Pre- and post-translocational stages in protein secretion by Bacillus subtilis
Zanen, Geeske Elisabeth

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2005

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
The *Bacillus* secretion stress response is an indicator for alpha-amylase production levels

Helga Westers, Elise Darmon, Geeske Zanen, Jan Willem Veening, Oscar P. Kuipers, Sierd Bron, Wim J. Quax, and Jan Maarten van Dijl

Abstract

Upon overproduction of certain secretory proteins, *Bacillus subtilis* displays a specific secretion stress response involving the CssRS two-component system. This system controls the expression of two housekeeping proteases, HtrA and HtrB. Secretion stress caused by overproduction of the α-amylase AmyQ is sensed by the CssS sensor. Previously, the *B. subtilis* TepA protein was implicated in high-level AmyQ secretion. The present studies were primarily aimed at investigating a possible role of TepA in secretion stress management, and characterising the intensity of the secretion stress response in relation to the levels of AmyQ production. For this purpose, the expression of a transcriptional *htrB-lacZ* gene fusion, and the cellular and secreted levels of AmyQ were monitored simultaneously using a collection of *tepA* mutant strains. The results show that TepA is dispensable for secretion stress management. Importantly, however, the levels of *htrB-lacZ* expression were shown to be correlated with the levels of AmyQ production. In conclusion, our observations show that the secretion stress response can serve as an indicator for AmyQ production levels. Conceivably, this stress response can be employed to monitor the biotechnological production of a variety of secretory proteins by the *Bacillus* cell factory.
1. Introduction

*Bacillus* species, such as *Bacillus subtilis*, are important commercial producers of enzymes and biopharmaceuticals, because of their capacity to secrete large quantities of proteins directly into their culture medium. Even though *B. subtilis* is efficient at secreting native proteins, it often exhibits a much lower efficiency for the secretion of heterologous proteins, such as human-Interleukin-3 (Bolhuis et al., 1999c). Upon translocation across the cytoplasmic membrane, most secretory proteins fold into their native, protease-resistant, conformation at the membrane-cell wall interface, after which they pass through the cell wall into the culture medium. However, in some cases, the translocated proteins do not fold effectively and are rapidly degraded by the various proteases (Tjalsma et al., 2000). In some cases, they may be rescued by folding catalysts that prevent their aggregation and/or degradation (Antelmann et al., 2003; Gottesman, 1996; Wickner et al., 1999).

The clearing of unfolded or malfolded translocated proteins is important because their extracytoplasmic accumulation can seriously harm the bacterial host cell (Hyyryläinen et al., 2001). Consequently, bacteria have evolved specific mechanisms to withstand the extracytoplasmic protein folding stress, resulting from the inefficient folding of translocated proteins or exposure to heat. Whereas *Escherichia coli* uses two partially overlapping signal transduction pathways, the Cpx two-component regulatory system and the σE-mediated stress response pathway (Danese and Silhavy, 1997; Connolly et al., 1997; Pogliano et al., 1997), *B. subtilis* employs the CssR-CssS two-component regulatory system (Control secretion stress Regulator and Sensor) to sense this type of stress and combat its detrimental effects (Darmon et al., 2002; Hyyryläinen et al., 2001).

Secretion stress in *B. subtilis* has been defined as any kind of stress that activates the CssR-CssS two-component system (Antelmann et al., 2003), one of them being the overproduction of certain secretory proteins, such as the α-amylase AmyQ from *Bacillus amyloliquefaciens*. It is thought that the presence of malfolded or aggregated proteins at the membrane-cell wall interface of *B. subtilis* is sensed with the help of the membrane protein CssS (Hyyryläinen et al., 2001). This sensor is also involved in the cellular response to heat stress (Noone et al., 2000; Darmon et al., 2002). Upon high-level production of a secretory protein, or a temperature upshift, CssS activates the response regulator CssR by phosphorylation. This in turn triggers increased transcription of htrA and htrB (Darmon et al., 2002) encoding, respectively, putative membrane-bound proteases, HtrA and HtrB. Interestingly, HtrA has a dual localisation, being detectable both in membrane-associated and soluble extracellular forms (Antelmann et al., 2003). By analogy to HtrA of *E. coli* (Spiess et al., 1999), both HtrA and HtrB of *B. subtilis* are
thought to have a “cleaning function” by degrading or refolding malfolded proteins at the membrane-cell wall interface (Antelmann et al., 2003; Tjalsma et al., 2000).

The cytoplasmic TepA (Translocation enhancing protein A) has been implicated in protein secretion in B. subtilis. Membrane translocation and processing of pre-AmyQ was shown to be retarded in a B. subtilis TepA-depleted strain (ItepA). TepA shows sequence similarities with a signal peptide degrading enzyme of E. coli (i.e. SppA) as well as the cytoplasmic ClpP protease. On the basis of these observations, three possible roles of TepA in protein translocation were proposed. TepA could be involved in the regulation of the secretion process, the cytosolic degradation of signal peptide fragments that are inhibitory to protein translocation, or act as a secretion-specific chaperone (Bolhuis et al., 1999a). Each of these proposed activities of TepA could impact on the management of the secretion stress response by cells of B. subtilis, irrespective of the precise function of TepA in the secretion process.

The aim of the present studies was to investigate whether TepA has a role in protein secretion stress management in B. subtilis and, if so, to what extent the AmyQ translocation defect in tepA mutant strains would impact on the intensity of this response. Unexpectedly, the secretion of AmyQ by a tepA deletion strain was not affected, suggesting either that TepA is not important in AmyQ secretion, or that a suppressor mutation was selected upon tepA deletion. Importantly however, these studies resulted in a collection of strains expressing AmyQ at different levels that served as models for low-producing strains that are encountered in daily practice. Analysis of these strains shows that the intensity of the CssRS-dependent secretion stress response in B. subtilis is correlated to the level of AmyQ production.

### 2. Materials and Methods

#### 2.1 Plasmids, bacterial strains, and media

Table 1 lists the plasmids and bacterial strains used. LB medium contained Bacto tryptone (1 %), Bacto yeast extract (0.5 %) and NaCl (0.5 %). TY medium contained Bacto tryptone (1 %), Bacto yeast extract (0.5 %) and NaCl (1 %). Minimal medium was prepared as described by Leskelä et al. (1996). Antibiotics were used in the following concentrations: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 5 µg/ml; erythromycin (Em), 100 µg/ml (E. coli) or 5 µg/ml (B. subtilis); and kanamycin (Km), 20 µg/ml. When required, media for B. subtilis were supplemented with 1 % (w/v) xylose. To visualise α-amylase activity of AmyE from B. subtilis or AmyQ from B. amyloliquefaciens, LB plates were supplemented with 1 % starch and stained with iodine vapor.
2.2 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.* (1989). Enzymes were obtained from Invitrogen Life Technologies. *B. subtilis* was transformed as described by Kunst and Rapoport (1995) or Leskelä *et al.* (1996). The nucleotide sequences of primers used for PCR are described below; nucleotides identical to genomic template DNA are printed in capital letters, restriction sites used for cloning are underlined, and nucleotides used for PCR-mediated coupling are in bold letters.

To construct a *B. subtilis tepA* deletion strain, two amplified fragments flanking the *tepA* gene were ligated into pUC19 after PCR-mediated splicing (Horton *et al.*, 1989). This construct was fused with the chromosomal integration and excision plasmid pORI280. The upstream fragment *tepA*front of 653 nucleotides was amplified with the primers ∆*tepA*front.1 (5’GGC GAC GTC GTT GAA TTC CGC GGC CAA AAC GTA AAA ATT GGG G 3’) and ∆*tepA*front.2 (5’ttg

### Table 1. Plasmids and strains

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant propertiesa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>Plasmid based on ColE1, Φ80dlacZ, lac promoter; Apr</td>
<td>Norrander <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>pORI280</td>
<td>Vector for chromosomal integration and excision allowing construction of markerless deletions; lacZ, ori+ of pWV01, replicates only in strains providing repA in trans; Emr</td>
<td>Leenhouts <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>pORI∆tepA</td>
<td>Vector based on pORI280 and pUC19 for chromosomal integration and excision allowing construction of a markerless <em>tepA</em> deletion; Emr</td>
<td>This work</td>
</tr>
<tr>
<td>pX</td>
<td>Vector for the xylose-inducible transcription of cloned genes by the xylA promoter; carries the xylR gene; Apr; Cmr</td>
<td>Kim <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>pX- tepA</td>
<td>pX derivative; carries <em>tepA</em> downstream of the xylA promoter; Apr; Cmr</td>
<td>This work</td>
</tr>
<tr>
<td>pX(his)6-htrA</td>
<td>pX derivative carrying the his6-htrA gene downstream of the xylA promoter; Apr, Cmr</td>
<td>Antelmann <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>pX(his)6-htrB</td>
<td>pX derivative carrying the his6-htrB gene downstream of the xylA promoter; Apr, Cmr</td>
<td>This work</td>
</tr>
<tr>
<td>pKTH10</td>
<td>Contains the amyQ gene of <em>B. amyloliquefaciens</em>; Km2</td>
<td>Palva, 1982</td>
</tr>
<tr>
<td>pKTH10L</td>
<td>pKTH10 derivative; Km2</td>
<td>Hyyrylainen <em>et al.</em>, 2001</td>
</tr>
</tbody>
</table>
a)Apr, ampicillin resistance marker; Em r, erythromycin resistance marker; Cm r, chloramphenicol resistance marker; Kmr, kanamycin resistance marker.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant propertiesa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td></td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td></td>
<td>F-  φdlacZΔM15 endA1 recA1 gyrA96 thi-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hsdR17 (K&lt;sup&gt;R&lt;/sup&gt; m&lt;sup&gt;K&lt;/sup&gt;) supE44 relA1 deoR Δ(lacZYA-argF) U169</td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Kunst et al., 1997</td>
</tr>
<tr>
<td>1tepA</td>
<td>trpC2; tepA::pMutin2; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Bolhuis et al., 1999a</td>
</tr>
<tr>
<td>1tepA</td>
<td>trpC2; ΔtepA</td>
<td>This work</td>
</tr>
<tr>
<td>htrB</td>
<td>also known as BFA3041; trpC2; htrB::pMutin4; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Darmon et al., 2002</td>
</tr>
<tr>
<td>htrB tepA</td>
<td>also known as BV2070; trpC2; ΔtepA; htrB::pMutin4; Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>168 X</td>
<td>trpC2; amyE::X; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>168 X-htrA</td>
<td>also known as BV2043; trpC2; amyE::X(his)&lt;sub&gt;6&lt;/sub&gt;-htrA; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>168 X-htrB</td>
<td>also known as BV2044; trpC2; amyE::X(his)&lt;sub&gt;6&lt;/sub&gt;-htrB; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>htrB tepA X</td>
<td>trpC2; ΔtepA; htrB::pMutin4; amyE::X; Em&lt;sup&gt;r&lt;/sup&gt;; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>htrB tepA X-</td>
<td>trpC2; ΔtepA; htrB::pMutin4; amyE::XtepA; Em&lt;sup&gt;r&lt;/sup&gt;; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

a)Apr<sup>r</sup>, ampicillin resistance marker; Em<sup>r</sup>, erythromycin resistance marker; Cm<sup>r</sup>, chloramphenicol resistance marker; Km<sup>r</sup>, kanamycin resistance marker.

**aat cat ceg ttc tte GCG GAA GCG TCG TTT CAC CAA GCT GCT GTc tag tea gct aAA TGC TGT CCT TCG CAT C 3’).** The downstream fragment tepAbacK of 1387 bp was amplified with the primers ΔtepAbacK.1 (5’tag ctg act aag cag cag ctt ggt gaa acg acg ctt ceg cGA AGG ACG GAT GAT TCA ATG ATT CCT TAT ACC GTG ATG CCT TAG G 3’) and ΔtepAbacK.2 (5’GGC CGG TAT GCT TCG GAT CTG CCA GCA AAT CCA GTG ACG GC 3’). The final construct pORIΔtepA was inserted into the chromosome of B. subtilis 168 by a Campbell-type integration. Upon growth in the absence of erythromycin, B. subtilis tepA was obtained due to spontaneous excision of the pORI plasmid from the chromosome together with the tepA gene. This deletion strategy leaves the tepA promoter region intact. Correct excision of tepA was verified by Southern hybridization.

To construct the B. subtilis htrB tepA (BV2070) double mutant, the B. subtilis tepA strain was transformed with chromosomal DNA isolated from B. subtilis htrB (BFA3041) encoding a transcriptional htrB-lacZ gene fusion.
To construct plasmid pX-\textit{tepA}, the entire \textit{tepA} gene was amplified by PCR with the primers ABp4a (5’ aat cta gaC ACA GAA GAG CGT CC 3’) and TepApX.2 (5’ CAC Ggg atc c AG AAT CAT TG A ATC ATC CGT CC 3’). The resulting PCR product was ligated into plasmid pX, resulting in pX-\textit{tepA}, which contains the \textit{tepA} gene under the transcriptional control of the xylose-inducible \textit{xylA} promoter (P\textit{xylA}). Sequence verification of the \textit{tepA} gene in three independently obtained constructs revealed three differences with the \textit{tepA} sequence as deposited in the SubtiList database after genome sequencing (http://genolist.pasteur.fr/SubtiList/). This implies that the chromosomal \textit{tepA} sequence in the region of the differences reads 355-ACA GCA ACG ATG-366, which would translate into the TepA residues: 119-TATM-122 (instead of TSTV). The xylose-inducible TepA-producing strain \textit{htrB} \textit{X-\textit{tepA}} was obtained by a double cross-over recombination event that resulted in the introduction of the P\textit{xylA} \textit{tepA} cassette (X-\textit{tepA}) into the chromosomally-located \textit{amyE} gene of \textit{B. subtilis} 168. Chloramphenicol resistant transformants were screened for an AmyE-negative phenotype on starch-containing plates.

\textit{B. subtilis} 168 \textit{X-htrA} (BV2043) was obtained by a double cross-over recombination event between the flanking regions of \textit{amyE} located on pX(his)\textit{6-htrA} and the chromosomal \textit{amyE} gene of \textit{B. subtilis} 168. Correct transformants carrying the P\textit{xylA}-(his)\textit{6-htrA} cassette (X-\textit{htrA}) in \textit{amyE} were chloramphenicol resistant and showed no \(\alpha\)-amylase activity on plates containing starch.

To construct a \textit{B. subtilis} strain with a xylose-inducible (his)\textit{6-htrB} gene (BV2044), the \textit{htrB} gene was amplified by PCR with primers N-YH1 (5’ gct cta gaC GTT AAA GGA GTG TAA GAA CAT Gca tea eca tea eca tea eca cga TTA TCG ACG TGA TGG CCA AAA CG 3’), specifying a hexa-histidine tag (sequence represented in bold) and hisyvtA02 (5’ cgg gat cc TAG CTT GAA CTG CTT TCT GTC 3’). The amplified fragment was cloned into plasmid pX, resulting in pX(his)\textit{6-htrB}. \textit{B. subtilis} 168 \textit{X-htrB} (BV2044) carrying the P\textit{xylA}-(his)\textit{6-htrB} cassette (X-\textit{htrB}) in \textit{amyE} was obtained as described above for BV2043. The control strain \textit{B. subtilis} 168 \textit{X} was obtained by integration of the empty P\textit{xylA} cassette of plasmid pX into the \textit{amyE} gene of the parental strain 168.

\textbf{2.3 \(\beta\)-galactosidase activity assay}

The \(\beta\)-galactosidase assay and the calculation of \(\beta\)-galactosidase units (Miller units: nmol/min/OD\textsubscript{600}) were performed as described by Hyyryläinen et al. (2001). Experiments were repeated at least twice, starting with independently obtained transformants, with relevant controls performed in parallel. Although some differences were observed in the absolute \(\beta\)-galactosidase activities, the ratios between these activities in the various strains tested were largely constant. A ratio of about 1.5 was generally reproducible.
2.4 Western blotting and immunodetection

Western blotting was performed as described by Kyhse-Andersen (1984). After separation by SDS-PAGE, proteins were transferred to a Protran® nitrocellulose transfer membrane (Schleicher and Schuell). To assay the presence of the precursor and mature forms of AmyQ in *B. subtilis*, cells were separated from the growth medium by centrifugation. If required, proteins in the growth medium were concentrated 8-fold upon precipitation with 5% trichloroacetic acid (TCA). AmyQ was visualised with specific antibodies and horseradish peroxidase-anti-rabbit IgG conjugates or alkaline phosphatase-anti-rabbit IgG conjugates (Biosource International).

3. Results

3.1 Deletion of *tepA* has no effect on AmyQ secretion

Previous studies with *B. subtilis* *tepA*, in which the expression of *tepA* is controlled by the IPTG-dependent *Pspac* promoter, indicated that TepA is required for efficient translocation of the α-amylase AmyQ (Bolhuis *et al.*, 1999a). To further characterise the function of TepA, the *tepA* gene was completely removed from the chromosome, using a plasmid-based chromosomal integration/excision system (Westers *et al.*, 2003). To study the translocation and processing of pre-AmyQ in cells lacking TepA, two independently obtained isolates of *B. subtilis* *tepA* were transformed with plasmid pKTH10, which directs the high-level production of AmyQ (Palva, 1982). Western blotting and pulse-chase experiments, in which *B. subtilis* 168 and *B. subtilis* *tepA* (both containing pKTH10) were used as parental and TepA-depleted control strains respectively, demonstrated that pre-AmyQ processing was not affected in the *B. subtilis* *tepA* deletion strain (unpublished observations). This unexpected finding suggests that TepA is either not important for pre-AmyQ translocation and subsequent processing, or that an unidentified *tepA* suppressor mutation was selected during the construction or subsequent cultivation of the *tepA* deletion strain in at least two independent cases.

Interestingly, pKTH10 was found to be genetically unstable upon prolonged cultivation of the *tepA* deletion and TepA depletion strains. Thus, derivatives of this pKTH10-transformed strain were obtained, which displayed varying levels of AmyQ production. Previous studies using strains containing pKTH10 suggested that variants of this plasmid, directing low-level or no synthesis of AmyQ, were rapidly selected when the cells were unable to respond to the secretion stress caused by high level production of this secretory protein (Hyyrylainen *et al.*, 2001). To investigate the role of TepA in the management of secretion stress, this was
Figure 1. Deletion of \textit{tepA} affects neither AmyQ secretion nor the AmyQ-induced secretion stress response

(A) AmyQ production and secretion. \textit{B. subtilis} \textit{htrB}, \textit{htrB} \textit{tepA}, \textit{htrB} \textit{tepA X-tepA} - xylose, and \textit{htrB} \textit{tepA X-tepA} + xylose, all containing pKTH10 for AmyQ production, were grown overnight in TY medium at 37 °C. Subsequently, the cultures were diluted to an OD$_{600}$ nm of 0.05 in fresh TY medium with or without xylose and incubated at 37 °C. Cells were harvested after overnight growth (ON), 2 hours before the transition between exponential and post-exponential growth ($t_{-2}$), and after 3 hours of post-exponential growth ($t_{3}$). The parental strains \textit{B. subtilis} 168 and \textit{B. subtilis} \textit{htrB} were used as negative controls (data not shown). Cells (C) were separated from the growth medium (M) by centrifugation. (Pre-)AmyQ was visualised by SDS-PAGE, Western blotting, and immunodetection, using AmyQ-specific antibodies. The positions of pre-AmyQ and mature AmyQ are indicated; note that several degradation products of AmyQ are detectable.
induced with high-level AmyQ production in a tepA deletion strain. An htrB-lacZ transcriptional gene fusion was introduced in the tepA mutant to monitor the secretion stress response via β-galactosidase activity. Notably, the activity of the htrB promoter in response to secretion stress is greatly enhanced, but not altered, by the disruption of the htrB gene in the reporter strain (Darmon et al., 2002; Noone et al., 2000; 2001). Moreover, the htrB-lacZ reporter gene fusion is more sensitive to secretion stress than a transcriptional htrA-lacZ reporter gene fusion (Darmon et al., 2002; Hyyrylainen et al., 2001). The resulting htrB-tepA strain was transformed with plasmid pKTH10. The expression of htrB-lacZ was also investigated in a pKTH10-containing htrB-tepA strain in which tepA was ectopically expressed with a xylose-inducible promoter (X-tepA). Overnight cultures of cells that had been freshly transformed with pKTH10 were diluted in fresh medium and grown until 3 hours after the transition between exponential and post-exponential phase. β-galactosidase activity was used to monitor the transcription of htrB, while AmyQ production was analysed by Western blotting. As shown in Figure 1A, neither AmyQ production nor secretion were affected by the deletion of tepA, irrespective of the xylose-induced ectopic expression of tepA. Accordingly, the intensity of the secretion stress response was similar in the tepA deletion strain and its ectopically complemented derivative (Fig. 1B; ▲, ○, and ▲). Notably, some variation in cellular or extracellular AmyQ levels can be observed for certain strains at the different time points. Nevertheless, the lack of effect of the tepA deletion on the secretion of AmyQ and the induction of the secretion stress response was reproducibly observed when cells, freshly transformed with pKTH10, were used. Taken together, these observations show that the deletion of tepA has no impact on the production and secretion of AmyQ. Furthermore, the tepA deletion strain displays an AmyQ-induced secretion stress response that is indistinguishable from that of the parental strain.

3.2 Correlation between AmyQ production levels and intensity of the secretion stress response
Prolonged cultivation of B. subtilis tepA strains containing pKTH10 resulted in the emergence of variants producing reduced levels of AmyQ compared to strains

(B) Cellular response to secretion stress. A transcriptional htrB-lacZ gene fusion was used to determine the time courses of htrB expression in the cells used for the experiment described in panel A. The strains included in the analyses were: B. subtilis htrB pKTH10 (■), htrB tepA pKTH10 (▲), htrB tepA X-tepA pKTH10 - xylose (●), and htrB tepA X-tepA pKTH10 + xylose (▲). The parental strains B. subtilis 168 (○) and B. subtilis htrB (△) were used as negative controls. Samples for the determination of β-galactosidase activities (indicated in nmol/min/OD₆₀₀) were withdrawn at the times indicated. Zero time (t = 0) indicates the transition point between the exponential and post-exponential growth phases.
freshly transformed with this plasmid. In all cases, this reduced AmyQ production could be attributed to the instability of pKTH10. Notably, plasmid instability is a frequently observed cause of reduced production levels of secretory proteins (Cordes et al., 1996). Thus, the ΔtepA strains with reduced AmyQ productivity are, to some extent, reminiscent of the genetically undefined low-producing variants of production strains in biotechnological applications. This prompted us to investigate whether the intensity of the B. subtilis secretion stress response was correlated with the level of AmyQ production. To this end, the secretion stress response and AmyQ production were measured in strains with different cultivation histories (i.e. strains that were kept on agar plates for different periods of time and strains that were kept frozen at -80 °C). Overnight cultures were diluted in fresh medium and grown until 3 hour after the transition between exponential and post-exponential growth (Fig. 2). A B. subtilis htrB strain, freshly transformed with pKTH10 and producing high levels of AmyQ, was used as a positive control (Fig. 2A; htrB control, x). A B. subtilis htrB strain that had been kept on an agar plate for 15 days showed a lower secretion stress response (i.e. htrB-lacZ expression) than the htrB control strain (Fig. 2A; compare ■ and x). The lower secretion stress response correlated with a reduced production level of AmyQ by the htrB strain (Fig. 2B). The reduced AmyQ production was reflected both by reduced cellular and extracellular levels of (pre-)AmyQ. Similarly, the very mild secretion stress response in the htrB tepA\(^1\) double mutant (Fig. 2A; ◊) was correlated with very low AmyQ production levels (Fig. 2B). In contrast, a strain with the same genotype producing high AmyQ levels (htrB tepA\(^2\)), displayed a high secretion stress response (Fig. 2A; ▲) that was comparable with the secretion stress response in the htrB control strain (Fig. 2A; x). Consistently, strains containing the X-tepA cassette that displayed strong secretion stress responses produced AmyQ at high levels (Fig. 2A; ● and ▲), whereas the one strain of this type that displayed a very mild secretion stress response produced AmyQ at a low level (Fig. 2A; ●). Significantly, the intensity of the secretion stress response seems to correlate better to the cellular levels than to the secreted levels of AmyQ. In conclusion, these findings show that, irrespective of the presence or absence of TepA, the intensity of the secretion stress response in cells of a particular AmyQ producing strain is indicative for the level of AmyQ production by this strain.

3.3 Overexpression of htrA or htrB has no effect on AmyQ production

It was previously shown that the induction of the secretion stress response in cells producing high amounts of AmyQ results in the synthesis of HtrA and, most likely, HtrB at significantly elevated levels (Antelmann et al., 2003). The induced expression of htrA and htrB was interpreted as a consequence of the triggering of the CssRS system by misfolded AmyQ (Darmon et al., 2002; Hyyrylainen et al.,
Figure 2. Correlation between AmyQ production levels and the intensity of the secretion stress response

(A) Cellular response to secretion stress. A transcriptional htrB-lacZ gene fusion was used to determine the time courses of htrB expression in cells producing AmyQ directed by pKTH10. Freshly constructed pKTH10 transformants were kept on plates for 15 days at room temperature. All strains were grown overnight at 37 °C in TY medium with or without xylose. Subsequently, the cultures were diluted to an OD_{600} nm of 0.05 in fresh TY medium with or without xylose and incubated at 37 °C. Strains included in the analyses were: B. subtilis htrB pKTH10 (■), htrB tepA^1 pKTH10 and htrB tepA^2 pKTH10 (○ and ▲, respectively) from two independent experiments, htrB tepA X-tepA pKTH10 - xylose (●), htrB tepA X-tepA pKTH10 + xylose (▲), and htrB tepA X-tepA pKTH10 ++ xylose (●) (++ xylose indicates that the strain was kept on plates supplemented with 1 % xylose and grown in the presence of 1 % xylose). The parental strains B. subtilis 168 (○) and B. subtilis htrB (▲) were used as negative controls. A B. subtilis htrB strain, freshly transformed with pKTH10, was used as a positive control (htrB control, x). Samples for the determination of β-galactosidase activities (indicated in nmol/min/OD_{600}) were withdrawn at the times indicated. Zero time (t = 0) indicates the transition point between the exponential and post-exponential growth phases.

(B) AmyQ production and secretion. Cells of B. subtilis htrB, htrB tepA^1 and htrB tepA^2 (two independent experiments), htrB tepA X-tepA - xylose, htrB tepA X-tepA + xylose, htrB tepA X-tepA ++ xylose, all containing pKTH10 for AmyQ production, were harvested after 3 hours of post-exponential growth (t_3) in the experiment described in
Although this is the most likely explanation, the possibility that increased levels of HtrA and HtrB synthesis are required to produce high levels of AmyQ could not be ruled out. To test this possibility, experiments were performed to determine the influence of HtrA or HtrB overexpression on the production of AmyQ. For this purpose, *B. subtilis* 168 X, *B. subtilis* htrA (BV2043), and *B. subtilis* htrB (BV2044) were transformed with the plasmid pKTH10L. The latter plasmid was used instead of pKTH10, because it is more stably maintained, which is probably due to the fact that it directs AmyQ production at moderate levels (Hyyrylainen et al., 2001). Overnight cultures were diluted in fresh medium with and without xylose, and growth was continued for 8 hours. The proteins in the medium fractions were precipitated with TCA, and AmyQ production and secretion was visualised by Western blotting. The results show that overexpression of neither htrA nor htrB affects the levels of produced and secreted AmyQ (Fig. 3). These observations imply that elevated levels of HtrA or HtrB are a consequence of, rather than a prerequisite for, high level AmyQ production. Thus, the expression levels of htrA and, as documented in this paper, htrB can be regarded as genuine indicators for the AmyQ production levels.

4. Discussion

The focus of the present studies was to investigate the extent to which the secretion stress response is correlated with α-amylase AmyQ production in *B. subtilis* in relation to the function of the TepA protein. The results indicate that the intensity of the secretion stress response reflects the AmyQ production level. The analysis of TepA function was hampered by two factors: firstly, the tepA deletion strain displayed a phenotype different from that of the original TepA depletion strain (Bolhuis et al., 1999a); and secondly, the genetic instability of plasmid pKTH10 that was used for overproduction of AmyQ. The latter phenomenon was, however, successfully exploited as a tool to monitor the relationship between the intensity of the secretion stress response and the level of AmyQ production. The resulting data have important biotechnological implications in relation to the discrimination between low- and high-level producing strains.
The reason why tepA deletion strains fail to display an AmyQ secretion defect is currently not clear. Notably, a great deal of effort was required to obtain two independent isolates of B. subtilis tepA, both of which lacked the TepA depletion phenotype of B. subtilis ItepA, as described by Bolhuis et al. (1999a). This suggests that a suppressor mutation could be required to obtain viable tepA deletion strains. An alternative explanation would be that downstream sequences of the tepA gene are somehow involved in the AmyQ secretion defect that is observed upon repressed tepA transcription in B. subtilis ItepA. Irrespective of the precise function of TepA, our present data indicate that this protein is dispensable for protein secretion stress management.

Plasmid pKTH10, which was used for high-level AmyQ production in B. subtilis, was found to be structurally unstable. This was reflected by the spontaneous emergence of variants that directed AmyQ production at lower levels, irrespective of the presence or absence of an intact tepA gene. Apparently, cells producing AmyQ at a reduced level have a selective growth advantage compared to cells containing the authentic pKTH10. In a population of cells, this will lead to a rapid replacement of the latter plasmid by mutant variants directing AmyQ production at lower levels. Moreover, the presence of pKTH10 was shown to have a negative impact on sporulation (unpublished observations). Consequently, a negative selection for the authentic pKTH10 will occur upon sporulation. This may provide an additional explanation for our observation that strains with a long history of cultivation display reduced levels of AmyQ production. Importantly, variants of pKTH10 that direct low-level production of AmyQ provoke a secretion

**Figure 3. No influence of overproduced HtrA or HtrB on AmyQ production**

Cells of B. subtilis 168 X, 168 X-htrA, and 168 X-htrB, all containing pKTH10L for AmyQ production, were grown in LB medium at 37 °C in the absence (-) or presence (+) of 1 % xylose for the induction of the xylA promoter. Cells (C) were harvested after 2 hours of post-exponential growth, and separated from the growth medium (M) by centrifugation. Proteins in the growth medium were concentrated 8-fold upon precipitation with 5 % trichloroacetic acid (TCA). AmyQ was visualised by SDS-PAGE, Western blotting, and immunodetection, using AmyQ-specific antibodies. The AmyQ-specific band is indicated; note that several degradation products of AmyQ are detectable.

The reason why tepA deletion strains fail to display an AmyQ secretion defect is currently not clear. Notably, a great deal of effort was required to obtain two independent isolates of B. subtilis tepA, both of which lacked the TepA depletion phenotype of B. subtilis ItepA, as described by Bolhuis et al. (1999a). This suggests that a suppressor mutation could be required to obtain viable tepA deletion strains. An alternative explanation would be that downstream sequences of the tepA gene are somehow involved in the AmyQ secretion defect that is observed upon repressed tepA transcription in B. subtilis ItepA. Irrespective of the precise function of TepA, our present data indicate that this protein is dispensable for protein secretion stress management.

Plasmid pKTH10, which was used for high-level AmyQ production in B. subtilis, was found to be structurally unstable. This was reflected by the spontaneous emergence of variants that directed AmyQ production at lower levels, irrespective of the presence or absence of an intact tepA gene. Apparently, cells producing AmyQ at a reduced level have a selective growth advantage compared to cells containing the authentic pKTH10. In a population of cells, this will lead to a rapid replacement of the latter plasmid by mutant variants directing AmyQ production at lower levels. Moreover, the presence of pKTH10 was shown to have a negative impact on sporulation (unpublished observations). Consequently, a negative selection for the authentic pKTH10 will occur upon sporulation. This may provide an additional explanation for our observation that strains with a long history of cultivation display reduced levels of AmyQ production. Importantly, variants of pKTH10 that direct low-level production of AmyQ provoke a secretion
stress response of limited intensity, which is in line with the view that production of high levels of AmyQ is stressful for *B. subtilis* cells.

Finally, the observation that the intensity of the CssRS-dependent secretion stress response can be correlated to the level of AmyQ production opens up the attractive possibility to use this stress response as an indicator for the production of secretory proteins by the *Bacillus* cell factory. Thus, *B. subtilis* strains with *htrA-lacZ* or *htrB-lacZ* transcriptional gene fusions can serve as highly useful tools for the rapid prediction of protein production levels, especially in those cases that involve secretory proteins for which no convenient assays are available. Importantly, this would also allow the identification and elimination of strains that display reduced production levels for secretory proteins. To further explore the application potential of *htrA* and *htrB* expression-based indicator strains, it will be important to define the range of proteins that can trigger the CssRS-dependent secretion stress response upon export from the cytoplasm.

**Acknowledgements**
The authors wish to thank Albert Bolhuis, Roland Freudl, Oliver Koeberling, Isabel Pérez-Arellano, Harold Tjalsma and other members of the Groningen and European *Bacillus* Secretion Groups for valuable discussions. Funding for the project, of which this work is a part, was provided by the CEU projects BIO4-CT98-0250, QLK3-CT-1999-00413, QLK3-CT-1999-00917, and LSH-503468. E.D. was supported by the Ubbo Emmius foundation of the University of Groningen. G.Z. was supported by the Stichting Technische Wetenschappen project VBI.4837.