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Production and Secretion Stress Caused by Overexpression of Heterologous α -Amylase Leads to Inhibition of Sporulation and a Prolonged Motile Phase in *Bacillus subtilis*^{∇†}

Andrzej T. Lulko, Jan-Willem Veening, Girbe Buist,[‡] Wiep Klaas Smits, Evert Jan Blom, Aaron C. Beekman, Sierd Bron, and Oscar P. Kuipers*

Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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Transcriptome analysis was used to investigate the global stress response of the gram-positive bacterium *Bacillus subtilis* caused by overproduction of the well-secreted AmyQ α -amylase from *Bacillus amyloliquefaciens*. Analyses of the control and overproducing strains were carried out at the end of exponential growth and in stationary phase, when protein secretion from *B. subtilis* is optimal. Among the genes that showed increased expression were *htrA* and *htrB*, which are part of the C_{ss}RS regulon, which responds to high-level protein secretion and heat stress. The analysis of the transcriptome profiles of a *cssS* mutant compared to the wild type, under identical secretion stress conditions, revealed several genes with altered transcription in a C_{ss}RS-dependent manner, for example, *citM*, *ylxF*, *yloA*, *ykoJ*, and several genes of the *flgB* operon. However, high-affinity C_{ss}R binding was observed only for *htrA*, *htrB*, and, possibly, *citM*. In addition, the DNA macroarray approach revealed that several genes of the sporulation pathway are downregulated by AmyQ overexpression and that a group of motility-specific (σ^D -dependent) transcripts were clearly upregulated. Subsequent flow-cytometric analyses demonstrate that, upon overproduction of AmyQ as well as of a nonsecretable variant of the α -amylase, the process of sporulation is severely inhibited. Similar experiments were performed to investigate the expression levels of the *hag* promoter, a well-established reporter for σ^D -dependent gene expression. This approach confirmed the observations based on our DNA macroarray analyses and led us to conclude that expression levels of several genes involved in motility are maintained at high levels under all conditions of α -amylase overproduction.

The gram-positive bacterium *Bacillus subtilis* is capable of secreting large amounts of endogenous proteins into the extracellular medium (41). Therefore, this bacterium and its relatives are often exploited as hosts for the production and secretion of heterologous industrially interesting enzymes. Secretion of heterologous proteins in large quantities has been shown to lead to the unfavorable condition of protein misfolding and subsequent degradation (36). In *B. subtilis*, accumulation of misfolded proteins at the membrane-cell wall interface is sensed by the C_{ss}RS two-component system, which consists of the membrane-embedded sensor kinase C_{ss}S and the response regulator C_{ss}R (14). This system responds to high-level protein secretion and heat stress by phosphorylation of C_{ss}R, which, in turn, activates transcription of the monocistronic *htrA* and *htrB* genes (6). The C_{ss}RS system has also been shown to regulate the expression of its own operon (6, 20). HtrA and HtrB are membrane-bound serine proteases whose major

functions are degradation of misfolded and aggregated proteins. Expression of both proteases is induced upon excessive expression of secretory proteins or a temperature increase (31). Thus, the C_{ss}RS regulon forms a quality control and defense system when cells are confronted with secretion stress. The members of the C_{ss}RS quality control system become involved at that stage and take action in two possible manners, either by acting as a chaperone aiding refolding or preventing unfolding or by HtrA/B-dependent proteolysis.

Recently, proteomic studies to investigate the global effects of secretion stress have been performed (3). These studies showed that HtrA is also present in the culture supernatant and, in addition to its protease activity, can act as a molecular chaperone. In addition, global effects of secretion stress in *B. subtilis* were also investigated by transcriptome analysis during the exponential growth phase. This study, however, revealed differential expression of only relatively few genes, including the genes encoding the HtrA and HtrB proteases (15).

Because most of protein secretion in *B. subtilis* takes place during the onset of the stationary phase of growth (5, 12), we set out to examine the global transcriptional response of *B. subtilis* under secretion stress conditions at this specific growth phase. Secretion stress was applied by overproducing the well-secreted AmyQ α -amylase from *Bacillus amyloliquefaciens*. Besides examining secretion stress in wild-type cells, we compared transcriptome profiles of a *cssS* mutant strain under conditions of high-level AmyQ production. In this work, we have identified and verified putative novel members of the

* Corresponding author. Mailing address: Molecular Genetics Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 31 50 3632093. Fax: 31 50 3632348. E-mail: o.p.kuipers@rug.nl.

† Supplemental material for this article may be found at <http://aem.asm.org/>.

‡ Present address: Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, Hanzeplein 1, P.O. Box 30001, 9700 RB Groningen, The Netherlands.

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TABLE 1. Bacterial strains and plasmids

<i>B. subtilis</i> strain or plasmid	Genotype	Reference or source
Strains		
168	<i>trpC2</i>	21
168::sp	168, <i>pks::sp</i> Sp ^r	10
IIA-gfp	168, P _{<i>spoIIA-gfp</i>} Cm ^r	39
hag-gfp	168, P _{<i>hag-gfp</i>} Cm ^r	This work
IIA/E	168, P _{<i>spoIIA-gfp</i>} Cm ^r pUB110	This work
IIA/Q	168, P _{<i>spoIIA-gfp</i>} Cm ^r pKTHM10	This work
IIA/QAla	168, P _{<i>spoIIA-gfp</i>} Cm ^r pKTHM102	This work
hag/E	168, P _{<i>hag-gfp</i>} Cm ^r pUB110	This work
hag/Q	168, P _{<i>hag-gfp</i>} Cm ^r pKTHM10	This work
hag/QAla	168, P _{<i>hag-gfp</i>} Cm ^r pKTHM102	This work
CB100	<i>sigD::cat</i> Cm ^r	27
Sik243	<i>spo0A::Em</i> Em ^r	16
BV2001	<i>cssS::sp</i> Sp ^r	14
Plasmids		
pUB110	Km ^r	
pKTH10	Km ^r , pUB110 derivative containing the α -amylase gene (<i>amyQ</i>) of <i>B. amyloliquefaciens</i>	32
pKTHM10	Km ^r , pKTH10 derivative	44
pKTHM102	Km ^r , pKTHM10 with the Ala-rich signal peptide of AmyQ	44

CssRS regulon and we dissected direct and indirect effects of α -amylase overproduction and protein secretion in stationary-phase cultures. Our study reveals that, upon overproduction of a nonsecreted α -amylase, as well as the secreted wild-type variant, the process of sporulation is severely inhibited. In addition, we show that expression levels of genes involved in motility are maintained at high levels under all conditions of α -amylase overproduction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are shown in Table 1. *B. subtilis* 168 with an integrated spectinomycin marker (*sp*) in the *pks* locus (*B. subtilis* 168::*sp*) (10) and *B. subtilis* 168 *cssS::sp* (14) were used

for transcriptional analyses. *B. subtilis* 168::*sp* was used to avoid possible effects on transcriptional profiling due to the presence of the spectinomycin gene alone, as was shown by Hamoen et al., and to be able to compare the data obtained for this strain with those for the *cssS* insertion mutant. Both strains were transformed with either the pUB110 (empty vector) (6) or the pKTH10 plasmid (pUB110 derivative containing the *amyQ* α -amylase gene of *B. amyloliquefaciens*) (32). *B. subtilis* strains were grown in TY medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing the appropriate antibiotics at 37°C and 250 rpm. Antibiotic concentrations were as follows: kanamycin, 10 μ g/ml; spectinomycin, 100 μ g/ml; chloramphenicol, 5 μ g/ml.

Strain construction. To construct plasmid pGFP-hag, a PCR with primer pair hag-F and hag-R (Table 2) was performed using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment containing the promoter region of the *hag* gene was subsequently cleaved with HindIII and EcoRI and ligated into the corresponding sites of pSG1151, in that way generating a fusion with the *gfpmut1* gene (22). *B. subtilis* strain hag-gfp was obtained by Campbell-type integration of plasmid pGFP-hag into the chromosome of *B. subtilis* 168. Transformants were selected on TY agar plates containing chloramphenicol (5 μ g/ml), after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

RNA isolation, preparation of labeled cDNA, and hybridization. Cells were grown overnight in 10 ml of TY medium with kanamycin (10 μ g/ml) and then diluted to an optical density at 600 nm of 0.1 in 40 ml of TY medium containing kanamycin. Samples for transcriptome analyses were collected at the late-exponential-growth stage (1 hour before the transition point) and 3 h after entry in the stationary-growth phase. Three independent cultures of each strain were used, and cells were sampled for macroarray experiments. RNA was isolated by spinning down cells from 4 ml of culture, with subsequent cell disruption, phenol-chloroform extraction, and then RNA purification with a High Pure RNA isolation kit from Roche, as described previously (10). RNA was eluted with 50 μ l of elution buffer and subsequently quantified with GeneQuant (Amersham), and the RNA integrity was checked on agarose gels. Four micrograms of RNA as a template and 1 pmol of open reading frame-specific primers (Eurogentec) were used for the reverse transcriptase reaction with SuperscriptII (Gibco BRL). The detailed protocols of reverse transcription and purification of ³²P-labeled cDNA are outlined by Hamoen et al. (10). Labeled cDNA was hybridized to *B. subtilis* Panorama arrays (Sigma-Genosys), according to the manufacturer's instructions. The membranes were exposed to Cyclone phosphorimager screens (Packard) for approximately 2.5 days, and signals were quantified with Array-Pro Analyzer 4.5 (Media Cybernetics).

Data analysis. Duplicate spots were averaged in Array-Pro software (Media Cybernetics, Inc.), and the signal was normalized after background subtraction by calculation of the percentage of total signal per gene using Microsoft Excel. Outstandingly high signals (for example, whole genomic DNA control spots, which act as a positive control and always show very strong signals) were excluded from calculations of the total signal. Statistical significance of the ob-

TABLE 2. Primer sequences

Primer	Sequence (5'→3')	Target fragment	Restriction site
hag-F	GGGATCAAGTGAAGCTTGAATTGACG	<i>hag</i>	HindIII
hag-R	CGGAATTCATTTCTCTCCTCCTTGAATATGTTGTAAAGGCACGTCC	<i>hag</i>	EcoRI
QE60- <i>cssR</i> -F	CATGCCATGGCATAACACCATTTATCTAGTTGAAGA	<i>cssR</i>	NcoI
QE60- <i>cssR</i> -R	CGGGATCCTGATGACATCATCTGTAGCCGAAACCGTA	<i>cssR</i>	BamHI
QE30- <i>cssR</i> -F	CGGGATCCTTGTCTACACCATTTATCTAGTTGAAG	<i>cssR</i>	BamHI
QE30- <i>cssR</i> -R	CCGAGCTCTTATCATGATGACATCATCTGTAGCCGAAA	<i>cssR</i>	SacI
htrA-EMSA-F	AACGGATCAGCCGATACGTT	<i>PhtrA</i>	None
htrA-EMSA-R	TCATCACGATAGTTATCCAT	<i>PhtrA</i>	None
htrB-EMSA-F	CGTCAGCAGTTCATTGAC	<i>PhtrB</i>	None
htrB-EMSA-R	CCATCACGTCGATAATCC	<i>PhtrB</i>	None
citM-EMSA-F	AGGTCACCTCCTCACCTGAA	<i>PcitM</i>	None
citM-EMSA-R	CATGAGAAAGCCTAAGATTGCTAAC	<i>PcitM</i>	None
flgB-EMSA-F	TGTATCGTTCCAGAAAATAAGC	<i>PflgB</i>	None
flgB-EMSA-R	TCAGGGCGAGAAATGTAGTTC	<i>PflgB</i>	None
ykoJ-EMSA-F	TGCCGATCAAATCAGCAG	<i>PykoJ</i>	None
ykoJ-EMSA-R	TTGTGAGCCCCTCCTTGT	<i>PykoJ</i>	None
ylmA-EMSA-F	CCAGAAGCACAAGCACCATA	<i>PyloA</i>	None
ylmA-EMSA-R	GTATGTAAACATGCCATCAAACG	<i>PyloA</i>	None
pK-F	AATCTATCGACATATCTGCAA	<i>PcomK</i>	None
K-FP-R	GGAATTCTTGCGCCGTTCACTTCATAC	<i>PcomK</i>	None

tained gene expression ratios was assessed by the Cyber-T program (23). Genes with a Cyber-T *P* value lower than 0.05 are discussed in this paper. Raw data and images are available at http://molgen.biol.rug.nl/publication/secstress_data/.

SDS-PAGE and Western blotting. To detect the precursor and mature forms of AmyQ in cells and growth medium, 1 ml of culture was centrifuged and the cell pellet was washed once with fresh medium. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to Immobilon polyvinylidene difluoride membranes. The visualization of the AmyQ protein was carried out with specific anti-AmyQ antibodies, horseradish peroxidase-anti-rabbit immunoglobulin G conjugates, and ECL immunoblotting detection reagents (Amersham).

Amylase activity assay. Samples from culture supernatants were diluted 100-fold and were subjected to amylase activity quantification with the EnzCheck Ultra amylase assay kit (Molecular Probes), according to the manufacturer's instructions. Assays were carried out in 96-well microtiter plates in a 100- μ l total volume using 50 μ l diluted supernatants. Reaction mixtures contained 200 μ g/ml DQ starch substrate and were carried out in 100 mM MOPS (morpholinepropanesulfonic acid) at pH 6.9. The degradation of the substrate by amylase yields highly fluorescent fragments, and fluorescence was monitored at room temperature every 5 min for 1 h with the microplate reader TECAN (GENios) using standard fluorescein filters. Samples were taken from three independent experimental replicates.

Flow cytometry. Cells were diluted 100-fold in 0.2 μ M of filtered minimal medium (2) and directly measured on an Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, The Netherlands), operating an argon laser (488 nm) as essentially described by Veening et al. (39). For each sample, at least 20,000 cells were analyzed. Data containing the green fluorescent signals were collected with a fluorescein isothiocyanate filter, and the photomultiplier voltage was set between 700 and 800 V. Data were captured using System II software (Beckman Coulter) and further analyzed using WinMDI 2.8 software (<http://facs.scripps.edu/software.html>). Figures were prepared for publication using WinMDI 2.8 and Corel Graphics Suite 11. To distinguish background fluorescence from green fluorescent protein-specific fluorescence, the parental strain *B. subtilis* 168 was also analyzed with each flow-cytometric experiment.

Construction and purification of six-His-tagged CsrR. Plasmids encoding CsrR protein with a six-His tag were constructed as follows. For C-terminally tagged proteins, the *csrR* open reading frame was amplified by PCR using primers QE60-*csrR*-F and QE60-*csrR*-R (Table 2) and Phusion polymerase (Finnzymes). Chromosomal DNA of *B. subtilis* strain 168 was used as a template. The product of this reaction was digested with NcoI and BamHI and cloned into the similarly digested pQE30 plasmid (QIAGEN), yielding plasmid pQE60-*csrR*.

To obtain an N-terminally tagged CsrR protein, the *csrR* open reading frame was amplified by PCR using primers QE30-*csrR*-F and QE30-*csrR*-R. The product of this reaction was digested with BamHI and SacI and cloned into the similarly digested pQE30 plasmid (QIAGEN), yielding plasmid pQE30-*csrR*.

To induce expression, *Escherichia coli* JM109 carrying pQE60-*csrR* or pQE30-*csrR* was diluted 1:100 to fresh medium from an overnight culture. At an optical density at 600 nm of 0.6, expression was induced by the addition of 1 mM of IPTG (isopropyl- β -D-thiogalactopyranoside). Cells were harvested after an additional 2 hours of growth, after which protein was purified as described elsewhere (37a). The purity of the protein was estimated to be >95% on the basis of Coomassie-stained SDS-PAGE gels. After purification, the protein was dialyzed against dialysis buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA, 10 mM MgCl₂, 0.2 M NaCl, 1 mM β -mercaptoethanol, 5 mM imidazole, 7% glycerol). The concentration of protein was determined on the basis of A280 using a Nanodrop machine (Nanodrop Technologies), with 0.1% (1 g/liter), giving a value of 1.272 (ExpASy ProtParam Tool; <http://www.expasy.org/tools/protparam.html>).

Electrophoretic mobility shift assays (EMSAs). To establish direct binding of CsrR to putative target promoters, gel shift experiments were carried out as described before (1). In our experiments, we observed no significant difference in affinity between C- and N-terminally His-tagged proteins. Subsequent experiments were therefore carried out using only N-terminally His-tagged proteins. The fragments obtained corresponded to base pairs -339 to +19 (*htrA*), -335 to +19 (*htrB*), -333 to +26 (*citM*), -191 to +25 (*flgB*), -320 to 0 (*ykoJ*), and -312 to +32 (*yloA*) compared to the start of the open reading frame, as annotated in SubtiList R16.1 (<http://genolist.pasteur.fr/SubtiList/>). As a negative control the promoter of *comK* (-201 to +47) was amplified. All primer sequences are available in Table 2.

Microarray data accession number. The array data have been deposited in NCBI's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE8014.

RESULTS

Transcriptomics of secretion stress in late-exponential and stationary-phase cells. AmyQ is the α -amylase from *Bacillus amyloliquefaciens* and contains a Sec-type signal sequence. This protein has been shown to trigger a specific secretion stress response when overexpressed in *B. subtilis* (14). DNA microarray analysis was used to compare transcriptional profiles of *B. subtilis* 168::*sp* containing plasmid pUB110 (empty vector) with those of *B. subtilis* 168::*sp* containing plasmid pKTH10 (AmyQ overexpression) grown in TY broth. Samples were taken for transcriptome analyses during late exponential (1 hour before the transition point) and stationary (3 h after the transition point) phases of growth. Amylase-dependent starch degradation on TY-agar plates and Western blot analysis of the growth media from the cells used for the microarray experiment verified the expected AmyQ overproduction for the pKTH10-containing cells (data not shown).

Differentially expressed genes in the late exponential phase are listed in Table S1 in the supplemental material. As expected, the most highly upregulated genes are *htrA* and *htrB*, encoding the Htr-like proteases. Also, moderately elevated transcription levels of the *cssRS* operon, encoding the secretion stress response regulator (CsrR) and histidine kinase (CsrS), were observed upon AmyQ overproduction, indicating that cells were clearly subjected to secretion stress. In addition, a stimulatory effect was observed for the transcripts of the ribosomal protein RpsB and the genes for the general stress proteins GroEL and DnaK, suggesting that, next to secretion stress, a cytosolic stress, most likely resulting from the presence of misfolded and aggregated proteins, is induced. Moreover, two members of the peroxide stimulon, *ahpF* and *mrgA*, next to the genes involved in cell wall homeostasis (*dltA*, *acpA*, and *accC*), showed elevated expression levels. Other genes with known function that were induced upon AmyQ overproduction include *pycA* and *citB* of the tricarboxylic acid cycle and *sodA* and *trxA*, which play an important role in maintaining the redox balance of the cell. The secretion stress directly or indirectly caused increased mRNA levels of four genes with an unknown or predicted function, including *yvfW*, encoding a putative iron-sulfur-binding protein, and *yvqH*, which has similarity to the gene encoding *E. coli* phage shock protein A, which was shown to be induced under anaerobic growth (42) and ethanol stress conditions (33). Also *ykoJ* and *ydbK* showed increased transcript levels. The former codes for a conserved protein of unknown function, and the latter encodes a putative ABC transport system permease protein. Besides the upregulation of a number of genes, some of which have been mentioned above, more than 40 genes were significantly downregulated upon AmyQ overproduction. These downregulated genes are classified within different functional categories including, among others, genes involved in the metabolism of lipids and sporulation and genes encoding transport/binding proteins (see Table S1 in the supplemental material).

Under natural conditions, most of the secretion takes place during the stationary-growth phase. Therefore, transcriptome analyses were also performed on AmyQ-overproducing and nonoverproducing cells in this phase. Again, a strong effect on the transcriptional levels of *htrA*, *htrB*, *cssS*, and *cssR* was observed (see Table S2 in the supplemental material). Thus,

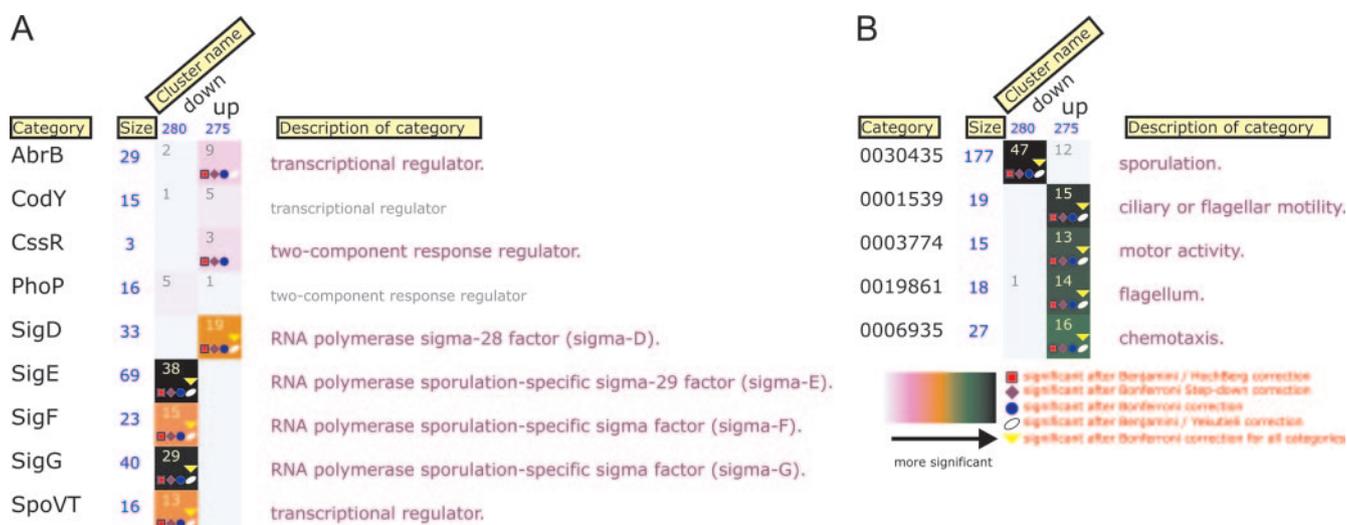


FIG. 1. FIVA analysis of interaction and GO (gene ontology) categories of the transcriptome data. Genes from the DNA macroarray data set were divided into up- and downregulated clusters [comparison of gene expression ratios between *B. subtilis* 168(pKTH10)/*B. subtilis* 168]. “Interaction” (A) and GO (B) categories are shown. The size of each cluster is presented in blue underneath the cluster name. Numbers in rectangles represent how many genes were up- or downregulated per cluster in each category. The colors of square boxes depict the significance of gene enrichment per category as defined in the key. Detailed information on significance analyses is available in Blom et al. (4).

secretion stress also occurs during later stages of growth. From the transcriptional profiling in this growth phase, two main novel findings can be deduced: several genes involved in motility and chemotaxis, including *sigD*, had increased mRNA levels, in contrast to a group of sporulation-related genes, which appeared to have decreased transcription levels (Fig. 1). The list of downregulated sporulation-related genes is more extensive, although for some of them the obtained expression ratios were slightly below the statistical cutoffs. The protein phosphatase encoded by *prpC* (*yloO*) was activated, and the product of this gene, together with the PrkC protein kinase, has been implicated in the sporulation process (25). Two sporulation genes, *spo0JA* and *spo0E*, had increased transcription levels under secretion stress, but, interestingly, both of them affect sporulation in a negative manner (34). Expression of several other genes seems also to be affected by AmyQ overproduction. The products of these genes are associated with different cellular processes such as metabolism of amino acids, lipids, and carbohydrates and transport and binding activity (see Table S2 in the supplemental material). For the genes with unknown function, *yloA* and *ykoJ* showed the strongest upregulation (11.2-fold and 5.3-fold, respectively). Both genes are well conserved among bacterial species. YloA bears similarity to a fibronectin-binding protein as well as to RNA-binding proteins that show homology to the eukaryotic snRNP's. YkoJ was also induced at the earlier time point. This protein contains a putative signal peptide and two PepSY domains, which, it has been suggested, have peptidase-regulatory activity in proximity to the cell wall (43).

Verification of putative members of the C_{ss}RS regulon. As secretion stress clearly affected the expression of the known members of the C_{ss}RS regulon, we attempted to identify novel members of this regulon, with a focus on the upregulated genes resulting from AmyQ overexpression. For this purpose we compared the transcriptome profiles of *B. subtilis* 168::sp (wild

type) and 168 *cssS*::sp (Δ *cssS*), both containing the pKTH10 plasmid. Previous research showed that *htrA* and *htrB* are regulated by the C_{ss}RS system and that disruption of *cssS* reduces *htrA* and *htrB* transcription (6, 14). Our transcriptome data confirmed these observations since both genes showed decreased mRNA levels in the *cssS* mutant strain. Besides *htrA* and *htrB*, similar expression patterns from our macroarray approach were observed for *citM* (secondary Mg-citrate transporter), the *flgB* operon (flagellar synthesis and chemotaxis), *ylxF*, *yloA*, and *ykoJ*.

Our analysis revealed several genes with altered transcription levels in response to overproduction of AmyQ, in a C_{ss}R-dependent manner. Though C_{ss}R is a two-component regulator, it is unknown if the observed effects are due to direct binding of C_{ss}R to the target promoters. In fact, the binding of C_{ss}R to target promoters has not been reported so far. Therefore, several candidate genes were selected and EMSAs were carried out using purified N-terminally His-tagged C_{ss}R protein. Phosphorylation of nontagged C_{ss}R by acetyl-phosphate enhances the affinity of the protein for DNA two- to fourfold, depending on the promoter fragment used (E. Darmon, unpublished observations). Figure 2 shows that unphosphorylated, six-histidine-tagged C_{ss}R has a high affinity for the DNA fragments containing the promoters of the *htrA* and *htrB* genes, with an apparent equilibrium dissociation constant (K_D) of ~125 nM; therefore, the tagged protein likely mimics the phosphorylated form of the protein. These observations are in good agreement with previous reporter studies that demonstrate the C_{ss}R-dependent induction of these genes upon protein overproduction (6, 14). In contrast, unphosphorylated, His-tagged C_{ss}R does not appear to bind strongly to any of the other fragments, with the possible exception of *citM*, for which consistently a reduced mobility was observed at 1 μ M of C_{ss}R protein. We conclude that, for the fragments used, only *htrA* and *htrB* contain a high-affinity C_{ss}R-binding site. As for the

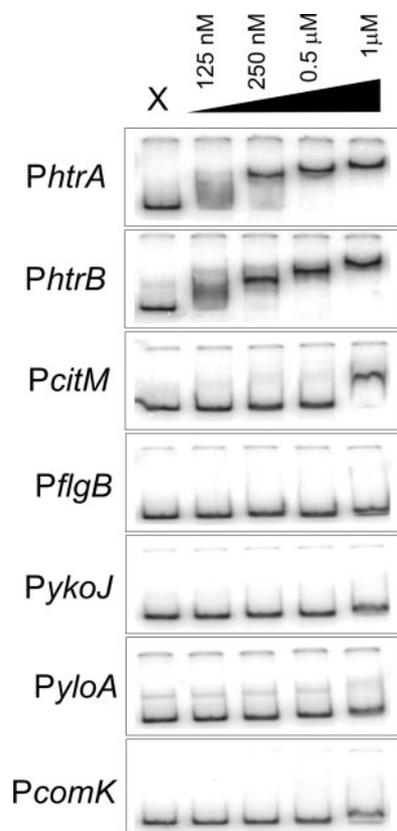


FIG. 2. Binding of CsrR-His₆ to the indicated promoter regions. Gel retardation reactions were performed with radiolabeled DNA fragments prepared by PCR and end labeled with ³²P. Promoter regions were incubated with increasing concentrations of purified CsrR-His₆ (see Materials and Methods), ranging from 0.125 μM to 1 μM of the protein. In each panel lane X corresponds to the reaction with no protein added.

other genes, the observed transcriptional changes most likely result from secondary effects.

Sporulation and motility are affected by AmyQ overproduction and secretion. Several genes that were significantly downregulated by AmyQ overexpression belong to the sporulation pathway. This suggests that secretion stress results in inhibition of spore formation. Interestingly, overexpression of a cytoplasmic protein did not affect early sporulation gene expression (18), indicating that secretion stress rather than overproduction stress might be the cause for the observed downregulation. To examine whether the observed downregulation of the sporulation pathway in our transcriptome analysis by AmyQ overproduction was specifically caused by secretion stress or was a result of an indirect effect caused by AmyQ overproduction, we made use of a sporulation-specific reporter strain carrying the *spoIIA* promoter in front of the gene encoding green fluorescent protein. The *spoIIA* promoter is directly activated by the key sporulation regulator, Spo0A, and was shown to be a good reporter for cells that initiate sporulation (39). Plasmids pKTHM10 (AmyQ overexpression), pKTHM102 (AmyQ-Ala overproduction), and pUB110 (empty vector) were introduced in the indicator strain, and cells were analyzed for their expression levels by flow cytometry. The pKTHM102

vector is a derivative of pKTHM10 (which is similar to pKTH10) and encodes the AmyQ protein with an Ala-rich signal sequence that renders it inactive in translocation across the cytoplasmic membrane (44). Importantly, this AmyQ variant does not evoke a secretion stress response (40). Strains were grown in TY medium, and samples were taken at hourly intervals and examined by flow cytometry. As depicted in Fig. 3, strain IIA/Q (*P_{spoIIA}-gfp* pKTHM10), harboring an AmyQ overexpression vector with the wild-type signal peptide, was slightly delayed in the activation of the *spoIIA* operon (Fig. 3). A more pronounced effect was observed in cells containing the pKTHM102 plasmid (strain IIA/QAla). Overall, these results show that both secretion stress and AmyQ overproduction (IIA/QAla) in particular inhibit the process of sporulation. In addition, this inhibition already occurs at the earliest stages in spore formation, at the level of phosphorylation of Spo0A, as shown by the single-cell analyses.

Another adaptive response that *B. subtilis* utilizes is motility. In this case, cells physically escape from adverse circumstances towards more promising ones to increase their survival chances. Regulation of synthesis of flagellum and motility gene expression is known to be orchestrated by alternative sigma factor σ^D (11, 27). As described above, we observed a strong upregulation of several genes involved in motility upon AmyQ overproduction. To investigate the increase of flagellar gene expression in *B. subtilis* as a result of protein overproduction, we constructed a strain in which the σ^D -dependent *hag* promoter was fused to the *gfp* gene (*hag-gfp*). The *hag* gene codes for the flagellin protein, and it was shown before that this gene can be used as a good reporter for studying environmental effects on σ^D -dependent gene expression (28). The AmyQ overproduction plasmids were introduced in this reporter strain, resulting in strains *hag/Q* (*P_{hag}-gfp* pKTHM10), *hag/QAla* (*P_{hag}-gfp* pKTHM102), and *hag/E* (*P_{hag}-gfp* pUB110). Cells were grown in TY medium, and samples were collected at hourly intervals for flow-cytometric analyses. As shown in Fig. 3, the promoter activity of the flagellin gene is the highest for the strain overproducing AmyQ with the Ala-rich signal peptide. This again suggests that the enhanced expression of motility genes is not a direct effect of secretion stress itself but rather results from AmyQ overproduction.

Effects of *sigD* and *spo0A* deletions on AmyQ overproduction and secretion. The results of the transcriptome analyses have shown that two cellular adaptive processes were clearly affected under secretion stress conditions. This raised the question whether disruption of these processes would affect heterologous protein secretion by *B. subtilis*.

It has been previously investigated how the yields of protein production can be improved by modification of the components engaged in the late stages of protein secretion, especially the ones influencing cell wall-associated protease activity or cell wall composition (41). To further study whether sporulation and/or motility affect protein secretion in *B. subtilis*, *sigD* (σ^D) and *spo0A* mutations were introduced into a strain containing the pKTHM10 plasmid (AmyQ overproduction). Mutants of *spo0A* are defective in sporulation since this transcriptional regulator plays a central role in the initiation of this developmental process (34). The null mutant of σ^D is non-motile and shows reduced levels of autolysins (27). The levels of production of secreted AmyQ in both mutant strains and the

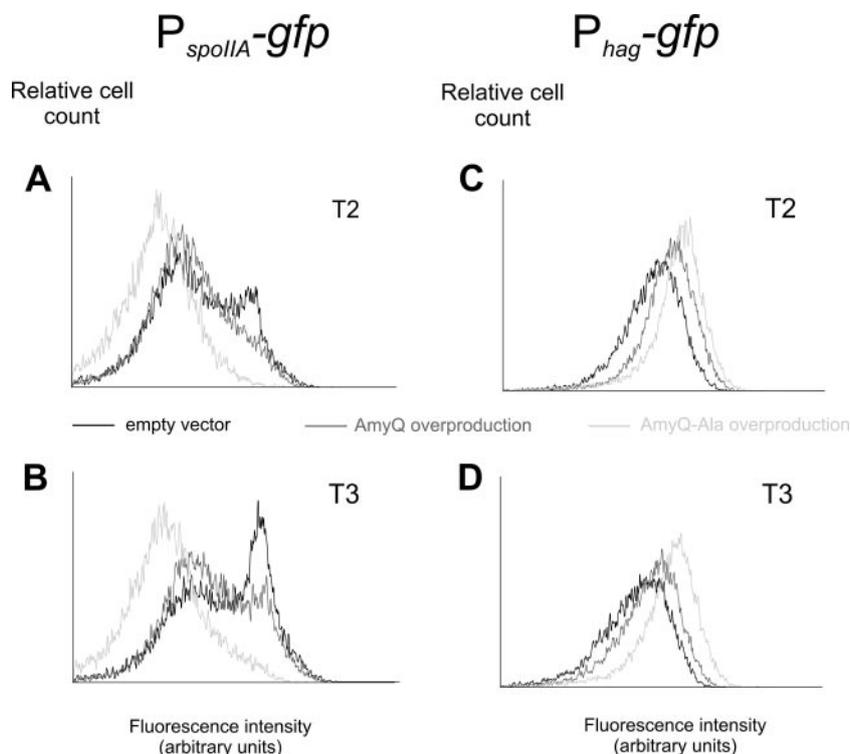


FIG. 3. Single-cell analysis of sporulation- and motility-specific gene expression. Strains were grown in TY medium at 37°C with shaking. Samples for flow-cytometric analysis were taken every hour during growth. Two time points are represented, T2 and T3, which correspond to 2 and 3 hours after the entry into stationary phase, respectively. The numbers of cells measured are indicated on the y axis, and their relative fluorescence levels are indicated on the x axis. (A and B) Strains IIA/E (P_{spoIIA} -gfp), IIA/Q (AmyQ overproduction, dark gray line), and IIA/QAla (AmyQ-Ala overproduction). (C and D) Strains hag/E (P_{hag} -gfp), hag/Q (AmyQ overproduction), and hag/QAla (AmyQ-Ala overproduction).

parental strain were determined at different time points of growth by means of the EnzCheck Ultra amylase assay kit as described in Materials and Methods. In all cases, the amount of active amylase in the culture medium increased from exponential phase (1 h after entry into stationary phase [T1]) and reached a maximum in the late stationary phase (T4) (Fig. 4). However, a clear delay was observed in the *spo0A* mutant, as this strain secreted significantly less amylase than the wild-type strain during exponential phase. In the late stationary phase the difference between the two strains became minimal. More interestingly, under our experimental setup, the *sigD* mutant reached slightly higher levels of the active enzyme at earlier growth phases compared to the wild-type strain. Again, 4 hours after entry into stationary phase the differences were negligible.

These results indicate that, when the cells are devoid of the possibility of initiating sporulation or entering the motile phase, the efficiency and timing of protein secretion by *B. subtilis* are modulated.

DISCUSSION

Protein secretion, like competence development and sporulation, is one of the postexponential processes that *B. subtilis* employs as a response to altering growth conditions. The secretion activity is rather low during exponential growth and increases substantially at the onset of stationary phase (35). It has been shown before that several components of the Sec machinery in *B. subtilis* reach maximum expression at the end

of exponential growth (12) or in the early postexponential phase (5). Based on these facts, we set out to study the global cellular response of *B. subtilis* to α -amylase (AmyQ) overproduction and secretion stress during late exponential and stationary phases. In all our macroarray analyses, we observed upregulation of the known targets of the secretion stress regulon, *htrA* and *htrB* (Fig. 1; see Tables S1 and S2 in the supplemental material). This validates the quality of the obtained transcriptome data and agrees well with previously published results (6, 14). The comparison of the transcriptional patterns of the control strain and the AmyQ-overproducing strain at the late exponential phase of growth disclosed a rather modest response of *B. subtilis* to the applied stress. This result is in a good accordance with the studies of Hyyryläinen et al., where the induction of only seven genes upon high-level AmyQ secretion was found (15). However, our transcriptome analysis at the late exponential phase revealed the activation of a higher number of genes, which most likely results from differences in the experimental approaches, especially the sampling at the later phase of growth in our case (i.e., late exponential). In addition to the stimulation of the components of the CsrRS quality control system, several other genes were weakly induced. Most of the gene products are involved in the stress response evoked by protein misfolding/aggregation events in the cytoplasm, detoxification, and fatty acid metabolism, indicating that cells sensed and tried to counteract the adverse conditions.

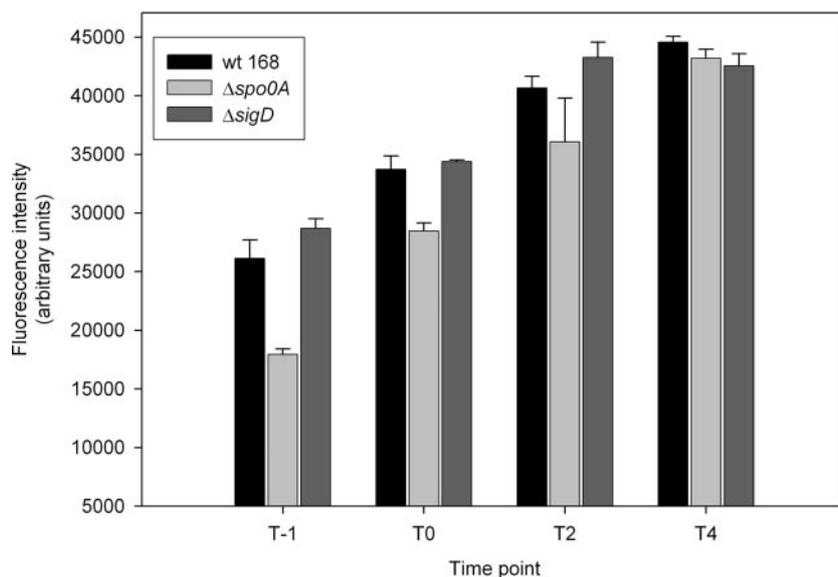


FIG. 4. Activity of secreted amylase from *B. subtilis* 168 wild-type (wt) and sporulation- and motility-deficient strains. Amylase activity was quantified in the culture medium of the wild-type strain, the *spo0A* mutant, and the *sigD* mutant. All strains were transformed with the pKTHM10 plasmid (AmyQ overproduction), and levels of activity of secreted AmyQ were determined at different time points of growth by using the EnzCheck Ultra amylase assay kit as described in Materials and Methods. T0 corresponds to the transition phase of growth. Error bars represent standard errors over three independent biological replicates.

Our data revealed the C_{ss}R-dependent regulation of several genes in response to AmyQ overproduction. However, for only two of the (putative) targets (*htrA* and *htrB*) could binding of the purified C_{ss}R protein to the DNA be demonstrated, indicating that C_{ss}R directly binds to and regulates expression of these operons. How can the apparent C_{ss}R-dependent expression of these genes then be explained? First, it is possible that some of the promoter regions tested by EMSA require additional cofactors in vivo for a proper binding of C_{ss}R. Second, phosphorylated C_{ss}R might bind different DNA sequences and with other affinities than those for unphosphorylated C_{ss}R. Since our EMSAs were carried out with unphosphorylated protein, we cannot exclude the possibility that in vivo some of the target genes are more strictly dependent on the phosphorylated form of C_{ss}R. Another explanation is that the observed effects are indirect and independent of C_{ss}R, for instance, via increased levels of HtrA, the activity of which was postulated to influence the regulatory effects of a two-component system in *Streptococcus pneumoniae* (37). Finally, data obtained from a *PykoJ-gfp* fusion at its native locus indicate that upregulation of this gene in response to protein overproduction is indeed C_{ss}R dependent but that promoter activity could not be demonstrated in an area of 500 bp upstream of the *ykoJ* open reading frame (30; R. Nijland, unpublished observations). The promoters of *htrA* and *htrB* are remarkably similar, making the in silico identification of a C_{ss}R binding motif very difficult. However, a Gibbs sampling method (38) identifies a CATT₅TTATC motif that is present in the 300-bp region upstream of both genes. In addition, close matches to this motif (GATTTTTTTC and CATTTTTTTC) can be identified 300 bp upstream of *citM*. The importance of this putative binding sequence remains to be established in future investigations.

The most striking outcome of the transcriptome analysis of

stationary-phase cells was the influence of protein overproduction on the processes of sporulation and motility. The effect on sporulation was much more pronounced at the later stages of growth, and reduced expression levels of many SigE, SigF, and SigG regulon members were observed in AmyQ-overproducing cells (Fig. 1). Interestingly, the level of *spo0E* transcript (phosphatase of Spo0A~P) in the AmyQ-overproducing cells is higher than in the wild type (pUB110), because of which the Spo0A~P pool might be drained. The inhibition of sporulation was confirmed by *gfp*-reporter experiments, and these analyses showed that sporulation is already blocked at the earliest stages (at the level of Spo0A~P) of amylase overproduction (Fig. 3). The differences between secretion stress and AmyQ production stress were examined using a nontranslocated AmyQ variant. This approach led us to conclude that the impaired-sporulation phenotype is most likely not an exclusive and direct effect of secretion stress but is rather caused by high-level cellular protein production and accumulation, possibly at the membrane. In fact, overproduction stress seems to inhibit sporulation more efficiently than secretion-specific stress (Fig. 3). It is plausible that the unprocessed AmyQ-Ala variant is still targeted to the Sec translocon, thereby leading to a kind of membrane congestion stress. This type of stress would account for the observed effect on sporulation and motility and would be less severe in the case of the wild-type AmyQ variant, which is translocated across the cytoplasmic membrane.

Sporulation and protein secretion are multistep and energy-consuming processes, and since protein production and secretion take place earlier in the life cycle of *B. subtilis* than endospore formation, they most likely inhibit the latter due to energy constraints. Our data suggest that *B. subtilis* cells sense a variety of environmental and intracellular signals under protein overproduction stress, which leads to the decision that

commencing energy-consuming spore development is not appropriate since it is highly unlikely that this process can be completed successfully.

Another survival strategy which *B. subtilis* employs as an adaptive response under unfavorable environmental conditions is motility. Remarkably, our transcriptome profiling revealed that a substantial part of the motility regulon was induced by high-level protein production and secretion. The alternative sigma factor σ^D orchestrates the transcription of many genes whose products are involved in flagellar assembly, motility, chemotaxis, and autolysis (11, 27). In a complex medium the amount of the σ^D protein increases during growth, reaching the maximum level at the transition point (28, 29). By single-cell flow-cytometric analysis, we show that AmyQ overproduction prolongs the motile phase of *B. subtilis* (Fig. 3). One of the explanations for this prolonged motile phase includes the putative replacement and competition of alternative sigma factors for core RNA polymerase (RNAP) during stationary phase, a phenomenon well documented for both *E. coli* (8, 26) and *B. subtilis* (13, 17, 24). The consecutive sporulation-specific sigma factors conduct developmental events during endospore formation (7). Since sporulation is blocked upon protein overproduction (Fig. 3), SigD does not have to compete with sporulation-specific sigma factors for free RNAP. However, it has been demonstrated that RNAP is present in excess in *B. subtilis* cells, and the engagement of anti-sigma factors or other mechanisms, which would give rise to deactivation of σ^A , could be conceived (9). An extended expression of the motility regulon can provide cells with a rescue machinery under the deficiency of sporulation, but, on the other hand, the members of yet uncharacterized σ^D -regulated genes could be also involved in helping cells to survive stress circumstances.

Our transcriptome results, combined with the single-cell analyses, predicted that both initiation of sporulation and motility play an important role in combating protein secretion stress. Indeed, when either *spo0A* or *sigD* was mutated, protein secretion was significantly affected (Fig. 4). It is demonstrated that the presence of a functional *spo0A* is required for efficient protein secretion, while the removal of *sigD* moderately but consistently increases secretion (Fig. 4). It has been shown before that a *sigD* mutation causes an increased extracellular accumulation of an artificial cell wall-binding lipase by an unknown mechanism (19). Likewise in our experiments, the secretion of enzymatically active AmyQ turned out to be enhanced in the *sigD* mutant, especially in earlier phases of growth, which renders this mutant strain a good host for efficient extracellular protein production (Fig. 4).

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