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Identification and partial characterization of transcriptional regulators involved in temperature-dependent expression of levansucrase in *Pseudomonas syringae*

by

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SUMMARY

In pathogenic bacteria, exopolysaccharides (EPS) play an important role during successful infection and colonization of the host. In the plant pathogen, *Pseudomonas syringae* pv. glycinea PG4180, synthesis of the EPS, levan, is mediated by two levansucrases, encoded by *lscB* and *lscC*. Both enzymes are 98% identical and exhibit a temperature-dependent mode of expression. Maximum mRNA synthesis for these genes occurs at the virulence-promoting temperature of 18°C and is repressed at the optimal growth temperature of 28°C. This process must be tightly regulated due to its demand for cellular energy and the potential loss of this energy once the enzymes are released under inappropriate conditions. Consequently investigation of regulatory links between polymer synthesis and internal metabolic pathways is important for understanding the ecological function(s) of levan and to develop strategies to efficiently combat the effects mediated by bacterial EPS. The main goal of this work was to better understand the regulatory system(s) governing levan formation in *P. syringae*. For this, transcriptional regulators mediating the temperature-dependent expression of *lscB* encoding levansucrase had to be identified and characterized in terms of function.

DNA affinity chromatography revealed that three repressor proteins, namely hexose metabolism repressor, HexR, and two H-NS-like repressor proteins termed MvaT and MvaU, were bound upstream of *lscB* at 28°C whereas at 18°C no such binding occured. Functional screening of a genomic library of *P. syringae* in the heterologous host *Pseudomonas putida* carrying *lscB* allowed the identification of the fourth transcriptional regulator named LscR, which gave rise to transcription of *lscB* in the same temperature-dependent manner as in its native host, *P. syringae*. The observed phenomenon was found to be due to removal of MvaT from the *lscB* upstream sequence by LscR. Surprisingly, the 9.8-kDa protein, LscR, was not binding to the *lscB* promoter region at any tested temperature. This result suggested that LscR might not interact with the DNA target but rather with MvaT directly. It was concluded that lack of *lscB* expression at 28°C is based on reversible binding of MvaT and MvaU to its upstream sequence and that this binding might in turn be regulated

by the activator protein, LscR, at 18°C. Analysis of the expression patterns of the *mvaT*, *mvaU* and *lscR* supported this hypothesis. The provided results give novel insights into mechanisms, by which certain H-NS like proteins might regulate their target genes.

From a micro-ecological point of view, sucrose utilization might be the dominant life style for plant-associated *P. syringae* strains. Carbohydrate metabolism in these strains relies on the Entner-Doudoroff pathway channeling intermediates into glycolysis and the TCA cycle and is controlled by the transcriptional regulator HexR. The herein for the first time observed involvement of HexR in regulation of levansucrase expression indicated a tight co-regulation of genes required for an extracellular function with those involved in central energy-obtaining metabolic pathways in a temperature-dependent manner in pseudomonads. This speculation was supported by the fact that growth of an Lsc$^-$ PG4180 mutant was impaired when cells were grown with sucrose as compared to glucose as the sole carbon source suggesting a central role of Lsc in hexose metabolism.

Complex regulation of the *lscB* expression by the H-NS-like proteins and the key regulator of the hexose metabolism HexR represent a unique and novel mechanism, which was not described for any levansucrase thus far. It might also reflect the necessity to tightly control *lscB* expression in plant pathogenic bacteria in order to ensure formation of a protective levan layer and simultaneous glucose release from sucrose at low virulence-promoting temperatures as well as to avoid energy loss in case of unfavorable conditions.

Aside of LscR, the herein identified regulators of levansucrase transcription in *P. syringae*, belong to groups of global regulatory factors wide-spread in bacteria. However for plant-pathogenic *Pseudomonas* species their role was demonstrated for the first time. Further studies of their impact on virulence and epiphytic fitness of *P. syringae* might provide a better and more detailed understanding on how to efficiently combat these plant pathogens.

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CONTENTS

1 INTRODUCTION

1.1. *Pseudomonas syringae*

Pseudomonads belong to the group of γ-proteobacteria and are characterized as Gram-negative, rod-shaped, aerobic bacteria, which are motile due to presence of polar flagella. Pseudomonads are nutritionally versatile and may use a wide range of organic compounds as carbon and energy sources. That is why these bacteria are able to colonize extremely diverse ecological niches. The group of pseudomonads includes a number of ecologically, economically, and medically important species, such as non-pathogenic bioremediation-conducting *Pseudomonas putida*, the rhizosphere-inhabiting bacterium *Pseudomonas fluorescens*, the opportunistic human pathogen *Pseudomonas aeruginosa*, and agriculturally important plant pathogens belonging to the group of *Pseudomonas syringae* species. The complete genomic sequences of the mentioned bacterial species are available online (Buell *et al.*, 2003; Nelson *et al*, 2002; Stover *et al.*, 2000; www.pseudomonas.com).

1.1.1. *Pseudomonas syringae* pv. glycinea

Pseudomonas syringae strains comprise a large and well-studied group of plant-pathogenic bacteria. They infect a broad range of host plants, thus being subdivided into pathogenic variants (pathovars). *P. syringae* pv.glycinea PG4180 causes bacterial blight disease of soybean plants (Glycine max (L.) Merrit). Typical symptoms are water-soaked lesions that develop into necrotic leaf spots surrounded by chlorotic halos (Fig. 1).

1.1.2. Traits contributing to the virulence and epiphytic fitness of *P. syringae*

Aerial parts of plants, also known as the ‘phyllosphere’, are exposed to ultraviolet radiation, periodic desiccation, as well as rapid changes in temperatures. Despite these

harsh conditions, the phyllosphere is colonized by microorganisms, some of which have a rather epiphytical life style whereas others can be pathogenic to the plant.

Successful pathogens have evolved specialized strategies to suppress plant defense and induce disease symptoms in otherwise resistant hosts. Depending on the conditions, pathogenic bacteria may have diverse relationships to the plant ranging from epiphytic growth to causing disease. Host processes, which are induced by phytopathogenic bacteria include programmed cell death, cell-wall based defense, hormone signaling, and other basal defense mechanisms (Abramovitch and Martin, 2004). The infection process involves epiphytic colonization of the leaf surfaces, establishment of infection sites via the penetration into the plant's apoplast, multiplication within the host, and finally development of disease symptoms (Hirano and Upper, 2000).

Fig. 1: Typical symptoms induced by *Pseudomonas syringae* on the soy bean leaf: necrotic spots are surrounded by chlorotic, yellowish halos (Source: http://www.forestryimages.org)

In order to successfully infect and colonize the host plant, *P. syringae* possesses a broad range of cellular traits, which contribute to its virulence and fitness on the plant leaves. First of all, production of different toxins is crucial for the infection process. Some of them, like e.g. syringomycin and syringopeptin, penetrate the plant cell membrane generating pores and causing disruption of the membrane potential thus leading to cell death and necrosis of the affected tissue (Hutchison and Gross, 1997). Other toxins, such as phaseolotoxin and coronatine, induce formation of chlorotic leaf spots. Coronatine is mimicking the plant hormone jasmonate thus misleading plant immune system and preventing a proper defense response (Feys *et al.*, 1994; Weiler *et al.*, 1994; Bender *et al.*, 1998; Bender *et al.*, 1999; Uppalapati *et al.*, 2005). In order to initiate and promote the infection, a type III protein secretion machinery termed Hrp (hypersensitive response and pathogenicity) system and corresponding so-called Hrp effector proteins are necessary

(Marco *et al.*, 2005). The effector proteins are injected via the Hrp system into plant cells, where they compromise plant defense reactions via transcriptional modifications or other processes (Collmer *et al.*, 2000). Besides this, to ensure disruption of cells and penetration into the leaf tissue, the ice nucleation protein, InaZ, is produced by *P. syringae* and was reported to cause serious tissue damage in citrus plants (Nemecek-Marshall *et al.*, 1993).

Virulence of the pathogen is often triggered by particular environmental conditions, such as pH fluctuations, plant-borne substances, or temperature changes. Prior to infection, bacteria have to adapt to their local environment, which is often rather harsh and hostile, and establish biofilms on the leaf surface. Consequently, virulence of plant pathogens is often controlled by cell-to-cell signaling, the so-called quorum sensing (Latifi *et al.*, 1995; Winson *et al.*, 1995; Surette *et al.*, 1999; Anand *et al.*, 2003; Von Bodman *et al.*, 2003; Dulla *et al.*, 2008). The ability of bacteria to survive and proliferate on the leaf at a given environmental regime is called epiphytic fitness. During the first stages of plant leaf colonization motility of bacteria due to the flagella movement is important. Stable attachment of bacterial cells to the plant surface dependent on type IV pili and/or exopolysaccarides is another crucial prerequisite for biofilm formation (Beattie *et al.*, 1994; Roine *et al.*, 1998). Besides this, in a highly competitive environment, bacteria have to ensure efficient supply of nutrients mediated by ABC transporters and different uptake systems, as well as acquisition of essential elements such as iron, which is mediated by siderophores and other iron-scavenging molecules (Expert *et al.*, 1996; Expert, 1999; Boughammoura *et al.*, 2007). Exopolysaccharides produced by plant pathogens are among the most important factors contributing to the bacterial epiphytic fitness and will be described in detail below.

1.2. The exopolysaccharides alginate and levan

Bacterial exopolysaccharides (EPS) are carbohydrate polymers which are secreted by the cell and may either form a tightly associated capsule or a loose slime layer surrounding the cell (Whitfield, 1988). They play important roles in the development of microbe-plant interactions. EPS provide the charged and highly hydrated matrix, which retains water thus being necessary to prevent desiccation of the bacteria. EPS also

contribute to the accumulation and retention of nutrients and minerals and are involved in protection of bacteria from plant-borne reactive oxygen species and antimicrobials (Denny, 1995). Finally, EPS may cover the cells in such a way, which prevents proper recognition of the pathogen by the plant. Two major EPS – termed alginate and levan – were previously demonstrated to be produced by different *P. syringae* pathovars (Gross and Rudolph, 1987).

Alginate is a copolymer of O-acetylated ß-(1,4) linked D-mannuronic acid and its C-5 epimer, L-guluronic acid. Expression of the alginate biosynthetic operon is regulated by the alternative sigma-factor AlgT, and is plant-inducible and temperature-dependent with maximum of expression at 18°C (Schenk *et al.*, 2008). *P. syringae's* alginate production is contributing to an increase epiphytic fitness, virulence, resistance to desiccation and plant-borne toxins, dissemination *in planta* and the induction of water-soaked lesions in infected leaves (Fett and Dunn, 1989; Rudolph *et al.*, 1994; Yu *et al.*, 1999).

Unlike other *P. syringae* pathovars, *P. syringae* pv. glycinea produces only negligible amounts of alginate because the *algT* gene carries a non-sense mutation in the fourth codon leading to lack of AlgT synthesis (Schenk *et al.*, 2006). This is one of the reasons why the best studied EPS of this species is actually levan. Levan is produced by the vast majority of *P. syringae* pathovars (Hettwer *et al.*, 1998), making it a suitable feature for taxonomical classification of these bacteria. Levan is a ß-(2,6)-polyfructan made up of single glucose head groups connected to numerously chained fructosyl residues with extensive branching through ß-(2,1) linkages (Osman *et al.,* 1986; Han, 1990). The size of levan polymers might reach several megaDalton. Bacterial levan accumulation is easily visualized in form of semi-translucent, whitish dome-shaped mucoid colony phenotypes when the microbes are grown on sucrose-containing agar media (Fig. 2). Levan has quite interesting biochemical properties being biodegradable, water soluble, resistant to certain organic solvents, such as jet fuel and *d*-limonene and being heat stable with a melting point of 225°C (Stivala and Bahary, 1978).

The proposed physiological role of levan during pathogenesis is the build-up of a diffusion-inhibiting barrier surrounding the bacteria thus masking and protecting the cells during early stages of infection while the pathogen proliferates inside the host tissue (Kasapis, Morris, 1994; Beattie, Lindow, 1999).

A

B

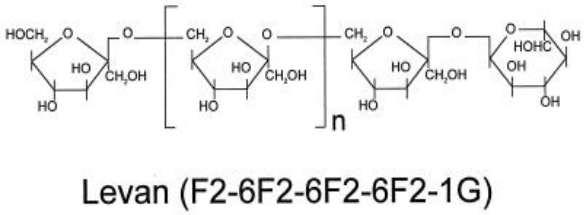

Fig. 2: Exopolysaccharide levan.

A) Appearance of levan-forming bacterial colonies on the MG plates supplemented with 5% sucrose; **B)** chemical structure of the EPS levan as a β-(2,6) polyfructan.

Recent results of Laue *et al.* (2006) demonstrated that neither alginate nor levan were essential for biofilm formation in *P. syringae* indicating a role for a yet-to-be identified additional EPS during biofilm formation. Besides this, levan formation might be of particular importance for plant pathogens, because plant-borne sucrose is a readily available carbon source for these microorganisms (Ullrich, 2009). Bacteria utilize the extra-cellular enzyme, levansucrase, to release glucose from sucrose and to polymerize fructosyl residues into the levan. Glucose is then consumed inside the cell while the polymerized fructosyl residues make up the extra-cellular mucus loosely associated with the cell envelope (Ullrich, 2009). Levan formation catalyzed by levansucrase will be discussed in detail below.

1.2.1. The extracellular enzyme levansucrase

Levansucrase (Lsc) (E.C.2.4.2.10.) is an extracellular enzyme produced by various Gram-positive and Gram-negative bacteria. It belongs to the superfamily of ß-fructosidase, has a five-bladed ß-propeller fold, and is able to cleave sucrose into glucose and fructosyl residues releasing glucose and polymerizing fructosyl residues into levan polymers (Han, 1990). Additionally, Lsc is able to hydrolyze levan and to exchange glucosyl head groups of levan polymers as demonstrated by feeding bacterial cells with ^{14}C-labelled glucose

(Kaspasi *et al.*, 1994). From the functional point of view, Lsc not only contributes to the formation of levan but as an extracellular saccharolytic enzyme is also thought to be an important component of primary metabolic pathways, which allows bacteria to utilize sucrose as an energy source.

Lsc were isolated from both Gram-positive and Gram-negative bacteria such as various *Streptococcus* species, *Leuconostoc mesenteroides*, different food-grade *Lactobacillus* species, *Bacillus subtilis*, *Acetobacter diazotrophicus*, *Erwinia amylovora,* and *Pseudomonas syringae* (Sato ei al., 1984; Morales-Arrieta *et al.*, 2006; Seibel *et al.*, 2006; Tieking *et al.*, 2005; van Hijum *et al.*, 2006; Dedonder, 1966, Arietta *et al.*, 1996; Gross *et al.*, 1992; Li and Ullrich, 2001; Lyness and Doelle, 1983; Hettwer *et al.*, 1998). Interestingly, Lsc from Gram-negative and Gram-positive bacteria differ dramatically in their biochemical and genetic characteristics, such as peptide sequences, enzymatic properties, protein location, and transcriptional regulation.

All studied Lsc of Gram-positive bacteria share some common features such as a typical N-terminal signal peptide for secretion via the type II protein secretion system (Benyahia *et al.*, 1988). Lsc of certain species, such as for example, *Lactobacillus sanfranciscensis* were found to be membrane-bound being linked to the membrane via C-terminal lipid anchors (Tieking *et al.*, 2005).

The Lsc proteins of Gram-negative bacteria have been studied to variable extent in *P. syringae*, *E. amylovora*, *Zymomonas mobilis*, *Gluconacetobacter diazototrophicus*, *Rahnella aquatilis*, and *Acetobacter xylinum* (Martinez-Fleites *et al.*, 2005; Seo et alo., 2002; Song *et al.*, 1999; Tajima *et al.*, 2000; Vigants *et al.*, 2001). These enzymes were shown to share higher similarities among each other as opposed to those from Gram-positive organisms. The Lsc protein, LevU, from *Z. mobilis* was found to be membrane-associated and mutational analysis revealed that its polymerizing activity could be uncoupled from the sucrolytic activity of this enzyme (Song *et al.*, 1999). In contrast to Lsc enzymes of other Gram-negative bacteria, Lsc of *G. diazotrophicus* (LsdA) possesses a typical N-terminal signal peptide sequence and is transported by a secretion system similar to the type II machinery (Arrieta *et al.*, 2004). The crystal structure of LsdA was resolved suggesting a strong overall similarity with SacB from *B. subtilis* but noticeable structural differences in the transfructosylation domain (Martinez-Fleites *et al.*, 2005).

Significant progress was achieved in investigation of the Lsc in *P. syringae* pv. glycinea strain PG4180 (Hettwer *et al.*, 1998; Li and Ullrich, 2001; Li *et al.*, 2006; Schenk *et al.*, 2008). It was shown, that this organism possesses three levansucrase genes, named *lcsA*, *lscB,* and *lscC* (Li and Ullrich, 2001). *LscA* and *lscC* reside on the chromosome whereas *lscB* was found to be located on one of the native plasmids of this organism. Interestingly, these three genes differ not only in their genomic location, but also in the expression status and secretion pattern. Sub-cellular fractionation technique uncovered differential localization of LscB and LscC - the former found predominantly in extracellular space and latter being retained in the periplasm (Li *et al.*, 2006) thus raising the speculation that this differential location might contribute to the utilization of sucrose with highest efficiency. Surprisingly, no product of *lscA* could be found. The corresponding gene was not expressed under any studied condition (Li *et al.*, 2006). In contrast, *lscB* and l*scC* were shown *in vitro* to be transcribed in a temperature- and growth phase-dependent manner with highest expression values at 18°C (Li *et al.*, 2006). The same tendency was observed for these two genes *in planta.* However, the exact mechanism ruling temperature-dependent expression of Lsc in *P. syringae* remained to be uncovered.

Lsc expression and regulation were studied to various extend in Gram-negative bacteria aside of *P. syringae*, such as *E. amylovora* (Bereswil and Geider,1997), *Z. mobilis* (Song *et al.*, 1999), *G. diazototrophicus* (Martinez-Fleites, 2005), *R. aquatilis* (Seo *et al.*, 2002), and *A. xylinum* (Tajima *et al.*, 2000). Obtained data suggested versatile mechanisms ruling these processes. For example, in the plant pathogen, *E. amylovora*, a single *lsc* gene was found to be plant-inducible (Zhao *et al.*, 2005), and its expression was shown to be down-regulated by overexpression of two regulatory proteins, RcsA and RcsB (Bereswill and Geider, 1997). In the enteric bacterium, *R. aquatilis*, expression of the levansucrase-encoding gene *lsrA* was found to be growth-phase dependent, with maximum expression in the stationary phase and was mediated by two antagonist regulators - an activator (LsrS) and a repressor (LsrR), both located upstream of the *lsrA* gene (Seo *et al.*, 2002). Interestingly, Lsc gene *levU* of *Z. mobilis* is transcribed as a bicistronic mRNA together with a sucrase gene possibly due to lack of a phosphoenolpyruvate-dependent carbohydrate phosphotransferase (PTS) system in this organism (Song *et al.*, 1999). Both, Lsc and sucrase, are constitutively transcribed at the basal level and the transcription of both genes

was significantly induced by sucrose (Song *et al.*, 1999). However, the exact regulatory mechanisms mediating this process are not known. In the plant endophyte, *G. diazotrophicus*, Lsc encoded by the *lsdA* gene is co-transcribed with a levanase gene, and the operon showed a growth phase-independent expression pattern. However, none of these regulatory patterns fit the temperature- and growth phase-dependent expression of Lsc in *P. syringae,* which consequently remained to be investigated.

1.3. Temperature-regulated expression of bacterial virulence genes

Many biological processes are optimized for particular temperature regimes and thus temperature changes induce remodeling of bacterial gene expression to adjust the physiology of bacterial cells to new environmental challenges (Ramos *et al.*, 2001). This is of particular importance for pathogenic microorganisms because coordinated regulation of bacterial virulence genes is critical for successful invasion and/or colonization of a host organism. This conclusion is obviously taking into consideration the high energy costs of unnecessary expression of virulence genes outside of the host (Konkel and Tilly, 2000). In order to ensure expression of only appropriate subsets of genes in a given environment, pathogens possess various mechanisms to sense environmental temperature changes and adapt their biochemical processes accordingly. On one hand, temperature alterations as small as 4 to 5°C may induce significant changes in gene expression. On the other hand, temperature ranges permissible for the microorganisms' growth can vary as much as 45°C as shown for e.g. *Listeria monocytogenes* (Seeliger and Jones, 1986).

To be converted into an expression regulating signal, temperature changes have to be sensed by cellular components. For this, bacteria utilize signals generated through changes in nucleic acid or protein conformation as well as changes in membrane composition and fluidity (Hurme and Rhen, 1998). Consequently, it is fair to state that temperature-mediated genetic regulation can take place at the levels of transcription, translation, or enzymatic activities.

1.3.1. Sensing of temperature changes at the DNA level

One level of temperature sensing is the alteration of the DNA structure, precisely, of DNA bending and supercoiling, which is reflecting the superhelical tension adopted by chromosomal or plasmid DNA. Negative supercoiling of bacterial DNA is a crucial determinant of gene expression patterns. Moreover, a given superhelicity is always controlled by homeostatically interconnected regulatory networks (Travers and Muskhelishvili, 2005b).

A number of bacterial proteins are known to be involved in creating and maintaining conformational DNA structures. First, this process is regulated by the interplay between two enzymes, topoisomerase I and DNA gyrase (topoisomerase II); these proteins have different binding affinities for the DNA depending on its curvature (Yang *et al.*, 1988; Rui *et al.*, 2003). Second, abundant nucleoid-associated proteins such as the well-characterized H-NS proteins can induce conformational shifts in local DNA topology and by this can influence transcription of the target genes (Travers and Muskhelishvili, 2005a). Third, RNA polymerase and effectors, which directly modify properties of RNA polymerase, are important parts of diverse regulatory networks (Travers and Muskhelishvili, 2005b).

Additionally, temperature-induced alterations of DNA conformation could directly result in changed H-NS binding affinities as demonstrated for the *hilC* gene of *Salmonella typhimurium* (Ono *et al.*, 2005) or the virulence regulator *virF* in *Shigella* (Falconi *et al.*, 1998; Prosseda *et al.*, 2004). Interestingly, genes, whose transcription is sensitive to local DNA supercoiling, are not randomly distributed along the bacterial chromosome as shown for *E. coli* (Willenbrock *et al.*, 2004). Close to the origin of replication there is a marked cluster of genes, which are repressed by DNA relaxation, whereas at distal locations there is a cluster of genes, whose expression is stimulated by DNA relaxation.

Generally, changes in DNA supercoiling are able to induce the global and simultaneous expression of unlinked genes in response to the environmental temperature stimulus (Konkel *et al.*, 2000). Conformational changes introduced by changes in DNA supercoiling may switch on or off whole sets of genes giving rise to differential developmental programs (Travers and Muskhelishvili, 2005a).

1.3.2. Sensing of temperature changes at the RNA level

Bacterial messenger RNA has a high potential to function as molecular thermometer due to its ability to form complex secondary and tertiary structures when temperature changes (Lease *et al.*, 2000; Narberhaus *et al.*, 2006). Most although not all RNA thermometers are located in 5'-non-translated regions of mRNA molecules and are capable to shield Shine-Dalgarno sequences by forming stable intra-molecular stem-loop structures at low temperatures (Hoe *et al.*, 1993). Melting of these secondary structures during temperature increase permits ribosome access and translation initiation. A striking example of this mechanism was the virulence regulator LcrF in the human pathogen, *Yersinia pestis* (Hoe *et al.*, 1993). Transcription of *lcrF* is indistinguishable at 25°C or 37°C. However, translation of the respective mRNA occurs only at 37°C when the mRNA structure is destabilized, its secondary structure melts, and the translational start becomes available for the respective ribosomal subunits to bind.

Thermo-responsive synthesis of antisense RNA may be another powerful mechanism to control gene expression as shown for the porins, OmpF and OmpC, of *E. coli* (Yigit *et al.*, 2002). Expression the respective genes is controlled by temperature-dependent binding of antisense RNA to the 5' end of the mRNA.

1.3.3. Sensing of temperature changes at the protein level

Proteins have a high potential to function as molecular thermometers due to thermoresponsive conformational changes. Coiled-coil structures, which can be formed by certain proteins, represent a versatile and flexible motif, which is crucial in mediating interactions between proteins (Lupas, 1996). A classical example for this is the TlpA protein of *S. typhimurium*. This autoregulatory transcriptional repressor occurs in a particular folding equilibrium to regulate its DNA binding activity (Hurme *et al.*, 1996; Hurme *et al.*, 1997). Coiled-coil structures constitute an oligomerization domain of this protein. Temperature-dependent expression of *tlpA* relies on the equilibrium between unfolded monomers and folded oligomers of TlpA thus functioning as the temperature sensor. Interestingly, unrelated proteins such as H-NS and the growing family of H-NS-like proteins have similar coiled-coil

structures, form oligomers, and are thought to be regulated in the similar manner (Ono *et al.*, 2005). The ability of H-NS to rapidly and reversibly self-associate and ultimately be organized in high-order oligomeric structures affects its cooperative binding to DNA. Elevation of temperature was reported to impede H-NS to form high-order structures and thus disable the repression of the target genes at higher temperatures (Ono *et al.*, 2005). This particular feature of H-NS also determines its involvement in cold repression of bacterial genes (Williams *et al.*, 1997).

Sensitivity of certain transcriptional regulators to proteolytic degradation varies with temperature as shown for small H-NS-like proteins of the Hha-Ymo family (Mikulskis *et al.*, 1994; Madrid *et al.*, 2007). These proteins were demonstrated to be involved in anti-silencing of some genes of the H-NS regulon with respect to environmental stimuli such as temperature or osmolarity (Cornelis *et al.*, 1991; Mourino *et al.*, 1994). Sensitivity of these proteins to proteolysis is modulated by growth temperature: at lower temperatures their stability increases (Jackson *et al.*, 2004).

Another type of protein-mediated thermoregulation of gene expression was reported for the chaperones, DnaK and DnaJ, which are involved in heat shock responses via modulating stability of the RpoH sigma-factor (Gamer *et al.*, 1996). At permissive temperatures, the well-characterized DnaK-DnaJ complex binds RpoH and disposes it for proteolytic degradation. Upon heat shock, DnaK-DnaJ binds misfolded proteins and simultaneously releases RpoH. Consequently, the balance between DnaK-DnaJ loaded with misfolded proteins or RpoH represents a molecular thermometer.

1.3.4. Alterations in membrane fluidity and thermosensing by bacterial two-component regulatory systems

Bacterial membrane composition and its physical state determined by its fluidity may change drastically in response to temperature shifts (Vigh *et al.*, 1998). The level of fatty acid saturation decreases at lower temperatures in order to maintain proper membrane fluidity. In consequence, membrane-embedded proteins such as histidine kinases, have been postulated to change their conformation and correspondingly their activities in further

signal transduction (Smirnova *et al.*, 2004). An example for such a sensory kinase is present in the modified two-component regulatory system required for phytotoxin synthesis in *P. syringae* PG4180. This system consists of two response regulators, CorR and CorP, and the histidine protein kinase, CorS, and regulates thermoresponsive expression of coronatine biosynthetic genes (Smirnova *et al.*, 2002; Smirnova *et al.*, 2004; Braun *et al.*, 2008; Smirnova *et al.*, 2008). This system is inducing gene expression at the virulence-promoting temperature of 18°C while it is inactive at the optimal growth temperature of *P. syringae*, 28°C. Detailed investigation of CorS membrane topology using various translational fusions with *phoA* and *lacZ* showed that the hydrophobic N-terminus of CorS contained six trans-membrane domains, from which one might apparently 'flip' into the periplasm at 18°C but not at 28°C. Interestingly, the flipping domain bears the essential catalytic histidine (H)-box for CorS autophosphorylation. Consequently, when this domain was looped into the periplasm and not placed into the membrane at the elevated temperature, it was inactive and autophosphorylation was disabled (Smirnova *et al.*, 2004). It was proposed that the degree of fatty acid saturation in phospholipids of the membrane might trigger conformational changes of CorS in dependence of temperature (Smirnova *et al.*, 2008).

1.4. Thermo-regulated traits in plant-pathogenic bacteria

For many animal and human pathogens, expression of genes involved in virulence is induced upon temperature upshift, which corresponds to the body temperature of the warm-blooded hosts. In contrast, for plant pathogenic bacteria almost all virulence genes studied with respect to temperature exhibited increased gene transcription at temperatures well below the optimal growth temperatures (Smirnova *et al.*, 2001). The list of thus-regulated genetic traits involved in virulence and epiphytic fitness includes those involved in bacteria-to-plant gene transfer, production of plant cell-wall-degrading enzymes, phytotoxin syntheses, ice nucleation activity, exopolysaccharide production, and the type III protein secretion machinery. Classical examples are the production of phytotoxins such as coronatine in *P.syringae* pv. PG4180 and phaseolotoxin in *P. syringae* pv. phaseolicola (Bender *et al.*, 1999). Biosynthetic genes encoding synthesis of either of these toxins

showed strong expression at 18°C, whereas no toxins were synthesized at 28°C (Palmer *et al.*, 1993; Ullrich *et al.*, 1995; Budde *et al.*, 1998). A very similar expression pattern was found for the *hrp* gene cluster encoding the type III secretion system of *P. syringae* (van Dijk *et al.*, 1999) and *E. amylovora* (Wei *et al.*, 1992). Synthesis of the EPS, alginate, was demonstrated to be diversely thermoresponsive in different *P. syringae* strains (Keith *et al.*, 2003; Schenk *et al.*, 2008). Alginate production is regulated by different two-component regulatory systems as well as by the alternative sigma factor, AlgT (Schenk *et al.*, 2008). Interestingly and important for the current study, *lsc* genes of *P. syringae* PG4180, which are thought to contribute to the overall epiphytic fitness of this bacterium exhibited strong expression at 18°C in comparison to 28°C (Li *et al.*, 2006).

For many *P. syringae* species low temperature and high humidity are playing a crucial role in triggering the infection process and disease symptoms. It was hypothesized that due to the high humidity and lower temperature of the plant's surface and interior as compared to ambient air temperatures, water films and aerosols are formed, which in turn may foster the motility of the phytopathogen and its transport into the plant's apoplast, where nutrients are available and the infection can proceed (Smirnova *et al.*, 2001). Since the array of low-temperature induced genes, which contribute to successful infection, is quite diverse, the pathogen must possess different regulatory mechanisms to coordinate host colonization and infection. As shown above, for some of the epiphytic fitness and virulence mediating traits such mechanisms were uncovered. However, for many other thermoresponsively expressed genes of *P. syringae* including those encoding Lsc detailed regulatory mechanism(s) still remain to be uncovered. Consequently, their in-depth investigation will provide novel insights to the global cold-induced developmental program of this plant-pathogenic bacterium.

2 AIMS OF THE WORK

Levansucrases of the plant pathogenic bacterium, *P. syringae* PG4180, catalyze formation of the EPS, levan, in sucrose-rich environments. This process must be tightly regulated at the level of transcription due to its demand for cellular energy and the potential loss of this energy once the enzymes are released under none-appropriate conditions. Consequently investigation of regulatory links between polymer synthesis and internal metabolic pathways is important for understanding the ecological function(s) of levan and to develop strategies to efficiently combat the effects mediated by bacterial EPS (Ullrich, 2009).

The main goal of this work was to better understand the regulatory system(s) governing levan formation in *P. syringae*. For this, transcriptional regulators mediating the temperature-dependent expression of *lscB* gene encoding levansucrase had to be identified and characterized in terms of function. DNA affinity chromatography and functional screening for positive regulators using a heterogenic bacterial host system were applied revealing four regulatory factors, HexR, MvaT, MvaU, and LscR. Since HexR, MvaT, and MvaU were found to be repressors of *lscB* expression while LscR had an activating function, another major goal was to investigate the molecular interplay of the regulatory proteins involved. Finally, the identified regulatory genes had to be analyzed in terms of their expression and mutants were to be obtained for some of the regulatory factors.

3 RESULTS

Results are represented by publications originated during this PhD thesis and were sorted in a thematic order as follows:

Levansucrase and levan formation in *Pseudomonas syringae* and related organisms (Review).

Abhishek Srivastava, Daria Zhurina and Matthias S.Ullrich

(M.Ullrich (ed.) ''Bacterial Polysaccharides – Current Topics and Innovations''; Horizon Scientific Press, Norwich, in press)

Thermo-responsive expression and differential secretion of the extracellular enzyme levansucrase in the plant pathogenic bacterium *Pseudomonas syringae*

Hongqiao Li, Alexander Schenk, Abhishek Srivastava, Daria Zhurina and Matthias S. Ullrich

(2006; FEMS Microbioogyl Letters; 265: 178-185.)

Interaction of LscR with H-NS-like proteins, MvaT and MvaU, determines temperature-dependent levansucrase expression in *Pseudomonas syringae*

Daria Zhurina, Helge Weingart, Annette Arndt, Melanie Brocker, Michael Bott, Bernhard J. Eikmanns and Matthias S. Ullrich

(to be submitted)

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

Daria Zhurina*, Abhishek Srivastava*, Helge Weingart, Annette Arndt, Melanie Brocker, Michael Bott, Bernhard J. Eikmanns and Matthias S. Ullrich

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(to be submitted)

3.1 Levansucrase and levan formation in *Pseudomonas syringae* and related organisms (Review)

Levansucrase and levan formation in *Pseudomonas syringae* and related organisms

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Abstract

Bacterial levan is formed wherever bacteria encounter moderate to high sucrose concentrations, i.e. in the plant environment, in the mammalian oral cavity, or during the fermentation of plant-borne substrates, i.e. for bio-ethanol production. Consequently, the transcriptional and translational regulation, synthesis, secretion, and enzymatic properties of levan-forming enzymes such as levansucrase (Lsc) from various bacterial species have raised substantial interest among researchers in plant pathology, dental medicine, food manufacturing, and renewable energy technologies. Experimental work with Lsc was accelerated by three major features, i.e. its intrinsically high protein stability as an extra-cellular enzyme, no need for reaction-catalyzing co-factors, and an enormous ease of enzymatic detection due to its glucose releasing activity. Aside of many possible applied aspects, Lsc enzymatic activity as well as its regulation and secretion are exciting biochemical and molecular model systems for the in-depth understanding of global carbon utilization, protein transport processes, and biofilm formation.

1. Levan as a fructosyl polymer

Bacterial and plant-borne levans are typical fructan polymers. The term fructan covers both inulin and levan. Levan is made up of numerous fructosyl residues derived from sucrose and single glucose head groups. The linear 2,6-linked levan molecules are flexible and tend to be left-handed twist, whereas the twist of inulin is right-handed (French, 1989). Sucrose (β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside) is a covalently (1→2-α, β-glycosidic bond) bonded compound consisting of glucose and fructose. Levan polymers can be several mega Dalton in size and levan polymers together become visible to the naked eye as a semi-translucent, whitish, homogenous mucus. Levan has the following interesting biochemical characteristics: It is biodegradable, water soluble, and forms liquid crystals being amorphously microcrystalline (Fuchs, 1956; Viney et al., 1993). While small fructans have a rather sweet taste, longer fructan chains such as those found in levan form emulsions with a fat-like texture and have a rather neutral taste (Vijn and Smeekens, 1999). Below 20% solids, levan solutions exhibit typical Newtonian behavior (Simms et al., 1990). Levan has a tensile strength of 991 pounds per square inch and possesses flexible five-membered rings packed in a dense, spheroid structure. At low concentrations, the spheres roll smoothly. When the density of spheres passes a critical point and come into contact with each other, a rapid increase in viscosity is observed (Stivala and Zweig, 1981). At 50% solids, the levan polysaccharide resembles chewing gum (Simms et al., 1990). Unlike most other polysaccharides, levan has a compact spherical structure and does not swell in water. The intrinsic viscosity of levan is only 0.14 dl/g

Figure 1. Typical colonies of *P. syringae* PG4180 grown on sucrose-containing minimal medium agar plates. The whitish mucus in form of dome-shaped colonies represents levan.

(Kasapis et al., 1994; Kasapis and Morris, 1994). Levan is resistant to organic solvents such as jet fuel and *d*-limonene (Stivala and Bahary, 1978) and is fairly heat stable with a melting point of 225°C.

Similar in structure to levan, fructan was first described by the German botanist, Rose, in boiling water extract from *Inula helenium* (Rose, 1804). This substance was later named inulin (Thompson, 1818). The term `levulan´ was first described as an undesirable by-product in sugar cane and liquor processing that caused increase in viscosity (Avigad, 1968; Lippmann, 1881). The term "levan" was introduced as analogous to "dextran" for a compound that was produced by an isolate of *Bacillus sp.* growing on sucrose (Greig-Smith, 1901). Gummy levan was later obtained from spores of *Aspergillus sydowi* (Kopeloff et al., 1920). *Pseudomonas pruni* was described as the first levan/dextran-forming plant pathogen (Lyne et al., 1940). In 1944, levan was first shown to be synthesized from sucrose by cell-free enzymes (Hestrin and Avineri-Shapiro, 1944). Shortly after this, levans from various micro-organisms were shown to be identical in terms of their immunological features (Hehre et al., 1945). Levan was then described as fructofuranose-containing polysaccharide synthesized by plants and many aerobic bacteria from sucrose or raffinose (Evans and Hibbert, 1946). In 1957, levan from *Corynebacterium* species was described to possess β-2,1 and β-2,6 linkages (Avigad and Feingold, 1957). Finally, the interaction between the lectin, concanavalin A, and levan was first demonstrated (So and Goldstein, 1968).

Levan formation is of particular importance in three major areas: The plant-borne disaccharide, sucrose, is a readily available carbon source for i) plant pathogens; ii) bacteria of the oral cavity; and iii) microbes associated with bio-ethanol production. These microorganisms use the extra-cellular enzyme, levansucrase, to release glucose from sucrose and to polymerize fructosyl residues into the branched polymer levan. Glucose is then consumed inside the cell while the polymerized fructosyl residues along with single glucosyl head groups make up the extra-cellular mucus loosely associated with the cell envelope. Bacterial levan accumulation in form of dome-shaped mucoid structures is easily visualized when those microbes are grown on sucrose-containing agar media (**Fig. 1**).

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2. Levansucrases – enzymatic properties and relatedness to other enzymes

Levansucrase (Lsc) (E.C.2.4.2.10.) belonging to the superfamily of ß-fructosidases and having a five-bladed ß-propeller fold is found in various gram-positive and gram-negative bacteria. This extra-cellular enzyme cleaves sucrose into glucose and fructosyl residues, releases glucose, and polymerizes the fructosyl residues to form the ß-

(2,6) linked and ß-(2,1) branched exopolysaccharide (EPS) levan, which has singular glucosyl head groups (Han, 1990). Additionally, Lsc is able to hydrolyze levan and to exchange glucosyl head groups of levan polymers as demonstrated by feeding bacterial cells with ^{14}C-labelled glucose (Kaspasi et al., 1994).

The mirror reaction of Lsc's forward reaction is performed by dextransucrase (EC 2.4.1.5), a kind of glucansucrase from *Leuconostoc mesenteroides,* which forms dextran, a glucose-containing polymer (Bailey et al., 1957). Alternansucrase (EC 2.4.1.140) - another variety of glucansucrase from *L. mesenteroides,* can synthesize alternan with alternating α-(1,6) linkages and α-(1,3) linkages (Cote and Robyt, 1982). Another glycosyltransferase named mutansucrase from *Streptococcus mutans* (Wenham et al., 1981) can synthesize mutans with mixed α-1,3 predominant, and α-1,6 linkages. A recently discovered glucansucrase-like enzyme termed reuteransucrase (EC 2.4.1.5) from *Lactobacillus reuteri* produces reuteran that contains mainly *α*-1,4 linkages together with *α*-(1,6) and α-(1,4,6) linkages (Kralj et al., 2004). Inulosucrase (EC 2.4.1.9) of Leuconostoc *citreum* results in inulin β- 2,1 linear fructans (Olivares-Illana et al., 2003). Amylosucrase (EC 2.4.1.4) from *Neisseria polysaccharea* has been shown to produce starch like amylan with α-1,4 linkages between glucosyl molecules (Sarcabal et al., 2000). Protein-Nπ phosphohistidine: sugar Nπ-phosphotransferase (EC2.7.1.69), which is an Enzyme II of the phosphor-transferase system can phosphorylate the fructosyl residue of sucrose thus generating sucrose 6-phosphate (Kornberg and Riordan, 1976; Postma and Roseman, 1976). In summary, one can clearly see that nature has evolved many different enzymatic reactions breaking down the most abundant plant transport sugar, sucrose.

Peptide sequences, enzymatic properties, protein location, and transcriptional regulation of Lsc differ most noticeably between gram-positive and gram-negative bacteria. In gram-positive microorganisms, Lsc has been studied in detail in *Bacillus subtilis* (SacB), in different food-grade *Lactobacillus* and oral *Streptococcus* species, and in *Leuconostoc mesenteroides* (Morales-Arrieta et al., 2006; Seibel et al., 2006; Tieking et al., 2005; van Hijum et al., 2006). The three-dimensional structure of SacB of *B. subtilis* had been resolved revealing interesting aspects of the enzymatic activity of this enzyme (Meng and Futterer, 2003). Most noticeably is that i) all studied Lsc of gram-positive bacteria possess typical N-terminal signal peptides and are secreted via a classical type II protein transport mechanism across the membrane (Benyahia et al., 1988); ii) some were found to be membrane-bound mediated by C-terminal lipid anchors (Tieking et al., 2005). ; and iii) levan formation by *Streptococcus* sp. contributed to oral biofilm formation associated with dental plaque (Tentuta et al., 2006).

For gram-negative bacteria, Lsc and its transport have been studied to variable extent in *Pseudomonas syringae* (see below for details), *Erwinia amylovora*, *Zymomonas mobilis*, *Gluconacetobacter diazotrophicus*, *Rahnella aquatilis*, and *Acetobacter xylinum* (Martinez-Fleites et al., 2005; Seo et alo., 2002; Song et al., 1999; Tajima et al., 2000; Vigants et al., 2001). Generally, the Lsc proteins of gram-negative bacteria share higher similarities among each other as compared to those enzymes from gram-positive organisms. The Lsc protein, LevU, from *Zymomonas mobilis* was found to be membrane-associated and mutational analysis had revealed that the polymerizing activity could be uncoupled from the sucrolytic activity of this enzyme (Song et al., 1999). In contrast to Lsc enzymes of other gram-negative bacteria, that of the plant endophyte, *G. diazotrophicus*, (LsdA) possesses a typical N-terminal signal peptide sequence and was shown to be transported by a type II protein secretion mechanism (Arrieta et al., 2004). The crystal structure of LsdA was resolved suggesting a strong overall similarity with SacB from *B. subtilis* but noticeable structural differences in the transfructosylation domain (Martinez-Fleites et al., 2005).

In the plant pathogen, *P. syringae*, at least two Lsc gene products, LscB and LscC, have been identified and analyzed in detail (Hettwer et al., 1998; Li and Ullrich, 2001; Li et al., 2006). A third allele coding for Lsc, *lscA*, was shown to be not expressed but its gene product was fully functional upon heterologous expression under control of an inducible promoter (Hettwer et al., 1998). The genes coding for LscB and LscC reside on an indigenous 60-Kb plasmid and the chromosome, respectively. In this context it is notable that *P. syringae* seems to be the only bacterial organism with multiple Lsc iso-enzymes. Remarkably, the presence of 2-3 very similar genes for Lsc iso-enzymes is highly conserved in the genome of strain PG4180 (pv. glycinea) as well as in all three fully sequenced genomes of additional *P. syringae* strains, which are strain DC3000 (pv. tomato), strain B728 (pv. syringae), and strain 1449A (pv. phaseolicola) (www.pseudomonas.com). This conservation may reflect a particular importance of Lsc for the life style of this foliar and biotrophic plant pathogen. Even more excitingly, the two expressed gene products accumulated in either the periplasmic space or the extra-cellular space, respectively, making it tempting to speculate that both enzymes may possess specific functions (Li et al., 2006). Differential localization of LscB and LscC was demonstrated in sub-cellular fractions derived from a reliable osmotic shock extraction procedure (see below). Sucrose acts as a major transport and storage sugar molecule in diverse crop plants. It is therefore tempting to speculate that the biotrophic plant pathogen, *P. syringae*, has evolved enzymatic mechanisms to particularly utilize this disaccharide with high efficiency.

3. Role of levan in biofilm formation and ecological fitness

Biofilm formation and EPS syntheses have always been tightly associated with each other due to the fact that the matrix surrounding cells in biofilms consists among others of diverse polysaccharides, (glyco-)proteins, and extra-cellular DNA (Branda et al., 2005; Flemming et al., 2007; Goller and Romeo, 2008). Numerous studies have shown that mutants defective in EPS synthesis show either no biofilm formation or significantly altered biofilm structures. For the current review, biofilms produced by pseudomonads and other bacteria occurring in sucrose-rich environments are of particular interest. Alginate, a poly-anionic EPS, is the best-investigated component of mucoid *P. aeruginosa* biofilms and its role in biofilm formation and infection has been established (Ryder et al., 2007). However, recent reports demonstrated that a cellulose-like fiber structure encoded by the *psl* and *pel* gene clusters of non-mucoid *P. aeruginosa* is essential for biofilm formation (Ma et al., 2007; Ryder et al., 2007). Expression of *psl* seems to depend on the *wsp* locus encoding for a chemosensory system and mediated a wrinkly spreader phenotype in *P. fluorescens* (Spiers et al., 2002) indicating the complexity of regulation of biofilm formation. Another important regulatory input for *P. aeruginosa* biofilms comes from the global two-component regulatory system, GacS/GacA (Goodman et al., 2004). Similar results were found for biofilms formed by *P. putida* (Chang et al., 2007) and those produced by *P. fluorescens* (Kives et al., 2006).

For *P. syringae*, our recent results demonstrated that neither alginate nor levan or the alternative sigma factor, AlgT, were essential for biofilm formation indicating a role for a yet-to-be identified additional EPS for biofilm structure formation (Laue et al., 2006; see below for more details). To elucidate this, mutants with defects in either, levan formation, alginate synthesis, or AlgT, were subjected to flow chamber experiments. Biofilms from the wild type of PG4180 as well as from levan- or alginate-deficient mutants showed indistinguishable formation patterns. However, alginate produced by *P. syringae* had an impact on initial cell attachment to artificial surfaces while levan accumulated in voids of bacterial colonies thus potentially representing a storage compound. Formation of biofilms on plant surfaces significantly enhances the epiphytic fitness of pathogenic *P. syringae* versus antagonistic epiphytes, such as

Pantoea agglomerans (Danhorn and Fuqua, 2007). An H-NS-like negative regulator, MvaT, controlled the fimbrial *cup* gene clusters and biofilm formation as well as exo-product formation in *P. aeruginosa* (Vallet et al., 2004) and in *P. fluorescens* (Baehler et al., 2006), respectively. Not surprisingly, MvaT also played a role in quorum sensing-associated N-acylhomoserine lactone formation in *P. aeruginosa* (Diggle et al., 2003) reflecting the complexity of regulatory effects mediated by this DNA binding protein.

The wild type of *P. syringae* strain PG4180 and its respective EPS mutants were analyzed with respect to surface attachment, lectin binding, and biofilm formation in flow chambers (Laue et al., 2006). Interestingly, neither alginate nor levan had a major impact on biofilm formation and its structure. In contrast to levan, presence of alginate seemed to improve cell-surface attachment. The lectin, concanavalin A (ConA), was identified to be specifically staining levan since its fluorescent signal was only detectable when Lsc was expressed and when sucrose was provided to the flow chamber medium. A third yet-to-be identified fibrous EPS structure was marked with a lectin from *Naja mossambica* (Laue et al., 2006). Surprisingly, ConA-stainable levan accumulated in large cell-free voids in the center of micro-colonies forming the biofilm. Since those centered voids are also formed when Lsc was absent or when no sucrose was provided, it may be speculated that these voids are the physical result of colony growth due to micro-aerobic and nutrient-deprived conditions inside colonies. We hypothesized that levan-filled voids in *P. syringae* biofilms might represent storage compartments being "loaded" during sucrose presence and being utilized by the bacterial cells during periods of starvation (Laue et al., 2006). This assumption was fueled by the idea that the poly-fructan, levan, is a side-product of glucose utilization from sucrose via Lsc activity and its fructosyl residues could potentially being used diauxically when glucose is used up and no other carbon sources are available. Consequently, it is logical to next test sucrose-grown PG4180 biofilms under starvation conditions. Should the accumulated levan disappear and thus been used up subsequently, our hypothesis would be proven. A potential direct or indirect linkage of Lsc activities with the general glucose metabolism should then be analyzed in detail.

The effects of sugars, particularly sucrose, on biofilm formation have been in-depth evaluated in oral (dental plaque) biofilms mainly formed by gram-positive bacteria (Tentuta et al., 2006; Vale et al., 2007). Presence of sucrose significantly enhanced biofilm build-up in comparison to fructose, galactose, glucose, or lactose for diverse organisms, which all produce Lsc (Yang et al., 2006). Furthermore, it was demonstrated that sucrose addition led to distinct population shifts when different streptococci and lactobacilli species were co-cultivated as dental plaque biofilms suggesting that this sugar gave advantage to lactobacilli (Tentuta et al., 2006). The same laboratory showed that sucrose addition to oral biofilms led to increased EPS synthesis, acid accumulation, and subsequent enamel demineralization in patients (Vale et al., 2007). When searching for differentially expressed genes during biofilm formation in *Staphylococcus aureus*, *S. mutans*, and *B. subtilis*, sugar uptake PTS genes were found (Sutrina et al., 2007). In an unrelated study, numerous bacterial contaminants were identified during corn (sucrose)-based fuel ethanol production by yeasts (Skinner-Nemec et al., 2007). Most of the identified bacterial contaminants were gram-positive and all of them contained Lsc genes allowing the speculation that levan synthesis negatively impacted ethanol production yields. One can easily envision that levan build-up in diverse production facilities such as bioreactors and tubes of any commercial (and thus non-sterile) high-throughput fermentation of sucrose-rich bio-fuel substrate will have a major negative impact on production yields.

4. Regulation and secretion of levansucrase

In the current review article most emphasis is brought to the transcriptional regulation and differential secretion of Lsc from *P. syringae* (see

below). However, the extensive work by others with different bacterial organisms should be considered at first place. Expression of the *sacB* gene in various Bacillus species is sucrose- and salt-inducible via the SacY anti-terminator and the DegSU two-component system (Biedendieck et al., 2007b; Li and Ferenec, 1997). In the plant pathogen, *E. amylovora*, which possesses a single *lsc* gene, overproduction of the regulatory proteins, RcsA and RcsB, strongly reduced Lsc synthesis (Bereswill and Geider, 1997). Moreover, *lsc* expression was found to be plant-inducible in this phytopathogenic organism (Zhao et al., 2005). In contrast to the *lsc* genes of *P. syringae* and other gram-negative bacteria, the Lsc-encoding *levU* gene of *Zymomonas mobilis* is transcribed as a bi-cistronic mRNA together with a sucrase gene (Song et al., 1999). This might possibly be due to the lack of an phosphoenolpyruvate-dependent carbohydrate phosphotransferase (PTS) system in this organism. In the enteric bacterium, *R. aquatilis*, expression of the Lsc-encoding *lsrA* gene

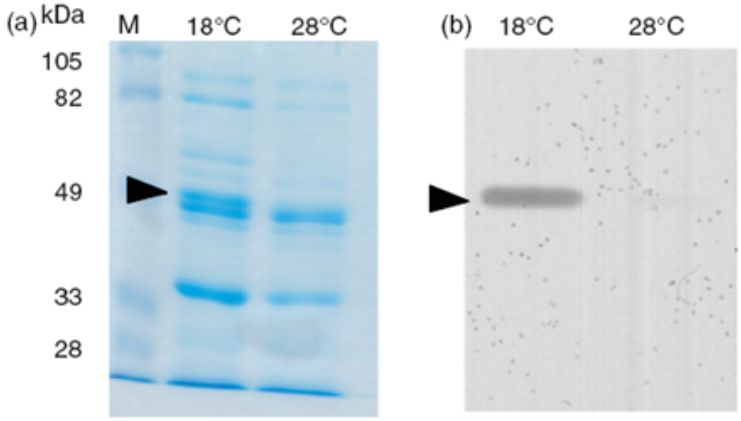

Figure 2. Extra-cellular protein profiles of P. syringae PG4180 as depicted by SDS-PAGE (left) and zymographic detection of levansucrase in by native PAGE (right). Levansucrase activity corresponds to a ~50 kDa protein found predominantly during bacterial growth at 18°C.

was shown to be stationary phase-dependent but *rpoS*-independent and was mediated by an activator (LsrS) and a repressor (LsrR) (Seo et al., 2002). The gene encoding for LsdA of *G. diazotrophicus* is encoded on a constitutively synthesized bi-cistronic mRNA together with a levanase gene (Arrieta et al., 2004). Interestingly, the *sacB* gene, its sucrose-inducibility, and the export characteristics of its gene product from *B. megaterium* were successfully used as biotechnological tools for heterologous protein recovery from bacterial supernatants (Biedendieck et al., 2007a and 2007b; Malten et al., 2006). It should also been noted that – for a long time – the signal peptide-deprived *sacB* gene of *B. subtilis* has been used as a lethal gene marker on plasmids used for homologous recombination experiments. This is due to the fact that intra-cellular Lsc activities lead to an accumulation of levan product inside the cell and subsequently to the bursting and disruption of cells expressing signal peptide-deprived *sacB*.

For years, our laboratory had been interested in the temperature-dependent gene expression of the plant pathogenic bacterium, *P. syringae* pv. glycinea PG4180 (for reviews see Braun et al., 2007; Ullrich et al., 2000). Several successful attempts were undertaken to identify low-temperature induced genetic loci or gene products. In this context, extra-cellular protein profiles were investigated and the 50-KDa protein, Lsc, was found to be secreted at the low and virulence-promoting temperature of 18°C in contrast to the optimal growth temperature of this bacterium, 28°C

(Li and Ullrich, 2001). This phenotype could be confirmed by zymographic detection of Lsc in native polyacryl amide gele electrophoresis (**Fig. 2**). In contrast to the well-studied Lsc of *B. subtilis* (SacB), *P. syringae* Lsc was not cleaved during export and secretion and did not possess a recognizable signal peptide suggesting that it was not transported via the general secretory pathway. This was proven by N-terminal sequencing of the extra-cellular protein. A total of three functional *lsc* genes were cloned and mutagenized in PG4180 (Hettwer et al., 1998; Li and Ullrich, 2001). The gene, *lscA*, was found to not be expressed under any condition tested (i.e. *in vitro*, *in planta*, or under numerous growth conditions) (Schenk et al., 2008) although it could be expressed under control of the P_{lac} promoter in *E. coli* and *P. syringae* (Hettwer et al., 1998; Li and Ullrich, 2001). In contrast, simultaneous mutation of both, *lscB* and *lscC*, led to a levan-negative phenotype in mutant PG4180.M6 while single mutation of either gene rendered the resulting mutants levan-positive. Levan syntheses by the two single locus mutants were indistinguishable for each other. These results suggested that both gene products were responsible for levan formation on sucrose-containing media. The predicted gene products of *lscB* and *lscC* showed 96% similarity towards each other indicating the potential of gene duplication. In line with this, *lscB* is located on an indigenous 60-

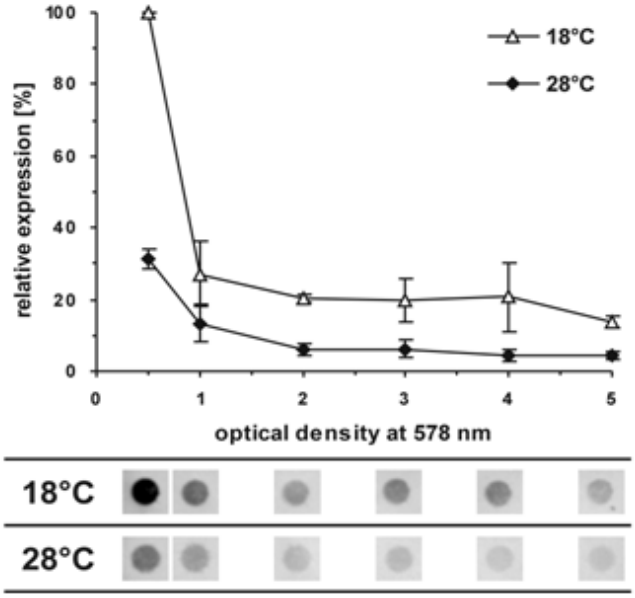

Figure 3. Transcriptional analysis of *lscB* and *lscC* by quantitation of transcript accumulation in P. syringae PG4180 during growth at 18°C and 28°C as determined by RNA dot blot analysis and as described by Li et al. (2006).

kb plasmid while *lscC* has a chromosomal location (Li and Ullrich, 2001). The two gene products differ in only five amino acyl residues, most of which represent conservative replacements.

Both gene products could be extracted from the periplasmic space suggesting that they were not exported and secreted by either, Type I, Type III, or Type IV transport systems (Li et al., 2006). In that study it was furthermore shown that both, *lscB* and *lscC*, are expressed *in vitro* in a temperature-dependent manner with maximum mRNA accumulation during the early logarithmic growth phase at 18°C (**Fig. 3**). During later growth stages, the thermo-responsive difference remained while

the overall amount of *lscB* and *lscC* mRNAs declined dramatically. The expression profiles of both genes were nearly identical. These results indicated a potentially particular role for Lsc during early logarithmic growth. Alternatively, it could be argued that early log phase synthesis of Lsc is sufficient for the bacterial cells since Lsc is an extremely stable protein. The respective *in vitro* results could be confirmed when *lsc* gene expression was analyzed during the *in planta* infection process using a modified and optimized total RNA extraction procedure (Schenk et al., 2008).

Excitingly, both gene products showed a differential secretion pattern: LscB was found in minor amounts in the periplasm but with large quantities in the supernatant while LscC mostly remained in the periplasm with only minor traces found in the supernatant (Li et al., 2006). Interestingly, four of the five divergent amino acyl residues from the predicted protein sequences were conservative replacements while the fifth was a cysteine-to-serine exchange allowing speculations about different secondary structure formation (one versus two disulfide bridges). The quality of the sub-cellular fractionation technique was verified by expression of a MucA-PhoA hybrid, which clearly resided in the periplasm (Li et al., 2006). A physiological rationale for this interesting differential secretion pattern remained to be elucidated but once again fueled reasonable speculations about distinct enzymatic functions for either protein.

In absence of direct experimental evidence, i.e. mutation of the particular transport system, exported and secreted proteins can be assigned as substrates for a given transport system by three major criteria: a) presence or absence in the periplasm of gram-negative bacteria; b) intrinsic amino acid sequence or mRNA sequence characteristics, which act as recognition signals for transport during or past translation; or c) signal peptide cleavage during transport. Interestingly, the extra-cellular Lscs of *P. syringae* can not be clearly assigned to any of the known protein transport system since these enzymes lack a signal peptide, are found in the periplasm, and do not possess any assignable amino acid sequences characteristic for any of the systems. Whether or not their mRNAs harbor specific signals – such as postulated for Type III secreted proteins – is yet unknown. With these features, Lscs of *P. syringae* clearly differ from Lscs of *B. subtilis* and *G. diazotrophicus*, which both possess signal peptides and were shown to be transported via the general secretory pathway (Arrieta et al., 2004; Benyahia et al., 1988). In a preliminary study, *P. syringae* mutants with defects in the *hrp* Type III protein secretion system and with a defect in the twin arginine transport (TAT) secretion pathway were checked for levan formation and extra-cellular as well as sub-cellular Lsc accumulation. Both mutants showed the normal wild type phenotype for both assays suggesting that neither the Type III secretion system nor the TAT pathway was responsible for Lsc export or secretion. It may be speculated that export and secretion of Lsc in *P. syringae* is mediated by an yet-to-be characterized novel protein translocation machinery or by a thus far unprecedented combination of known protein transport systems.

Currently ongoing work in our laboratory allowed the determination of the transcriptional start sites of *lscB* and *lscC* as well as the characterization of the respective upstream sequences in *P. syringae* (Srivastava et al, unpublished data). Not surprisingly, variable length of upstream sequence gave rise to variable expression levels for *lscB* suggesting the potential for several (independent) regulatory factors affecting transcription. Regulators such as those found in the enterobacterium, *E. amylovora*, i.e. RcsA and RcsB (Bereswill and Geider, 1997) could not be identified in the genome of *P. syringae*. Virulence assays with a *lsc*-deficient mutant of *P. syringae* indicated no particular role of levan formation for virulence or pathogenicity. However, the ecological fitness and the general survival efficiency were affected. Heterologous expression in a related levan-minus *Pseudomonas* strain with the help of a genomic library derived from *P. syringae* was used to identify a potential activator protein required for Lsc expression (Zhurina et al., unpublished data). Finally, potential repressor proteins involved in the thermoregulation of Lsc expression have been identified using DNA affinity

chromatography. These preliminary results will help to further elucidate the likely complex regulatory system involved in *lsc* gene expression in future studies.

5. Conclusions

The plant-borne disaccharide, sucrose, is a ubiquitously occurring and readily available carbon source for plant pathogens, bacteria of the oral cavity, and microbes associated with bio-ethanol production. Many of these microorganisms use the extra-cellular enzyme, levansucrase, to release glucose for consumption from sucrose and to polymerize fructosyl residues into the multiply branched polymer levan. Our previous studies showed that two levansucrases of the gram-negative bacterium, *P. syringae* are expressed in a temperature- and growth phase-dependent manner with maximal levan formation at the virulence-promoting temperature of 18°C as opposed to the optimal growth temperature, 28°C. They showed distinct export and secretion patterns distinguishable from any classical protein secretion pathway. Expression of levansucrase led to an accumulation of levan in 'storage compartments' of biofilms. In our future work, it will have to be determined in detail how levansucrase gene expression is regulated, how the enzymes are transported via inner and outer membrane, and what the actual physiological role of levan formation is in context of carbon metabolism. The role of a newly identified regulator and its potential interaction with repressor proteins will be investigated. The export/secretion pathways of both levansucrases need to be identified and characterized. The hypothesis that levan formation might be a form of bacterial 'waste disposal' for later carbon recycling will be tested using an array of divergent and complementary experiments. Finally, the individual enzymatic activities of both iso-enzymes need to be elucidated in detail.

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3.2 Thermo-responsive expression and differential secretion of the extracellular enzyme levansucrase in the plant pathogenic bacterium *Pseudomonas syringae*

Thermoresponsive expression and differential secretion of the extra-cellular enzyme levansucrase in the plant pathogenic bacterium *Pseudomonas syringae* pv. glycinea

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ABSTRACT

In the plant pathogen *Pseudomonas syringae*, production of the exopolysaccharide levan is mediated by extra-cellular levansucrase (Lsc), which is encoded by two functional genes, *lscB* and *lscC*. Comparison of extra-cellular protein profiles of *P. syringae* pv. glycinea PG4180 grown at 18 and 28°C and Western blots revealed that Lsc was predominantly found in the supernatant at 18°C, a temperature fostering virulence of this pathogen. Northern blot analysis indicated that transcription of *lscB* and *lscC* was temperature-dependent. Quantification of Lsc in supernatants and cellular protein samples of mutants defective in either *lscB* or *lscC* confirmed that LscB secretion at low temperature was due to a combination of thermo-regulated transcription and secretion. In contrast, LscC accumulated in the periplasmic space. LscB and LscC differ in only five amino acid residues, one of which is a cysteine residue. Temperature shift experiments suggested that *de novo* synthesized protein(s) at 18°C might be responsible for differential LscB secretion and that the presumed secretory machinery was stable when cells were shifted to 28°C. Our results imply that Lsc export and secretion may occur by yet-to-be identified novel mechanism(s).

INTRODUCTION

The phytopathogenic bacterium, *Pseudomonas syringae* pv. glycinea PG4180, causes blight of soybeans by formation of water-soaked lesions developing into necrotic spots surrounded by chlorotic halos. Symptoms are most severe during periods of cold and humid conditions (Dunleavy, 1988). Temperature has a significant impact on cellular processes of *P. syringae* pv. glycinea such as virulence gene expression, protein stability, and protein secretion (Smirnova *et al.*, 2001). A number of genetic loci or gene products that were differentially expressed at 18 or 28°C, respectively, were identified in PG4180 (Rohde *et al.*, 1999; Ullrich *et al.*, 2000). While lower temperatures promote virulence, 28°C is the optimal growth temperature for this pathogen.

Effects of temperature on protein expression and secretion in pathogenic bacteria have been reported in several studies. *Yersinia* species secrete virulence proteins preferentially at 37-40°C via type III secretion systems whose assembly depends on the temperature-regulated transcription of so-called *yop* genes (Hurme & Rhen, 1998). Virulence-associated protein secretion in *Escherichia coli* and *Fusobacterium necrophorum* was induced at 37°C (Amoako et al., 1996; Ebel *et al.*, 1996; Kenny *et al.*, 1997). In the plant pathogens, *Erwinia chrysanthemi*

and *E. carotovora*, expression and secretion of extra-cellular pectolytic enzymes occur in temperature-dependent manner with maximal secretion at 25°C (Hugouvieux-Cotte-Pattat *et al.*, 1996; Housby *et al.*, 1998). *P. syringae* secretes more Avr effector proteins via the type III *hrp* secretion apparatus at low temperatures (Van Dijk *et al.*, 1999). Avr protein secretion in the fire blight pathogen, *E. amylovora*, might be thermoresponsive since *hrp* gene expression was induced at 18°C (Wei *et al.*, 1992). Temperature also affects synthesis of the extra-cellular polysaccharides (EPS) amylovoran and levan in *E. amylovora* (Bereswill *et al.*, 1997). A particular role of temperature for protein secretion mechanisms in plant-associated pathogens and symbionts, respectively, has been implied by Bladergroen *et al.* (2003).

The synthesis of EPS and their role in virulence have been described in detail in many plant pathogenic bacteria (Denny, 1995; Leigh & Coplin, 1992; Saile *et al.*, 1997). *P. syringae pv. glycinea* produces two major EPS, alginate and levan. Levan is a β-(2,6) polyfructan with branching through β-(2,1) linkages and is produced by a single gene product, levansucrase (Lsc). Like other EPS, levan might be particularly important during early stages of infection by masking and protecting the pathogen in the host tissue (Kasapis *et al.*, 1994). Levan may be essential for attachment of *P. syringae pv. glycinea* to plant surfaces during epiphytic growth. A previous report on the variability of levan formation in various *P. syringae* isolates (Fett *et al.*, 1989) showed that environmental factors, such as temperature, influence the composition of total bacterial EPS.

The extra-cellular enzyme Lsc (EC 2.4.1.10) is produced by various gram-negative and gram-positive bacteria and catalyzes synthesis of levan from sucrose by transfructosylation (Gross & Rudolph, 1987). Carbon compounds such as plant-borne sucrose are often limiting factors for bacterial growth on plant leaves (Wilson & Lindow, 1994). Since epiphytic bacterial population sizes on leaves depend on the amount of sugar present (Mercier & Lindow, 2000), bacterial saccharolytic enzymes such as Lsc or sucrase constitute a major metabolic pathway to utilize sucrose as the prime energy source.

In gram-negative bacteria, secreted proteins have to traverse the cell envelope consisting of two membranes separated by the periplasm. Based on their distinctive features, five protein secretion pathways (type I to IV and *tat*) have been reviewed in detail (Hanekop *et al.*, 2006; Johnson *et al.*, 2006; Mota *et al.*, 2005; Christie *et al.*, 2005; Yen *et al.*, 2002). Lsc from PG4180 has been demonstrated to follow a two-step secretion pathway since it accumulates in the periplasm (Li & Ullrich, 2001). However, translocation of Lsc across the inner membrane must be mediated by a yet-unknown signal peptide-independent mechanism since no N-terminal cleavage was observed. In combination, the characteristics of Lsc secretion do not match any of the secretion pathways known for gram-negative bacteria.

Previously, three genetic loci coding for Lsc in PG4180 were identified and mutational analysis of the respective genes, *lscA*, *lscB*, and *lscC*, was carried out (Li & Ullrich, 2001). Interestingly, although the gene products of *lscB* and *lscC* were highly homologous, only LscB was secreted to the exterior while LscC accumulated in the periplasm. The gene product of *lscA* was not detectable suggesting that this gene was not expressed.

Aims of the current study were the gene-specific analysis of temperature-dependent expression and secretion of Lsc in *P. syringae* pv. glycinea, respectively, and a time- and temperature-dependent analysis of the synthesis and stability of the postulated secretion machinery.

MATERIALS & METHODS

Bacterial strains, plasmids, and growth conditions

P. syringae pv. glycinea PG4180 (R.E. Mitchell, 1975) as the wild type produces levan. Its two levan-deficient mutants, PG4180.M3 and PG4180.M5 (Li & Ullrich, 2001), carry antibiotics resistance cassettes in *lscB* and *lscC*, respectively. Mutant PG4180.M3 produces only LscC while mutant PG4180.M5 produces only LscB. *Pseudomonas* strains were maintained on mannitol-glutamate medium (Keane *et al.*, 1970) at 28°C. For liquid cultures, bacteria were incubated in HSC medium (Li & Ullrich,

2001), or King's B (KB) medium (King *et al.*, 1954) at 18 and 28°C as described by Hettwer *et al.* (1998). Microbial growth was monitored by measuring the optical density at 578 nm (OD_{578}). The following antibiotics were added to the media when needed (in µg/ml): ampicillin, 50; chloramphenicol, 25; kanamycin, 25; tetracyclin, 25; spectinomycin, 25; and streptomycin, 25. gentamicin, 2.

DNA procedures

Restriction digestions, electrophoresis, purification of DNA from agarose gels, electroporation, PCR, and plasmid DNA preparation were performed by standard techniques (Sambrook *et al.*, 1989). Subclones were generated in pBluescript SKII(+) (Stratagene, Heidelberg, Germany) or in pRK415 (Keen *et al.*, 1988).

Northern Blot and Dot Blot Analyses

PG4180 and its mutants were incubated in HSC medium at 18 and 28°C and grown to an OD_{578} of 1. Subsequently, aliquots of these cultures were adjusted to an OD_{578} of 0.1 in fresh HSC medium and incubated until early- and mid-logarithmic growth (OD_{578} of 0.5 and 1.0, respectively).Total RNA was isolated from bacterial cells by acid phenol/chloroform extraction as described by Majumdar *et al.* (1991). For Northern Blot, electrophoresis of 20 µg of total RNA was carried out on a 1.2% glyoxal-denaturing agarose gel and the RNA was subsequently blotted to positively-charged nylon membranes (Pall, Dreieich, Germany). For Dot Blot, total RNA samples (500 ng / dot) were applied to a Bio-Dot apparatus (BioRad, Munich, Germany) according to the manufacturer's recommendations. The membranes were incubated in hybridization solution for 2 h, next hybridized with DIG-labeled RNA probes which were synthesized by the Strip-EZ^{TM} RNA Probe Synthesis & Removal kit (Ambion Europe, Cambridgeshire, UK) at 68°C for overnight. The hybridization probe for *lsc* was generated by in *vitro* transcription using a PCR product of *lsc*. PCR was used to add the T7 promoter by including its sequence at the 5' end of the reverse PCR primer. The primers were lsc-fwd 5'-GTCAGTGCGGACTTTCCGGTCATG-3' and lsc-rev-T7 5'- TAATACGACTCACTATAGGGA GGGATCGCGAAAGTTCCAGCT-3'. After hybridization, filters were washed twice for 5 min at room temperature in 2 × SSC/0.1% SDS, followed by two 15-min washes in 0.2 × SSC / 0.1% SDS at 68°C. Finally, hybridization signals were detected by incubation with anti-digoxygenin-AP Fab fragments (Roche, Mannheim, Germany) and ECF substrate (Amersham, Freiburg, Germany) using a Storm phosphoimager (Amersham). Signals were quantified using the manufacturer's image analysis software package.

Subcellular fractionation, enzymatic assays, and immuno-detection of Lsc

Subcellular fractionation of bacterial proteins, qualitative assays for Lsc activity by zymograms, photometric quantification of Lsc activity, and Western blot analysis of Lsc using polyclonal antibodies were carried out as described previously (Li & Ullrich, 2001). A translational fusion of the N-terminal signal peptide of the periplasmic serine protease, MucD, from PG4180 (Schenk et al., in revision), with alkaline phosphatase, PhoA, from *E. coli* (Gutierrez & Devedjian, 1989) served as control for the integrity of sub-cellular fractions. The fusion was constructed by PCR amplification of the N-terminus of *mucD* using oligonucleotide primers mucD-fw 5'–CAGAATTCAATTTTCATCCTGGGTTTC-3' and mucD-rev 5'-TAAGGTACCAAATCCGGCAGGTTCTC-3' and subsequent ligation of the 362-bp product via *Kpn*I/*EcoR*I-digestion into vector pBBR1MCS-2 yielding plasmid pBBR-mucD. Next, a 2.6-kb *Kpn*I insert containing *phoA* from pPH07 (Gutierrez & Devedjian, 1989) was ligated into pBBR-mucD. The resulting plasmid, pBBR-mucD-phoA, was introduced to PG4180 by conjugation and the sub-cellular fractions of the transconjugant were subjected to Western blot using anti-alkaline phosphatase antibodies (Smirnova & Ullrich, 2004).

RESULTS

Detection of Lsc in extra-cellular protein fractions of PG4180 at 18° and 28°C

PG4180 cells were grown in HSC medium at 18° and 28°C and extra-cellular protein

fractions were collected in the exponential growth phase (OD_{578} = 1.5). Analysis of secreted proteins was carried out by separation of 50-fold-concentrated cell-free supernatants on a 10 % SDS polyacrylamide gel. In samples of 18°C grown cultures a distinct protein band of approximately 50 kDa was detected which was hardly visible in samples of 28°C grown cells (Fig. 1A). Zymograms with 10 % sucrose and electrophoretically separated native extra-cellular protein samples demonstrated that the corresponding protein exhibited levan formation activity (Fig. 1B). This result suggested that Lsc activities of PG4180 accumulated in supernatants when cells were grown at 18°C. The experiment was repeated with complex KB medium; however, a clear difference in protein profiles of samples derived from 18 and 28°C cells could not be observed suggesting that the observed effect was nutrient-dependent (data not shown).

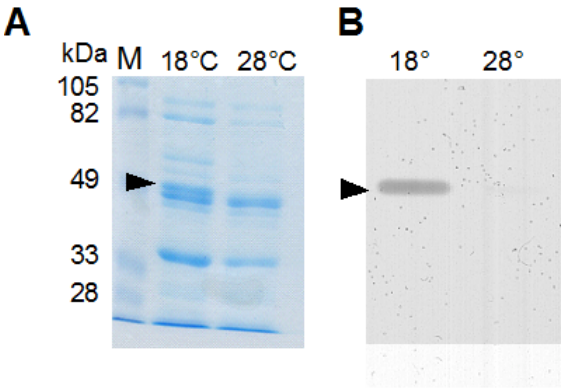

Fig. 1. Thermoresponsive secretion of Lsc in *P. syringae* PG4180. (A) SDS-PAGE analysis of extra-cellular proteins from supernatants of PG4180 cultures. Extra-cellular proteins from 18° and 28°C grown cultures were precipitated with TCA and separated by 10% SDS-PAGE. The black arrow marks a ca. 50-kDa protein band, predominately visible in samples derived from 18°C incubated cultures. (B) Zymogram with extra-cellular proteins of PG4180. Extra-cellular protein samples from 18° and 28°C grown cultures were loaded on a native polyacrylamid gel, separated under non-denaturing conditions, and incubated in a 5% sucrose solution. The whitish swelling of the gel corresponds to levan formation.

Immunological detection of Lsc in extra-cellular protein fractions at 18° and 28°C

To rule out the possibility that differences in extra-cellular Lsc activity between 18°C- and 28°C-incubated PG4180 cultures were due to malfunction of the enzyme at 28°C rather than differential secretion, Western blot experiments were carried out with 50-fold-concentrated supernatants and cell lysates of PG4180 and its *lsc* mutants, PG4180.M3 and PG4180.M5, grown at 18 and 28°C. Due to their genotypes, both mutants produce only one iso-form of Lsc: PG4180.M3 produces only LscC whereas PG4180.M5 produces only LscB (Li & Ullrich, 2001). Polyclonal antiserum against LscB/LscC was used to visualize the enzyme. Signals specific for Lsc could be detected in the cell lysates of both, 18°C- and 28°C-incubated PG4180 cultures, whereas Lsc was only detected in the supernatant of 18°C-incubated cells but not in that of 28°C-grown cells (Fig. 2A). As shown in Fig. 2B, signals for Lsc were only found in the supernatants of PG4180 and PG4180.M5 (*lscA*$^-$ *lscC*$^-$) incubated at 18°C but not in those derived from cultures grown at 28°C. No signal was detected in the supernatant from PG4180.M3 (*lscA*$^-$ *lscB*$^-$) regardless of temperature. The MucD-PhoA fusion expressed in PG4180 (pBBR-mucD-phoA) used as control was found predominantly in the periplasmic while only minor amounts were detected in the supernatant fraction regardless of temperature (Fig. 2C) confirming the integrity of sub-cellular fractions. These results clearly demonstrated that LscB but not LscC is secreted in a temperature-dependent manner**.**

Transcriptional analysis of *lsc* genes in dependence of temperature

In order to study expression of *lscB* and *lscC*, total RNA samples were isolated from PG4180 and its mutants PG4180.M3 and PG4180.M5 incubated in HSC medium at 18 and 28°C during early- and mid-logarithmic growth (OD_{578} of 0.5 and 1.0, respectively). Identical amounts (2.5 µg) of total RNA samples were analyzed by electrophoresis and expression of the *lsc* genes was examined by Northern blot analysis with an RNA probe derived from both *lsc* genes (Fig. 3A). A signal of roughly 1.4 kb corresponding to the predicted size of the *lsc*

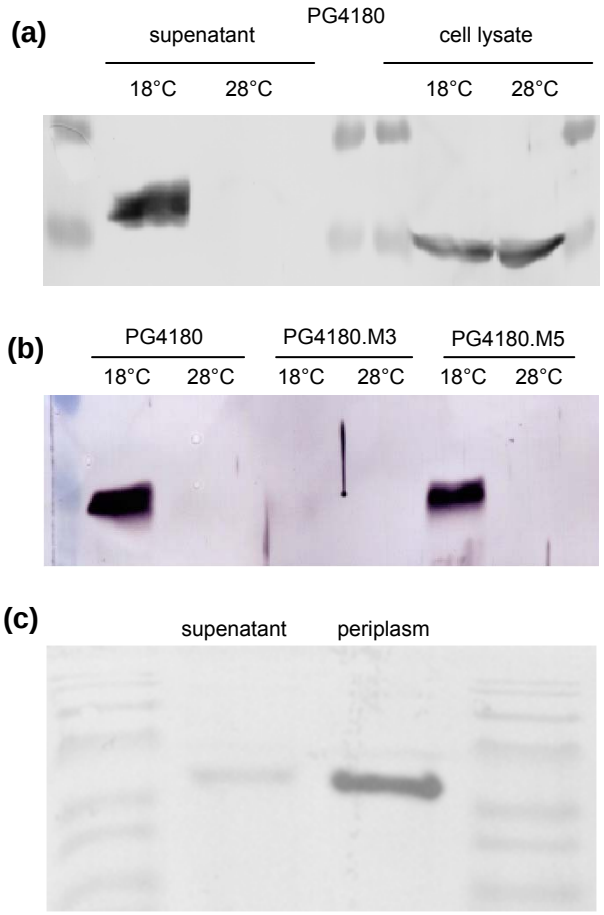

Fig. 2. Western blot analysis (A) of extra-cellular proteins in the cell-free supernatant and of total protein samples from cell lysates of PG4180 grown at either 18° or 28°C. (B) Extra-cellular proteins in cell-free supernatants of PG4180, PG4180.M3 (*lscB*$^-$), and PG4180.M5 (*lscC*$^-$) grown at 18° and 28°C, respectively. (C) Detection of a periplasmic MucD-PhoA fusion expressed in PG4180 (pBBR-mucD-phoA) at 18°C (supernatant and periplasmic fraction). Protein samples (5 μg per lane) were electrophoretically separated, transferred to nitrocellulose filters, and then hybridized with Lsc-specific or PhoA-specific polyclonal antibodies. Bands in unlabeled lanes represent proteins from the molecular weight marker.

transcripts (Li & Ullrich, 2001) could be detected in all samples. Signal intensity was much more pronounced in samples from 18°C grown cultures as compared to those from 28°C cultivation. Likewise, considerably more *lsc* transcript accumulated during the early logarithmic phase (OD_{578} of 0.5) as contrasted by samples taken during mid-logarithmic phase (OD_{578} of 1.0). In samples from 28°C incubated cells, transcript intensity was stronger for *lscB* at OD_{578} = 0.5 (mutant

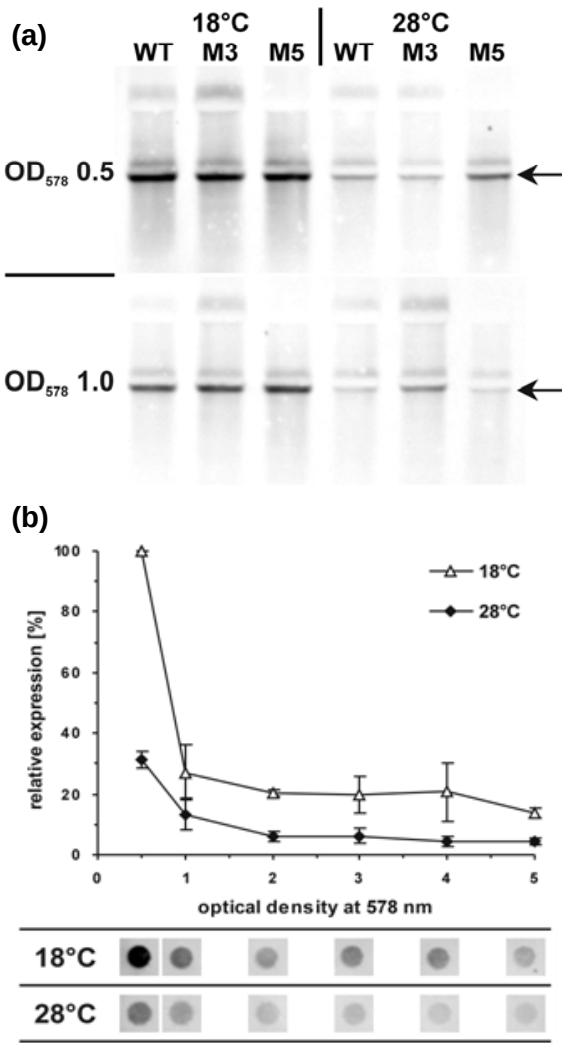

Figure 3. Transcriptional analysis of *lscB* and *lscC.* (A) Northern blot analysis of total RNA samples derived from PG4180 wild type (WT) and its mutants PG4180.M3 (*lscB*$^-$) (M3) and PG4180.M5 (*lscC*$^-$) (M5). Ribonucleic acids were separate by agarose gelelectorphoresis and hybridized with an *lsc*-specific DIG labeled RNA probe. RNA samples were taken when the bacterial cultures had reached an OD_{578} of 0.5 or 1.0, respectively. The arrow indicates the ~1.4-kb *lsc* transcript. (B) Quantitation of *lsc* transcript accumulation in dependence of growth phase and temperature as determined by RNA dot blot analysis. Representative samples are shown in the bottom of the figure. Data were derived from three individual experiments with each four replicates.

PG4180.M5), while *lscC* was more expressed at OD_{578} = 1.0 (mutant PG4180.M3).

To substantiate thermo-responsiveness of *lsc* expression, RNA dot blot experiments were conducted with RNA samples derived from batch cultures of PG4180 incubated at 18 and 28°C, respectively. Fig. 3B clearly shows that

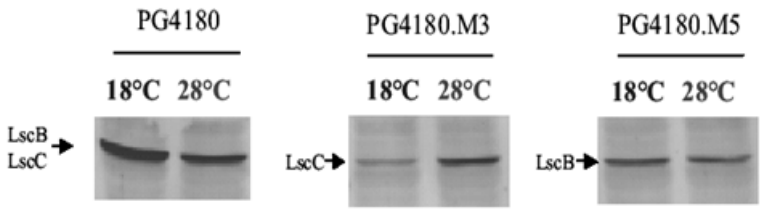

Fig. 4. Western blot analysis of cell lysates of PG4180, PG4180.M3 (*lscB*$^-$), and PG4180.M5 (*lscC*$^-$) grown at 18° and 28°C, respectively. Cells were grown to an OD_{578} of 1.5 and subjected to total protein extraction. Protein samples (5 μg per lane) were electrophoretically separated, transferred to nitrocellulose filters, and then hybridized by Lsc-specific polyclonal antibodies.

lsc expression is strongest during growth in early logarithmic phase (OD_{578} = 0.5) at 18°C confirming the above mentioned results. Signal intensity for the *lsc* transcript was roughly three-fold higher at 18 as compared to 28°C at OD_{578} of 0.5. This ratio remained effective even at higher optical densities although the overall mRNA abundance declined significantly. These results suggested that transcription of both, *lscB* and *lscC*, was temperature-dependent. Our findings also indicated that temperature-mediated differences in *lsc* transcript accumulation and/or stability were most pronounced during the onset of logarithmic growth and that *lsc* transcript signals diminished during later stages of the bacterial growth.

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

To determine whether the observed temperature effect on transcription of *lscB* and *lscC* is directly responsible for the observed thermo-responsive Lsc secretion, additional Western blot analyses were carried out with cell lysates of PG4180 and its mutants impaired in either *lscB* or *lscC*. We had previously shown that most of the cellular Lsc can be found in the periplasm (Li & Ullrich, 2001). Thus, Western blotting of cellular protein samples is indicative for periplasmic accumulation of this enzyme. Equal amounts (5 μg) of cell lysates from mutants, PG4180.M3 (*lscA*$^-$ *lscB*$^-$) and PG4180.M5 (*lscA*$^-$ *lscC*$^-$), as well as the wild type grown at 18 and 28°C were subjected to 10 % SDS-PAGE and blotted with antiserum raised against Lsc (Fig. 4). The amount of Lsc detected in lysates of PG4180 wild type and the LscB-producing mutant PG4180.M5 were slightly higher at 18°C while mutant PG4180.M3 showed more cellular LscC accumulation at 28°C. These results indicated that periplasmic LscB accumulation at the lower temperature can be attributed to the significantly increased cellular *lscB* mRNA abundance (and its translation) and a simultaneously selective secretion of LscB at 18°C. Interestingly, the increased periplasmic abundance of LscC at 28°C contradicts our Northern blot results with RNA samples from mutant PG4180.M3.

Analysis of Lsc secretion after temperature shifts

The fact that Lsc secretion by PG4180 was more pronounced at 18°C as compared to 28°C raised the question which pathway this bacterium might utilize for Lsc secretion. Therefore, the influence of temperature shifts on Lsc secretion was investigated in two sets of experiments. First, PG4180 was grown at 28°C until OD_{578} of 1.0. Cultures were then divided into four aliquots. Two of these aliquots were treated with the protein synthesis inhibitor, chloramphenicol (400 μg/ml), and subsequently incubated at 18 and 28°C, respectively. The two remaining aliquots were incubated without chloramphenicol at 18 and 28°C, respectively. At various time points, samples of supernatants were collected, applied to native PAGE, and subjected to zymographic detection of Lsc activity. As shown in Fig. 5A, Lsc activities could be detected in chloramphenicol-free samples 6-10

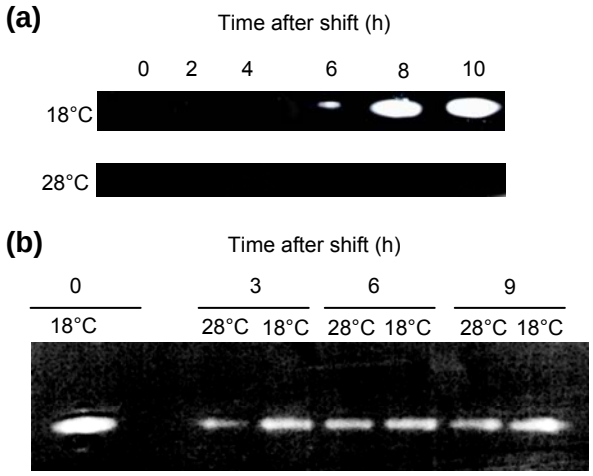

Figure 5. Temperature shift experiments with PG4180 cells. (A) Zymographic analysis of extra-cellular Lsc activities upon shift from 28° to 18°C. Cells were grown to an OD_{578} = 1.0 at 28°C, aliquoted four-fold, and then two parallels were treated with chloramphenicol. Two aliquots were subsequently incubated at 18°C while the other two remained at 28°C. Cell-free supernatant samples were collected at various time points, electrophoretically separated, and subjected to zymographic Lsc analysis. Chloramphenicol-treated samples resembled 28°C incubated ones and are not shown. (B) Zymographic analysis of extra-cellular Lsc activities upon a shift from 18° to 28°C. Cells were grown to an OD_{578} = 1.0 at 18°C, centrifuged, re-suspended in fresh medium, and divided into two aliquots which were subsequently incubated at 18° and 28°C, respectively. Cell-free supernatant samples were collected at various time points, subjected to native PAGE, and analyzed zymographically for Lsc activities.

hours after the temperature shift. In contrast, no Lsc activities were found in the supernatant at 28°C or when cultures were treated with chloramphenicol regardless of the temperature to which the cultures were shifted (Fig. 5A and data not shown). These results indicated that *de novo* protein synthesis is required for Lsc secretion at 18°C and that this secretion process is not proceeding rapidly after a temperature down-shift.

Next, we estimated the stability of protein(s) responsible for Lsc secretion at 28°C. Initially, PG4180 cultures were grown at 18°C until they had reached an OD_{578} of 1.0 and cells were centrifuged down at 4,000 × g for 5 min. Pellets were re-suspended in fresh HSC medium, aliquoted two-fold, and subsequently cultured at 18 and 28°C, respectively. At various time points, supernatant samples were collected and subjected to native PAGE and zymographic Lsc analysis. As shown in Fig. 5B, Lsc activities were detected in cell-free supernatant samples over an extended period of time at either temperature. This result suggested that the putative protein(s) required for Lsc secretion – once synthesized at 18°C – might be rather stable at 28°C. In summary, synthesis of protein(s) required for Lsc secretion might only occur at lower temperatures such as 18°C but might remain stable even at high temperatures such as 28°C.

DISCUSSION

For the first time, we provide evidence for a thermo-responsive expression and secretion of Lsc in the plant pathogen *P. syringae* pv. glycinea. PG4180 possesses three *lsc* alleles but only two of them, *lscB* and *lscC*, were expressed under the tested conditions (Li & Ullrich, 2001). In that former study, knock-out mutations were generated for the two expressed *lsc* genes and a respective double mutant showed a clear levan-minus phenotype. In the present study, single mutants defective in *lscB* or *lscC* allowed us to separately investigate secretion of either gene product at 18° and 28°C. It was demonstrated that extra-cellular accumulation of LscB was responsible for the temperature phenotype. This was shown by extra-cellular protein profiling, compartment-specific enzymatic and Western blot detection of Lsc. In a subsequent preliminary experiment, we additionally tested the *P. syringae* strains, B728a and 1448A, which are known from genomic sequencing to also possess more than one allele of Lsc, in terms of thermoresponsiveness of Lsc secretion (data not shown). Both strains showed similar phenotypes as observed for PG4180. This might hint towards a general phenomenon in *P. syringae* but deserves further investigations.

P. syringae pv. glycinea is known to be a "cold-weather" pathogen because bacterial blight symptoms preferential occur during and following periods of cold and humid weather (Dunleavy, 1988). Ecologically it makes sense that levan formation, which contributes to the overall fitness of the pathogen, is preferentially occurring at low temperatures. Respective plant inoculation studies are currently underway to elucidate what particular function levan formation might play in the plant-microbe interaction and/or during biofilm formation. Our results confirmed previous results of Fett *et al.* (1989) who had shown, that the type of EPS produced by various plant-associated fluorescent pseudomonads differed with the applied temperature and that levan-producing *P. syringae* produced more EPS at lower temperatures.

Interestingly, thermo-responsive Lsc secretion was more pronounced when PG4180 was incubated in minimal medium as compared to complex medium suggesting that additional environmental factors might influence the extra-cellular accumulation of Lsc. Biosynthesis of various secondary metabolites, fitness determinants, and virulence factors of *P. syringae* was previously shown to be dictated by nutritional factors (Lindow, 1991).

In previously reported cases where bacterial proteins occur in the extra-cellular space in a temperature-dependent manner (Ebel *et al.*, 1996; Housby *et al.*, 1998; Hugouvieux-Cotte-Pattat *et al.*, 1996; Wei *et al.*, 1992), expression of the respective genes was shown to be influenced by temperature as well. This general concept seems to hold true for Lsc. The clear temperature dependence of LscB secretion can be attributed to the differential expression of its gene. However, much harder to interpret, an 18°C-induced expression of *lscC* is accompanied by a 28°C-mediated accumulation of its gene product in the periplasmic space of *P. syringae pv. glycinea*. We speculate that the low expression of *lscB* and *lscC* at 28°C might still lead to a considerable accumulation of their respective gene products in the periplasm, where LscC might be needed for yet-to-be determined function(s). In order to test this hypothesis, alternative enzymatic functions of Lsc need to be considered in a compartment-specific manner. In future studies, we will investigate whether the catalytic activities of periplasmic versus extracellular Lsc differ. Recently, it was shown that mutants of *Z. mobilis* which carried cysteine-to-serine substitutions in Lsc lacked levan forming activity but retained sucrose hydrolysis activity (Senthikumar *et al.*, 2003). The different number of cysteine residues of LscB and LscC might account for different enzymatic functions, which can not be detected with the Lsc assays utilized in this study. In future studies, structural analyses of the polymeric levans produced by respective mutants and mutant analysis in virulence assays will help to determine the particular role of periplasmic and extra-cellular levan formation in PG4180.

LscB and LscC differ in only five amino-acyl residues from which four conservative changes (amino acid residues 92, 327, 329, and 429) might not be important for the structure and physicochemical characteristics of those enzymes. However, the alteration in position 119, a serine residue in LscB contrasts a cysteine residue in LscC, could impact the number of putative disulfide bridges the respective proteins might form. Accordingly, LscC might contain two disulfide bonds whereas LscB might only possess one and a free cysteine in its structure. Such an alteration could significantly influence the overall structure of the enzyme, possibly leading to a selective transport across the outer membrane. Genetic and biochemical evidence is needed to support our assumption and respective experiments (i.e. site-directed mutagenesis) are currently underway.

Results of temperature shift experiments demonstrated that secretion of LscB correlated with the expression of *lscB*. Our experiments furthermore suggested that LscB is being secreted even at the elevated temperature once synthesized at 18°C. Additionally, results of shift experiments allowed us to speculate that protein(s) required for the thermo-responsive secretion of LscB might be synthesized *de novo* at 18°C.

We can only speculate by which particular secretion mechanism LscB is actually trans-ported across the inner and outer membrane.

Compared to the five widely accepted models for protein secretion in gram-negative bacteria (type I-IV, *tat*), secretion of LscB does not fit into any of these: periplasmic occurrence of LscB and LscC contradicts type I, III, and IV while absence of a typical signal peptide sequence and the experimentally determined unmodified N-terminus of extra-cellular LscB (Li and Ullrich, 2001) ruled out that Lsc is transported by a classical type II mechanism. Our results are in contrast to a recent report on levansucrase export in *Gluconoacetobacter diazotrophicus* (Arrieta *et al.*, 2004) where it was shown that levansucrase contains a classical type II signal peptide sequence and thus is exported via a type II-based mechanism. Neither LscB nor LscC possess signature sequences typical for the twin arginine translocator (*tat*) pathway (Yen *et al.*, 2002). In a preliminary experiment, secretion of Lsc in a *tat* mutant of *P. syringae* DC3000 (Dr. Caldelari, John Innes Center, Norwich, UK) was not impaired at all (data not shown) thus further substantiating our assumption that the twin arginine translocation system is not involved in Lsc export or secretion in *P. syringae* pv. glycinea.

In future studies, we aim at the identification of the particular (novel) secretory system for Lsc by use of translational fusions of Lsc to reporter enzymes and saturated random mutagenesis of the genome of PG4180. Similar emphasis will be put on our understanding of the transcriptional regulation of *lsc* genes. Both approaches will be particularly useful in order to dissect the mode(s) of action of thermoresponsiveness of Lsc expression and secretion, respectively, as well as their particular functions during pathogenic or saprophytic growth of *P. syringae pv. glycinea*.

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3.3 Interaction of LscR with H-NS-like proteins, MvaT and MvaU, determines temperature-dependent levansucrase expression in *Pseudomonas syringae*

Interaction of LscR with H-NS-like proteins, MvaT and MvaU, determines temperature-dependent levansucrase expression in *Pseudomonas syringae*

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Summary

In pathogenic bacteria, exopolysaccharides (EPS) play an important role during successful infection and colonization of the host. Therefore, enzymes involved in EPS production need to be tightly regulated at the level of transcription. In the plant pathogen, *Pseudomonas syringae* pv. glycinea PG4180, synthesis of the EPS, levan, is mediated by two levansucrases, encoded by *lscB* and *lscC*. Both enzymes exhibit a temperature-dependent mode of expression with maximum mRNA synthesis occurring at the virulence-promoting temperature of 18°C and repression at the optimal growth temperature of 28°C. DNA affinity chromatography revealed two H-NS-like repressor proteins, termed MvaT and MvaU, bound upstream of *lscB* at 28°C whereas no such binding occurred at 18°C. When *lscB* was introduced to the heterologous host, *Pseudomonas putida* KT2440, it was not expressed from its native promoter. Functional screening of a genomic library of *P. syringae* PG4180 in *P. putida* carrying *lscB* identified a 9.8-kDa transcriptional regulator, LscR, which gave rise to *lscB* transcription in the same temperature-dependent manner as in its native host, *P. syringae*. The observed phenomenon was due to removal of MvaT from the *lscB* upstream sequence by LscR at 18°C. Interestingly, LscR did not bind to the *lscB* promoter region at any tested temperature suggesting that LscR might not interact with the DNA target but rather with MvaT directly. It was concluded that lack of *lscB* expression at 28°C is based on reversible binding of MvaT and MvaU to its upstream sequence and that this binding might be controlled by the activator protein, LscR, in a temperature-dependent manner. Analysis of the expression patterns of the three identified regulators supported this hypothesis. The results give novel insights into mechanisms, by which H-NS like proteins might regulate their target genes.

Introduction

Changes in temperature are known to induce remodeling of bacterial gene expression patterns in order to adjust cellular physiology to the new environmental challenges. For pathogenic bacteria temperature is often a crucial factor, which controls expression of genes contributing to virulence and epiphytic fitness, and consequently to the successful invasion and colonization of the host organism. For plant pathogenic bacteria many of these traits are triggered by temperatures below their respective growth optima, presumably because at these conditions water films and aerosols are formed and do foster the penetration of phytopathogens into the plant's apoplast where infection can proceed (Smirnova *et al.*, 2001).

For the soybean-infecting plant pathogen, *Pseudomonas syringae* pv. glycinea PG4180, the optimal growth temperature was shown to be 28°C whereas the virulence-promoting temperature optimum lies at approximately 18°C (Smirnova *et al.*, 2001). Genes of PG4180, which were found to be induced at 18°C, included biosynthetic genes for production of the phytotoxin coronatine (Ullrich *et al.*, 1995; Rohde *et al.*, 1999; Budde *et al.*, 2000; Ullrich *et al.*, 2000), for the exopolysaccharide (EPS), alginate (Penaloza-Vazquez *et al.*, 1997; Schenk *et al.*, 2008), for ice nucleation protein InaZ (Nemecek-Marshall *et al.*, 1993), for the *hrp* type III secretion system (van Dijk *et al.*, 1999), and for levansucrase genes required for formation of the EPS, levan (Li *et al.*, 2006).

P. syringae PG4180 possesses three levansucrase genes, named *lcsA*, *lscB* and *lscC* (Hettwer *et al.*, 1998; Li *et al.*, 2001; Li *et al.*, 2006). Genes, *lscA* and *lscC*, reside on the chromosome whereas *lscB* was found to be located on a 60-kb native plasmid. The three genes differ in their expression status. Surprisingly, *lscA* was found not to be expressed under any studied condition (Li *et al.*, 2001). In contrast, *lscB* and *lscC* were shown to be transcribed in a temperature- and growth phase-dependent manner with highest expression values in the early exponential phase at 18°C (Li *et al.*, 2006). A similar temperature-dependent expression was observed for these two genes during the infection process *in planta (Schenk et al., 2008).* However, the exact regulatory mechanism ruling the *lsc* gene expression remained to be uncovered.

Strong *lsc* gene expression at 18°C suggested its tight connection with the global low-temperature induction of virulence and pathogenicity in *P. syringae.* Hence, it must be regulated at the transcriptional level to prevent energy-demanding levan formation at inappropriate conditions. However, known regulatory systems required for other cold-induced genes in PG4180 did not show any impact on *lsc* expression. These systems include the alternative sigma factor, AlgT, (Schenk *et al.*, 2008), and the two-component system CorRP/S (Ullrich *et al.*, 1995). Additionally, no impact on levansucrase regulation was found for the two-component system, GacS/GacA (Li and Ullrich, unpublished data), which is known to control the production of secondary metabolites and extracellular enzymes and is involved in pathogenicity, ecological fitness and tolerance to stress in various *Pseudomonas* species including *P. syringae* (Gaffney *et al.*, 1994; Altier *et al.*, 2000).

Lsc expression was studied in several other gram-negative bacteria such as *Erwinia amylovora (Bereswill et al., 1997)*, *Zymomonas mobilis (Song et al., 1999)*, *Gluconacetobacter diazototrophicus* (Martinez-Fleites *et al.*, 2005), *Rahnella aquatilis (Seo et al., 2002)*, and *Acetobacter xylinum* (Tajima *et al.*, 2000) suggesting versatile regulatory mechanisms. In the plant pathogen, *E. amylovora*, *lsc* was found to be plant-inducible (Zhao *et al.*, 2005) and its expression was shown to be repressed by two regulatory proteins, RcsA and RcsB (Bereswill *et al.*, 1997). In *R. aquatilis* expression of the Lsc-encoding gene *lsrA* was shown to be growth-phase dependent with maximum transcription during stationary phase and being mediated by an activator, LsrS, and a repressor, LsrR (Seo *et al.*, 2002). Interestingly, the Lsc gene, *levU*, of *Z. mobilis* is transcribed as a bicistronic mRNA together with a sucrase gene possibly due to lack of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase (PTS) system in this organism (Song *et al.*, 1999). In the plant endophyte, *G. diazotrophicus*, the Lsc-encoding *lsdA* gene is co-transcribed with a levanase gene showing a growth phase-independent expression pattern (Martinez-Fleites *et al.*, 2005).

The predicted proteins of *P. syringae* PG4180, LscB and LscC, are 98% identical towards each other and there is also a high degree of similarity of the respective upstream DNA regions. Consequently, in the present study a 680-bp fragment upstream of the *lscB* translational start site (Zhurina et al., unpublished) was used to isolate transcription factors applying DNA affinity chromatography (Cramer *et al.*, 2006). Two H-NS like repressors, MvaT and MvaU, showed tight binding to the *lscB* promoter at 28°C but not at 18°C. Subsequently, a positive regulator, LscR, was isolated by functional screening of a genomic library of *P. syringae* in the heterologous host, *P. putida* KT2440. LscR was shown to not bind to the *lscB* upstream sequence but rather to promote removal of MvaT, thus allowing *lscB* expression at 18°C. Expression pattern of the three identified regulators were analyzed with respect to temperature and growth phase giving rise to an exciting novel mechanism on how H-NS-like proteins might be removed from their target sequences under particular conditions.

Results

Binding of MvaT and MvaU to lscB upstream sequence

DNA affinity chromatography with protein extracts from *P. syringae* PG4180 cultures grown at either 18°C or 28°C was used to purify proteins specifically binding to the 680-bp *lscB* upstream sequence as a bait. For this, cells were harvested at an OD_{578} of 0,5, at which *lscB* expression differed most notably (Li et al.,

2006). Comparison of DNA bait-bound proteins revealed that no significant protein binding occurred when extracts of 18°C-grown cells were applied. However, all protein elution

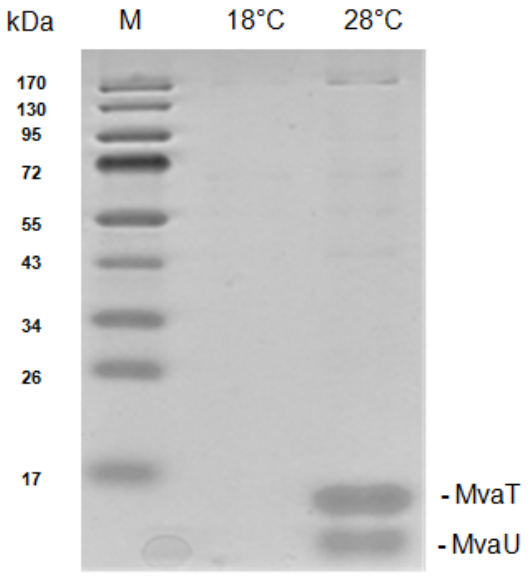

Fig. 1. DNA affinity chromatography with *lscB* upstream sequence and *P. syringae* proteins. SDS-PAGE of proteins isolated from *P. syringae* grown at 18 and 28°C, respectively, incubated with *lscB* upstream DNA linked to magnetic beads, and eluted with 1M NaCl. Protein bands for MvaT and MvaU as determined by MALDI-TOF MS and peptide mass fingerprint analysis are indicated.

fractions derived from samples of 28°C-incubated cells reproducibly exhibited two dominant protein bands of approximately 15 and 16 kDa regardless of the stringency of elution (300 mM, 500 mM, and 1 M NaCl) indicating a high strength of DNA binding (Fig. 1). Subsequently, the two protein bands were subjected to MALDI-TOF and peptide mass fingerprint analyses. The 16-kDa protein showed 82% identity to MvaT, an H-NS-like repressor of *P. aeruginosa* PAO1 (PA4315) (Vallet *et al.*, 2004). The 15-kDa protein in turn was homologous and 61% identical to MvaU, a cognate H-NS-like repressor of *P. aeruginosa* PAO1 (PA2667). Similarly to the situation in *P. aeruginosa*, the 15- and 16-kDa MvaT and MvaU proteins of *P. syringae* exhibited 53 % identity to each other. The corresponding genes for these proteins were present in the three published genomes of *P. syringae* but had not been annotated thus far. Consequently, the 16-kDa protein was designated MvaT and 15-kDa protein was named MvaU.

Heterologous screening for transcriptional activator(s) of lscB expression

Since DNA affinity chromatography did not revealed any protein binding to the *lscB* upstream sequence under inducing conditions, i.e. when PG4180 cells were grown at 18°C, an alternative approach was chosen to identify potential transcriptional activator proteins for *lscB* expression. *Pseudomonas putida* KT2440 does not contain any levansucrase genes and does not form levan when grown on sucrose-containing medium. Consequently, *lscB* along with its 680-bp upstream sequence was introduced to *P. putida* KT2440.

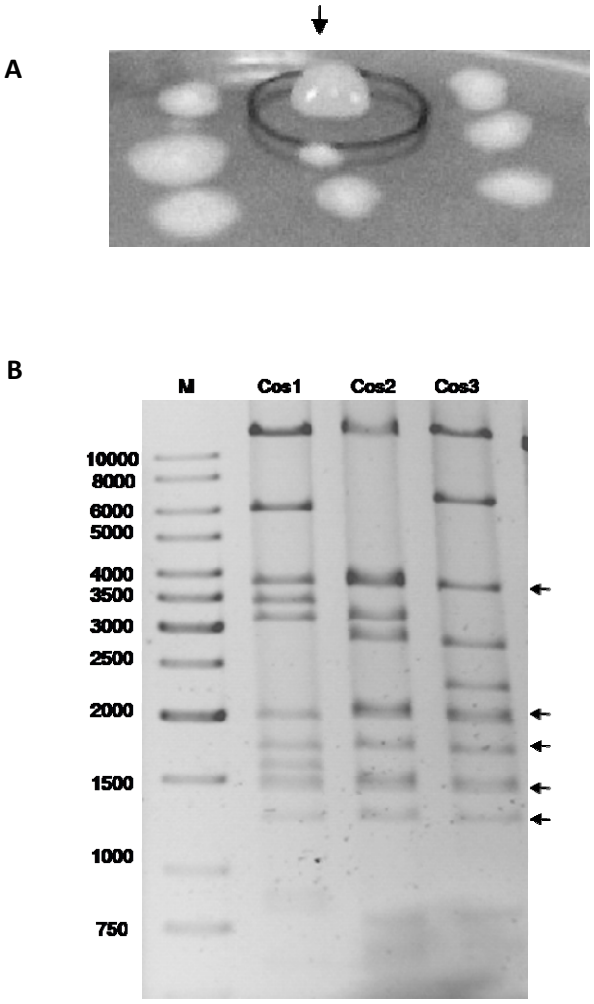

Fig. 2. Heterologous screening for levan-promoting gene(s) in a genomic cosmid library of *P. syringae* PG4180 in *P. putida*. A. Growth of *P. putida* (*lscB*) transconjugants harboring PG4180 genomic library cosmids on sucrose-containing MG agar. The arrow indicates the dome-shaped levan-positive phenotype of *P. putid*a (*lscB*, cos3). B. Electrophoretic comparison of *lscB* expression-promoting cosmids, cos1, cos2, and cos3, after treatment with endonuclease *PstI*. The arrows indicate fragments common to all three cosmids.

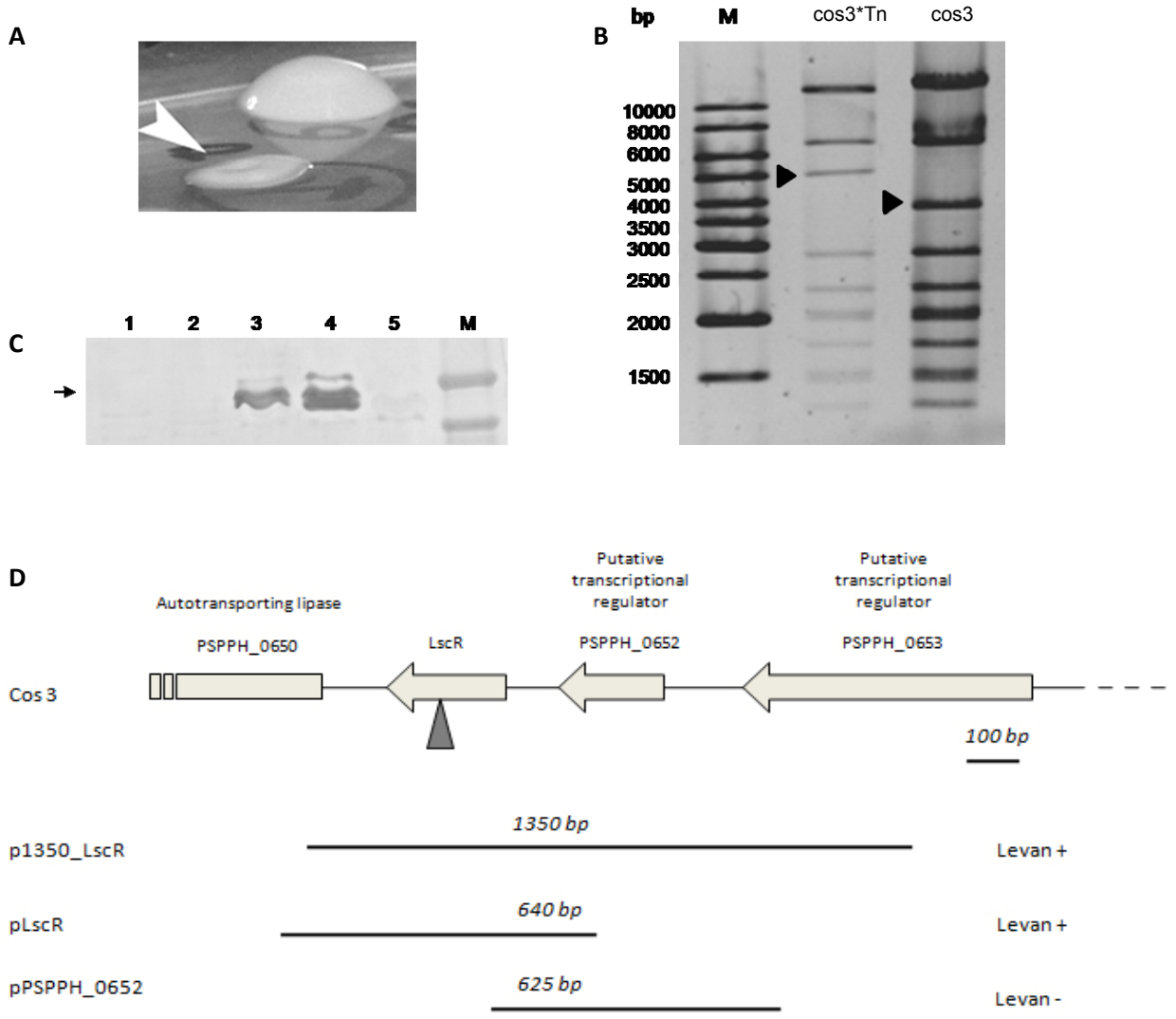

Fig. 3. Identification of *lscR* on cosmid cos3 by *in vitro* mutagenesis. **A.** Growth of *P. putida* (*lscB*) transconjugants harboring cos3 on sucrose-containing MG agar after *in vitro* mutagenesis. The arrow indicates the levan-minus transconjugant carrying mutagenized cosmid, cos3*Tn. **B.** Electrophoretic comparison of cosmids, cos3 and cos3*Tn, after treatment with endonuclease *PstI*. The arrows indicate the fragment containing the entranceposon insertion in cos3*Tn. **C.** Western blot analysis of cell lysates using LscB-specific antibodies. Cells were grown to an OD_{578} of 1.6 at 18°C and subjected to total protein extraction. Lanes: (1) *P. putida* WT, (2) *P. putida* (*lscB*), (3) *P. putida* (*lscB*, *lscR*), (4) *P.syringae* PG4180.WT, (5) *P. syringae* PG4180.M6. **D.** Genetic map of the *lscR* harboring DNA region common to cos1, cos2, and cos3. Sub-cloned fragments and their impact on Lsc expression *P. putida (lscB)* are indicated for p1350_LscR, pLscR and pPSPPH_0652. The inverted triangle marks the entraceposon insertion in cos3*Tn.

The respective transconjugant exhibited a levan-negative phenotype on sucrose-containing agar and a lack of LscB expression as observed by Western blotting (Data not shown) indicating that KT2440 does not possess any regulatory protein(s) required for *lscB* expression. The *lscB*-carrying KT2440 transconjugant was supplemented with a pooled genomic cosmid library of *P. syringae* PG4180 via conjugation and grown on sucrose-containing agar plates. Three individual transconjugants showed the typical levan-positive phenotype, i.e. a dome-shaped colony morphology and translucent slime formation, indicating that *lscB* was expressed (Fig. 2A). The corresponding cosmid clones, designated cos1, cos2, and cos3, were isolated, treated with restriction endonucleases, and the resulting digestion patterns were compared (Fig. 2B). As expected, the cosmid inserts exhibited several common bands although they were not identical with each other suggesting that they contained partially overlapping genomic DNA regions. In order to confirm the mediated levan-positive phenotype,

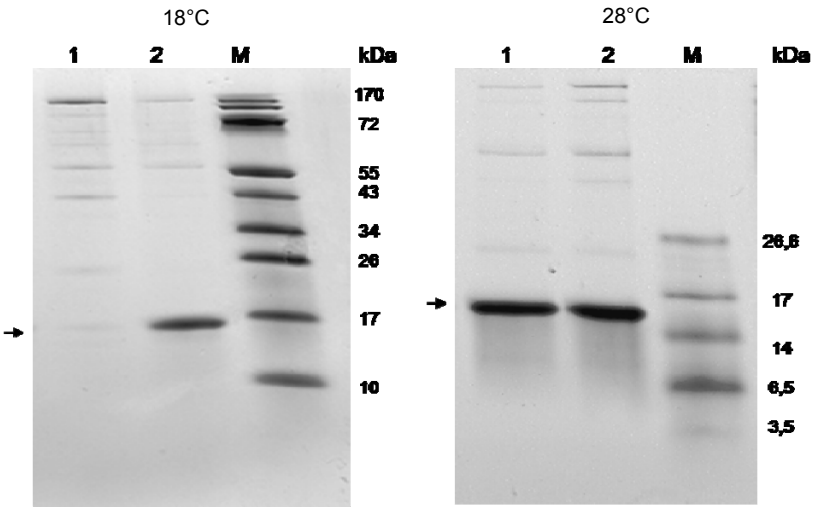

Fig. 4. DNA affinity chromatography with *lscB* upstream sequence and proteins from *P. putida* transconjugants. SDS-PAGE of proteins isolated from *P. putida* transconjugants grown at 18 and 28°C, respectively, incubated with *lscB* upstream DNA linked to magnetic beads, and eluted with 1 M NaCl. Lanes: (1) *P. putida* (*lscB*, *lscR*); (2) *P. putida* (*lscB*). The protein band for MvaT (PP1366) as determined by MALDI-TOF MS and peptide mass fingerprint analysis is indicated by the arrow.

the individual cosmid clones were re-introduced to *E. coli*, the wild type of *P. putida* and *P. putida* containing *lscB*. None of the cosmid clones induced levan formation in *E. coli* or *P. putida* wild type (data not shown). In contrast, when reintroduced into *P. putida* (*lscB*), typical levan formation was observed and presence of LscB in the cellular extracts was confirmed by Western blotting (data not shown). These results indicated that cosmids, cos1, cos2, and cos3 were not sufficient to mediate levan formation on their own but require *lscB* for this suggesting that these cosmids might encode for a transcriptional activator of this gene.

Functional cloning of the transcriptional activator LscR

Transposon *in vitro* mutagenesis was conducted with cosmid, cos3, in order to identify the gene(s) required for the *lsc* expression phenotype. The mutagenized cos3 pool was re-introduced to *P. putida* (*lscB*) and transconjugants were screened for lack of levan formation. The mutagenized cosmid, cos3*Tn, was re-isolated from one levan-negative *P. putida* (*lscB*) transconjugant (Fig. 3A), its DNA extracted, treated with endonuclease, and compared with correspondingly treated DNA of cos3 (Fig. 3B). The resulting digest pattern revealed a potential insertion of the transposon in one of the DNA fragments, which was then subjected to nucleotide sequencing using primers derived from the transposon (Table 2). The transposon of cos3*Tn had inserted in a 267-bp ORF, which is 99% identical to ORF PSPPH_0651 from the genome of *P. phaseolicola* 1448A and was annotated as a putative transcriptional regulator. Consequently, the ORF was designated *lscR* (*lsc* regulator). Interestingly, *lscR* is conserved only in genomes of *P. syringae* species but absent in any other organism's genome including any other *Pseudomonas* species.

A 1,350-bp *Sac*I fragment of cos3 common to all three cosmids was sufficient to induce levan formation in *P. putida* (*lscB*) (Fig. 3C). However, a smaller sub-clone, pLscR, comprising only 640 bp and containing *lscR* in opposite direction to the vector-borne P_{lac} promoter also rendered *P. putida* (*lscB*) levan-positive ruling out any involvement of the upstream located ORF, PSPH_0652, which had also been annotated as a putative transcriptional regulator (www.pseudomonas.com). Confirming this, the 625-bp fragment containing ORF PSPH_0652 did not mediate levan formation to the heterologous host. *P. putida* (*lscB*, *lscR*) clearly expressed LscB as demonstrated by Western blot analysis (Fig. 3D).

Investigation of LscR's mode of action

When *P. putida* transconjugants harboring *lscB* and *lscR* were grown on sucrose-containing agar plates at 18 and 28°C, respectively, significantly more levan was formed at the lower

temperature (data not shown). This interesting observation gave rise to the assumption that

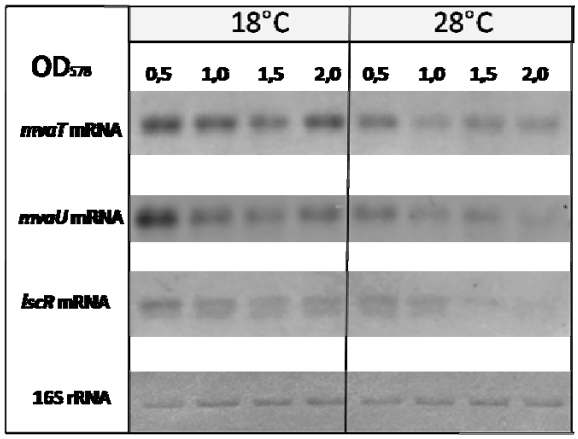

Fig. 5. Transcriptional analysis of *mvaT*, *mvaU*, and *lscR*. *P. syringae* PG4180 cells were grown in HSC medium at 18°C and 28°C to various OD_{578} during logarithmic growth, and total RNA was extracted and subjected to Northern blot analysis with gene-specific probes. The signal obtained for 16S rRNA was used to normalize signals and proved equal loading.

LscR might govern a thermo-responsive *lscB* expression in *P. putida* similar to that found in *P. syringae* (Li et al., 2006). Consequently, DNA affinity chromatography 680-bp *lscB* upstream sequence as bait was conducted using protein extracts from 18°C- or 28°C-grown *P. putida* either carrying *lscB* only or harboring *lscB* and *lscR* (Fig. 4). A 9.8-kDa protein band potentially representing LscR could neither be found in 18°C- or 28°C-derived *P. putida* protein extracts indicating that LscR did not bind to the DNA bait. In contrast to the results for *P. syringae* protein extracts, only one 16-kDa protein band but not the previously observed 15-kDa band representing MvaU bound to the DNA bait when *P. putida* (*lscB*) protein extracts derived from either temperature were used. MALDI-TOF and peptide mass fingerprint analyses revealed that the 16-kDa protein band represented MvaT of *P. putida* (ORF PP1366) indicating that this H-NS-like protein bound to the *lscB* upstream sequence. MvAT of *P. putida* is 86% identical to its previously identified homolog in *P. syringae*. Excitingly, the MvaT binding was observed when LscR either was absent or when protein extracts were derived from *P. putida* (*lscB*, *lscR*) grown at 28°C. In contrast, MvaT did not bind when protein extracts were derived from 18°C-grown and *lscR*-harboring cells (Fig. 4). This result suggested that presence of LscR at 18°C inhibited binding of MvaT to DNA. It furthermore indicated that the potentially repressive mechanism of MvaT binding for *lscB* expression in *P. putida* or in *P. syringae* grown at 28°C could be overcome by presence of LscR at 18°C.

Transcriptional analysis of mvaT, mvaU, and lscR genes

To address the question whether differential binding of MvaT/MvaU to the *lscB* upstream sequence in *P. syringae* was due to thermo-responsive *mvaT*, *mvaU*, and *lscR* expression or rather was due to protein-protein interactions, expression profiles with total mRNA 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_{578} 0,5-2) and gene-specific probes were hybridized with the blotted mRNA (Fig. 5). The approximate sizes of the corresponding transcripts were 500 bp for *mvaT*, 520 bp for *mvaU*, and 540 bp for *lscR*. The determined transcript size for *lscR* substantiated our finding that a 640-bp fragment was sufficient for *lscB* expression in *P. putida* (Fig. 3C). Interestingly, expression of all three genes was more pronounced at 18°C as compared to 28°C. While *mvaT* mRNA was abundant throughout the tested growth phase (i.e. from OD_{578} of 0.5 to 2.0), expression of *mvaU* and *lscR* was significantly stronger in the early logarithmic phase and declined as bacterial growth proceeded (Fig. 5). These results clearly ruled out that lack of MvaT/MvaU binding to the *lscB* upstream sequence at 18°C was due to lack of the expression of the corresponding genes. Consequently, these results suggested a direct or indirect protein interaction of MvaT and LscR at 18°C in *P. syringae*.

Characterization of mutants lacking mvaT, mvaU and lscR

This part of the study is not finished yet. Knock-out mutants lacking *lscR*, *mvaT*, and *mvaU* as well as *ΔmvaTΔmvaU* double mutant are currently being created. A *ΔmvaU* mutant was successfully generated in *P. syringae* PG4180 by homologous recombination and was genetically verified by PCR analysis (data not shown). All mutants will be characterized in terms of phenotypic levan formation and by

Western blot with an LscB-specific antibody as wells as by Northern hybridization with an lscB-specific probe in dependence of temperature and growth phase. At the time being the following phenotypes may be speculated on: the *ΔlscR* mutant might exhibit a levan-minus or levan-reduced phenotype on sucrose-containing agar plates. Expression of *lscB* mRNA might be reduced or absent. Moreover, this mutant might either lack LscB or produce significant lower amounts of the enzyme. For the *ΔmvaT* and *ΔmvaU* single mutants one might speculate on an overproduction of levan and LscB or lack of thermoresponsiveness of *lscB* expression. Eventually, such a phenotype might require the simultaneous knock-out of *mvaT* and *mvaU*.

Discussion

Interactions between bacterial pathogens and their hosts are dynamic and require adaptation to rapid changes of the environment. To succeed, pathogens bring into play sophisticated networks which modulate the transcriptional profile of the cell (Dorman *et al.*, 1999). Increasing evidence indicates that structural and temporal re-organization of the chromosome via H-NS and its homologs by modulating DNA packing plays a crucial role in this process (Travers and Muskhelishvili, 2005; Fang *et al.*, 2008). H-NS and H-NS-like proteins, such as MvaT and MvaU, enable silencing of extended regions of the bacterial chromosome by binding first to high-affinity sites, then spreading along AT-rich sequences, and consequently forming high-order structures, which are responsible for selective silencing of bacterial genes (Dame *et al.*, 2005; Grainger *et al.*, 2006; Navarre *et al.*, 2006; Lang *et al.*, 2007; Castang *et al.*, 2008). Many of these genes are associated with pathogenicity or are responsive to environmental signals (Fang *et al.*, 2008).

Δhns mutants of *E. coli* exhibit pleiotropic phenotypes such as e.g. extensive exoplysaccharide production (Sledjeski *et al.*, 1995) or lack of motility (Bertin *et al.*, 1994; Soutourina *et al.*, 1999; Ko *et al.*, 2000). Interestingly, these mutant phenotypes may be reversed by the H-NS-like protein, MvaT, first discovered in different pseudomonads (Goyard *et al.*, 1997; Tendeng *et al.*, 2003). MvaT and its homologous proteins show low sequence similarities to enterobacterial H-NS. However, their two-module organization, predicted three dimensional structures, and demonstrated functions are similar to those of H-NS (Dorman *et al.*, 1999; Tendeng *et al.*, 2003).

For the first time, herein a molecular interplay between a novel transcriptional regulator, LscR, required for levan formation in the plant pathogen, *P. syringae*, and the H-NS-like proteins, MvaT and MvaU, has been proposed based on an array of experimental evidences. Results of this study consequently help to explain the thermo-responsive mode of expression of *lscB* encoding for levansucrase, the unique extra-cellular enzyme synthesizing levan polymers (Li *et al.*, 2006).

It is intriguing to speculate that synthesis of an extra-cellular enzyme requires a particularly tight mode of regulation since cellular investments and energy expenses are ultimately lost once such a protein is secreted (Ullrich, 2009). In this context, results of this study are in line with previous observations that MvaT-like proteins function as global regulators for exo-product formation (Baehler *et al.*, 2006) and for colonization of host tissue (Vallet *et al.*, 2004; Baehler *et al.*, 2006) by regulating genes involved in virulence, quorum sensing, and biofilm formation (Diggle *et al.*, 2002; Westfall *et al.*, 2004; Castang *et al.*, 2008).

H-NS of enterobacteria and, as recently shown, MvaT and MvaU of *P. aeruginosa*, bind and silence 'xenogenic' elements in the genome such as prophages or pathogenicity islands (Navarre *et al.*, 2006; Navarre *et al.*, 2007; Navarre *et al.*, 2007; Navarre *et al.*, 2007; Castang *et al.*, 2008; Stoebel *et al.*, 2008). This binding is due to the increased AT-content of these elements as compared to the characteristic AT/GC ratio for a given bacterial host genome. Interestingly, the plasmid-borne *lscB* gene of *P. syringae* is flanked by transposase genes and prophage-associated sequences (Hattman,1999; Braid *et al.*, 2004). Likewise, upstream of the chromosomally located *lscC* gene there is a prophage-borne gene cluster (www.pseudomonas.com). Consequently, it was concluded that binding of MvaT and MvaU to the *lscB* upstream sequence might be mediated by 'xenogenic' elements.

Stoebel et al. (2008) recently summarized mechanisms, by which gene silencing via H-NS-like proteins could be reversed, such as changes in DNA topology and expression of activators or H-NS modulating factors. The

current study demonstrated that repression of *lscB* transcription by MvaT might be reversed by presence of LscR.

Herein, MvaT proteins from two different *Pseudomonas* species were demonstrated to be functionally interchangeable. In line with this, *mvaT* genes were predicted to be wide-spread in the genomes of different pseudomonads (Tendeng *et al.*, 2003). Interestingly, in *P. putida* only MvaT bound to the *lscB* upstream sequence. Unlike MvaU, MvaT might have significantly more pronounced effects on target gene expression as demonstrated by comparing *ΔmvaT* with *ΔmvaU* / *ΔmvaV* double mutants of *P. aeruginosa* and *P. fluorescens* (Vallet *et al.*, 2004; Baehler *et al.*, 2006). In absence of its heterodimeric partner, MvaT might form homodimers when binding to DNA as recently shown for *P. aeruginosa* (Castang *et al.*, 2008).

In the current study, a clear thermo-responsive DNA binding of MvaT and MvaU to the upstream sequence of *lscB* was observed thereby enlarging the number of MvaT targets and directly demonstrating its role for gene silencing in *P. syringae*. Involvement of both, MvaT and MvaU, in *lscB* regulation suggested heterodimer formation as previously shown for *P. mevalonii* (Rosenthal *et al.*, 1998) and might reflect a fine-tuned regulation contributing to *P. syringae*'s ability to rapidly adapt its gene expression to subtle changes encountered during the infection processes inside the host plant. Interestingly, another MvaT-like protein, TurA, was shown to be involved in temperature-dependent modulation of *Pu* promoter activity in *P. putida*, where it represses expression of an operon encoding for toluene-degrading enzymes at the suboptimal temperature of 16°C as opposed to 30°C (Rescalli *et al.*, 2004). Cold repression of target genes was found to be typical for H-NS possibly due its structure being stabilized at lower temperatures (Ono *et al.*, 2005). Results of the current study are in contrast to this assumption since more MvaT and MvaU binding was observed at the elevated temperature. This striking difference might be due to an overall ''inversed'' virulence mechanism in plant pathogens. Here, virulence is triggered by low temperature associated with infection-promoting high humidity on plant surfaces in contrast to the high-temperature conditions in warm-blooded hosts (Hurme *et al.*, 1998; Smirnova *et al.*, 2001).

Herein it was excluded that lack of MvaT and MvaU target DNA binding at 18°C might be due to lack of expression of *mvaT* and *mvaU* at this temperature. Additionally, there was no competitive DNA binding by LscR observed. Consequently, it is proposed that direct or indirect interactions between MvaT and LscR are the major reason for thermo-responsive *lscB* expression. LscR might inhibit attachment of MvaT to or promote its removal from the *lscB* upstream sequence by molecularly mimicking the DNA and thus 'scavenging' it away. While higher expression of the *lscR* gene at 18°C is fully in line with such a hypothesis, simultaneously increased expression of *mvaT* and *mvaU* might indicate the necessity to tightly control expression of an energy-consuming cellular trait at certain growth stages.

Our future research will aim at the in-depth characterization of *ΔmvaT*, *ΔmvaU* and *ΔlscR* mutants not only with respect to *lscB* expression but also regarding other cellular features being suspected to be regulated by H-NS-like proteins such as swarming ability, chemotaxis, quorum sensing, exo-product release, and biofilm formation. The impact of these regulators on virulence of *P. syringae* will be analyzed in-depth. Detailed DNA binding studies and pull-down experiments with MvaT and MvaU in presence or absence of LscR will allow a better insight into the molecular interplay discovered.

Experimental Procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was maintained on Luria-Bertani (LB) medium at 37°C (Sambrook, J., Fritsch, E.F., Maniatis, T.,1989). *P. syringae* was routinely maintained on mannitol-glutamate (MG) medium *(*Keane *et al.*,1970) at 28°C. For liquid *P. syringae* cultures at 18 or 28°C, bacteria were grown in 200 ml of Hoitink-Sinden (HSC) minimal medium (Palmer *et al.*, 1993) in 1-liter Erlenmeyer flasks. Bacterial growth was continuously monitored by measuring the optical density at 578 nm (OD_{578}). Antibiotics were added to the media at the following concentrations (μg/ml): ampicillin, 50; spectinomycin, 25; kanamycin, 25; tetracycline, 25; gentamicin, 2.

Molecular genetics techniques

All routine molecular methods were performed using standard protocols (Sambrook *et al.*, 1989). Nucleotide sequencing was carried out commercially (MWG Biotech, Ebersberg, Germany). Restriction enzymes (Fermentas, Burlington, Canada or New England Biolabs, Ipswich, USA) were used in accordance with the manufacturer's instructions. The Gluniversal DNA Minispin Kit (Molzym, Bremen, Germany) was employed for isolation of chromosomal DNA from *P. syringae.* Cosmids were extracted from *P. putida* cells with phenol and chlorophorm; and from *E.coli* cells by alkaline lysis with subsequent isopropanol precipitation (Sambrook et al., 1989). Plasmid constructs were electroporated into *E.coli* DH5α. Conjugation of replicating and suicide vector constructs was done by tri-parental mating using helper plasmid pRK2013 (Figurski *et al.*, 1979). Phenotypic assessment of *P. syringae* and *P. putida* transconjugants was done by streaking the cells on MG agar medium containing 5 % sucrose as described by Li *et al*. (2001).

Transcriptional analyses

Total RNA was isolated from bacterial cells by acid phenol/chloroform extraction as described by Schenk et al. (2008). For Northern blot analysis, aliquots of total RNA (500 ng per lane), unlabelled 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 to 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 (Herrin *et al.*, 1988). The membranes were incubated in hybridization solution for 2 h and subsequently hybridized with DIG labeled RNA probes. The hybridization probes for *mvaT, mvaU, lscR* 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 2 × SSC / 0.1% SDS followed by two 15-min washes in 0.2 × SSC / 0.1% SDS at 68°C. Finally, hybridization signals were detected by incubation with anti-digoxygenin-AP Fab fragment (Roche) and ECF substrate (Amersham) using a FLA3000 phosphoimager (Amersham, Buckinghamshire, England) and the manufacturer's image analysis software package.

Levansucrase isolation

Sub-cellular fractionation of bacterial proteins and qualitative assays for levansucrase by Western blot analysis using polyclonal antibodies were carried out as described previously (Li *et al.*, 2001; Li *et al.*, 2006).

DNA affinity chromatography and peptides mass fingerprinting analysis

Two liters of *P. syringae* culture were grown in HSC medium at 18 and 28°C, respectively. Cells were harvested at an OD_{578} 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* and 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 (Gabrielsen *et al.*, 1989; Cramer *et al.*, 2006). As the DNA bait the region from nucleotides -341 to +339 with respect to the transcriptional start site of *lscB* was generated by PCR using plasmid pRB7.2 (Li *et al.*, 2001) as a template and primers lscB-fw-DAP and LscB-rev-DAP, the latter tagged with biotin via TEG linker (MWG-Biotech).

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

Bacterial strain	**Relevant characteristics**	**Reference or source**
Escherichia coli		
DH5α	*supE*44 Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Sambrook *et al.*, 1989
Pseudomonas syringae pv. glycinea		
PG4180	wild type, levan$^+$	(Bender *et al.*, 1993)
PG4180.M6	*lscB lscC* mutant of PG4180, Sp^r, Gm^r, levan$^-$	(Li *et al.*, 2001)
PG4180.*ΔmvaU*	*mvaU* deletion mutant of PG4180	This study
Pseudomonas putida		
KT2440	wild type, levan$^-$	(Ramos-Diaz *et al.*, 1998)
Plasmid		
pRK2013	Km^r, helper plasmid	(Figurski *et al.*, 1979)
pRK7813	Tc^r; cosmid vector used for generation of genomic libraries	(Jones *et al.*, 1987)
pBBR1MCS-2	Km^r ,broad-host-range cloning vector	(Kovach *et al.*, 1995)
pFKm1	Ap^r,Km^r, source of Km^r cassette flanked with *FRT* sequences	H. Schweizer
pK18mobGII	Km^r, mobilizable suicide vector	(Katzen *et al.*, 1999)
pLscB7.2	Km^r, contains *lscB* on 7.2-kb *Eco*RV insert	(Li *et al.*, 2001)
cos1, cos2, cos3	Tc^r, genomic library clones of PG4180; approx. 25-to-35-kb inserts in pRK7813, promote *lscB* expression in *P. putida* (*lscB*)	This study
cos3*Tn	Km^r,Tc^r, cos3 with entranceposon insertion in *lscR*; does not promote *lscB* expression in *P. putida* (*lscB*)	This study
pGEM_LscR	Ap^r, 640-bp fragment containing *lscR* inserted in pGEM (<>p*lac*)	This study
pLscR	Km^r, 640-bp fragment containing *lscR* from pGEM_LscR cloned into *SacII-SpeI* sites of pBBR1MCS-2, levan positive in *P. putida (lscB)*	This study
p1350_LscR	Km^r, 1350-bp *SacI* fragment of cos 3, containing LscR, levan positive in *P. putida (lscB)*	This study
pPSPPH_0652	Km^r, *EcoRI/KpnI* 625-bp fragment containing the ORF *PSPPH0652* in pBBR1MCS-2, levan negative in *P. putida (lscB)*	This study
pGEM.MvaU1	Ap^r, contains 445-bp upstream region of *mvaU*	This study
pGEM.MvaU2	Ap^r, contains 470-bp downstream region of *mvaU*	This study
pGEM.MvaU2-Km	Ap^r, Km^r , 1230-bp *Kpn*I fragment containing Km^r-*FRT* cassette from pFKm1 in pGEM.MvaU2	This study
pGEM.MvaU-Km	Ap^r, Km^r, 1700-bp *Bam*HI-*Spe*I fragment from pGEM.MvaU2-Km in pGEM.MvaU1	This study
pK18mob.MvaU-Km	Km^r, 2145-bp *Eco*RI fragment of pGEM_MvaU-Km in pK18mob	This study

[a] *Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline*

Table 2. Oligonucleotide primers used in this study.

Oligonucleotide primer	Nucleotide sequence (5' to 3')[a]
lscB-fw-DAP	GTTAGCCGGACTCGCTCA
lscB-rev-DAP	ATAGCTTTGGGAGGCAGCAG
mvaU_1f_B	GGATCCCTGATTGGCCGATTCGCTCAC
mvaU_1r	CTGCAACAGGGACTGTAG
mvaU_2f	GATACCTGCTTTTTCGCAG
mvaU_2r_BK	GGATCC*GGTACC*GAAGCCCTGCACTTGCAG
lscR_1f_B	GGATCCGTTCATTCCATGACTCC
lscR_1r	GAA CAC CAC CAT GGT
lscR_2f	GAA GGT ATC AGC GTG AC
lscR_2r_BK	GGATCC*GGTACC*CATCGTGACCAACAGGGCAG
mvaT-fwd	ATGTCCCTGATCAACGAATACC
mvaT-rev-T7	**TAATACGACTCACTATAGGGA**GGAGTGGCCCAGCTTTCGACGTCGTC
mvaU-fwd	TTGTCCAGACTTGCCGAGTTTC
mvaU-rev-T7	**TAATACGACTCACTATAGGGA**GGTCAGGCGCGAAGCCACGAATCAAC
LscR-fwd	CCACAGGGAGTCATGGAATG
LscR-rev-T7	**TAATACGACTCACTATAGGGA**GGCTGCCCTGTTGGTCACGATG
LscR_pBBR_fw	GACGAGCTCGAACACCACCATGGT
LscR_pBBR_rev	GACGAGCTCGAAGGTATCAGCGTGAC
PSPPH_0652_fw	GACGAATTCCGAATTCTTTGCGAAGTTGATC
PSPPH_0652_rev	GACGGTACCCCTTGCGGTGACCGAGGAGTG
mvaU_check_s_f	GTGCAGACGCTCAGTTGAC
mvaU_check_s_r	GTACAGGGCAGCATCGAG
mvaU_check_l_f	CCAGACACTGGTAGCGGCTG
mvaU_check_l_r	GTTGACGCAGATTGTTCA

[a] Restriction sites incorporated in primers are underlined, GGATCC – *BamH*I, GAATTC – *EcoRI*, GGTAAC – *KpnI,* GAGCTC – *SacI*; T7 RNA polymerase promoter sequences incorporated in primers are indicated in bold.

Screening of a genomic library of P. syringae in P. putida

A pooled genomic cosmid library of *P. syringae* was introduced to *P. putida* (*lscB*) by triparental mating, and transconjugants were selected on MG plates, supplemented with kanamycin and tetracycline. Approximately 2000 of the resulting transconjugants were picked on MG agar plates supplemented with 5 % sucrose and incubated at 18°C for 10 days. Transconjugants exhibiting a dome-shaped colony morphology were considered levan-positive and were verified by Western blot analysis.

Transposon in vitro mutagenesis of cosmid DNA and lscR identification

Transposon *in vitro* mutagenesis was performed with the TGS II Template Generation System (Finnzymes, Espoo, Finland). Briefly, genomic

library cosmid cos3 was *in vitro* mutagenized by insertion of the entranceposon encoding for kanamycin resistance and then electroporated into *E. coli*. Resulting transformants were pooled and their cosmids being conjugated into *P. putida* (*lscB*). 100 transconjugants were picked on MG agar supplemented with 5% sucrose, incubated at 18°C for 10 days, and screened for lack of levan formation. One transconjugant did not produce levan, and its cosmid termed cos3*Tn was extracted and sequenced using primers SeqW and SecE (Table 2) to obtain nucleotide sequence information for the entranceposon insertion site. The entranceposon-carrying 267-bp ORF was termed *lscR* (**Fig. 3**). From cos3, a 640-bp DNA fragment containing *lscR* was removed using endonucleases *Sac*II and *Spe*I and cloned into the *Sac*II/*Spe*I-treated broad host-rang vector pBBR1MCS-2 (Kovach et al., 1994) in opposite orientation to the vector-borne p_{lac} promoter yielding plasmid pLscR. Subsequently, pLscR was conjugated into *P. putida* (*lscB*). The resulting transconjugant, *P. putida* (*lscB*, *lscR*), was grown on sucrose-containing MG agar and verified to be levan-positive. Its cellular protein extract was further subjected to Western blot analysis using polyclonal antibodies against levansucrase.

Generation of mvaU mutant in PG4180

A *P. syringae* PG4180 *mvaU* mutant was generated by homologous recombination using suicide vector pK18mob (Katzen *et al.*, 1999). 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_KB (Table 2). PCR products were cloned into vector pGEM-T Easy (Promega, Mannheim, Germany) yielding plasmids pGEM.MvaU1 and pGEM.MvaU2. A 1230-bp *Kpn*I fragment containing a Kanamycin resistance cassette flanked with *FRT* sequences was removed from plasmid pFKm1 (kind gift from Prof. H. Schweizer, Colorado State University, USA) and ligated into *Kpn*I-digested pGEM.MvaU2 yielding pGEM.mvaU2-Km. A 1700-bp *Spe*I-*Bam*HI fragment derived from pGEM.MvaU2-Km was ligated into *Spe*I-*Bam*HI-digested plasmid pGEM.MvaU1 yielding plasmid pGEM.MvaU-Km. Finally, a 2145-bp *Eco*RI fragment was removed from pGEM.MvaU-Km and ligated into *Eco*RI-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 subsequently plated on MG agar supplemented with kanamycin. Genotypes of mutants were screened by PCR using primers mvaU_checks_f and mvaU_checks_r (Table 2). Gene replacement was verified when a 1317-bp PCR product was amplified. Next, the kanamycin resistance cassette was excised by means of the FRT-system (Hoang *et al.*, 1998) resulting in the unmarked Δ*mvaU* mutant, PG4180.ΔmvaU, whose genotype was ultimately verified by PCR amplification of 195-bp and 1390-bp products using primer pairs, mvaU_check_s_f / mvaU_check_s_r and mvaU_check_l_f / mvaU_check_l_r (Table 2), respectively.

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3.4 Expression of extra-cellular levansucrase in *Pseudomonas syringae* is controlled by metabolic repressor HexR

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

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Summary

The opportunistic plant pathogen *Pseudomonas syringae* pv. glycinea PG4180 causes bacterial blight on soybean plants. This pathogen resides epiphytically on plant surfaces and enters the leaf tissue through stomata or open wounds where it encounters a sucrose-rich milieu. Sucrose can be utilized either extracellularly via the secreted enzyme, levansucrase (Lsc) thus liberating glucose and forming the polyfructan, levan, or intracellularly via catabolic enzymes. *P. syringae* PG4180 possesses three *lsc* alleles, two of which are transcribed in a temperature-dependent manner with maximal expression at the virulence-promoting temperature of 18°C whilst the third one seems to be cryptic. Herein, the transcriptional start sites for *lscB* and *lscC* were mapped suggesting a bicistronic transcription with *com*, presumably encoding for a translational regulator, which was confirmed by nested deletion experiments. However, site-directed mutagenesis of *com* did not reveal any impact on levansucrase expression. DNA affinity chromatography and MALDI-TOF analysis demonstrated binding of the hexose metabolism repressor, HexR, to the upstream sequence of *lscB* at 28°C but not at the *lsc* expression-promoting temperature of 18°C. Furthermore, it was shown that growth of an *lscB lscC* double mutant of PG4180 was impaired when cells were grown with sucrose as compared to glucose as the sole carbon source suggesting a central role of Lsc in hexose metabolism. Our data suggest a tight co-regulation of genes required for an extracellular function with those involved in central energy-obtaining metabolic pathways in a temperature-dependent manner in pseudomonads.

Introduction

Fructan or glucan polymers are formed wherever microbes encounter sucrose-rich conditions, would it be in plant association, in the oral cavity, or in food manufacturing or bio-fuel production processes (Ullrich, 2009). When sucrose is present, the soybean-infecting bacterial blight pathogen, *Pseudomonas syringae* pv. glycinea, uses levansucrase (Lsc) to synthesize the extracellular high molecular fructofuranan, levan, thereby releasing glucose for primary metabolism. Grown epiphytically on plants, bacterial communities 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 *et al.*, 2003). Stomatal openings and wounds provide the site of entry for *P. syringae*, at which under favorable micro-environmental conditions the bacterial cells live

in the endophytic phase of the disease cycle and initiate the infection process possibly using the phytotoxin coronatine (Budde *et al.*, 2000; Melotto *et al.*, 2008). 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).

Once inside the leaf tissue, *P. syringae* attaches to plant cells, following a complex sequence of events mediated by injection of *hrp* effector proteins into plant cells (Collmer *et al.*, 2000), which activates plant-borne K^+ efflux and H^+ influx, the apoplastic pH increases from 5.5 to 7.5 (Atkinson *et al.*, 1987; Gross,1991). Subsequently, this high extracellular pH induces efflux of sucrose, the dominant photoassimilate (Atkinson *et al.*, 1987). Apoplastic sucrose concentrations in *P. syringae*-infected bean leaves were estimated to range from 20 µM to 1-5 mM (Atkinson *et al.*, 1987).

Three levansucrase-encoding genes, *lscA*, *lscB*, and *lscC*, were identified in *P. syringae* pv. glycinea PG4180, from which only *lscB* and *lscC* were expressed since a mutant lacking *lscB* and *lscC* but containing *lscA* was levan-deficient (Li *et al.*, 2001). Mutant analyses furthermore suggested a differential secretion of Lsc gene products with LscB being the predominant extracellular variant and LscC being the cell-bound, periplasmic form (Li *et al.*, 2006). Both enzymes were synthesized maximally at 18ºC *in vitro* and *in planta* and their expression was the highest at the early logarithmic growth stage (Li *et al.*, 2006; Schenk *et al.*, 2008).

Regarding glucose metabolism, bacteria of the genus *Pseudomonas* metabolize glucose exclusively by the Entner-Doudoroff pathway due to lack of 6-phosphofructokinase and hence do not catabolize sugars via the Embden-Meyerhof-Parnas pathway usually found and well-studied in *Enterobacteriaceae (Lessie et al., 1984; Conway,1992; Portais et al., 2002)*.

The major aim of the present study was to gain better insight into the transcriptional regulation of *lsc* genes. For this, the transcriptional start sites of *lscB* and *lscC* were mapped and the upstream sequences analyzed using nested deletion and bioinformatics approaches. DNA affinity chromatography revealed binding of the hexose metabolism repressor, HexR, in a temperature dependent manner and implying its role in *lsc* transcriptional regulation. Furthermore, the bicistronically transcribed *com* gene was analyzed however revealing that this gene did not influence expression of *lscB*.

Results

Determination of transcriptional start sites of lscB and lscC

Both, the coding regions and the upstream sequences of *lscB* and *lscC* are highly similar (98.1% identity for the coding sequence and 97.5% identity for the –61 bp upstream to +340 bp downstream sequence). Consequently, primer extension experiments using total RNA from PG4180 cells and a set of reverse oligonucleotide primers including primer pe.BC.PG~150bp were carried out to determine the transcriptional start sites (TSS) of *lsc* genes. This resulted in a clear signal at nucleotide position -339 upstream of the translational start codon of *lscB* and *lscC* when oliognucleotide primer pe.BC.PG~150bp was used (Fig. 1). Application of other primers located up- and downstream did not yield in an extension product suggesting that position -339 is the only TSS and is identical for both, *lscB* and *lscC*. Since upstream regions of *lscB* and *lscC* are highly homologous to those of two *lsc* genes, termed *lsc1* and *lsc3*, from *P. syringae* DC3000, the experiment was repeated with total RNA of DC3000 and a primer analogous to the one above revealing the same result (Data not shown). The predicted long non-translated region(s) prompted a BLAST-P search for potential additional coding sequences revealing the presence of a 192-bp ORF co-directed with

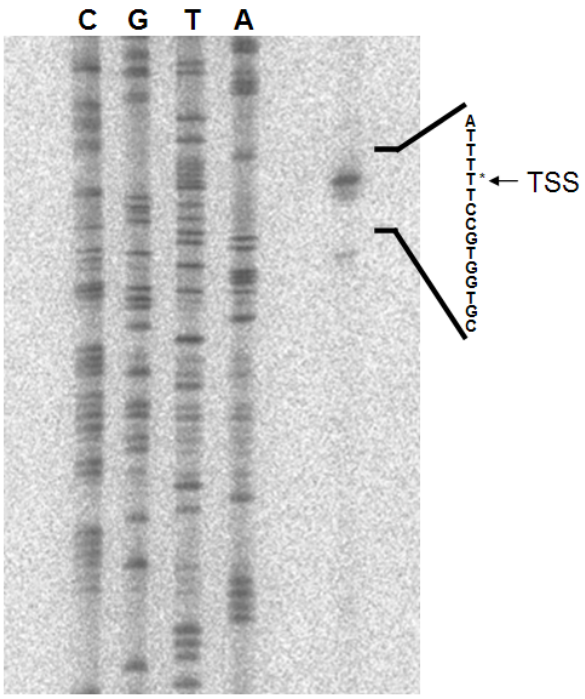

Fig. 1. Determination of the transcriptional start site (TSS) of *lscB* in *P. syringae* PG4180 by electrophoresis of nucleotide sequencing reaction and primer extension on 6% polyacrylamide gel. Nucleotide sequence of the TSS (*) is shown at the right.

lscB and *lscC* and being homologous to the *com* gene encoding for a translational regulator in various bacteriophage genomes (Braid *et al.*, 2004; Morgan *et al.*, 2002) (Fig. 2). The predicted amino acid sequence of the *com*-like ORF exhibited a high degree of similarity to Com translational regulators in bacteriophages, including DVM 2008 of *P. fluorescens Pf-5* (97.6% similarity), Mu-like phage of *P. entomophila* L48 (85.4% similarity), and phage B3 of *P. aeruginosa* (78.3% similarity), all belonging to the Com super family. The *P. syringae com*-like gene additionally possessed conserved Shine-Dalgarno sequences (5'-AAGGAG-3') 4 bp upstream of its translational start site which might indicate its efficient translation.

Bioinformatics analysis of lscB upstream region

The nucleotide sequence spanning from -339 bp to -1100 bp with respect to the translational start site of *lscB* of *lscB* was analyzed for potential transcription motifs, ORFs, and conserved binding sites for transcriptional regulators. According to the annotated genomes of three *P. syringae* strains (www.pseudomonas.com), position -354 bp with respect to the *lscB* TSS coincided with the translational stop site of an ORF termed 1448A homolog, which does not show any conserved sequence motifs and is only found in the respective genome regions of *P. syringae* strains PG4180, 1448A and DC3000 designated PSPPH_A0029 and PSPTO_A0034, respectively (Fig. 2). Nucleotide sequence comparisons revealed no classical -10 or -35 bp boxes but presence of an additional 477-bp ORF divergently oriented to *lscB* and annotated to encode for a conserved hypothetical protein, *chp* (www.pseudomonas.com). Interestingly, *chp* is fully present in the upstream sequence of *lscB* but is truncated in that of *lscC* (Data not shown). However, the *chp* gene could be identified upstream of the *lscB* homologs in all five fully annotated genomes of *P. syringae*. The predicted amino acid sequence of *chp* indicated two possible membrane spanning domains with a large cytoplasmic or periplasmic loop suggesting a membrane association of this gene product.

Analysis of the nucleotide sequences in close upstream proximity of the TSS of *lscB* and *lscC* excitingly revealed the presence of a motif (nucleotides -21 to +5) with high similarity to the conserved motif GntTtTaN_{12}TAAAAnC, previously predicted to be the binding site of HexR, a global repressor of hexose metabolic genes in *Pseudomonas putida* (Petruschka *et. al.*, 2002; Castillo et al., 2008) (Fig. 3). This sequence could potentially form a stem loop structure thus possibly exposing the TSS of *lscB* to binding of regulatory proteins.

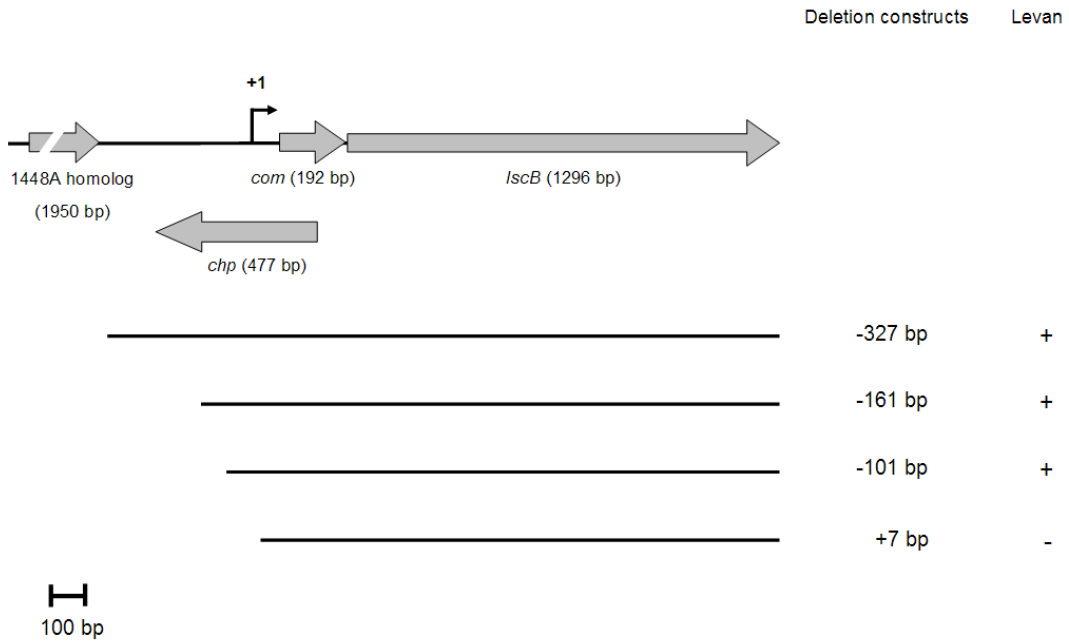

Figure 2. Schematic presentation of *lscB* and its upstream nucleotide sequence containing the potential ORFs, *com* and *chp*. +1 represents the transcriptional start site. Thin black bars represent deletion constructs, for which levan phenotypes after complementation of *lscB lscC* double mutant, PG4180.M6, are given at the right. Gene *chp* (conserved hypothetical protein) is located divergently to *lscB* while gene *com* is organized bicistronically with *lscB*. The ORF termed 1448A homolog is conserved in different *P. syringae* strains but has no assigned function.

Deletion analysis of upstream sequence of lscB and lscC

To substantiate the above finding, a nested deletion analysis of the *lscB* and *lscC* upstream sequence ranging from –327 to +7 of the respective TSS was conducted. For this, the *lscB*- and *lscC*-deficient mutant PG4180.M6 (Li and Ullrich, 2001) was complemented with various plasmid-borne deletion constructs and the phenotypes of the transconjugants were analyzed with respect to levan production, quantitative Lsc production, and compartment-specific accumulation of Lsc using Western blotting (Fig. 4). As expected, deletion constructs ending 5' at position -101 upstream of the TSS as well as any larger upstream sequence complemented mutant PG4180.M6 with respect to levan formation while the deletion construct ending at position +7 did not complement this mutant thereby confirming the above results. The experiment was repeated with respective deletion constructs for *lscC* giving the same results (data not shown).

An unexpected observation was made when the compartment-specific accumulation of LscB was compared for various deletion constructs complementing mutant PG4180.M6 (Fig. 4). Any nucleotide sequence larger than -161 bp upstream of the TSS of *lscB* mediated the typical LscB secretion pattern with the majority of this enzyme accumulating in the supernatant and minor amounts found in the periplasm (Li *et al.*, 2006). However, the smallest levan formation-mediating deletion construct, termed -101, showed a remarkably different pattern. For the respective transconjugant, a complete absence of LscB in the periplasmic fraction was observed aside of the typical extracellular accumulation (Fig. 4). Since deletion construct -

101 contained a 3'-truncated *chp* ORF, it was hypothesized that Chp might play a role in periplasmic retention of LscB.

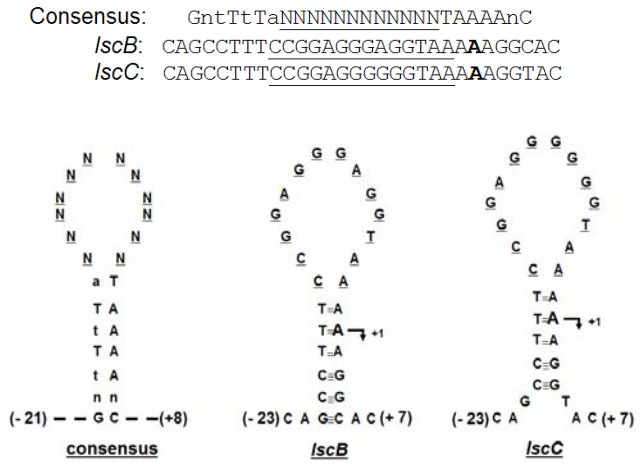

Fig. 3. Conservation of the *Pseudomonas putida* consensus HexR binding site (del Castillo *et al*., 2008) in *P. syringae lscB* and *lscC* upstream regions. Variable nucleotides in the loop regions are underlined. Bold letters and arrows represent the transcriptional start sites of *lscB* and *lscC*. Numbers in brackets are nucleotides relative to the TSS of *zwf-1* (Castillo *et al*., 2008), *lscB*, and *lscC*.

To test this, an additional deletion construct ending at position -161 bp upstream of the *lscB* TSS and containing the full size *chp* ORF was constructed and tested for compartment-specific Lsc complementation in mutant PG4180.M6. However, this construct exhibted the same phenotype as construct -101 thereby ruling out any involvement of Chp in periplasmic retention of LscB and suggesting that the total length of upstream sequence determines lscB expression and that lowered expression levels might lead to lack of periplasmic accumulation of LscB.

Site-directed mutagenesis of the com *gene*

Using heterologous expression of *lscB* in a transconjugant of *P. putida* KT2440 carrying a potential activator gene termed *lscR* from *P. syringae* PG4180 (Zhurina et al., unpublished data) the wild type and a site-directed mutant *com* gene of PG4180 were compared with respect to levan formation. *P. putida* KT2440 carrying the non-mutated com gene showed the same level of levan formation and LscB

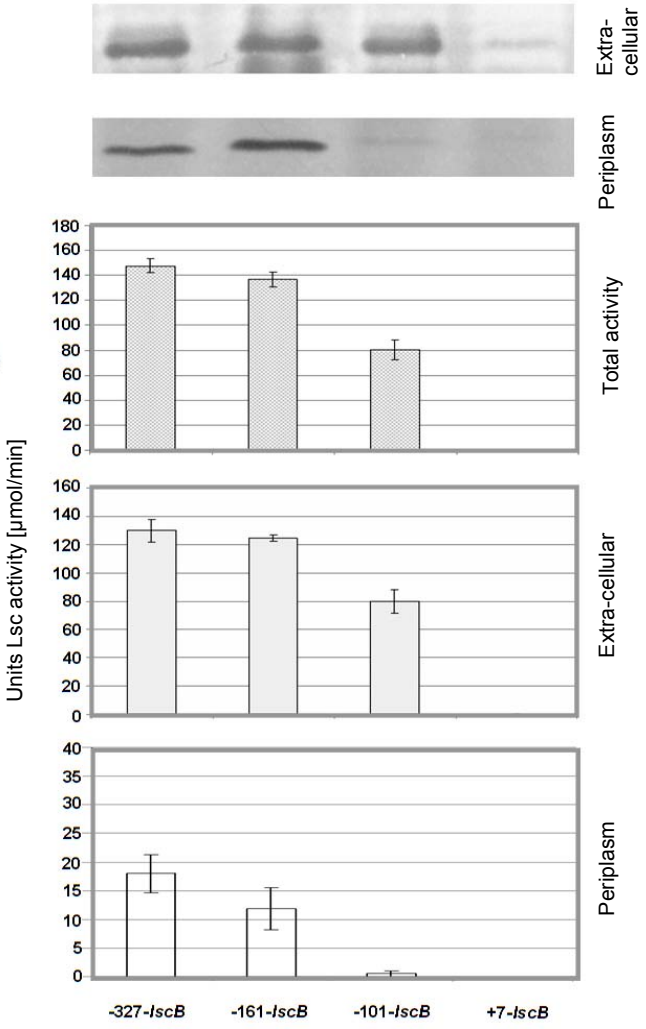

Fig. 4. Cell compartment-specific accumulation of LscB and LscC in the levan-deficient mutant, PG4180.M6, after complementation with *lscB* upstream deletion constructs as determined by Western blotting (top panels) and quantitative enzymatic activity measurements (bottom panels). Cells were grown at 18°C and harvested at OD_{600} of 1.5 to 2.0. Cell-free extra-cellular fractions were concentrated 30-fold. Data represent average values from three independent experiments with three replicates each. Error bars represent standard deviation.

secretion as its pendant harboring a mutated *com* gene. Consequently, any potential involvement of the putative translational regulator, Com, in LscB production could be ruled out (data not shown).

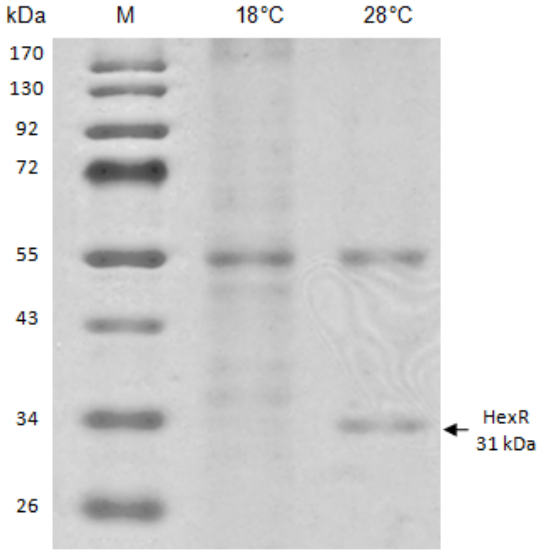

Figure 5. SDS-PAGE of proteins isolated from crude protein extracts of *P. syringae* grown at 18°C and 28°C, respectively, using DNA affinity chromatography with the *lscB* upstream region as bait. The arrow indicates a 31-kDa protein found in protein extracts of 28°C-grown cells and identified as HexR by MALDI-TOF MS and peptide mass fingerprint analysis.

Binding of HexR to the lscB upstream sequence

To identify regulatory proteins binding to the *lscB* upstream region, a respective 681-bp DNA fragment was immobilized on magnetic streptavidin beads and incubated with concentrated protein extracts of *P. syringae* PG4180 cells grown at 18°C or 28°C and harvested at an OD_{600} of 0.5. Sequential elution of DNA-bound proteins revealed the presence of a 31-kDa protein, which was found in the protein extract of 28°C-grown cells but was absent in the respective extracts of cells grown at 18°C (Fig. 5). Next, the protein was subjected to proteolytic cleavage and MALDI-TOF peptide mass fingerprint analysis. Interestingly, the 31-kDa protein was identified as the transcriptional repressor protein, HexR (Petruschka *et al.*, 2002; del Castillo *et al.*, 2008). Since HexR was found binding upstream of *lscB* only at 28°C but not at the *lscB* expression-promoting temperature, 18°C, this result suggested that HexR might be involved in transcriptional repression of *lscB* at 28°C.

Hexose utilization of P. syringae PG4180 and its lsc mutant

The above results suggested a regulatory link of intracellular hexose metabolism and expression of Lsc leading to extracellular sucrose cleavage. To substantiate this, growth of PG4180 and its *lscB lscC* double mutant, PG4180.M6, in liquid minimal medium containing either glucose or sucrose as sole carbon source was monitored (Fig. 6). Determined OD_{600} values corresponded to cell numbers as confirmed by determination of CFU/ml for representative time points (data not shown). For both, wild type and mutant, sucrose apparently allowed for a faster adaptation as sucrose-supplemented cultures grew faster during early logarithmic growth. However, during mid-logarithmic growth a significant difference between sucrose-supplemented wild type and mutant was observed. While the wild type showed the most optimal growth with sucrose reaching a cell density higher than that for glucose-grown cells, mutant PG4180.M6 exhibited a decreased growth rate and reached significantly lower final cell densities with sucrose as compared to those in glucose-supplemented medium (Fig. 6). This result clearly demonstrated that presence of Lsc enhanced growth on sucrose. No significant difference was observed when wild type and mutant were grown on glucose. The data showed that PG4180 can use both carbon sources and that Lsc activity is not the sole entry route of sucrose to hexose metabolism. However, our results also suggested that presence of Lsc gave *P. syringae* a significant growth advantage if only sucrose was provided.

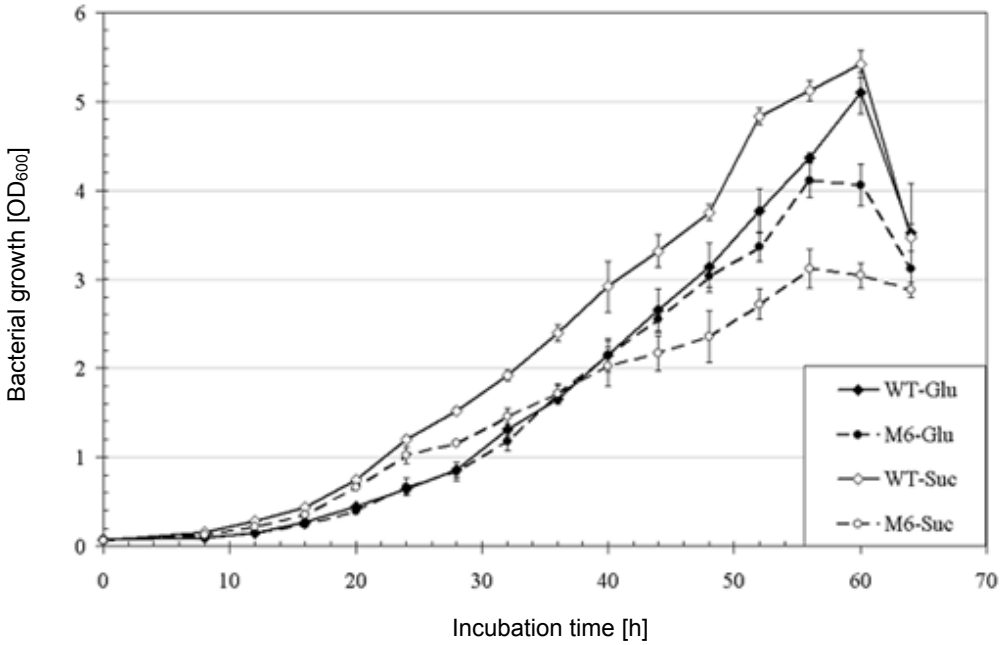

Fig. 6. Growth of PG4180 (WT) and its *lscB lscC* double mutant, PG4180.M6 (M6), in HSC minimal medium containing glucose or sucrose as sole carbon source at 18°C.

Generation and phenotypic characterization of hexR mutant

To investigate the impact of the hexose metabolism respressor, HexR, on expression of *lscB* and *lscC*, a *hexR*-deficient mutant of *P. syringae* PG4180 will be generated in the near future. The mutant will be obtained by homologous recombination, genetically verified by PCR analysis, and tested in comparison to the wild type for visual levan formation, *lscB* and *lscC* expression using Northern blot analysis, and accumulation of Lsc in cellular and extra-cellular fractions using immunological detection. The outcome of this experiment is pending. However, the following scenario could be envisioned: since HexR is supposed to repress transcription of hexose metabolism-associated genes, a Δ*hexR* mutant of *P. syringae* PG4180 could potentially overexpress *lscB* or might lose the typical thermoresponsive expression pattern of this gene.

Discussion

This is the first study reporting that expression of *lsc* genes in a gram-negative bacterium is controlled by the global hexose metabolic repressor, HexR (Petruschka *et al.*, 2002; del Castillo *et al.*, 2008). This finding opens the way to a fundamentally new understanding on how intracellular and extracellular enzymatic activities might be co-regulated. Determination of the TSS of *lsc* genes in *P. syringae* PG4180 and DC3000 as well as a nested deletion approach in PG4180 allowed for an in-depth bioinformatics analysis of the upstream sequences of *lsc* genes. Moreover, the currently determined length of *lsc* transcripts matched that previously reported by Northern blot analyses (Li *et al.*, 2006; Schenk *et al.*, 2008), indicating that *com* is cotranscribed with *lscB*. Here by mutational analysis it was demonstrated that *com* is co-transcribed with *lscB* however potential presence of its gene product has no impact on proper translation of LscB. Com homologues in bacteriophages such

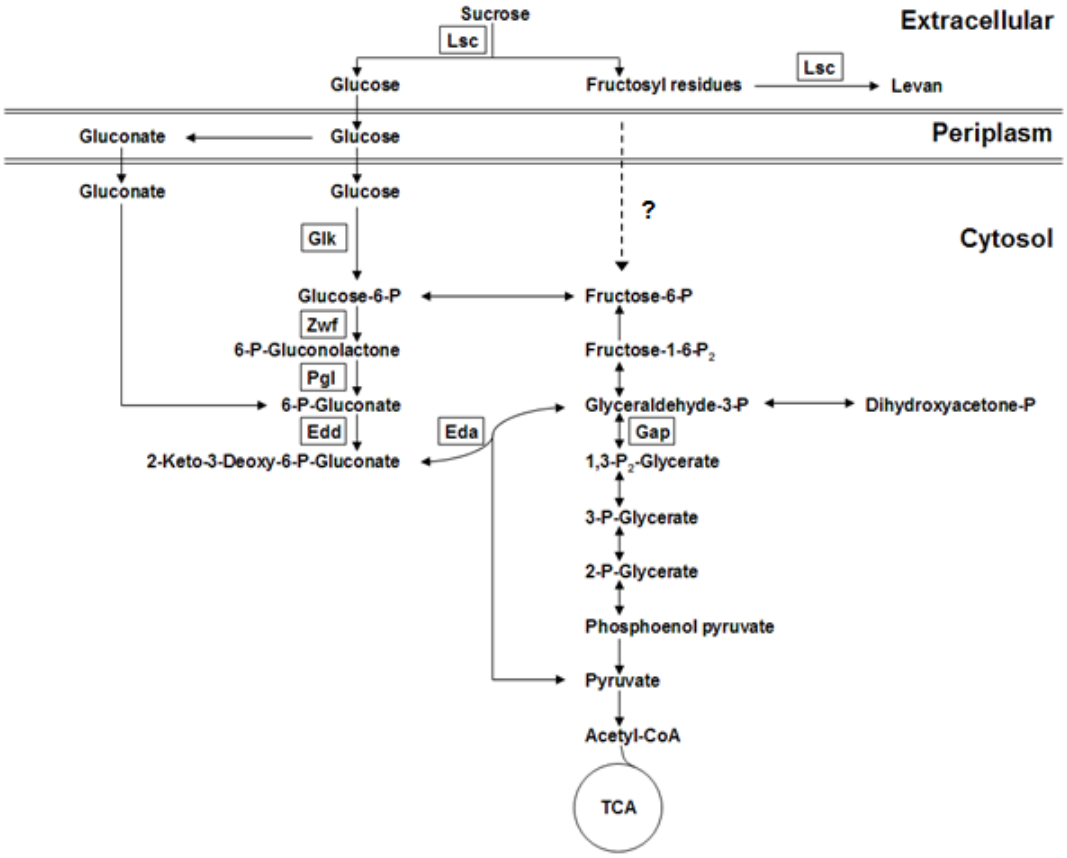

Fig. 7. Schematic presentation of putative sucrose utilization pattern in *P. syringae* PG4180. Enzymes put in rectangular blocks appear to be regulated by HexR (del Castillo *et al.*, 2008; and present study). Lsc, levansucrase; Glk, glucose kinase; Zwf, glucose-6-phosphate dehydrogenase; Pgl, 6-phosphogluconolactonase; Edd, 6-phosphogluconate dehydratase; Eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; Gap, glyceraldehyde 3-phosphate-dehydrogenase; TCA, tricarboxylic acid cycle.

as B3 or Mu were shown to act *in trans* as translational activators or anti-terminators at the mRNA level for *dam* of B3 and *mom* of Mu involved in DNA modification by methylation and acetamidoadenine modification, respectively (Hattman,1999; Braid *et al.*, 2004). Homologues of *com* were found in all five sequenced genomes of *P. syringae* (www.ncbi.nlm.nih.gov). Interestingly, the *lsc* genes found in these *P. syringae* strains were always associated with *com* homologues suggesting similar genomic arrangements, which in the future might improve our understanding how lsc genes were distributed among different *P. syringae* pathovars.

A particular role for the divergently located *chp* gene in expression or translocation of LscB was initially suspected but could be ruled out. Instead, it is likely that the length of the upstream sequence directly influences the level of expression and thus the compartment-specific accumulation of LscB by yet-to-be determined mechanism(s).

At the TSS of *P. syringae lsc* genes palindromic sequences were identified, which resemble those of predicted HexR binding sites in the genome of *P. putida* (del Castillo *et al.*, 2008). Identical nucleotide sequences surrounding the TSS for *lscB* and *lscC* of PG4180 as well as for the homologous, *lsc1* and *lsc3*, of *P. syringae* strain DC3000

(www.pseudomonas.com) suggested similar regulatory patterns for at least two *P. syringae* pathovars. HexR was reported to act by binding to inverted repeats that partly or fully overlap the RNA-polymerase binding site (Rojo,1999). In contrast to enterobacteria (Lessie *et al.*, 1984), pseudomonads utilize a distinct peripheral glucose pathway, so called the Entner-Doudoroff pathway, channeling intermediates into glycolysis and the TCA cycle for energy conservation (Conway,1992) (Fig. 7). The transcriptional regulator HexR controls key steps of this process (del Castillo *et al.*, 2007; del Castillo *et al.*, 2008). HexR responds to a broad range of effectors including glucose, gluconate, ketogluconate, and fructose, and controls glucose flux to the TCA cycle via transcriptional regulation of the *edd* and *eda* genes required for the Entner-Doudoroff pathway and the pentose phosphate pathway genes, *glk* and *zwf-1* genes (del Castillo *et al.*, 2008). Herein, the binding of HexR to the upstream sequence of *lscB* was demonstrated using DNA affinity chromatography. It makes perfect sense that intracellular hexose metabolism is co-regulated with an enzyme, which apparently provides the vital precursor for glucose metabolism if the cells encounter moderate or high sucrose concentrations in the environment (Fig. 7).

From a micro-ecological point of view, sucrose utilization might be the dominant life style for plant-associated *P. syringae* strains (Atkinson *et al.*, 1987; Gross,1991) as supported by growth experiments conducted herein. However, this might also hold true for other bacterial species, which possess similar enzymes for cleavage of the disaccharide, sucrose, in order to obtain readily usable glucose for primary metabolism. Consequently, it may be advised to screen some of the most important oral cavity inhabitants (Bergeron *et al.*, 2001) as well as bacteria, which cause mucus formation in sucrose-based food manufacturing industry (Bekers *et al.*, 2003) or bio-fuel production (Rojers *et al.*, 1980) for presence of this regulatory linkage.

Of particular interest was the finding that HexR appeared to bind to the *lscB* upstream region only at 28°C but not at 18°C, which is the *lsc* expression-fostering temperature (Li *et al.*, 2006). This might not only explain the thermo-responsiveness of *lsc* expression (Smirnova *et al.*, 2001) but may also shed some light into a potentially temperature-mediated pattern of central glucose metabolism in *P. syringae*. At the optimal growth temperature, 28°C, *P. syringae* metabolizes nutrients at maximum rate thereby increasing the intracellular pool of activated glucono-phosphates, which in turn induce the repressive activities of HexR (**Fig. 7**). Under those conditions and in contrast to somewhat slower metabolism at 18°C, HexR may be required for repression of genes under its control including *lsc* genes.

In order to check this hypothesis, further experiments on expression of *lsc* as well as other known genes of the HexR regulon in the wild type *P. syringae* PG4180 strain as well as in its *ΔhexR* mutants will be conducted.

Whether HexR-controlled genes, such as *edd*, *eda*, *glk*, *pgl*, *zwf*-1, or *gap*-1 (Castillo et al, 2008) are indeed co-regulated with *lsc* and show similar temperature dependence in *P. syringae* remains to be studied in the future. Likewise, the expression pattern of *hexR* itself needs to be investigated. This study revealed exciting options for an in-depth analysis of intracellular and extracellular sucrose metabolism in the plant pathogen, *P. syringae*, and may allow us to better understand the epiphytic and pathogenic behavior of this organism. Results of this study may also open the field for respective analyses in other sucrose-utilizing gram-negative organisms with relevance in dental medicine and bio-fuel production.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was

maintained and grown at 37°C on Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). *P. syringae* was routinely maintained at 28°C on mannitol-glutamate (MG) medium (Keane *et al.*, 1970). For liquid cultures at 18 or 28°C, bacteria were grown in 200 ml of Hoitink-Sinden minimal medium (HSC) (Palmer *et al.*, 1993) in 1-liter Erlenmeyer flasks. Bacterial growth was continuously monitored by measuring the optical density at 600 nm (OD_{600}). Antibiotics were added to the media at the following concentrations (µg/ml), respectively: ampicillin, 50; spectinomycin, 25; kanamycin, 25; tetracycline, 25; gentamicin, 2.

Molecular genetic techniques and transconjugant studies

All routine molecular methods were performed using standard protocols (Sambrook *et al.*, 1989). Nucleotide sequencing was carried out commercially (MWG Biotech, Ebersberg, Germany). Nested deletion analysis for the upstream region of *lscB* gene in pRB7.2 (Li *et al.*, 2001) was conducted using the Erase-a-Base® kit (Promega, USA). Similar lengths of deletion constructs were generated for *lscC* gene by PCR amplification and cloning into vector pBBR1MCS-3. All constructs were electroporated into *E.coli* DH5α followed by tri-parental mating (Gerhardt *et al.*, 1994) into the *lscB lscC* mutant, PG4180.M6. Transconjugants were streaked on 5% sucrose-containing MG agar media as described by Li *et al*. (2001).

Determination of transcriptional start site

Bacteria were cultured in HSC medium at 18°C to an OD_{600} of 0.5 and harvested by mixing 15 ml of culture with an equal volume of chilled killing buffer (20 mM Tris-HCl [pH7.5], 20 mM NaN_3). This mixture was centrifuged at 4°C for 15 min at 3,220 × g. Total RNA was isolated from the cell pellets by acid phenol/chloroform extraction as described previously (Schenk *et al.*, 2008). RNA concentrations were determined by measuring the absorbance at 260 nm, (Sambrook *et al.*, 1989). For primer extension analysis, (^{32}P)-labeled primers, pe.BC.PG~150bp, pe.1.DC~150bp, and pe.3.DC~150bp, (4 pmol) were annealed with 10 µg of total RNA and reverse transcription was performed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Nucleotide sequencing of 5 µg plasmid pLB7.2, -327-*lsc1*, and -327-*lsc3* with primers, pe. BC.PG~150bp, pe.1.DC~150bp, and pe3.DC~150bp, respectively, was done with the Sequenase Version 2.0 DNA Sequencing Kit (USB) according to the manufacturer's recommendation. The extension product and sequencing reaction were resolved on a 6% polyacrylamide sequencing gel. Signal detection was performed using a FLA-3000 phosphoimager (Raytest, Straubenhardt, Germany) according to standard procedures (Sambrook *et al.*, 1989) and the manufacturer's recommendations.

Cell compartment-specific activities and immunological detection of Lsc

Sub-cellular fractionation of PG4180.M6 transconjugants, use of polyclonal antibodies, and quantitative measurement of compartment-specific Lsc activities were carried out as described previously (Li *et al.*, 2006). Sub-cellular protein fractions were loaded equally (5µg/lane) and separated by 10% SDS-PAGE. Electrophoresis, electroblotting on nitrocellulose membranes, and immunodetection were conducted by standard procedures (Sambrook *et al.*, 1989). 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.

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

Strain	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*supE*44 Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Sambrook *et al.*, 1989)
Pseudomonas syringae pv. glycinea		
PG4180	wild type, levan+	(Bender *et al.*, 1993)
PG4180.M6	*lscB lscC* mutant of PG4180, Sp, Gm, levan-	(Li *et al.*, 2001)
Pseudomonas syringae pv. tomato		
DC3000	wild type, levan+	(Moore *et al.*, 1989)
Pseudomonas putida		
KT2440	wild type, levan$^-$	(Ramos-Diaz *et al.*, 1998)
Plasmid		
pRK2013	Km^r, helper plasmid	(Figurski *et al.*, 1979)
pLB7.2	Ap^r, contains *lscB* on 7.2-kb EcoRV insert	(Li *et al.*, 2001)
pRB7.2	Cm^r, contains *lscB* on 7.2-kb *Eco*RV insert	(Li *et al.*, 2001)
pLC5.5	Ap^r, contains *lscC* on 5.5-kb *Pst*I insert	(Li *et al.*, 2001)
-327-*lscB*	Cm^r, *lscB* gene with -327bp upstream sequence to/with tss, (*lscB*>P_{lac})	This study
-161-*lscB*	Cm^r, *lscB* gene with -161bp upstream sequence to/with tss, (*lscB*>P_{lac})	This study
-101-*lscB*	Cm^r, *lscB* gene with -101bp upstream sequence to/with tss, (*lscB*>P_{lac})	This study
+7-*lscB*	Cm^r, *lscB* gene with +7bp downstream sequence to/with tss, (*lscB*>P_{lac})	This study
-327-*lscC*	Tc^r, *lscC* gene with -327bp upstream sequence to/with tss, (*lscC*>P_{lac})	This study
-161-*lscC*	Tc^r, *lscC* gene with -161bp upstream sequence to/with tss, (*lscC*>P_{lac})	This study
-101-*lscC*	Tc^r, *lscC* gene with -101bp upstream sequence to/with tss, (*lscC*>P_{lac})	This study
+7-*lscC*	Tc^r, *lscC* gene with +7bp downstream sequence to/with tss, (*lscC*>P_{lac})	This study
-327-*lsc1*	Tc^r, *lsc1* gene with -327bp upstream sequence to/with tss, (*lsc1*>P_{lac})	This study
-327-*lsc3*	Tc^r, *lsc3* gene with -327bp upstream sequence to/with tss, (*lsc3*>P_{lac})	This study
cos2	Tc^r, genomic library clone of PG4180; approx. 30-kb inserts in pRK7813, promotes *lscB* expression in *P. putida* (*lscB*)	Zhurina *et al.*, unpublished
pGEM.HexR1	Ap^r, contains 456-bp upstream region of the *hexR* gene	This study
pGEM.HexR2	Ap^r, contains 360-bp downstream region of the *hexR* gene,	This study
pGEM.HexR1-Km	Ap^r, Km^r , 1230-bp *Kpn*I fragment containing Km^r-*FRT* cassette from pFKm subcloned into pGEM.HexR1	This study
pGEM.HexR-Km	Ap^r, Km^r, 360-bp *Bam*HI-*Spe*I fragment from pGEM.HexR2 subcloned into pGEM.HexR1-Km	This study
pEX.HexR-Km	Ap^r, Km^r, 2046-bp *Eco*RI fragment of pGEM_HexR-Km subcloned into pEX18Ap	This study
pFKm1	Source of Km^r cassette flanked with *FRT* sequences	H.Schweizer
pEX18Ap	Ap^r, oriT+ sacB+ gene replacement vector	(Hoang *et al.*, 1998)
-327-*lscB*.com1	Km^r, *lscB* gene with -327bp upstream sequence to/with tss, (*lscB*>P_{lac}) and nucleotide deletion in com gene	This study

[a] *Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; Tc, tetracycline*

Table 2. Oligonucleotide primers used in this study.

Oligonucleotide primer	Nucleotide sequence (5' to 3')[a]
lscB_PG-327_fwd	GATGAGCTCCTAAGGCAGTCGCATTAA
lscB_PG-161_fwd	GATGAGCTCAGTCGCAATTAATGCGAG
lscB_PG-101_fwd	GATGAGCTCCCAGGTCAATGGCGCAGC
lscB_PG+7_fwd	GATGAGCTCCACGATATGCGATTTGCG
lscB_PG_rev	CGATCTAGATCAGCTTAGCGTCACGTC
lscC_PG-327_fwd	GATGAGCTCAGCTCTGCCAGAAACAGG
lscC_PG-161_fwd	GATGAGCTCTCATAGGAAATTCCTTTT
lscC_PG-101_fwd	GATGAGCTCCCGGGTCAATTGCGCAAC
lscC_PG+7_fwd	GATGAGCTCCACGATATGCGATTTGCG
lscC_PG_rev	CGATCTAGATCAGCTCAGTTGCACGTC
pe.BC.PG~150bp	GTCACCCATGCGGGCCAGCAG
pe.1.DC~150bp	GTCACCCATGCGGGCCAGCAG
pe.3.DC~150bp	GTCACCCATGCGAAGCAGCAG
lscB-fw-DAP	GTTAGCCGGACTCGCTCA
lscB-rev-DAP	ATAGCTTTGGGAGGCAGCAG
HR_1f	GGATCC GTTCAACTCATCGAGTC
HR_1r	CAGATGCGACTGTTCGTC
HR_2f	GACCCCCGGATCAGTGCCAG
HR_2r	GGATCC GGTACC CAGCCGCTATCCGATCGAG
com1	GCAAATGTTGAAAGACTACCGATGCGGGCAGTGC

[a] Restriction sites incorporated in primers are underlined, GAGCTC – *Sac*I, TCTAGA – *Xba*I, GGATCC – *BamHI*, GGTACC – *KpnI*

DNA affinity chromatography and protein identification by MALDI-TOF and peptide mass fingerprint analysis

Two liters of *P. syringae* culture were grown in HSC minimal medium at 18 and 28°C, harvested at 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. Cellular debris was removed by centrifugation at 45,000 x *g* and 4°C for 60 min. Total protein amount in the crude extracts was determined using a Nanodrop apparatus (Thermo scientific, Wilmington, USA) and verified using SDS-PAGE.

Purification of DNA-binding proteins was performed as described previously (Cramer et al., 2006; Gabrielsen et al., 1989). Briefly, the *lscB* upstream sequence probe covering the region from -341 to +339 with respect to the TSS was generated by PCR using plasmid pRB7.2 (Li *et al.*, 2001) as a template and primers lscB-fw-DAP and LscB-rev-DAP, the latter tagged with biotin via TEG linker (MWG-Biotech, Ebersbach, Germany). Unincorporated oligonucleotides were removed during PCR product purification using Nucleospin Extract II kit (Macherey-Nagel, Bethlehem, USA). About 100 pmol of biotin-labeled PCR product was coupled to 3 mg of magnetic Dynabeads coated with streptavidin (Dynal, Oslo, Norway). Uncoupled DNA was removed by magnetic separation with a magnet particle concentrator (New England Biolabs) according to the manufacturer's recommendations. Prior to incubation with protein extracts, the DNA-loaded Dynabeads were equilibrated in 300 µl of binding buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol [DTT], 100 mM NaCl, 10% glycerol, and 0.05% Triton X-100) for two minutes.

Equal amounts of protein extracts were added to DNA-loaded Dynabeads suspensions for 2 h at room temperature with gentle mixing in a Hybaid rolling shaker (ThermoFisher

Scientific, Langenselbold, Germany). Unbound proteins were removed by four-fold washing with 100 µl of binding buffer containing 10 mM NaCl and subsequent magnetic separations. Subsequent elution of DNA-bound proteins was done with 15 µl of binding buffer containing 0.3 M and 1 M NaCl, respectively, followed by magnetic separation. Eluted fractions were collected and separated on 10% SDS-PAGE using 10-20% Tris-Tricine pre-casted protein gels (BioRad) stained with the colloidal blue Coomassie staining (Kang *et al.*, 2002). Protein identification by means of MALDI-TOF and peptide mass fingerprint analysis was performed as described by Cramer et al. (2006).

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. PCR products were cloned into pGEM-T Easy (Promega, Mannheim, Germany) yielding plasmids pGEM.HexR1 and pGEM.HexR2. A 1230-bp *Kpn*I fragment containing the Km^R cassette flanked with *FRT* sequences was removed from plasmid pFKm1 (kind gift of prof. H.Schweizer, Colorado State University, USA) and ligated into *Kpn*I-digested pGEM.HexR1, yielding pGEM.HexR1-Km. A 360-bp *Spe*I-*Bam*HI fragment digested from pGEM.HexR2 was ligated into *Spe*I-*Bam*HI-digested pGEM.HexR1-Km, yielding plasmid pGEM.HexR-Km. Finally, a 2046-bp *Eco*RI fragment was removed from pGEM.HexR-Km and ligated into *Eco*RI-digested pEX18Ap, yielding the *hexR* gene replacement plasmid pEX.HexR-Km. This plasmid is being mobilized into *P. syringae* PG4180 by tri-parental mating with helper plasmid pRK2013 (Figurski et al., 1979). Putative mutants will be screened on MG media supplemented with kanamycin and their genotype will be subsequently confirmed by PCR.

Site-directed mutagenesis of com and heterologous expression in P. putida

To analyze the influence of *com* on expression of *lscB*, a single-nucleotide change at position 15 which resulted in a nonsense (amber) mutation in the fifth codon of the *com* gene and absence of a functional Com gene product was introduced into plasmid -327 using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, Holland) and the mutagenic primer, com1. The resulting plasmid -327-*lscB*.com1 was mobilized into the *com*-negative *P. putida* strain KT2440 by tri-parental mating with helper strain pRK2013. Since KT2440 is natively not expressing *lscB* when plasmid -327-*lscB* is inserted, KT2440 (-327-*lscB*.com1) and KT2440 (-327-*lscB*) were additionally supplemented with the PG4180 genomic library clone cos2, which mediates levan formation and *lscB* expression in *P. putida* (-327) (Zhurina et al., unpublished).

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4 CONCLUSIONS

More and more data point to the importance of exopolysaccharides (EPS) in colonization of host tissues by bacterial pathogens. For example, it was recently demonstrated that due to their polyanionic nature bacterial EPS are able to chelate divalent calcium ions (Aslam *et al.*, 2008). Thus, EPS suppress the host immune response, which is induced by pathogen-borne conserved oligomeric and polymeric molecules also called MAMPs (microbe-associated molecular patterns). This also points to a potential novel role of the EPS, levan, which is produced by *Pseudomonas syringae* pathovars in sucrose-rich environments such as on the plant surface and is synthesized by levansucrase (Lsc). Strong expression of *lsc* genes encoding Lsc at 18°C suggested its tight connection with global low-temperature induction of virulence and pathogenicity in *P. syringae.* Hence, Lsc production must be regulated at the transcriptional level to prevent energy-demanding levan formation under inappropriate conditions.

In the current work, four transcriptional regulators were identified, which control cold-induced expression of the *lsc* genes in *P. syringae* PG4180. Successful application of DNA affinity chromatography and subsequent peptide mass fingerprint analysis revealed presence of three repressor proteins named HexR, MvaT, and MvaU, which bound upstream of the *lscB* gene at 28°C, when expression of Lsc is low. In contrast, no such binding occurred at the *lscB* expression-promoting temperature of 18°C. The identified repressors fall into two functional categories: MvaT and MvaU belong to the growing family of H-NS-like proteins (Tendeng *et al.*, 2003), whereas HexR is the central regulator of genes, which are involved in glucose metabolism via the Entner-Doudoroff pathway (del Castillo *et al.*, 2008). Interestingly, the positive regulator, LscR, was identified to control reversible binding of MvaT and MvaU to the *lscB* promoter region. This is a fundamentally novel type of Lsc regulation as compared to previously published data by others. Besides this, the expression patterns of *mvaT*, *mvaU*, and *lscR* with respect to temperature and growth phase were characterized in detail. A summarizing schematic presentation of how *lscB* transcriptional regulation may occur at different temperatures is provided in Fig. 3.

Involvement of MvaT, MvaU and LscR in regulation of *lsc* expression

Although the role of MvaT-like proteins has already been investigated to variable extent in other *Pseudomonas* species (Diggle *et al.*, 2002; Vallet *et al.*, 2004; Westfall *et al.*, 2004; Baehler *et al.*, 2006; Castang *et al.*, 2008), identification of MvaT and MvaU as transcriptional repressors of Lsc synthesis enlarges the number of pathogenicity- and ecological fitness-asssociated MvaT targets. For the first time, the current study directly demonstrated the role of MvaT-like proteins for gene silencing in a plant pathogen bacterium, i.e. *P. syringae.* Moreover, Lsc fits into the list of known MvaT targets, as it was demonstrated that H-NS-like proteins function as global regulators for exo-product formation (Baehler *et al.*, 2006) and for colonization of host tissue (Vallet *et al.*, 2004; Baehler *et al.*, 2006) by regulating genes involved in virulence, quorum sensing, and biofilm formation (Diggle *et al.*, 2002; Westfall *et al.*, 2004; Castang *et al.*, 2008).

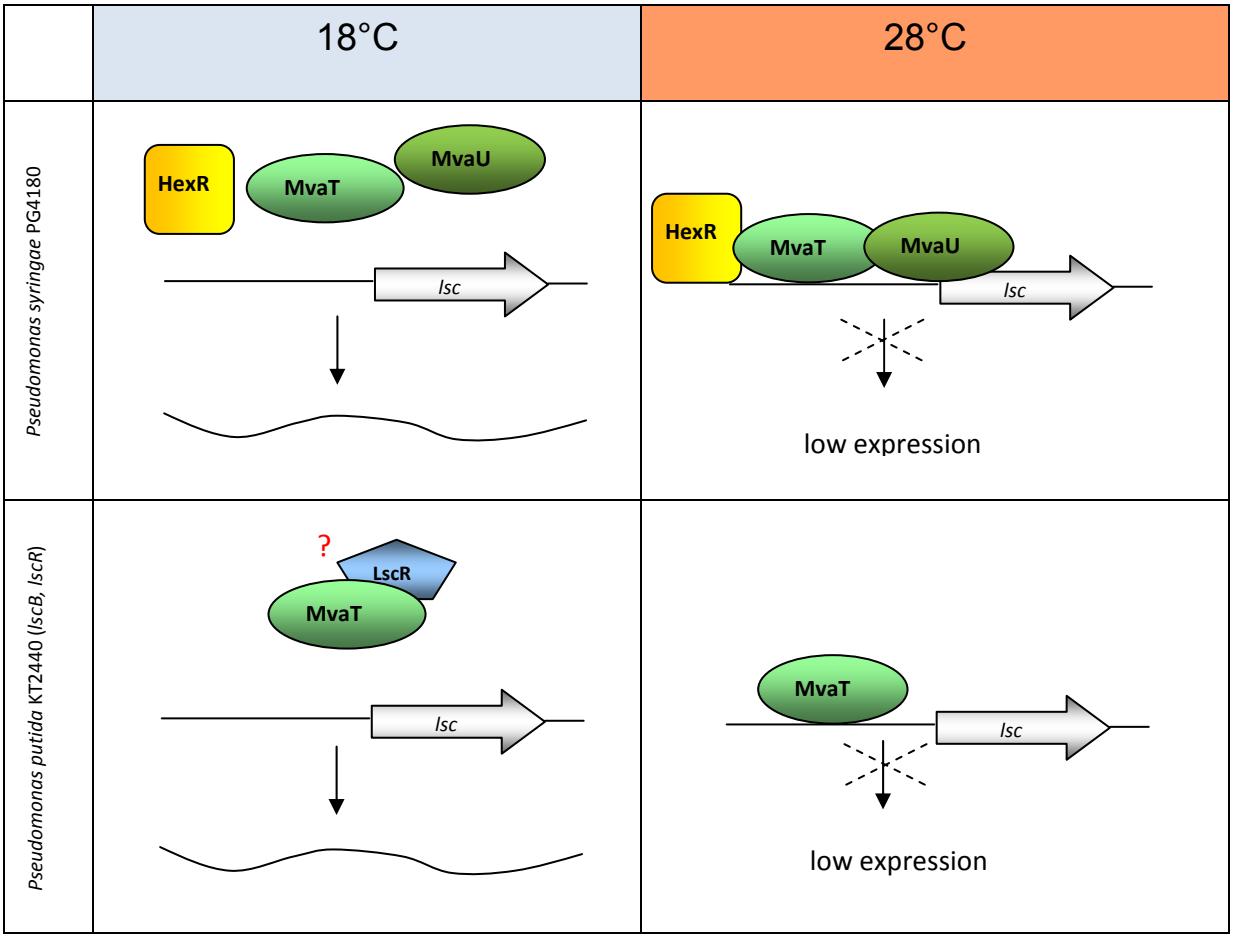

Fig. 3: Schematic presentation of the involvement of transcriptional regulators in temperature-dependent expression of *lsc* in *P. syringae* PG4180 and in the heterologous host, *P. putida*.

Thus far, there has been only one example for a MvaT-like protein reported, in which an involvement in temperature-dependent gene repression was shown: the TurA protein of *P. putida* modulates *Pu* promoter activity and thereby represses expression of an operon encoding for toluene-degrading enzymes at the suboptimal temperature of 16°C as opposed to 30°C (Rescalli *et al.*, 2004). Results of the current study are in contrast to those findings since herein more MvaT and MvaU binding to the target DNA was observed at elevated temperature. This striking difference might be due to an overall ''inversed'' virulence in plant pathogens, where virulence is induced by low-temperature conditions associated with infection-promoting high humidity on plant surfaces in contrast to the high-temperature conditions in warm-blooded hosts (Hurme *et al.*, 1998; Smirnova *et al.*, 2001).

Since DNA affinity chromatography did not reveal any protein binding to the *lscB* upstream sequence in *P. syringae* under inducing conditions, i.e. 18°C, potential positive regulator(s) had to be searched for by an alternative approach. Functional screening of a genomic cosmid library of *P. syringae* PG4180 in the heterologous host, *P. putida* KT2440, carrying *lscB* identified a 9.8-kDa transcriptional regulator, termed LscR, which gave rise to *lscB* transcription in the same temperature-dependent manner as in the native host, *P. syringae*. Interestingly, the observed phenotype was likely due to removal of *P. putida*-borne MvaT from the *lscB* upstream sequence by LscR at 18°C. In consequence, MvaT molecules of two different *Pseudomonas* species turned out to be interchangeable. Moreover, the mechanism of *lscB* transcriptional regulation as observed for *P. syringae* appeared to be fully restored in *P. putida* in presence of LscR. Analysis of *lsc* expression in a heterologous host therefore represents a promising experimental set up for simultaneous studies on *lscB* and *lscC* regulatory mechanisms due to the *lsc*-negative background, which was also successfully applied for mapping of the transcriptional start sites of both *lsc* genes.

P. syringae PG4180 bears two functional cold-induced levansucrase genes, *lscB* and *lscC,* in its genome. Considering that these genes and their corresponding upstream regions are ~98% identical with each other, the present study focused on *lscB* and used its upstream region as bait for DNA affinity chromatography. Experimental evidence, that *lscC* might indeed follow the same type of regulation, was obtained when LscR provided on a cosmid was shown to induce expression of the otherwise silent *lscC* in *P. putida*. However, to finally prove an identical or highly

similar type of regulation of *lscC as compared to lscB* DNA affinity chromatography experiments with the *lscC* upstream sequence as the DNA bait will have to be performed in future studies.

The observed temperature-dependent effect of LscR on *lscB* expression raised the interesting question about the mechanism, by which this protein might impact reversible binding of MvaT (and MvaU) proteins to the target upstream sequences. A recent review by Stoebel *et al.* (2008) summarized mechanisms, by which gene silencing via H-NS (and presumably H-NS-like proteins such as MvaT) could be reversed. This process might either occur due to changes in local DNA topology, the expression of promoter-specific activators, or expression of H-NS-modulating factors. Examples for H-NS-modulating factors are histone-like ~8-kDa proteins of the Hha-Ymo family, which were shown to modulate expression of some virulence genes in pathogenic *E. coli*, *Yersinia enterolitica,* and other enterobacteria (Mikulskis *et al.*, 1994; Madrid *et al.*, 2007). In support of the herein described findings, those modulatory factors affected H-NS target binding in dependence of different environmental stimuli including temperature (Cornelis *et al.*, 1991; Mourino *et al.*, 1994). The underlying processes are thought to rely on the high similarities, which these histone-like proteins show towards the N-terminal oligomerization domain of H-NS. It has been postulated that the H-NS-modulatory factors might interact directly with H-NS. This interaction results in heteromers, which fail to participate in DNA-protein-DNA bridging thus compromising the repressor complex structures (Nieto *et al.*, 2000; Nieto *et al.*, 2002). An additional interesting feature of some H-NS-modulating proteins is their thermoresponsive sensitivity to proteolysis with increased stability at lower temperatures (Jackson *et al.*, 2004).

Herein it was demonstrated that the H-NS-like MvaT/MvaU-mediated repression of *lscB* transcription in *P. syringae* can be reversed by presence of LscR, which probably interacts with MvaT directly. Thus it may be assumed that the principal mechanism mediating expression of genes belonging to the MvaT regulon in *P. syringae* might undergo similar rules as previously demonstrated for H-NS targets. More experimental data is required to confirm this speculation and to identify the exact mechanism(s), by which LscR, MvaT and MvaU interact and mediate Lsc expression in *P. syringae*.

H-NS of enterobacteria as well as MvaT and MvaU of *P. aeruginosa* silence ‘xenogenic’ elements in the genome such as prophage DNA or so-called

pathogenicity islands (Navarre *et al.*, 2006; Navarre *et al.*, 2007; Castang *et al.*, 2008; Stoebel *et al.*, 2008). These interactions are due to increased AT-contents of these genetic elements as compared to the characteristic AT/GC ratio for a given bacterial host genome. Interestingly, the plasmid-borne *lscB* gene of *P. syringae* is flanked by transposase genes and prophage-associated sequences (Hattman,1999; Braid *et al.*, 2004). Likewise, upstream of the chromosomally located *lscC* gene there is a prophage-borne gene cluster (www.pseudomonas.com). Consequently, it may be concluded that binding of MvaT and MvaU to the *lscB* upstream sequence might at least in part be mediated by 'xenogenic' DNA elements. Interestingly, although it had been annotated as a transcriptional regulator LscR also shows high similarity to certain bacteriophage proteins (Braid *et al*., 2004). There is a 11-kDa bacteriophage protein, encoded by gene 5.5, which is thought to be involved in target gene de-repression by interacting with H-NS (Liu *et al.*, 1993). Interestingly, the genomic positions of *mvaT*, *mvaU,* and *lscR* are conserved in three different *P. syringae* strains. Likewise, thermoresponsiveness of *lscB* expression seems to be conserved as well (H. Weingart, unpublished). Future studies will in-depth assess the potential of LscR to act as a putative bacteriophage-borne 'anti-repressor' of H-NS-like proteins.

Expression of *mvaT*, *mvaU* and *lscR* is temperature-dependent

Analysis of transcription of genes encoding for the three *lscB*-regulating proteins, MvaT, MvaU, and LscR was initiated in the current study. To address the question whether binding of MvaT and MvaU to the *lscB* upstream sequence and the interaction of those proteins with LscR were due to either thermo-responsive gene expression or temperature-dependent protein-protein interactions, Northern blot analyses were performed. As expected and similar to *lscB* expression (Li *et al*., 2006), *lscR* showed maximum expression levels at 18°C and during early exponential growth of *P. syringae*. Surprisingly, expression of *mvaT* and *mvaU* was also more pronounced at 18°C as compared to 28°C. Moreover, both genes showed distinct growth phase-dependent expression patterns. This might indicate the necessity to tightly control expression of energy-consuming cellular traits such as levan formation at certain growth stages. Expression of *mvaT* and *mvaU* might be important for many different cellular functions aside of *lscB* repression as shown for

other *Pseudomonas* species (Castang et al., 2008) while presence of the *lscR* gene is restricted to levan-forming *P. syringae* species. Consequently, the genetic interplay investigated herein might be an interesting composite of phylogenetically conserved regulatory traits with relatively recent modifications in the plant pathogen. Besides this, it is speculated, that *mvaT* and *mvaU* mRNAs might be sensitive to temperature alterations. To prove this, one should look for particular secondary structures as they were shown for comparable molecular mRNA thermometers. Consequently, the transcriptional start sites for both genes will be determined and RNA modeling will be performed in the near future.

The hexose metabolism repressor, HexR, is involved in regulation of *lscB* transcription

Besides MvaT, MvaU, and LscR, herein the global metabolic repressor, HexR, was shown to be involved in thermoresponsive regulation of the *lscB* gene. This is the first report about an extra-cellular enzyme, e.g. levansucrase, to be part of the well-studied HexR regulon. HexR is a global repressor, which controls glucose metabolic flux to the Krebs cycle via transcriptional repression of key enzymes required for the glucokinase and Entner-Doudoroff pathways (del Castillo *et al.*, 2008) (Fig. 4). Since pseudomonads can utilize many carbon sources aside of glucose, this pathway is inducible although the actual inducing molecules show considerable species variations (Conway, 1992). For example, for *P. putida* such inducing carbohydrates were glucose, gluconate, fructose, and glycerol (Petruschka *et al.*, 2002). HexR has a typical two-domain structure: a helix-turn-helix domain is responsible for DNA binding whereas a sugar isomerase domain is thought to bind phosphosugars. Presumable DNA target sequences of HexR in *P. putida* were predicted by del Castillo *et al.* (2008) and contain the conserved motif GntTtTaN_{12}TAAAAnC. Speculating that HexR binding sites might be conserved in different pseudomonads, the genome of *P. syringae* 1448A, which is a very close relative of *P. syringae* PG4180, was screened for potential HexR targets. Interestingly, besides the expected targets such as the *zwf* operon, such HexR binding sites were predicted to be located upstream of *lscB* and *lscC*, as well as in close proximity to *lscR* (I. Kostadinov and F.O.Glöckner, personal communications). This exciting preliminary result will be investigated in detail in future experiment and

might imply an overall substrate-dependent coordination of these genes in *P. syringae*.

From an ecological point of view it makes perfect sense that intracellular hexose metabolism is co-regulated with an enzyme, which provides the vital precursor for glucose metabolism if the cells encounter moderate or high sucrose concentrations in the environment. This is clearly true for plant-associated microorganisms but might also be applicable for other microbes residing in sucrose-rich environments such as the human oral cavity or sugar cane-driven bioethanol manufacturing (Ullrich, 2009). Of particular interest and yet puzzling was the finding that HexR appeared to bind to the *lscB* upstream region only at 28°C but not at 18°C, which is the *lscB* expression-fostering temperature (Li *et al.*, 2006). Consequently, future studies on this interesting topic may shed important new light onto a potentially temperature-mediated pattern of central glucose metabolism in *P. syringae*.

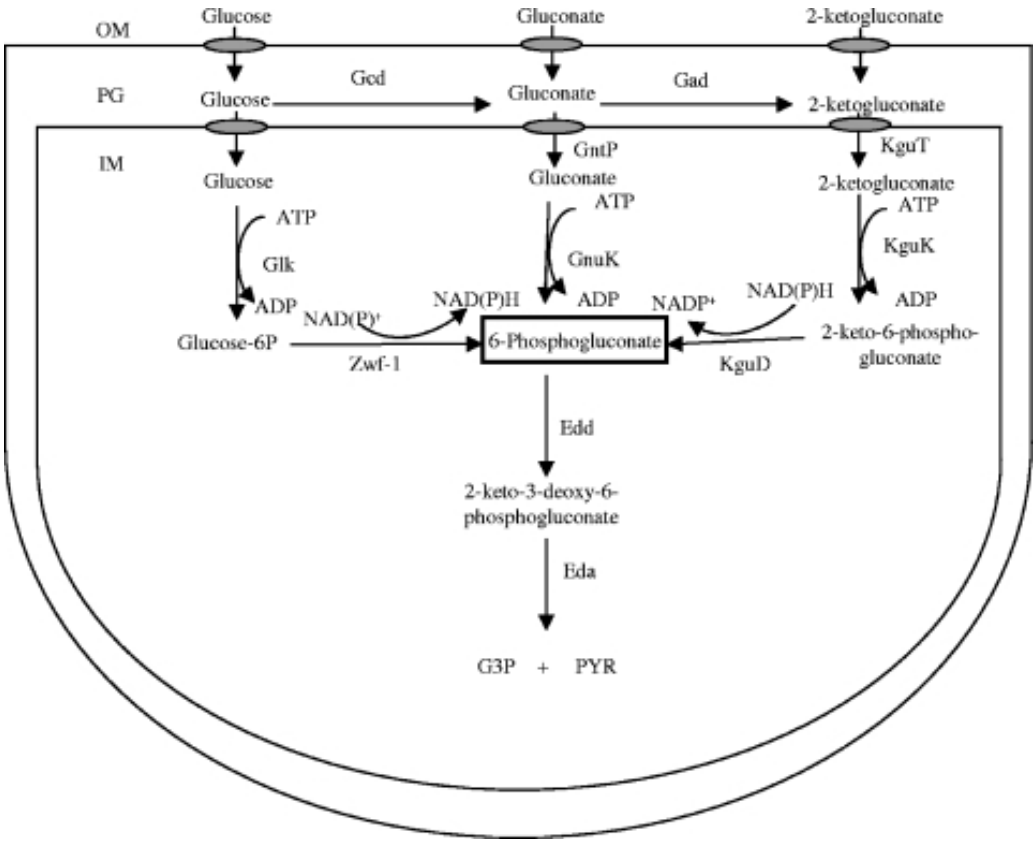

Fig. 4: Schematic presentation of the channeling of glucose into the Krebs cycle in *P. putida* KT2440 (del Castillo *et al.*, 2008).

5 FUTURE SCOPE

Transcriptional regulators, identified in the current work, gave new insights how the temperature-dependent expression of levansucrase is mediated in *P. syringae* PG4180. This has raised several exciting new questions, which are planned to be studied in future:

Individual mutants defective in the four genes of interest will be generated. The first of those mutants, *ΔmvaU*, has been successfully obtained. Knock-out constructs for the remaining three genes have been prepared and the attempts to obtain homologous recombinations are underway. Once obtained, mutant phenotypes will be characterized with respect to *lscB* expression in context of sugar metabolism and temperature changes. Other genes, which might be under control of HexR, MvaT, or MvaU will be analyzed in mutant backgrounds as well. Results of these experiments will indicate the embedding of *lsc* expression in higher-order regulatory mechanisms.

DNA binding experiments using electrophoretic mobility shift assays with overexpressed and purified regulator proteins will have to be performed. Currently, all four regulators were successfully overexpressed as cleavable hybrid proteins fused to maltose binding protein. Additionally, MvaT was overexpressed as a GST-fusion protein. Purified proteins will also be used in pull-down assays to clarify the nature of LscR interaction with MvaT.

A further exiting question is that of the ecological and metabolic function of levan. For this, biofilm studies using confocal laser scanning microscopy and sucrose-grown cells will be conducted with the wild type as well as with various mutants.

Ultimately, *in planta* studies are planned in order to estimate the overall contribution of each regulator in virulence and epiphytic fitness of *P. syringae*.

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