Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the \textit{Bacillus subtilis} expression system

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\(\text{INTRODUCTION}\)

\textit{Bacillus subtilis} and the closely related bacilli \textit{Bacillus amyloliquefaciens} and \textit{Bacillus licheniformis} are capable of secreting enzymes into the culture supernatant at concentrations in excess of 10 g L\(^{-1}\) (Ferrari et al., 1993), and these species are currently used in the large-scale manufacture of industrial enzymes. However, attempts to use \textit{B. subtilis}, for which an extensive knowledge of physiology and molecular biology exists, to secrete heterologous proteins at commercially significant concentrations have, with few exceptions, been disappointing (Harwood, 1992).

Today’s industrial enzymes are frequently subjected to protein engineering to obtain products which exhibit improved characteristics with respect to specific industrial applications. Whilst such approaches may result in enzymes with significantly improved enzymic performance under application conditions, the introduced modifications are not always beneficial with regard to production and secretion from the intended host strain. It is not uncommon for the development of new products with commercial potential to be abandoned due to adverse production economics. One factor contributing to low yields is proteolytic degradation of secreted enzymes by extracellular proteases produced by the host. Strains in which genes encoding extracellular proteases have been deleted have been successful in reducing the proteolysis of certain products in the culture medium (Sloma et al., 1989, 1991; Wu et al., 1991), although the integrity of such strains can be compromised (Stephenson et al., 1999). An alternative approach has been to identify protease-labile sites in the product and to modify these sites by protein engineering (Boguslawski et al., 1996). However, these approaches may only improve the yield of products which are already efficiently secreted.

Conceptually, the \textit{Bacillus} secretory pathway (Simonen & Palva, 1993) can be divided into three functional stages: (i) early stages involving the synthesis of secretory pre-proteins, their interaction (if any) with chaperones and binding to the secretory translocase; (ii) translocation across the cytoplasmic membrane via the

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A series of chimeric \(\alpha\)-amylase genes derived from \textit{amyL}, which encodes the liquefying \(\alpha\)-amylase from \textit{Bacillus licheniformis}, were constructed \textit{in vitro} using gene splicing techniques. The gene constructs were cloned in \textit{Bacillus subtilis}, where their ability to direct the synthesis and secretion of active \(\alpha\)-amylase was determined. Detectable \(\alpha\)-amylase activity was observed for some, but not all, of the chimeric proteins. Studies on the secretion of wild-type \textit{AmyL} and its chimeric derivatives revealed that, whilst these proteins were stable in the extracellular milieu, all were subject to some degree of degradation during secretion. The chimeric enzymes were degraded to a greater extent than the native enzyme. These findings suggest that cell-associated proteolysis is a significant problem affecting the use of \textit{B. subtilis} as host bacterium for the production of heterologous proteins.

Keywords: \textit{Bacillus subtilis}, chimeric proteins, protein secretion, proteolysis, industrial enzymes
translocase; (iii) late stages, including removal of the signal peptide, release from the translocase, folding on the trans side of the cytoplasmic membrane and passage through the cell wall.

A rate-limiting step in the secretion of certain proteins from \textit{B. subtilis} is release from the cytoplasmic membrane and concomitant folding into the native conformation (Petit-Glatron \textit{et al}., 1993; Chambert \textit{et al}., 1995; Bolhuis \textit{et al}., 1999a). The folding step is modulated by ions such as Ca\(^{2+}\), Fe\(^{3+}\) and H\(^{+}\) at the outer surface of the cytoplasmic membrane (Chambert \textit{et al}., 1995; Leloup \textit{et al}., 1997) which promote the rapid folding of the secretory proteins. In addition, folding may be assisted by PrsA, a lipoprotein located at the outer surface of the cytoplasmic membrane (Kontinen & Sarvas, 1993). Although its precise role in secretion is not clear, it is thought to act as an extracellular chaperone, assisting the folding of a subset of secretory proteins as they emerge from the translocase on the trans side of the cytoplasmic membrane (Kontinen \& Sarvas, 1993; Jacobs \textit{et al}., 1993). In addition to these extrinsic factors, the efficiency of secretory protein folding is also dependent on the characteristics of the individual secretory proteins.

To facilitate studies on the influence of secretory protein charge on passage through the negatively charged cell wall of \textit{B. subtilis}, we have constructed a series of chimeric \(\alpha\)-amylases with different isoelectric points (pl). AmyL, the \(\alpha\)-amylase of \textit{B. licheniformis} (Ortlepp \textit{et al}., 1983; Laoide \textit{et al}., 1989) is efficiently expressed and secreted in \textit{B. subtilis}, and we took advantage of the observations that functional chimeric proteins could be constructed using \(\alpha\)-amylases from \textit{B. licheniformis} (AmyL), \textit{B. amyloliquefaciens} (AmyQ) and \textit{Bacillus stearothermophilus} (AmyS) (Gray \textit{et al}., 1986; Diderichsen \textit{et al}., 1987; Jørgensen \textit{et al}., 1990) to construct genes encoding chimeric \(\alpha\)-amylases with modified net charge. This was achieved by replacing segments of the \textit{amyL} gene with corresponding segments from the \textit{amyQ} and \textit{amyS} genes. This paper reports the design and construction of genes encoding chimeric \(\alpha\)-amylases with altered pl values, and the enzymic characterization and production in terms of transcription and yield of their protein products.

Pulse–chase and immunoprecipitation techniques were used to follow the secretion of wild-type and chimeric enzymes from \textit{B. subtilis}, as well as the secretion of wild-type AmyL from \textit{B. licheniformis}. These studies revealed a significant cell-associated degradation of amylase synthesized in \textit{B. subtilis}, a degradation which was not observed when wild-type AmyL was produced in \textit{B. licheniformis}.

**METHODS**

**Bacterial strains and growth conditions.** \textit{Escherichia coli} S\textsubscript{J}2 (Diderichsen \textit{et al}., 1990) and \textit{E. coli} XL-1 Blue (Stratagene) were grown at 37 °C in TY medium (Diderichsen \textit{et al}., 1990). \textit{B. subtilis} DN1885 (amy\textsubscript{E} amy\textsubscript{R}2), a derivative of \textit{B. subtilis} 168 strain RUB200 (Yoneda \textit{et al}., 1979), was grown at 37 °C in TY medium containing 0-4 % (w/v) glucose. \textit{B. licheniformis} ATCC 9789 was obtained from the American Type Culture Collection, Manassas, VA, USA.

Strains of \textit{B. subtilis} DN1885 harbouring plasmids were maintained on LB agar plates (Maniatis \textit{et al}., 1982) supplemented with 0-4 % glucose and 6 µg chloramphenicol ml\(^{-1}\). Strains of \textit{E. coli} S\textsubscript{J}2 harbouring plasmids were maintained on LB agar plates supplemented with 100 µg ampicillin ml\(^{-1}\). To compare the yields of \(\alpha\)-amylase released from \textit{B. subtilis}, the strains were also grown in a complex liquid medium (Jørgensen \textit{et al}., 1991b) for 7 d at 37 °C with shaking. For pulse–chase experiments, the \textit{B. subtilis} strains were grown in Spizizen’s minimal medium (Spizizen, 1958) modified by the replacement of glucose with 1 % (w/v) ribose, a non-catabolite-repressing carbon source, and 1 % (w/v) xylose to induce the synthesis of \(\alpha\)-amylase.

The plasmids used throughout the work are given in Table 1.

**Construction of plasmids.** pSX63, containing a xylose-inducible \textit{amyL} gene, was used as the basis for the development of an expression system for the production of chimeric proteins. To facilitate the cloning of the engineered \(\alpha\)-amylase genes as \textit{Pst}I–\textit{Hind}III fragments, an unwanted \textit{Psfl} site between the \textit{cat} and \textit{xylR} gene was removed in three stages: (i) pSX63 was digested with \textit{ClaI} and the large fragment purified, religated and transformed into \textit{B. subtilis} DN1885, resulting in plasmid pKS200; (ii) pKS200 was digested at the now unique \textit{Psfl} site and the overhanging ends trimmed with \textit{S1} nuclease to form blunt ends. The blunt ends were ligated prior to transforming into \textit{B. subtilis} DN1885, resulting in plasmid pCJ72; (iii) finally, the \textit{EcoR}I–\textit{Hind}III \textit{amyL} fragment from pSX63 was cloned into the \textit{EcoR}I and \textit{Hind}III restriction sites in pCJ72 to produce pCJ92 (Fig. 1a).

To generate an \textit{E. coli}/\textit{B. subtilis} shuttle vector, the \textit{Sppl} to \textit{SacI} (positions 2501 to 683) fragment of pUC19, containing the origin of replication and \(\beta\)-lactamase gene (Yanisch-Perron \textit{et al}., 1985), was amplified by PCR using oligonucleotide primers with \textit{Bgl}II restriction sites incorporated into their 5\textsuperscript{′} ends. The PCR product was digested with \textit{Bgl}II and cloned into the unique \textit{Bgl}II site of pCJ92. The resulting plasmid, pKS301, was able to replicate in both \textit{E. coli} and \textit{B. subtilis} (Fig. 1b).

**DNA manipulation.** Plasmid DNA was prepared from strains of \textit{E. coli} and \textit{B. subtilis} by an alkaline-phenol extraction method (Kieser, 1984). Enzymes for restriction, ligation, end-filling and exonuclease treatments were used as recommended by the supplier (New England Biolabs). DNA fragments were purified from agarose gels using either Spin-X centrifuge tube filters (Costar) or a QIAquick Gel Extraction kit (Qiagen), as recommended by the manufacturers.

**Transformation.** Competent cells of \textit{B. subtilis} were prepared and transformed with plasmid DNA by the procedure of Yasbin \textit{et al}., (1975) and transformants selected on LB agar plates containing chloramphenicol (6 µg ml\(^{-1}\)).

\textit{E. coli} cells were electro-transformed in a Bio-Rad Gene Pulser according to the manufacturer’s instructions. Transformants were selected on plates of LB agar supplemented with 100 µg ampicillin ml\(^{-1}\).

**PCR.** Oligonucleotide primers used for the construction of genes encoding chimeric \(\alpha\)-amylases were synthesized on an Applied Biosystems DNA synthesizer and purified before use. \textit{Tag} DNA polymerase (Super\textit{Taq}, HT Biotechnology) was used for PCR amplifications according to the supplier’s instructions. The reaction cycles were as follows: 1 × (2 min at
94 °C); pause for the addition of 0.5 μl SuperTaq DNA polymerase; 15 x (30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C); 1 x (5 min at 72 °C).

Splicing by overlap extension (SOE; Horton et al., 1989), a PCR-based in vitro method, was used for fusing specific fragments of DNA with the need for restriction sites. Briefly, four oligonucleotide primers were synthesized; primers at the fusion junction included non-annealing extensions at their 5’ ends which were complementary to each other, whilst the flanking primers were complementary to their respective template DNA. The DNA regions were amplified in separate PCR reactions and the products mixed.

Because SOE steps was dependent on the number of fragments purified from agarose gels using Spin-X columns. The number of SOE steps was dependent on the number of fragments required for the construction of the final spliced product.

The following primers were used in the construction of amylQ55 chimera.

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<thead>
<tr>
<th>Primers</th>
<th>Characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pUC19</td>
<td>General purpose E. coli cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pSX63</td>
<td>Derivative of the S. aureus plasmid pUB110 containing the B. licheniformis amyl. gene</td>
<td>S. Hastrup, Novo Nordisk A/S</td>
</tr>
<tr>
<td>pDN1528</td>
<td>Derivative of pUB110 with the amyl. gene from B. subtilis xyl operon</td>
<td>Jørgensen et al. (1991a)</td>
</tr>
<tr>
<td>pDN1681</td>
<td>Derivative of pUB110 with the amylQ gene from B. amyloliquefaciens DN1552</td>
<td>B. Diderichsen, Novo Nordisk A/S</td>
</tr>
<tr>
<td>pPL1235</td>
<td>Derivative of pUB110 with the amylS gene from B. stearothermophilus DN1792</td>
<td>Jørgensen et al. (1990)</td>
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Table 1. Plasmids used in this work

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</table>

Chimeric block 1 was constructed using primers 50–1/50–4, and wild-type block 1 using 50/1+50/4. Chimeric block 2 was constructed using primers 50/5–50/8, and wild-type block 2 using 50/5+50/8. Chimeric block 3 was constructed using primers 50/9–50/14, and wild-type block 3 using 50/9+50/14. Chimeric block 4 was constructed using primers 50/15–50/18, and wild-type block 4 using 50/15+50/18. Chimeric block 5 was constructed using primers 50/19–50/24, and wild-type block 5 using 50/19+50/24.

The following primers were used in the construction of amylQ55 chimera sequence 5’–3’):

55/1, AAACCGGTCGCCCGCCGGATTGGCTGACG; 55/2, GCATATTGTACGAGTGTCGTCGAC; 55/3, GGAAAGCTTGGAGAGAGGAGGAG; 55/4, GGAAAATTCAAGGTGTCGAC; 55/5, GGGAAGCTTGGAGAGGAGGAG; 55/6, GGGAAGCTTGGAGAGGAGGAG; 55/7, GTGAAGCTTGGAGAGGAGGAG; 55/8, and wild-type block 4 using 55/15–55/50.

Chimeric block 3 was constructed using primers 55/10–50/1, and wild-type block 3 using 55/10+50/1. Chimeric block 4 was constructed using primers 55/15–50/18, and wild-type block 4 using 55/15+50/18. Chimeric block 5 was constructed using primers 55/19–50/24, and wild-type block 5 using 50/19+50/24.

The following primers were used in the construction of amylQ55 chimera sequence 5’–3’):

55/1, AAACCGGTCGCCCGCCGGATTGGCTGACG; 55/2, GCATATTGTACGAGTGTCGTCGAC; 55/3, GGAAAGCTTGGAGAGAGGAGGAG; 55/4, GGAAAATTCAAGGTGTCGAC; 55/5, GGGAAGCTTGGAGAGGAGGAG; 55/6, GGGAAGCTTGGAGAGGAGGAG; 55/7, GTGAAGCTTGGAGAGGAGGAG; 55/8, and wild-type block 4 using 55/15–55/50.

Chimeric block 3 was constructed using primers 55/10–50/1, and wild-type block 3 using 55/10+50/1. Chimeric block 4 was constructed using primers 55/15–50/18, and wild-type block 4 using 55/15+50/18. Chimeric block 5 was constructed using primers 55/19–50/24, and wild-type block 5 using 50/19+50/24.

The following primers were used in the construction of amylQ55 chimera sequence 5’–3’):

55/1, AAACCGGTCGCCCGCCGGATTGGCTGACG; 55/2, GCATATTGTACGAGTGTCGTCGAC; 55/3, GGAAAGCTTGGAGAGAGGAGGAG; 55/4, GGAAAATTCAAGGTGTCGAC; 55/5, GGGAAGCTTGGAGAGGAGGAG; 55/6, GGGAAGCTTGGAGAGGAGGAG; 55/7, GTGAAGCTTGGAGAGGAGGAG; 55/8, and wild-type block 4 using 55/15–55/50. 55/9, GTGAAGCTTGGAGAGGAGGAG; 55/10, and wild-type block 5 using 55/19+50/24.

Chimeric block 1 was constructed using primers 55/1–55/4, and wild-type block 1 using 55/1+55/4. Chimeric block 2
<table>
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<th>Block 2 using 55 primers using primers 55</th>
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<td>The various encodes the elements required for the inducible expression of</td>
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<td>and pKS301 (b). The large</td>
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<td>B. subtilis). The shuttle vector pKS301 was</td>
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<tr>
<td>Vectors pCJ92 (a) and pKS301 (b). The large \textit{EcoR}I–\textit{Bgl} II DNA fragment of pCJ92 was constructed using a fragment containing the origin of replication and the \textit{\beta}-lactamase gene from pUC19 into the unique \textit{Bgl} II restriction site.</td>
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<td>DNA sequencing. DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase 2.0 kit (United States Biochemicals) and [\textsuperscript{35}S]dATP;S [&gt; 1000 Ci mmol(^{-1}) (37 TBq mmol(^{-1})); DuPont NEN].</td>
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<td>\textit{\alpha}-Amylase assay. \textit{\alpha}-Amylase activity was determined using the Phadebas Amylase Test kit (Pharmacia Upjohn), using the protocol provided with the assay kit and purified AmyL as a standard. One unit of activity was defined as the amount of enzyme catalysing the hydrolysis of 1 \mu mol glycoside linkage min(^{-1}) at 37 °C.</td>
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<td>Dot-blot hybridization of \textit{\alpha}-amylase-specific mRNA. Cultures of \textit{B. subtilis} were grown in 30 ml Speizen’s minimal medium with 1% ribose as the carbon source and 1% xylose to induce the synthesis of \textit{\alpha}-amylase. Samples were removed for the determination of growth and \textit{\alpha}-amylase activity, and for the isolation of total RNA.</td>
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<td>RNA was extracted from the cells of 1 ml culture samples using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. RNA samples were stored at −75 °C in the presence of RNase inhibitor (RNasin; Promega). Contaminating DNA was removed with RNase-free DNase I (Boehringer Mannheim). RNA was quantified by spectrometry (Gene-Quant II; Pharmacia Upjohn). For each sample, 3 \mu g total RNA was dot-blotted onto a positively charged nylon membrane (Boehringer Mannheim) using standard protocols (Sambrook et al., 1989).</td>
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<td>The DNA probes, corresponding to common regions in the 5' ends of \textit{amyL}, \textit{amyLQS50} and \textit{amyLQS55}, were amplified from \textit{amyL} by PCR. Probe 1, used to probe RNA from strains DN1885 \textit{xyl}R::pKS401 (expressing AmyL) and DN1885 \textit{xyl}R::pKS405B (expressing AmyLQS50-5), was amplified using oligonucleotide primers LQS50/27 (5’-G\textsuperscript{+}GTG GCT TAC GAG CTT TAT \textsuperscript{3}G-3’) and LQS50/28 (5’-AA\textsuperscript{−}TGA AAA TG\textsuperscript{−}GTG TGC CAG GC-3’). Probe 2, used to probe RNA from strains DN1885 \textit{xyl}R::pKS401 (expressing AmyL), DN1885 \textit{xyl}R::pC\textsuperscript{−}CJ201 (expressing AmyLQS55-3) and DN1885 \textit{xyl}R::pC\textsuperscript{−}CJ272 (expressing AmyLQS55-6), was amplified using oligonucleotide primers KS15 (5’-ACT GCA ATT GGA CGG TTT CCG \textsuperscript{−}G-3’) and KS16 (5’-CTG TGT CGA TGC AGC ATG G-3’).</td>
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<td>The probes were radiolabelled with [\textsuperscript{32}P]dATP (Amersham) using the Rediprime kit (Amersham) and were hybridized to immobilized RNA as described by Sambrook et al. (1989). The amount of probe bound to total RNA samples was quantified by phosphor-imaging (Phosphor-Imager; Molecular Dynamics) and analysed using the ImageQuant software (version 3.22; Molecular Dynamics).</td>
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<tr>
<td>Pulse–chase labelling and immunoprecipitation. Cultures of \textit{B. subtilis} growing exponentially to an optical density at 660 nm of approximately 0.5 were pulse-labelled with L-\textsuperscript{\textsuperscript{35}S}methionine as described previously (Jensen, 1997; Stephenson &amp; Harwood, 1998) and samples removed at the following time intervals: 0, 0.5, 1, 2, 3, 4, 5, 10 and 15 min. Labelled \textit{\alpha}-amylases in whole-culture samples and released into the growth medium [obtained by filtration through 0.45 um PVDF filters (Whatman)] were immunoprecipitated and subjected to SDS-PAGE and fluorography. The relative amounts of \textit{\alpha}-amylase present at each time interval were determined by phosphor-imaging (Phosphor-Imager; Molecular Dynamics) using the associated software (ImageQuant version 3.22; Molecular Dynamics).</td>
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</table>

\textbf{RESULTS}

\textbf{Computer-aided design of chimeric \textit{\alpha}-amylases with altered net charge}

Chimeric proteins with pI values higher (AmyLQS50) and lower (AmyLQS55) than that of AmyL were designed in silico (\texttt{pc/gene} version 5.16; Genofit) by swapping specific segments of the mature portion of AmyL with the corresponding segments from AmyQ or
AmyS (Palva, 1982; Takkinen et al., 1983; Gray et al., 1986; Jørgensen et al., 1991a). The segments were chosen so that each swap led to an increase or decrease in the pI, as calculated by the ChargPro programme in the PC/GENE software package. For example, the carboxy-terminal extension of AmyS includes a large number of positively charged amino acid residues so the inclusion of this ‘tail’ in the chimeric α-amylase AmyLQS50 increases its predicted pI.

A strategy was developed that involved a relatively small number of segment swops to generate variants of AmyL, AmyLQS50 and AmyLQS55, with predicted pI values of 6–0, 8–1 and 4–7, respectively (Fig. 2).

**Construction of genes encoding chimeric α-amylases**

The native amyl gene has an unique PstI site located within the signal peptide coding region, and an unique HindIII site downstream (3′) of its transcription terminator. Consequently, the chimeric genes were designed as in-frame PstI–HindIII DNA fragments, and the AmyL signal peptide was used to direct the secretion of all the chimeric α-amylases and the reconstructed AmyL.
The chimeric genes were subdivided into blocks bordered by unique restriction sites already present in *amyL* (Fig. 2) and synthesized using a PCR-based gene splicing method (Horton et al., 1989). As well as naturally occurring unique restriction sites, the gene constructions were facilitated by additional sites introduced as silent mutations: a *Mlu*I site in the case of *amyLQS50* and a *Bam*HI site in the case of *amyLQS55*. *amyLQS50* and *amyLQS55* were constructed from five and four blocks, respectively, with regions of *amyL* being replaced by corresponding regions of either *amyQ* or *amyS* (Fig. 2). In addition to the chimeric DNA blocks, equivalent wild-type *amyL* blocks were also constructed. This allowed individual chimeric blocks to be replaced with the corresponding wild-type blocks as and when required.

Individual DNA blocks were cloned into pUC19 using the unique restriction sites generated at their ends. A number of independently isolated pUC19 clones were sequenced to detect errors generated during the PCR and to ensure the fidelity of the splicing reactions. *amyLQS55* block 2 included a base substitution that changed the expected amino acid residue at position 115 from alanine to glycine, whilst *amyLQS55* block 4 had two base substitutions changing the expected residues at positions 403 (from serine to alanine) and 485 (from glycine to serine). These changes were accepted since they were unlikely to affect the overall charge of the respective amylases.

**Assembly of chimeric *β*-amylase genes**

The individual DNA blocks were assembled stepwise in pUC19 to generate *Pst*I–*Hind*III gene fragments encoding the mature protein of the chimeric *β*-amylases. As illustrated in Fig. 2, a number of intermediate genes were assembled using combinations of chimeric and wild-type DNA blocks.

The assembled *amyLQS55* gene fragments were cloned into plasmid pCJ92 (Fig. 1a) using *B. subtilis* DN1885 as the host, whilst *amyLQS50* gene fragments were cloned into an *E. coli*/*B. subtilis* shuttle vector plasmid, pKS301 (Fig. 1b), using *E. coli* XL-1 Blue as an intermediate host prior to transfer to *B. subtilis* DN1885. In both cases, the assembled *Pst*I–*Hind*III gene fragments replaced the pre-existing wild-type *amyL* gene. The presence of a *Mlu*I or *Bam*HI restriction site within the cloned *α*-amylase genes was taken as evidence that the wild-type *amyL* gene had been replaced.

The *α*-amylase genes were cloned downstream of the promoter/operator (*P*O) from the *B. subtilis* *xyl* operon, which enabled *α*-amylase production to be induced in the presence of xylose. In the absence of xylose, repression is mediated by the XylR repressor, which is also encoded by the expression plasmids pCJ92 and pKS301 (Fig. 1).

The transformants were screened on starch plates for the secretion of a functional *α*-amylase by staining with iodine vapour. Neither of the strains encoding the full chimeric *α*-amylases AmyLQS50 and AmyLQS55 showed detectable *α*-amylase activities. Data on intermediate chimeric *α*-amylases indicated that the presence of block 1 in *amyLQS50* and block 2 in *amyLQS55* prevented the production of detectable amounts of *α*-amylase activity. Constructs in which these two blocks were replaced with wild-type blocks showed *α*-amylase activities that could be detected on the starch plates, although there were marked differences in the sizes of their zones of hydrolysis compared to that for the strain encoding AmyL (data not shown).

The plasmid-encoded expression systems (Fig. 1) were based on the replicon of the *Staphylococcus aureus* plasmid pUB110, which can be relatively unstable in *B. subtilis* (Bron & Luxen, 1985; Bron, 1991). A more stable expression system was obtained by integrating the *α*-amylase genes into the chromosome of *B. subtilis*. The integration vectors were constructed by cloning *EcoRI–BglII* fragments of pCJ92 and pKS301 (Fig. 1), containing *α*-amylase, *xylR* and *cat* genes, into pUC19. The resulting plasmids, which lack an origin of replication that is functional in *B. subtilis*, were transformed into *B. subtilis* DN1885 and chloramphenicol resistance was used to select for cells containing integrated plasmids. Integration occurred by single, homologous recombination between the plasmid and chromosomally encoded copies of the *xylR* gene, resulting in the tandem duplication of this gene. The integration events were verified by Southern blot hybridization (data not shown).

**Production of chimeric *β*-amylases in *B. subtilis***

*B. subtilis* DN1885 strains containing integrated chimeric constructs were grown in shake flasks for 7 d at 37 °C in a complex medium containing 0.2% xylose and 6 μg chloramphenicol ml⁻¹ (conditions chosen to mimic industrial fed-batch fermentations), and the accumulation of *α*-amylase in the culture medium was determined by Western blotting and by enzyme activity assays. The data confirmed that strains containing the full chimeric constructs encoding AmyLQS50 and AmyLQS55 produced no detectable amylase activity and that no amylase protein could be detected in the culture supernatant by Western blotting. The active variant with highest PI, AmyLQS50-5, was present in the fermentation medium at a concentration that was about half that of AmyL, whereas the active variant with lowest PI, AmyLQS55-6, was present at a concentration that was less than 1% that of AmyL (Table 2). Western blot analysis (data not shown) confirmed the results obtained from the enzyme activity assays: reduced amounts of chimeric *α*-amylases accumulated in the culture supernatants as compared to AmyL.

Following fermentation, stability of the chimeric enzymes in the spent culture medium was determined. The chimeric amylases were as stable as AmyL during incubation for more than 140 h, retaining more than 85% of their initial activity over this time. This indicates that the observed differences in accumulated *α*-amylase
**Table 2.** Amount of accumulated α-amylase secreted by *B. subtilis* DN1885 containing wild-type or chimeric α-amylase genes integrated into the chromosome

Strains were grown with shaking in complex medium supplied with chloramphenicol (6 µg ml⁻¹) and xylose (0.2%) for 7 d at 37 °C. Expression was driven by the *B. subtilis* xyl promoter/operator.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Protein</th>
<th>Specific activity [units (mg protein)⁻¹]*</th>
<th>α-Amylase accumulation</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>α-Amylase activity (% of AmyL)†</td>
</tr>
<tr>
<td>DN1885</td>
<td>xylR::pKS405B AmyLQS50-5</td>
<td>4,570</td>
<td>33</td>
</tr>
<tr>
<td>DN1885</td>
<td>xylR::pKS401/pCJ199 AmyL</td>
<td>6,530</td>
<td>100</td>
</tr>
<tr>
<td>DN1885</td>
<td>xylR::pCJ201 AmyLQS55-3</td>
<td>7,300</td>
<td>93</td>
</tr>
<tr>
<td>DN1885</td>
<td>xylR::pCJ272 AmyLQS55-6</td>
<td>10,500</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* Purification and specific activity determination are described elsewhere (Stephenson *et al.*, 2000).
† α-Amylase activity was determined in the culture medium using the Phadebas Amylase Test (Pharmacia Upjohn). The 100% AmyL value corresponded to an activity of 1000 units ml⁻¹.
‡ The amount of α-amylase protein was calculated using the specific activity of the α-amylase proteins.

**Fig. 3.** Transcriptional activity of *amyL* and chimeric α-amylase genes during growth. (a–c) *amyL* (c) and low pI variants *amyLQS55-6* (a) and *amyLQS55-3* (b) hybridized with probe 2. (d–e) *amyL* (e) and high pI variant *amyLQS50-5* (d) hybridized with probe 1. □, Growth; ◊, α-amylase specific activity, normalized to the OD₆₀₀ of the culture; bars, relative amounts of α-amylase-specific mRNA.
Fig. 4. Secretion of wild-type and chimeric α-amylases. (a, c, e, g) Autoradiographs from representative pulse–chase experiments. 'Whole culture' indicates α-amylase immunoprecipitated from whole-culture samples. 'Medium' indicates α-amylase immunoprecipitated from culture medium only. (b, d, f, h) Quantification by phosphor-imaging of α-amylases at intervals following the chase, expressed as relative amounts. □, Whole culture; ○, medium. (a, b) *B. subtilis* expressing wild-type AmyL; (c, d) *B. subtilis* expressing AmyLQ55S-3; (e, f) *B. subtilis* expressing AmyLQ55S-5; (g, h) *B. licheniformis* expressing AmyL.
were not due to increased susceptibilities of the folded forms of these enzymes to the extracellular proteases produced by *B. subtilis*. Selected chimeric amylases were purified and, relative to AmyL, no major differences were observed with respect to thermostability and pH and temperature profiles, whilst the measured pl values showed the desired differences, being 5.0, 7.0 and 10.0 for enzymes AmyLQS55-6, AmyL and AmyLQS50-5, respectively (Stephenson *et al.*, 2000).

The production of the chimeric α-amylases were also tested under other conditions, for example during growth in minimal medium and TY medium (data not shown). *B. subtilis* DN1885 strains encoding the chimeric α-amylases grew at identical growth rates as compared to the wild-type strain. However, when the yields of secreted α-amylases were compared, the strains encoding the chimeric α-amylases produced a significantly reduced level of α-amylase activity. This was observed for the production of α-amylase in both exponential and stationary phases.

**Transcription of genes encoding AmyL and chimeric enzymes**

Quantitative dot-blot hybridization was used to determine whether the observed variations in the amount of secreted chimeric enzymes were due to differences in the transcriptional activities of their genes. Although all the genes were transcribed from the same promoter system, differences in mRNA levels might have arisen from the unwitting incorporation of secondary structures or RNase processing sites during chimeric gene construction, or from variations in gene copy number. Cultures of *B. subtilis* DN1885, expressing AmyL or representative high and low pl α-amylases from chromosomally located expression cassettes, were sampled in exponential and stationary phases to determine α-amylase activities and to quantify α-amylase-specific mRNA. Separate probes were designed for genes encoding high and low pl α-amylases, using regions that were conserved in both *amyL* and the constructed genes.

The amount of α-amylase activity detected in the culture medium varied from <60 (the minimum detection limit) to >1400 enzyme units per culture OD unit whilst the relative amount of mRNA remained relatively constant, varying by no more than about twofold (Fig. 3). In the case of the strain encoding AmyLQS55-6 (Fig. 3a), it was never possible to detect any α-amylase activity, despite the fact that the gene was transcribed at a level similar to that of *amyL*. These data confirm that the reduced levels of α-amylase activity observed for the chimeric enzymes were due to post-transcriptional events.

**Pulse–chase analysis of wild-type and chimeric α-amylase secretion**

The secretion of the chimeric α-amylases was studied using a combination of pulse–chase and immuno-precipitation techniques. Strains of *B. subtilis* with chromosomally integrated α-amylase genes were used since they provided an expression system that was stable in the absence of antibiotic selection.

The precursor of AmyL was processed rapidly into the mature form (Fig. 4a, b); immediately after the addition of the chase (*T*₀ min) >70 % of the labelled AmyL was already in the mature form. The amount of labelled mature AmyL peaked at about 1 min after the addition of the chase and then declined until, at about 6 min, it reached a constant level at around 25 % of the total α-amylase synthesized during the pulse (Fig. 4a, b). This indicates that a significant fraction of newly synthesized AmyL was degraded during or shortly after translocation across the cytoplasmic membrane, and possibly before release into the culture medium. AmyL was detected in the culture medium within 1 min of the addition of the chase and reached a constant level after about 5 min (Fig. 4a, b). AmyL appeared to be stable once it had reached the culture medium.

For the low pl chimeric α-amylase, AmyLQS55-3, degradation of the newly synthesized protein was much more pronounced than was observed for AmyL (Fig. 4c, d). Approximately 90 % of the labelled α-amylase was rapidly degraded. As with AmyL, AmyLQS55-3 was detected in the culture supernatant after about 2 min of chase. Similar observations were made for the high pl chimeric α-amylase, AmyLQS50-5; newly synthesized AmyLQS50-5 was degraded to a similar extent as compared with the low pl α-amylase, AmyLQS55-3 (Fig. 4e, f).

The degradation of wild-type AmyL in *B. subtilis* might reflect the fact that this protein is not native to this strain. We therefore studied its secretion in *B. licheniformis* strain ATCC 9778 (Fig. 4g, h) and observed that, whilst the AmyL precursor was rapidly processed, the amount of newly synthesized α-amylase remained constant throughout the 15 min chase. This implies that newly synthesized AmyL is stable in its native host, *B. licheniformis*, and that the observed degradation in *B. subtilis* might reflect the fact that production of AmyL in *B. subtilis* takes place in a heterologous expression and secretion system.

**DISCUSSION**

Gene splicing techniques and the related genes from *B. amyloliquefaciens, B. licheniformis* and *B. stea
thermophilus* encoding liquefying α-amylases were used to construct a series of genes encoding α-amylases with pl values that were either higher or lower than the pl of the wild-type *B. licheniformis* amylase, AmyL. Whilst no activity was detected from either of the full chimeric constructs, in each case the replacement of one of the constituent blocks with a wild-type block led to the production of active enzymes. The purification and characterization of these enzymes is described elsewhere (Stephenson *et al.*, 2000).

Chimeric and wild-type α-amylases were expressed in *B. subtilis* from constructs in which the transcriptional and translational regulatory regions, including the signal peptide coding sequence, were identical. Additionally,
the amounts of α-amylase mRNA synthesized from the genes were comparable. Despite this, large differences were observed in terms of amylose activity and amylase protein accumulating in growth medium from strains expressing different chimeric enzymes. These differences were observed irrespective of the particular growth medium and growth stage investigated. The observed differences could not be ascribed to differences in susceptibility of the fully folded, secreted amylases to the *B. subtilis* extracellular proteases as both chimeric amylases and wild-type AmyL were fully stable during incubation in spent growth medium at 37 °C for at least 7 d. However, pulse–chase experiments showed that, even in the case of AmyL, substantial amounts of the newly synthesized enzymes were degraded within a few minutes of their synthesis. Because the fully folded proteins were stable in growth medium, it is likely that the observed degradation occurs in a cell-associated location, possibly before or during post-translocational folding into their native conformations. When the native *B. licheniformis* host was used, newly synthesized AmyL was stable. We therefore propose that the instability of AmyL and its chimeric derivatives in *B. subtilis* reflects different degrees of mismatch between their folding behaviour and this bacterium’s systems for screening the authenticity and/or fidelity of secreted proteins. The likely role of these screening systems is to prevent blockage of the presecretory protein translocases and/or the formation of protein aggregates within the matrix of the cell wall.

Subtilisin–alkaline phosphatase fusion proteins have been shown to be degraded extensively in a *prsA3* mutant of *B. subtilis* (Jacobs et al., 1993), presumably because the limited amount of the folding effector PrsA reduces the rate at which the fusion proteins are folded. Furthermore, increased amounts of PrsA have a positive effect on the accumulation of AmyL when the *amyL* gene is expressed at high rates from a multicopy plasmid. The increased AmyL accumulation coincides with a strong reduction in the extent to which newly synthesized AmyL is degraded in the PrsA-over-expressing strain, indicating that co- or post-translocational folding is an important factor for efficient secretion (Jensen, 1997). The increased cell-associated degradation observed for the chimeric enzymes is therefore likely to be a consequence of changes in their folding kinetics (Stephenson et al., 1998).

An intriguing observation, consistent with the notion that folding kinetics is an important parameter in determining secretory protein yield, was the finding that production of one of the chimeric α-amylases was significantly affected by growth temperature. The production of AmyLQS55-6, which at 37 °C was produced at about 1% of the level of AmyL, was increased 50-fold when the growth temperature was lowered to 26 °C (Jensen, 1997); at this temperature, AmyL production decreased twofold.

The *E. coli* outer-membrane protein A (*ompA*) has also been shown to be susceptible to proteolytic degradation during secretion from both *B. subtilis* and *Staphylococcus carnosus* (Meens et al., 1997). There were strong indications that the degradation of OmpA was caused by membrane- and/or cell-wall-associated protease(s). Whilst problems might have been expected when attempting to secrete an outer-membrane protein from a Gram-negative bacterium in a Gram-positive bacterium, our observations that a protein from a closely related species is also susceptible to cell-associated degradation are more surprising, and point to an efficient mechanism for detecting and removing non-native protein structures. We have recently shown that CWBP52, a cell-wall-associated serine protease, is responsible for only 15% of the observed cell-associated degradation of AmyL (Stephenson & Harwood, 1998; Bolhuis et al., 1999b). This and other wall- or membrane-associated proteases may act to prevent defective or foreign proteins from blocking secretory translocases, a potentially lethal event.

An implication of these studies is that, even for apparently robust *Bacillus* enzymes such as AmyL, cell-associated degradation is a factor limiting the yield of secretory proteins and is an important parameter that needs to be addressed when *Bacillus* strains are being optimized for high-level production systems. Our work also demonstrates that engineering changes in native proteins, for example to optimize their enzymic properties for a specific industrial application, could have secondary effects that reduce their suitability for secretion into the culture medium. Therefore, when protein engineering is used to modify the specific characteristics of a enzyme of commercial interest, it should be borne in mind that the introduced modifications have to be compatible with the secretion system of the production host. Even small modifications within an enzyme can dramatically affect the yield of secreted product.

**ACKNOWLEDGEMENTS**

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Cell-associated degradation of chimeric α-amylases


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