The Role of Lipoprotein Processing by Signal Peptidase II in the Gram-positive Eubacterium *Bacillus subtilis*

**SIGNAL PEPTIDASE II IS REQUIRED FOR THE EFFICIENT SECRETION OF α-AMYLASE, A NON-LIPOPROTEIN**

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Harold Tjalsma, Vesa P. Kontinen, Zoltán Prágai, Hongyan Wu, Rob Meima, Gerard Venema, Sierd Bron, Matti Sarvas, and Jan Maarten van Dijl

From the Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, Kerklaan 30, 9751 NN Haren, The Netherlands, the Vaccine Development Laboratory, National Public Health Institute, Mannerheimintie 166, SF-00300 Helsinki, Finland, the Department of Microbiology, The Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom, and the Department of Pharmaceutical Biology, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Computer-assisted analyses indicate that *Bacillus subtilis* contains approximately 300 genes for exported proteins with an amino-terminal signal peptide. About 114 of these are lipoproteins, which are retained in the cytoplasmic membrane. We have investigated the importance of lipoprotein processing by signal peptidase II (SPase II) for cellular homeostasis, using cells lacking SPase II. The results show that lipoprotein processing is important for cell viability at low and high temperatures, suggesting that lipoproteins are essential for growth under these conditions. Although certain lipoproteins are required for the development of genetic competence, sporulation, and germination, these developmental processes were not affected in the absence of SPase II. Cells lacking SPase II accumulated lipid-modified precursor and mature-like forms of PrsA, a folding catalyst for secreted proteins. These forms of PrsA seem to have a reduced activity, as the secretion of α-amylase was strongly impaired. Unexpectedly, type I signal peptidases, which process secretory preproteins, were not involved in alternative amino-terminal processing of pre-PrsA in the absence of SPase II. In conclusion, processing of lipoproteins by SPase II in *B. subtilis* is not strictly required for lipoprotein function, which is surprising as lipoproteins and type II SPases seem to be conserved in all eubacteria.

One of the most commonly used eubacterial sorting (retention) signals for proteins that are exported from the cytoplasm is an amino-terminal lipid-modified cysteine residue (see Refs. 1 and 2). In Gram-positive eubacteria, such as *Bacillus subtilis*, lipid-modified proteins (lipoproteins) are retained in the cytoplasmic membrane. In Gram-negative eubacteria, such as *Escherichia coli*, these proteins are retained in the cytoplasmic or the outer membrane; retention in the cytoplasmic membrane depends on the presence of an additional sorting signal in the form of an aspartic acid residue at the +2 position relative to the amino-terminal cysteine residue (see Refs. 3–6). Even the organism with the smallest known genome, *Mycoplasma genitalium*, seems to make use of lipid modification to retain proteins in the cytoplasmic membrane (7). The number of putative lipoprotein-encoding genes per eubacterial genome seems to range from approximately 18 in *M. genitalium* (http://www.tigr.org/db/mdb/mgd) to approximately 89 in *E. coli* and 114 in *B. subtilis* (Table I). Thus, lipoproteins appear to represent about 1–3.5% of the proteome of eubacteria.

Lipoproteins are directed into the general (Sec) pathway for protein secretion by their signal peptides, which show similar structural characteristics as the signal peptides of secretory proteins: a positively charged amino terminus, a hydrophobic core region, and a carboxyl-terminal region containing the cleavage site for signal peptidase (SPase) (2). The major difference between signal peptides of lipoproteins and secretory proteins is the presence of a well conserved “lipobox” of four residues in the former signal peptides, which constitutes the cleavage site for the lipoprotein-specific SPase, also known as SPase II. Invariably, the carboxyl-terminal residue of the lipobox is cysteine, which, upon lipid modification, forms the retention signal of the mature lipoprotein (for details, see Refs. 1 and 8). Modification of this cysteine residue by the diacylglycerol transferase is a prerequisite for processing of the lipoprotein precursor by SPase II. Processing by SPase II can be inhibited with globomycin, a reversible and noncompetitive peptide inhibitor (2, 9, 10). In *E. coli*, the processed lipoprotein is further modified by aminocytolysis of the diacylglycerol-cysteine amino group (11, 12). It is presently not known whether the latter lipid modification step is conserved in all eubacteria. For example, *B. subtilis* and *M. genitalium* lack an *int* gene for the lipoprotein aminocytoltransferase (1). In Gram-negative eubacteria, the outer membrane confines numerous proteins to the periplasm. In Gram-positive eubacteria, which lack an outer membrane, lipid modification of exported proteins prevents their loss into the environment, as these proteins remain anchored to the cytoplasmic membrane. This may explain why *B. subtilis* contains more putative lipoproteins than *E. coli* and why, for example, 32 lipoproteins of *B. subtilis* are homologues of periplasmic high affinity sub-

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¶ To whom correspondence should be addressed. Tel.: 31-50-3633079; Fax: 31-50-3632348; E-mail: j.m.van.dijl@farm.rug.nl.
Lipoprotein Processing in B. subtilis

A hexahistidine tag, stop codon, and ClaI cleavage site were added to the 3′-end of the prsA gene. The tagged prsA, PrsA, is essential for the efficient secretion of various proteins and cell viability (14–17). Notably, no specific function can presently be assigned to the majority of putative lipoproteins of B. subtilis (about 75%); Table I and other Gram-positive eu-bacteria.

The present studies were aimed at the evaluation of the importance of B. subtilis lipoproteins for cellular homeostasis in general, and their processing by SPase II in particular. For this purpose, SPase II-depleted cells were used. Unexpectedly, the results show that lipoprotein processing is required for growth at low and high temperatures and the efficient secretion of the α-amylase AmyQ (a non-lipoprotein) but not for growth and cell viability at 37 °C, development of competence for DNA binding and uptake, sporulation, or spore germination.

EXPERIMENTAL PROCEDURES

Plasmids, Bacterial Strains, and Media—Table II lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). S7 media 1 and 3, used for labeling of B. subtilis proteins with [35S]methionine (Amersham Pharmacia Biotech), were prepared as described in Refs. 21 and 22. When required, media for E. coli were supplemented with ampicillin (50 μg/ml), erythromycin (100 μg/ml), or kanamycin (40 μg/ml); media for B. subtilis were supplemented with chloramphenicol (5 μg/ml), erythromycin (1 μg/ml), kanamycin (10 μg/ml), tetracyclin (6 μg/ml), spectinomycin (100 μg/ml), globomycin (50 μg/ml), and/or IPTG (1 mM). DNA Techniques—Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of E. coli were carried out as described in Ref. 30. Enzymes were from Boehringer Mannheim. B. subtilis was transformed as described in Ref. 25. Correct integration of plasmids into the chromosome of B. subtilis was verified by Southern blotting. PCR was carried out with Vent DNA polymerase (New England Biolabs) as described in Ref. 31. pMutin2-MIL was constructed by PCR amplification of the 5′-region of the lsp gene with the primers lsp-m1(5′-ATAAGCTTACGTTAAATGTGAGG-3′) and lsp-m2 (5′-GCGGATCCAAAGAAGCCTTTATG-3′) and subsequent cloning in pMutin2. pMutin2-MIL was constructed by PCR amplification of an internal fragment of the lsp gene with the primers lsp-m5 (5′-ATGTCGAGCGATGATGGAATGATG-GATGATTACGTTAAATGTGAGG-3′) and lsp-m2 and subsequent cloning in pMutin2. To construct pKTH3409, the prsA gene was amplified by PCR with a primer containing a ClaI site and 5′ sequences of prsA (starting at position −27 relative to the start codon) and a primer, which adds the sequence CACGTCGAGCGATGATGGAATGATG-GATGATTACGTTAAATGTGAGG to the 3′-end of prsA, specifying a hexahistidine tag, stop codon, and SalI cleavage site. The tagged prsA was placed under the control of the xylose-inducible xylA promoter of plasmid pX50, using the ClaI and SalI restriction sites. The resulting plasmid was designated pKTH3409.

Competence and Sporulation—Competence for DNA binding and uptake was determined by transformation with plasmid or chromosomal DNA (28). The efficiency of sporulation was determined by overnight growth in Schaeffer’s medium (32), killing of cells with 0.1% volume of chloroform, and subsequent plating.

β-Galactosidase Activity Assay—Overnight cultures were diluted 100-fold in fresh medium, and samples were taken at hourly intervals for A500 readings and β-galactosidase activity determinations. The assay and the calculation of β-galactosidase units (expressed as units per A500) were carried out as described in Ref. 33.

Protein Labeling, Immunoprecipitation, SDS-PAGE, and Fluorography—Pulse-chase labeling of B. subtilis, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography were performed as described previously in Refs. 21 and 22. Palmitic acid labeling of (pre-)PrsA was performed as described in Ref. 16. Western Blot Analysis—Western blotting was performed as described in Ref. 34. After separation by SDS-PAGE, proteins were transferred to Immobilon-PVDF membranes (Millipore Corporation). To detect PrsA or the α-amylase AmyQ, B. subtilis cells were separated from the growth medium by centrifugation (5 min, 12,000 rpm, room temperature), and samples for SDS-PAGE were prepared as described in Ref. 25. PrsA and (pre-)AmyQ were visualized with specific antibodies and horseradish peroxidase-anti-rabbit-IgG conjugates (Amersham Pharmacia Biotech). Hexahistidine-tagged (pre-)PrsA was visualized with hexahistidine-specific monoclonal antibodies (Amersham Pharmacia Biotech), and biotinylated (pre-)AmyQ-PSBT was visualized with streptavidine-horseradish peroxidase conjugates (Amersham Pharmacia Biotech).

Trypsin Accessibility Assay—The preparation of protoplasts from exponentially growing cells of B. subtilis and the testing of the protease accessibility of membrane proteins was performed as described in Ref. 29.

RESULTS

The lsp Gene for SPase II Is Not Essential for Cell Viability—To determine whether the lsp gene for the SPase II of B. subtilis (35) is essential for growth and cell viability, two lsp mutant strains were constructed, using derivatives of the integration vector pMutin2. B. subtilis M/L was constructed by integration of pMutin2-M/L within the structural lsp gene, resulting in the disruption of this gene. B. subtilis MIL was constructed by integration of pMutin2-MIL at the 5′-end of lsp in such a way that the original lsp promoter region was replaced by the IPTG-inducible Papac promoter (Fig. 1A). The fact that B. subtilis M/L could be obtained shows that SPase II is not essential for cell viability, at least when cells are grown in TY or minimal medium at 37 °C. Under these conditions, the growth of B. subtilis M/L was only slightly reduced, compared with the parental strain 8G5. Similarly, the growth rate of B. subtilis MIL was slightly reduced in the absence of IPTG, as compared with that of B. subtilis MIL in the presence of IPTG, or the parental strain. Unexpectedly, the disruption of the lsp gene did not inhibit the development of competence for DNA binding and uptake, sporulation, and subsequent spore germination (data not shown), although at least one lipoprotein is required both for competence development and sporulation (OppA) (38), three for sporulation (DppE, also known as DciA, SpoIIIJ, and SpoIVB) (37–39), and five for germination (GerA, GerB, GerKC, GerM, and GerD) (40–44). Interestingly, SPase II appeared to be essential for growth at 15 °C (data not shown) and 48 °C. Upon a temperature shift from 37 to 48 °C, cells lacking SPase II stopped growing and lysed (Fig. 1B, Δlsp). These findings imply that some as yet unidentified lipoproteins of B. subtilis are required for growth at low and high temperatures or that the accumulation of lipoprotein precursors causes cold and heat sensitivity. In what follows, we show that the cold and heat sensitivity of cells lacking SPase II must be due to the malfunction of certain lipoproteins.

Maximal lsp Transcription in the Exponential Growth Phase—Both in B. subtilis M/L and B. subtilis MIL, the transcription of the lacZ gene, present on pMutin2, is directed by the lac promoter region (Fig. 1A). To study the transcription of the lsp gene, B. subtilis M/L was grown in the absence of IPTG, and samples withdrawn at hourly intervals were assayed for β-galactosidase activity. The results showed that the β-galactosidase levels increased during exponential growth, reaching a maximum in the transition phase between exponential and postexponential growth. In contrast, the β-galactosidase levels were strongly decreased in the postexponen-
Putative lipoprotein signal peptides were identified in two ways. First, the presence of a lipobox was determined by a search for cysteine residues relative to the cleavage site for SPase II, are indicated in boldface. Note that aspartic acid residues are present of the annotated proteins of \textit{B. subtilis} in the SubtiList database (http://www.pasteur.fr/BioSubtiList.html). The highly conserved leucine residue at position –3 and the strictly conserved cysteine at position +1 relative to the cleavage site for SPase II, are indicated in boldface. Note that aspartic acid residues are absent from position +2. KapB, which seems to be a lipoprotein (20), is not listed in this table because its amino terminus is atypical for signal peptides of lipoproteins of \textit{B. subtilis} due to the presence of two lysine residues in the hydrophobic core region.

### TABLE I

| Lipoprotein Processing in \textit{B. subtilis} |
|-----------------------------------------------|
| **Signal peptide** | **Lipobox** | **Signal peptide** | **Lipobox** |
|---------------------|-------------|---------------------|-------------|
| **Name** | **Signal peptide** | **Name** | **Signal peptide** |
| AppA<sup>*</sup> | MRRKRTALMLSLMVLLAFLGACG | YhQ<sup>*</sup> | MKRLTVLLSSSLLTACG |
| AraN<sup>*</sup> | MKMTTCFLVLFMLLTVIAGCG | YgB | MKMTSAITAAVAVSCG |
| BagC | MKRFPYALVLLGCG | YkJA | MKKLYFLVTLVISACG |
| CccB | MSDKLISLIGTLSLVAAG | YkoI | MKTITLVSSAAALVIVCT |
| CtaC | MKHHRLAILVPLLGGCG | YkUH | MKGKLLKVLVLSLIVIFNYWICTA |
| DacA | MMKCCQLMILSLVTVCT | YlaJ | MLRLITIQLTLSCAG |
| DppE<sup>*</sup> | MKRGKRMVKHLWMLGALGSLACG | YmaC | MFSEAEILRIRALVIFLFGAC |
| FucA<sup>*</sup> | MKKSIITVLLVLALTTACG | YnC | MKIISSMTAVLIFTACG |
| FuhD<sup>*</sup> | MTHYKGLAFAFLLIALAAG | YndF | MKSLLKRGFLPAMVCLLMICTG |
| GerAC | MKIRICMFCTLLGGCG | YnoJ | MKISSAFGMVMLITFC |
| GerBC | MKTASFKSMFOMKLGLCGCG | YnoO | MKRRKNIKLWILAGLIC |
| GerD | MSKATKLMCSLLVTVTACA | YobA | MKPIGVLSSLLVIFLAAACG |
| GerK<sup>*</sup> | MKKLPVLMLSLVTVIAGCG | YodJ | MKKGGPSLASLAAASVTVAGCS |
| GerM | MKLGGPAGICATSLASLLCG | YojM | MLHHLLMLTIALVAGC |
| GlnH<sup>*</sup> | MKFISSALISLVAAG | YobK | MNIASFLMVC5IFFTGAC |
| LplA | MKIRKKMMLALAMMIAGCG | YofK | MRRVLGFAFFTSLSACG |
| LytA<sup>*</sup> | MKFIALFILLIALLACG | YonS<sup>*</sup> | MKAPRNKLILLILSIALACG |
| MprE<sup }}> | MKVIIFPFLTFVAGCG | YoeF | MRHLFVLFVFAGCG |
| Oppi<sup>*</sup> | MKNKSTVFLTIVLASS | YofH | MKGLCAIAFVAFILVSSC |
| Opuc<sup>*</sup> | MKLIIIGVSAALILALAG | YpmQ | MKVIKTLAGLFLFTACG |
| Opuc<sup>*</sup> | MKKLMGAPAFVVMGCACG | YpmR | MKLRFLAIMSLLITACG |
| PbcC | MKKCIILPLCGLGACG | YqmF | MKHIFLILLFLFTACG |
| PrsA | MKKIAAATITISSIALACG | YqmO | MKRFLAIMPMMLACG |
| QoxA | MIFLFRALKPLVLAFLTVFVGCG | YqsoU | MLRSCFVFLLAVLFISSACG |
| RbsB<sup>*</sup> | MKRASVVLSSLLTACG | Yqso | MKALMAYAFLATACG |
| Sle | MKRASYVFMLIIFLACG | YlaA | MKMLVLIFLLALACG |
| SpoIVB | MDPNIRKHVLVLVSSLGCL | YppR | MKKKLGLAGATLVTGAC |
| XenD | MKRKCSCVILLLVILGGC | YpeR | MNILSFLRLGILITISSLLAVACG |
| YacD <sup>*</sup> | MKSKRTIITLIALGVC | YtgA | MKPGLAYMAVFAPACGAC |
| YbbD | MKRFVFLASLVLSCG | YikA | MKMLVLILSLLACG |
| YedC | MKSTFNIQGKTVKCATPMGTSACG | YikA | MKRKLGLGAFVCMAPACG |
| YedC<sup>*</sup> | MKKQRLMTLFTALLVFTGC | YmJ<sup>*</sup> | MKKMLGLLVSFLAAGC |
| YedD | MFQKTYAVFILLLLMTFAACG | YtnK<sup>*</sup> | MKTKTAIFSLSTTLVACG |
| YedD | MNLAPKTVLCLLLDFCG | YtfF | MKMRKLTVMTLATTACG |
| YedH<sup>*</sup> | MKRFNGLSVFAACLALLAVG | YuoO<sup>*</sup> | MKMLNFLLAVAFMVISLACG |
| YoeH<sup>*</sup> | MKKLFLALFVPLAFCG | YuaA | MKMLFLALFVPLAFCG |
| YckB<sup>*</sup> | MKSMHISKAVSTMFAFILLALACG | Yuc | MKTKVASCLLITGSLACG |
| YckK<sup>*</sup> | MKKALLFVMISVAACG | YveA | MKKILIIFCSSLALLTACG |
| YelQ<sup>*</sup> | MKFPLAFFALTVVAGACG | YvG<sup>*</sup> | MVLKKRFGLAIAASPFLACG |
| YedA | MRHVLAVIFLFLISLSCG | YvcK | MKVPSFVMCLPMVALACG |
| YedC | MKKSFVLLGVLVLAGGC | YvdO | MKMPFAAVAACG |
| YedH | MSDKVVLPLFSAPFIVFGACG | YygL<sup>*</sup> | MKFKYSIFIALTAPACG |
| YedJ | MKKRRCKICYNTALLML1LACGT | YvrC<sup>*</sup> | MKKRIAGAAILLLAVMGLACG |
| YedF | MKRLISIFVFAIMACG | Yybm | MNFKARVASCIGAC |
| YidK | MKSFKYGLMLASLYFLACG | YynA | MKNLGAVWIFILVSCG |
| YidK | MRFVWYMTFVVACG | YxeA | MKMKAMAAVIAAAACG |
| YerB | MKKMTVCAFCFVLLVSSCG | YxeB<sup>*</sup> | MKKNLAVMLVLVMSACG |
| YerH | MKKTALAAATVMACG | YxeF | MKPILRNKYGIFLIVACG |
| Yf<sup>+</sup> | MKHISLFLFVMAVMVLSACG | Yxm<sup>*</sup> | MKMKTVLTVALAVLSACG |
| Yj<sup>i</sup> | MKKLFGLVALFLFGCG | Yxm | MKMKAVFVMVGLACG |
| Ymc<sup>+</sup> | MKSTNSKLLASVLLACG | Yxp | MKRLCSLGLVTLVMSACG |
| YgbA<sup>*</sup> | MKKGLIVAVFLALLACG | YxkH | MKKFLISIFLGSCALACG |
| YhaA | MKKVTIAAIHGAAAGGLGSLACG | YybM | MESHYIIRIKKLITIFTIILIPC |
| YhcF<sup>+</sup> | MKKWCSFVFLVLVTSACG | YycO | MKLRKVRMFLVTLNACG |
| YhcN | MKGERQLASVLLIMTSAG | Yyes | MKFRWKLFLVWLALLACG |

# Notes

- Signals of lipoproteins that are homologues of known periplasmic high affinity substrate binding proteins from Gram-negative eubacteria.
SPase II for lipoprotein processing in the transition and post-exponential growth phases.

Alternative Amino-terminal Processing of Pre-PrsA in Cells Lacking SPase II—To examine the effects of the absence of SPase II, the processing of (pre-)PrsA, the major lipoprotein of _B. subtilis_ (15, 16) was studied by pulse-chase labeling experiments with _B. subtilis_ MAŁ. As shown in Fig. 2A (∆lsp), pre-PrsA processing was strongly impaired in the absence of SPase II, and even after a long chase period of 15 min, no mature PrsA was detectable. In contrast, pre-PrsA was rapidly processed to the mature form in the parental strain. As expected, Western blotting experiments showed that _B. subtilis_ MAŁ, accumulated pre-PrsA, but surprisingly, this concerned only about 50% of the total PrsA present in the cells. In addition to pre-PrsA, cells of _B. subtilis_ MAŁ also contained mature-like forms of PrsA, which, compared with mature PrsA, had a slightly lower mobility on SDS-PAGE (Fig. 2B, ∆lsp). This difference in mobility could only be visualized clearly when proteins were separated on long gels (40 cm). In what follows, standard gel systems (15 cm) were used, resulting in a less pronounced separation of mature PrsA (strains containing SPase II) and mature-like forms of PrsA (strains lacking SPase II). As shown in Fig. 2C, the mature-like forms of PrsA were also detected when cells of _B. subtilis_ MAŁ (∆lsp) or the parental strain (8G5) were grown in the presence of globomycin. Western blotting experiments with a xylene-inducible mutant form of PrsA, containing a carboxy-terminal hexahistidine tag, showed that at least one of the mature-like forms of PrsA was cleaved at the amino terminus (Fig. 2D). Taken together, these findings show that in the absence of SPase II, pre-PrsA is subject to alternative amino-terminal processing at a low rate.

It has been shown previously that lipoprotein precursors from which the cleavage site for SPase II was removed by site-directed mutagenesis can be cleaved at alternative sites (for review, see Ref. 45). Type I SPases, which are required for the processing of secretory precursor proteins (for review, see Ref. 46) have been invoked in this alternative processing. To investigate whether the five type I SPases of _B. subtilis_ (i.e. SipS, SipT, SipU, SipV, and SipW) (25, 47) might be involved in the amino-terminal cleavage of pre-PrsA in the absence of SPase II, multiple sip mutants were used. First, the _lsp_ gene of _B. subtilis_ strains ∆SUVW (lacks SipS, SipU, SipV, and SipW) and ∆TU VW (lacks SipT) was disrupted by transformation with chromosomal DNA of _B. subtilis_ MAŁ. As shown by Western blotting, mature-like forms of PrsA were detected in both resulting strains (Fig. 2E). As the sipS and sipT genes can not be disrupted simultaneously (25), the involvement of SipS and SipT was investigated with the _B. subtilis_ strain ∆SXTsS-D146A, which lacks wild-type copies of sipS and sipT but contains a mutant sipS gene specifying the temperature-sensitive SipS-D146A protein. The transcription of the latter gene is controlled by the xylene-inducible _xylA_ promoter. _B. subtilis_ ΔSXTsS-D146A was transformed with chromosomal DNA of _B. subtilis_ MIL, resulting in _B. subtilis_ ΔSXTsS-D146A MIL, in which the synthesis of SPase II depends on the presence of IPTG. As shown in Fig. 2F, alternative processing of pre-PrsA was barely affected in cells depleted of SipS, SipT and SPase II by incubation at 48 °C in the absence of xylene (no activity of SipS-D146A and IPTG). Taken together, these findings indicate that type I SPases are not involved in the alternative processing of pre-PrsA in the absence of SPase II.

Membrane Topology and Lipid Modification of PrsA Are Not Affected in the Absence of SPase II—As PrsA is an essential protein for growth and viability of _B. subtilis_ (16), pre-PrsA and/or the mature-like forms of PrsA that are observed

### Table II

| Plasmids | Relevant properties | Ref. |
|----------|---------------------|------|
| pMutin2 | pBR322-based integration vector for _B. subtilis_; contains a multiple cloning site downstream of the Papomoter (23), and a promoter less lacZ gene preceded by the RBS of the spoVG gene; Ap<sup>+</sup>; Em<sup>+</sup> | 59 |
| pMutin2-MIL | pMutin2 derivative; carries the 5′ part of the _B. subtilis_ lsp gene | This paper |
| pMutin2-MIL | pMutin2 derivative; carries an internal fragment of the _lsp_ gene | This paper |
| pKTH10 | Encodes the o-amylase (AmyQ) of _B. amyloplactonicus_; 6.8 kb; Em<sup>+</sup> | 24 |
| pKTH10-3 | Like pKTH10, encodes the AmyQ-PFB fusion protein, 7.0 kb | 25 |
| pKTH3409 | Vector for xylene-inducible transcription of cloned genes in _B. subtilis_; carries the xylA promoter and the xylR gene; 3.8 kb; Cm<sup>+</sup> | 26 |
| pKTH3409 | pX50 derivative; encodes a carboxyl-terminally hexahistidine-tagged PrsA protein; 4.7 kb | This paper |
| Strains | | |
| E. coli | F<sup>+</sup>; araD139; ∆ (ara-leu7696); Δ (lacX74); galU; galK; hsdR2; mcrA; mcrB1; rapL | 27 |
| MC1061 | | |
| _B. subtilis_ | | |
| 8G5 | trpC2; tyr; his; nic; ured; rib; met; ade | 28 |
| MAŁ (Δlsp) | Like 8G5, _lsp_; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| MIL (lisp) | Like 8G5, _Papac-lisp_; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| ∆SUVW | Like 8G5, rib<sup>+</sup>; sipS, sipU; sipV; sipW; Tc<sup>+</sup> | 25 |
| ∆SUVW MIL | Like 8G5, rib<sup>+</sup>; sipS, sipU; sipV; sipW; Tc<sup>+</sup>; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| ∆TU VW | Like 8G5, _sipT_; _Cm<sup>+</sup>; sipU; sipV; sipW; Tc<sup>+</sup>; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| ∆TU VW MIL | Like 8G5, _sipT_; _Cm<sup>+</sup>; sipU; sipV; sipW; Tc<sup>+</sup>; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| ∆STxsS-D146A | Like 8G5, rib<sup>+</sup>; sipT; Sp<sup>+</sup>; _PcyAla-sipS-D146A; amyE_; Cm<sup>+</sup>; preprotein processing by SipS and SipT is severely impaired at 48 °C in the absence of xylene | 25 |
| ∆STxsS-D146A-MIL | Like 8G5, rib<sup>+</sup>; sipT; Sp<sup>+</sup>; _PcyAla-sipS-D146A; amyE_; Cm<sup>+</sup>; _Papac-lisp_; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| IH6538 | trpC2; hisA1; _glyB133_ | 14 |
| IH6510 | trpC2; thr-5; lgt (prs-11) | 14 |
| IH7327 | trpC2; hisA1; _glyB133_; _lsp-lacZ_; Em<sup>+</sup> | This paper |
| M1F | trpC2; secDF::pMIC; Em<sup>+</sup> | 29 |

3 V. P. Kontinen and M. Sarvas, unpublished results.
in cells lacking SPase II must be (partially) active. This implies that at least one of the latter forms of PrsA is correctly localized at the external surface of the membrane. To determine the topology of pre-PrsA and the mature-like forms of PrsA in the absence of SPase II, protoplasts of \textit{B. subtilis} 8G5 \textit{M} \textit{D} were incubated with trypsin. Like the mature PrsA of \textit{B. subtilis} 8G5, pre-PrsA and the mature-like forms of PrsA of \textit{B. subtilis} 8G5 \textit{M} \textit{D} were associated with protoplasts and accessible to trypsin (Fig. 3A, \textit{Δlsp}). In contrast, the cytosolic protein GroEL was only accessible to

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**Fig. 1. Construction and properties of \textit{lsp} mutant strains of \textit{B. subtilis}**. A, schematic presentation of the construction of \textit{lsp} mutant strains of \textit{B. subtilis}. \textit{B. subtilis} MIL (I\textit{sp}) was constructed by Campbell-type integration of pMutin2-MIL in the \textit{ileS-pyrR} locus of \textit{B. subtilis} 8G5 in such a way that the \textit{lsp} promoter region was replaced by the IPTG-dependent \textit{Pspac} promoter. \textit{B. subtilis} M\textit{AL} (\textit{Δlsp}) was constructed by Campbell-type integration of pMutin2-M\textit{AL} in the \textit{ileS-pyrR} locus of \textit{B. subtilis} 8G5 in such a way that the \textit{lsp} gene was disrupted, and downstream genes were placed under the control of the \textit{Pspac} promoter. Due to the integration of pMutin2-MIL and pMutin2-M\textit{AL}, \textit{B. subtilis} MIL and M\textit{AL} both contain the \textit{spoVG-lacZ} reporter gene of pMutin2 under the transcriptional control of the \textit{lsp} promoter region. The relative positions of open reading frames in the \textit{ileS-pyrR} locus are shown. Restriction sites relevant for the construction are indicated: Ba, BamHI; Be, BclI; Bg, BglII; Nd, NdeI; Hi, HindIII. Ori pBR322, replication functions of pBR322; Apr, ampicillin resistance marker; Emr, erythromycin resistance marker; \textit{lsp}, 3' truncated \textit{lsp} gene; T,T<sub>p</sub>, transcriptional terminators on pMutin2; \textit{lsp}, 5' truncated \textit{lsp} gene. B, temperature-sensitive growth of \textit{B. subtilis} lacking SPase II. Overnight cultures of \textit{B. subtilis} M\textit{AL} (\textit{Δlsp}) (■) and the parental strain 8G5 (○) grown in TY medium at 37 °C were diluted 100-fold in fresh TY medium and incubated at 37 °C. When the cells reached an \textit{A}_{600} of about 1.2, the temperature was shifted to 48 °C. Zero time (\textit{t} = 0) indicates the transition point between the exponential and postexponential growth phases. C, time courses of the transcription of the \textit{lsp-lacZ} gene fusion in \textit{B. subtilis} MIL were determined in cells growing at 37 °C in TY (●) or minimal (▲) medium, both supplemented with 1 mM IPTG. \textit{β}-Galactosidase activities were determined in units per \textit{A}_{600}. Zero time (\textit{t} = 0) indicates the transition point between the exponential and postexponential growth phases.
methionine for 1 min prior to chase with excess nonradioactive
noprecipitation, SDS-PAGE, and fluorography. Cells were labeled
was analyzed by pulse-chase labeling at 37 °C and subsequent immu-
pre-PrsA in cells of
B. subtilis
pre-PrsA in cells lacking SPase II and various type I SPases.

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Fig. 2. Processing of PrsA in lsp mutant strains. A, processing of
pre-PrsA in cells of B. subtilis MΔL (Δlsp) and the parental strain 8G5
was analyzed by pulse-chase labeling at 37 °C and subsequent immu-
nonprecipitation, SDS-PAGE, and fluorography. Cells were labeled
with [35S]methionine for 1 min prior to chase with excess nonradioactive
methionine. Samples were withdrawn at 0, 1, and 15 min after the
chase. The positions of pre-PrsA and mature PrsA are indicated. B, accu-
ulation of pre-PrsA in cells of B. subtilis MΔL (Δlsp) and the parental strain 8G5. Samples were withdrawn after overnight growth
and analyzed by SDS-PAGE (long gels of 40 cm) and Western blotting.
The positions of PrsA, pre-PrsA, and two mature-like forms of PrsA
(PrsA*) are indicated. C, effects of globomycin on the accumulation of
pre-PrsA in cells of B. subtilis 8G5 and MΔL (Δlsp). Cells were grown
in TY medium at 37 °C in the presence (+) or absence (−) of 80 μM
globomycin, and samples for SDS-PAGE and Western blotting were
withdrawn after overnight growth. The positions of PrsA, pre-PrsA, and
mature-like forms of PrsA* are indicated. D, alternative amino-termi-
nal processing of pre-PrsA in B. subtilis MΔL was demonstrated by
SDS-PAGE and Western blotting using the xylose-inducible, carboxy-
terminally hexahistidine-tagged PrsA protein (PrsA-His) specified by
pKTH3409. Cells of B. subtilis MΔL (Δlsp) and the parental strain 8G5
containing pKTH3409 were grown overnight in the presence (+) or
absence (−) of 1% xylose. The positions of PrsA-His, pre-PrsA-His, and
mature-like forms of PrsA-His* are indicated. E and F, accumulation of
pre-PrsA in cells lacking SPase II and various type I SPases. E, accu-
trypsin when the protoplasts were lysed with Triton X-100
(Fig. 3B). Because the latter findings show that pre-PrsA and
the mature-like forms of PrsA are correctly localized in cells
lacking SPase II, we also addressed the question whether
these forms are lipid-modified. To this purpose, palmitic acid
labeling experiments were performed with strains lacking
SPase II or, as a control, the diacylglycerol transferase,
specified by the lgt gene (i.e. prs-11) (14).4 Cells of the latter
strain (Δlgt) accumulated non-lipomodified pre-PrsA,
whereas the strain lacking SPase II (Δlsp) accumulated lipomodified pre-PrsA and mature-like PrsA (Fig. 4, A and B). In
conclusion, these data show that, in the absence of SPase II,
the precursor and mature-like forms of PrsA are lipid-modified,
displaying a similar membrane topology as the mature
PrsA in SPase II-proficient cells. Thus, all of these forms
might, in principle, be active and responsible for the viability
of cells lacking SPase II.

Lsp Is Required for the Efficient Processing and Secretion of
the Non-lipoprotein Pre-AmyQ—It was previously shown that
PrsA sets a limit for high level secretion of the Bacillus
amylobacteriaceus a-amyrase AmyQ and that it is required for
the folding of AmyQ into a protease-resistant conformation
(16). To examine the effect of SPase II depletion on PrsA activity,
AmyQ secretion was monitored in B. subtilis 8G5 MΔL
(Fig. 5, Δlsp) and 8G5 MIL (Fig. 5, Δlsp). To this purpose, both
strains were transformed with pKTH10, which contains the
amyQ gene (24). Next, the secretion of AmyQ was analyzed by
Western blotting. As shown in Fig. 5, the accumulation of
pre-PrsA and mature-like forms of PrsA in cells depleted of
SPase II (Fig. 5A, Δlsp and Δlsp in the absence of IPTG) was
paralleled by the secretion of about 5-fold reduced amounts of
mature AmyQ into the growth medium (Fig. 5C). The latter

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mulation of pre-PrsA in cells of B. subtilis MΔL (Δlsp), B. subtilis
ΔSUWW MIL (ΔSUWW las), and B. subtilis ΔTUVW MIL (ΔTUVW
lsp). Samples were withdrawn after overnight growth and analyzed by
SDS-PAGE and Western blotting. The positions of pre-PrsA and
mature-like forms of PrsA* are indicated. F, exponentially growing cells of
B. subtilis ΔSTxs-D146A MIL in TY medium with 1 mM IPTG (37 °C)
cultures were washed and resuspended in fresh TY medium. Upon incubation
for 1 h in the presence (+) or absence (−) of 1 mM IPTG and/or 1% xylose
at 37 °C or 48 °C, samples were taken for SDS-PAGE and Western
blotting. The positions of PrsA, pre-PrsA, and the mature-like forms of
PrsA* are indicated.
observation is diagnostic for reduced levels of PrsA activity. In addition, SPase II-depleted cells accumulated increased levels of pre-AmyQ (Fig. 5B), which is atypical for PrsA mutants (14). As shown by pulse-chase labeling experiments, the rate of pre-AmyQ processing by type I SPase(s) was slightly (but reproducibly) reduced in cells lacking SPase II (Fig. 5D). As mutations in PrsA do not cause the accumulation of pre-AmyQ, this effect of the absence of SPase II must be attributed to the accumulation of lipoprotein precursors or the malfunction of an as yet unidentified lipoprotein. The reason why the kinetic effects of the absence of SPase II on pre-AmyQ processing (Fig. 5D) are mild in comparison to the strong accumulation of pre-AmyQ at steady state (Fig. 5B) is not clear. One explanation could be that the growth conditions in both types of experiments are not identical.

To determine whether the accumulation of pre-AmyQ in SPase II-depleted cells reflects a reduced rate of translocation of pre-AmyQ, which might be caused by the accumulation of lipoprotein precursors, we made use of an AmyQ variant (AmyQ-PSBT) (25) containing the biotinylation domain of the lipoprotein precursors, we made use of an AmyQ variant of pre-AmyQ, which might be caused by the accumulation of SPase II-depleted cells reflects a reduced rate of translocation of pre-AmyQ at steady state (Fig. 5A). As shown by pulse-chase labeling experiments, the rate of pre-AmyQ processing by type I SPase(s) was slightly (but reproducibly) reduced in cells lacking SPase II (Fig. 5D). As mutations in PrsA do not cause the accumulation of pre-AmyQ, this effect of the absence of SPase II must be attributed to the accumulation of lipoprotein precursors or the malfunction of an as yet unidentified lipoprotein. The reason why the kinetic effects of the absence of SPase II on pre-AmyQ processing (Fig. 5D) are mild in comparison to the strong accumulation of pre-AmyQ at steady state (Fig. 5B) is not clear. One explanation could be that the growth conditions in both types of experiments are not identical.

To determine whether the accumulation of pre-AmyQ in SPase II-depleted cells reflects a reduced rate of translocation of pre-AmyQ, which might be caused by the accumulation of lipoprotein precursors, we made use of an AmyQ variant (AmyQ-PSBT) (25) containing the biotinylation domain of the pyruvate decarboxylase of Propionibacterium shermanii (48). The rationale of this experiment is that pre-AmyQ-PSBT can only be biotinylated by the cytoplasmic biotin-ligase if the PSBT domain folds into its native three-dimensional structure in the cytoplasm. This will only happen if the rate of translocation of pre-AmyQ-PSBT across the membrane is significantly reduced. As shown in Fig. 6, cells lacking SPase II(Δlsp) did not accumulate biotinylated pre-AmyQ-PSBT, irrespective of the growth temperature (15, 37, or 48 °C), although AmyQ-PSBT was produced under these conditions (data not shown). In contrast, B. subtilis cells with a disrupted secDF gene (B. subtilis MIF) (29) accumulated biotinylated forms of AmyQ-PSBT at all growth temperatures tested (Fig. 6, ΔsecDF), whereas cells lacking SipS and SipT but expressing the temperature-sensitive SipS-D146A protein (Fig. 6, ΔStxS-D146A) (25) accumulated biotinylated forms of AmyQ-PSBT only at 48 °C. These observations indicate that the accumulation of pre-AmyQ in SPase II-depleted cells is not due to impaired translocation across the membrane and that the cold sensitivity of these cells is not related to protein translocation defects. Instead, the accumulation of pre-AmyQ must be attributed to

**Fig. 4. Lipid modification of PrsA.** B. subtilis IH6538 (parental strain), IH6538 with an integrated copy of pMutin2-MAL, disrupting the lsp gene (Δlsp), and IH6538 with the prs-11 mutation, inactivating the lgt gene (Δlgt), were grown in TY medium at 37 °C. Exponentially growing cells were labeled with 50 μCi of [3H]palmitic acid for about 45 min. Membranes were isolated and used for SDS-PAGE, Western blotting, and immunodetection with PrsA-specific antibodies (A) or for SDS-PAGE and fluorography (B). B. subtilis IH6538 and derivatives of this strain were used in this experiment because they incorporate higher levels of [3H]palmitic acid than B. subtilis 8G5. The positions of nonmodified pre-PrsA (pre-PrsA*), lipid-modified pre-PrsA (pre-PrsA**), lipid-modified mature PrsA (PrsA*), and mature-like forms of PrsA (PrsA**) are indicated.

**Fig. 5. Impaired secretion of AmyQ in the absence of SPase II.** Cells of B. subtilis MIL(Δlsp), MIL(Δlsp), and the parental strain 8G5 were grown in TY medium at 37 °C in the presence (+) or absence (−) of 1 mM IPTG. Samples for SDS-PAGE and Western blotting were prepared from cells and their growth medium. Cells were harvested 2 h after the transition between exponential and postexponential growth (t = 2) (Fig. 5B). Specific antibodies were used to detect the cellular levels of PrsA (A) and AmyQ (B) and the levels of secreted AmyQ in the growth medium (C). The positions of pre-PrsA, the mature form of PrsA, the mature-like form of PrsA (PrsA**), AmyQ, and pre-AmyQ are indicated. D, processing of pre-AmyQ in B. subtilis MIL(Δlsp) and the parental strain 8G5, was analyzed by pulse-chase labeling at 37 °C and subsequent immunoprecipitation, SDS-PAGE, and fluorography. Cells were labeled with [35S]methionine for 1 min prior to chase with excess nonradioactive methionine. Samples were withdrawn after the chase at the times indicated. The positions of pre-AmyQ and mature AmyQ are indicated.

**DISCUSSION**

We have previously shown that five paralogous type I SPases are involved in the processing of secretory precursor proteins in B. subtilis (25, 47, 49, 50). Two of these, designated SipS and SipT, are of major importance for protein secretion, and cells depleted of both SipS and SipT stop growing and lyse. The other type I SPases (SipU, SipV, and SipW) are of minor importance for protein secretion and viability. Thus, B. subtilis is representative for Gram-positive eubacteria, archaea, and eukaryotes, many of which contain paralogous type I SPases (25). In contrast to the type I SPases, B. subtilis (35, 51) and other eubacteria seem to contain only one gene for SPase II, whereas type II SPases appear to be absent from archaea and eukaryotes. Here, we document four unexpected observations with respect to SPase II function in B. subtilis. First, unlike the SPase II of E. coli, the SPase II of B. subtilis is not essential for

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2 The product of the yaaT gene, which has been annotated as a putative SPase II-encoding gene of B. subtilis (51), does not show sequence similarity to known type II SPases, and, moreover, is predicted to be a soluble cytoplasmic protein (see Footnote 1). This makes a role of the YaaT protein in lipoprotein processing highly unlikely.
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Notably, the lipoproteins of *B. subtilis* (Table I) and other Gram-positive eubacteria (13) seem to lack aspartic acid residues at the +2 position. The latter observation suggests that this aspartic acid residue has specifically evolved as a retention signal for lipoproteins of Gram-negative eubacteria. Furthermore, the lack of *Int* genes from *B. subtilis* and *M. genitalium* suggests that the lipoproteins of these organisms are not aminoacylated. Consistent with the latter hypothesis, a macrophage-stimulating lipoprotein with a non-acylated amino terminus has been isolated from *Mycoplasma fermentans* (54). The latter observations raise the intriguing question of whether aminoacylation has a particular function in Gram-negative eubacteria; for example, in the sorting of lipoproteins.

Cold sensitivity seems to be a general property of *E. coli* (55) and *B. subtilis* (29) strains, which are defective in protein translocation via the Sec-machinery. Thus, the cold sensitivity of *B. subtilis lsp* mutants might reflect a general defect in protein translocation, which could be caused primarily by the accumulation of lipoprotein precursors. However, this possibility is unlikely, as SPhase II-depleted cells did not show a translocation defect for AmyQ-PSBT, irrespective of the growth temperature. Instead, our results indicate that the observed cold and heat sensitivity of *B. subtilis* mutants lacking SPhase II is caused by the malfunction of certain lipoproteins, which are required for cell viability at low and high temperatures.

The observation that the development of competence, sporulation, and germination are not affected by the absence of SPhase II shows that the precursors, or (putative) alternatively processed forms of the lipoproteins required for these primitive developmental processes are active. Similarly, the fact that the strain lacking SPhase II is viable at 37 °C shows that pre-PrsA and/or the mature-like forms of PrsA are active, because PrsA is essential for cell viability (16). Nevertheless, as indicated by the reduced secretion of AmyQ in *lsp* mutants *B. subtilis*, lipoprotein processing is important for the full functionality of lipoproteins, such as PrsA. Our current working model for the effects of SPhase II limitation on the processing of pre-PrsA and the secretion of AmyQ is shown in Fig. 7. Although SPhase II-depleted cells contain similar amounts of PrsA protein as wild-type cells, processing by SPhase II seems to be important for the stable maintenance of certain other lipoproteins, suggesting that processing by SPhase II is required to protect these proteins against proteolytic degradation at the membrane-cell wall interface.

The fact that pre-PrsA is subject to alternative processing in the absence of SPhase II is reminiscent of the previously reported observation that mutants of the penicillinase PenP of *Bacillus licheniformis*, which lack the cysteine residue at the +1 position, are subject to alternative processing. As these mutant proteins are not lipid-modified and contain putative SPhase I cleavage sites, type I SPhases have been invoked in their processing (57, 58). In contrast, our results indicate that type I SPhases are not involved in the alternative amino-terminal processing of pre-PrsA, which is consistent with the fact that this precursor is lipid-modified. Surprisingly, the nonmodified pre-PrsA produced by the *lgt* mutant is not processed, even though putative SPhase I cleavage sites are present in this precursor, suggesting that it contains an as yet unidentified “SPhase I avoidance signal.” Important challenges for future research are the identification of the protease(s) involved in the alternative processing of pre-PrsA and the SPhase I avoidance signal in this precursor.

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[Ref. 53 and this paper], this antibiotic displays no cytotoxic activity against *E. coli* as an antibiotic for *B. subtilis* (Ref. 53 and this paper), this antibiotic displays no cytotoxic activity against *E. coli* as an antibiotic for *B. subtilis* (Ref. 53 and this paper), this antibiotic displays no cytotoxic activity against *E. coli* as an antibiotic for *B. subtilis* (Ref. 53 and this paper), this antibiotic displays no cytotoxic activity against *E. coli* as an antibiotic for *B. subtilis* (Ref. 53 and this paper), this antibiotic displays no cytotoxic activity against *E. coli* as an antibiotic for *B. subtilis* (Ref. 53 and this paper), this antibiotic displays no cytotoxic activity against.

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*J. Bengtsson and L. Hederstedt, personal communication.*
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Finally, the reason why SPase II-depleted cells accumulate pre-AmyQ is presently not completely clear. First, as shown with AmyQ-PsBT, the rate of AmyQ translation in these cells is not detectably affected. Second, as PrsA mutants do not accumulate pre-AmyQ (14), this effect cannot be attributed to PrsA malfunction. Consequently, the accumulation of pre-AmyQ must be due to the malfunction of at least one as yet unknown lipoprotein, which affects the stability or processing of pre-AmyQ. This hypothesis is supported by our observation that the half-life of pre-AmyQ and at least one other precursor, pre(A13i)-β-lactamase (50) (data not shown) is slightly increased in the absence of SPase II. We are presently investigating which of the putative lipoproteins of B. subtilis could be responsible for this effect.

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