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Non-functional expression of Escherichia coli signal peptidase I in Bacillus subtilis

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The Escherichia coli lep gene, encoding signal peptidase I (SPase I) was provided with Bacillus subtilis transcription/translation signals and expressed in this organism. When present on a low-copy-number plasmid, the amount of E. coli SPase I produced (per mg cell protein) in B. subtilis was half that produced in wild-type E. coli cells. The production of E. coli SPase I in B. subtilis was increased approximately fivefold by cloning the gene, encoding signal peptidase I (SPase I) and the cellular export machinery (for reviews, see Saier et al., 1989). So far, three different chaperones have been described in E. coli: (1) SecB, (2) GroEL and (3) GroES (see Kusukawa et al., 1989; Lecker et al., 1989). Other cellular components required for protein translocation across the cytoplasmic membrane in E. coli are SecA, SecD, SecE and SecY (see Saier et al., 1989). The final step in protein translocation is the release of the mature part of the protein from the membrane, which requires the proteolytic removal of the signal peptide. This processing step is catalysed by signal peptidases (SPases; for review, see Ray et al., 1986). Two E. coli SPases have been cloned and sequenced: SPase I (synonymous for leader peptidase) is encoded by the lep gene (Date & Wickner, 1981; Wolfe et al., 1983a) and SPase II (synonymous for prolipoprotein signal peptidase) is encoded by the lsp gene (Innis et al., 1984; Yu et al., 1984). SPase I is responsible for the processing of the precursor of bacteriophage M13 coat protein and the majority of exported pre-proteins (Dalbey & Wickner, 1985; Wolfe et al., 1982). SPase II exclusively processes glycerolipid-modified lipoproteins (Tokunaga et al., 1982; Yamada et al., 1984).

Both SPase I and II are essential for viability, as demonstrated in mutant strains of E. coli producing low levels of either SPase I or II under certain conditions.

Introduction

The efficiency of bacterial protein export depends on properties of both the exported protein (for reviews, see Bankaitis et al., 1987; Pollit & Inouye, 1987) and the cellular export machinery (for review, see Saier et al., 1989).

Exported proteins are usually synthesized as precursors with an N-terminal extension, called the signal peptide (Watson, 1984). This signal peptide is involved in guiding the protein into the export pathway by interacting with the membrane and other components of the cellular export machinery (for reviews, see Pollit & Inouye, 1987; Randall & Hardy, 1989; Saier et al., 1989). Moreover, the signal peptide retards the folding of the precursor, which appears to be essential for export-competence (Laminet & Plückthun, 1989; Liu et al., 1988; Park et al., 1988; for review, see Saier et al., 1989). Cytoplasmic components, denoted as chaperones, are required for maintaining the export-competence of precursor proteins, either by stabilizing an unfolded conformation (see Saier et al., 1989), or by preventing their aggregation (Lecker et al., 1990; Mitraki & King, 1989).
(Dalbey & Wickner, 1985; van Dijl et al., 1988; Yamagata et al., 1982). SPase limitation resulted in the accumulation of precursors of exported proteins.

Recently, we demonstrated that the availability of SPase I in E. coli could also be limiting under standard conditions (van Dijl et al., 1991a): certain (hybrid) precursors of TEM-β-lactamase fused to signal peptides which had been randomly selected from the B. subtilis chromosome were inefficiently processed (Smith et al., 1987, 1988, 1989; H. Smith and others, unpublished; van Dijl et al., 1991b). The processing and export efficiencies of several of these precursors could be considerably improved by SPase I overproduction (van Dijl et al., 1991a). In contrast, the processing rates of wild-type pre-β-lactamase and hybrid precursors, already high under standard conditions, were not detectably altered by SPase I overproduction.

In B. subtilis the processing of certain hybrid precursors containing randomly selected signal peptides was not efficient either (H. Smith and others; unpublished; van Dijl et al., 1991b). We reasoned that the availability of SPase I might also be a limiting factor in the processing of these precursors in this organism. However, since lep genes from bacilli have not been cloned so far, it was not possible to address this question directly in a homologous system. Since B. subtilis-exported proteins are usually efficiently processed in E. coli, we reasoned that the E. coli SPase I might function in B. subtilis. This consideration prompted us to study the effects of E. coli SPase I production on the processing of export proteins in B. subtilis. The results show that, although the E. coli SPase I was produced by B. subtilis, in vivo processing efficiencies were not increased.

Methods

Bacteria and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and plates. TY medium contained (g l⁻¹): Bacto tryptone, 10; Bacto yeast extract, 5; and NaCl, 10. S7 medium used in the pulse-chase labelling of B. subtilis DB114(pSB-A2d) was made according to Vasantha & Freese (1980) with the modification that MOPS was replaced by 20 mM-potassium phosphate (S7 medium-1). Since the synthesis of B. licheniformis α-amylase is subject to glucose repression in B. subtilis, the synthesis of this protein was achieved by growth of B. subtilis 8G5(amy)(psA13) in S7 medium-2, in which glucose was replaced by 2% (w/v) starch (Merck) (H. Smith and others, unpublished). S7 media-3 and -4 were methionine-free variants of S7 media-1 and -2, respectively. In S7 medium-4 the amount of starch was reduced to 0·1% (w/v). If required, the media were supplemented with chloramphenicol (2 μg ml⁻¹), erythromycin (2 μg ml⁻¹) and kanamycin (10 μg ml⁻¹).

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent E. coli cells were carried out as described in Maniatis et al. (1982). Transformation of competent B. subtilis cells was performed as described by Bron & Venema (1972). Enzymes (Boehringer) were used as indicated by the supplier.

Western blot analysis. The expression of SPase I was assayed by Western blotting (Towbin et al., 1979) on nitrocellulose membranes (BA 85, Schleicher and Schuell). SPase I production was monitored with specific antibodies (obtained from Dr R. Zimmermann, Universität München, FRG) and subsequent visualization of the bound antibodies was achieved with alkaline phosphatase anti-rabbit IgG conjugates (Protoblot, Western Blot AP system, Promega). Reference SPase I was purified from an overproducing strain as described by Wolfe et al. (1983b).

Protein assay. Protein was quantified by the method of Bradford (1976).

Pulse-chase protein labelling. Exponential phase cells in S7 medium-1 or -2 were washed once with methionine-free S7 medium-3 or -4, respectively, and incubated for 45 min at 37 °C in these media. Labelling with [35S]methionine (1330 Ci mmol⁻¹, 49·2 TBq mmol⁻¹; Amersham) for the times indicated, chasing with excess (2·5 mg ml⁻¹) non-radioactive methionine and sampling, followed by the immediate precipitation of proteins with trichloroacetic acid (TCA) at 0 °C, were performed as described previously (van Dijl et al., 1988). Precipitates were resuspended in 100 μl 10 mM-Tris/HCl (pH 8·0), 25 mM-MgCl₂, 200 mM-NaCl and 5 mg lysozyme ml⁻¹ (Boehringer). After 10 min at 37 °C, lysis was completed by the addition of 10 μl 10% (v/v) SDS and heating for 10 min at 70 °C.

Immunoprecipitation, SDS-PAGE and fluorography. Immunoprecipitation was carried out as described by Edens et al. (1982) with specific antisera. SDS-PAGE was performed according to Laemmli (1970). 

14C-Methylated molecular mass reference markers were obtained from American. Fluorography was performed as described by Skinner & Griswold (1983). Relative amounts of radioactivity (pulse-chase experiments), or of alkaline phosphatase staining (Western blot analysis) were estimated by densitometer scanning with an LKB Ultrascan XL enhanced laser densitometer.

Isolation of B. subtilis membranes. Exponential phase cells in TY medium were concentrated 40-fold in 100 mM-potassium phosphate buffer (pH 6·6) and 10 mM-EDTA. The cell suspension was incubated for 10 min at 37 °C in the presence of lysozyme (1 mg ml⁻¹). Lysis was completed using a French press at 6000 p.s.i. (41·3 MPa) (0 °C). Unlysed cells were removed by low-speed centrifugation (SW 50.1 rotor, 1500 r.p.m., 15 min, 4 °C). Membranes were subsequently separated from cytoplasmic contents by centrifugation (150000 g, 1 h, 4 °C) and resuspended in one-third vol. of the same buffer, with or without 1% (v/v) Triton X-100.

Spheroplasting of E. coli. Exponential phase cells of E. coli in TY medium were concentrated 10-fold in spheroplast-buffer [30 mM-Tris/HCl buffer (pH 8·0), 20% (w/v) sucrose, 10 mM-EDTA, 0·5 mg lysozyme ml⁻¹] and incubated for 30 min at 37 °C. Spheroplasts were directly used for further experiments.

Protoplasting of B. subtilis. Exponential phase B. subtilis DB114-15 cells in TY medium were concentrated 10-fold in spheroplast-buffer and incubated for 30 min at 37 °C. Protoplasts were directly used for further experiments.

In vitro transcription/translation. 35S-Labelled precursors of the bacteriophage M13 coat protein and the outer-membrane protein PhoE were synthesized in vitro as described by de Vrije et al. (1987).

In vitro processing. Exponential phase cultures of E. coli or B. subtilis in TY medium were concentrated 10-fold in 50 mM-triethanolamine/ HCl (pH 8·0), 1 mM-EDTA, 20% (w/v) sucrose and 1 mg lysozyme ml⁻¹, and the suspension was incubated for 20 min at room temperature. Lysis was completed using a French press at 6000 p.s.i. (41·3 MPa) in the same buffer containing 1 mM-diethiothreitol. Triton X-100 was added to the extracts to a final concentration of 1% (v/v). Processing reactions were carried out by incubating 4 μl of a five-times
To examine whether the hybrid Spase I protein was active, a complementation analysis was performed in *E. coli* N4156::pGD28. In this strain the chromosomally-

dilated **in vitro** transcription/translation mixture in 50 mM-triethanol-
amine/HCl; (pH 8.0), 1 mM-EDTA, 1% (v/v) Triton X-100, 1 mM-
PMSF with various amounts of extract, or with purified Spase I at
37 °C for 60 min (total reaction volume 14 μl).

**Results**

**Fusion of the E. coli lep gene to B. subtilis gene expression sequences**

To express the *E. coli* lep gene in *B. subtilis*, it was placed under the control of efficient transcription/translation signals for this organism. This was accomplished by using the bacteriophage SPO2 promoter and the Shine–Dalgarno sequence of the *B. pumilus* cat-86 gene (Williams et al., 1981), which are present on the low-
copy-number plasmid pHPS4 (five copies per chromosome equivalent; Fig. 1a). The resulting plasmid, pHPL1, contained an in-frame fusion between the first two codons of the cat-86::lep gene fusion of pHPL1; 6.4 kb, Km

Reference:

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Properties and genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTD101</td>
<td>pBR322 carrying the <em>Escherichia coli</em> lep operon; 8.9 kb, Ap&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Date &amp; Wickner (1981)</td>
</tr>
<tr>
<td>pUC9/pUC18</td>
<td>2.8 kb, Ap&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vieira &amp; Messing (1982)</td>
</tr>
<tr>
<td>pHPI3</td>
<td>pTA1060-pUC9 derivative; 4.9 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Haima et al. (1987)</td>
</tr>
<tr>
<td>pHPI3-2</td>
<td>Deletion derivative of pHPI3; 4.7 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Williams et al. (1981)</td>
</tr>
<tr>
<td>pPL608</td>
<td>5.1 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Km&lt;sup&gt;g&lt;/sup&gt;; bacteriophage SPO2 promoter</td>
<td>Laboratory collection (P. Haima)</td>
</tr>
<tr>
<td>pHPS4</td>
<td>pHPI3-2 derivative carrying a cat-86::lacZa gene fusion; bacteriophage SPO2 promoter; 5.4 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Em&lt;sup&gt;g&lt;/sup&gt;; five copies per chromosome equivalent</td>
<td></td>
</tr>
<tr>
<td>pHPS4l</td>
<td>pHPS4 derivative; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>This paper</td>
</tr>
<tr>
<td>pHPL0</td>
<td>pUC18 carrying an 811 bp <em>NcoI</em> fragment encoding the 5'-end of the <em>lep</em> gene; 3' end of <em>lep</em> gene is truncated; 6.1 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>This paper</td>
</tr>
<tr>
<td>pHPL1</td>
<td>pHPS4 carrying a cat-86::lep gene fusion; 7.9 kb, Cm&lt;sup&gt;g&lt;/sup&gt; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>This paper</td>
</tr>
<tr>
<td>pGD40</td>
<td>pSC105-derived cloning vector; 8.4 kb, Km&lt;sup&gt;g&lt;/sup&gt; Tc&lt;sup&gt;c&lt;/sup&gt; cI857&lt;sup&gt;+&lt;/sup&gt;</td>
<td>van Dijl et al. (1990)</td>
</tr>
<tr>
<td>pGD28</td>
<td>pGD40 carrying a cat-86::lep gene fusion; 12.3 kb, Km&lt;sup&gt;g&lt;/sup&gt; Tc&lt;sup&gt;c&lt;/sup&gt; cI857&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This paper</td>
</tr>
<tr>
<td>pTZ12</td>
<td>2.5 kb, Cm&lt;sup&gt;g&lt;/sup&gt;; 150-200 copies per chromosome equivalent</td>
<td>Aoki et al. (1987)</td>
</tr>
<tr>
<td>pGDV1</td>
<td>pTZ12-derived cloning vector; 2.6 kb, Cm&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Bron (1990)</td>
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<td>pGDL24</td>
<td>pGDV1 containing the cat-86::lep gene fusion of pHPL1; 6.4 kb, Cm&lt;sup&gt;g&lt;/sup&gt;</td>
<td>This paper</td>
</tr>
<tr>
<td>pGDL2</td>
<td>pSC101-derived plasmid carrying the <em>lep</em> gene under the control of the <em>tac</em> promoter; 9.8 kb, Km&lt;sup&gt;g&lt;/sup&gt;</td>
<td>van Dijl et al. (1991a)</td>
</tr>
<tr>
<td>pGPA14</td>
<td>α-Amylase based signal sequence selection vector carrying the pTA1060 replication functions</td>
<td>Smith et al. (1987)</td>
</tr>
<tr>
<td>pSPA13</td>
<td>pGPA14 carrying signal sequence A13; amylase* Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>Smith et al. (1988)</td>
</tr>
<tr>
<td>pSA13</td>
<td>pSPA13 with the pTA1060 replication functions replaced by those from the lactococcal plasmid pWVO1; compatible with pHPS41- and pGDV1-derived plasmids</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pGBP14</td>
<td>β-Lactamase based signal sequence selection vector carrying the pTA1060 replication functions</td>
<td>Smith et al. (1987)</td>
</tr>
<tr>
<td>pSPB-A2d</td>
<td>pGBP14 carrying signal sequence A2d; Em&lt;sup&gt;g&lt;/sup&gt;</td>
<td>H. Smith and others (unpublished)</td>
</tr>
<tr>
<td>pSB-A2d</td>
<td>pSPB-A2d with the pTA1060 replication functions replaced by those of pWVO1; compatible with pHPS41- and pGDV1-derived plasmids</td>
<td>Laboratory collection</td>
</tr>
</tbody>
</table>

**Strains**

**Bacillus subtilis**

8G5  | trpC2 tyr his nic ura rib met ade | Bron & Venema (1972) |
8G5(amy)  | α-Amylase-negative derivative of 8G5 | Laboratory collection |
6G3M15  | trpC2 tyr his ura rib met lacZAM15 Km<sup>g</sup> | Haima et al. (1990b) |
DB104  | his nprR2 nprE18 aprA3 | Kawamura & Doi (1984) |
DB114  | met nprR2 nprE18 aprA3 | Laboratory collection |
DB114-15  | Derivative of DB114; lacZAM15 Km<sup>g</sup> | This paper |

**Escherichia coli**

C600  | thr leu thi lacY tonA phx supE str | Phabagen collection, State University, Utrecht, The Netherlands |

JM83  | ara Nac-proAB thi strA φ80 lacZAM15 | Vieira & Messing (1982) |
N4156::pGD28  | polyA and thy gyrA::lep gene under transcriptional control of Ap<sup>φ</sup>; Ap<sup>φ</sup> | van Dijl et al. (1988) |
N4156::pGD28 (c1857)  | Unable to grow at 28 °C; Ap<sup>φ</sup> | van Dijl et al. (1988) |

Ap, ampicillin; Cm, Chloramphenicol; Em, erythromycin; Km, kanamycin; Tc, tetracycline.
located lep gene is transcribed from the repressible phage λ pL promoter (van Dijl et al., 1988). SPase I synthesis in this strain can be controlled by the λ1857 temperature-sensitive repressor, provided in trans via the signal peptidase probe vector pGD40. Under conditions of repression of SPase I synthesis (28 °C), strain N4156::pGD28 is unable to grow. Cloning of lep genes into pGD40 results in the restoration of growth at 28 °C,
provided that the product of the cloned lep gene is functional (van Dijl et al., 1990). In the test system described here, the 3.9 kb BclI-BamHI fragment from plasmid pHPL1, containing the hybrid lep gene, was ligated into the unique BamHI site of pGD40. This resulted in pGD20 (Fig. 1a). The viability of E. coli N4156::pGD28 after transformation with pGD40 and pGD20 was tested at 28 and 42 °C. As expected, at 42 °C (chromosomal lep gene expressed) transformants carrying pGD40 or pGD20 showed normal growth properties (data not shown). In contrast, at 28 °C (chromosomal lep gene repressed) transformants carrying pGD40 had lost their colony-forming capacity when transferred to fresh plates, whereas transformants carrying pGD20 did form colonies (Fig. 2). This test has been used successfully before (van Dijl et al., 1990) to demonstrate the presence of a functional lep gene product. Similar results were obtained when transformants were transferred to liquid medium:

**Expression of E. coli SPase I in B. subtilis**

The production of E. coli SPase I in B. subtilis DB114(pHPL1) was monitored by Western blotting (Fig. 3, lane 2), which showed that the production of the enzyme (per mg total protein) in exponential phase cells was approximately 50% of the enzyme produced in wild-type (plasmid-free) cells of E. coli C600 (data not shown). In an attempt to raise the production of E. coli SPase I in B. subtilis, the 3.9 kb BclI-BamHI fragment carrying the hybrid lep gene was ligated into the unique BamHI site of the high-copy-number plasmid pGDV1 (150–200 copies per chromosome equivalent). This resulted in pGD24 (Fig. 1). As compared to B. subtilis cells containing pHPL1, cells containing pGD24 produced approximately five-times more SPase I (Fig. 3, lane 3).

**Effects of E. coli SPase I production on the in vivo processing of hybrid precursor proteins in B. subtilis**

The effects of the production of E. coli SPase I in B. subtilis on the processing kinetics of two hybrid precursors, pre(A13)-α-amylase and pre(A2d)-β-lacta-

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**Fig. 1.** (on facing page) (a) Scheme of the construction of pHPL1, pGD20 and pGD24. Only restriction sites relevant for the construction and properties of the plasmids are shown. To provide the lep gene with the Shine–Dalgarno sequence of the B. subtilis cat-86 gene, resulting in a cat-86::lep gene fusion, several construction steps were required. First, an 811 bp pTD101-derived NcolHI fragment, containing the 3' truncated E. coli lep gene (lep'), was ligated into the unique SphI site of pUC18. This resulted in plasmid pHPL0. Plasmid pHPS4 was obtained by cloning a 700 bp Dral fragment, derived from plasmid pPL608 (Williams et al., 1981), in plasmid pHPl3-2, which resulted in an in-frame fusion between the first two codons of cat-86 and α-lacZ (P. Haima, unpublished results). From plasmid pHPL0 the 5' end of the lep gene, present on a 672 bp Psrl–EcoRI fragment, was isolated. This fragment and a 1.8 kb pTD101-derived EcoRI fragment, containing the 3' end of the lep gene were ligated into plasmid pHPS4, cleaved with Psrl and EcoRI, which resulted in pHPL1. pHPL1 contained an in-frame fusion of the 5' end of the lep gene to the first two codons of the cat-86 gene (the sequence of the fusion site is shown in b). pGD20 and pGD24 were constructed by ligating the 3.9 kb BamHI–BclI fragment of pHPL1 containing the cat-86::lep gene fusion into the unique BamHI sites of pGD40 and pGDV1, respectively. MCS, multiple cloning site; RBS, ribosome binding site. (b) Nucleotide sequence of the cat-86::lep gene fusion and the deduced amino acid sequence.
mase, were studied by pulse-chase labelling experiments. Both hybrid precursors contain signal peptides which had been randomly selected previously from the *B. subtilis* chromosome (Smith et al., 1987, 1988, 1989). Under standard conditions pre(A13)-α-amylase was processed relatively slowly in *B. subtilis*: the time required to process 50% of the precursor (t50) was approximately 5 min (H. Smith and others, unpublished; van Dijl et al., 1991b). In contrast, the same precursor was processed very rapidly in *E. coli* (no precursor could be detected; H. Smith and others, unpublished; van Dijl et al., 1991b). Assuming that the different processing rates in these two organisms reflect differences in the interaction of pre(A13)-α-amylase with the respective SPases, rather than differences in translocation efficiencies, we reasoned that pre(A13)-α-amylase might be a suitable precursor to detect possible activity of *E. coli* SPase I in *B. subtilis*. Pre(A2d)-β-lactamase was also processed relatively slowly in *B. subtilis* (t50 = 2 min; H. Smith and others, unpublished; van Dijl et al., 1991b). In contrast to pre(A13)-α-amylase, this precursor was processed extremely slowly in *E. coli* under standard conditions of SPase I production (H. Smith and others, unpublished; van Dijl et al., 1991a). Interestingly, overproduction of SPase I in *E. coli* increased the rate of pre(A2d)-β-lactamase processing drastically (t50 < 1 min; van Dijl et al., 1991a). Based on this result, we reasoned that in addition to pre(A13)-α-amylase, pre(A2d)-β-lactamase might be a suitable substrate for detecting the activity of *E. coli* SPase I in *B. subtilis*.

Strains of *B. subtilis* producing either pre(A13)-α-amylase or pre(A2d)-β-lactamase were transformed with pHPS41 or pGDV1 (no production of *E. coli* SPase I), pHPL2 (low level of *E. coli* SPase I production) or pGDL24 (high level of *E. coli* SPase I production). Plasmids pHPS41 and pHPL2 are similar to pHPS4 and pHPL1, respectively, but lack a functional erythromycin-resistance gene. This enabled the co-selection of transformants containing both pHPS41 (or pHPL2) and the erythromycin-marked plasmid encoding the precursor of interest (pSA13 or pSB-A2d). The results of the pulse-chase labelling experiments showed that the processing rates of both precursors were not increased in the presence of pHPL2 (Fig. 4) or pGDL24 (data not shown), suggesting that the activity of the *E. coli* SPase I was too low to be detected in this assay.

**Localization of *E. coli* SPase I in *B. subtilis***

In *E. coli*, SPase I spans the cytoplasmic membrane twice and most of the protein (the C-terminal part) is exposed to the outer surface of the membrane (Dalbey et al., 1987; Moore & Miura, 1987). Treatment of spheroplasted *E. coli* cells with trypsin caused the digestion of the exposed SPase I moiety. An N-terminal fragment of SPase I, designated TRF II, remained protected against degradation (Wolfe et al., 1983a; Moore & Miura, 1987). Since incorrect membrane assembly is a possible explanation for the observed lack of *E. coli* SPase I activity in *B. subtilis*, we studied the localization properties of this enzyme in *B. subtilis* membranes in two different ways. First, membrane and cytoplasmic fractions of *B. subtilis* DB114 containing either pGDV1 or pGDL24 were isolated. No *E. coli* SPase I was detectable in the cytoplasmic fractions (data not shown). The enzyme was detectable only in the membrane fraction of cells containing pGDL24. Like in *E. coli*, the SPase I was solubilizable in 1% (v/v) Triton X-100 (Fig. 5, lane 5). Fig. 5 also shows that antibodies directed against the *E. coli* SPase I cross-react weakly with a membrane protein of *B. subtilis* of a higher molecular mass than that of *E. coli* SPase I (Fig. 5, lanes 2–5). Since this protein was not detected with a different batch of antibodies directed against SPase I (data not shown) we concluded that this cross-reactivity was not SPase I-specific.

The question as to how the *E. coli* SPase I was present in the *B. subtilis* membrane was addressed by trypsin treatment of protoplasts of *B. subtilis* DB114-15 cells containing either pHPL1 (data not shown) or pGDL24 (Fig. 6a). Cells containing pGDV1 were used as controls. The *LacZΔM15* protein produced by DB114-15 served as a cytoplasmic reference protein (Fig. 6a2). The results showed that, unlike the situation described for *E. coli*, digestion of SPase I was not detectable upon treatment of *B. subtilis* protoplasts with trypsin (Fig. 6a1, lane 3).
Signal peptidase I in Bacillus subtilis

**Fig. 5.** Membrane association and solubilization of E. coli SPase I in B. subtilis DB114. Membranes of B. subtilis DB114(pGDV1) and B. subtilis DB114(pGDL24) were prepared as described in Methods. Membrane proteins were solubilized by the addition of 1% (v/v) Triton X-100 and incubation for 15 min (0 °C). Solubilized membrane proteins were separated from non-solubilized membrane proteins by centrifugation (1 h, 4 °C, 150,000 g). The non-solubilized membrane proteins were resuspended in an equal volume of the same buffer. Equal amounts of both fractions were used for SDS-PAGE and the presence of SPase I was monitored by Western blotting. Lanes: 1 and 6, reference SPase I; 2, non-solubilized proteins of B. subtilis DB114(pGDV1); 3, solubilized proteins of B. subtilis DB114(pGDV1); 4, non-solubilized proteins of B. subtilis DB114(pGDL24); 5, solubilized proteins of B. subtilis DB114(pGDL24).

Treatment of the protoplasts with trypsin did not cause the disruption of the protoplast, since no digestion of the LacZAM15 protein was detectable (Fig. 6a2, lane 3). Only when the protoplasts were disrupted with 1% (v/v) Triton X-100 and then treated with trypsin were the SPase I (Fig. 6a1, lane 4) and the LacZAM15 protein (Fig. 6a2, lane 4) degraded to any extent. These results suggest that the E. coli SPase I was not incorporated into the B. subtilis membrane in such a way that potential sites for cleavage by trypsin were exposed on the outside of the protoplasts, suggesting that SPase I was incorrectly inserted in the membrane. In a control experiment, intact and disrupted spheroplasts of E. coli C600(pGDL2), which overproduces SPase I (van Dijl et al., 1991a), were also treated with trypsin (Fig. 6b). As expected, in this case treatment of intact spheroplasts resulted in the partial degradation of SPase I to TRF II (Fig. 6b, lane 2). In the presence of 1% (v/v) Triton X-100, SPase I was completely degraded (Fig. 6b, lane 3).

**Fig. 6.** Localization of E. coli SPase I in B. subtilis (a) and in E. coli (b). (a) Cells of B. subtilis DB114-15 were protoplasted as described in Methods. In separate experiments, intact and disrupted protoplasts of cells containing either pGDV1 or pGDL24, were incubated with trypsin (10 μg ml⁻¹) for 30 min at room temperature. Protoplasts were disrupted in 1% (v/v) Triton X-100 prior to treatment with trypsin. Prior to analysis by SDS-PAGE and Western blotting, samples were treated with trypsin-inhibitor (30 μg ml⁻¹). The presence of SPase I (a1) or the LacZAM15 protein (a2) in samples (approximately 0.03 mg of total protein) was monitored with specific antibodies. Lanes: 1, unprotoplasted cells; 2, intact untreated protoplasts; 3, protoplasts incubated with trypsin; 4, protoplasts incubated with trypsin in the presence of 1% (v/v) Triton X-100; 5(a), reference SPase I; 5(a2), reference LacZ protein. (b) Cells of E. coli containing plasmid pGDL2 were spheroplasted as described in Methods. Intact and disrupted spheroplasts were incubated with trypsin (10 μg ml⁻¹) for 30 min at room temperature. Spheroplasts were disrupted in 1% (v/v) Triton X-100 prior to treatment with trypsin. Prior to analysis by SDS-PAGE and Western blotting, samples were treated with trypsin-inhibitor (30 μg ml⁻¹). The presence of SPase I and TRF II in samples (approximately 0.02 mg total protein) was monitored with specific antibodies. Lanes: 1, intact untreated spheroplasts; 2, spheroplasts incubated with trypsin; 3, spheroplasts incubated with trypsin in the presence of 1% (v/v) Triton X-100; 4, reference SPase I.
In vitro activity of the E. coli SPase I produced in B. subtilis

To examine whether the E. coli SPase I produced in B. subtilis was active, in vitro synthesized bacteriophage M13 coat protein precursor (procoat) was incubated with cell-free extracts of B. subtilis containing pHPS4 (Fig. 7, lane 3) or pGDV1 (Fig. 7, lane 5) and with a cell-free extract of E. coli C600 producing standard amounts of SPase I (Fig. 7, lane 1). The results show that only the cell-free extract of E. coli C600 was able to process significant amounts of procoat (Fig. 7, lane 1). None of the B. subtilis extracts were able to process M13 procoat, even under conditions in which 10-fold higher amounts of procoat (4 mg protein ml\(^{-1}\)) were added in comparison to the analogous experiment with E. coli C600 (Fig. 7, lane 4) or pGDV1 (Fig. 7, lane 5). This suggests that the enzyme synthesized in B. subtilis was not active.

It was apparent from Fig. 7 that the endogenous B. subtilis SPase I was likewise unable to process M13 procoat under the conditions used (lanes 3–6). This might be due to the presence in B. subtilis of unknown components interfering with productive processing of procoat by both the E. coli and the B. subtilis SPase I. To test this, cell-free extracts from B. subtilis were mixed with extracts from E. coli C600 (producing standard levels of SPase I; Fig. 8, lane 5) and with cell-free extracts from E. coli C600(pTD101), which overproduces SPase I (Fig. 8, lane 6). These mixtures were subsequently incubated with procoat and the effects on the efficiency of processing were analysed. Purified E. coli SPase I was included as a control (Fig. 8, lane 7). The results show that in vitro processing of procoat by E. coli SPase I was not affected by the presence of cell-free extract of B. subtilis (Fig. 8, lanes 5–7). This indicates that the B. subtilis extracts did not interfere with E. coli SPase I activity and, therefore, it is concluded that the E. coli SPase I produced in B. subtilis was not active.

Discussion

Recently, we demonstrated that among a collection of bacterial secretory proteins, which contained signal peptides randomly selected from the B. subtilis chromosome, several were processed relatively slowly in B. subtilis and E. coli (Smith et al., 1989; H. Smith and others, unpublished; van Dijl et al., 1991b). We have demonstrated before that the rates of processing of two of these precursors [pre(A2d)-\(\beta\)-lactamase and pre(A13i)-\(\beta\)-lactamase] could be increased by overproduction of SPase I in E. coli (van Dijl et al., 1991a). This indicated that in E. coli, under standard conditions, the availability of SPase I was rate-limiting for the processing of these precursors. By analogy, we reasoned that the inefficient processing of two precursors [pre(A13)-\(\alpha\)-amyrase and pre(A2d)-\(\beta\)-lactamase] in B. subtilis might also be due to limited availability of SPase.

To test this, we expressed the E. coli lep gene in B. subtilis by fusing it to appropriate transcription/translation signals. As a consequence of this construction, the E. coli SPase I contained seven additional amino acids at its N-terminus. Apparently, this extension did not interfere with the activity of the enzyme in E. coli. In contrast, our results suggest that activity of the E. coli SPase I was absent in B. subtilis in vivo. Even in vitro, after solubilization in Triton X-100, the activity could not be recovered. This lack of processing activity did not appear to result from inhibiting components in B. subtilis. This indicates that the E. coli SPase I itself was inactive in B. subtilis. Two explanations may be considered to underlie this phenomenon. The first is that the inactivity of the E.
coli SPase I produced in B. subtilis is due to incorrect folding of the enzyme. We can only speculate about possible reasons for inappropriate folding. One is that particular cellular components, such as chaperones (see Lecker et al., 1989; Saier et al., 1989), are necessary to guide the correct folding of the E. coli SPase I. It is conceivable that the corresponding components of B. subtilis are not able to effect the correct folding of the heterologous protein. An alternative reason is that in B. subtilis the seven additional N-terminal amino acids of the E. coli SPase I fusion protein interfered with the folding of the protein into the conformation required for activity. However, this explanation is not easily reconciled with the observation that in E. coli the fusion protein was active in the complementation assay.

As a second possible explanation for the lack of E. coli SPase I activity in B. subtilis, incorrect insertion of the protein into the membrane can be considered. In E. coli the correct assembly of SPase I results in the exposure of the C-terminal part (two-thirds of the total protein) on the outer surface of the cytoplasmic membrane. As a consequence, after spheroplasting the enzyme is sensitive to trypsin (Moore & Miura, 1987; Wolfe et al., 1983a). Mechanistically, the membrane insertion of SPase I appears, at least in part, to share features with the export of proteins. It requires the (internal) signal peptide of the protein (Dalbey et al., 1987; Zhu & Dalbey, 1989) and the activities of the SecA and SecY proteins (Wolfe et al., 1985).

The present results indicated that the E. coli SPase I was associated with the membrane when expressed in B. subtilis. However, in protoplasts, the protein was protected against degradation by trypsin, indicating that the enzyme was not inserted correctly into the B. subtilis cytoplasmic membrane. Although a positive control showing that trypsin under these conditions could degrade other B. subtilis membrane proteins was not carried out, these data suggest that the SPase I was either inserted across the membrane without exposing a proper cleavage site for trypsin at the external surface, or that it was associated with the cytoplasmic side of the membrane. Obviously, this would prevent the correct interaction between precursors and SPase I. Alternatively, the SPase I may have formed aggregates in the cytoplasm, which fractionated with the membranes. If defective membrane insertion has occurred in this system, this might be of more general importance. It could mean that the apparatus for membrane insertion is host-specific and is crucial for the final structure of proteins in the membrane. The causes of the presumed defective membrane insertion are not clear. One possibility is that the heterologous protein does not interact efficiently with essential components of the B. subtilis export machinery (e.g. chaperones, SecA or SecY), preventing the correct translocation of the protein across the membrane.

The incorrect membrane assembly of the E. coli SPase I in B. subtilis is reminiscent to the defective export of the E. coli outer-membrane protein A (OmpA) in B. subtilis (Kallio et al., 1986). OmpA, when fused to the signal peptide of B. amyloliquefaciens α-amylase, was not translocated across the cytoplasmic membrane of B. subtilis. Kallio et al. (1986) suggested that this may be due to the absence in B. subtilis of a specific factor required for the translocation of OmpA across the membrane.

One interesting outcome of the present work is that the B. subtilis and E. coli SPase I proteins appear to differ in two aspects. First, antibodies raised against E. coli SPase I did not cross-react with B. subtilis membrane proteins. Second, the B. subtilis SPase I did not show in vitro processing activity under conditions appropriate for E. coli SPase I. Two other lines of evidence also suggest that the SPases of Gram-positive bacteria may be rather distinct from those of Gram-negative bacteria. First, the lep genes of E. coli and Salmonella typhimurium showed no similarity with the B. licheniformis lep gene (J. M. van Dijl and others, unpublished). Second, B. steaerothermusophilus α-amylase is processed at different sites in B. subtilis and in E. coli (Suominen et al., 1987).

For a better understanding of the differences between SPases from Gram-positive and Gram-negative bacteria, the cloning and analysis of a Bacillus SPase I is required. This will also enable us to address the question whether the availability of this enzyme can be limiting for the secretion of proteins by this organism.

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