

University of Groningen

## Replication and maintenance of plasmids in *Bacillus subtilis*

Meijer, Wilhelmus Johannes Jozef

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

*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

1995

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Meijer, W. J. J. (1995). *Replication and maintenance of plasmids in Bacillus subtilis*. s.n.

### Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

### Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

# Chapter VI

The endogenous *Bacillus subtilis* (natto) plasmids pTA1015 and pTA1040 contain signal peptidase-encoding genes: identification of a new structural module on cryptic plasmids

Wilfried J.J. Meijer, Anne de Jong, G.Bea.A. Wisman, Harold Tjalsma, Gerard Venema, Sierd Bron and Jan Maarten van Dijl

Accepted for publication in: *Molecular Microbiology*

## SUMMARY

Various strains of *Bacillus subtilis* (natto) contain small cryptic plasmids that replicate via the rolling circle mechanism. Like plasmids from other Gram-positive bacteria, these plasmids are composed of several distinct structural modules. A new structural module was identified on the *B. subtilis* plasmids pTA1015 and pTA1040. It is composed of two genes: one specifies an unidentified protein with a putative signal peptide; the other (sipP) specifies a functional type I signal peptidase (SPase). The homologous, but non-identical, sipP genes of the two plasmids are the first identified plasmid-specific SPase-encoding genes. With respect to structure and activity, the corresponding enzymes (denoted SipP) are highly similar to the chromosomally encoded SPase, SipS, of *B. subtilis* and several newly identified SPases of other bacilli. Our findings suggest that plasmid-encoded SPases have evolved because under certain conditions SPase can be a limiting factor for protein secretion in *B. subtilis*.

## INTRODUCTION

Most small plasmids of Gram-positive bacteria replicate via single-stranded DNA intermediates, probably by rolling circle replication (RCR). For this process, three distinct structural modules are required: (1), a rep gene, encoding a protein (Rep) required for the initiation of plasmid replication at (2), the double-strand origin (DSO); and (3), a single-strand origin (SSO) for the conversion of single-stranded replication intermediates to double-stranded plasmid DNA. Based on sequence similarity, the Rep proteins, DSO's and SSO's from different plasmids have been grouped into several distinct families (Gruss and Ehrlich, 1989; Bron, 1990; Meijer et al., 1995). Frequently, RCR-type plasmids also contain two other structural elements: (1), a mob gene (also called pre gene), specifying a protein (Mob/Pre) required for conjugative plasmid mobilisation and site-specific recombination; and (2), an antibiotic resistance marker. In different plasmids, the

relative order of these structural modules is not always the same (Gruss and Ehrlich, 1989; Novick, 1989; Bron, 1990).

The existence of cryptic endogenous *Bacillus subtilis* (natto) plasmids has already been known for a long time (Tanaka and Koshikawa, 1977; Uozumi et al., 1980; Hara et al., 1983). Based on their size and restriction patterns, these plasmids could be classified into six different groups, of which pTA1015, pTA1020, pTA1030, pTA1040, pTA1050, and pTA1060 are representatives (Uozumi et al., 1980) (our unpublished data). All of these pTA-plasmids appear to replicate by RCR (Meijer et al., 1995). Only plasmids of the pTA1020-type (Devine et al., 1989; Seery and Devine, 1993) and the pTA1060-type (Bron et al., 1987; Chang et al., 1987) were analysed in some detail. The latter studies focused on the functions for plasmid replication since these plasmids are stably maintained in *B. subtilis*. Thus, it seemed attractive to use their replication functions for the construction of cloning vectors. Indeed, for cloning in *B. subtilis*, the

pTA1060-based vectors were superior to cloning vectors derived from *Staphylococcus aureus* RCR plasmids, which are frequently unstable (Gruss and Ehrlich, 1989; Bron, 1990; Bron et al., 1991).

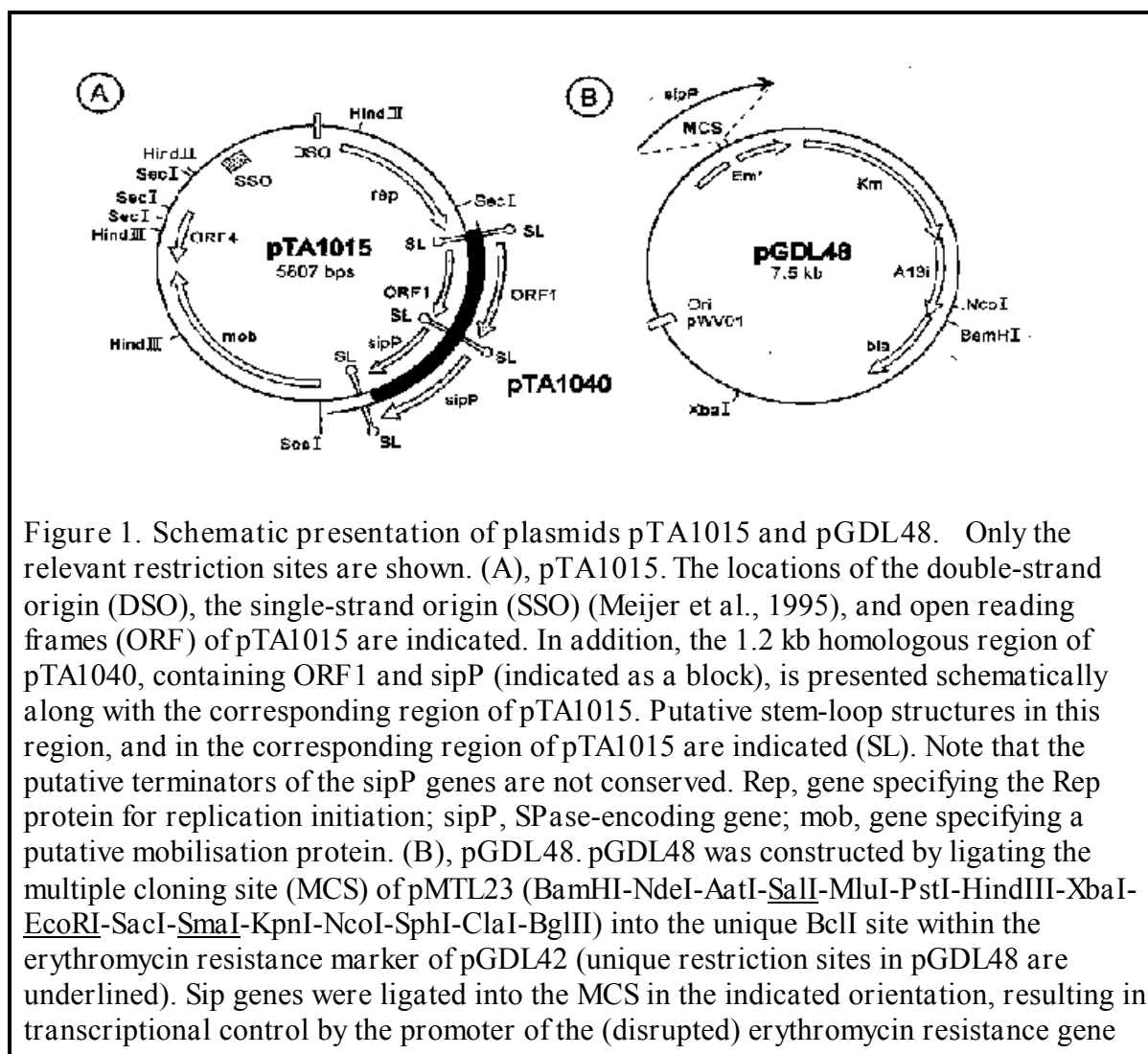
Thusfar, little is known about the biological function of cryptic *B. subtilis* plasmids. The fact that these plasmids are stably maintained suggests that they may confer, as yet unidentified, beneficial properties to the host. To identify functions that may account for their high stability, and to approach the question of the biological function of cryptic *B. subtilis* plasmids, we recently sequenced the *B. subtilis* plasmids pTA1015 and pTA1060

(our unpublished data). In the present paper, we report on the surprising finding that pTA1015 and pTA1040 contain type I signal peptidase (SPase)-encoding genes, which appear to be part of a novel structural module of certain plasmids from *B. subtilis*.

## RESULTS

Plasmid pTA1015 carries a gene for a functional type I signal peptidase.

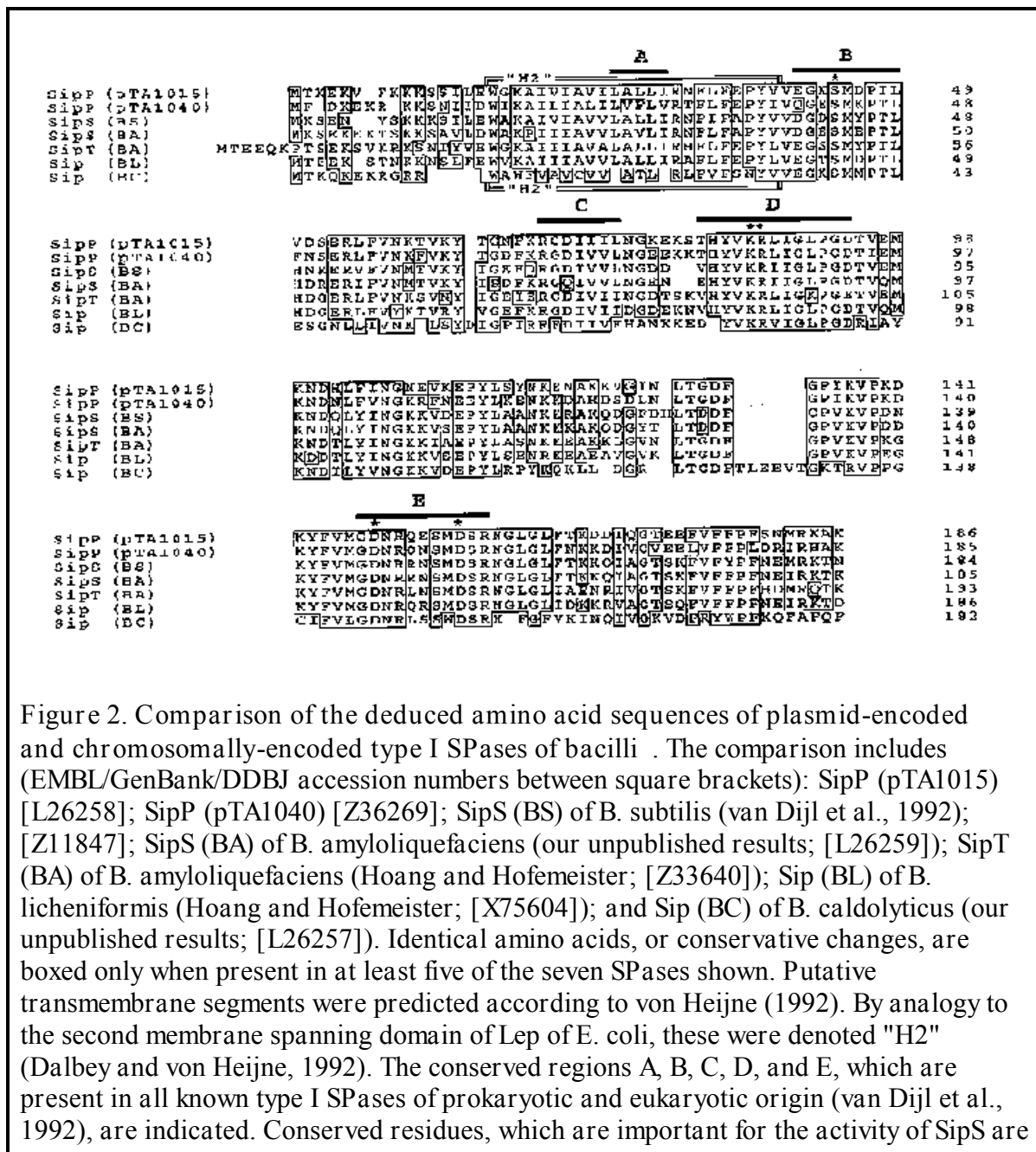
The sequence analysis of pTA1015 revealed the presence of five open reading frames (ORF's), a putative DSO, and a putative SSO (Figure 1).



Two of the ORF's, ORF1 (146 codons) and ORF4 (159 codons), did not show any significant similarity to previously identified sequences. Two other ORF's, ORF3 (482 codons) and ORF5 (339 codons) showed a high degree of similarity to mob and rep genes, respectively (data not shown).

Interestingly, the nucleotide

sequence of ORF2 showed 65% identity with that of the chromosomal sipS gene of *B. subtilis*, encoding a type I SPase (van Dijnl et al., 1992). The deduced amino acid sequence of ORF2 (186 residues) showed 70% identity with that of the *B. subtilis* SipS protein. Like SipS, the pTA1015-encoded SPase homologue contained the patterns of conserved amino acids that can



be found in all other known type I SPases of prokaryotic and eukaryotic origin (Figure 2). These findings strongly indicated that pTA1015 specifies a type I SPase, which is able to remove signal peptides from secretory proteins (processing).

To test the functionality of the pTA1015 (ORF2)-encoded SPase homologue, the hybrid precursor pre(A13i)- $\beta$ -lactamase was used. This precursor is processed by SipS, but not by the type I SPase of *Escherichia coli*. In the latter organism, processing of pre(A13i)- $\beta$ -lactamase by SipS can be visualised in a halo assay on plates (van Dijl et al., 1991b;

van Dijl et al., 1992). Thus, the activity of the pTA1015-encoded SPase homologue could be monitored similarly in *E. coli*.

To this purpose, a 671 bp fragment of pTA1015, containing ORF2 with 39 bp of its upstream sequence, and 72 bp of its downstream sequence, was amplified by PCR using primers 15-2 and 15-5 (Table 3). The unique Sall and EcoRI sites of primers 15-2 and 15-5, respectively, were used to ligate the amplified fragment into the multiple cloning site of plasmid pGDL48 (Figure 1B), encoding pre(A13i)- $\beta$ -lactamase. The resulting plasmid was denoted pGDL61. Transformants of *E. coli*

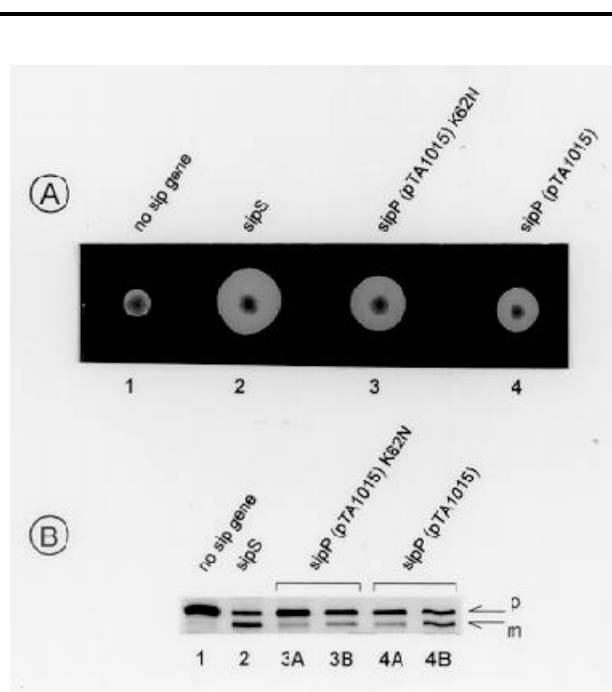


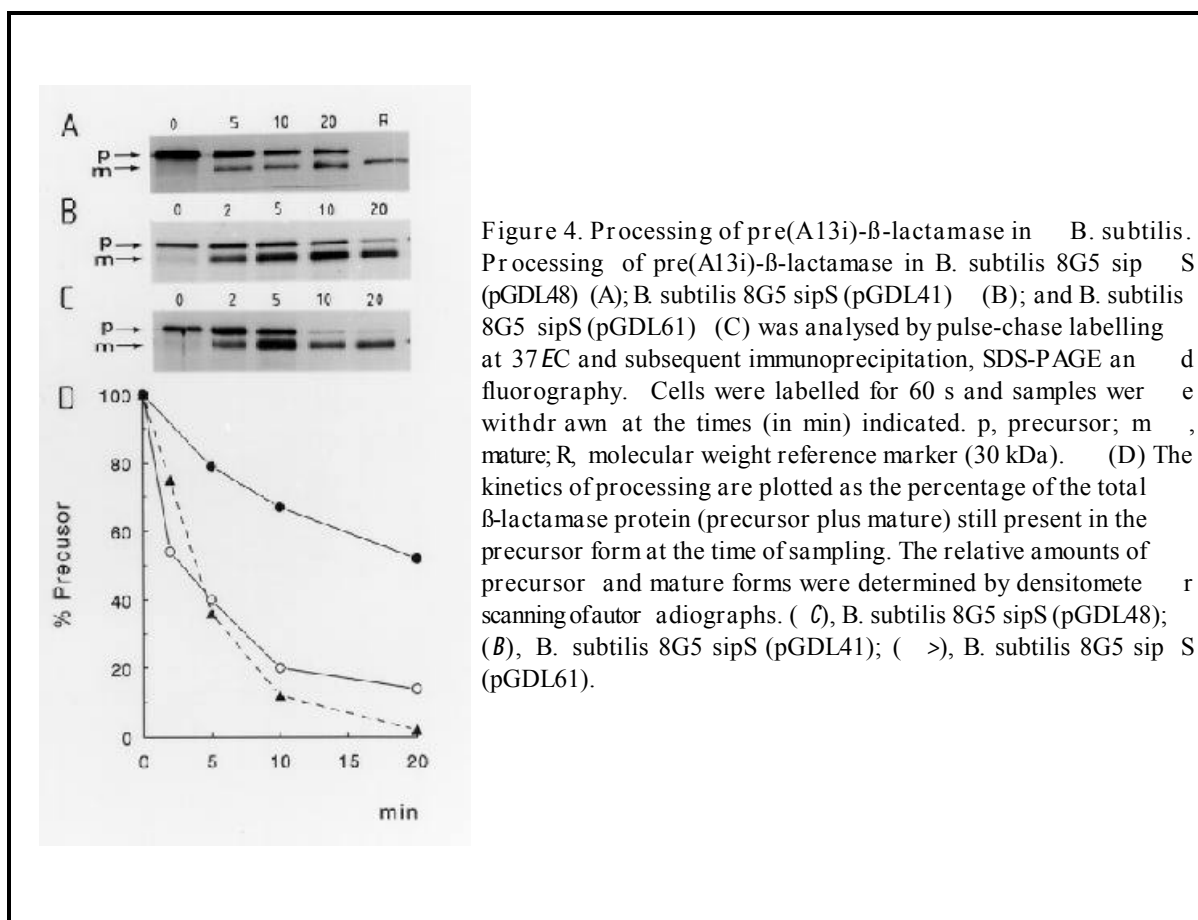
Figure 3. Processing of pre(A13i)- $\beta$ -lactamase by SipP (pTA1015) in *E. coli*. (A), Leakage of mature (A13i)- $\beta$ -lactamase into the medium. Colonies of *E. coli* MC1061 harbouring pGDL48 (1; no sip gene), pGDL41 (2; sipS), pGDL60.1 (3; sipP [pTA1015] K62N), and pGDL61 (4; sipP [pTA1015]) were transferred to fresh plates and incubated overnight at 37 °C.  $\beta$ -Lactamase activity was assayed as described in Materials and Methods. Due to a slight difference in the thickness of the deep-blue coloured overlayer on the plate used for this experiment, the halos in lanes 2, 3 and 4 are not of the same size. (B), Pulse-chase analysis of pre(A13i)- $\beta$ -lactamase processing. Processing of pre(A13i)- $\beta$ -lactamase in *E. coli* MC1061 harbouring pGDL48 (lane 1; no sip gene), pGDL41 (lane 2; sipS), pGDL60.1 (lanes 3A and 3B; sipP [pTA1015] K62N), or pGDL61 (lanes 4A and 4B; sipP [pTA1015]) was analysed by pulse-chase labelling at 37 °C and subsequent immunoprecipitation, SDS-PAGE and fluorography. Cells were labelled with [<sup>35</sup>S] methionine for 60 sec prior to chase with excess non-radioactive methionine. Samples were withdrawn 1 min (lanes 3A and 4A), or 10 min (lanes 1, 2, 3B, and 4B) after the chase. p, precursor; m, mature

MC1061 with pGDL61 were tested for their ability to produce halos in the plate assay for pre(A13i)- $\beta$ -lactamase processing. *E. coli* MC1061, containing pGDL41, which carries the sipS gene, was used as a positive control, and *E. coli* MC1061 (pGDL48) as a negative control. Like *E. coli* MC1061 (pGDL41), *E. coli* MC1061 (pGDL61) clearly formed halos, whereas *E. coli* MC1061 (pGDL48) was unable to do so (Figure 3A; pGDL60.1 is discussed in a later section).

To verify that the observed halo formation was due to processing of pre(A13i)- $\beta$ -lactamase and to estimate relative efficiencies of processing (halo size is not directly related to the level of SPase activity), pulse-chase labelling experiments were performed. The results (Figure 3B) showed that pre(A13i)- $\beta$ -lactamase was processed to the mature form in *E. coli* MC1061 (pGDL61). The

efficiency of pre(A13i)- $\beta$ -lactamase processing in *E. coli* MC1061 (pGDL61) was comparable to that observed in *E. coli* MC1061 (pGDL41) (Figure 3B; lanes 4B and 2, respectively); in both cases approximately 90% of the precursor was processed after 30 min of chase (data not shown).

A similar result was obtained when pre(A13i)- $\beta$ -lactamase processing was analysed in *B. subtilis*. Since pre(A13i)- $\beta$ -lactamase is processed at a relatively high rate in wild-type *B. subtilis* (van Dijl et al., 1992), we used the *B. subtilis* strain 8G5 sipS, which lacks the chromosomal sipS gene. In *B. subtilis* 8G5 sipS (pGDL48), pre(A13i)- $\beta$ -lactamase was processed relatively slowly (Figure 4A). We attribute the residual processing activity in this control to the presence of a second sip gene on the chromosome (van Dijl et al., 1992) (our unpublished results). Compared to *B.*



*subtilis* 8G5 sipS (pGDL48), pre(A13i)- $\beta$ -lactamase was processed at a much higher rate in *B. subtilis* 8G5 sipS containing pGDL41, or pGDL61 (Figures 4B, C and D).

These data show that pTA1015 encodes a type I SPase, which is functional both in *E. coli* and *B. subtilis*. Because this type I SPase is plasmid-encoded, it was denoted SipP (pTA1015) (Signal peptidase from Plasmid pTA1015). The nucleotide sequence of the 1.7 kb *SecI* fragment of pTA1015, which contains the sipP gene (Figure 1A), has been deposited in the EMBL/Genbank/DDBJ nucleotide sequence database and was assigned the accession number L26258.

Plasmid pTA1040 also carries a sipP gene specifying a functional type I SPase

The sequence analysis of pTA1060 revealed that this plasmid does not contain an SPase-encoding (sip) gene (data not shown). To investigate whether the presence of the sipP gene is a unique feature of pTA1015, or whether other cryptic *B. subtilis* plasmids also contain sip genes, Southern hybridisation experiments were performed. The plasmids pTA1015 (positive control), pTA1020, pTA1030, pTA1040, pTA1050, and pTA1060 (negative control) were screened using a sipP-containing PCR fragment, which was obtained with primers 15-2 and 15-5, as a probe. As expected, a strong hybridisation signal was obtained with pTA1015. However, weaker hybridisation signals were also obtained with pTA1020 (6.6 kb) and pTA1040 (7.8 kb) (not shown). A 1.4 kb *EcoRI* - *HindIII* fragment of pTA1040, which hybridised with the sipP probe of pTA1015 was further analysed by sequencing. Indeed, this fragment contained an ORF, the deduced amino acid sequence of which (185 residues) showed 60% and 71% identity to that of SipS of *B. subtilis* and SipP of pTA1015, respectively (Figure 2).

To test the functionality of the putative pTA1040-encoded SPase homologue, the corresponding gene was introduced into pGDL48, similar to sipP (pTA1015). This resulted in plasmid pGDL71 (not shown). Subsequently, processing of pre(A13i)- $\beta$ -lactamase was analysed in *E. coli* MC1061 (pGDL71), and in *B. subtilis* 8G5 sipS (pGDL71). In both organisms, the efficiency of pre(A13i)- $\beta$ -lactamase processing, as measured by pulse-chase labellings assays, was essentially the same as that observed with SipS, and SipP (pTA1015) (not shown). These data showed that also pTA1040 specifies a functional type I SPase. By analogy to the sipP (pTA1015) gene, the gene for this SPase was denoted sipP (pTA1040) (EMBL/Genbank/DDBJ accession number Z36269).

#### Comparison of *B. subtilis* sipP genes

The two sipP genes described above appeared to be highly similar to the chromosomal sipS gene of *B. subtilis*. Firstly, sipS, sipP (pTA1015) and sipP (pTA1040) were preceded by a potential RBS with the sequence GGAGG. Secondly, like sipS, both sipP genes possess an atypical start codon (TTG for sipS; TTG for sipP [pTA1015]; and GTG for sipP [pTA1040]). Only the spacing between the RBS and the putative start codon of sipS and the sipP genes was slightly different (10 nucleotides for sipS; 11 nucleotides for the two sipP genes). Thirdly, sipS (555 nucleotides), sipP (pTA1015) (561 nucleotides), and sipP (pTA1040) (558 nucleotides) are highly similar: sipS and sipP (pTA1015) show 66% sequence identity; sipS and sipP (pTA1040) 64%; and sipP (pTA1015) and sipP (pTA1040) 73%. Fourthly, no sequence corresponding to the major classes of *B. subtilis* promoters was present in the sequences upstream of the putative RBS of sipS (van Dijl et al., 1992), sipP (pTA1015), and sipP (pTA1040). Finally, downstream of sipS



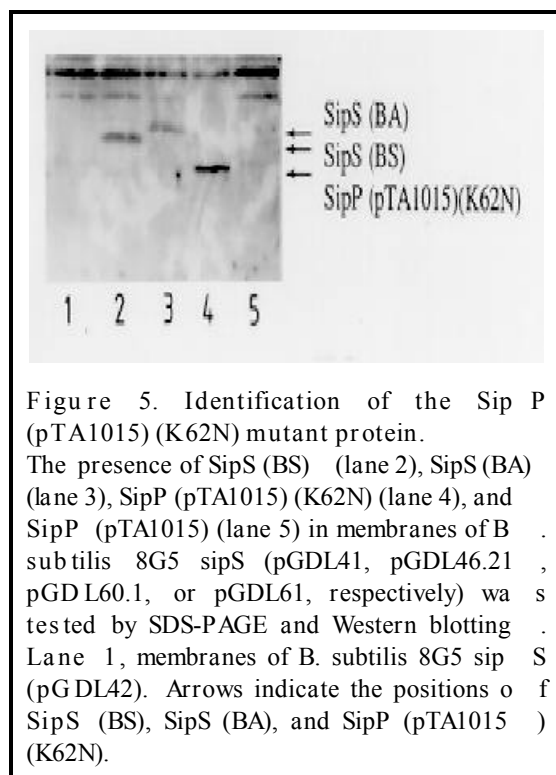


Figure 5. Identification of the Sip P (pTA1015) (K62N) mutant protein.

The presence of SipS (BS) (lane 2), SipS (BA) (lane 3), SipP (pTA1015) (K62N) (lane 4), and SipP (pTA1015) (lane 5) in membranes of *B. subtilis* 8G5 sipS (pGDL41, pGDL46.21, pGDL60.1, or pGDL61, respectively) was tested by SDS-PAGE and Western blotting. Lane 1, membranes of *B. subtilis* 8G5 sipS (pGDL42). Arrows indicate the positions of SipS (BS), SipS (BA), and SipP (pTA1015) (K62N).

(unpublished results), sipP (pTA1015), and sipP (pTA1040) inverted repeats were identified, which are potential rho-independent transcriptional terminators (Figure 1).

Since the sipS gene of *B. subtilis* 8G5 is not essential (van Dijk et al., 1992), it was conceivable that the chromosome of this strain, which does not carry endogenous plasmids, contained a second sip gene identical to one of the two identified sipP genes. To identify potential chromosomal copies of sipP (pTA1015), or sipP (pTA1040) of *B. subtilis* 8G5, PCR techniques and Southern hybridisation were used. For PCR, primers 15-3 and 15-4 (Table 3) were used. These primers, which correspond to sipP (pTA1015) sequences, show no significant similarity to the chromosomal sipS gene. Under stringent conditions for primer annealing to chromosomal *B. subtilis* DNA, no DNA fragments were amplified by PCR. DNA fragments were amplified only when annealing conditions of low-stringency were used. However, sequence analysis

revealed that the amplified fragments showed no similarity to SPase-encoding genes (not shown). Similarly, Southern hybridisations with PCR-generated probes, corresponding either to sipP (pTA1015), or to sipP (pTA1040) sequences, revealed no clear similarities with chromosomal DNA of *B. subtilis* (not shown). Taken together, these findings demonstrate that the sipP genes of pTA1015 and pTA1040 are plasmid-specific.

#### Identification of a SipP (pTA1015) mutant protein

To detect the SipP (pTA1015) and SipP (pTA1040) proteins, solubilised membrane proteins of *B. subtilis* strains containing pTA1015, pTA1040, pGDL61, or pGDL71 were subjected to SDS-PAGE and Western blotting, using antibodies against a SipS-based synthetic peptide (sippep 1) with the sequence NKKRAKQD. Although the SipS protein of *B. subtilis* (SipS [BS]; calculated mol.wt 21.032 Da), and its homologue SipS (BA) from *B. amyloliquefaciens* (calculated mol.wt 21.086 Da) could be detected in this assay, it was not possible to demonstrate the presence of wild-type SipP (pTA1015) (calculated mol.wt 21.234 Da) (Figure 5), or SipP (pTA1040) (calculated mol.wt 21.536 Da) (not shown). The latter finding might be due to the fact that the target sequence of the SipS-specific antibody (NKKRAKQD; residues 115 to 122) is not completely conserved in SipP (pTA1015) (NKENAKKV; residues 118 to 125) and SipP (pTA1040) (NKKDAHDS; residues 117 to 124). Nevertheless, the same antibody could be used to detect a SipP (pTA1015) mutant protein in which the lysine residue at position 62 was replaced by asparagine (Figure 5). The corresponding mutant gene was

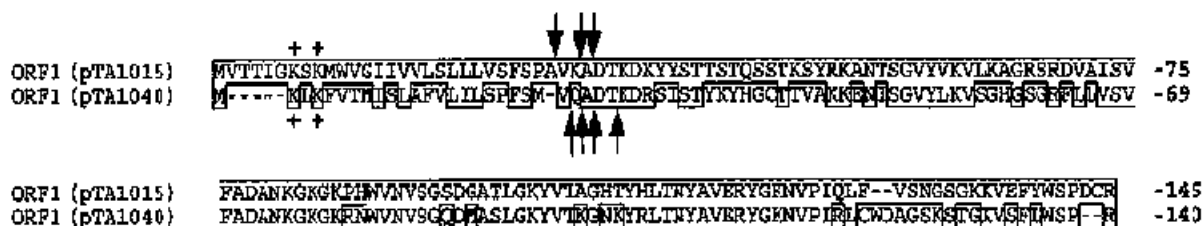


Figure 6. Comparison of the ORF1-encoded proteins of pTA1015 and pTA1040.

Only identical amino acids or conservative changes are boxed in the aligned deduced amino acid sequences of the ORF1-encoded proteins of pTA1015 (ORF1 pTA1015) and pTA1040 (ORF1 pTA1040). Positively charged residues (+) in the putative signal peptide n-regions are indicated. Potential SPase I cleavage sites, indicated by arrows, were

serendipitously obtained upon amplification of sipP (pTA1015) by PCR with primers 15-1 and 15-2, and cloning of amplified fragments in pGDL48. The resulting plasmid with the mutant sipP gene was denoted pGDL60.1. Pulse-chase labelling experiments with *E. coli* MC1061 (pGDL60.1) revealed that lysine 62 was not critical for the functionality of SipP (pTA1015) since the K62N mutant enzyme was still able to process pre(A13i)- $\beta$ -lactamase (Figure 3B, lanes 3A and 3B). The rate of processing was only slightly lower than that observed with the wild-type enzyme (Figure 3B, lanes 4A and 4B). A possible explanation for these findings is that the K62N mutation caused only a slight conformational change of SipP (pTA1015), allowing the antibodies against sippep 1 to bind the mutant protein more efficiently than the wild-type protein.

ORFs1 of pTA1015 and pTA1040 specify putative secretory proteins

Since SPases, such as SipP of pTA1015, are required for the processing of precursors of exported proteins, we conceived that potential target proteins might also be specified by this plasmid. Therefore, we investigated whether other pTA1015-encoded proteins contained putative signal peptides. Only the ORF1-encoded protein appeared to possess a

putative signal peptide with a positively charged n-region, a hydrophobic h-region, and a c-region containing three potential SPase cleavage sites (Figure 6). Interestingly, the 1.4 kb EcoRI - HindIII fragment of pTA1040 contains an ORF, the deduced amino acid sequence of which was highly similar to that of the ORF1 (pTA1015)-encoded protein (56% identity; Figure 6). This ORF was denoted ORF1 (pTA1040). The ORF1 (pTA1015/pTA1040)-encoded proteins show no similarity to previously identified proteins. The two proteins share two features: they have a remarkably high calculated isoelectric point (10.4 and 10.7, respectively); and both have a putative signal peptide (Figure 6). Since neither of the two proteins contains a potential cell-wall anchor, the proteins specified by ORF1 (pTA1015) and ORF1 (pTA1040) are likely to be exported from the cytoplasm, either to the membrane, or to the growth medium. This we tried to demonstrate by SDS-PAGE, or by Western blotting with sera of rabbits, immunised with ORF1-based synthetic peptides. Unfortunately, irrespective of the type of growth medium used (TY or minimal medium), or the growth phase of cells (exponential phase, transition phase, or stationary phase), ORF1-encoded proteins could not be detected in the growth medium, cytoplasmic

membranes, or even in unfractionated cellular extracts of *B. subtilis* 8G5 containing pTA1015, or pTA1040. The same was the case for *B. subtilis* WB600, which lacks most of the extracellular proteases of *B. subtilis*. Therefore, it seems unlikely that the ORF1-encoded proteins were not detected due to proteolytic degradation in the medium.

ORF1 and sipP form one structural module in pTA1015 and pTA1040

Both in pTA1015 and in pTA1040, ORF1 is located at the 5' end of the sipP gene, and both genes have the same transcriptional orientation. The intergenic region between ORF1 and sipP is short: 102 bp and 124 bp for pTA1015 and pTA1040, respectively, and it contains inverted repeats (Figure 1A). In fact, the entire ORF1 plus sipP-containing regions of pTA1015 and pTA1040 (1210 bp and 1212 bp, respectively) are highly similar (68% identity). In both plasmids, this region of similarity starts 100 bp upstream of ORF1, and it ends 10 bp downstream of the stopcodon of the sipP gene. Taken together, these findings indicate that ORF1 and sipP form one structural module of pTA1015 and pTA1040 and that there may exist a functional relation between the products of these genes.

## DISCUSSION

Many endogenous *B. subtilis* plasmids contain sipP genes

In the present manuscript we report the identification of a new structural module which is present on at least two cryptic RCR plasmids (pTA1015 and pTA1040) of *B. subtilis*. The module contains two genes: one, ORF1, specifies an unidentified protein with a putative signal peptide; the other, sipP, specifies a functional type I SPase. In particular, the

identification of SPase-encoding genes on cryptic plasmids is novel. Nevertheless, the presence of SPase-encoding genes on cryptic plasmids of *B. subtilis* appears to be fairly common: firstly, by using either sipP (pTA1015), or sipP (pTA1040) as a probe in Southern hybridisations, we obtained evidence that pTA1020 possesses yet another SPase-encoding gene (results not shown). Secondly, among 29 independently isolated endogenous *B. subtilis* plasmids (Tanaka and Koshikawa, 1977; Uozumi et al., 1980; Hara et al., 1983), 18 were of the pTA1015 type, five of the pTA1020 type, and one of the pTA1040 type, suggesting that many endogenous *B. subtilis* plasmids contain SPase-encoding genes (our unpublished results). Only two of the 29 independently isolated plasmids were of the pTA1060 type. These do not contain a sip gene.

Functional and structural (dis)similarities between SPases from bacilli and other organisms

In several aspects, the plasmid-encoded SPases SipP (pTA1015) and SipP (pTA1040) are similar to the chromosomally encoded type I SPase, SipS, of *B. subtilis* (van Dijn et al., 1992), and to several recently identified type I SPases of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus caldolyticus*: (1), all these enzymes are able to process pre(A13i)- $\beta$ -lactamase (this study and our unpublished results); (2), their genes possess atypical putative startcodons (TTG or GTG); (3) their deduced amino acid sequences show a high degree of similarity (Table 1); and (4), they possess only one putative membrane spanning domain (Figure 2). The latter feature is shared with related type I SPases of the cyanobacterium *Phormidium laminosum* (Packer et al., 1995), the mitochondrial inner membrane, and the endoplasmic reticular membrane (van Dijn et al., 1992; Dalbey and von Heijne, 1992).

The known type I SPases of bacilli are quite distinct from the type I SPases which were identified in *E. coli*, *Salmonella typhimurium* and *Pseudomonas fluorescens* (Wolfe et al., 1983; van Dijn et al., 1990; Black et al., 1992): the latter enzymes, also known as leader peptidases (Lep), possess two membrane spanning domains (Dalbey and von Heijne, 1992), and they are much larger (323, 324 and 284 residues, respectively) than the *Bacillus* SPases (on average 186 residues). Furthermore, Lep of *E. coli* is unable to process pre(A13i)- $\beta$ -lactamase (van Dijn et al., 1992). Why the known type I SPases of bacilli and Gram-negative bacteria have evolved so differently is a matter of speculation.

However, the structural differences and the differences in specificity may relate to species-specific differences in the design of signal peptides. In this respect, the observation that signal peptides from Gram-positive bacteria possess longer h- and c-regions than signal peptides from Gram-negative bacteria may be important (von Heijne and Abrahmsén, 1989), in particular, since the c-region specifies the SPase cleavage site (von Heijne, 1986).

Like SipS of *B. subtilis*, the two plasmid-encoded SPases and the four newly identified chromosomally-encoded type I SPases of *B. amyloliquefaciens*, *B. caldolyticus*, and *B. licheniformis* contain the patterns of conserved amino acids that

Table 1. Similarities between known *Bacillus* type I SPases

		% IDENTICAL RESIDUES + CONSERVED REPLACEMENTS							
	A	B	C	D	E	F	G		
A	-	78	77	77	76	77	53	A = SipP (pTA1015); (L26258)*	
B	71	-	72	74	71	64	49	B = SipP (pTA1040); (Z36269)*	
C	70	60	-	91	78	79	46	C = SipS (BS); (Z11847)*	
D	68	64	84	-	77	81	47	D = SipS (BA); (L26259)*	
E	68	61	69	67	-	82	57	E = SipT (BA); (Z33640)*	
F	69	60	69	70	74	-	51	F = Sip (BL); (X75604)*	
G	44	40	35	36	47	40	-	G = Sip (BC); (L26257)*	

% IDENTICAL RESIDUES

\* EMBL/GenBank/DDBJ accession numbers; see also the legend to Figure 2

are present in all other known type I SPases of prokaryotic and eukaryotic organisms (van Dijl et al., 1992; van Dijl et al., 1993; Dalbey and von Heijne, 1992; Packer et al., 1995). In a recent study we showed that of the 30 conserved residues, only five are important for the functionality of SipS of *B. subtilis*: arginine 84 and aspartic acid 146 appeared to be important conformational determinants; serine 43, lysine 83 and aspartic acid 153 were critical for activity (van Dijl et al., 1995). Serine 43 and lysine 83 correspond, respectively, to serine 90 and lysine 145 of Lep of *E. coli*, which are essential residues for the activity of the latter enzyme (Sung and Dalbey, 1992; Black et al., 1992; Black, 1993; Tschantz et al., 1993). Based on these findings, and on the observation that SipS showed significant similarity with LexA-like proteases, we hypothesised that SipS and the other prokaryotic type I SPases make use of a serine-lysine catalytic dyad. Aspartic acid 153 might be part of a highly conserved structural element (conserved region E; Figure 2) of type I SPases (van Dijl et al., 1995). Several new findings are in good agreement with this hypothesis: (1), the five residues which are important for the functionality of SipS are conserved in all six newly identified *Bacillus* SPases; (2), of the 25 other residues, which were not essential for the activity of SipS, eight (ie. aspartic acid 42, glycine 69, aspartic acid 70, leucine 74, valine 79, leucine 88, tyrosine 141, and asparagine 150) are not conserved in the *Bacillus* SPases (Figure 2); and (3), like SipS of *B. subtilis*, the newly identified SPases of bacilli show similarity with LexA-like proteases (data not shown)

Why is the ORF1- and sipP-encoding module present on plasmids of *B. subtilis*?

With respect to the findings presented in this study, two major questions remain to be answered: why are ORF1 and sipP linked in one structural module, and why is this module located on plasmids? Since we were unable to detect the ORF1-encoded proteins, we can only speculate. Both pTA1015 and pTA1040 were isolated from *B. subtilis* (natto) strains that are used for the production of natto, a traditional Japanese food product based on fermented soy beans. This process requires the production of large amounts of extracellular polymers, such as polyglutamate and levan. Cryptic plasmids, in particular those of the pTA1015-type, have been implicated in this process (Hara et al., 1983). Since the ORF1-encoded proteins, which possess a (putative) signal peptide, are likely to be exported from the cytoplasm, it is conceivable that they are directly or indirectly involved in the production of extracellular polymers during fermentation on soy beans. If indeed exported, these proteins are probably processed by a type I SPase, like most other proteins which are exported through the general protein secretion pathway (Wolfe et al., 1983; Dalbey and Wickner, 1985; Pugsley, 1993). Thus, SipP (pTA1015) and SipP (pTA1040) may be involved in the secretion of ORF1-encoded proteins. One possibility would be that these SPases are specifically required for processing of ORF1-encoded proteins. However, since our results showed that SipP (pTA1015) and SipP (pTA1040) can process at least one other precursor (ie. pre[A13i]- $\beta$ -lactamase), it seems unlikely that these plasmid-specified SPases will exclusively process ORF1-encoded proteins. An alternative possibility is that for processing of the ORF1-encoded proteins, the availability of the chromosomally encoded SPases is insufficient. This view is supported by the

observation that the chromosomally-encoded SPases of *B. subtilis* are produced in very low amounts (unpublished results). In addition, it was previously shown that the availability of SPase can be a limiting factor for the processing of certain mutant, or hybrid precursors, both in *B. subtilis* and in *E. coli* (Date and Wickner, 1981; van Dijl et al., 1991b; van Dijl et al., 1992). Our present findings suggest that SPase can be a limiting factor for protein secretion also in natural systems. Thus, SPase may not only be limiting for the secretion of ORF1-encoded proteins, but also for chromosomally-encoded secretory proteins. For *B. subtilis*, the localisation of ORF1 and sipP on plasmids could have two advantages: (1), the presence in multiple copies gives the cell a possibility to express these genes at a high level; and (2), by conjugation, plasmids can be easily spread throughout a *B. subtilis* population. In fact, we were able to show that pTA1015 contains a functional mob gene, facilitating the conjugative transfer of this plasmid to other cells (unpublished results).

Although our findings indicate that ORF1 and sipP form one functional module in at least two endogenous plasmids of *B. subtilis*, we can presently not exclude the possibility that other endogenous plasmids of this organism contain only ORF1, or sipP. Variants of pTA1015 containing either ORF1, or sipP were stably maintained in *B. subtilis* 8G5 and other *B. subtilis* strains. Moreover, the disruption of these genes did not appear to affect the growth properties of pTA1015-containing *B. subtilis* cells (unpublished results). Obviously, these findings may relate to the fact that under our laboratory conditions the products of ORF1 (pTA1015/pTA1040) were not produced at detectable (high) levels.

We have recently shown that the sipS gene of *B. subtilis* is not essential. Since cells lacking SipS were still able to process and secrete proteins, albeit at

reduced efficiency, we concluded that *B. subtilis* possesses at least two chromosomal sip genes (van Dijl et al., 1992). This was recently confirmed by the identification of a second chromosomal sip gene of *B. subtilis*, denoted sipT (our unpublished results), and by the identification of homologous chromosomal sipS and sipT genes in the closely related bacterium *B. amyloliquefaciens* (our unpublished results; van Dijl, EMBL/Genbank/DDBJ accession number L26259; Hoang and Hofemeister, EMBL/Genbank/DDBJ accession number Z33640). Since only one type I SPase has been identified in *E. coli*, the observation of plasmid-specific sipP genes in addition to the two chromosomal sip genes was rather surprising. It is the major challenge of our present studies to identify the reason why *B. subtilis* has acquired so many distinct SPase-encoding genes during its evolution. To this purpose, we are currently performing expression studies with the different sip genes of *B. subtilis*, and we are trying to analyse the substrate specificities of the corresponding enzymes.

## MATERIALS AND METHODS

Bacteria and plasmids. Table 2 lists the bacterial strains and plasmids used.

Media. Minimal media for *B. subtilis* were as described by Smith et al. (1983). Media for protoplast transformation of *B. subtilis* were as described by Chang and Cohen (1979). TY medium contained Bacto Tryptone (1%), Bacto yeast extract (0.5%) and NaCl (1%). TY plates contained in addition 2% agar. M9 media 1 and 2, used for labelling of *E. coli* were prepared as described by van Dijl et al. (1991b). Both M9 media contained ampicillin (40 µg/ml). S7 media 1 and 3, for the labelling of *B. subtilis* were prepared as described by van Dijl et al. (1991a). Both S7 media contained kanamycin (10 µg/ml).

DNA techniques. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis and Southern transfer of DNA to GeneScreen Plus filters (Dupont NEN, Boston, MA, USA) were carried out as described by Sambrook et al. (1989). Probe labelling, DNA hybridisation, and washing steps were performed using the enhanced chemiluminescence DNA labelling and detection system (Amersham International plc, Amersham, UK). Enzymes were obtained from Boehringer Mannheim (FRG). DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). [<sup>35</sup>S] dATP (8 µCi/µl; > 1000 Ci/mmol) was obtained from Amersham International. DNA and protein sequences were analysed using version 6.7 of the PCGene Analysis Program (Intelligenetics Inc., Mountain View, CA, USA). The FASTA algorithm of Lipman and Pearson (1985) was used for protein comparisons in the Atlas of protein and genomic sequences, release september 1993 (MIPS, Martinsried, FRG). PCR was carried out with the Vent DNA polymerase

(New England Biolabs, Beverly, USA). The template DNA was denatured for 1 min at 94°C. The primers listed in Table 3 were used to amplify DNA fragments in 30 cycles of denaturation (30 sec; 94°C), primer annealing (1 min; 50°C) and DNA synthesis (3 min; 73°C). When low-stringency conditions for the annealing of primers to template DNA were required, the temperature for annealing was lowered to 37°C. In the latter reactions, the Supertaq DNA polymerase was used (Sphaero-Q, Leiden, the Netherlands). The sequences of all fragments obtained through PCR were verified. Electrotransformation of *E. coli* MC1061 was carried out with a Bio-Rad Gene Pulser™. Competent *B. subtilis* cells were transformed as described by Bron and Venema (1972). *B. subtilis* protoplasts were transformed as described by Chang and Cohen (1979).

Plate assay for the processing of (A13i)-β-lactamase precursor. The plate assay for processing of the hybrid precursor pre(A13i)-β-lactamase in *E. coli* was carried out as described by van Dijl et al., (1992). Briefly, this assay is based on the observation that pre(A13i)-β-lactamase is not processed when produced in *E. coli*, which is due to the insertion of 10 hydrophobic residues in the h-region of signal peptide A13. Thus, pre(A13i)-β-lactamase remains attached to the outer side of the cytoplasmic membrane. Mature (A13i)-β-lactamase can only reach the growth medium if the precursor is processed by SPase. The presence of the mature enzyme in the agar medium around colonies of *E. coli* is visualised with a β-lactamase activity assay, which is based on decoloration (halo formation) of the blue starch-iodine complex upon hydrolysis of ampicillin. Transformed *E. coli* MC1061 cells were transferred to TY agar plates. After an overnight incubation at 37°C, a soft agar (TY) assay medium containing

Table 2. Strains and plasmids

Bacterial strain	Genotype	Reference
E.coli		
MC1061	F <sup>-</sup> araD139 Δ(ara-leu )7696 galE15 galK16 )(lac)X743 rpsL hsdR2 mcrA mcrB 1	Wertman et al., 1986
B.subtilis		
8G5	trpC2 tyr 1 met his ura nic pur A rib	Bron and Venema, 1972
8G5 sipS	trpC2 tyr his nic ura met ade sipS	van Dijl, unpublished
WB600	aprE, nprE, nprB, bpr, epr, mpr	Wu et al., 1991
Plasmids	Relevant properties	Reference
pTA1015	Cryptic plasmid from B. subtilis IAM1028; 5.8 kb	Uozumi et al., 1980
pTA1020	Cryptic plasmid from B. subtilis IAM1076; 6.6 kb	Uozumi et al., 1980
pTA1040	Cryptic plasmid from B. subtilis IAM1232; 7.8 kb	Uozumi et al., 1980
pGDL41	Contains the replication functions of the Lactococcus lactis WG2 plasmid pWVO1 (Kok et al., 1984); replicates also in E. coli and B. subtilis ; contains the sipS gene of B. subtilis ; encodes pre(A13i)- β-lactamase; 8.1 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	van Dijl et al., 1992
pGDL42	pGDL41 derivative; does not contain the sipS gene; 7.5 kb; Ap <sup>r</sup> ; Em <sup>r</sup> ; Km <sup>r</sup>	van Dijl et al., 1992
pGDL46.21	pGDL42 derivative; contains the sipS gene of B. amyloliquefaciens ; 8.8 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	van Dijl, unpublished
pGDL48	pGDL42 derivative; contains a multiple cloning site; 7.5 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	This paper
pGDL60.1	pGDL48 carrying a mutant sipP (pTA1015) gene; 8.3 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	This paper
pGDL61	pGDL48 carrying the wild-type sipP (pTA1015) gene; 8.2 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	This paper
pGDL71	pGDL48 carrying the sipP (pTA1040) gene; 8.3 kb; Ap <sup>r</sup> ; Em <sup>s</sup> ; Km <sup>r</sup>	This paper
pMTL23	pBR322 derivative; 2.8 kb; Ap <sup>r</sup>	Chambers et al., 1988



Table 3. Primers used

Oligonucleotide	Sequence (5'-3') *	Sequence (5'-3') *
15-1	acgcgtcgaCCCGATTGTAGATAGTCC	766 - 783
15-2	tatgaattCTTTGATCTGTGTGCTA	1515 - 1499
15-3	CTATGGATCCCACTTTGGTTGATTC	1014 - 1038
15-4	gctagatccAAGGTTTATAACCCACTTTC	1270 - 1252
15-5	GTGATAAGCGGGTTTTG	845 - 861

\*Nucleotides identical to pTA1015/pTA1040 sequences are in upper case letters.

Lower case letters represent 5' extensions specifying restriction sites for cloning.

# The position of oligonucleotides 15-1 to 15-5 is numbered according to the sipP (pTA1015) sequence (EMBL/GenBank/ DDBJ accession number L26258).

iodine and ampicillin was poured on the plates with transformants. After solidification of the overlayer, the plates were incubated for 15 min at 30°C. During this time halos appeared around colonies that released processed mature  $\beta$ -lactamase. The size of individual halos relates mainly to colony size,  $\beta$ -lactamase production levels, and to the thickness of the overlayer, but not to differences in SPase activity.

Pulse-chase protein labelling, immunoprecipitation, SDS-PAGE and fluorography. Pulse-chase labelling experiments with *E. coli* and in *B. subtilis* were performed as described by van Dijl et al. (1991b; 1991a). Immunoprecipitation was carried out as described by Edens et al. (1982). SDS-PAGE was performed according to Laemmli (1970). [<sup>14</sup>C]-methylated molecular weight reference markers were from Amersham International. Fluorography was performed as described by Skinner and Griswold (1983). Relative amounts of precursor and mature forms of (A13i)- $\beta$ -lactamase were estimated by film scanning with an LKB ultrascan XL laser densitometer.

Western blot analysis. The expression of SPases in *B. subtilis* was assayed by

Western blotting (Towbin et al., 1979). Membranes of *B. subtilis* were prepared as described by van Dijl et al. (van Dijl et al., 1991a). Membrane proteins were solubilized in 2% Triton X100 (30 min, 0°C). Non-solubilized material was removed by centrifugation (135.000 g; 20 min; 4°C). The solubilized membrane proteins were separated by SDS-PAGE and, subsequently, transferred to Immobilon-PVDF membranes (Millipore Corporation, Bedford, MA, USA). The presence of SipS and related SPases was visualised with antibodies against a SipS-based synthetic peptide, denoted sippep 1 (van Dijl et al., 1995) and alkaline phosphatase-anti-rabbit-IgG conjugates (Western-light™, Tropics, Bedford, MA, USA). The antibodies against sippep 1 show some aspecific reactions with membrane proteins of *B. subtilis*, which are larger than approximately 30 kDa. This did not interfere with the detection of Bacillus SPases (approximately 21 kDa).

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

## ACKNOWLEDGEMENTS

We thank Dr. J. Hofemeister for informing us about ongoing studies in his laboratory on *Bacillus* signal peptidase genes; Dr. P. Terpstra for performing most of the sequencing work; H. Mulder for preparing figures; and A. Bolhuis for helpful discussions. W. Meijer was supported by STW (Stichting Technische Wetenschappen, the Netherlands) and Gist-brocades B.V. (Delft, the Netherlands); H. Tjalsma was supported by Gist-brocades B.V.; J.M. van Dijl, A. de Jong and S. Bron were supported by Gist-brocades B.V. and, in part, by CEC Biotech Grants (BIOT-CT910268 and BIO2-CT 920254).

## REFERENCES

- Black, M.T., Munn, J.G.R., and Allsop, A. (1992). On the catalytic mechanism of prokaryotic leader peptidase I. *Biochem. J.* 282: 539-543.
- Black, M.T. (1993). Evidence that the catalytic activity of prokaryotic leader peptidase depends upon the operation of a serine-lysine catalytic dyad. *J. Bacteriol.* 175: 4957-4961.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.
- Bron, S., Bosma, P., Van Belkum, M.J., and Luxen, E. (1987). Stability function in the *Bacillus subtilis* plasmid pTA1060. *Plasmid* 18: 8-15.
- Bron, S. (1990). Plasmids. In *Molecular Biological Methods for Bacillus*. Harwood, C.R. and Cutting, S.M. (eds.) Chichester, UK: John Wiley & Sons Ltd. pp. 75-174.
- Bron, S., Meijer, W., Holsappel, S., and Haima, P. (1991). Plasmid instability and molecular cloning in *Bacillus subtilis*. *Res. Microbiol.* 142: 875-883.
- Bron, S. and Venema, G. (1972). Ultraviolet inactivation and excision repair in *Bacillus subtilis*. I. Construction and characterization of a transformable eightfold auxotrophic strain and two ultraviolet-sensitive derivatives. *Mutat. Res* 15: 1-10.
- Chambers, S.P., Prior, S., Barstow, D.B., and Minton, N.P. (1988). The pMTL series of cloning vectors: Improved polylinker regions to facilitate the generation of sonicated DNA for nucleotide sequencing. *Gene* 68: 139-149.
- Chang, S., Chang, S., and Gray, O. (1987). Structural and genetic analysis of a par locus that regulates plasmid partition in *Bacillus subtilis*. *J. Bacteriol.* 169: 3952-3962.
- Chang, S. and Cohen, S.N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol. Gen. Genet.* 168: 111-115.
- Dalbey, R.E. and von Heijne, G. (1992). Signal peptidases in prokaryotes and eukaryotes: a new protease family. *Trends Biochem. Sci.* 17: 474-478.
- Dalbey, R.E. and Wickner, W. (1985). Leader peptidase catalyzes the release of exported proteins from the outer surface of the *Escherichia coli* plasma membrane. *J. Biol. Chem.* 260: 15925-15931.
- Date, T. and Wickner, W. (1981). Isolation of the *Escherichia coli* leader peptidase gene and effects of leader peptidase overproduction in vivo. *Proc. Natl. Acad. Sci. USA* 78: 6106-6110.
- Devine, K.M., Hogan, S.T., Higgins, D.G., and McConnell, D.J. (1989). Replication and segregational stability of the *Bacillus* plasmid pBAA1. *J. Bacteriol.* 171: 1166-1172.
- Edens, L., Heslinga, L., Klok, R., Ledebroer, A.M., Maat, J., Toonen, M.Y., Visser, C., and Verrips, C.T. (1982). Cloning of the cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*. *Gene* 18: 1-12.
- Gruss, A. and Ehrlich, S.D. (1989). The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol. Rev.* 53: 231-241.
- Hara, T., Zhang, J., and Ueda, S. (1983). Identification of plasmids linked with polyglutamate production in *Bacillus subtilis* (natto). *J. Gen. Appl. Microbiol.* 29: 345-354.
- Kok, J., van der Vossen, J.M.B.M., and Venema, G. (1984). Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*.

## Chapter VI

- Appl. Environ. Microbiol. 48: 726-731.
- Lammli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Lipman, D.J. and Pearson, W.R. (1985). Rapid and sensitive protein similarity searches. *Science* 227: 1435-1441.
- Meijer, W. J. J., Venema, G., and Bron, S. (1995). Characterization of single strand origins of cryptic rolling-circle plasmids from *Bacillus subtilis*. *Nucleic Acids Res.* 23: 612-619.
- Novick, R.P. (1989). Staphylococcal plasmids and their replication. *Annu. Rev. Microbiol.* 43: 537-565.
- Packer, J.C.L., André, D., and Howe, C.J. (1995). Cloning and sequence analysis of a signalpeptidase I from the thermophilic cyanobacterium *Phormidium laminosum*. *Plant Mol. Biol.* 27: 199-204.
- Pugsley, A.P. (1993). The complete general secretory pathway in Gram-negative bacteria. *Microbiol. Rev.* 57: 50-108.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463-5467.
- Seery, L. and Devine, K.M. (1993). Analysis of features contributing to activity of the single-stranded origin of *Bacillus* plasmid pBAA1. *J. Bacteriol.* 175: 1988-1994.
- Skinner, M.K. and Griswold, M.D. (1983). Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenylloxazole in acetic acid and its comparison with existing procedures. *Biochem. J.* 209: 281-284.
- Smith, H., de Vos, W., and Bron, S. (1983). Transformation in *Bacillus subtilis*: properties of DNA-binding-deficient mutants. *J. Bacteriol.* 153: 12-20.
- Sung, M. and Dalbey, R.E. (1992). Identification of potential active-site residues in the *Escherichia coli* leader peptidase. *J. Biol. Chem.* 267: 13154-13159.
- Tanaka, T. and Koshikawa, T. (1977). Isolation and characterization of four types of plasmids from *Bacillus subtilis* (natto). *J. Bacteriol.* 131: 699-701.
- Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.
- Tschantz, W.R., Sung, M., Delgado-Partin, V.M., and Dalbey, R.E. (1993). A serine and a lysine residue implicated in the catalytic mechanism of the *Escherichia coli* leader peptidase. *J. Biol. Chem.* 268: 27349-27354.
- Uozumi, T., Ozaki, A., Beppu, T., and Arima, K. (1980). New cryptic plasmid of *Bacillus subtilis* and restriction analysis of other plasmids found by general screening. *J. Bacteriol.* 142: 315-318.
- van Dijl, J.M., van den Bergh, R., Reversma, T., Smith, H., Bron, S., and Venema, G. (1990). Molecular cloning of the *Salmonella typhimurium* lep gene in *Escherichia coli*. *Mol. Gen. Genet.* 223: 233-240.
- van Dijl, J.M., de Jong, A., Smith, H., Bron, S., and Venema, G. (1991a). Non-functional expression of *Escherichia coli* signal peptidase I in *Bacillus subtilis*. *J. Gen. Microbiol.* 137: 2073-2083.
- van Dijl, J.M., de Jong, A., Smith, H., Bron, S., and Venema, G. (1991b). Signal peptidase I overproduction results in increased efficiencies of export and maturation of hybrid secretory proteins in *Escherichia coli*. *Mol. Gen. Genet.* 227: 40-48.
- van Dijl, J.M., de Jong, A., Vehmaanperä, J., Venema, G., and Bron, S. (1992). Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. *EMBO J.* 11: 2819-2828.
- van Dijl, J.M., de Jong, A., Smith, H., Venema, G., and Bron, S. (1993). Protein secretion in *Bacillus subtilis*. In *DNA transfer and gene expression in microorganisms*. Balla, E., Berencsi, G., Farkas, J., and Szentirmai, A. (eds.) Andover, U.K. Intercept Ltd, pp. 251-261.
- van Dijl, J.M., de Jong, A., Venema, G., and Bron, S. (1995). Identification of the potential active site of the signal peptidase SipS of *Bacillus subtilis*: structural and functional similarities with LexA-like proteases. *J. Biol. Chem.* In press

- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14: 4683-4690.
- von Heijne, G. (1992). Membrane protein structure prediction: Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* 225: 487-494.
- von Heijne, G. and Abrahmsén, L. (1989). Species-specific variation in signal peptide design. *FEBS Lett.* 244: 439-446.
- Wertman, K.F., Wyman, A.R., and Botstein, D. (1986). Host/vector interactions which affect the viability of recombinant phage lambda clones. *Gene* 253: 253-262.
- Wolfe, P.B., Wickner, W., and Goodman, J.M. (1983). Sequence of the leader peptidase gene of *Escherichia coli* and the orientation of leader peptidase in the bacterial envelope. *J. Biol. Chem.* 258: 12073-12080.
- Wu, X., Lee, W., Tran, L., and Wong, S.-L. (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* 173: 4952-4958.



