associated promoter element (PAPE) upstream of lscB/C could be the decisive factor for expression of lsc in P. syringae. Insertion sequence (IS) elements were also seen to be neighbouring the PAPE and lscB/C. With the help of different combinations of promoter-ORF fusions of lscA and lscB we could, for the first time, express lscA with a native P. syringae lsc promoter in P. syringae. Another important observation of this study was that lscA produced levan in the phylogenetically unrelated plant pathogen E. amylovora. With both these results we could draw a putative scenario of the evolution of Lsc in P. syringae as follows:

The lscA gene could have been obtained by horizontal gene transfer from enterobacteria such as E. amylovora. However, perhaps due to its native promoter not suited for expression in P. syringae, the gene remained inactive. Gene duplication followed by insertion of PAPE upstream of the gene could have led to an lsc gene with a functional promoter sequence. Such a phenomenon in which an insertion of an IS element activates a gene has been reported previously [15, 79]. Most notable of these observations was one in E. coli K12, where the insertion of IS elements IS1 and IS5 activated the previously cryptic bgl operon [74]. It should be noted that between the transcriptional and the translational start sites of lscB/C, resides a gene called com. Deletion of this gene did not influence synthesis of Lsc indicating that this gene is not directly involved in Lsc synthesis (Helge Weingart, unpublished data). A second gene duplication, this time of the functional lsc onto a native plasmid or vice versa, could have caused the presence of two functional lsc genes, lscB and lscC.

One can only speculate about this peculiar feature of P. syringae. Organisms which possess Lsc include bacteria from different niches including, but not limited to, nitrogen-fixing symbionts (G. diazotrophicus and Azotobacter), enterobacteria (Erwinia), normal gut flora (Bacillus and Lactobacillus), human pathogens (Streptococcus and Rahnella) etc. The common factor in the otherwise diverse group of organisms is the availability of sucrose in their surrounding habitat. The presence of two functional copies of lsc could be related to pathogenic fitness of the organism. Lsc with two different localizations, periplasmic and extra-cellular, could increase the probable availability of glucose for the cell. It has been proposed that
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gene duplication and innovation occurs in ancillary genes rather than those with established functions [44]. This could be true in case of Lsc since alginate negative but levan positive P. syringae did not survive well in planta indicating that levan alone cannot act as a virulence or pathogenicity factor [80]. The gene duplication could also have been a result of environmental stress as in the case of cold-shock protein CspA in E. coli [101]. The csp gene family in E. coli contains nine members formed by repeated gene duplication over the course of evolution. The multiple copies, probably having overlapping functions, could be an adaptive response to environmental stress like temperature fluctuation.

The current study also showed that expression of lscA is cryptic not only in PG4180 but in other P. syringae pathovars as well. This was not surprising since these strains despite their different host ranges are still belonging to the same bacterial species. Chromosomally encoded proteins are difficult to integrate or eliminate in the evolutionary process as opposed to plasmid-encoded proteins [69, 98, 39]. Thus, the cell could still be harbouring a functional albeit transcriptionally inactive lscA in its genome, unlike the situation in pv. syringae B728a where lscB is absent due to the absence of a corresponding native plasmid [28].

4.2 Regulation of levansucrase

Possession of two or more gene copies coding for the same protein makes it imperative to optimally control their potentially differential expression. The most ergonomic type of control of gene expression for any cell is the initiation of transcription itself. Due to limited availability of RNA polymerase molecules, promoters display different molecular mechanisms to ensure optimal transcription. These include bending-prone DNA sequences, σ-factors, small ligands, transcription factors and the folded bacterial chromosome structure [6]. In P. syringae pv. phaseolicola 1448A, the phylogenetically closest relative to strain PG4180, 535 ORFs corresponding to 10% of total ORFs have a predicted or proven regulatory role [47]. In this study, three transcription factors of Lsc were identified. They included the metabolism regulator HexR and two small nucleoid-associated proteins MvaT and MvaU, which were characterized for their effect on expression of Lsc.
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4.2.1 Hexose metabolism regulator HexR

The compound, 6-phosphogluconate (6-PG), is a common intermediate in glucose metabolism in *Pseudomonas*. The tight control of the enzyme 6-PG-dehydratase is extremely important for proper distribution of the 6-PG in the ED and the Pentose Phosphate pathway [59]. This enzyme, along with enzymes involved in key glucose metabolism processes, is controlled by HexR. In-spite of having such background information about HexR, it is safe to assume that such a key glucose metabolism regulator will likely have a wider role in bacterial metabolism. Levansucrase is involved in degradation of sucrose and hence, is an integral part of the sugar metabolism, particularly glucose metabolism, if the disaccharide is present in the bacterial surrounding environment. Hence, finding the conserved HexR binding site in the promoter regions of *lscB* and *lscC* was very interesting [18] but not totally surprising. Herein it is shown that an extra-cellular enzyme is controlled by a key intra-cellular regulator. The rationale behind this seems simple: once an enzyme is secreted, it is lost by the cell and hence, a better regulation of its production will prevent loss of energy for the cell.

Control of *lsc* by HexR could indicate that procurement of glucose is the primary function of Lsc while levan formation might be a secondary, but nonetheless beneficial, side-effect. This could represent a particular survival strategy of a phytopathogen like *P. syringae*, since Lsc is not present in the close relative, *P. putida*, the soil-borne model organism of most of the previous HexR studies. PG4180 cells with a *hexR* deletion exhibited a severely hampered growth in media containing glucose or sucrose, respectively, as the carbon source. Since HexR is a repressor, its deletion from the cell would result in a continuous glucose utilization due to the de-repression of the corresponding genes. However, due to lack of repression after glucose has been metabolized by the cell, the cell metabolism could be disturbed by an increased redox potential generated by the ED pathway leading to hampered growth. Using an *in silico* mathematical modelling approach, Knorr and co-workers showed that minimizing the reactions generating redox potential is of a higher priority to the *E. coli* cells than growth itself [51]. This indicates that even though *Pseudomonas* has likely adapted the ED pathway over EMP pathway to overcome environmental
oxidative stress [13], disruption of a key glucose metabolism regulator makes the increase in redox potential due to the same ED pathway a hindrance for the survival of the cell.

On one hand the hexR mutant was severely impaired in *in planta* growth. On the other hand, deletion of hexR did not affect the organism’s ability to cause a hypersensitive reaction on non-host plant *Nicotiana tabacum* or the secretion of hypersensitive response pathogenicity (hrp)-associated proteins. This indicates that the low level of the mutant’s survival *in planta* was only due to its altered sugar metabolism and not due to any secondary effects associated with the T3SS.

Future studies on HexR should include to test whether genes of the hex regulon namely, *edd*, *eda*, *glk*, *pgl*, *zwf-1*, or *gap-1* [20] and the newly found member of this regulon, *lsc*, are co-regulated or not. Co-regulation seems likely since glucose intake and utilization should, logically, be controlled in a tandem for optimal ergonomy of both in the cells of *P. syringae*.

### 4.2.2 H-NS-like proteins MvaT-MvaU

MvaT and MvaU belong to a family of small DNA-binding proteins called histone-nucleoid structuring (H-NS)-like proteins. MvaT was originally identified in *P. mevalonii* as a regulator of mevalonate catabolism [77]. Further studies have shown its importance as regulator of virulence genes and in altering the expression of a multidrug operon in *P. aeruginosa* [23, 100]. Later, it was discovered that MvaT is associated with another protein, MvaU, and together they control similar set of target genes [11, 94]. Addition of *lsc* to this set of target genes added to the repertoire of these global regulators. This also seems to be in accordance with some earlier studied functions of these proteins like control of biofilm formation [93, 94]. The temperature sensitive binding of these proteins, however, was the hitherto unknown property of these proteins. The role of H-NS and H-NS-like proteins in temperature-dependent regulation of genes has been reported before like H-NS of *E. coli* or *Shigella* [27, 24] and H-NS-like protein TurA protein of *P. putida* [73]. Maximum expression of *lsc* was found at 18°C corresponding to the organism’s optimal temperature for pathogenesis. The observation that the binding of MvaT-MvaU to the promoter
region of \textit{lscB} was at 28°C might explain the basal level expression of \textit{lsc} observed at this temperature [53].

H-NS proteins of enterobacteria and H-NS-like proteins of \textit{Pseudomonas} are involved in binding to AT-rich pathogenicity islands, especially of foreign DNA, acquired via horizontal gene transfer [11, 55, 68, 67]. These regions have a characteristic GC-content which is usually different from that of the host DNA. The upstream region of \textit{lscB/C} is pro-phage-borne with a GC-content of 54.4\% which is lower than the average GC content of \textit{P. syringae} which is 59-61\% [83]. This indicates that the promoter region of \textit{lscB/C} might be an excellent candidate for MvaT-MvaU binding and regulation. In a previous finding of this PhD study, we proposed that \textit{lsc} was acquired by lateral transfer by \textit{P. syringae}. Thus, it is tempting to speculate that repression of \textit{lsc} by MvaT-MvaU could have been affected by its mode of acquisition by the cell. Expression of genomically integrated foreign DNA has to be correctly silenced as in \textit{Salmonella}, where it was observed that deletion of H-NS had deleterious effects on bacterial fitness due to uncontrolled expression of several pathogenicity islands [55]. Mutations in \textit{hns} can be lethal unless accompanied by compensatory mutations in such pathogenicity islands [68].

Additional experiments should be done to determine whether MvaU has a similar effect as MvaT since it has been shown that they regulate similar set of genes [11]. \textit{In planta} studies with corresponding mutants would also give an idea about the effect of MvaT-MvaU proteins on virulence and pathogenicity of \textit{P. syringae}. Since few studies including this one have showed that a \textit{mvaTmvaU} double knockout is lethal to the cell [11, 10], it could be worthwhile investigating the effect of their over-expression on \textit{lsc} synthesis.

### 4.2.3 Levansucrase activator LscR

An interesting aspect not included in the results of this study was the role of another transcription factor of Lsc, namely LscR. The detailed identification of this protein was reported in the PhD thesis of Daria Zhurina [104]. To summarize, a genomic cosmid library of PG4180 was introduced into transformants of a heterologous host, \textit{P. putida} KT2440, already containing \textit{lscB/C} on a plasmid.
4. DISCUSSION

The resulting transformants were plated on nutrient agar medium containing 5% sucrose. Four colonies showed formation of levan indicating that their cosmids contained a potential activator of \( lscB/C \). Further sub-cloning identified the gene as PSPPH\_0651 from the genome sequence of \( P. syringae \) pv. phaseolicola 1448A (www.pseudomonas.com database). There, it was annotated as ‘prophage PSPPH01 transcriptional regulator’ and hence was named LscR (Levansucrase Regulator). The phenotype of the deletion mutant of \( lscR \) was indistinguishable from that of the wild-type of PG4180 with respect to growth rate, levan formation, Lsc synthesis and mRNA quantification (Data not shown). LscR was purified using MBP-tag using the same protocol as for MvaT-MvaU. Subsequent EMSA using \( lscB \) promoter region as bait did not show any shift or retardation in the migration of DNA. The reasons for this could be many. Firstly, the EMSA protocol can be modified for optimization of additives, buffer composition or increase in sensitivity by fluorescent labelling of DNA bait [41]. Co-operative binding of transcriptional activators has been reported in which both activators bind the promoter region but one activator alone is unable to bind in the absence of the other activator [99]. However, in this case, a more profound effect would be expected for the \( \Delta lscR \) mutant on \( lsc \) synthesis. It can also be that the protein purification process led to a purified but non-functional protein. Thus, in this case, future studies should focus on over-expression of LscR in PG4180 to check its effect on \( lsc \) synthesis. It would also be interesting to check binding of LscR to other transcription factors like MvaT or MvaU through competitive binding assays [31].
Bibliography


BIBLIOGRAPHY


BIBLIOGRAPHY


Appendix

Note:

- Methods given in this section are step-by-step protocols used to perform the experiments during the course of the PhD study.
- Add the buffer components in the order as mentioned in the tables.
- Always make-up the volume of the buffers with distilled water unless otherwise mentioned.
- RT = Room Temperature

(I) 1-2-3 plasmid prep

Buffers and Reagents

<table>
<thead>
<tr>
<th></th>
<th>Buffer P1</th>
<th>Buffer P2</th>
<th>Buffer P3</th>
</tr>
</thead>
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<td>Potassium acetate</td>
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</tr>
<tr>
<td></td>
<td>Store at 4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Inoculate 1 ml LB-medium in 2 ml tubes with the required bacterial strain. Grow bacteria overnight at 37°C for E. coli or 28°C for P. syringae.

2. Harvest cells by centrifugation at 16,000 g for 1 min at RT. Discard the supernatant completely. Tap on tissue paper to remove the last drop.
3. Resuspend pellet completely in 150 µL of cold P1 buffer by flicking the tubes over a tube rack.

4. Add 150 µL of P2 lysis buffer. Mix gently, but thoroughly, by inverting the tubes. Incubate at RT for 1 min.

5. Add 150 µL of cold neutralisation Buffer P3. Mix immediately but gently by inverting the tubes until phases are mixed completely.

6. Centrifuge at 16,000 g for 8 min. During the centrifugation: Fill new 1.5 ml tubes with 50 µL 3M sodium acetate (pH 5.8) and 350 µL of isopropanol.

7. Transfer 450 µL of the supernatant into the tubes containing sodium acetate and isopropanol. (Don’t take any of the white protein/SDS pellet). Mix by inverting the tubes.

8. Centrifuge for 30 min at 16,000 g.

9. Slowly discard the supernatant. Add 500 µL 70% ethanol. (Do not resuspend the pellet, do not vortex or flick!)

10. Centrifuge at 16,000 g for 1 min. (Align the tubes as during the previous centrifugation step).

11. Slowly discard supernatant. Use a pipette to remove residual alcohol. Dry the pellet in a heat block (50°C) until no ethanol is visible (approximately 5-7 min).

12. Add 35 µL of ddH₂O for high-copy plasmids or 20 µL of ddH₂O for low-copy plasmids to dissolve the DNA. Incubate for 10 min in a heat block at 50°C. Vortex shortly and spin down.

13. Use 3 µL of the plasmid DNA for restriction enzyme digestion and subsequent agarose gel electrophoresis analysis.

14. Store the DNA at -20°C.
(II) Electrophoretic Mobility Shift Assay

Buffers and Reagents

<table>
<thead>
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<th>Loading buffer</th>
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<td>1 mM</td>
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<td>pH</td>
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</tr>
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</tbody>
</table>

- Prepare purified protein fractions as 10, 8, 6, 4, 2, 1 μL. Prepare dilutions as 1:2, 1:5, 1:10, 1:50, 1:100 etc. with ddH₂O

Reaction composition

<table>
<thead>
<tr>
<th>DNA bait (250-300 ng/μL)</th>
<th>1 μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein fractions and dilutions</td>
<td>1-20 μL</td>
</tr>
<tr>
<td>Binding buffer</td>
<td>Upto 20 μL</td>
</tr>
</tbody>
</table>

1. Incubate at RT for 15 min.
2. Mix 7.5 μL of Loading Buffer with the reaction mix.
3. Carefully load the mix in the respective wells of the gel.
4. Add 2 μL of normal loading dye (containing Bromophenol Blue + Xylene Cyanol) in one or both of the flanking empty wells to observe the running behaviour of the gel. Loading of the DNA ladder is not necessary.
5. Carry out the electrophoresis for 1 hour or until the running front reaches the end of the gel.
6. Stain with ethidium bromide for atleast 10 min and visualize under Gel-Doc system.
(III) Growth curve analysis of *P. syringae*

**Pre culture**

1. Inoculate a loopful of bacteria from MG/KB agar plate into 10 mL growth medium in 50 mL flask for first pre-culture.

2. Incubate at 18°C or 28°C for 24-36 hours with 250 rpm shaking.

3. Make second pre-culture by inoculating 3 mL of first pre-culture into 47 mL of growth medium in 250 mL flask. (If unsure about growth rate of the organism, adjust OD$_{600}$=0.1 to get a general idea as to how fast the organism is growing.)

**Main culture**

1. Harvest required amount of cells from pre-culture so as to start the main culture at an OD$_{600}$=0.1 for 100 mL final volume in 1 litre flask.

2. If pre-culture and main culture have different media, then centrifuge the cells (equivalent of OD$_{600}$=0.1) at 3000 g for 10 min. Discard the supernatant and resuspend the cells in 5 mL fresh medium. Add 195 mL sterile culture medium to the cells to start the main culture.

3. Take zero hour OD$_{600}$ reading.

4. For CFU/mL measurement, take 200 µL culture in a sterile 96-well plate with a multichannel pipette. (Use 96-well plate only if you have more than 4 cultures; otherwise use microcentrifuge tubes.) Dispense 180 µL of diluent in the rest of the wells. Dilute 10-fold upto the concentration of desire from A-H or 1-12 (20+180=200). Take all dilutions of a single sample with the multichannel pipette and spot 10 µL on the agar plate. Use 8.5 cm plate for 1-6 samples, 14 cm plate for 7-14 samples. Before the spots dry out, tilt the plate such that the liquid runs to the bottom of the plate in a straight line! Dry the ‘smears’ and incubate at 28°C. Colonies will appear from 48 upto 96 hours. Check regularly to so as to obtain colonies of countable size.

5. Measure the OD$_{600}$ every 6 hours. If required, take samples for CFU/mL count every 12 hours.

6. Stop taking the measurements when two consecutive OD$_{600}$ readings show a progressive decline.
(IV) Preparation of electrocompetent cells of *E. coli*

**Buffers and Reagents**

All solutions should be autoclaved and chilled prior to use!

- 0.1 M CaCl$_2$ solution
- 0.1 M CaCl$_2$ + 10% glycerol solution

**Procedure**

Always keep the cells on ice after harvesting!

1. Streak-out *E. coli* strain on the LB plate and incubate at 37°C.

2. Prepare an overnight culture of 5 mL in test-tube or 10 mL in 50 mL flask.

3. Dilute the overnight culture 1:200 in 200 mL LB a 1 L flask and incubate at 37°C at 250 rpm.

4. Harvest cells at OD$_{600}$=0.350 - 0.375 (∼3 hours) in 4 pre-chilled 50 mL centrifuge tubes.

5. Keep tubes on ice for 10 min.

6. Centrifuge cells at 3000 g for 10 min at 4°C.

7. Discard supernatant of all tubes and resuspend the pellet of each tube in 10 mL ice-cold 0.1 M CaCl$_2$.

8. Mix the resuspended cells of two tubes into one (continue with two tubes).

9. Centrifuge cells at 3000 g for 5 min at 4°C.

10. Discard supernatant of all tubes and resuspend the pellet of each tube in 10 mL ice-cold 0.1 M CaCl$_2$.

11. Keep on ice for 30 min. (While waiting, bring liquid nitrogen!)

12. Centrifuge cells at 3000 g for 5 min at 4°C.

13. Discard supernatant of all tubes and and resuspend the pellet of each tube in 1 mL ice-cold 0.1 M CaCl$_2$ + 10% glycerol.
14. Unify both solutions into one tube.

15. Measure the optical density of a 1:100 dilution (dilute with CaCl$_2$ + 10% glycerol).

   - Blank: CaCl$_2$ + 10% glycerol; The optical density should be between 0.3 and 0.6.
   - OD$_{600}$$<$0.3: Centrifuge again at 3000 g for 5 min, discard the supernatant and resuspend in 1 - 1.5 mL CaCl$_2$ + 10% glycerol.
   - OD$_{600}$$>$0.6: Add 0.5 - 1 mL of CaCl$_2$ + 10% glycerol

16. Dispense cells into pre-chilled microcentrifuge cups (75 - 100 µL aliquots).

17. Snap-freeze them in liquid nitrogen and put them in a pre-chilled cryo box.

18. Store at -80°C immediately.

**Transformation**

1. Use 1 ng of a high copy plasmid (E.g. pBluescript or pUC18) per vial of chemically competent cells.

2. Mix the plasmid DNA with competent cells and incubate on ice for 5-10 min.

3. Warm the heating block to 42°C and dYT or SOC medium to 37°C

4. Heat shock the cells at 42°C for 2 min 30 sec.

5. Immediately add pre-warmed 1 mL dYT or SOC medium.

6. Incubate for 1 hour at 37°C with shaking at 250 rpm.

7. Make 10-fold dilutions from $10^{-1}$ to $10^{-4}$.

8. Spread on LB-agar plates with appropriate antibiotic using sterile glass beads

9. Incubate overnight at 37°C until transformants are visible (If using blue-white screening, check for the appropriate colour for the colonies).
(V) Preparation of electrocompetent cells of *P. syringae*

Buffers and Reagents
- Ice cold 300 mM sucrose solution - filter sterilized

Procedure
Always keep the cells on ice after harvesting!
1. Streak *P. syringae* strain on MG agar plate and incubate at 28°C for 2 days.
2. Take two sterile 2 mL microcentrifuge tubes and resuspend the bacteria in 1 mL ice-cold 300 mM sucrose each.
3. Spin down for 8 min on 3000 g at 4°C.
4. Discard supernatant and resuspend again in 1 mL ice-cold 300 mM sucrose.
5. Repeat step 2 and 3 two more times (3 times in total).
6. Resuspend each pellet in 75 µL ice-cold 300 mM sucrose.

Transformation
1. Take 75 µL of cells and add your plasmid for immediate electroporation.
2. After the pulse, add 1 mL of King’s B liquid medium.
3. Incubate the cells at 28°C for at least 3 hours (preferably 6 hours) for recovery of the cells in 2 mL microcentrifuge tube with shaking a 250 rpm.
4. Spread 100 µL on MG agar plate with respective antibiotic.
5. Centrifuge the remaining cells at 3000 g for 6 min at RT.
6. Discard the supernatant and leave approximately 100-150 µL in the tube.
7. Resuspend the cells in the remaining supernatant.
8. Spread plate on MG agar plate with appropriate antibiotic
9. Incubate at 28°C until transformants are visible.
(VI) Protein purification

Buffers and reagents

- 1M glucose (Filter sterilized, store at RT)
- 1M IPTG (Filter sterilized, store at -20°C)

<table>
<thead>
<tr>
<th>Sonication buffer</th>
<th>Elution buffer</th>
<th>Dialysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES 20 mM</td>
<td>HEPES 20 mM</td>
<td>HEPES 20 mM</td>
</tr>
<tr>
<td>NaCl 500 mM</td>
<td>NaCl 500 mM</td>
<td>NaCl 200 mM</td>
</tr>
<tr>
<td>DTT 0.2 mM</td>
<td>DTT 0.2 mM</td>
<td>DTT 0.2 mM</td>
</tr>
<tr>
<td>EDTA 1 mM</td>
<td>EDTA 1 mM</td>
<td>EDTA 1 mM</td>
</tr>
<tr>
<td>Glycerol 10%</td>
<td>Glycerol 10%</td>
<td>Glycerol 10%</td>
</tr>
<tr>
<td></td>
<td>Maltose 20 mM</td>
<td></td>
</tr>
</tbody>
</table>

Bacterial culture

1. Streak out the *E. coli* strain on LB-Amp plate and incubate overnight at 37°C.
2. Start pre-culture in 50 ml LB-Amp for a 2-3 hours.
3. Set up the main culture of approximately 2 litres with a starting OD₆₀₀=0.05 - 0.1 in LB-Amp containing 0.2% glucose using the cells from the pre-culture.
4. Induce the cultures with 0.3 mM IPTG at OD₆₀₀=0.5 and grow the culture for another 2 hours on 37°C.
5. After 2 hours of induction, change the temperature to 18°C and grow the culture overnight.

Harvesting of proteins

Prepare 2x15ml sonication buffer with half a tablet of a protease inhibitor cocktail.

1. Harvest the cells with the high speed centrifuge at 5000 g for 20min.
2. Discard the supernatant and resuspend each cell pellet (equivalent of 1 litre culture) in 15ml of sonication buffer (plus protease inhibitor cocktail).
3. Incubate on ice for 10 min.
4. Sonicate 15 times for 10 sec. Cool for at least 15 sec between two sonication steps.

5. After sonication, sediment the cell debris for 45 min at 10,000 g.

**Purification preparation**

Set-up the chromatography column in the cold room. Avoid bubbles in the tube since it changes the speed of the flow through.

1. Clean the syringe, tubes and column with ethanol followed by H₂O.
2. Equilibrate the column with 30 ml of sonication buffer. Close the valve and make sure that the pipe is completely filled with buffer without bubbles.
3. Resuspend the amylose resin and load 600 µL with 1 ml sonication buffer directly onto the column.
4. Load the cell lysate into the syringe and let it slowly run through the column (1 drop every 2-3 sec).
5. Collect the flow-through and keep 20 µL for SDS-PAGE.
6. Before the syringe is empty, pass the flow-through two more times through the column. Keep 20 µL each time for analysis.
7. Wash 2 times with 30 ml sonication buffer. Keep 20 µL of each wash.
8. Remove the syringe and the tube. Open the valve and discard the first 4 drops.
9. Load 3 ml of elution buffer directly onto the column.
10. Incubate for 10 min.
11. Collect the eluate in microcentrifuge tubes or 15 mL tubes.

**Qualitative test**

1. Spot 2 µL of each collected sample on a clean Whatman filter paper.
2. Dip the paper into Coomassie Brilliant Blue stain.
3. Rinse it with water for 5-10 sec.
4. Dry the paper for 10-15 seconds using a microwave oven.
5. Observe the amount of protein in each sample (size of the spots).
6. Proceed only if there is any staining in the elution fraction!
APPENDIX

Dialysis
1. Use a dialysis bag and wet it with H₂O.
2. Take at least 600 mL of dialysis buffer in a 1 litre beaker.
3. Close one end of the bag with a clamp and equilibrate the bag with dialysis buffer.
4. Remove the buffer and load the eluate (0.5 - 1 ml).
5. Close the bag after removing all air bubbles.
6. Stir for 2-3 hours at RT or 4°C.

Analysis
1. Analyse all the collected fractions as well as 20 µL the dialysed eluate by SDS-PAGE.
2. Stain the polyacrylamide gel with Coomassie Brilliant Blue stain.

(VII) Quantitative Reverse Transcriptase (qRT)-PCR

Buffers and reagents
Store buffers at RT unless otherwise mentioned.

- 3M Sodium azide (Store in Safety cabinet!)
- 1 M DTT (Store at -20°C)
- 10% SDS (w/v)
- 8 M Lithium chloride (Autoclave before use!)

1. For each sample, aliquot 15 mL of killing buffer in a 50 mL tube and freeze at 4°C.
2. Harvest the cells by centrifugation at 3000 g for 15 min at 4°C.
3. Discard the supernatant in appropriate container (toxic!).
4. Resuspend the pellet in 3 mL ice-cold killing buffer.
5. Aliquot the samples into 3 x 1.5 mL micro-centrifuge tubes.
6. Centrifuge at 6,550 g for 5 min at 4°C.
7. Discard the supernatant completely using a pipette.
8. Snap-freeze the pellets in liquid nitrogen and store at -80°C.

### Killing buffer

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<th>Concentration</th>
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<td>Sodium azide</td>
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### RP-CTAB

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</tr>
<tr>
<td>DEPC-H₂O</td>
<td></td>
</tr>
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<td>Before using add:</td>
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### CTAB buffer

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### LP-CTAB

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<tr>
<td>SDS</td>
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</tr>
<tr>
<td>DEPC-H₂O</td>
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</tbody>
</table>

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**RNA isolation - Cell lysis by hot SDS**

**Setup**

1. Set the Thermomixer to 95°C.
2. Prepare a RP-CTAB (resuspension buffer) aliquot with DTT as given in Buffers and Reagents section.
3. Briefly heat the LP-CTAB (lysis buffer) in the microwave to dissolve precipitate.
4. Briefly heat the CTAB in the microwave for 7 sec and place it in the 65°C oven.
APPENDIX

Procedure

1. Label four optimized 2 mL micro-centrifuge tubes with 1 to 4 and pipette 375 µL LP-CTAB.

2. Heat tubes in the thermomixer to 95°C.

3. Thaw the at frozen RNA cell pellets on ice.

4. Add 375 µL RP-CTAB (with DTT) to the first cell pellet.

5. Resuspend the pellet, add it to the first tube with the hot LP-CTAB and start shaking at 900 rpm for exactly 3 min (the solution should be clear after the cell lysis).

6. Change the tip and resuspend the next sample.

7. Add it to the hot LP-CTAB exactly 20 seconds later (2:40).

8. Change the tip and resuspend the next sample.

9. Add it to the hot LP-CTAB exactly 20 seconds later (2:20).

10. Change the tip and resuspend the next sample.

11. Add it to the hot LP-CTAB exactly 20 seconds later (2:00).

12. Prepare the water-saturated phenol.

13. After exactly 3 min, take the first sample and add 750 µL phenol.

14. Invert the tube and place it in the Labinco shaker and shake for at least 5 min at level 7.

15. Every 20 seconds (exactly!) take the next tube, add phenol, invert and place it in the shaker.

If you have more than four samples, you can start the next lysis step of another four samples at this point (Hint: stop the shaker after each centrifugation in order to avoid mixing of the phases).

1. Centrifuge the mixed samples for 7 min at 16,000 g.

2. Prepare four new 2 mL tubes with 675 µL phenol.

3. As soon as the centrifugation if cone, also stop the shaker in order to avoid mixing of the separated phases.
4. Carefully take 675 µL of the top aqueous phase using a broader (or cut) tip and add it to the phenol.

5. Transfer the tubes in the Labinco shaker and shake for 5 min at Level 7.

6. Centrifuge the mixed samples (should be whitish) for 7 min at 16,000 g.


8. After centrifugation carefully take 575 µL of the top aqueous phase using a broader (or cut) tip and add it to the phenol:chloroform:isoamylalkohol mixture.

9. Transfer the tubes in the Labinco shaker and shake for 5 min at Level 7.

10. Centrifuge the mixed samples (should be whitish) for 6 min at 16,000 g.

11. Prepare four 2 mL tubes with 550 µL chloroform:isoamylalkohol (24:1) and add 55 µL CTAB to each tube.

12. After centrifugation carefully take 495 µL of the top aqueous phase using a broader (or cut) tip and add it to the chloroform:isoamylalkohol mixture.

13. Transfer the tubes in the Labinco shaker and shake for 5 min at Level 7.

14. Centrifuge the mixed samples (should be whitish) for 6 min at 16,000 g.

15. Prepare four 1.5 mL tubes with 145 µL lithium chloride.

16. After centrifugation carefully take 435 µL of the top aqueous phase using a broader (or cut) tip, add it to the lithium chloride. Invert it several times to mix.

17. In order to precipitate the RNA, incubate the tubes for 30 min at -80°C (precipitate always for the same time).

18. Pre-cool the centrifuge to 4°C.

19. Centrifuge the samples at 16,000 g for 20 min at 4°C.

20. Carefully take the supernatant taking care not to disturb the loosely sticking white pellet.

21. Wash the samples with 800 µL 75% ice-cold ethanol.

22. Centrifuge the samples at 16,000 g for 1 min at 4°C.
APPENDIX

23. Carefully take the supernatant.
24. Air-dry the pellet at RT.
25. Solve pellets in 50 µL DEPC treated H$_2$O.
26. Shake the tubes at 1000 rpm for 5 min at RT.
27. Spin down and store at -80°C.

DNA removal

Turbo DNAfree kit from Ambion

1. Add 10x DNase I buffer to a final concentration of 1x and 1 µL rDNase I (2U/µL) to each RNA sample.
2. Mix the sample and incubate at 37°C for at least 30 min.
3. Add 0.1 volume of resuspended DNase Inactivation Reagent.
4. Incubate for 2 min at RT and shake at 1000 rpm in heating block.
5. Spin at 16,000 g for 90 sec at RT.
6. Carefully transfer the supernatant into a new tube (do not touch the pellet)

RNA quality check

1. Measure the RNA concentration with the Nanodrop spectrophotometer (determine the 260/280 ratios)
2. Dilute extracts to a final concentration of 50 ng/µL with DEPC treated H$_2$O.
3. If in doubt about the amount of RNA, load 500 ng of RNA extracts on the gel and run it for 30 min on 110 V (do not overrun because this might cause RNA degradation).
4. Visually verify that the extracts have the same amount of RNA
(VIII) Western blot

Preparation of protein samples

- Grow the cultures as described earlier. Once the OD\textsubscript{600} of the cultures has been obtained, collect 15 mL of culture in centrifuge tubes. Centrifuge at 3,000 g for 20 min at 4°C.

Buffers and Reagents

<table>
<thead>
<tr>
<th></th>
<th>10x SDS PAGE buffer</th>
<th>5x Electrotransfer buffer</th>
<th>Wash Buffer (WB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>250 mM</td>
<td>Tris-base</td>
<td>Tris-HCl</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.92 M</td>
<td>Glycine</td>
<td>NaCl</td>
</tr>
<tr>
<td>SDS</td>
<td>1%</td>
<td>SDS</td>
<td>Tween-20</td>
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<tr>
<td>pH</td>
<td>8.3</td>
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<td>8.3</td>
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<td></td>
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<td>100 mM</td>
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<td></td>
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<td>0.9%</td>
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<td></td>
<td></td>
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<td>0.1%</td>
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<td>7.5</td>
</tr>
</tbody>
</table>

Supernatant

1. Pass the supernatant through 0.2 µm filter to prepare cell-free supernatant. Collect in 50 mL falcon tubes.
2. Cover the mouths of the tubes with soft tissue paper.
3. Freeze the supernatant at -80°C for at least 30 min or till the liquid is completely frozen (opaque).
4. Put the frozen supernatant for lyophilization in the Freeze-Dryer.
5. Stop the lyophilization process after all the liquid has sublimed (approx. 48-60 hours).

Cell lysate

1. Resuspend the cell pellet in 5 mL Tris-HCl (pH 8.0).
2. Freeze the cells at -80°C for ~20 min.
3. Thaw cells at 37°C with shaking for ~20 min or till they are completely thawed.
4. Perform freeze-thawing 3-5 times.
5. After the final thawing, sediment the cell debris at 3,000 g for 45 min at 4°C.
6. Collect 2 mL of supernatant containing the cell lysate.
APPENDIX

SDS-PAGE

Settings are for Mini-PROTEAN Tetra cell vertical gel electrophoresis system (Bio-Rad Laboratories).

1. Prepare equal amounts of protein samples (usually 10-20 µg). Add loading dye and boil for 5 min at 95°C. Spin down the samples after boiling.

2. Load all samples to be analysed in the polyacrylamide gel.

3. Add Protein Ladder preferably in an asymmetric manner with respect to the samples for ease of orientation.

4. Run the gel at 150 V for initial 15 min till the proteins enter the resolution gel.

5. Increase voltage to 180 V and continue till the running front reaches the end or passes out of the gel (usually 45-60 min).


Blotting

Settings are for Mini Trans-Blot electrophoretic transfer cell system (Bio-Rad Laboratories).

Preparation

1. Soak 2 filter pads and 2 Whatman filter papers in 1x electrotransfer buffer (1 part buffer + 1 part methanol + 3 parts H₂O) for at least 10 min.

2. Activate PVDF membrane by soaking in methanol for ∼1 min and then subsequently soaking in 1x electrotransfer buffer for at least 10 min.

Set-up

1. Remove gel from the glass plate sandwich and put into H₂O to wash-off residual SDS-PAGE running buffer.

2. Transfer into 1x electrotransfer buffer for equilibration until starting of the Western blot.

3. Make the blot assembly in the following order: black side of plastic cast, filter pad, Whatman filter paper, polyacrylamide gel, PVDF membrane, Whatman filter paper, filter pad, white side of plastic cast. (Note the orientation of the gel)
APPENDIX

4. Transfer at 400 mA for 1.5 hours at RT with constant stirring using magnetic stir-bar.

Detection using antibody and development

1. After transfer of proteins, incubate the membrane in blocking buffer (5% milk powder in WB) for 45-60 min at RT with constant shaking. (Alternatively: Membrane can be kept in the Blocking Buffer overnight at 4°C.)

2. Incubate the membrane in anti-Lsc antibody (1:10,000 diluted) in WB for 1 hour at RT. (Alternatively: Membrane can be kept in the primary antibody overnight at 4°C. In this case, seal the container with Parafilm to prevent evaporation of buffer)

3. Wash the membrane 4 times for 10 min each with WB.

4. Incubate the membrane in alkaline-phosphatase labelled anti-rabbit IgG (Sigma Aldrich) antibody (1:10,000 dilution) in WB for 1 hour at RT.

5. Wash the membrane 4 times for 10 min each with WB.

6. Dissolve one SigmaFast BCIP/NBT tablet in 10 mL ddH₂O by vortexing vigourously for 2 min.

7. Remove from WB and incubate the membrane in the BCIP/NBT solution in the dark to develop the blot.

8. Check periodically until the signals for the Lsc (54 kDa) are strong.

9. Stop the reaction by immersing the membrane in normal tap H₂O.

10. Dry the blot on a soft tissue in the dark for atleast 1 hour.

11. Scan the blot or take a photograph to digitalize and record the result.

(IX) Zymogram

Native-PAGE

1. Prepare polyacrylamide gel of required percentage without SDS.

2. Prepare equal amounts of total proteins samples (usually 100 µg). Mix with loading dye not containing SDS or β-metaptoethanol.
3. Load samples in the gel without boiling. Add 5 μL loading dye in one or both of the extreme-end lanes for better visualization of the running behaviour.

4. Run the gel at 150 V for initial 15 min till the proteins enter the resolution gel.

5. Increase voltage to 180 V and continue till the running front reaches the end or passes out of the gel (usually 45-60 min).

**Enzymatic development**

1. Remove gel from the glass plate sandwich and put into H₂O to wash-off residual SDS-PAGE running buffer.

2. Put the gel into 5% sterile sucrose solution.

3. Incubate overnight or till levan formation is visible in form of bands in the gel. Observe against black paper or background for better visualization.
Statutory Declaration

I, **Shaunak Khandekar**, hereby declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned. This PhD thesis has not been submitted for conferral of degree elsewhere.

Bremen, March 15, 2013