

A Novel Class of Heat and Secretion Stress-Responsive Genes Is Controlled by the Autoregulated C_{ss}RS Two-Component System of *Bacillus subtilis*

Elise Darmon,¹ David Noone,² Anne Masson,³ Sierd Bron,¹ Oscar P. Kuipers,¹ Kevin M. Devine,^{2*} and Jan Maarten van Dijl⁴

Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, 9751 NN Haren,¹ and Department of Pharmaceutical Biology, University of Groningen, 9713 AV Groningen,⁴ The Netherlands; Department of Genetics, Smurfit Institute, Trinity College, Dublin 2, Ireland²; and Institut de Génétique et Microbiologie, Université Paris-Sud, 91405 Orsay Cedex, France³

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Bacteria need dedicated systems that allow appropriate adaptation to the perpetual changes in their environments. In *Bacillus subtilis*, two HtrA-like proteases, HtrA and HtrB, play critical roles in the cellular response to secretion and heat stresses. Transcription of these genes is induced by the high-level production of a secreted protein or by a temperature upshift. The C_{ss}R-C_{ss}S two-component regulatory system plays an essential role in this transcriptional activation. Transcription of the *cssRS* operon is autoregulated and can be induced by secretion stress, by the absence of either HtrA or HtrB, and by heat stress in a HtrA null mutant strain. Two start sites are used for *cssRS* transcription, only one of which is responsive to heat and secretion stress. The divergently transcribed *htrB* and *cssRS* genes share a regulatory region through which their secretion and heat stress-induced expression is linked. This study shows that C_{ss}RS-regulated genes represent a novel class of heat-inducible genes, which is referred to as class V and currently includes two genes: *htrA* and *htrB*.

Since the conditions in natural environments are highly variable and unpredictable, eubacteria need systems that support their adaptation to perpetual changes. For this purpose, relevant stimuli must be sensed and identified by the cells and subsequently the resulting information must be transformed into appropriate transcriptional or behavioral responses.

In many cases, two-component systems are used for signal transduction (18, 24). The presence of multiple two-component systems is a prerequisite to adequately respond to diverse stimuli received by the cells. Such systems consist of a sensor histidine kinase and a cognate response regulator. In many cases, the sensor kinase is located in the cytoplasmic membrane. It is responsible for sensing environmental or nutritional stimuli and transferring this information to the second protein of the system through autophosphorylation and phosphotransfer reactions. When the cognate response regulator is phosphorylated, it either activates or represses the transcription of specific genes, thereby eliciting a cellular response appropriate to the original stimuli. The genome of the gram-positive bacterium *Bacillus subtilis* encodes 34 two-component systems (8). The functions of most of them have not yet been determined. However, one of these two-component systems, C_{ss}R-C_{ss}S (for “control of secretion stress regulator and sensor”) (11), has recently been reported to respond to secretion stress generated by overproduction of the α -amylase AmyQ of *Bacillus amyloliquefaciens*. The *cssR* gene and the downstream *cssS* gene form a bicistronic operon, but little is known about its regulation.

In *B. subtilis*, heat-inducible genes can be divided into four different classes on the basis of their regulatory mechanisms (5). The transcription of class I genes, which code for classical chaperones, is σ^A dependent and regulated by the HrcA repressor (14, 29, 31). The class II genes respond not only to heat but also to other stresses such as exposure to ethanol or salt, starvation for glucose or phosphate, or growth under anaerobic conditions. Transcription of these genes is regulated by σ^B (3, 10, 19). The class III genes also respond to general stress, but they are regulated by the CtsR transcriptional repressor (5, 6). All the heat-responsive genes that do not belong to one of these three classes have been collectively termed class IV genes (*ahpC*, *clpX*, *fisH*, *htpG*, *lonB*, *ykda*, *yvtA*, and *trxA*) (6).

The *B. subtilis* genome contains three genes encoding putative membrane-bound HtrA-like proteases: YkdA, YvtA, and YyxA (16, 25). Transcription of *ykda* and *yvtA* is inducible by heat stress or α -amylase overproduction, whereas *yyxA* is insensitive to these stimuli (15, 16). Furthermore, transcription of the *ykda* and *yvtA* genes is negatively auto- and cross-regulated. Because of their similarity to the HtrA protein of *Escherichia coli* and their induction upon heat stress, YkdA and YvtA are referred to as HtrA and HtrB, respectively (for “high-temperature requirement”). At present, *htrA* and *htrB* are listed as class IV heat stress responsive genes since their respective promoters have -10 regions (but not -35 regions) typical of σ^A -type promoters and lack both σ^B -type promoters and the consensus binding sites for HrcA or CtsR. Instead, the control regions of *htrA* and *htrB* have a fourfold-repeated octameric consensus sequence positioned in the vicinity of the -35 regions, suggesting a novel regulatory mechanism of expression (15). Interestingly, it has been established that the *htrA* gene is one of the targets of the C_{ss}RS two-component

* Corresponding author: Mailing address: Department of Genetics, Smurfit Institute, Trinity College, Dublin 2, Ireland. Phone (353)-1-6081872. Fax: (353)-1-6798558. E-mail: kdevine@tcd.ie.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	1
BFA3041	168 <i>htrB(yvtA)::pMutin4</i>	This study
BFA2461	168 <i>cssR(yvqA)::pMutin4</i>	11
BV2001	168 <i>cssS::Sp</i>	11
BV2006	168 <i>cssS::pMutin2</i>	11
BV2015	168 <i>cssS::Sp htrB(yvtA)::pMutin4</i>	This study
BV2016	168 <i>cssS::Sp amyE::P cssRS(yvqAB)-bgaB</i>	This study
BV2017	168 <i>htrA(ykdA)Δ439 amyE::P cssRS(yvqAB)-bgaB</i>	This study
BV2018	168 <i>cssS::Sp htrA(ykdA)Δ439 amyE::P cssRS(yvqAB)-bgaB</i>	This study
BV2019	168 <i>htrB(yvtA)::Km amyE::P cssRS(yvqAB)-bgaB</i>	This study
BV2020	168 <i>cssS::Sp htrB(yvtA)::Km amyE::P cssRS(yvqAB)-bgaB</i>	This study
BV2021	168 <i>htrA(ykdA)Δ439 htrB(yvtA)::Km amyE::P cssRS(yvqAB)-bgaB</i> (suppressed)	This study
BV2022	168 <i>cssS::Sp htrA(ykdA)Δ439 htrB(yvtA)::Km amyE::P cssRS(yvqAB)-bgaB</i> (suppressed)	This study
BV2023	168 <i>cssS::pMutin2 amyE::XcssS</i>	This study
BV2024	168 <i>cssS::Sp amyE::P htrA(ykdA)-bgaB</i>	This study
BV2025	168 <i>cssS::Sp amyE::P htrB(yvtA)-bgaB</i>	This study
DN2	168 <i>amyE::P htrA(ykdA)-bgaB</i>	16
DN26	168 <i>htrA(ykdA)Δ439</i>	16
DN110	168 <i>amyE::P htrB(yvtA)-bgaB</i>	15
DN111	168 <i>htrB(yvtA)::Km</i>	15
DN112	168 <i>htrB::kan amyE::P htrB(yvtA)-bgaB</i>	15
DN113	168 <i>htrAΔ439 amyE::P htrB(yvtA)-bgaB</i>	15
DN119	168 <i>htrAΔ439 amyE::P* htrB(yvtA)-bgaB</i>	This study
DN120	168 <i>htrB::kan amyE::P* htrB(yvtA)-bgaB</i>	This study
DN220	168 <i>amyE::P cssRS(yvqA/B)-bgaB</i>	This study
DN225	168 <i>amyE xylR::xylR-amyL P cssRS(yvqAB)::pDN221</i>	This study
DN226	168 <i>amyE xylR::xylR-amyL cssRS(yvqA)::pMutin4</i>	This study
DN227	168 <i>P cssRS(yvqAB)::pDN221</i>	This study
DN228	168 <i>amyE xylR::xylR-amyL *P cssRS(yvqAB)::pDN222</i>	This study
KS408	168 <i>amyE xylR::xylR-amyL</i>	23
<i>E. coli</i>		
MC1061	F ⁻ <i>araD139 Δ(ara-leu)7696 Δ(lac)X74 galU galK hsdR2 mcrA mcrB1 rspL</i>	28
TG-1	<i>supE hsdΔ 5 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^a lacZΔM15]</i>	22

system (11). However, a comparison of the phenotypes of *cssS* and *htrA* mutant strains indicated that at least one other protease is controlled by C_{ss}RS.

The present studies were aimed at the identification of additional C_{ss}RS-controlled genes and the establishment of a possible dual role of C_{ss}RS in heat and secretion stress responses. The results show that expression of *htrA*, *htrB*, *cssR*, and *cssS* is responsive to secretion stress in a manner dependent on the C_{ss}RS two-component system. In addition, the induction of *htrA* and *htrB* expression by heat stress in a wild-type background is C_{ss}RS dependent, signifying that this is a new class of heat-inducible genes, termed class V.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. Tables 1 and 2 list the bacterial strains and plasmids, respectively, used in this study. Bacteria were grown in TY medium (1% tryptone, 0.5% yeast extract, 1.0% NaCl). Minimal medium was prepared as described by Leskela et al. (13). Antibiotics were used in the following concentrations: ampicillin, 50 μg/ml; erythromycin, 1 μg/ml; kanamycin, 10 μg/ml; spectinomycin, 100 μg/ml; chloramphenicol, 5 μg/ml. To visualize α-amylase activity, TY plates were supplemented with 1% starch.

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described by Sambrook et al. (22). *B. subtilis* was transformed as described by Leskela et al. (13). PCR was carried out as described by van Dijk et al. (27) using chromosomal DNA of *B. subtilis* 168 as a template. The nucleotide sequences of primers used for PCR are listed below; restriction sites used for cloning are underlined. Enzymes were from Roche Molecular Biochemicals (Mannheim, Germany). Constructs were first made in *E. coli* MC1061 or TG-1 and then introduced into *B. subtilis*.

To construct the *htrB::pMutin4* mutation and the transcriptional *htrB-lacZ* fusion, an internal fragment of the *htrB* (*yvtA*) gene was amplified by PCR with the oligonucleotides *yvtAH3* (5'-GGC CAA GCT TCA ACA TCA AAC TGA ACC-3') and *yvtAB1* (5'-GGC CGG ATC CAC AGC CGT TTC TTG C-3'). The amplified fragment (180 nucleotides) was cloned into plasmid pMutin4, using *Bam*HI and *Hind*III. The *B. subtilis* 168 *htrB::pMutin4* (BFA3041) strain was obtained by Campbell-type (single-crossover) integration of the resulting plasmid into the chromosome of *B. subtilis* 168. This mutant was verified by Southern hybridization. The *B. subtilis* 168 *cssS::Sp htrB::pMutin4* (BV2015), *B. subtilis* 168 *cssS::Sp amyE::PhtrA-bgaB* (BV2024), and *B. subtilis* 168 *cssS::Sp amyE::PhtrB-bgaB* (BV2025) strains were, respectively, constructed by transformation of *B. subtilis* 168 *htrB::pMutin4* (BFA3041), *B. subtilis* 168 *amyE::PhtrA-bgaB* (DN2), and *B. subtilis* 168 *amyE::PhtrB-bgaB* (DN110) with chromosomal DNA of *B. subtilis* 168 *cssS::Sp* (BV2001) and selection for spectinomycin resistance.

To construct plasmid pDN220, the *cssRS* promoter region was amplified by PCR with the oligonucleotides YVQAPF (5'-CCG GAA TTC GTT CTT ACA CTC CTT AAC G-3') and YVQAPR (5'-CGG GAT CCG CAG TTC ATT

TABLE 2. Plasmids used in this study

Plasmid	Characteristics	Reference
pUB110	Km ^r	9
pKTH10	pUB110 derivative; encodes the α-amylase (AmyQ) of <i>B. amyloliquefaciens</i> ; Km ^r	17
pKTH10L	pKTH10 derivative; Km ^r	11
pMutin4	pBR322-based integration vector for <i>B. subtilis</i> ; contains a multiple-cloning site downstream of the Pspac promoter and a promoterless <i>lacZ</i> gene preceded by the RBS ^a of the <i>spoVG</i> gene; Ap ^r /Em ^r	26
pDL	Vector for the integration of transcriptional promoter- <i>bgaB</i> gene fusions in the <i>amyE</i> locus of <i>B. subtilis</i> ; Ap ^r Cm ^r	30
pDN220	pDL containing the complete <i>cssR-csS</i> control region on a PCR-amplified fragment; Ap ^r Cm ^r	This study
pDN221	pMutin4 containing the complete <i>cssR-csS</i> control region on a PCR-amplified fragment; Ap ^r Em ^r	This study
pDN222	pMutin4 containing the complete <i>cssR-csS</i> control region, with the A → G mutation at position -60 relative to the start codon of <i>cssR</i> ; Ap ^r Em ^r	This study
pDN223	pDL containing the <i>htrB</i> control region with the T → C change at position -218 relative to the start codon of <i>htrB</i> ; Ap ^r Cm ^r	This study
pX	Vector for the integration of genes in the <i>amyE</i> locus; the integrated gene will be transcribed from the <i>xylA</i> promoter; carries the <i>xylR</i> gene; Ap ^r Cm ^r	12
pXcssS	pX derivative; carries <i>cssS</i> downstream of the <i>xylA</i> promoter; Ap ^r Cm ^r	This study

^a RBS, ribosome-binding site

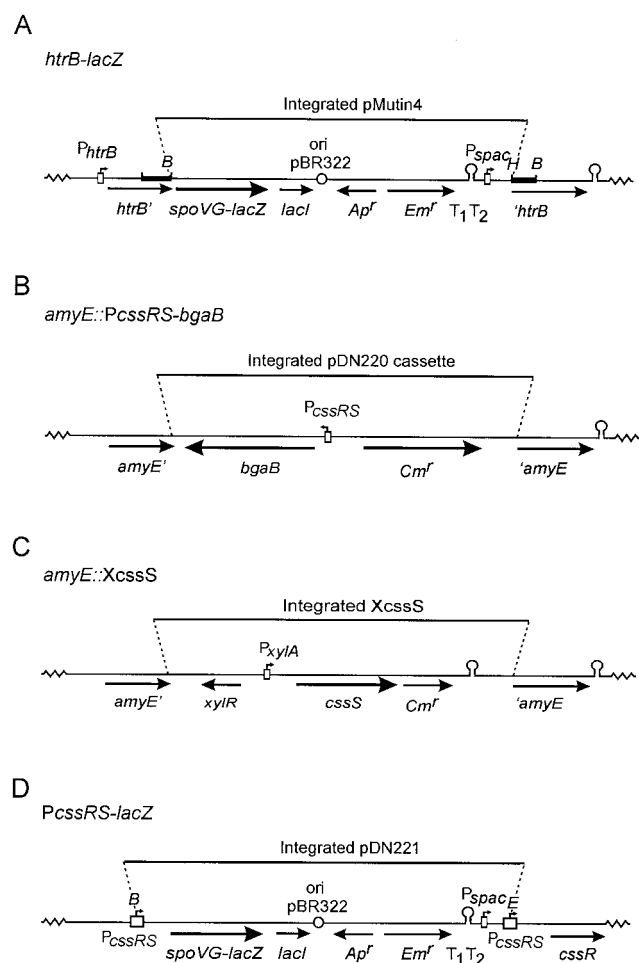


FIG. 1. Construction of mutant strains. (A) Schematic presentation of the *htrB*::pMutin4 mutation. The *htrB* gene was disrupted with pMutin4 by a single-crossover event (Campbell-type integration). Simultaneously, the *spoVG-lacZ* reporter gene of pMutin4 was placed under the transcriptional control of the *htrB* promoter region (*P_{htrB}*). The chromosomal fragment from the *htrB* region, which was amplified by PCR and cloned into pMutin4, is indicated by black bars. Only the restriction sites relevant for the construction are shown (*B*, *Bam*HI; *H*, *Hind*III). *lacI*, *E. coli lacI* gene; ori pBR322, replication functions of pBR322; *Ap^r*, ampicillin resistance marker; *Em^r*, erythromycin resistance marker; *T₁T₂*, transcriptional terminators on pMutin4; *P_{spac}*, isopropyl- β -D-thiogalactopyranoside (IPTG)-dependent promoter; *htrB'*, 3'-truncated *htrB* gene; *'htrB*, 5'-truncated *htrB* gene. (B) Schematic presentation of the *amyE* region of the chromosome of strains containing an *amyE*::PcssRS-*bgaB* mutation. By a double-crossover event, the *amyE* gene was disrupted with a pDN220-derived cassette containing the *bgaB* reporter gene placed under the transcriptional control of the *cssRS* promoter region (*P_{cssRS}*); *Cm^r*, chloramphenicol resistance marker; *amyE'*, 3' truncated *amyE* gene; *'amyE*, 5' truncated *amyE* gene. (C) Schematic presentation of the *amyE* region of the chromosome of strains containing the *amyE*::XcssS mutation. By a double-crossover event, the *amyE* gene was disrupted with a pXcssS-derived cassette (XcssS), which contains the *cssS* gene placed under the transcriptional control of a xylose-inducible promoter (*P_{xylA}*). *Cm^r*, chloramphenicol resistance marker; *xylR*, gene specifying the XylR repressor protein; *amyE'*, 3' truncated *amyE* gene; *'amyE*, 5' truncated *amyE* gene. (D) Schematic presentation of the *PcssRS*::pDN221 reporter strain. The promoter region of the *cssRS* genes was duplicated by the insertion of pDN221 into the chromosome via a single-crossover event (Campbell-type integration). Consequently, both the *cssRS* operon and the *spoVG-lacZ* reporter gene of pDN221 are placed under the transcriptional control of a *cssRS* promoter region (*P_{cssRS}*). Only the restriction sites relevant for the construction are shown (*B*, *Bam*HI; *E*,

CAG GTT ATC C-3'). The amplified fragment (337 nucleotides) was cloned into plasmid pDL, using *Eco*RI and *Bam*HI. To determine the *P_{cssRS}* promoter activity in different backgrounds, the strains *B. subtilis* 168 *amyE*::PcssRS-*bgaB* (DN220), *B. subtilis* 168 *htrA* Δ 439 *amyE*::PcssRS-*bgaB* (BV2017), *B. subtilis* 168 *htrB*::Km *amyE*::PcssRS-*bgaB* (BV2019), and *B. subtilis* 168 *cssS*::Sp *amyE*::PcssRS-*bgaB* (BV2016) were, respectively, constructed by transformation of *B. subtilis* 168, *B. subtilis* 168 *htrA* Δ 439 (DN26), *B. subtilis* 168 *htrB*::Km (DN111), and *B. subtilis* 168 *cssS*::Sp (BV2001) with plasmid pDN220, selection for chloramphenicol resistance, and screening for an *AmyE*⁻ phenotype. Two of these strains, BV2017 (*htrA*) and BV2019 (*htrB*), were transformed with chromosomal DNA of the BV2001 (*cssS*) strain; selection for spectinomycin resistance resulted in *B. subtilis* 168 *cssS*::Sp *htrA* Δ 439 *amyE*::PcssRS-*bgaB* (BV2018) and *B. subtilis* 168 *cssS*::Sp *htrB*::Km *amyE*::PcssRS-*bgaB* (BV2020). Strain BV2017 (*htrA*) was also transformed with chromosomal DNA from the DN111 strain (*htrB*); selection for kanamycin resistance resulted in the strain *B. subtilis* 168 *htrA* Δ 439 *htrB*::Km *amyE*::PcssRS-*bgaB* (BV2021; suppressed). Finally, BV2021 (*htrA* *htrB*; suppressed) was transformed with chromosomal DNA of the BV2001 (*cssS*) strain; selection for spectinomycin resistance resulted in *B. subtilis* 168 *cssS*::Sp *htrA* Δ 439 *htrB*::Km *amyE*::PcssRS-*bgaB* (BV2022; suppressed).

To construct plasmid pDN221, the *cssRS* promoter region was amplified by PCR with the oligonucleotides YVQAPF and YVQAPR. The amplified fragment was cloned into plasmid pMutin4, using *Eco*RI and *Bam*HI. Strain *B. subtilis* 168 *PcssRS*::pDN221 (DN227) was generated by transforming *B. subtilis* 168 with plasmid pDN221 and selection for erythromycin resistance. Plasmid pDN222 is identical to plasmid pDN221 except for the introduction of a single PCR-generated A-to-G base change at bp -60 relative to the TTG start codon of *cssR*. Strain DN228 was constructed by transforming *B. subtilis* 168 *amyE xylR*::*xylR-amyL* (KS408) with plasmid pDN222 and selecting for erythromycin resistance. Plasmid pDN223 was constructed by PCR amplifying the insert of plasmid pDN222 with primers YVTAPF and YVTAPR (15) and cloning the product into pDL as an *Eco*RI-*Bam*HI fragment. Strains DN119 and DN120 were generated by transforming strains DN26 (*htrA*) and DN111 (*htrB*), respectively with plasmid pDN223 with selection for chloramphenicol resistance. Strain *B. subtilis* 168 *amyE xylR*::*xylR-amyL PcssRS*::pDN221 (DN225) was created by transformation of KS408 with chromosomal DNA from strain DN227 and selection for erythromycin resistance. Strain *B. subtilis* 168 *amyE xylR*::*xylR-amyL cssRS*::pMutin4 (DN226) was generated by transformation of strain KS408 with chromosomal DNA from strain *B. subtilis* 168 *cssRS*::pMutin4 (BFA2461) and selection for erythromycin resistance.

To construct a strain with a xylose-inducible *cssS* gene, the complete *cssS* region was amplified with the oligonucleotides *cssS3* (5'-GCT CTA GAA TTG CCG TCT CCT CGT ATC G-3') and *cssS4* (5'-CGC GGA TCC AGC AGA CCT TGT CAG AGA A-3'). The amplified fragment (1,755 nucleotides) was cleaved with *Xba*I and *Bam*HI and ligated into the *Spe*I and *Bam*HI sites of plasmid pX, resulting in pXcssS. The mutant *B. subtilis* 168 *cssS*::pMutin2 *amyE*::XcssS (BV2023) was obtained by a double-crossover integration of the XcssS cassette from plasmid pXcssS into the *amyE* locus of *B. subtilis* 168 *cssS*::pMutin2 (BV2006); chloramphenicol-resistant transformants were screened for an *AmyE*⁻ phenotype.

β -Galactosidase activity assay. To assay β -galactosidase activities, overnight cultures were diluted in fresh medium and samples were taken at different intervals for optical density readings at 600 nm (*OD*₆₀₀) and β -galactosidase activity determinations. For strains containing a transcriptional *lacZ* fusion, the β -galactosidase assay and the calculation of β -galactosidase units (Miller units: nanomoles per *OD*₆₀₀ unit per minute) were performed as described by Hyyryläinen et al. (11). To investigate the effects of heat stress, cells containing a transcriptional *bgaB* fusion were first grown at 37°C to an *OD*₆₀₀ of 0.3 to 0.4 and then divided into two cultures: one remained at 37°C, while the other was transferred to a prewarmed flask and incubated at 48°C. To assay BgaB activity, the LacZ activity assay was used with minor modifications: cell lysis was followed by an incubation at 70°C for 15 min and the subsequent β -galactosidase activity assays were performed at 55°C. Experiments were repeated at least twice, starting with independently obtained transformants. In all experiments, the relevant controls were performed in parallel. Although some differences were observed in the absolute β -galactosidase activities, the ratios between these activities in the

*Eco*RI). *lacI*, *E. coli lacI* gene; ori pBR322, replication functions of pBR322; *Ap^r*, ampicillin resistance marker; *Em^r*, erythromycin resistance marker; *T₁T₂*, transcriptional terminators on pDN221; *P_{spac}*, IPTG-dependent promoter.

various strains tested were largely constant. A ratio of about 1.5 was generally reproducible. Differences in absolute β -galactosidase levels reported in this paper and those reported by Noone et al. (15, 16) are due to different methods for calculating specific activities: in this study β -galactosidase units are calculated as nanomoles per minute per OD₆₀₀ unit, and in references 15 and 16 they were calculated as as nanomoles per minute per milligram of protein.

Transcriptional analysis. For the analysis of the effects of heat stress, total RNA was isolated from *B. subtilis* 168 *htrA* Δ 439 (DN26) at various times before and after heat stress at 48°C. For the analysis of the effects of secretion stress, total RNA isolated at different time points from *B. subtilis* 168 *xylR::xylR-amyL* (KS408), grown in TY medium with or without 1% xylose, was used as a template for primer extension. ³²P-radiolabeled primers YVQA-RT1 (5'-CTA GAT AAA TGG TGT ATG ACA AGG C-3') and YVQA-RT2 (5'-GAT GTA ATG TTC CAG CCC-3') were annealed to 25 μ g of total RNA, and primer extension analysis was carried out as previously described (16).

RESULTS

Transcription of *htrB* is *CssS* dependent. Since the transcription of *htrB* responds to the same stimuli as the *CssRS*-dependent transcription of *htrA*, a transcriptional *htrB-lacZ* fusion (Fig. 1A) was used to investigate a possible role of *CssS* in the regulation of *htrB*. In view of the negative autoregulation of *htrB*, it is important to note that the chromosomal pMutin4 insertion used to obtain the *htrB-lacZ* fusion results in the inactivation of this gene. The β -galactosidase activities of *B. subtilis* 168 *htrB::pMutin4* (BFA3041) and the *B. subtilis* 168 *cssS::Sp htrB::pMutin4* strain (BV2015) were measured as a function of growth in TY medium at 37°C. As shown in Fig. 2A, the *htrB* transcription peaked at about 1 h before the transition ($t = 0$) between exponential and postexponential growth. The disruption of *cssS* reduced *htrB-lacZ* transcription more than threefold and significantly changed the expression profile. To test whether the previously reported induction of *htrB* by α -amylase overproduction (15) is *CssS* dependent, plasmid pKTH10L was introduced into the *htrB-lacZ* strains. This plasmid imposes secretion stress on the cells by overexpression of the α -amylase AmyQ (11). The presence of pKTH10L resulted in a more than eightfold increase in *htrB* transcription in the *cssS*⁺ background without affecting the expression profile (Fig. 2B). In contrast, a strain containing the control vector pUB110 did not affect *htrB* transcription (data not shown). The disruption of *cssS* reduced *htrB-lacZ* transcription in cells subject to AmyQ secretion stress to a level similar to that observed in nonstressed cells (Fig. 2B). Remarkably, the *cssS* disruption did not reduce *htrB-lacZ* transcription to background levels. Essentially the same effects were observed when cells were grown in minimal medium (data not shown). Disruption of *cssS* in the *htrB* mutant strain containing pKTH10L caused a reduced growth rate during the exponential phase and a reduced cell density in the postexponential phase of growth. For example, *cssS htrB* mutant cells containing pKTH10L reached an OD₆₀₀ of about 2, while control cells containing pUB110 reached an OD₆₀₀ of about 5 (data not shown). These observations demonstrate that both the basal level of transcription and secretion stress induction of *htrB* are *CssS* dependent.

***CssS* is essential for the heat stress induction of *htrA* and *htrB*.** To study whether the *CssRS* system is involved in the increased transcription of *htrA* and *htrB* in response to heat stress, the activities of their respective promoters were monitored using transcriptional fusions with the *bgaB* reporter gene

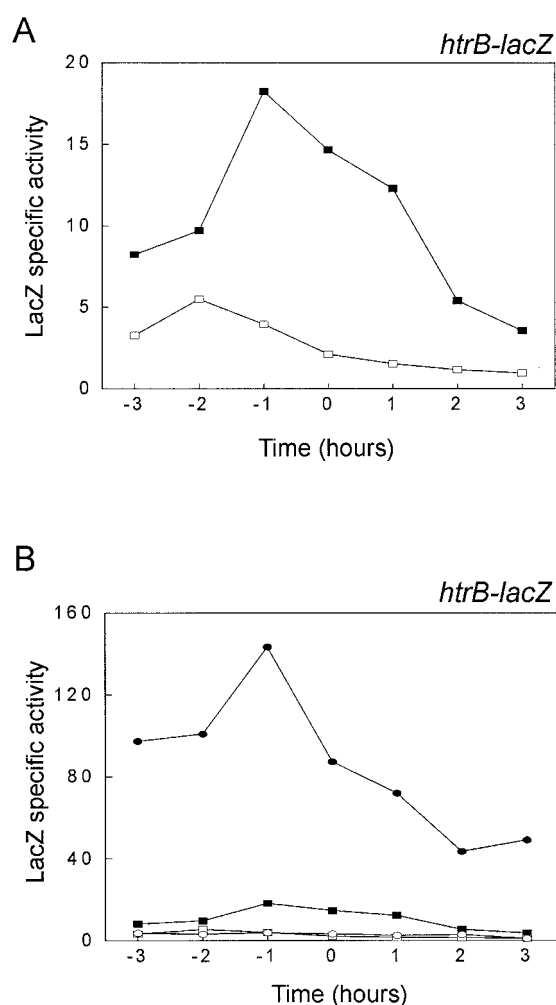


FIG. 2. Analysis of *htrB* expression in a *cssS* mutant background. The *CssS* dependence of *htrB-lacZ* transcription was analyzed in the absence (A) or presence (B) of pKTH10L-induced secretion stress. The transcriptional *htrB-lacZ* gene fusion schematically shown in Fig. 1A was used to determine the time courses of *htrB* expression in cells grown at 37°C in TY medium. The strains used for the analyses were *B. subtilis* 168 *htrB::pMutin4* (BFA3041; solid rectangles), *B. subtilis* 168 *cssS::Sp htrB::pMutin4* (BV2015; open rectangles), *B. subtilis* 168 *htrB::pMutin4* containing pKTH10L (solid ellipses), and *B. subtilis* 168 *cssS::Sp htrB::pMutin4* containing pKTH10L (open ellipses). Samples for the determination of β -galactosidase activities (indicated in nanomoles per minute per OD₆₀₀ unit) were withdrawn at the times indicated. Zero time ($t = 0$) indicates the transition point between the exponential and postexponential growth phases. The parental strain *B. subtilis* 168 was used as a negative control (data not shown).

(which encodes a thermostable β -galactosidase). To avoid interference with normal HtrA and HtrB functions, the promoter-*bgaB* fusions were positioned at the *amyE* locus of the *B. subtilis* chromosome. This resulted in *B. subtilis* 168 *amyE::PhrA-bgaB* (DN2) and *B. subtilis* 168 *amyE::PhrB-bgaB* (DN110). Subsequent disruption of *cssS* in these strains resulted in *B. subtilis* BV2024 and *B. subtilis* BV2025, respectively. To analyze the transcription of *htrA-bgaB* and *htrB-bgaB* in response to heat stress, the four strains were first grown in TY medium at 37°C to an OD₆₀₀ of 0.3 to 0.4. Subsequently, half of the culture was incubated at 48°C and half remained at

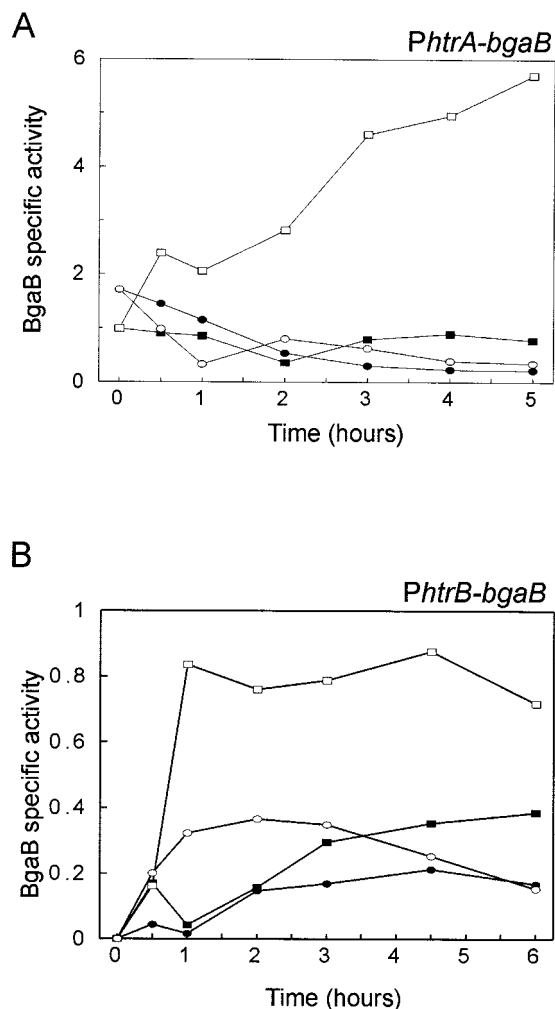


FIG. 3. Analysis of *htrA* and *htrB* transcription under heat shock conditions. Transcriptional *PhtrA-bgaB* and *PhtrB-bgaB* promoter-gene fusions in the *amyE* locus were used to determine the time courses of *htrA* and *htrB* promoter activity in cells grown in TY medium. The cells were first grown at 37°C to an OD₆₀₀ of 0.3 to 0.4 and then divided into two cultures: one culture remained at 37°C, and the other was transferred to 48°C. Samples for the determination of β -galactosidase (BgaB) activities (indicated in nanomoles per minute per OD₆₀₀ unit) were withdrawn as a function of time after the temperature shift ($t = 0$). The parental strain *B. subtilis* 168 was used as a negative control (data not shown). (A) *B. subtilis* 168 *amyE::PhtrA-bgaB* (DN2) at 37°C (solid rectangles) and at 48°C (open rectangles). *B. subtilis* 168 *cssS::Sp amyE::PhtrA-bgaB* (BV2024) at 37°C (solid ellipses) and at 48°C (open ellipses). (B) *B. subtilis* 168 *amyE::PhtrB-bgaB* (DN110) at 37°C (solid rectangles) and at 48°C (open rectangles). *B. subtilis* 168 *cssS::Sp amyE::PhtrB-bgaB* (BV2025) at 37°C (solid ellipses) and at 48°C (open ellipses).

37°C. Samples for β -galactosidase measurements were withdrawn from the cultures at specified times after the temperature shift ($t = 0$). As shown in Figure 3A, *htrA-bgaB* transcription in the *cssS*⁺ background was constant at a low level at 37°C but increased progressively after the temperature upshift to 48°C. When *cssS* was disrupted, the *htrA-bgaB* transcription was no longer induced by heat stress (Fig. 3B, 37 and 48°C), showing the critical role of *CssS* in this process. The *htrB-bgaB* transcription was low at 37°C (Fig. 3B). In contrast to *htrA-*

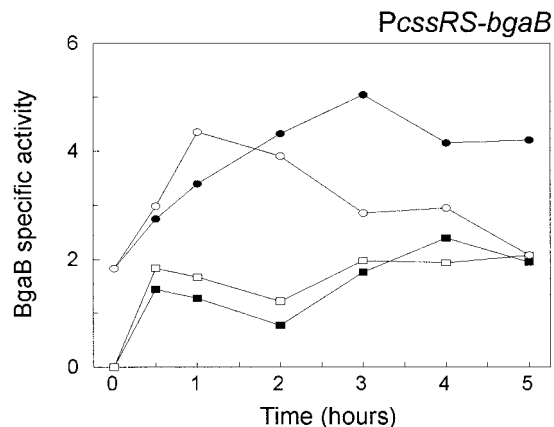


FIG. 4. Analysis of *cssRS* expression under various stress conditions. A transcriptional *bgaB* gene fusion in the *amyE* locus was used to determine the time course of the *PcssRS* activity in cells grown in TY medium. *B. subtilis* 168 *amyE::PcssRS-bgaB* (DN220) was first grown at 37°C to an OD₆₀₀ of 0.3 to 0.4 and then divided into two cultures: one remained at 37°C (solid rectangles), and the other was transferred to 48°C (open rectangles). Similarly, cultures of this strain containing pKTH10 were incubated at 37°C (solid ellipses) or 48°C (open ellipses). Samples for the determination of β -galactosidase (BgaB) activities (indicated in nanomoles per minute per OD₆₀₀ unit) were withdrawn as a function of time after the temperature shift ($t = 0$). The parental strain *B. subtilis* 168 was used as a negative control (data not shown).

bgaB, the *htrB-bgaB* transcription increased rapidly after heat stress (open rectangles) and then remained constant. Furthermore, when *cssS* was mutated, the effect of the heat stress on *htrB-bgaB* transcription was still detectable but was significantly reduced (Fig. 3B, 37 and 48°C). It was established previously that *htrA* expression is not induced by puromycin (16). Similar experiments have demonstrated that puromycin addition has no effect on *htrB* expression (data not presented). These findings indicate that heat stress induction of both *htrA* and *htrB* is controlled by the *CssRS* system. The inducing signal for *htrA* and *htrB* expression is probably not misfolded cytosolic proteins, since expression of neither gene is induced by puromycin addition.

Response of the *cssR-cssS* operon to heat and secretion stress. To investigate whether the transcription of the *cssRS* operon responds to heat stress and secretion stress, a fusion between the promoter region of this operon and the *bgaB* reporter gene was introduced into the *amyE* locus of the *B. subtilis* 168 chromosome, resulting in strain DN220 (Fig. 1B). The *cssRS* promoter activity was determined in TY medium at 37 and 48°C, as described in the previous section for the *PhtrA-bgaB* and *PhtrB-bgaB* constructs. At 37 and 48°C, the activity of the *cssRS* promoter was low and constant, with little difference in expression between the two temperatures (Fig. 4). Therefore, *cssRS* expression appeared to be insensitive to heat stress at 48°C. In contrast, secretion stress caused by the overproduction of AmyQ, encoded by plasmid pKTH10, resulted in a more than twofold increase of *PcssRS-bgaB* transcription at 37°C. An elevated level of *PcssRS-bgaB* transcription was observed at 48°C in the presence of high α -amylase levels. However, on reaching a maximum at approximately 1 h after the temperature upshift, *PcssRS-bgaB* transcription started to de-

TABLE 3. Expression of transcriptional fusions between the *cssR-cssS* promoter region and the *bgaB* reporter gene in various genetics background at 37 and 48°C

Background strain	Presence of:			BgaB activity ^a at:	
	<i>htrA</i>	<i>htrB</i>	<i>cssS</i>	37°C	48°C
DN220	+	+	+	1.2	1.8
BV2017	-	+	+	3.6	6.9
BV2019	+	-	+	2.6	3.9
BV2021; suppressed	-	-	+	3.7	8.2
BV2016	+	+	-	0.8	1.0
BV2018	-	+	-	0.6	0.7
BV2020	+	-	-	1.6	1.5
BV2022; suppressed	-	-	-	0.8	1.1

^a All values represent BgaB activities that were determined 1 h after the cultures were split. One activity unit is defined as 1 nmol of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per min per OD₆₀₀ unit. All values represent the average of two independent experiments.

crease slowly, reaching the level of the control strain 4 to 5 h after heat induction. Thus, it is apparent that the transcription of *cssRS* is upregulated in response to secretion stress. In contrast, under the conditions tested, *cssRS* transcription is not responsive to heat stress in the absence of secretion stress.

Effect of *htrA* and *htrB* mutations on *cssRS* transcription at 37°C. Since HtrA and HtrB are involved in a negative autoregulation and reciprocal cross-regulation of their own genes (15), the possible influences of *htrA* and *htrB* mutations on *cssRS* transcription were investigated. For this purpose, the transcriptional *PcssRS-bgaB* promoter-gene fusion was introduced into the *amyE* locus of *B. subtilis* 168 *htrA* Δ 439 (DN26) and *B. subtilis* 168 *htrB*::Km (DN111), resulting in strains BV2017 and BV2019, respectively. In addition, a strain (BV2021) containing mutations in both *htrA* and *htrB* and also harboring the *PcssRS-bgaB* fusion was constructed. Such *htrA-htrB* double-mutant strains are severely impaired in growth and rapidly accumulate suppressor mutations (15). Therefore, strain BV2021 used in the present studies carried an uncharacterized suppressor mutation that affected growth. The results summarized in Table 3 show the expression levels that persist in each strain at the midexponential phase of the growth cycle in TY medium. Compared to the *htrA*⁺ *htrB*⁺ *cssS*⁺ control strain, the transcription level of the *cssRS* operon was increased threefold in the *htrA* single mutant and twofold in the *htrB* single mutant when cells were grown at 37°C. At this temperature, the *cssRS* transcription in the *htrA htrB* double mutant was similar to that in the *htrA* single mutant, showing that under these conditions, the effects of the mutations were not additive. On closer examination, the transcription of the *cssRS* operon in the *htrA htrB* double mutant (BV2021; suppressed) increased during exponential growth and reached its maximum in the postexponential phase (Fig. 5, transition point around 3 h). At that point, the *cssRS* expression in the *htrA htrB* double mutant was about fivefold higher than that in the *htrA*⁺ *htrB*⁺ control strain (DN220). Interestingly, the profile of the *cssRS* expression in the *htrA htrB* double mutant was nearly identical to that observed for the *htrA* single mutant (data not shown). However, in contrast to the midexponential growth phase (Table 3, 37°C), the level of *cssRS* expression in

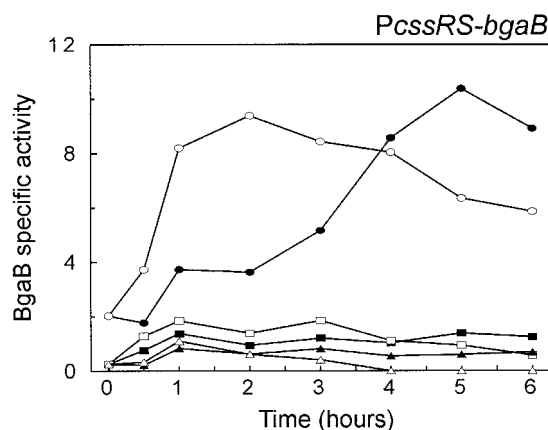


FIG. 5. Analysis of *cssRS* operon expression in the *htrA-htrB* double mutant. The transcriptional *PcssRS-bgaB* gene fusion in the *amyE* locus was used to determine the time courses of the *cssR-cssS* operon expression in cells grown in TY medium. The cells were first grown at 37°C to an OD₆₀₀ of 0.3 to 0.4 and then divided into two cultures: one remained at 37°C, and the other was transferred to 48°C. The strains used for the analyses were *B. subtilis* 168 *amyE*::*PcssRS-bgaB* (DN220) at 37°C (solid rectangles) and 48°C (open rectangles), *B. subtilis* 168 *htrA* Δ 439 *htrB*::Km *amyE*::*PcssRS-bgaB* (BV2021; suppressed) at 37°C (solid ellipses) and 48°C (open ellipses), and *B. subtilis* 168 *cssS*::Sp *htrA* Δ 439 *htrB*::Km *amyE*::*PcssRS-bgaB* (BV2022; suppressed) at 37°C (solid triangles) and 48°C (open triangles). β -Galactosidase (BgaB) activities are indicated in nanomoles per minute per OD₆₀₀ unit. Zero time ($t = 0$) indicates the time point of the heat shock. The parental strain 168 was used as a negative control (data not shown).

the postexponential phase was about twofold higher in the double mutant than in the *htrA* single mutant (data not shown).

Effect of *htrA* and *htrB* mutations on *cssRS* transcription under heat stress conditions. Expression of *htrA* and *htrB* are both upregulated by heat stress while that of *cssRS* is elevated in the absence of HtrA and/or HtrB. Since expression of *cssRS* in the wild-type (*htrA*⁺ *htrB*⁺) background was insensitive to heat stress, the effects of heat stress on *cssRS* expression were examined in *htrA* and *htrB* mutant backgrounds. Table 3 shows the *cssRS* transcription levels determined at 37°C and 1 h after a temperature upshift to 48°C. The response of *PcssRS-bgaB* expression to heat stress differed, depending on the genetic background. In the *htrA*⁺ *htrB*⁺ *cssRS*⁺ control strain (DN220) and in the *htrB* single mutant (BV2019), there was little difference in *cssRS* transcription at 37 and 48°C. However, there was an approximately twofold difference in *cssRS* expression levels in the *htrA* single mutant (BV2017) and in the *htrA htrB* double mutant (BV2021; suppressed). Notably, at 48°C the double mutant grew to a lower OD₆₀₀ (less than 2) than did the single mutants or the parental strain (around 5 [data not shown]). As shown in Fig. 5, the transcription of *cssRS* in the *htrA htrB* background increased rapidly for 1 h after heat stress and decreased slowly after 2 h. The maximal levels of *cssRS* transcription were reached much earlier after heat stress than at 37°C. Since the transcription of *cssRS* in the *htrA* or *htrB* single mutants was lower than that in the double mutant at 48°C, it seems that mutations in both *htrA* and *htrB* have an additive effect on *cssRS* transcription after heat stress (Table 3 and data not shown).

Transcription of *cssRS* is affected by levels of CssR, CssS,

HtrA, HtrB, and secretory proteins. To investigate whether expression of the *cssRS* operon is subject to autoregulation, the *cssS* gene was disrupted in the parental strain and in a series of *htrA/B* mutants generating the *cssS* (BV2016) single mutant, the *htrA cssS* (BV2018) and *htrB cssS* (BV2020) double mutants, and the *htrA htrB cssS* (BV2022; suppressed) triple mutant. Expression of the *cssRS* operon was measured with the *bgaB* fusion as described in the previous paragraphs. Interestingly, many of these strains did not grow normally at 48°C. The *cssS htrA* double mutant (BV2018) stopped growing at an OD₆₀₀ of about 3 instead of 5, the *cssS htrB* double mutant (BV2020) ceased growth at an OD₆₀₀ of approximately 2, while the *cssS htrA htrB* triple mutant (BV2022; suppressed) ceased growth at an OD₆₀₀ of below 2. Table 3 shows the expression levels of the *cssRS* operon in these mutant strains at 37°C and after 1 h of heat stress at 48°C. Expression of *cssRS* was very marginally affected by mutation of *cssS* at 37°C (1.2 U reduced to 0.8 U) and at 48°C (1.8 U reduced to 1 U). The strongest effect was observed in the *htrA* mutant background, where the level of *cssRS* expression decreased approximately 10-fold (6.9 U reduced to 0.7 U) at 48°C and 6-fold (3.6 U reduced to 0.6 U) at 37°C compared with the levels in the *cssS*⁺ background. Mutation of *cssS* in the *htrB* background had a milder effect (reduced approximately twofold) on *PcssRS-bgaB* expression. However as shown in Fig. 5, transcription of *PcssRS-bgaB* in the *htrA htrB* double mutant dropped to a low level in the absence of *cssS*.

To investigate whether the response of *cssRS* transcription to secretion stress is dependent on the C_{ss}R_S system, a transcriptional *cssR-lacZ* fusion was constructed by insertion of pMutin4 into the *cssR* gene of *B. subtilis* KS408, which contains a xylose-inducible gene for the α -amylase AmyL of *Bacillus licheniformis* in the *xyIR* locus. The resulting *cssR* mutant strain DN226 was grown in TY medium at 37°C in the presence or absence of 1% xylose. In contrast to the *cssR*⁺ parental strain, the induction of *amyL* did not result in increased *cssR* transcription in the *cssR* mutant background (data not shown). In fact, the *cssR* transcription was about 10-fold reduced compared to that of the non-secretion-stressed *cssR*⁺ control strain (data not shown). Taken together, these data show that the C_{ss}R_S system regulates its own expression in response to secretion stress. Under conditions of heat stress, C_{ss}R_S has little effect on *cssRS* expression in a wild-type background, but this two-component system does regulate its own expression in *htrA* or *htrB* single mutants and *htrA htrB* double-mutant backgrounds.

Increased *cssRS* transcription on overexpression of C_{ss}S. To study the effects of C_{ss}S overexpression on the transcription of *cssRS*, the *cssS* gene was placed under the control of a xylose-inducible promoter in the *amyE* locus of a strain containing a *cssS-lacZ* fusion (BV2006), resulting in strain BV2023 (Fig. 1C). It should be noted that the *cssS* gene is disrupted by pMutin2 in the latter strain. As shown in Fig. 6, expression of the *cssRS* operon was up to eightfold higher when *cssS* was overexpressed after induction with 1% xylose in cells grown at 37°C. In contrast, the transcription of *cssS* remained low and unaffected in the absence of xylose. As a control, it was established that the presence of xylose had no effect on *cssS* transcription in the *cssS* mutant control strain (BV2006) (data not shown). Similar results were observed in minimal medium.

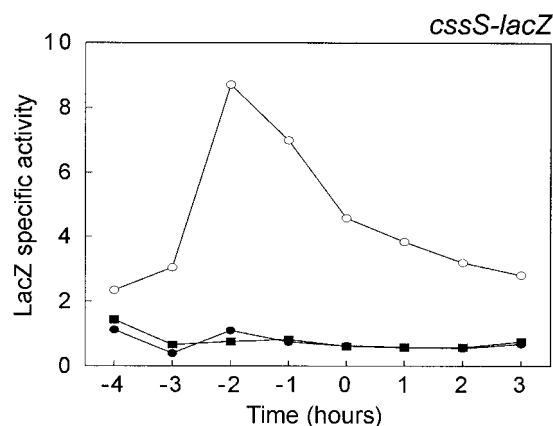


FIG. 6. Analysis of *cssS* transcription on C_{ss}S overexpression. A transcriptional *cssS-lacZ* gene fusion was used to determine the time courses of *cssS* expression in cells grown at 37°C in TY medium. The strains used for the analyses were *B. subtilis* 168 *cssS*::pMutin2 (BV2006; solid rectangles) and *B. subtilis* 168 *cssS*::pMutin2 *amyE*::X_{cssS} (BV2023) in the absence (solid ellipses) or presence (open ellipses) of 1% xylose. The parental strain 168 was used as a negative control (data not shown). β -Galactosidase activities are indicated in nanomoles per minute per OD₆₀₀ unit. Zero time ($t = 0$) indicates the transition point between the exponential and postexponential growth phases.

These observations imply that the expression of the *cssRS* operon, encoding the C_{ss}R_S two-component system, is autoregulated.

Mapping of the transcriptional start sites of *cssRS*. To determine the transcriptional start points of the *cssRS* operon by primer extension analysis, two different primers were used. Primer YVQA-RT1 is complementary to a region spanning the putative translational start site of *cssR*. Primer YVQA-RT2 is located further downstream in the coding sequence of *cssR*, with its 3' end being located at bp +63 relative to the putative translational start site (+1).

Because the expression levels of *cssRS* are very low in the parental strain and significantly higher in a *htrA* mutant background, the effects of heat stress on *cssRS* transcription were determined with the *htrA* Δ 439 mutant strain (DN26). Using RNA from this latter strain, two transcriptional start points were identified upstream of the *cssRS* operon. A transcriptional start site designated S1 was identified at a cytosine nucleotide positioned 99 bp upstream of the putative translational start codon. The intensity of this reverse transcript was the same in all RNA samples and did not change in response to heat stress (Fig. 7A, left panel; Fig. 7C). A second transcriptional start site designated S2 was detected at a guanine nucleotide 18 bp upstream of the putative translational start site (Fig. 7B, left panel; Fig. 7C). In contrast to start site S1, the intensity of the band corresponding to start site S2 was clearly increased in samples from heat-stressed cells. This result using primer extension is consistent with the data presented in Table 3 using a *PcssRS-bgaB* transcriptional fusion; both results show that the *cssRS* operon is heat stress inducible in a *htrA* mutant background. Both transcriptional start sites are located downstream of -10 motifs typical of σ^A -dependent promoters (Fig. 7C). However, a canonical σ^A -type -35 motif is not apparent in either promoter.

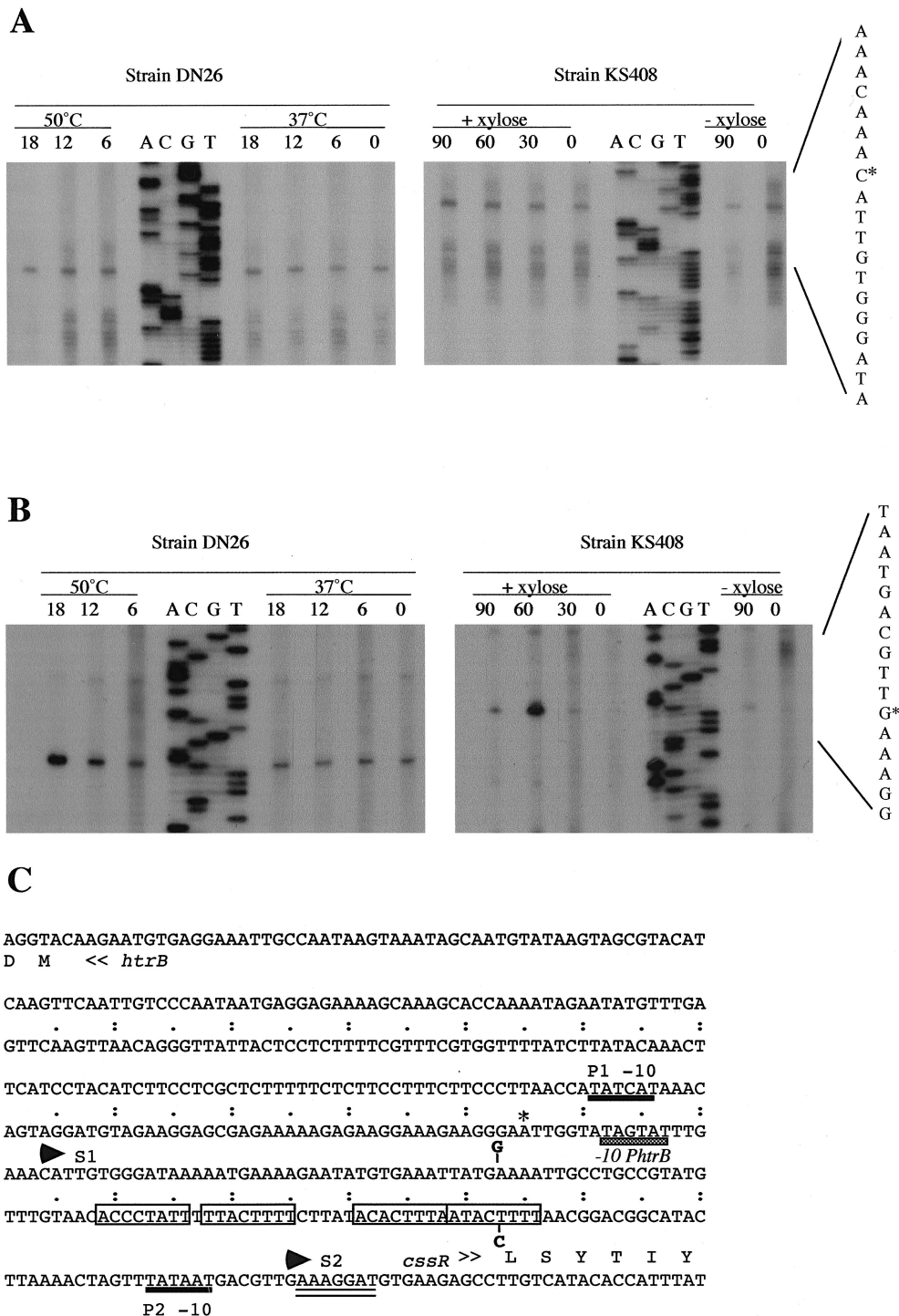


FIG. 7. Transcriptional analysis of *cssRS*. The transcription of *cssRS* under heat shock and secretion stress conditions was analyzed by primer extension. RNA was isolated from the DN26 mutant (*htrA*Δ439) under heat shock conditions and the KS408 strain (*xylR-amyL*) under secretion stress conditions. The time points of sampling are indicated in minutes above each lane. For DN26, zero time represents the time point at which cells grown in TY medium were subject to heat shock. For KS408, zero time represents the beginning of exponential growth ($t = 0$; OD_{550} about 0.5) in TY medium with or without 1% xylose. (A) Primer extension of total RNA using primer YVQA-RT1. Equivalent amounts of RNA (12.5 μ g) were used from all samples. The sequencing ladders were generated with primer YVQA-RT1. The start point of transcription is indicated (*). (B) Primer extension of total RNA using primer YVQA-RT2. Equivalent amounts of RNA (12.5 μ g) were used from all samples. The sequencing ladders were generated with primer YVQA-RT2. The start point of transcription is indicated (*). (C) Nucleotide sequence of the intergenic region between the *cssRS* operon and the divergently transcribed *htrB* gene. The transcriptional start sites, S1 and S2, are indicated by arrowheads above the sequence. Heavy black bars below the sequence indicate two σ^A -type -10 motifs, denoted P1 and P2, upstream of the S1 and S2 start sites, respectively. The double-underlined sequence represents the putative ribosome binding site of *cssR*. The repeated motif (TTTTCA) common to the inducible promoters of *cssRS* and *htrB* are boxed. The grey-shaded box represents the -10 motif of the inducible *htrB* promoter (-10 *PhrB*), and the initiation point of s1 transcription is indicated by an asterisk.

TABLE 4. Comparison of the levels of expression of P *htrB-bgaB* and of the fusion containing a point mutation P**htrB-bgaB* in *htrA* and *htrB* null mutant backgrounds at 37°C and after heat stress for 1 h at 48°C

Background		Strain	BgaB activity ^a P <i>htrB-bgaB</i> at:		Strain	BgaB activity P* <i>htrB-bgaB</i> at:	
<i>htrA</i>	<i>htrB</i>		37°C	48°C		37°C	48°C
-	+	DN113	2.4 (17)	40.6 (268)	DN119	0.5 (7)	2.4 (24)
+	-	DN112	2.5 (12)	31.7 (184)	DN120	0.5 (5)	1.3 (15)

^a Units of β -galactosidase are nanomoles per minute per OD₆₀₀ unit. To allow a comparison with previously reported data (15, 16), units of β -galactosidase are also indicated in nanomoles per minute per milligram of protein in parentheses.

To study the effects of secretion stress on *cssRS* transcription, the transcription start sites of *cssRS* were mapped with RNA from strain KS408, which contains a xylose-inducible *amyL* gene at the *xylR* locus. Similar to the heat stress experiment, the two transcriptional start sites S1 and S2 were detected with RNA from KS408 with or without xylose induction. While the level of mRNA with the S1 start site was mildly increased in cells grown in the presence of xylose (Fig. 7A, right panel), the level of mRNA with the S2 start site was significantly increased after 60 min of exponential growth under these conditions (Fig. 7B, right panel). In summary, these data indicate that the *cssRS* operon has two transcriptional start sites: a noninducible S1 site that might correspond to a maintenance promoter and an inducible S2 site for a promoter that is responsive to heat and secretion stress in a *htrA* mutant background.

The inducible *htrB* and *cssRS* promoters share a regulatory sequence. The *htrB* and *cssRS* genes are juxtaposed on the chromosome and are divergently transcribed (Fig. 7C). Furthermore, the inducible promoters of *htrB* and *cssRS* have good consensus σ^A -type -10 regions but do not have σ^A -type -35 regions. Instead, overlapping with and/or upstream of the -35 regions of both promoters is a 40-bp sequence that contains four copies of the TTTTCACA sequence, shown to be essential for heat-inducible expression of *htrA* (16). To investigate the possibility that these motifs may participate in inducible expression of both *htrB* and *cssRS*, strains were generated in which a single point mutation was introduced into one motif, changing it from TTTTCATA to TTTCCATA at position -60 relative to the *cssR* start codon (Fig. 7C). To test the effects of this point mutation on the activity of the *htrB* promoter in response to heat stress, the original or mutant *htrB-*cssRS** regulatory regions were placed in front of the *bgaB* gene and integrated in the *amyE* locus of *htrA* or *htrB* mutant strains (Table 4). The comparison of the *bgaB* expression levels directed by the original (P*htrB-bgaB*) and the mutant (P**htrB-bgaB*) regulatory sequences at 37°C shows that the point mutation reduces the *htrB* promoter activity in both the *htrA* (DN113 and DN119) and *htrB* (DN112 and DN120) mutant backgrounds. More significantly, this mutation also reduces the heat stress-induced activity (1 h at 48°C) of the *htrB* promoter by 10- to 20-fold in both genetic backgrounds.

To investigate the effects of the point mutation on the transcription of the *cssRS* operon in relation to secretion stress, P*cssRS-lacZ* fusions with and without the mutation (Fig. 1D) were introduced at the *cssRS* locus of *B. subtilis* KS408. This

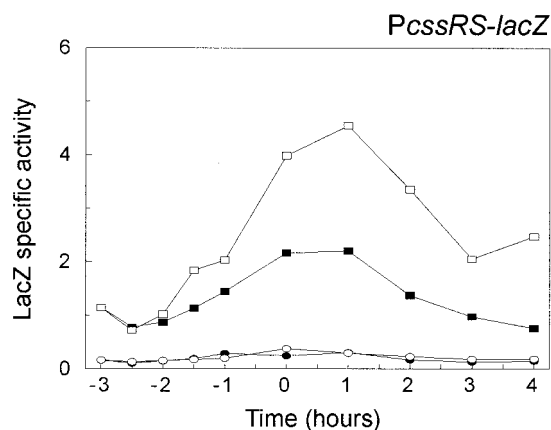


FIG. 8. Effect of a point mutation in the *cssRS* promoter on its induction by secretion stress. The transcriptional P*cssRS-lacZ* fusion in *B. subtilis* 168 *xylR::xylR-amyL* P*cssRS::pDN221* (DN225), which contains a xylose-inducible *amyL* gene, was used to determine the time courses of *cssRS* promoter activity in cells grown at 37°C in TY medium with 1% xylose (open rectangles) or without xylose (solid rectangles). In parallel, the transcriptional P**cssRS-lacZ* fusion in *B. subtilis* 168 *xylR::xylR-amyL* P*cssRS::pDN222* (DN228) was grown in the presence (open ellipses) or absence (solid ellipses) of 1% xylose. β -Galactosidase activities were measured in nanomoles per minute per OD₆₀₀ unit. Zero time ($t = 0$) indicates the transition point between the exponential and postexponential growth phases.

strain contains a xylose-inducible *amyL* gene at the *xylR* locus. The resulting strains, DN225 and DN228, were grown in TY medium in the presence or absence of 1% xylose, and the β -galactosidase levels were measured throughout the growth cycle (Fig. 8). The promoter activity of the intact P*cssRS-lacZ* fusion was about twofold increased in postexponential DN225 cells expressing AmyL due to xylose induction. In contrast, the mutated P**cssRS-lacZ* displayed significantly reduced levels of promoter activity irrespective of the induction of AmyL synthesis with xylose. It is interesting that a mutation in *cssRS* or a point mutation in the *htrB-*cssRS** regulatory region reduced the transcription of *htrB* and *cssRS* in a similar manner. These data show that through a shared regulatory sequence that participates in heat and secretion stress induction, the expression of *htrB* and *cssRS* are linked.

DISCUSSION

The synthesis of proteases at elevated levels is one of a variety of cellular responses that counteract the detrimental effects of the presence of misfolded proteins. The present studies show that the C*ssR*-C*ssS* two-component system responds to heat and secretion stress by activating the expression of two H*trA*-like proteases encoded by *htrA* and *htrB*. Misfolded proteins can accumulate in the cell through thermal denaturation or from a limited availability of appropriate folding catalysts at the extracytoplasmic side of the membrane. The latter mechanism would operate particularly on high-level production of secreted proteins. In this respect, it is important to bear in mind that most proteins of *B. subtilis* are transported across the membrane in an unfolded conformation via the Sec translocation channel (25). The C*ssRS* two-component regulatory system detects secretion stress by sensing the accumu-

lation of misfolded proteins at the membrane-cell wall interface (11). The C_{ss}RS-inducing signal is not cytosolic misfolded proteins, since neither *htrA* nor *htrB* expression is induced by puromycin addition. The present observation that the expression of C_{ss}RS-controlled genes is, in principle, responsive both to heat and secretion stress indicates that the C_{ss}RS system can sense misfolded proteins extracytosolically, irrespective of the cause that leads to their accumulation.

It is interesting that C_{ss}RS-dependent heat stress induction of the *cssRS* operon is observed under these conditions in the absence of *htrA* and not in a wild-type background. However, *cssRS* is induced by secretion stress in a C_{ss}RS-dependent manner. Therefore, *cssR* and *cssS* are both members of the C_{ss}RS regulon, but only under secretion stress conditions. Perhaps the level of misfolded proteins generated by heat stress in a wild-type background is sufficient to stimulate the expression of *htrA* and *htrB* but insufficient to stimulate the expression of *cssRS*. The higher level of misfolded protein that persists after heat stress in the absence of HtrA, or overexpression of α -amylase, is necessary to stimulate C_{ss}RS-dependent expression of the *cssRS* operon. This would imply that *cssRS* is responsive to different levels of misfolded proteins.

These results, together with previous studies, justify the regrouping of heat-inducible genes of *B. subtilis* into five distinct classes that differ in their regulatory mechanisms. The class I genes are regulated by σ^A and the HrcA repressor, the class II genes are regulated by σ^B , and the class III genes are regulated by the CtsR transcription factor. Since the *htrA* and *htrB* genes are not regulated by any of the known regulatory systems of the class I, II, or III genes, the present studies indicate that they belong to a separate class of heat stress-inducible genes, which are controlled by the C_{ss}RS two-component regulatory system. Since only genes induced in a wild-type background can be considered bona fide members of a class of heat stress genes, it is evident that in sensu strictu, only *htrA* and *htrB* are members of this new C_{ss}RS-dependent grouping of heat stress genes, termed class V. However, since *cssRS* is autoregulated under certain genetic and environmental conditions, there are consequently four genes in the C_{ss}RS regulon: *cssS*, *cssR*, *htrA*, and *htrB*.

The *htrA* and *htrB* genes were previously shown to have one transcriptional start site (15), whereas the *cssRS* operon has two transcriptional start sites, only one of which is heat and secretion stress responsive. The three stress-inducible promoters have canonical -10 regions typical of σ^A -type promoters but none have canonical -35 regions. All three stress-inducible promoters have repeated octameric motifs (TTTTTCATA) in the vicinity of the -35 regions, and it has been demonstrated that deletion of one copy of this motif from the *htrA* promoter results in a dramatic (>20 -fold) decrease in heat stress induction (16). This study shows that a point mutation in repeat I of the octameric consensus sequence (the point mutation is in the *htrB* copy corresponding to the one deleted in the *htrA* promoter [see the discussion of promoter alignment in reference 16]) affects heat and secretion stress induction of both the *htrB* and *cssRS* genes. These data show that stress-induced expression of *htrB* and *cssRS* are linked through this common regulatory sequence, perhaps to make the levels of protease (HtrA and HtrB) and regulator (C_{ss}R and C_{ss}S) responsive to the prevailing stress conditions. It is tempting to speculate that

C_{ss}R binds to these repeated motifs to modulate gene expression in response to stress. The identification of a noninducible promoter activity upstream of the *cssRS* operon suggests the existence of a second mode of regulation for this operon.

The C_{ss}RS system bears some resemblance to the CpxA-CpxR two-component system from *E. coli*. First, CpxA and C_{ss}S show amino acid sequence similarities, and the same is true for CpxR and C_{ss}R (11). Second, these two systems control the transcription of genes encoding HtrA-like proteases: *htrA* (*degP*) of *E. coli* is regulated by the CpxAR system (4), and *htrA* and *htrB* of *B. subtilis* is regulated by the C_{ss}RS system. Finally, like the *cpxAR* operon (7, 20), the transcription of the *cssRS* operon is autoregulated. Notwithstanding these similarities, the *htrA*, *htrB*, and *cssRS* promoter regions lack the CpxR consensus binding site. This work unveils an even more remarkable difference, since CpxAR and C_{ss}RS seem to respond to different stimuli. While the C_{ss}RS two-component system can respond to heat stress, the equivalent task in *E. coli* is accomplished by σ^E and not by CpxAR. This could be because the σ^E -mediated stress response pathway senses misfolded outer membrane or periplasmic proteins whereas the CpxAR pathway senses inner membrane-associated aggregates or misfolded protein (21). In contrast to *E. coli*, the gram-positive *B. subtilis* lacks an outer membrane, having a thick matrix of cell wall polymers instead (2). Thus, it seems as if one system might be sufficient to sense and respond to the accumulation of misfolded proteins in the cell envelope of organisms that lack an outer membrane.

The fact that the growth of the *htrA-htrB* double-mutant strain was severely affected underscores the importance of the proteases encoded by these genes and demonstrates a redundancy in their functions (15). Due to an as yet unidentified suppressor mutation, this strain displayed similar growth characteristics to the parental strain at 37°C. Nevertheless, the suppressor mutation did not completely restore the growth at 48°C. In fact, the growth at 48°C of all strains with a double mutation in *cssS*, *htrA*, or *htrB* was affected. Notably, the strongest effect on growth at 48°C was observed when the two protease genes were mutated, and this was not exacerbated by the introduction of an additional mutation in *cssS*. This implies that the C_{ss}RS system combats the detrimental effects of heat stress primarily by the upregulation of *htrA* and *htrB*. Strikingly, the *cssS-htrA* double mutant grew better at 48°C than did the *cssS-htrB* double mutant, indicating that HtrA and HtrB have at least partly different functions. The latter view is supported by the observation that *htrB* transcription showed a faster response to heat stress than did that of *htrA*. Interestingly, the expression of *htrB* was still upregulated by heat stress in a *cssS* mutant background whereas the expression of *htrA* was no longer heat inducible in the absence of C_{ss}S. Thus, it seems that heat-induced expression of *htrB* may not be exclusively C_{ss}S dependent.

In conclusion, this paper documents the existence of a novel class of heat stress-responsive genes in *B. subtilis*. These class V genes (*htrA* and *htrB*) are regulated by the C_{ss}RS two-component system. The C_{ss}RS two-component system can also be induced by secretion stress, and therefore the regulon comprises four genes (*cssR*, *cssS*, *htrA*, and *htrB*) under these conditions. Ongoing research is focused on the determination of

the precise binding site for C_{ss}R and the complete definition of the C_{ss}RS regulon.

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E.D. and D.N. contributed equally to this work.

REFERENCES

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741–746.
2. Archibald, A. R., I. C. Hancock, and C. R. Harwood. 1993. Cell wall structure, synthesis and turnover, p. 381–410. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. ASM Press, Washington, D.C.
3. Benson, A. K., and W. G. Haldenwang. 1993. The sigma B-dependent promoter of the *Bacillus subtilis* sigB operon is induced by heat shock. *J. Bacteriol.* **175**:1929–1935.
4. Danese, P. N., W. B. Snyder, C. L. Cosma, L. J. Davis, and T. J. Silhavy. 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes Dev.* **9**:387–398.
5. Derré, I., G. Rapoport, and T. Msadek. 1999. CtsR, a novel regulator of stress and heat shock response, controls clp and molecular chaperone gene expression in gram-positive bacteria. *Mol. Microbiol.* **31**:117–131.
6. Derré, I., G. Rapoport, K. Devine, M. Rose, and T. Msadek. 1999. ClpE, a novel type of HSP100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. *Mol. Microbiol.* **32**:581–593.
7. De Wulf, P., O. Kwon, and E. C. Lin. 1999. The CpxRA signal transduction system of *Escherichia coli*: growth-related autoactivation and control of unanticipated target operons. *J. Bacteriol.* **181**:6772–6778.
8. Fabret, C., V. A. Feher, and J. A. Hoch. 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *J. Bacteriol.* **181**:1975–1983.
9. Gryczan, T. J., S. Contente, and D. Dubnau. 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. *J. Bacteriol.* **134**:318–329.
10. Hecker, M., and U. Völker. 1990. General stress proteins in *Bacillus subtilis*. *FEMS Microbiol. Ecol.* **74**:197–214.
11. Hyyryläinen, H. K., A. Bolhuis, E. Darmon, L. Muukkonen, P. Koski, M. Vitikainen, M. Sarvas, Z. Prágai, S. Bron, J. M. van Dijk, and V. P. Kontinen. 2001. A novel two-component regulatory system of *Bacillus subtilis* for the survival of severe secretion stress. *Mol. Microbiol.* **41**:1159–1172.
12. Kim, L., A. Mogk, and W. Schumann. 1996. A xylose-inducible *Bacillus subtilis* integration vector and its application. *Gene* **181**:71–76.
13. Leskela, S., V. P. Kontinen, and M. Sarvas. 1996. Molecular analysis of an operon in *Bacillus subtilis* encoding a novel ABC transporter with a role in exoprotein production, sporulation and competence. *Microbiology* **142**:71–77.
14. Mogk, A., G. Homuth, C. Scholz, L. Kim, F. X. Schmid, and W. Schumann. 1997. The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. *EMBO J.* **16**:4579–4590.
15. Noone, D., A. Howell, R. Coltery, and K. M. Devine. 2001. YkdA and YvtA, HtrA-like serine proteases in *Bacillus subtilis*, engage in negative autoregulation and reciprocal cross-regulation of *ykdA* and *yvtA* gene expression. *J. Bacteriol.* **183**:654–663.
16. Noone, D., A. Howell, and K. M. Devine. 2000. Expression of *ykdA*, encoding a *Bacillus subtilis* homologue of HtrA, is heat shock inducible and negatively autoregulated. *J. Bacteriol.* **182**:1592–1599.
17. Palva, I. 1982. Molecular cloning of alpha-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. *Gene* **19**:81–87.
18. Parkinson, J. S., and E. C. Kofoid. 1992. Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**:71–112.
19. Price, C. P. 2000. Protective function and regulation of the general stress response in *Bacillus subtilis* and related gram-positive bacteria, p. 179–198. In G. Storz and R. Hengge-Aronis (ed.), *Bacterial stress responses*. ASM Press, Washington, D.C.
20. Raivio, T. L., D. L. Popkin, and T. J. Silhavy. 1999. The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J. Bacteriol.* **181**:5263–5272.
21. Raivio, T. L., and T. J. Silhavy. 1999. The sigmaE and Cpx regulatory pathways: overlapping but distinct envelope stress responses. *Curr. Opin. Microbiol.* **2**:159–165.
22. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. Stephenson, K., and C. R. Harwood. 1998. Influence of a cell-wall-associated protease on production of alpha-amylase by *Bacillus subtilis*. *Appl. Environ. Microbiol.* **64**:2875–2881.
24. Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol. Rev.* **53**:450–490.
25. Tjalsma, H., A. Bolhuis, J. D. Jongbloed, S. Bron, and J. M. van Dijk. 2000. Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol. Mol. Biol. Rev.* **64**:515–547.
26. Vagner, V., E. Dervyn, and S. D. Ehrlich. 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**:3097–3104.
27. van Dijk, J. M., A. de Jong, G. Venema, and S. Bron. 1995. Identification of the potential active site of the signal peptidase SipS of *Bacillus subtilis*. Structural and functional similarities with LexA-like proteases. *J. Biol. Chem.* **270**:3611–3618.
28. Wertman, K. F., A. R. Wyman, and D. Botstein. 1986. Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene* **49**:253–262.
29. Yuan, G., and S. L. Wong. 1995. Isolation and characterization of *Bacillus subtilis* *groE* regulatory mutants: evidence for orf39 in the *dnaK* operon as a repressor gene in regulating the expression of both *groE* and *dnaK*. *J. Bacteriol.* **177**:6462–6468.
30. Yuan, G., and S. L. Wong. 1995. Regulation of *groE* expression in *Bacillus subtilis*: the involvement of the sigma A-like promoter and the roles of the inverted repeat sequence (CIRCE). *J. Bacteriol.* **177**:5427–5433.
31. Zuber, U., and W. Schumann. 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. *J. Bacteriol.* **176**:1359–1363.