Appl Microbiol Biotechnol (2005) 67: 389–396
DOI 10.1007/s00253-005-1898-1

GENOMICS AND PROTEOMICS

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Transcriptome analysis of the secretion stress response of *Bacillus subtilis*

Received: 1 November 2004 / Revised: 22 December 2004 / Accepted: 27 December 2004 / Published online: 27 January 2005
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Abstract Transcription profiling of all protein-encoding genes of *Bacillus subtilis* was carried out under several secretion stress conditions in the exponential growth phase. Cells that secreted AmyQ α-amylase at a high level were stressed only moderately: seven genes were induced, most significantly *htrA* and *htrB*, encoding quality control proteases, and *yqxL*, encoding a putative CorA-type Mg^{2+} transporter. These three genes were induced more strongly by severe secretion stress (*prsA3* mutant secreting AmyQ), suggesting that their expression responds to protein misfolding. In addition, 17 other genes were induced, including the *liaIHGFSR* (*yvqIHGFEC*) operon, *csaA* and *ffh*, encoding chaperones involved in the pretranslocational phase of secretion, and genes involved in cell wall synthesis/modification. Severe secretion stress caused down-regulation of 23 genes, including the *prsA* paralogue *yacD*. Analysis of a *cssS* knockout mutant indicated that the absence of the CssRS two-component system, and consequently the absence of the HtrA and HtrB proteases, caused secretion stress. The results also suggest that the *htrA* and *htrB* genes comprise the CssRS regulon. *B. subtilis* cells respond to secretion/folding stress by various changes in gene expression, which can be seen as an attempt to combat the stress condition.

Introduction

The secretion of heterologous proteins at high levels is an adverse condition for bacterial cells due to the imbalance between protein synthesis, translocation, and quality control, leading to the accumulation of misfolded proteins in the cell envelope. The secretion (envelope) stress is sensed by membrane-bound sensors and signal transduction systems, which are two-component systems (TCS) and extracytoplasmic function (ECF) sigma factors (Helmann 2002; Raivio and Silhavy 1999, 2001). In *Bacillus subtilis*, secretion stress is sensed at least by the CssRS TCS (a homologue of the *Escherichia coli* CpxRA). The CssRS TCS regulates the *htrA* and *htrB* genes, which encode the cell envelope-associated quality control proteases HtrA and HtrB (Darmon et al. 2002; Hyyryläinen et al. 2001; Noone et al. 2001). Overexpression of a secretory protein and the resulting misfolding activates the CssRS TCS, resulting in induction of the *htrA* and *htrB* genes (Darmon et al. 2002; Hyyryläinen et al. 2001). The HtrA and HtrB proteases degrade misfolded proteins and thereby rescue the cell—accumulation of misfolded proteins in the cell envelope is lethal (Hyyryläinen et al. 2001). The PrsA lipoprotein, a parvulin-type peptidyl-prolyl cis/trans isomerase, is anchored to the cell membrane and catalyses folding of secretory proteins (Vitikainen et al. 2001, 2004). The *prsA3* mutation decreases the level of PrsA and increases misfolding of secretory proteins at the membrane/cell wall interface, increasing secretion stress and induction of the *htrA* and *htrB* genes (Hyyryläinen et al. 2001). PrsA is an essential protein in *B. subtilis*, suggesting that it also catalyses the folding of some essential protein in the cell envelope (Vitikainen et al. 2001).

It is important to characterise stress responses of industrial micro-organisms to obtain knowledge that can be used to optimise industrial production strains and processes. In this study we used DNA macroarrays to characterise secretion stress responses of *B. subtilis*. Bacterial cells were subjected to secretion stress by expressing a secretory heterologous α-amylase (AmyQ) at a high level in both a wild-type strain and the *prsA3* mutant (severe secretion stress). We also characterised the CssRS regulon and genes that were differentially expressed when cells were depleted of the PrsA foldase. Severe secretion stress induced numerous genes, most significantly *htrA*, *htrB*, *liaIHG* (*yvqIHG*) and *yqxL*. The patterns of differentially expressed genes suggest roles for both functionally known and unknown proteins under stress conditions.

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Materials and methods

Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used are listed Table 1. Strains that express AmyQ α-amylase harbour the plasmid pKTH3339 in which the *amyQ* gene is under the control of a xylose-inducible promoter (P_{xyn}-*amyQ*) (Vitikainen et al. 2001). Bacteria were cultured in modified 2×L-broth (2% tryptone, 1% yeast extract and 1% NaCl). Erythromycin (Em) and chloramphenicol (Cm) were used at concentrations of 1 and 5 μg ml^{-1}, respectively. Expression of P_{xyn}-*amyQ* was induced in early exponential growth phase by adding either 0.2 or 0.02% xylose. P_{spac}-*prsA* was induced at the beginning of growth to express PrsA either at a low sublethal level (24 μM IPTG) or at a near wild-type level (1 mM IPTG) (Vitikainen et al. 2001).

Isolation of total RNA

Expression of P_{xyn}-*amyQ* was induced with xylose in early exponential growth phase (Klett~60), and the cells in 4 ml culture were harvested after 30 min of induction (cell density of about Klett 100). The cells were resuspended in 400 μl culture medium and mixed with a mixture containing 1.5 g glass beads (0.1 mm diameter; Sigma, St. Louis, Mo.), 50 μl 10% SDS, 50 μl sodium acetate pH 5.2, and 500 μl phenol/chloroform/isoamylalcohol (25:24:1). The mixture was briefly vortexed and then frozen in liquid nitrogen. Bacteria were allowed to thaw in a horizontal shaker at room temperature for 6 min with vigorous shaking. After centrifuging at 4°C for 2 min, the aqueous phase was withdrawn and extracted with chloroform. The aqueous phase was again separated by centrifugation and RNA was isolated using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Synthesis of ^{33}P-labelled cDNA and hybridisation

For cDNA synthesis, 23 μl RNA (10 μg), 4 μl primer mix (specific primers for all *B. subtilis* genes, Sigma Genosys, Haverhill, UK), and 3 μl 10× hybridisation buffer (100 mM Tris-HCl pH 7.9, 10 mM EDTA, 2.5 M KCl) were mixed and heated to 95°C for 10 min. The temperature was slowly decreased to 42°C over 20 min. A solution containing 12 μl 5× first strand buffer (Sigma Genosys), 6 μl 0.1 M DTT, 2 μl dATP (10 mM), 2 μl dTTP (10 mM), 2 μl dGTP (10 mM), 4.5 μl [α-^{33}P]-dCTP (10 μCi ml^{-1}) and 1.5 μl Superscript II (Gibco BRL, Paisley, UK) reverse transcriptase (RTase) was mixed with the RNA solution. The RTase reaction mixture was incubated at 42°C for 90 min and then the reaction was stopped by adding 2 μl 0.5 M EDTA and 6 μl 3 M NaOH, and incubating at 65°C for 30 min and at 22°C for 15 min. The reaction mixture was neutralised by adding 20 μl 1 M Tris-HCl pH 8.0 and 6 μl HCl. Unincorporated labelled nucleotides were removed by using MicroSpin G-25 columns (Amersham, Piscataway, N.J.) according to the manufacturer's instructions. The ^{33}P-labelled cDNA was used to hybridise Panorama *B. subtilis* gene array filters (Sigma Genosys) according to the manufacturer's instructions. The Panorama arrays contain duplicate spots of 4,107 open reading frames, representing all *B. subtilis* protein-coding genes. The array filters were exposed to phosphoimager screens to analyse the transcriptome profiles by phoshoimagery using a Fluorescent Image Analyzer FLA-2000 (Fujifilm).

Quantification of gene expression signals

The gene expression signals were quantified by using ArrayVision software (http://www.imagingresearch.com). Spot intensities were corrected by subtracting the background intensity and normalised by dividing with the mean spot intensity of all the genes. Experiments were carried out either two or three times with biologically independent samples. Microsoft Excel software was also used in data analysis. Genes with at least 2-fold differential expression, and signal-to-noise ratios greater than three in at least two independent experiments were considered induced. Reduced gene expression was considered significant if the difference in expression was at least 3-fold. Some smaller differences in gene expression are also listed in the tables if the genes belong to operons in which at least one gene fulfilled the criteria.

Table 1 Bacterial strains/plasmid

Strain	Relevant characteristics	Reference
IH7574	*cssS*::pMutin2 pKTH3339	Hyyryläinen et al. 2001
IH7576	*prsA3cssS*::pMutin2 pKTH3339	Hyyryläinen et al. 2001
IH7663	*prsA3* pKTH3339	Hyyryläinen et al. 2001
IH7665	pKTH3339	Hyyryläinen et al. 2001
IH7211	p_{spac}-*prsA*	Vitikainen et al. 2001
Plasmid		
pKTH3339	p_{xyn}-*amyQ*	Vitikainen et al. 2001

Results

DNA macroarray analysis of secretion stress response

B. subtilis cells were subjected to secretion stress by inducing the P_{xyn}-*amyQ* gene construct on plasmid pK TH3339 with 0.2% xylose in the exponential growth phase (Klett 60). DNA macroarray analysis was carried out with exponentially growing cells to avoid the bacterial heterogeneity of later growth phases and consequent variation of data. Another set of DNA array experiments was carried out with a strain that additionally contained the *prsA3* mutation (severe secretion stress).

In the *prsA*$^{+}$ strain secreting AmyQ, the *htrA* and *htrB* (*yvtA*) genes, encoding extracytoplasmic quality-control proteases, were induced 4.3- and 5.8-fold, respectively, and *yqxL*, encoding a putative protein similar to CorA-type Mg^{2+} transporters, was induced 2.3-fold as compared to the non-secreting strain (Table 2). Four other genes (*plsX*, *rpoB*, *rpsJ* and *yesO*) were moderately induced (1.6- to 4.8-fold). These seven genes were also all upregulated in the *prsA3* mutant secreting AmyQ (*prsA3*+AmyQ vs *prsA3*) (Table 2). In the *prsA3* mutant the induction ratios of *htrA*, *htrB* and *yqxL* were clearly higher than in the *prsA*$^{+}$ strain, at 12.3-fold, 15.2-fold and 13.8-fold, respectively (Table 2), suggesting that these genes respond to protein misfolding in the cell envelope. Interestingly, *yvqI* (renamed *liaI* in Mascher et al. 2004) and its downstream genes *liaH* (*yvqH*) and *liaG* (*yvqG*) were upregulated 2.1- to 10.3-fold (Table 3). Several of the induced genes encode components involved in protein secretion or general adaptation to atypical conditions. The *csaA* gene (3.5-fold) encodes a chaperone-like factor involved in early stages of protein secretion (Muller et al. 2000) and *ffh* (2.1-fold) encodes a signal recognition particle (SRP)-like component. The *ylxM* gene of the bicistronic *ylxM-ffh* operon, encoding a conserved functionally unknown protein, was also induced (2.5-fold). Some of the induced genes are involved in cell wall biosynthesis (Table 3). The *dlt* operon (1.6- to 2-fold) encodes the D-alanylation system of teichoic and lipoteichoic acids, and the *pbpA* (2.8-fold) gene encodes a penicillin-binding protein. The *exuR* and *uxaB* (alternative names *yjmH* and *yjmI*, respectively) genes, encoding a putative repressor of hexuronate utilization and a putative tagaturonate reductase, respectively, were strongly induced (about 5-fold) in a *prsA3*-dependent manner (Table 3). One of the induced genes was *cssS* (2-fold), which encodes the sensor histidine kinase of the CssRS TCS, a regulator of *htrA* and *htrB*. In the absence AmyQ secretion, the *prsA3* mutation did not affect gene expression (*prsA3* vs *prsA*$^{+}$, data not shown).

AmyQ oversecretion alone did not reduce expression of any gene, but severe secretion stress (*prsA3*+AmyQ) re-

Table 2 Genes induced by oversecretion of AmyQ α-amylase in both the *prsA* wild type and the *prsA3* mutant

Gene	Induction±SD[a]		Function
	prsA^{+} AmyQ	*prsA3* AmyQ	
htrA	4.36±0.81	12.34±1.65	Serine protease homologue
htrB	5.82±2.30	15.28±1.30	Serine protease homologue
plsX	2.07 (1.57, 2.56)	1.74±0.31	Involved in fatty acid/ phospholipid synthesis
rpoB	1.97 (1.53, 2.41)	2.26±0.54	RNA polymerase
rpsJ	1.66 (1.45, 1.86)	3.09±0.50	Ribosomal protein S10
yesO	4.81 (7.59, 2.02)	4.06±3.67	Similar to sugar-binding protein
yqxL	2.35±0.64	13.85±5.43	Similar to Mg^{2+}-transporter

[a]Induction ratios are means from two or three independent experiments

Table 3 Genes induced by severe secretion stress in the *prsA3* mutant

Gene	Down/ upstream genes	Induction ±SD[a]	Function
Protein secretion and folding/adaptation to atypical conditions			
csaA		3.55±0.92	Molecular chaperone
ffh		2.16±0.28	Signal recognition particle (SRP)-like component
	ylxM	2.58±0.31	Unknown
htrA		12.34±1.65	Serine protease homologue
htrB		15.28±1.30	Serine protease homologue
ywtA		2.38±0.66	Similar to capsular polyglutamate biosynthesis
liaG (*yvqG*)		2.19±0.78	Unknown
	liaH (*yvqH*)	6.03±2.17	Similarity to *Escherichia coli* phage shock protein PspA
	liaI (*yvqI*)	10.31±2.76	Unknown
Cell wall synthesis			
dltA		1.62±0.13	D-Alanyl-D-alanine carrier protein ligase (Dcl)
	dltC	2.00±0.20	D-Alanyl carrier protein (Dcp)
	dltD	1.83±0.48	D-Alanine esterification of lipoteichoic acid and wall teichoic acid
	dltE	1.72±0.09	Similar to ketoacyl reductase
pbpA		2.86±0.76	Penicillin-binding protein 2A spore outgrowth
	racX	2.5	Amino acid racemase
Regulation of RNA synthesis			
cssS		2.00±0.30	Two-component sensor histidine kinase *yvqB*
rho		2.02±0.53	Transcriptional terminator Rho
rpoB		2.26±0.54	RNA polymerase (beta subunit)
	rpoC	1.94±0.32	RNA polymerase (beta subunit)
yjmH		4.70±2.55	Repression of the hexuronate utilization operon, *exuR*
	yjmI	4.41±1.71	Tagaturonate reductase, *uxaB*
Other			
clpC		2.63±0.44	Unknown
fabD		2.02±0.19	Unknown
glpD		2.14±0.43	Unknown
rpsJ		3.09±0.50	Unknown
yesO		4.06±3.67	Unknown

Table 3 (continued)

Gene	Down/ upstream genes	Induction ±SD[a]	Function
yjaY		2.03±0.36	Unknown
yqxL		13.85±5.43	Unknown
yxbG		2.13±0.23	Unknown

[a]Induction ratios are means from three independent experiments

duced the expression of 23 genes at least 3-fold (Table 4). Interestingly, *yacD*, which encodes a PrsA-like protein, was strongly downregulated (0.22-fold) in the secretion-stressed *prsA3* mutant (Table 4). Several multicistronic operons were also repressed. These include *flhOP* (0.15- to 0.22-fold), encoding flagellar proteins, *lytABC* (0.33- to 0.56-fold), encoding proteins involved in autolysis, and *lonB* (0.19-fold), encoding an ATP-dependent protease.

The CssRS regulon and stress response to the absence of HtrA-type proteases

Inactivation of the CssRS TCS increases protein misfolding in the cell envelope (Hyyryläinen et al. 2001). The increased misfolding due to the absence of CssRS and Htr proteases may be sensed by other signal transduction mechanisms. In order to study this stress response and to identify genes belonging to the CssRS regulon, gene expression patterns of a *cssS* knockout mutant and its wild-type parent were compared by DNA array (*cssS*$^-$ vs *cssS*$^+$) in the exponential phase of growth. In both strains, P_{xyn}-*amyQ* was induced with 0.02% xylose and consequently AmyQ was synthesised and secreted at a low level. The only genes that were expressed at clearly lower levels in the *cssS* mutant were *htrA* and *htrB* (0.20-fold and 0.15-fold, respectively), suggesting that only these two genes are directly regulated by the CssRS TCS. Another prominent finding was the induction of the *liaIHG* (*yvqIHG*) genes (2.2- to 5.3-fold). Nine other functionally unknown genes were also moderately (about 2-fold) induced, including *ydjF*, which encodes a phage shock protein A homologue, and downstream genes of the operon (data not shown).

Sublethal depletion of PrsA foldase results in induction of genes involved in cell wall synthesis

We also used the DNA macroarray method to analyse changes in gene expression pattern in cells depleted of PrsA foldase to a sublethal level. These DNA arrays were carried out with cultures with a cell density of Klett 100 and the cells did not overproduce AmyQ. PrsA depletion might result in compensatory induction of a gene that encodes an essential component whose folding is dependent on PrsA (cascade effect) if such a gene is expressed from a reg-

Table 4 Genes with reduced expression level in the *prsA3* mutant oversecreting AmyQ

Gene	Up/ downstream genes	Reduction ±SD[a]	Function
flhO		0.15±0.03	Flagellar basal-body rod protein
	flhP	0.22±0.03	Flagellar hook-basal body protein
lonB		0.19±0.07	Lon-like ATP-dependent protease
lytA		0.33±0.04	Involved in secretion of major autolysin LytC (amidase)
	lytB	0.46±0.08	Modifier protein of major autolysin LytC (CWBP76)
	lytC	0.56±0.02	*N*-Acetylmuramoyl-L-alanine amidase (major autolysin)
	lytR	0.49±0.09	Attenuator role for *lytABC* and *lytR* expression
moaB		0.23±0.03	Molybdopterin precursor biosynthesis protein B
pyrB		0.16±0.26	Aspartate carbamoyltransferase, pyrimidine biosynthesis
	pyrC	0.39±0.24	Dihydroorotase, pyrimidine biosynthesis
	pyrAA	0.21±0.24	Carbamoyl-phosphate synthetase (glutaminase subunit), pyrimidine biosynthesis
	pyrAB	0.28±0.25	Carbamoyl-phosphate synthetase (catalytic subunit), pyrimidine biosynthesis
	pyrDII	0.46±0.14	Dihydroorotate dehydrogenase (electron transfer subunit), pyrimidine biosynthesis, *pyrK*
	pyrD	0.51±0.10	Dihydroorotate dehydrogenase (catalytic subunit), pyrimidine biosynthesis
	pyrF	0.32±0.12	Orotidine 5′-phosphate decarboxylase, pyrimidine biosynthesis
	pyrE	0.29±0.09	Dihydroorotate dehydrogenase (electron transfer subunit), pyrimidine biosynthesis
splB		0.21±0.02	Spore photoproduct (thymine dimer) lyase
spoIID		0.16±0.03	Required for complete dissolution of the asymmetric septum
sspF		0.10±0.05	Small acid-soluble spore protein (alpha/beta-type SASP)
yabD		0.42±0.03	Unknown
	yabE	0.25±0.12	Similar to cell wall-binding protein
yacB		0.50±0.01	Unknown
	yacD	0.22±0.03	Similar to protein secretion PrsA homolog
	cysK	0.41±0.03	Cysteine synthetase A
yhbD		0.30±0.06	Unknown
	yhbF	0.27±0.17	Unknown
yonS		0.32±0.03	Unknown
	yonU	0.60±0.04	Unknown

Table 4 (continued)

Gene	Up/ downstream genes	Reduction ±SD[a]	Function
yqiX		0.21±0.04	Similar to amino acid ABC transporter (binding protein)
	yqiZ	0.41±0.03	Similar to amino acid ABC transporter (ATP-binding protein)
ytfJ		0.22±0.03	Unknown
	ytfI	0.23	Unknown
ytgA		0.18±0.01	Manganese ABC transporter (membrane protein)
yyaG		0.56±0.08	Transcriptional regulator involved in catabolite repression, *ccpB*
	yyaH	0.23±0.22	Unknown

[a]Ratios are means from three independent experiments

ulatable promoter. This could also be an autoregulatory phenomenon. The above secretion stress results suggested that such feedback compensatory mechanisms exist in the cell. PrsA depletion may also cause a secretion stress response.

The CssRS-regulated quality-control protease gene *htrB* was induced 6.3-fold in PrsA-depleted cells (Table 5), suggesting that PrsA depletion causes accumulation of misfolded native proteins in the cell envelope. Several genes involved in cell wall synthesis were also moderately upregulated. These were *dltBCE* (1.7- to 2.3-fold), *ddlA* (1.9-fold) encoding the D-alanyl-D-alanine ligase A, and *murF* (1.8-fold), which probably forms an operon with *ddlA*. The *maf* (2.0-fold) *mreB* (1.5-fold) and *mreD* (1.5-fold) genes, which all belong to the *mre* operon and encode proteins involved in septum formation and cell-shape determination, respectively, and *pbpX* (2.7-fold), which encodes a penicillin-binding protein, were induced. Also induced were the σ^X-regulated genes, suggesting a stress response via this extracytoplasmic function sigma factor (Table 5).

Table 5 Genes induced in cells depleted of PrsA foldase

Gene	Up/ downstream genes	Induction[a]	Function
htrB		6.74 (8.82, 4.65)	Serine protease
ywaC		5,42 (7.10, 3.73)	Similar to GTP-pyrophosphokinase
Cell wall synthesis			
ddlA		2.09 (2.24, 1.93)	D-Alanyl-D-alanine ligase A
	murF	1.86 (2.18, 1.53)	UDP-*N*-acetylmuramoylalanyl-D-glutamyl-2.6-diaminopimelate-D-alanyl-D-alanine ligase
	ydbO	3.49 (3.58, 3.40)	Similar to cation efflux system
dltB		2.05 (2.38, 1.72)	D-Alanine esterification of LTA and WTA, σ^S-regulated
	dltC	2.66 (3.32, 2.00)	D-Alanyl carrier protein, σ^S-regulated
	dltE	1.75 (1.78, 1.72)	Unknown, σ^S-regulated
maf		1.98 (2.13, 1.83)	Septum formation
	radC	3.94 (5.04, 2.83)	Similar to DNA repair protein, σ^M-regulated
	mreB	1.57 (1.59, 1.55)	Cell-shape determining protein
	mreD	1.46 (1.47, 1.45)	Cell-shape determining protein
murG		2.00 (2.38, 1.62)	UDP-*N*-acetylglucosamine-*N*-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol *N*-acetylglucosamine transferase
pbpX		2.75 (3.05, 2.45)	Penicillin binding protein
σ^X-regulated			
abh		4.19 (5.61, 2.77)	Transcriptional regulator of transition state genes
abrB		1.47 (1.49, 1.44)	Transcriptional regulator of transition state genes

Discussion

The secretion stress response in *B. subtilis* was studied by DNA macroarray analysis. Our approach was to induce secretion/folding stress by various means in order to identify components upregulated or downregulated by protein oversecretion and misfolding (the secretion stress regulon), and which may be involved in combatting the harmful effects of secretion stress. In addition to previously known components of the secretion stress regulon several new factors were identified.

High-level AmyQ secretion induced only seven genes in exponentially growing *B. subtilis*, suggesting that secretion stress was moderate under these conditions. The upregulated genes included *htrA* and *htrB*, consistent with previously published results (Hyyryläinen et al. 2001).

Table 5 (continued)

Gene	Up/ downstream genes	Induction[a]	Function
bcrC (*ywo-A*)		3.55 (4.24, 2.86)	Similar to bacteriocin transport permease, σ^X/σ^M-regulated
csbB		2.51 (3.20, 1.82)	Stress response protein
divIC		1.71 (1.81, 1.60)	Cell division initiation protein, σ^X/σ^M-regulated
ycgR		1.63 (1.55, 1.71)	Unknown
ypuA		2.84 (3.35, 2.33)	Unknown
yrhH		3.81 (4.63, 2.98)	Similar to methyltransferase
Other			
radA		2.69	DNA repair protein homologue
yetG		2.68	Unknown
yhdK		2.39	Unknown
yjbC		3.25	Unknown
yjbD		2.54	Unknown
ypuD		2.43	Unknown

[a]Induction ratios are means from two independent experiments

The severe secretion stress in the *prsA3* mutant caused a clearly stronger induction of *htrA* and *htrB* than mere AmyQ oversecretion alone, consistent with the fact that the degree of induction of these genes correlates with the degree of protein misfolding in the cell envelope (Hyyryläinen et al. 2001). A proteomic analysis has also revealed elevated levels of HtrA protease in the culture medium under secretion stress conditions (Antelmann et al. 2003). We also identified a third gene, *yqxL*, which responded to the secretion stress in a manner similar to that of the *htr* genes. The *yqxL* gene was weakly activated by mere AmyQ oversecretion (2.4-fold) but strongly by the severe secretion stress in the *prsA3* mutant (13.9-fold). The YqxL protein belongs to the family of CorA-type Mg^{2+} transporters. In *Salmonella typhimurium* and *E. coli*, CorA is the primary influx system for extracellular magnesium (Kehres et al. 1998). In contrast to YqxL, the *S. typhimurium* and *E. coli* homologues are constitutively expressed. Some of the CorA-like proteins may have a function other than Mg^{2+} transport (Kehres et al. 1998). How does secretion stress and accumulation of misfolded proteins at the membrane-cell wall interface induce *yqxL* expression? Misfolded proteins could directly activate some still unknown signal transduction pathway that regulates *yqxL*, or indirectly affect the microenvironment at the membrane-cell wall interface and thereby affect activation of a regulatory mechanism controlling *yqxL* expression.

In addition to *htrA*, *htrB* and *yqxL*, severe secretion stress induced 17 genes or operons (at least 2-fold), among them the 10-fold induced *liaI* (*yvqI*) and downstream *liaH* (*yvqH*) and *liaG* (*yvqG*). Induction of *lia* (*yvq*) genes is mediated by the LiaRS (YvqCE) TCS that is encoded by the *liaSR* (*yvqEC*) genes in the same gene cluster as *liaIHG* (*yvqIHG*) (Kobayashi et al. 2001; Mascher et al. 2003; H.-L. Hyyryläinen et al. unpublished). Cell wall antibiotics that interfere with the lipid II cycle in the cytoplasmic membrane, and thus peptidoglycan synthesis, are strong inducers of LiaRS (YvqCE) (Mascher et al. 2004). We have shown that LiaRS (YvqCE) is also strongly activated (>100-fold induction ratio) by cationic antimicrobial peptides such LL-37 and protegrin-1 (Milla Pietiäinen et al., MS submitted). This study shows that secretion stress activates LiaRS (YvqCE), possibly due to the accumulation of misfolded proteins in the cell envelope and possibly causing perturbation in the membrane, although as an activator it is clearly weaker than cell wall antibiotics and cationic antimicrobial peptides. The roles of these proteins in the stress response remain unclear, but LiaH (YvqH) shows significant homology with the *E. coli* phage shock protein A (PspA).

DNA array analysis of the CssRS regulon suggested that this TCS is probably dedicated to regulating only the *htrA* and *htrB* genes. Fujita and his collaborators found that overexpression of *cssR* (*yvqA*) induced, in addition to the *htr* genes, two functionally unknown genes, *ygxB* and *yjgD* (Kobayashi et al. 2001). In our analysis, these two genes were not found to belong to the CssRS regulon. The inactivation of *cssS* and consequent absence of HtrA and HtrB proteases also caused stress, as evidenced by the induction of the stress-sensitive *liaIHG* (*yvqIHG*) genes via the LiaRS (YvqCE) TCS.

Several of the gene induction patterns suggest that cells combat the harmful effects of secretion/folding stress by compensatory up- or down-regulation of genes having a role in the affected functions. Components of the secretion apparatus are typically expressed from constitutive housekeeping genes. Our results, however, show that the CsaA molecular chaperone and the Ffh signal recognition particle-like component, which are involved in the early phase of protein secretion, are significantly induced to combat secretion stress. The upregulation of genes involved in cell wall synthesis/modification probably has a similar role. Among the induced operons was *dlt*, which encodes a system for the D-alanylation of teichoic acids and consequently modulates the charge density of the cell envelope. The *dlt* operon is regulated by the extracytoplasmic function sigma factor σ^X (Cao and Helmann 2004), suggesting that σ^X senses secretion/misfolding stress. There is a signal transduction pathway for sensing of misfolded proteins also in eukaryotic cells. The accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER) activates the unfolded protein response, consequently upregulating the expression of several ER foldases (Patil and Walter 2001). Genes involved in many other cellular

functions were also induced. Interestingly, the *exuR* (*yjmH*) and *uxaB* (*yjmI*) genes, which encode the ExuR repressor of the hexuronate utilization operon (*exu* locus) and the UxaB tagaturonate reductase, respectively, both involved in the utilization of galacturonate, were induced almost 5-fold. These genes are upregulated by galacturonate and repressed by glucose (Mekjian et al. 1999). The upregulation of *exuR* and *uxaB* expression was not caused by the xylose added to the culture medium to induce P_{xyn}-*amyQ*, since in the absence of *prsA3*, mere xylose-induced *amyQ* expression did not result in the upregulation of these genes. It has been shown that there are two promoters that control gene expression in the *exu* locus, the σ^{A}-dependent exuP1 and the σ^{E}-dependent exuP2, and the latter promoter has been suggested to be responsible for the expression of *exuR* and *uxaB* (Mekjian et al. 1999). Our DNA array analysis, however, revealed that the *yjmF* and *exuT* genes of the *exu* locus, which are located upstream from *exuR* and *uxaB*, were expressed at significantly lower levels (>10-fold) than *exuR* and *uxaB* (data not shown). This suggests that a third promoter (exuP3) exists between *exuT* and *exuR*, which controls expression of downstream genes. Our transcriptome analysis suggests that posttranslocational protein misfolding induced *exuR* and *uxaB* from the predicted exuP3 by an unknown mechanism.

The reduced expression levels of numerous genes during severe secretion/folding stress can also be seen as an attempt by the cell to cope with the harmful effects of this stress. The expression of autolysins was decreased, possibly affecting the rate of turnover of the cell wall, together with increased expression of genes involved in cell wall synthesis. The *yacD* gene, which is the only *prsA* paralogue in *B. subtilis*, was strongly repressed, whereas *prsA* was expressed constitutively, suggesting that these parvulin-type folding factors have distinctly different roles in the cell wall.

The most prominent groups of genes induced by sublethal PrsA depletion were those involved in cell wall synthesis, including the *ddl*, *dlt* and *maf*-*mre* operons, and the σ^{X} regulon. Do these differentially expressed genes suggest a mechanism for the lethality of PrsA depletion? PrsA catalyses protein folding, and is probably required for the folding of some essential cell wall or membrane protein (s) (Vitikainen et al. 2001, 2004). A possible PrsA target could be the essential MreC cell-shape determining protein (Lee and Stewart 2003; Kobayashi et al. 2003). MreC is predicted to have one transmembrane segment at the N-terminus, and the large C-terminal domain is probably located at the membrane/cell wall interface (topology prediction at the TMHMM server http://www.cbs.dtu.dk/services/TMHMM) in the same compartment where PrsA is located. Furthermore, sublethal and lethal PrsA depletion cause similar changes in cell morphology as MreC depletion (Lee and Stewart 2003; Hyyryläinen et al. 2001).

Acknowledgements This work was supported by grants from European Union (Bio4-CT96-0097 and QLK3-CT-1999-00413), and Academy of Finland (Life2000).

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