The density of negative charge in the cell wall influences two-component signal transduction in *Bacillus subtilis*

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The Dlt system modulates the density of negative charge in the cell wall of Gram-positive bacteria by substituting anionic polymers (wall and lipoteichoic acids) with D-alanine. The *htrA* and *htrB* genes, regulated by the CssRS two-component system (TCS) and encoding membrane-associated protein quality control proteases, were expressed at strongly decreased levels in a mutant with defective Dlt (*dltD*::miniTn10) as compared to the *dlt*⁺ wild-type strain under a secretion stress condition (hypersecretion of AmyQ α -amylase). The level of HtrA protein in the extracellular proteome of the *dltD* mutant was decreased consistently. Expression from the promoter of the *liaIHGFSR* (*yvqIHGFEC*) operon (*P_{liaI}*) is dependent on the LiaRS TCS. The Dlt defect increased the expression from *P_{liaI}* under two stress conditions, AmyQ hypersecretion and treatment with a cationic antimicrobial peptide (LL-37), but decreased the expression in vancomycin-treated cells. Furthermore, Dlt inactivation enhanced the expression of the YxdJK-regulated *yxdL* gene in LL-37-treated cells. The increased net negative charge of the cell wall seems to cause varied and opposite effects on the expression of CssRS-, LiaRS- and YxdJK-regulated genes under different stress conditions. The results suggest that TCSs which sense misfolded proteins or peptides are modulated by the density of negative charge in the cell wall. The density of negative charge on the outer surface of the cell membrane did not have a similar effect on TCSs.

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INTRODUCTION

Two-component signal transduction is involved in controlling the quality of exported proteins in bacteria (Raivio & Silhavy, 1999; Sarvas *et al.*, 2004). In *Bacillus subtilis*, the CssRS two-component system (TCS), the *B. subtilis* functional orthologue of the *Escherichia coli* CpxRA TCS (Connolly *et al.*, 1997; Danese & Silhavy, 1997; Missiakas & Raina, 1997; Pogliano *et al.*, 1997), responds to the accumulation of misfolded proteins at the membrane–cell wall interface (Hyryläinen *et al.*, 2001; Darmon *et al.*,

2002). CssRS-mediated signalling induces the *htrA* and *htrB* genes, which encode serine-type surface proteases involved in quality control. The expression of these quality-control proteases is strictly dependent on CssRS (Hyryläinen *et al.*, 2001; Darmon *et al.*, 2002). Severe secretion stress and consequent accumulation of misfolded proteins at the membrane–cell wall interface also induces several other genes in *B. subtilis* (Hyryläinen *et al.*, 2005), including the *liaIHGFSR* operon, the expression of which is also induced by several other stress conditions such as alkaline shock, detergents and cationic antimicrobial peptides (Wiegert *et al.*, 2001; Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005). The last two genes of the *lia* operon encode a TCS, LiaRS, which regulates expression from the *P_{liaI}* promoter in front of the *liaI* gene (Mascher *et al.*, 2004). The *liaF* gene encodes a membrane-bound

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Abbreviations: CAMP, cationic antimicrobial peptide; QRT-PCR, quantitative real-time RT-PCR; TCS, two-component system.

negative regulator of the LiaRS TCS (Jordan *et al.*, 2006). LiaH is similar to *E. coli* phage shock protein A (PspA). It negatively modulates (weakly) the activity of LiaR-dependent promoters (Jordan *et al.*, 2006), whereas the functional roles of LiaI and LiaG are less well known. In addition to P_{liaH} , there is a weak promoter in the immediate upstream region of *liaG*, P_{liaG} , from which the *liaGFSR* genes are expressed in unstressed cells (Jordan *et al.*, 2006).

PrsA3 is a mutant variant of the PrsA lipoprotein of *B. subtilis*, a membrane-bound peptidyl-prolyl *cis/trans*-isomerase (Hyyryläinen *et al.*, 2000; Kontinen *et al.*, 1991). We have shown previously that PrsA3 is highly sensitive to cell-associated proteolytic degradation and consequently its cellular level is decreased to only about 10 % of the wild-type level (Hyyryläinen *et al.*, 2000). We have observed that PrsA3 was stabilized at the membrane-cell wall interface in a similar manner in both *cssS* and *dltD* mutants (Hyyryläinen *et al.*, 2000, 2001). Mutations in *dlt* increase the density of negative charge in the cell wall matrix. In addition to PrsA3, some other mutant and heterologous proteins are also stabilized and their secretion improved when Dlt is inactivated (Craynest *et al.*, 2003; Hyyryläinen *et al.*, 2000; Thwaite *et al.*, 2002; Vitikainen *et al.*, 2005).

Four of the five proteins encoded by the *dlt* operon (DltA, B, C and D) comprise the Dlt system (Perego *et al.*, 1995). Dlt modifies both lipoteichoic and wall teichoic acids with D-alanine and thereby decreases the density of negative charge in the cell wall. The *dlt* operon is under the transcriptional control of σ^X , a sigma factor with an extracytoplasmic function, which belongs to the *Bacillus* cell envelope stress response network (Cao & Helmann, 2004). The improved stability of PrsA3 and other abnormal proteins in *dlt* mutants raises the question of whether the altered charge of the wall affects post-translocational folding of these proteins and thereby stabilizes them, as suggested previously (Hyyryläinen *et al.*, 2000). Alternatively, Dlt could modulate the quality control of exported proteins. The increased net negative charge of the wall in *dlt* mutants may increase the binding of divalent cations to the wall and the bound cations may improve post-translocational folding of exported proteins (Hyyryläinen *et al.*, 2000). Alternatively, the inactivation of Dlt could 'desensitize' the CsrRS TCS to misfolded proteins, decrease CsrRS-mediated signal transduction, and consequently decrease the expression of the HtrA and HtrB quality control proteases. Indeed, we could demonstrate in this study that in the absence of Dlt, *htrA* and *htrB* were expressed at significantly lower levels than in its presence. It was found that Dlt modulates TCS activity in a manner that is dependent on TCS and the type of inducer/stress.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in either Luria-Bertani broth (LB; 1 % tryptone, 0.5 % yeast extract, 1 % NaCl) or modified 2 × LB broth (2 × LB₅; 2 % tryptone, 1 % yeast

extract, 1 % NaCl) supplemented with appropriate antibiotics, unless otherwise indicated. The final concentrations of the antibiotics were 10 µg kanamycin ml⁻¹, 5 µg chloramphenicol ml⁻¹ and 1 µg erythromycin ml⁻¹. For gene expression measurements, bacteria were grown in the absence of antibiotics to avoid putative antibiotic effects on gene expression. Expression from the P_{spac} and P_{syn} promoters was induced by adding 1 mM IPTG and 0.2 % xylose, respectively, to the growth medium.

Two plasmids, pKTH10 and pKTH3339, were used to cause secretion stress. The pKTH10 plasmid is a derivative of pUB110 with an insert containing the *amyQ* gene of *Bacillus amyloliquefaciens* (Palva *et al.*, 1982). In pKTH3339 (a derivative of pSX50; Hastrup & Jacobs, 1990), *amyQ* has been placed under P_{syn} control (Vitikainen *et al.*, 2001). Strains harbouring these plasmids secrete AmyQ at high levels into the extracellular medium. The LL-37 antimicrobial peptide (purchased from Dr Ale Näränen, University of Kuopio, Finland, or from EzBiolab) and vancomycin were added in the early exponential growth phase (~60 Klett units, OD₅₆₀=0.6) to cause cell envelope stress, and were used at the subinhibitory concentrations of 4 or 6 µg ml⁻¹ and 2 µg ml⁻¹, respectively. Triton X-100 was added at the density of 100 Klett units (to cause stress) and used at a concentration of 0.005 %.

Transcription analysis by quantitative real-time RT-PCR (QRT-PCR). Total cellular RNA was isolated for QRT-PCR as described previously (Pietäinen *et al.*, 2005) or as follows. RNA was extracted by using the Roche High Pure RNA isolation kit according to the manufacturer's instructions. Bacteria from 2 ml of an exponential-phase culture were resuspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0, 1 mg lysozyme ml⁻¹) and incubated at 37 °C for 10 min. The subsequent steps were performed as described by the kit manufacturer. If cells were treated with LL-37, vancomycin or Triton X-100 to cause stress, the treatments were continued for 10 or 20 min, followed by harvesting of the cells and extraction of the RNA. Secretion stress was caused by overexpressing the α -amylase of *B. amyloliquefaciens* (AmyQ) from the pKTH3339 plasmid (Hyyryläinen *et al.*, 2001). P_{syn} -*amyQ* in pKTH3339 was induced with 0.2 % xylose at the density of 60 Klett units for 30 min, followed by RNA extraction. cDNA synthesis was carried out by using the high capacity cDNA archive kit (Applied Biosystems) with an additional step of DNase I treatment. Each reaction mixture consisted of 5 µl 10 × buffer, 2 µl 25 mM dNTP, 5 µl hexamer, 11 µl 25 mM MgCl₂, 5 µl 20 U DNase (Roche) and 1 µg RNA in 50 µl total volume. The mixture was divided into two equal portions and 1.3 µl reverse transcriptase (MultiScribe) was added to one. The cDNA synthesis reaction was carried out in a thermal cycler with the following steps: (i) DNase I treatment at 37 °C for 30 min, (ii) inactivation of DNase I by incubating at 75 °C for 5 min, (iii) cooling to 4 °C, (iv) addition of reverse transcriptase, (v) cDNA synthesis at 25 °C for 10 min, and (vi) at 37 °C for 120 min. QRT-PCR was carried out as described previously (Pietäinen *et al.*, 2005) using the SYBR Green PCR master mix (Applied Biosystems) and a 7500 real-time PCR system (Applied Biosystems). The cDNA values were normalized with the value of *gyrA* (Pietäinen *et al.*, 2005). Sequences of the QRT-PCR primers for the genes studied are shown in Table 2.

pMUTIN constructions and β -galactosidase assay. The pMUTIN2 (Vagner *et al.*, 1998) inserts in the *liaI* and *mprF* (*yfiW* and *yfiX*) genes constructed in the *Bacillus subtilis* Functional Analysis (BSFA) program (Kobayashi *et al.*, 2003) were used to study expression of these genes. Due to a sequencing error (an extra cytidine nucleotide) at position 916935 of the *B. subtilis* genome sequence, the *mprF* gene has been misidentified as two different genes, *yfiW* and *yfiX* (Staubitz & Peschel, 2002). The pMUTIN2 insertion sites in the *yfiW*::pMUTIN2 and *yfiX*::pMUTIN2 mutants are located at 248 and 977 (1 is the first nucleotide of the initiation

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and characteristics	Reference
Strains		
IH6531	<i>trpC2 glyB133 hisA1</i> pKTH10	Leskelä <i>et al.</i> (1999)
IH6538	<i>trpC2 glyB133 hisA1</i>	Leskelä <i>et al.</i> (1999)
IH7144	<i>prsA3 glyB133 hisA1</i> pKTH10	Hyryläinen <i>et al.</i> (2000)
IH7171	<i>prsA3 glyB133 hisA1</i>	Hyryläinen <i>et al.</i> (2001)
IH7375	<i>dltD::miniTn10 trpC2 glyB133 hisA1</i>	Vitikainen <i>et al.</i> (2005)
IH7378	<i>dltD::miniTn10 trpC2 glyB133 hisA1</i> pKTH10	This study
IH7558	<i>prsA3 glyB133 hisA1</i> pKTH3339	Hyryläinen <i>et al.</i> (2001)
IH7612	<i>htrA::pMUTIN2 trpC2 glyB133 hisA1</i> pKTH10	This study
IH7663	<i>yhcT::pMUTIN2 prsA3 glyB133 hisA1</i> pKTH3339	Hyryläinen <i>et al.</i> (2001)
IH7988	<i>htrB::pMUTIN4 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8112	<i>liaR::pMUTIN2 prsA3 glyB133 hisA1</i> pKTH3339	This study
IH8122*	<i>trpC2 hisA1 glyB133</i> pKTH10; parental strain	Leskelä <i>et al.</i> (1999)
IH8123*	<i>trpC2 hisA1 glyB133</i> ; parental strain	Leskelä <i>et al.</i> (1999)
IH8149	<i>htrA::pMUTIN2 dltD::miniTn10 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8205	<i>liaI::pMUTIN2 dltD::miniTn10 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8207	<i>liaI::pMUTIN2 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8219	<i>liaI::pMUTIN2 trpC2 glyB133 hisA1</i>	This study
IH8220	<i>liaI::pMUTIN2 dltD::miniTn10 trpC2 glyB133 hisA1</i>	This study
IH8268	<i>htrB::pMUTIN4 dltD::miniTn10 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8394	<i>mprF(yfiX)::pMUTIN2 trpC2 glyB133 hisA1</i>	This study
IH8396	<i>mprF(yfiX)::pMUTIN2 trpC2 glyB133 hisA1</i> pKTH10	This study
IH8429	<i>psd1::neo trpC2 glyB133 hisA1</i>	This study
IH8430	<i>mprF(yfiW)::pMUTIN2 trpC2 glyB133 hisA1</i>	This study
IH8434	<i>psd1::neo prsA3 glyB133 hisA1</i> pKTH3339	This study
Plasmids		
pKTH10	<i>P_{amyQ}-amyQ</i> ; Km ^r	Palva <i>et al.</i> (1982)
pKTH3339	<i>P_{xym}-amyQ</i> ; Cm ^r	Vitikainen <i>et al.</i> (2001)

*IH8122 and IH8123 are reisolutions of IH6531 and IH6538, respectively (Leskelä *et al.* 1999).

codon) in the 2568 nt *mprF* gene, respectively. The *htrA::pMUTIN2* and *htrB::pMUTIN4* constructs used in this study have been described previously (Hyryläinen *et al.*, 2001; Darmon *et al.*, 2002). β -Galactosidase activity was determined with ONPG (Merck) substrate as described previously (Msadek *et al.*, 1990) and the activity was expressed in Miller units per milligram total protein.

2D gel electrophoresis analysis. To prepare extracellular protein extracts, *B. subtilis* strains were grown at 37 °C under vigorous agitation in LB medium. After 1 h of post-exponential growth, cells

were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were precipitated with 10 % TCA and prepared for 2D PAGE, as described previously (Antelmann *et al.*, 2001). IEF was performed as described previously (Buttner *et al.*, 2001) using commercially available IPG Strips (Amersham Biosciences) in the pH range 3–10 for extracellular proteins. Separation in the second dimension was performed using 12 % polyacrylamide gels. Analytical gels were loaded with 200 μ g protein. Gels were stained with either silver nitrate (O'Connell & Stults, 1997) or SYPRO Ruby protein gel stain (Molecular Probes) according to the

Table 2. Specific primer pairs for QRT-PCR

Gene	Forward primer	Reverse primer
<i>gyrA</i>	5'-GATTATTAACCTGCTGGAGGTAGAAAA-3'	5'-AGGTAAAAGCTCCGCATTGAATTC-3'
<i>liaI</i> (<i>yvqI</i>)	5'-CATCTGCTCACTTCCGTTTGTC-3'	5'-TTCCAGCCGTAATACACCATAGC-3'
<i>liaH</i> (<i>yvqH</i>)	5'-TCCAAAGGTGATGCTGAATCAA-3'	5'-GTTTCACAATTGTTTGCTTTGCTT-3'
<i>liaG</i> (<i>yvqG</i>)	5'-GCCGGGAATGACCATCTTG-3'	5'-CGTCCGTCACGCTCGTATTT-3'
<i>liaF</i> (<i>yvqF</i>)	5'-CCGACATGCGCAGCTTTT-3'	5'-TCAGGTCAAACGGCTGCTT-3'
<i>liaS</i> (<i>yvqE</i>)	5'-GATCAGGATGGTCGAGCATATG-3'	5'-GCAGCGCCCTCATCTCATT-3'
<i>liaR</i> (<i>yvqC</i>)	5'-CGCGCCGCAAGCAA-3'	5'-CGCAGCCTGGATAATACTTTTCC-3'
<i>yxdL</i>	5'-GGTCATTCTAAGCCGTCCTCA-3'	5'-TTGTCGCTTTGGAATCAAGGT-3'

manufacturer's instructions. Proteins were identified using MALDI-TOF MS, as described previously (Antelmann *et al.*, 2002). Quantitative image analysis was performed by using the false-colour image analysis and quantification tool of the DECODON Delta 2D software (<http://www.decodon.com>).

RESULTS

Induction of *htrA* and *htrB* by secretion stress is abolished in the absence of D-alanylated teichoic acids

In order to find out whether the increased net negative charge of the cell wall in *dlt* mutants causes down-regulation of expression of the *htrA* and *htrB* genes and thereby stabilization of the PrsA3 protein, a *lacZ* reporter was placed under the control of the protease gene promoters and β -galactosidase activity was measured. The *lacZ* reporter was placed under the transcriptional control of the *htrA* and *htrB* genes by inserting pMUTIN plasmids (Vagner *et al.*, 1998) into the protease genes. The strains expressed AmyQ α -amylase from the pKTH10 plasmid and secreted it at a high level (hypersecretion). This caused secretion stress, activated signal transduction via the CssRS TCS and induced the CssRS-regulated P_{htrA} and P_{htrB} promoters (Hyryläinen *et al.*, 2001).

In the wild-type strain, AmyQ hypersecretion resulted in about 10-fold induction of P_{htrA} expression (Fig. 1a), consistent with previously published results (Hyryläinen *et al.*, 2001). Secretion stress caused a similar induction of expression from the P_{htrB} promoter (Fig. 1b). In contrast, hardly any induction of P_{htrA} and P_{htrB} was observed in the *dltD* mutant (Fig. 1a, b; representative results of two experiments are shown in each panel). These results suggest that the D-alanylation of teichoic acids modulates signal transduction via CssRS.

Extracellular proteomes of the *dltD* mutant and the wild-type strain were compared using 2D gel electrophoresis. False-colour images of the proteomes (Fig. 2; representative results of two experiments are shown) revealed that the majority of the protein spots were yellow, suggesting that most extracellular proteins were secreted at similar levels from the two strains. A significant fraction of the proteins in the extracellular proteome were cytoplasmic proteins, probably released into the culture medium by cell lysis (Tjalsma *et al.*, 2004). The cytoplasmic proteins included CitH, Eno, SodA and PdhD. The false-colour images of the spots for these proteins suggested that the two strains were not significantly different in their degree of cell lysis (Fig. 2).

The normally cell membrane/wall-associated HtrA protease is also present in the extracellular proteome in abundant amounts (Tjalsma *et al.*, 2004). A prominent finding in this study was that HtrA accumulated at a significantly lower level in the culture medium of the *dltD* mutant than in that of the wild-type strain (Fig. 2). This is in good agreement

Fig. 1. The effect of *dltD::miniTn10* on the expression of *htrA* and *htrB*. (a) Strains IH8149 (*dltD::miniTn10 htrA::pMUTIN2* pKTH10) and IH7612 (*dlt*⁺ *htrA::pMUTIN2* pKTH10) were cultivated in 2× LB₅, and β -galactosidase activities of cell samples taken at the indicated time points during growth were determined. The strains secreted AmyQ α -amylase at high levels due to the presence of the pKTH10 plasmid. Squares, *dlt*⁺; diamonds, *dltD::miniTn10*; filled symbols, growth; open symbols, β -galactosidase activity. (b) Experiment performed with strains IH7988 (*dlt*⁺ *htrB::pMUTIN4* pKTH10) and IH8268 (*dltD::miniTn10 htrB::pMUTIN4* pKTH10) as in (a).

with the decreased transcription of the *htrA* gene in the *dltD* mutant. Consistent with the decreased amount of HtrA in the proteome of the *dltD* mutant, we also detected decreased secretion of YqxI, which is probably stabilized post-transcriptionally by the chaperone-like activity of HtrA in response to secretion stress (Antelmann *et al.*, 2003). The extracellular proteome analysis further suggested that some other proteins were also present at decreased levels in the culture medium of the *dltD* mutant, including the PbpX penicillin-binding protein, and YrpD and YxkC, whose functions are unknown.

Fig. 2. Comparison of extracellular proteomes of the *dltD::miniTn10* mutant and its wild-type (*dlt*⁺) parent. Superimposed false-colour images of extracellular proteomes of IH6531 (*dlt*⁺ pKTH10) and IH7378 (*dltD::miniTn10* pKTH10) are shown. Extracellular proteins were TCA-precipitated from the culture medium of late-exponential-phase cultures and separated by 2D gel electrophoresis. The colour code of the spots indicates the relative abundance of the proteins in the extracellular proteomes of the two strains. The spot for HtrA protease is indicated by blue type.

Induction of expression from the LiaRS-regulated *P_{lial}* promoter in secretion stress and stress caused by cationic antimicrobial peptides is dependent on the cell wall charge

The *liaIHGFSR* gene cluster is strongly induced in an unspecific manner by antibiotics that interfere with the lipid II cycle, membrane-active cationic antimicrobial peptides (CAMPs) such as LL-37, detergents such as Triton X-100 (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005) and severe secretion stress (AmyQ secretion at a high level and a defect in the PrsA petidyl-prolyl *cis/trans* isomerase; Hyryläinen *et al.*, 2005). Expression from the *liaI* promoter (P_{liaI}) is strictly dependent on the LiaRS TCS, encoded by the *liaR* and *liaS* genes in the same gene cluster (Mascher *et al.*, 2003). Severe secretion stress (*amyQ* expressed from pKTH3339 and impaired post-translocational folding due to the *prsA3* mutation) caused no induction of *liaI* expression in a knockout mutant of *liaR*, whereas a 77-fold induction (QRT-PCR measurement) was seen in the wild-type. A similar result was obtained with

LL-37: *liaI* was induced 55-fold in wild-type cells and not at all in *liaR* mutant cells. These results are similar to the LiaRS-dependent induction of *liaIH* in cells treated with bacitracin (Mascher *et al.*, 2003).

In order to find out whether the Dlt system, and consequently the density of negative charge in the cell wall, modulates stress responses mediated by two-component sensory systems other than CssRS, the effect of *dltD::miniTn10* on the expression from the LiaRS-regulated P_{liaI} promoter was determined. The pMUTIN2 plasmid (Vagner *et al.*, 1998) was inserted into the *liaI* gene. The plasmid insert probably inactivated *liaI*, placed a *lacZ* reporter under the control of P_{liaI} and placed the downstream genes of the *lia* operon under the control of the IPTG-inducible P_{spac} promoter. The P_{liaI} promoter was induced by either subjecting the cells to AmyQ hypersecretion or treating them with LL-37.

The effect of the P_{spac} induction on the expression of the downstream genes, *liaHGFSR*, was determined in the

absence and presence of IPTG by using QRT-PCR. In the absence of IPTG, they were expressed at low levels (Table 3). However, the expression levels of *liaHGFSR* were clearly higher than that of *liaH* (>10-fold higher levels of cDNA), consistent with the presence of the weak P_{liaG} promoter in the upstream region of *liaG* (Jordan *et al.*, 2006). When P_{spac} was induced with 1 mM IPTG, all five downstream genes were strongly induced. In the case of *liaH*, the induction was about 500-fold as compared to the level in the absence of IPTG. The expression levels were similar in the *dlt*⁺ (wild-type) strain (IH8207) and the *dltD*::miniTn10 mutant (IH8205).

P_{liaI} expression was first determined in the absence of IPTG (the P_{spac} -*liaHGFSR* genes expressed at low levels) by measuring β -galactosidase activity. In wild-type cells secreting AmyQ, P_{liaI} was induced only slightly (Fig. 3a). This is consistent with our previous DNA macroarray results (Hyyryläinen *et al.*, 2005) showing that the *lia* operon is not significantly induced by mere AmyQ hypersecretion, but it is induced if there is also a mutation in the gene encoding the post-translocational folding factor PrsA. In the present study, about fivefold higher P_{liaI} expression levels (LacZ measurement) were observed in the *dltD*::miniTn10 mutant than in the wild-type strain (Fig. 3a). In the presence of IPTG and thus the downstream P_{spac} -*liaHGFSR* genes expressed at higher levels than in its absence, the results were distinctly different. In the wild-type strain, the overall P_{liaI} expression levels were similar to those in the absence of IPTG (Fig. 3a, b). However, the *dltD*::miniTn10 mutation conferred no expression-enhancing effect on P_{liaI} (Fig. 3b).

When cells were treated with LL-37, the expression from P_{liaI} was clearly upregulated (up to ~10-fold) in the *dltD*::miniTn10 mutant under both conditions (with or without IPTG; Fig. 4a, b). The P_{liaI} expression returned to the low initial level in about 4 h from the addition of

Fig. 3. Induction of P_{liaI} in the *dltD*::miniTn10 mutant by secretion stress. (a) Strains IH8205 (*dltD*::miniTn10 *liaI*::pMUTIN2 pKTH10) and IH8207 (*dlt*⁺ *liaI*::pMUTIN2 pKTH10) secreting AmyQ α -amylase at high levels were cultivated in 2× LB₅, and β -galactosidase activities of cell samples taken at the indicated time points during growth were determined. The *lia* genes downstream of *liaI* (P_{spac} -*liaHGFSR*) were not induced. Squares, *dlt*⁺; triangles, *dltD*::miniTn10; filled symbols, growth; open symbols, β -galactosidase activity. (b) As in (a), but P_{spac} -*liaHGFSR* was induced with 1 mM IPTG.

Table 3. Expression levels of the *lia* genes downstream of the *liaI*::pMUTIN2 insertion site and under the control of the P_{spac} promoter

Gene	Expression levels of P_{spac} - <i>liaHGFSR</i> *			
	<i>dlt</i> ⁺		<i>dltD</i> ::miniTn10	
	-IPTG	+IPTG	-IPTG	+IPTG
<i>liaH</i>	0.002	1.065	0.004	2.396
<i>liaG</i>	0.081	3.008	0.155	2.082
<i>liaF</i>	0.038	0.526	0.041	0.294
<i>liaS</i>	0.173	2.014	0.305	1.758
<i>liaR</i>	0.131	1.055	0.190	0.784

*Strains (IH8205 and IH8207) were grown in either the absence or the presence of IPTG to a cell density of 60 Klett units, and P_{spac} -*liaHGFSR* gene expression levels were determined by QRT-PCR. Values are ng DNA.

LL-37, possibly due to elimination of the peptide in the cells. In the presence of IPTG, the overall P_{liaI} expression levels were lower (about fourfold reduced) as compared to the expression levels in the absence of IPTG, and the induction was also more transient (Fig. 4a, b). In the wild-type strain (in the absence of IPTG) only a small induction peak was observed (Fig. 4a). These results are similar to and consistent with those obtained for secretion stress.

The pMUTIN2 insertion in the strains of the above experiments probably inactivated the *liaI* gene. In order to ensure that *liaI* disruption did not modulate the response patterns of P_{liaI} under stress, we also determined the expression levels of the *lia* genes in the strain pair IH8123 (*dlt*⁺) and IH7375 (*dltD*::miniTn10), which do not contain the pMUTIN2 insertion in *liaI*, by using QRT-PCR. Cells of

Fig. 4. Induction of P_{liaI} in the $dltD::miniTn10$ mutant by LL-37. (a) Strains IH8220 ($dltD::miniTn10$ $liaI::pMUTIN2$) and IH8219 (dlt^+ $liaI::pMUTIN2$) were cultivated in $2\times$ LB₅ and treated with LL-37 at a cell density of 60 Klett units for 20 min, followed by determination of β -galactosidase activities of cell samples taken at the indicated time points during growth. The lia genes downstream of $liaI$ (P_{spac} - $liaHGFSR$) were not induced. Squares, dlt^+ ; triangles, $dltD::miniTn10$; filled symbols, growth; open symbols, β -galactosidase activity. (b) As in (a), but P_{spac} - $liaHGFSR$ was induced with 1 mM IPTG.

these strains were treated with LL-37 for 10 or 20 min, and lia gene expression levels were determined and compared with those in non-treated cells (Table 4). The fold induction values were significantly higher than those obtained with the β -galactosidase activity measurements due to differences in the sensitivities of the methods. However, consistent with the β -galactosidase activities (P_{spac} not induced), LL-37 caused a much stronger induction of P_{liaI} in the $dltD::miniTn10$ mutant than in the wild-type. This stronger induction was evident in the fold induction values of all six lia genes at both time points. The $liaI$ and $liaH$ genes were induced in both strains more strongly than the other lia genes, consistent with the presence of a potential transcription termination loop after $liaH$ (Jordan *et al.*, 2006).

Induction of P_{liaI} by vancomycin or Triton X-100 is not enhanced in the $dltD::miniTn10$ mutant

We also studied whether the density of negative charge in the cell wall modulates the response of LiaRS to another cationic-type inducer, the glycopeptide antibiotic vancomycin. Furthermore, we used a non-ionic inducer of LiaRS, the detergent Triton X-100, to find out if the Dlt effect is restricted to cationic inducers such as peptides or if the response to a non-ionic inducer is also affected by the Dlt system. Cells of the $dltD::miniTn10$ mutant and the wild-type strain were treated with vancomycin ($2\text{ }\mu\text{g ml}^{-1}$) or 0.005 % Triton X-100 for 10 or 20 min and P_{liaI} expression was determined using QRT-PCR.

In the wild-type strain, vancomycin enhanced P_{liaI} expression (QRT-PCR measurements) hundredsfold as compared to the expression level in non-treated cells (Table 5), consistent with the published results (Mascher *et al.*, 2004) and showing that vancomycin is a strong inducer of the LiaRS TCS. In the $dltD::miniTn10$ mutant, the response to vancomycin was significantly lower (at least sixfold reduced expression of P_{liaI}), in contrast to the stronger response in

Table 4. Induction of the expression of the lia genes in the $dltD::miniTn10$ mutant and the dlt^+ wild-type strain by LL-37

Gene	Fold induction for strain:*			
	IH8123 (dlt^+)		IH7375 ($dltD::miniTn10$)	
	10 min	20 min	10 min	20 min
<i>liaI</i>	19.7 (20.6, 18.7)	13.4 (12.0, 14.8)	379.1 (532.3, 225.9)	318.8 (123.0, 514.5)
<i>liaH</i>	17.9 (14.9, 20.8)	15.3 (12.9, 17.6)	300.4 (391.1, 209.7)	479.7 (140.5, 818.9)
<i>liaG</i>	1.7 (1.4, 2.0)	1.6 (1.6, 1.6)	29.8 (42.9, 16.7)	7.3 (5.6, 9.0)
<i>liaF</i>	2.4 (2.0, 2.7)	1.5 (1.8, 1.1)	14.9 (10.1, 19.7)	6.7 (4.1, 9.2)
<i>liaS</i>	1.2 (1.1, 1.3)	1.4 (1.6, 1.2)	20.9 (29.6, 12.1)	2.9 (2.3, 3.4)
<i>liaR</i>	1.3 (1.1, 1.4)	1.0 (1.0, 0.9)	8.3 (3.7, 12.9)	2.0 (2.5, 1.5)

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by QRT-PCR. The induction ratios are means from two independent experiments.

the LL-37-treated *dltD::miniTn10* mutant cells. The fold induction values were similar at both time points.

In the cells treated with Triton X-100, the induction time-course of P_{liaI} was such that at the 10 min time point the fold induction was about 60, and it increased to about 150 at the 20 min time point (Table 5). There were no significant differences in the induction patterns of the two strains under this stress condition.

Dlt modulates the response of the YxdJK TCS to LL-37

The results of the above experiments suggested that cell wall charge can modulate TCS activity in different ways depending on the TCS and properties of the inducer molecule. YxdJK is a TCS which is activated by LL-37 in a highly specific manner (Pietäinen *et al.*, 2005). We used YxdJK as the third sensory system to study the Dlt dependence of TCS activation. It was observed that Dlt affects the activity of YxdJK in a similar manner to that of LiaRS, i.e. the activity was enhanced in the *dltD::miniTn10* mutant. There was about a threefold higher induction of the YxdJK-regulated *yxdL* gene in the *dltD::miniTn10* mutant as compared to the wild-type strain at both the 10 and 20 min time points (Table 5).

Modulating the negative charge of the outer surface of the cell membrane does not affect the activity of TCSs

In a similar manner to the negative charge of the cell wall, the negative charge of the cell membrane surface could also

affect the activity of TCSs. We used mutations that decrease positively charged amines in the membrane and thereby increase the negative charge of the membrane surface. The inactivated components were MprF, which modifies membrane lipids with L-lysine (Peschel *et al.*, 2001; Staubitz & Peschel, 2002), and Psd, which encodes phosphatidylserine decarboxylase and is involved in the biosynthesis of phosphatidylethanolamine (Cao & Helmann, 2004; Matsumoto *et al.*, 1998). Both *Staphylococcus aureus* and *B. subtilis* synthesize lysylphosphatidylglycerol, and it has been shown with *S. aureus* that MprF is responsible for the synthesis (Peschel *et al.*, 2001; Staubitz & Peschel, 2002). Two pMUTIN2 insertion mutations of the *B. subtilis* mprF orthologue, *yfiW::pMUTIN2* and *yfiX::pMUTIN2*, were used in this study to characterize the putative role of MprF in the activation of LiaRS and YxdJK. The *psd1::neo* mutation (Matsumoto *et al.*, 1998) was used to determine the putative role of Psd in the activation of LiaRS, YxdJK and CsrRS.

We first determined the sensitivities of the *yfiX::pMUTIN2* (IH8394) mutant and the wild-type parent (*mprF*⁺, IH8123) to LL-37. LL-37 completely inhibited the growth of the wild-type *B. subtilis* strain in a liquid culture at a concentration of 12 µg ml⁻¹, delayed the start of growth at 10 µg ml⁻¹ and had no effect on growth at 8 µg ml⁻¹. The LL-37 sensitivity of the mutant did not differ from that of the wild-type strain, in contrast to the increased sensitivity of analogous mutants of *S. aureus* to defensins (Peschel *et al.*, 2001). We used a sub-inhibitory concentration of LL-37 (4 or 6 µg ml⁻¹) and determined the induction of P_{liaI} and P_{yxdL} in the *yfiW::pMUTIN2*, *yfiX::pMUTIN2* and *psd1::neo* mutants and the wild-type

Table 5. Effects of the inactivation of *dltD*, *mprF* and *psd* on P_{liaI} and P_{yxdL} expression

Strain	Genotype	Stress	Fold induction*			
			P_{liaI}		P_{yxdL}	
			10 min	20 min	10 min	20 min
IH8123	wt	LL-37	21 (17, 25)	14 (13, 14)	55 (53, 56)	47 (35, 58)
IH7375	<i>dltD</i>	LL-37	417 (395, 438)	316 (122, 509)	166 (105, 226)	169 (75, 262)
IH8123	wt	Vancomycin	749 (415)	910 (325)	ND	ND
IH7375	<i>dltD</i>	Vancomycin	83 (30)	150 (77)	ND	ND
IH8123	wt	Triton X-100	64 (57, 71)	135 (107, 163)	ND	ND
IH7375	<i>dltD</i>	Triton X-100	68 (55, 80)	188 (147, 229)	ND	ND
IH8123	wt	LL-37	18 (16, 20)	12 (8, 16)	55 (53, 56)	47 (35, 58)
IH7375	<i>mprF</i> (<i>yfiX::pMUTIN2</i>)	LL-37	33 (44, 22)	18 (20, 16)	28 (20, 36)	44 (32, 55)
IH8123	wt	LL-37	1281 (192)†	866 (351)†	323 (181)†	301 (37)†
IH8430	<i>mprF</i> (<i>yfiW::pMUTIN2</i>)	LL-37	935 (198)†	901 (279)†	258 (78)†	216 (58)†
IH8123	wt	LL-37	614 (137)†	191 (55)†	219 (84)†	135 (25)†
IH8429	<i>psd1::neo</i>	LL-37	704 (162)†	447 (113)†	287 (66)†	234 (30)†

*The strains were exposed to the indicated stresses, and fold inductions of the *liaI* and *yxdL* genes were determined by QRT-PCR after 10 and 20 min exposure to stress. The fold inductions are means of two or three independent experiments. The fold inductions of the independent experiments (two experiments) or SD (three experiments) are shown in parentheses. ND, Not determined; wt, wild-type.

†In these experiments LL-37 was used at a concentration of 6 µg ml⁻¹, whereas in the others the concentration was lower (4 µg ml⁻¹), explaining the significant difference in the fold induction values.

parental strain by QRT-PCR. The mutations had no significant effects on the induction levels of the two promoters (Table 5). Furthermore, *psd1::neo* did not modulate the activation of P_{htrA} by secretion stress [fold inductions: 21.1 (wild-type) versus 19.9 (*psd1::neo*)]. These results suggest that the membrane surface charge does not modulate the TCSs.

DISCUSSION

In this study transcription and proteome analyses were used to demonstrate the role of the Dlt D-alanylation system and the net negative charge of the cell wall in two-component signal transduction and gene expression regulation. The transcription measurements and extracellular proteomes of the *dltD* mutant and its wild-type parent revealed that the *htrA* and *htrB* genes were expressed at strongly decreased levels in the *dltD::miniTn10* mutant. This finding is consistent with the stabilization of the PrsA3 protein in the *dltD::miniTn10* mutant, since the HtrA and HtrB quality-control proteases are partially responsible for the degradation of PrsA3 (Hyöryläinen *et al.*, 2001). It was suggested previously that the increased net negative charge of the cell wall in *dlt* mutants may improve the post-translocational folding of heterologous and mutant proteins that are particularly sensitive to proteolytic degradation (Hyöryläinen *et al.*, 2000). Improved protein folding could decrease stress in the cell envelope and consequently decrease the activity of CssRS and the expression of *htrA* and *htrB*, explaining the findings of the present study and the stabilization of heterologous and mutant proteins in *dlt* mutants. Alternatively, interactions between misfolded secretory proteins and CssS may be different in the absence and presence of D-alanylated teichoic acids due to changes in electrostatic affinity/repulsion forces at the cell wall matrix. Such changes could also affect interactions between lipoteichoic acids and CssS, possibly modulating the activity of the TCS. It is evident, however, that the reason for the improved stability of PrsA3 and some heterologous proteins in *dlt* mutants is probably the decreased levels of the HtrA and HtrB quality control proteases, and consequently decreased degradation. Consistent with the decreased level of HtrA, the level of YqxI (in the extracellular proteome) was also decreased. The secretion and stability of YqxI is probably dependent on HtrA (Antelmann *et al.*, 2003).

The LiaRS system also exhibited a Dlt effect in cells subjected to severe secretion stress or treated with LL-37, but compared to the CssRS system (secretion stress), its direction was the opposite, i.e. a clearly stronger induction of LiaRS, as measured by the expression from P_{liaI} , was observed in the *dltD::miniTn10* mutant compared with the wild-type strain (*dlt*⁺). The enhanced induction was observed with both a P_{liaI} -*lacZ* reporter and by measuring P_{liaI} expression with QRT-PCR. The enhanced LiaRS activity may be due to stronger cell envelope stress in the mutant. Most probably, cationic peptides bind to cell

envelopes of *dlt* mutants of Gram-positive bacteria in higher amounts than to those of *dlt*⁺ strains (Peschel *et al.*, 1999). The increased affinity of *dlt* mutant cell walls to CAMPs subjects the mutant cells to higher stress and increases their sensitivity to CAMPs (Cao & Helmann, 2004; Kristian *et al.*, 2005; Peschel *et al.*, 1999; Poyart *et al.*, 2003).

The glycopeptide antibiotic vancomycin, also a cationic molecule, is one of the strongest inducers of LiaRS (Mascher *et al.*, 2004), but Dlt modulated the response to vancomycin in a different manner than the response to secretion stress or LL-37. It was found that P_{liaI} expression was attenuated in the *dltD* mutant as compared to the *dlt*⁺ strain. In contrast to the responses to these cationic inducers, the LiaRS response to a non-ionic inducer, Triton X-100, was not affected by Dlt.

These results suggest that Gram-positive bacterial cells can modulate two-component signal transduction and gene expression under stress by modulating the density of negative charge in the wall. The expression of the *dlt* operon is dependent on the extracytoplasmic sigma factor σ^X (Cao & Helmann, 2004), and under some growth conditions probably also on σ^D and the Spo0A and AbrB sporulation proteins (Perego *et al.*, 1995; Cao & Helmann, 2004). Environmental stress conditions, which activate σ^X , modulate the net negative charge of the wall by increasing *dlt* expression and thereby affect the stress response of TCSs. However, the modulation of the stress response may be limited to stress caused by cationic molecules and accumulation of misfolded proteins, and TCSs responding to these stress conditions. Consistent with this conclusion, the YxdJK TCS, which is activated highly specifically by the human cathelicidin LL-37, was also induced in a Dlt-dependent manner. The induction pattern was similar to that of the LiaRS TCS: stronger induction in the *dltD::miniTn10* mutant.

The LiaRS and YxdJK TCSs most probably sense a very different type of stress than does CssRS. In addition to our results, such a difference is also suggested by the predicted transmembrane topology of the two-component sensors. The CssS protein has a 137 aa soluble periplasmic region which may have a role in the sensing of misfolded proteins at the membrane-cell wall interface. It is a fairly specific sensor, since membrane-active compounds such as CAMPs or detergents do not activate it. In contrast, LiaS and YxdK do not have a periplasmic domain, suggesting that they are dedicated to sensing intramembrane stress. This may explain why membrane-active CAMPs activate LiaS and YxdK much more strongly than secretion stress. This model may also explain the effect of vancomycin on LiaRS. Since vancomycin strongly activates LiaRS, it probably causes intramembrane stress (e.g. accumulation of cell wall precursors in the membrane), which is alleviated in the absence of D-alanylated teichoic acids.

We also studied whether the degree of lysinylation of the negatively charged phospholipid head groups of the cell

membrane and the proportion of phosphatidylethanolamine in the membrane lipids could modulate the stress response to LL-37 or protein secretion in a similar manner to the D-alanylation of teichoic and lipoteichoic acids. This was not the case. The induction of LiaRS, YxdJK and CssRS was independent of these factors.

The induction of $P_{\text{spac-liaHGFSR}}$ expression (pMUTIN2 insert at *liaI*) with IPTG repressed P_{liaI} expression. Thorsten Mascher's group (University of Göttingen, Germany) has found that inactivating *liaF* increases expression from the P_{liaI} promoter, indicating that LiaF is a negative regulator of P_{liaI} (Jordan *et al.*, 2006). LiaF is a putative integral membrane protein and may therefore not repress P_{liaI} by directly interacting with the promoter but rather by negatively regulating the activity of LiaRS. LiaH also moderately down-regulates P_{liaI} (Jordan *et al.*, 2006). When the *lia* genes are expressed at low levels (in non-stressed cells), the P_{liaI} promoter is capable of responding strongly if the cells are exposed to stress. Under stress conditions, when the *lia* genes are expressed at high levels, an auto-control mechanism starts to down-regulate P_{liaI} expression. The Lia proteins, which are repressors of P_{liaI} , may have a role in this auto-control, particularly LiaH, since the stoichiometry between LiaH and LiaRS changes dramatically during the activation of the operon.

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