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# Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archaeal and eukaryotic signal peptidases

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Approximately 47% of the genes of the Gram-positive bacterium *Bacillus subtilis* belong to paralogous gene families. The present studies were aimed at the functional analysis of the *sip* gene family of *B. subtilis*, consisting of five chromosomal genes, denoted *sipS*, *sipT*, *sipU*, *sipV*, and *sipW*. All five *sip* genes specify type I signal peptidases (SPases), which are actively involved in the processing of secretory preproteins. Interestingly, strains lacking as many as four of these SPases could be obtained. As shown with a temperature-sensitive SipS variant, only cells lacking both SipS and SipT were not viable, which may be caused by jamming of the secretion machinery with secretory preproteins. Thus, SipS and SipT are of major importance for protein secretion. This conclusion is underscored by the observation that only the transcription of the *sipS* and *sipT* genes is temporally controlled via the DegS–DegU regulatory system, in concert with the transcription of most genes for secretory preproteins. Notably, the newly identified SPase SipW is highly similar to SPases from archaea and the ER membrane of eukaryotes, suggesting that these enzymes form a subfamily of the type I SPases, which is conserved in the three domains of life.

[Key Words: *Bacillus subtilis*; paralogous gene family; protein secretion; signal peptidase; leader peptidase]

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Since 1995, at least 12 microbial genomes have been completely sequenced and annotated (for a recent update, see the microbial database at <http://www.tigr.org/tdb/mdb/mdb.html>). A remarkable common property of these genomes is that many genes are present in multiple (paralogous) copies. A good example is the genome of *Bacillus subtilis*, which contains ~4200 genes, 47% of which belong to paralogous gene families (Kunst et al. 1997). Close examination of the corresponding proteins of *B. subtilis* has indicated that many of these are involved in transcription regulation, and the transport of compounds into and out of the cell. Interestingly, most genes for components of the protein secretion machinery are present in only one copy, despite the fact that *B. subtilis* has a large capacity for protein secretion. The only known exception concerns the genes for type I sig-

nal peptidases (SPases; Tjalsma et al. 1997). These SPases remove amino-terminal signal peptides from secretory preproteins during, or shortly after their translocation across the cytoplasmic membrane, to release these proteins from the *trans* side of this membrane (for review, see Dalbey et al. 1997).

Homologous type I SPases have been identified in archaea, Gram-positive, and Gram-negative eubacteria, the inner membrane of yeast mitochondria, the thylakoid membrane of chloroplasts, and the endoplasmic reticular (ER) membranes of yeast and higher eukaryotes. Despite the fact that considerable similarities exist between the known type I SPases when amino acid sequences are compared pairwise, only few residues are strictly conserved in all known enzymes of this family (Dalbey et al. 1997). In particular, the type I SPases of eubacteria, mitochondria, and chloroplasts differ considerably from their homologs in archaea and the ER membrane, indicating that these enzymes belong to distinct

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subfamilies of SPases. Below, we have referred to these subfamilies as the P (prokaryotic)- and ER-type SPases, respectively. Most P-type SPases contain conserved serine and lysine residues that are essential for enzymatic activity, possibly by forming a catalytic dyad (Black 1993; Tschantz et al. 1993; van Dijn et al. 1995; Paetzel and Dalbey 1997). The putative active site serine residue of the P-type SPases is also conserved in the ER-type SPases. In contrast, the putative active site lysine residue of the P-type SPases is replaced with a histidine residue in the ER-type SPases (Dalbey and von Heijne 1992; van Dijn et al. 1992).

In many eubacteria, such as *Escherichia coli* (Blattner et al. 1997), *Haemophilus influenzae* (Fleischmann et al. 1995), *Helicobacter pylori* (Tomb et al. 1997), and *Mycobacterium tuberculosis* (Philipp et al. 1996), one P-type SPase seems to be sufficient for the processing of secretory pre-proteins. Similarly, the archaea *Methanococcus jannaschii* (Bult et al. 1996) and *Methanobacterium thermoautotrophicum* (Smith et al. 1997), and the yeast *Saccharomyces cerevisiae* (Goffeau et al. 1997) contain only one ER-type SPase. As shown for *E. coli* (Dalbey and Wickner 1985; van Dijn et al. 1988) and yeast (Böhni et al. 1988), these SPases are likely to be essential for cell viability. In contrast, two paralogous P-type SPases are found in *Synechocystis* PCC 6803 (Kaneko et al. 1996) and the inner membrane of yeast mitochondria (Behrens et al. 1991; Nunnari et al. 1993), whereas the presence of two paralogous ER-type SPases appears to be characteristic for most eukaryotic species (Dalbey et al. 1997). Finally, the largest numbers of paralogous SPases appear to be present in the archaeon *Archaeoglobus fulgidus*, which contains three genes for ER-type SPases (Klenk et al. 1997), and the Gram-positive bacterium *B. subtilis* in which seven *sip* genes for type I SPases have been identified. Five of the *sip* genes of *B. subtilis* (denoted *sipS*, *sipT*, *sipU*, *sipV*, and *sipW*) are located on the chromosome (van Dijn et al. 1992; Tjalsma et al. 1997); two additional *sip* genes (denoted *sipP*) are located on plasmids which were, thus far, only found in natto-producing strains of *B. subtilis* (Meijer et al. 1995).

Thus far, the presence of both P- and ER-type SPases in one organism was only evident for eukaryotes, which contain P-type SPases in their organelles and ER-type SPases in the ER (Dalbey et al. 1997). In this paper we document our surprising observation that one of the seven type I SPases of *B. subtilis* (ie. SipW) belongs to the ER-type subfamily of SPases. Thus, *B. subtilis* is the first organism known to contain SPases of the P- and the ER-type in one membrane. Furthermore, our studies, aimed at the functional analysis of the type I SPases of *B. subtilis*, show that SipS and SipT are the most important SPases of *B. subtilis*, whereas SipU, SipV, and SipW appear to have a minor role in precursor processing.

## Results

### *SipW* belongs to the subfamily of ER-type SPases

To make an inventory of the known type I SPases, database searches were performed with various P- and ER-

type SPases. Unexpectedly, similarity searches with the ER-type SPase of *M. jannaschii* showed that the *yqhE* gene of *B. subtilis* (GenBank accession no. D84432) specifies a protein with a high degree of sequence similarity to the known ER-type SPases from archaea and eukaryotes. One of the SPases of *A. fulgidus* (GenBank locus AF1655) exhibited the highest degree of similarity with the deduced YqhE protein sequence (57% identical residues and conservative replacements in 131 residues). This observation strongly suggested that the *yqhE* gene specifies an ER-type SPase of *B. subtilis*. By analogy to other SPase-encoding genes of bacilli, we therefore renamed the *yqhE* gene *sipW* (Fig. 1A). As shown previously for the ER-type SPases of yeast (i.e., Sec11p) and *Canis familiaris* (i.e., SPC18 and SPC21; see Dalbey et al. 1997), SipW also shows amino acid sequence similarity with P-type SPases, but this similarity is mainly limited to the conserved domains B-E, which are present in all known SPases (Fig. 1B). Like the other known ER-type SPases, SipW contains a conserved histidine residue in domain D instead of the conserved lysine residue in domain D of the P-type SPases (Fig. 1A, B). Finally, SipW seems to contain one amino-terminal (AI), and one carboxy-terminal (AII) membrane anchor (Fig. 1C), as suggested previously for the P-type SPases of *Rhodobacter capsulatus* and yeast mitochondria (Dalbey et al. 1997).

The availability of the sequences of SipW of *B. subtilis* and recently identified SPases from archaea allowed the detailed comparison of the ER-type SPases, which is documented in Figure 1A. The comparison shows that these enzymes belong to a heterogeneous group of proteins. As documented previously for the P-type SPases, only the domains B-E are conserved in the ER-type SPases (Fig. 1A,B). A striking difference with the P-type SPases is that the domains B and C are separated by only one residue, and the domains D and E are separated by only 2-11 residues (Fig. 1A,C). The corresponding domains of the P-type SPases are separated by 19-42, and 23-118 residues, respectively (see Dalbey et al. 1997). Finally, the number of putative membrane spanning domains seems to vary in the ER-type SPases. All known enzymes of this subfamily have one amino-terminal membrane anchor (AI, Fig. 1A). In addition, some of these SPases, such as SipW of *B. subtilis* and SPC21 of *A. fulgidus*, seem to have a carboxy-terminal membrane anchor (AII, Fig. 1A,C), and a second SPase of *A. fulgidus* [Sip (Afu)] seems to have even three carboxy-terminal membrane anchors (data not shown).

### Two quadruple SPase mutants of *B. subtilis* are viable

To determine whether SipW is required for growth or viability of the cell, an internal *SacI*-*NdeI* fragment of the chromosomal *sipW* gene, specifying the conserved domains C-E, was replaced with a tetracycline resistance (*Tc<sup>r</sup>*) marker (schematically shown in Fig. 2). As shown previously for strains lacking intact *sipS*, *sipT*, *sipU*, or *sipV* genes (Bolhuis et al. 1996; Tjalsma et al. 1997), the disruption of the *sipW* gene had no detectable effect on cell growth or viability (data not shown).

A

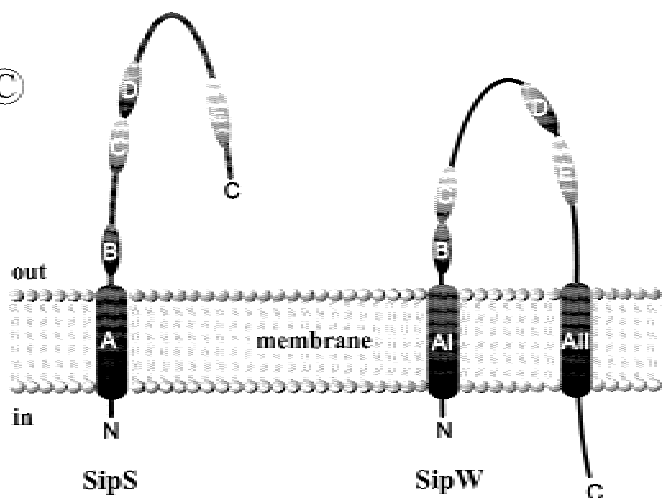
		A(nchor) I	B	C					
SipW (Bsu)	M	-----KI-----	<b>LSNIIAYWIIIFPLIIIVLTUVIST</b>	<b>RSGGEP</b> AWFGYTLAS	<b>VLSGSMEP</b> EPF	<b>WGSLSHAK</b> KKITD-----	<b>VKELQ</b> HGWIDF--HMDAN	87	
SPC21 (Afu)	M	-----RPK-----	<b>LYLLSAILLTILALYL--SG</b>	-----WR--VTK	<b>VLTITSMI</b> EPF	<b>WGDITFD</b>	FPKNPS ---	<b>LDKAV</b> GULVQYKRIIDGR	68
Sip (Mth)	M	-----MOKK-----	<b>VIEARAYLLLVLAVVASSG</b>	-----Y-----	<b>VLSGSMEP</b> EPF	<b>WGDITFD</b>	KNTSFPQVQMDPSIRAGDI	IYDAIWEPE	77
Sec11p (Sce)	M	-----DLRF--ELQML-----	<b>LVAVCFI</b> FASAYHEWQGLAIQNS	SASPIWV	<b>VLSGSMEP</b> EPF	<b>WGDITFD</b>	ANAN-----	TFNIVGUVVYVEVGGQI	82
SPC18 (Cfa)	MC	-----SLDFLDVSRPNNKRIQI	<b>VYQVIVN</b> IFGMIVNSALMIWEGLMWIDG	SGSEPIWV	<b>VLSGSMEP</b> EPF	<b>WGDITFD</b>	INAVH-----	CDIRAGEIIVFRIDGREI	95
SPC18 (Rno)	MC	-----SLDFLDVSRPNNKRIQI	<b>VYQVIVN</b> IFGMIVNSALMIWEGLMWIDG	SGSEPIWV	<b>VLSGSMEP</b> EPF	<b>WGDITFD</b>	INAVH-----	CDIRAGEIIVFRIDGREI	95
SPC21 (Cfa)	MV	RAGAVGTHLDASGLDIFDQVRYNNKRII	<b>VYQVIVN</b> IFGMIVNSALMIWEGLMWIDG	SGSEPIWV	<b>VLSGSMEP</b> EPF	<b>WGDITFD</b>	INAVH-----	CDIRAGEIIVFRIDGREI	107
Sip (Mja)	M				<b>VLSGSM</b> EPF	<b>WGDITFD</b>		36	
Sip (Afu)	M				<b>VLSGSM</b> EPF	<b>WGDITFD</b>		49	
Sec11 (Afu)	M				<b>VLSGSM</b> EPF	<b>WGDITFD</b>		58	
SPC21 (Cpe)	M				<b>VLSGSM</b> EPF	<b>WGDITFD</b>		41	

	D	E	A(nchor) II	
SipW (Bsu)	<b>TAVT</b> HRIT	WDI--TRQCDHLI	<b>FTTRGDNNA</b> ARD	SAPVSDI-----SVAAGDQGGFQCLPYAGYML--HFAS
SPC21 (Afu)	<b>KLII</b> TRHW	VEK-----TSEA	<b>FLTRGDNL</b> PRAD	E--PVSYD-----SVAQGVV--ARI
Sip (Mth)	<b>FWIE</b> --HRW	IGVETDRAGAY	<b>FLTRGDNN</b> QDPD	PAPVYSQVEARVLTGSGQLMI--
Sec11p (Sce)	<b>EIV</b> --HRV	LRGQNHADQGE	<b>FLTRGDNN</b> AGMD	ISLYANSKIYLNKSKELVGTWGGY--
SPC18 (Cfa)	<b>RM</b> --HRW	LRTHESQDGHK	<b>FLTRGDNN</b> AVDD	PCLYNQQGEMLEK--KDWVGRARGF--
SPC18 (Rno)	<b>RM</b> --HRW	LRTHESQDGHK	<b>FLTRGDNN</b> AVDD	PCLYNQQGEMLEK--KDWVGRARGF--
SPC21 (Cfa)	<b>EIV</b> --HRV	IKVHERDNGDIM	<b>FLTRGDNN</b> AVDD	PCLYNQQGEMLEK--KDWVGRARGF--
Sip (Mja)	<b>FWI</b> --HRW	IKVWFNMSI--Y	<b>FTTRGDN</b> NPHND	106
Sip (Afu)	<b>SWIE</b> HRWV	---EII--CDDY	<b>FTTRGD</b> IVPDD	97
Sec11 (Afu)	<b>FTI</b> --HRV	-----A	<b>VTRGDI</b> TRAVI	117
SPC21 (Cpe)	<b>RAM</b> --HRW	LEKMEEC	<b>FTTRGDN</b> NAARD	71

B

	B	C
P-type	--sgSM-pt1	r-gd-i###
ER-type	vlagSMEP-f	r-Gd-l###
	D	E
P-type	-FA-ER##	---GDnr--D
ER-type	p#w-ERr#	F-tKGDnn--d

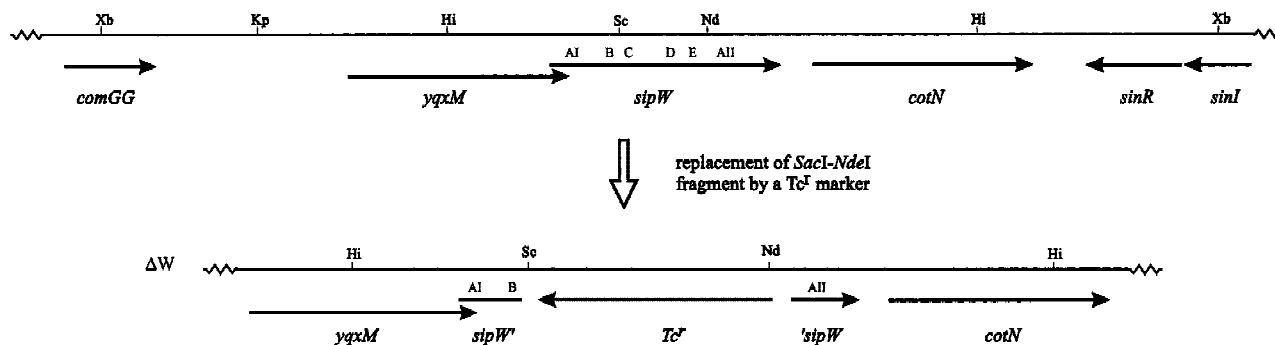
C



**Figure 1.** Conserved domains in ER-type SPases from eubacteria, archaea and eukaryotes. (A) Identification of conserved domains in the ER-type SPases from *B. subtilis* [SipW (Bsu); GenBank accession no. D84432], *A. fulgidus* [SPC21 (Afu), Sec11 (Afu) and Sip (Afu); GenBank loci AF1657, AF1791 and AF1655, respectively], *M. thermoautotrophicum* [Sip (Mth); GenBank locus MTH1448], *S. cerevisiae* [Sec11p (Sce); Böhni et al. 1988], *C. familiaris* [SPC18 (Cfa) and SPC21 (Cfa); Shelness and Blobel 1990; Greenburg et al. 1989], *Rattus norvegicus* [SPC18 (Rno); Signs and Jacquet 1994], *M. jannaschii* [Sip (Mja), GenBank locus MJU67481], and the partial amino acid sequence of a protein from *Clostridium perfringens* [SPC21 (Cpe); GenBank accession no. X86488]. The conserved domains B–E, which are present in all known type I SPases (van Dijl et al. 1992; Dalbey et al. 1997) are indicated. Because of limited similarities in the regions between conserved domains, only the four conserved domains B–E of Sip (Mja), Sip (Afu), Sec11 (Afu), and SPC21 (Cpe) are shown. Identical amino acids are indicated in bold when present in at least 6 of the 11 sequences shown. Putative transmembrane segments, indicated with A(nchor) I, or A(nchor) II, were predicted as described by Sipoos and von Heijne (1993). (B) Consensus sequences of the conserved domains B–E of the P- and ER-type SPases. Strictly conserved residues are indicated by uppercase letters, conservative substitutions by lowercase letters, and conserved hydrophobic residues by a number sign (#). The serine and lysine residues that are critical for the activity of P-type SPases (Dalbey et al. 1997) are indicated (\*). (C) Comparison of the predicted membrane topologies of SipS and SipW of *B. subtilis*. Membrane anchors (A, AI, AII) and the conserved domains B–E are indicated. (N) Amino terminus; (C) carboxyl terminus; (in) cytoplasmic side of the membrane; (out) extracytoplasmic, cell wall-exposed side of the membrane.

To investigate which SPases are needed for growth and cell viability, various chromosomal *sip* gene deletions or disruptions were combined. The double, triple, and quadruple *sip* mutants obtained are listed in Table 1, which

shows that most *sip* gene mutations could be combined, with one exception: It was not possible to construct strains lacking both *sipS* and *sipT*. Interestingly, the quadruple mutants jointly lacking *sipS*, *sipU*, *sipV*, and



**Figure 2.** Disruption of the *B. subtilis* *sipW* gene. Schematic presentation of the construction of *B. subtilis* 8G5 *sipW*-Tc<sup>r</sup> ( $\Delta$ W). The chromosomal *sipW* gene was disrupted with a Tc<sup>r</sup> marker by replacement recombination. To this purpose, *B. subtilis* 8G5 was transformed with the linearized plasmid pKW34dT, in which the 190-bp *SacI*-*NdeI* fragment (specifying the conserved domains C-E of SipW) was replaced with a Tc<sup>r</sup> marker. Only restriction sites relevant for the constructions are shown: (Hi) *HindIII*; (Kp) *KpnI*; (Nd) *NdeI*; (Sc) *SacI*; (Xb) *XbaI*. The relative positions of the *comGG*, *yqxM*, *sipW*, *cotN*, *sinR*, and *sinI* genes are indicated according to Kunst et al. (1997). Regions of *sipW* specifying the conserved domains B-E and the putative membrane anchors AI and AII are indicated. (*sipW'*), 3'-Truncated *sipW* gene; (*'sipW*) 5' truncated *sipW* gene.

*sipW* (*B. subtilis*  $\Delta$ SUVW), or *sipT*, *sipU*, *sipV*, and *sipW* (*B. subtilis*  $\Delta$ TUVW) showed similar growth rates as the parental strain *B. subtilis* 8G5, irrespective of the growth medium used (data not shown). Thus, it seems that the secretory precursor processing machinery is functionally redundant, and that one SPase (i.e., SipS or SipT) is sufficient for growth and cell viability of *B. subtilis*.

#### Functional SipS is essential for viability of a *B. subtilis* strain lacking SipT

To evaluate the importance of SipS and SipT, a strain was constructed that produces a temperature-sensitive SipS mutant protein, but lacks wild-type SipS and SipT. To this purpose, we first selected temperature-sensitive variants from a large collection of previously constructed site-specific mutants of SipS (van Dijl et al. 1995). By use of a plate assay for SipS activity (van Dijl et al. 1992), five temperature-sensitive mutants were identified, which are active at 30°C, but not at 48°C (Table 2). These contain the L74A, Y81A, R84A, R84H, or D146A mutations, respectively. In a second step, *B. subtilis* 8G5 *sipS*, which lacks the chromosomal *sipS* gene (Bolhuis et al. 1996), was transformed with plasmids specifying the L74A, Y81A, R84A, R84H, or D146A SipS mutant proteins. In a third and final step, the *sipT* gene of the latter transformants was disrupted with a chloramphenicol resistance (Cm<sup>r</sup>) marker, as described previously (Tjalsma et al. 1997). The resulting mutant strains ( $\Delta$ ST *sipS*<sup>ts</sup>) showed no growth defects at 30°C, 37°C, and 42°C (data not shown). However, after a temperature shift to 48°C during the exponential growth phase, all five  $\Delta$ ST *sipS*<sup>ts</sup> strains stopped growing when they reached the transition phase between exponential and postexponential growth and, on continued incubation at 48°C, they even started to lyse. In contrast, the control strain  $\Delta$ ST (pGDL41), producing wild-type SipS, continued to grow in the postexponential growth phase (Fig. 3A). Because all  $\Delta$ ST *sipS*<sup>ts</sup> mutant strains showed similar characteristics with respect to growth and cell viability, only one

of these strains [i.e.,  $\Delta$ ST (pS-D146A)] was selected for further analyses. As shown by Western blotting, the cellular levels of wild-type SipS were not affected by the temperature shift from 37°C to 48°C. In contrast, the cellular levels of SipS-D146A, which were already reduced at 37°C, were decreased to levels below detection at 48°C (Fig. 3B). In summary, these observations show that for growth and cell viability of *B. subtilis*, a functional SipS is required in the absence of SipT.

#### Accumulation of secretory preproteins in the absence of functional SipS and SipT

As a first approach to monitor the effects of the absence of functional SipS and SipT on the processing of secretory preproteins, we analyzed the processing of pre(A13i)- $\beta$ -lactamase. This hybrid precursor is most efficiently processed by SipS and SipT (van Dijl et al. 1992; Tjalsma et al. 1997). As shown in Figure 3, B and C, the disappearance of the SipS-D146A mutant protein in *B. subtilis*  $\Delta$ ST (pS-D146A) at 48°C was paralleled by the accumulation of pre(A13i)- $\beta$ -lactamase. In contrast, cells of *B. subtilis*  $\Delta$ ST (pGDL41; wild-type SipS) contained much smaller amounts of this precursor and, in addition, they contained the corresponding mature protein (Fig. 3C). These findings show that processing of pre(A13i)- $\beta$ -lactamase is strongly affected in the absence of functional SipS and SipT.

To investigate the effects of depletion of SipS in cells lacking SipT, two strains with a disrupted *sipT* gene were constructed in which the transcription of *sipS* or *sipS*-D146A is controlled by the xylose-inducible *xylA* promoter. In these strains, denoted  $\Delta$ STxS and  $\Delta$ STxS-D146A, respectively, the wild-type *sipS* gene was removed from its original locus and the *xylA*-*sipS* or *xylA*-*sipS*-D146A constructs were integrated into the *amyE* locus (Fig. 4A). Unexpectedly, even in the absence of xylose, both strains showed normal growth at 37°C and 42°C, suggesting that the *xylA* promoter was not completely repressed in the absence of xylose (data not

**Table 1.** *Plasmids and bacterial strains*

Plasmids	Relevant properties	Reference
pGDL41	encodes pre(A13i)- $\beta$ -lactamase and SipS of <i>B. subtilis</i> ; replicates in <i>E. coli</i> and <i>B. subtilis</i> ; 8.1 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	van Dijl et al. (1992)
pGDL48	lacks the <i>sipS</i> gene and contains a multiple cloning site; otherwise identical to pGDL41; 7.5 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	Meijer et al. (1995)
pMO	encodes pre(A13i)- $\beta$ -lactamase, used for the site-directed mutagenesis of <i>sipS</i>	van Dijl et al. (1995)
pS-x	pMO derivatives carrying mutant <i>sipS</i> genes (x indicates the position and type of amino acid substitution in the corresponding mutant proteins); otherwise identical to pGDL41; 8.1 kb; Ap <sup>r</sup> ; Km <sup>r</sup>	van Dijl et al. (1995)
pKTH10	encodes the $\alpha$ -amylase (AmyQ) of <i>B. amyloliquefaciens</i> ; 6.8 kb; Km <sup>r</sup>	Palva (1982)
pCY66	encodes the PSBT domain of the <i>P. shermanii</i> transcarboxylase 1.3 S subunit	Cronan (1990)
pBR322amyQ	encodes the $\alpha$ -amylase (AmyQ) of <i>B. amyloliquefaciens</i>	this paper
pBR322amyQ-BT	encodes the AmyQ-PSBT fusion protein	this paper
pKTH10-BT	as pKTH10, encodes the AmyQ-PSBT fusion protein; 7.0 kb	this paper
pX	vector for the integration of genes in the <i>amyE</i> locus of <i>B. subtilis</i> ; integrated genes will be transcribed from the <i>xylA</i> promoter; carries the <i>xylR</i> gene; 7.5 kb; Ap <sup>r</sup> ; Cm <sup>r</sup>	Kim et al. (1996)
pXS	pX derivative; carries <i>sipS</i> downstream of the <i>xylA</i> promoter; 8.2 kb; Cm <sup>r</sup>	this paper
pXS-D146A	pX derivative; carries <i>sipS-D146A</i> downstream of the <i>xylA</i> promoter; 8.2 kb; Cm <sup>r</sup>	this paper
pLGW200	integration vector for <i>B. subtilis</i> with a promoterless <i>lacZ</i> gene fused to the ribosome-binding site of the <i>spoVG</i> gene; 6.8 kb; Cm <sup>r</sup>	van Sinderen et al. (1990)
pLGW201	pLGW200 derivative with a transcriptional <i>sipW-lacZ</i> fusion; 7.8 kb	this paper
pUK21	cloning vector; 2.8 kb; Km <sup>r</sup>	Vieria and Messing (1990)
pKW34dT	pUK21 derivative for the disruption of <i>sipW</i> ; 6.6-kb Km <sup>r</sup> ; Tc <sup>r</sup>	this paper
<b>Strains</b>		
<i>E. coli</i>		
MC1061	F; <i>araD139</i> ; $\Delta$ ( <i>ara-leu</i> )7696; $\Delta$ ( <i>lac</i> )X74; <i>galU</i> ; <i>galk</i> ; <i>hsdR2</i> ; <i>mcrA</i> ; <i>mcrB</i> ; <i>rspL</i>	Wertmann et al. (1986)
<i>B. subtilis</i>		
8G5	<i>trpC2</i> ; <i>tyr</i> ; <i>his</i> ; <i>nic</i> ; <i>ura</i> ; <i>rib</i> ; <i>met</i> ; <i>ade</i> ; lacks the <i>sipP</i> genes	Bron and Venema (1972)
8G5 <i>sipS</i> ( $\Delta$ S)	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i>	Bolhuis et al. (1996)
8G5 <i>sipT</i> -Cm ( $\Delta$ T)	like 8G5; <i>sipT</i> ; Cm <sup>r</sup>	Tjalsma et al. (1997)
8G5 <i>sipT</i> -Sp	like 8G5; <i>sipT</i> ; Sp <sup>r</sup>	this paper
8G5 <i>sipU</i>	like 8G5; <i>sipU</i>	Tjalsma et al. (1997)
8G5 <i>sipV</i> -Em	like 8G5; <i>sipV</i> ; Em <sup>r</sup>	Tjalsma et al. (1997)
8G5 <i>sipV</i> -Sp	like 8G5; <i>sipV</i> ; Sp <sup>r</sup>	this paper
8G5 <i>sipW</i> -Tc ( $\Delta$ W)	like 8G5; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ SU	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipU</i>	this paper
$\Delta$ SV	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipV</i> ; Em <sup>r</sup>	this paper
$\Delta$ SW	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ SUV	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipU</i> ; <i>sipV</i> ; Em <sup>r</sup>	this paper
$\Delta$ SUW	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipU</i> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ SVW	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipV</i> ; Em <sup>r</sup> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ SUVW	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipS</i> ; <i>sipU</i> ; <i>sipV</i> ; Em <sup>r</sup> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ TU	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipU</i>	this paper
$\Delta$ TV	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipV</i> ; Em <sup>r</sup>	this paper
$\Delta$ TW	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ TUV	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipU</i> ; <i>sipV</i> ; Em <sup>r</sup>	this paper
$\Delta$ TUW	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipU</i> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ TUVW	like 8G5; <i>sipT</i> ; Cm <sup>r</sup> ; <i>sipU</i> ; <i>sipV</i> ; Sp <sup>r</sup> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ UV	like 8G5; <i>sipU</i> ; <i>sipV</i> ; Em <sup>r</sup>	this paper
$\Delta$ UW	like 8G5; <i>sipU</i> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ UW	like 8G5; <i>sipU</i> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ VW	like 8G5; <i>sipV</i> ; Sp <sup>r</sup> ; <i>sipW</i> ; Tc <sup>r</sup>	this paper
$\Delta$ ST (pGDL41)	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipT</i> ; Cm <sup>r</sup> ; contains pGDL41	this paper
$\Delta$ ST (pS-D146A)	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipT</i> ; Cm <sup>r</sup> ; contains pS-D146A	this paper
$\Delta$ STxS	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipT</i> ; Sp <sup>r</sup> ; <i>PxylA-sipS</i> ; <i>amyE</i> ; Cm <sup>r</sup>	this paper
$\Delta$ STxS-D146A	like 8G5; <i>rib</i> <sup>+</sup> ; <i>sipT</i> ; Sp <sup>r</sup> ; <i>PxylA-sipS-D146A</i> ; <i>amyE</i> ; Cm <sup>r</sup>	this paper
8G5::pGDE22	8G5 carrying pGDE22 ( <i>sipS-lacZ</i> ) in the chromosome; Cm <sup>r</sup>	Bolhuis et al., (1996)
8G5::pLGT207	8G5 carrying pLGT207 ( <i>sipT-lacZ</i> ) in the chromosome; Cm <sup>r</sup>	Tjalsma et al. (1997)
8G5::pLGU202	8G5 carrying pLGU202 ( <i>sipU-lacZ</i> ) in the chromosome; Cm <sup>r</sup>	Tjalsma et al. (1997)
8G5::pLGV201	8G5 carrying pLGV201 ( <i>sipV-lacZ</i> ) in the chromosome; Cm <sup>r</sup>	Tjalsma et al. (1997)
8G5::pLGW201	8G5 carrying pLGW201 ( <i>sipW-lacZ</i> ) in the chromosome; Cm <sup>r</sup>	this paper

**Table 1.** (Continued)

8G5 <i>degS-degU</i>	like 8G5; <i>degS-degU</i> ; <i>Km<sup>r</sup></i>	Bolhuis et al. (1996)
8G5 <i>degU32(Hy)</i>	like 8G5; <i>degU32(Hy)</i> ; <i>Km<sup>r</sup></i>	Bolhuis et al. (1996)
8G5::pGDE22 <i>degU32(Hy)</i>	derivative of 8G5::pGDE22; <i>degU32(Hy)</i> ; <i>Cm<sup>r</sup></i> ; <i>Km<sup>r</sup></i>	Bolhuis et al. (1996)
8G5::pLGT207 <i>degU32(Hy)</i>	derivative of 8G5::pLGT207; <i>degU32(Hy)</i> ; <i>Cm<sup>r</sup></i> ; <i>Km<sup>r</sup></i>	this paper
8G5::pLGU202 <i>degU32(Hy)</i>	derivative of 8G5::pLGU202; <i>degU32(Hy)</i> ; <i>Cm<sup>r</sup></i> ; <i>Km<sup>r</sup></i>	this paper
8G5::pLGV201 <i>degU32(Hy)</i>	derivative of 8G5::pLGV201; <i>degU32(Hy)</i> ; <i>Cm<sup>r</sup></i> ; <i>Km<sup>r</sup></i>	this paper
8G5::pLGW201 <i>degU32(Hy)</i>	derivative of 8G5::pLGW201; <i>degU32(Hy)</i> ; <i>Cm<sup>r</sup></i> ; <i>Km<sup>r</sup></i>	this paper

shown). In fact, the inhibition of growth and subsequent cell lysis was only observed for *B. subtilis*  $\Delta$ STxS-D146A on incubation at 48°C in the absence of xylose (data not shown), similar to *B. subtilis*  $\Delta$ ST (pS-D146A) (Fig. 3A). As shown by Western blotting experiments with cells in the postexponential growth phase, the cellular levels of SipS or SipS-D146A in *B. subtilis*  $\Delta$ STxS and  $\Delta$ STxS-D146A, respectively, depended on the presence or absence of xylose. Compared with the parental strain *B. subtilis* 8G5, *B. subtilis*  $\Delta$ STxS contained slightly reduced levels of SipS in the absence of xylose, whereas SipS was about five-fold overproduced in the presence of xylose. In contrast, in *B. subtilis*  $\Delta$ STxS-D146A, only very low levels of SipS-D146A were detectable in the presence of xylose, whereas SipS-D146A was not detectable in the absence of xylose (Fig. 4B). The latter observation suggests that very small amounts of SipS-D146A are sufficient for growth and cell viability.

To determine the effects of various levels of SipS (-D146A) activity on the accumulation of secretory precursor proteins in cells lacking SipT, *B. subtilis*  $\Delta$ STxS and  $\Delta$ STxS-D146A were transformed with plasmid pKTH10 that specifies the *Bacillus amyloliquefaciens*  $\alpha$ -amylase AmyQ. The presence of pKTH10 results in the accumulation of pre-AmyQ, even in wild-type cells

(Palva 1982; Kontinen and Sarvas 1988). As shown by Western blotting, cells of *B. subtilis*  $\Delta$ STxS-D146A, which were incubated at 48°C in the absence of xylose, accumulated strongly increased amounts of pre-AmyQ (Fig. 4C). In contrast, at 37°C, cells of the latter strain accumulated similar amounts of pre-AmyQ as *B. subtilis*  $\Delta$ STxS, producing close to wild-type levels of SipS (37°C and 48°C; Fig. 4C), or overproducing SipS (data not shown). Taken together, our observations show that the presence of SipS and SipT is required for efficient processing of pre-AmyQ, and that in cells lacking SipT, very small amounts of SipS, which are below the level of detection, are sufficient to prevent the accumulation of pre-AmyQ at high levels.

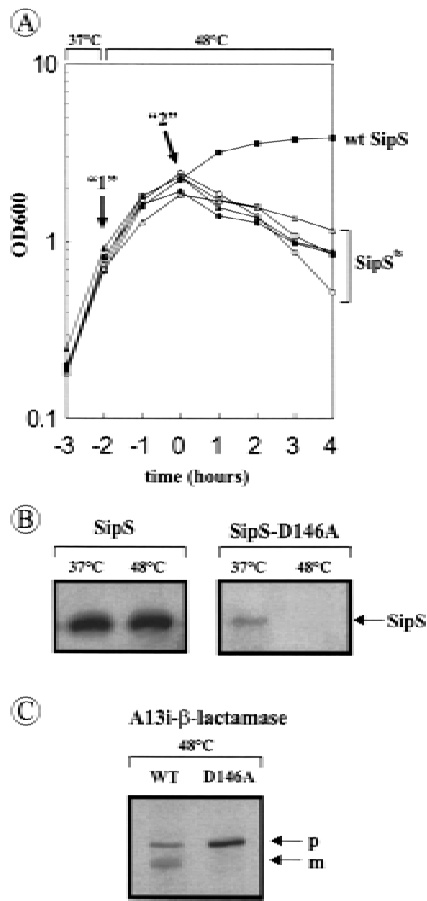
#### Impaired translocation of pre-AmyQ in cells lacking functional SipS and SipT

To investigate whether SipS and SipT depletion affects the translocation of pre-AmyQ, the sequences specifying the biotin-accepting domain of a transcarboxylase from *Propionibacterium shermanii* (PSBT; Jander et al. 1996) were fused to the 3' end of the *amyQ* gene on pKTH10. The resulting plasmid pKTH10-BT was used to transform *B. subtilis*  $\Delta$ STxS-D146A and, subsequently, the accumulation of biotinylated (pre-)AmyQ-PSBT was determined. 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 translocation of pre-AmyQ-PSBT across the membrane is impaired. As shown in Figure 5A, processing of pre-AmyQ-PSBT occurred efficiently in cells of *B. subtilis*  $\Delta$ STxS-D146A growing at 37°C in the absence of xylose, as only small amounts of pre-AmyQ-PSBT were detectable. Under these conditions, mature nonbiotinylated AmyQ-PSBT was secreted into the growth medium (data not shown). Similar results were obtained with the parental strain 8G5, both at 37°C and 48°C (data not shown). In contrast, high levels of pre-AmyQ-PSBT accumulated in cells of *B. subtilis*  $\Delta$ STxS-D146A incubated in the absence of xylose at 42°C (permissive temperature for growth) or 48°C (non-permissive temperature) (Fig. 5B, top panel). As shown with a streptavidin-horseradish peroxidase (HRP) conjugate, significant amounts of the accumulating pre-

**Table 2.** Identification of temperature-sensitive SipS mutants

Plasmid	Temperature			
	22°C	30°C	37°C	48°C
pGDL48 (empty vector)	-	-	-	-
pGDL41 ( <i>sipS</i> )	+	+	+	+
pS-L74A	+	+	+/-	-
pS-Y81A	-	+	-	-
pS-R84A	+	+	-	-
pS-R84H	+	+	-	-
pS-D146A	+	+	-	-

A plate (halo)-assay for the processing of the hybrid precursor pre(A13i)- $\beta$ -lactamase (van Dijl et al. 1992) was used to examine the temperature sensitivity of mutant SipS proteins. *E. coli* MC1061 strains containing plasmids that specify wild-type SipS (pGDL41) or SipS mutant proteins (pS-x) were analyzed for halo formation at 22°C, 30°C, 37°C, and 48°C respectively. Halo formation is indicated with +.



**Figure 3.** Temperature-sensitive growth of *B. subtilis*  $\Delta$ ST containing a *sipS<sup>ts</sup>* gene. (A) Growth curves of *B. subtilis* strains that lack intact chromosomal copies of *sipS* and *sipT* but contain plasmids specifying the wild-type SipS or temperature-sensitive SipS mutant proteins. The names of the latter plasmids refer to the respective mutation in SipS. (○) *B. subtilis*  $\Delta$ ST (pS-L74A); (□) *B. subtilis*  $\Delta$ ST (pS-Y81A); (●) *B. subtilis*  $\Delta$ ST (pS-R84A); (▲) *B. subtilis*  $\Delta$ ST (pS-R84H); (△) *B. subtilis*  $\Delta$ ST (pS-D146A); (■) *B. subtilis*  $\Delta$ ST (pGDL41; specifies wild-type SipS). Overnight cultures were diluted 100-fold in fresh TY medium and incubated at 37°C. When the cells reached an OD<sub>600</sub> of ~0.6, the temperature was shifted to 48°C. Samples were withdrawn shortly before the temperature shift (arrow "1") and 2 hr after the temperature shift (arrow "2"). Zero time ( $t = 0$ ) indicates the transition point between the exponential and post-exponential growth phases. (B) The presence of wild-type SipS [*B. subtilis*  $\Delta$ ST (pGDL41)] and SipS-D146A [*B. subtilis*  $\Delta$ ST (pS-D146A)] in cells growing at 37°C (arrow "1" in A) and 48°C (arrow "2" in A) was analyzed by SDS-PAGE and Western blotting. The position of SipS/SipS-D146A is indicated. (C) Accumulation of pre(A13i)- $\beta$ -lactamase in cells of *B. subtilis*  $\Delta$ ST (pGDL41; wild-type SipS) and  $\Delta$ ST(pS-D146A) at 48°C (arrow "2" in A) was analyzed by SDS-PAGE and Western blotting. (p) Precursor; (m) mature.

AmyQ-PSBT were biotinylated, showing that the translocation of these precursor molecules was impaired (Fig. 5B, bottom panel). Compared with cells of *B. subtilis*  $\Delta$ STxS-D146A, which were grown in the absence of xylose at 37°C, cells grown at 42°C, or 48°C accumulated

significantly reduced levels of mature AmyQ-PSBT; although mature AmyQ-PSBT was produced in the absence of xylose at 42°C or 48°C, it was not detectably biotinylated (Fig 5A,B).

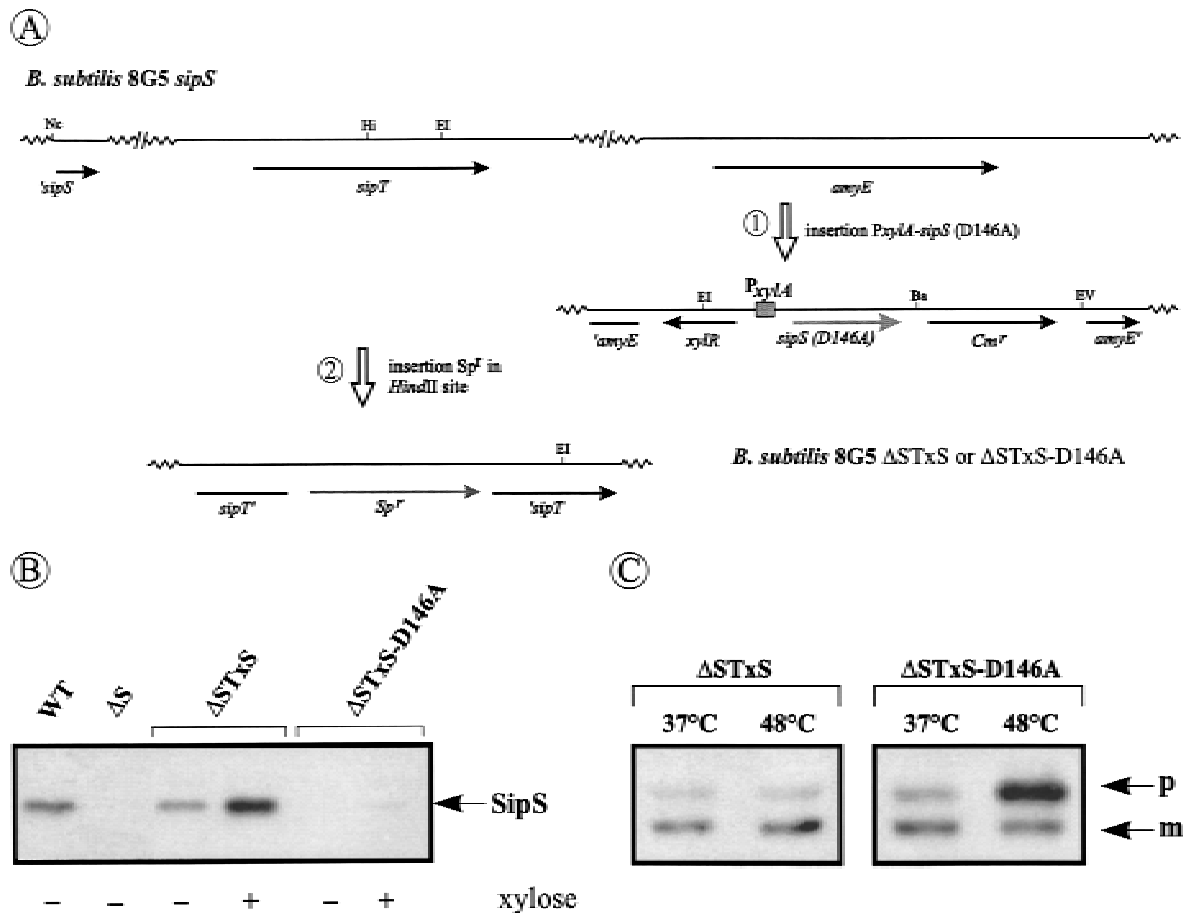
#### Relative contributions of SipU, SipV, and SipW to the processing of pre-AmyQ

We have reported previously that the processing of pre-AmyQ is stimulated in the absence of SipS, and retarded in the absence of SipT, indicating that the production of SipS interferes with pre-AmyQ processing, and that this precursor is a preferred substrate of SipT (Tjalsma et al. 1997). To examine the relative contributions of SipU, SipV, and SipW to the processing of pre-AmyQ in the absence of SipS or SipT, Western blotting and pulse-chase labeling experiments were performed with strains lacking multiple SPases. The parental strain 8G5 was used as a control. As the results obtained by pulse-chase labeling confirmed those obtained by Western blotting, only the latter are documented. As shown in Figure 6A, cells of *B. subtilis*  $\Delta$ S, or  $\Delta$ SU, accumulated only very low levels of pre-AmyQ. Similar results were obtained with cells of *B. subtilis*  $\Delta$ SV and  $\Delta$ SW (data not shown). The level of pre-AmyQ was slightly increased in cells of *B. subtilis*  $\Delta$ SUV, but remained lower than in the parental strain (Fig. 6A). The levels of pre-AmyQ accumulation in *B. subtilis*  $\Delta$ SUW and  $\Delta$ SVW were similar to those in *B. subtilis*  $\Delta$ SUV (data not shown). Compared with *B. subtilis*  $\Delta$ T, which accumulates more pre-AmyQ than the parental strain, cells of *B. subtilis*  $\Delta$ TU and  $\Delta$ TUV showed no additional accumulation of pre-AmyQ (Fig. 6A), and similar results were obtained with *B. subtilis*  $\Delta$ TV,  $\Delta$ TW, and  $\Delta$ TUW (data not shown). The levels of pre-AmyQ in cells of *B. subtilis*  $\Delta$ SUV or  $\Delta$ TUV could only be increased further by disruption of the *sipW* gene, showing that SipW is actively involved in the processing of pre-AmyQ (Fig. 6B). In conclusion, these observations show that the accumulation of pre-AmyQ in cells lacking either SipS or SipT was significantly exacerbated only by the simultaneous removal of SipU, SipV, and SipW.

#### Transcription of only *sipS* and *sipT* is temporally regulated by the *DegS-DegU* system

The transcription of the *sipS* and *sipT* genes is temporally controlled, whereas the *sipU* and *sipV* genes are constitutively transcribed (Bolhuis et al. 1996; Tjalsma et al. 1997). To analyze the transcription of the *sipW* gene, a transcriptional *sipW-lacZ* fusion was constructed, and introduced into the chromosome of *B. subtilis* 8G5 (schematically shown in Fig. 7A). Cells of the resulting strain were grown in TY or minimal medium, and samples withdrawn at hourly intervals were assayed for  $\beta$ -galactosidase activity. The results show that, irrespective of the growth medium used, *sipW* is constitutively transcribed at a low level (only the results for minimal medium are shown in Fig. 7B, ▲).

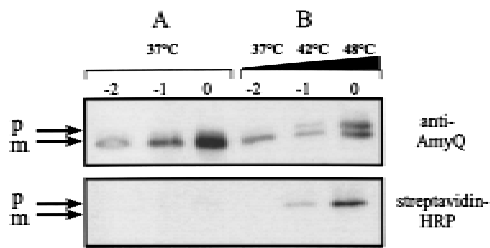




**Figure 4.** Accumulation of pre-AmyQ in *B. subtilis* cells lacking functional SipS and SipT. (A) Schematic presentation of the construction of *B. subtilis*  $\Delta$ STxS and  $\Delta$ STxS-D146A. For this purpose, the *sipS* and *sipS-D146A* genes were amplified by PCR with the primers EX-1 (5'-ACTCTAGATGCCATTGAAATAGACC-3') and EX-2 (5'-ATGGATCCTTGTTTAAAACCTTGG-3') with the plasmids pGDL41 and pS-D146A as templates. The amplified fragments were cleaved with *Xba*I and *Bam*HI and ligated to the *Spe*I-*Bam*HI sites of the plasmid pX (Kim et al. 1997), resulting in pXS and pXS-D146A. Next, both plasmids were linearized and used to transform *B. subtilis* 8G5 *sipS*. Thus, the *amyE* gene was replaced with the xylose-inducible *sipS* or *sipS-D146A* genes and a *Cm<sup>r</sup>* marker. The inactivation of the *amyE* gene was tested and confirmed by lack of halo formation during growth on plates containing 1% starch. Finally, the chromosomal *sipT* gene was disrupted with a *Sp<sup>r</sup>* marker by homologous recombination, resulting in the strains  $\Delta$ STxS (*sipS*, *sipT*-*Sp<sup>r</sup>*, *P<sub>XylA</sub>-sipS*, *Cm<sup>r</sup>*) and  $\Delta$ STxS-D146A (*sipS*, *sipT*-*Sp<sup>r</sup>*, *P<sub>XylA</sub>-sipS-D146A*, *Cm<sup>r</sup>*). Only restriction sites relevant for the constructions are shown: (Ba) *Bam*HI; (EI) *Eco*RI; (EV) *Eco*RV; (Hi) *Hind*II; (Nc) *Nco*I. (*'sipS*) 5'-Truncated *sipS* gene (Bolhuis et al. 1996); (*'amyE*) 3'-truncated *amyE* gene; (*'sipT*) 3'-truncated *sipT* gene; (*'sipT*) 5'-truncated *sipT* gene; (*xyIR*) gene specifying the XylR repressor protein. (B) Detection of wild-type SipS or SipS-D146A in cells of *B. subtilis* 8G5 (WT), 8G5 *sipS* ( $\Delta$ S),  $\Delta$ STxS and  $\Delta$ STxS-D146A. Cells were grown in TY medium at 37°C in the presence (+) or absence (-) of 1% xylose, and sample for SDS-PAGE and Western blotting were withdrawn 4 hr after the transition from exponential to postexponential growth. (C) Accumulation of pre-AmyQ in cells of *B. subtilis*  $\Delta$ STxS and  $\Delta$ STxS-D146A. Overnight cultures were diluted 100-fold in fresh TY medium without xylose and incubated at 37°C. When the cells reached an OD<sub>600</sub> of ~0.6, the temperature was shifted to 48°C. Samples withdrawn shortly before ( $t = -1$ ; see Fig. 3A), and 30 min after the temperature shift ( $t = -0.5$ ) were analyzed by SDS-PAGE and Western blotting. (p) Precursor; (m) mature.

Because it was shown previously that the temporal control of *sipS* transcription is exerted by the DegS-DegU two-component regulatory system (Bolhuis et al. 1996), which is also required for the transcription of most genes for secreted degradative enzymes (for review, see Ferrari et al. 1993), we investigated whether this regulatory system is also involved in the transcription of the *sipT*, *sipU*, *sipV*, and *sipW* genes. To this purpose, the *degU32(Hy)* mutation, which leads to increased transcription of genes for degradative enzymes (Ferrari et al.

1993; Msadek et al. 1993) and *sipS* (Bolhuis et al. 1996), was introduced in strains carrying chromosomal *sipS-lacZ*, *sipT-lacZ*, *sipU-lacZ*, *sipV-lacZ*, or *sipW-lacZ* gene fusions. The resulting strains were grown in minimal medium and samples, withdrawn at hourly intervals, were assayed for  $\beta$ -galactosidase activity. The results show that only the transcription of *sipS* and *sipT* was stimulated in strains containing the *degU32(Hy)* mutation (Fig. 7B,C; indicated with  $\square$  and  $\blacksquare$  respectively). In contrast, the *degU32(Hy)* mutation had no ef-



**Figure 5.** Accumulation of biotinylated pre-AmyQ-PSBT in the absence of functional SipS and SipT. An overnight culture of *B. subtilis*  $\Delta$ STxS-D146A (pKTH10-BT) was diluted 100-fold in fresh TY medium without xylose and incubated at 37°C. When the culture reached an OD<sub>600</sub> of ~0.6, a sample ( $t = -2$ ) was taken. Subsequently, the culture was divided in two equal moieties A and B. Culture A was kept for 2 hr at 37°C, and samples were taken at  $t = -1$  and  $t = 0$ . Culture B was incubated for 1 hr at 42°C and, after a sample ( $t = -1$ ) had been taken, incubation was continued for 1 hr at 48°C before a second sample ( $t = 0$ ) was taken. Cells were collected by centrifugation and analyzed by SDS-PAGE and Western blotting. The presence of AmyQ-PSBT was visualized with specific antibodies. Biotinylated pre-AmyQ-PSBT was visualized with streptavidin-HRP. (p) Precursor; (m) mature.

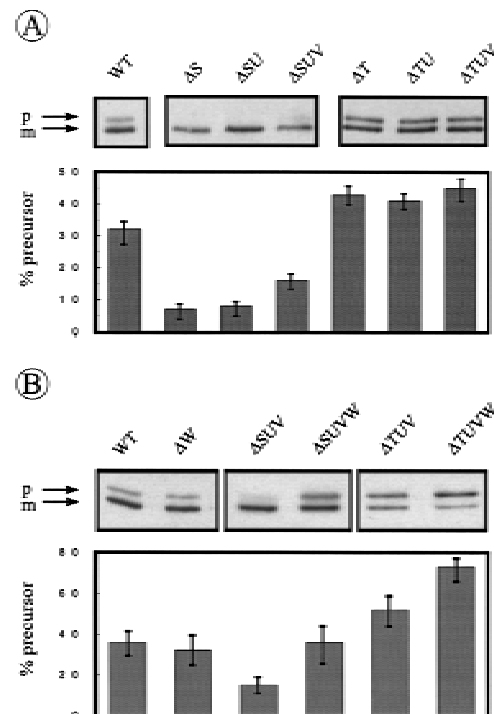
fect on the transcription of *sipU*, *sipV*, and *sipW* (Fig. 7B,C; indicated with  $\circ$ ,  $\bullet$ ,  $\blacktriangle$ , respectively).

To verify the involvement of the DegS-DegU system in the transcription of the *sipT* gene, Northern hybridization experiments were performed with total RNA from the *B. subtilis* strains 8G5, 8G5 *degU32*(Hy), and 8G5 *degS-degU*. In contrast to *sipS*, for which two transcripts were detectable (Bolhuis et al. 1996), only one, monocistronic, *sipT*-specific transcript was detected. Compared with the parental strain 8G5, the amount of the *sipT*-specific transcript was about twofold increased in cells with the *degU32*(Hy) mutation, although it was hardly detectable in cells containing the *degS-degU* deletion (Fig. 7D). These findings show that the transcription of the *sipT* gene is controlled by the DegS-DegU system. Consistent with this conclusion, we identified the sequence: 5'-TATGATAAAGTA-3' at a position 46 nucleotides upstream of the translational start of *sipT*. This sequence contains one mismatch and one base insertion compared with a consensus sequence (AMTGAAMAKWW in which K = G or T; M = A or C; W = A or T), which has been implicated in the DegU-dependent control of gene expression (Jacobs 1995). Taken together, these results show that the transcription of *sipS* and *sipT* is concerted with that of the genes for secreted degradative enzymes. In contrast, the *sipU*, *sipV*, and *sipW* genes are constitutively transcribed at a low level.

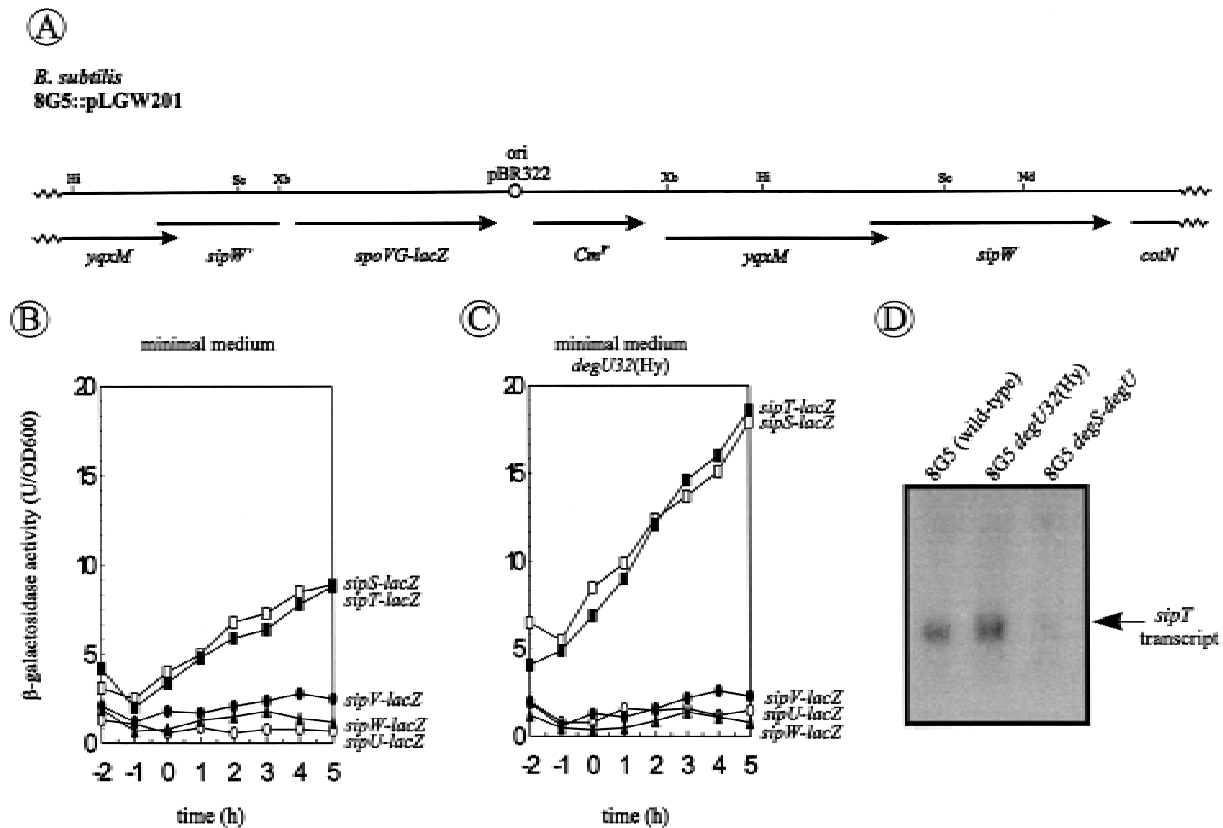
## Discussion

Various microorganisms contain paralogous type I SPases. Interestingly, this seems to be a common feature of Gram-positive eubacteria in particular. For example, *B. amyloliquefaciens* (Hoang and Hofemeister 1995; Meijer et al. 1995), *Staphylococcus aureus* (Cregg et al. 1996) and *Streptomyces lividans* (S. Schacht and J. Anné,

pers. comm.) contain at least two paralogous SPases of the P-type, whereas *Deinococcus radiodurans* seems to contain at least three of these enzymes (sequences are available via ftp://ftp.tigr.org/pub/data/d\_radiodurans). Notably, we have identified one ER-type and six P-type SPases in *B. subtilis*. In the present studies, which were aimed at the functional analysis of the chromosomally encoded SPases of *B. subtilis*, we show that, in principle, the presence of one SPase (i.e., SipS or SipT) is sufficient for precursor processing, growth, and cell viability. Thus, the secretory precursor processing machinery of *B. subtilis* is functionally redundant, ensuring that this organism can avail of backup SPases. Under natural condi-



**Figure 6.** Relative contribution of SipU, SipV, and SipW to pre-AmyQ processing in the absence of SipS or SipT. (A) The accumulation of pre-AmyQ in pKTH10-transformed cells of the parental strain *B. subtilis* 8G5 (WT) and in pKTH10-transformed cells lacking SipS ( $\Delta$ S), SipS, and SipU ( $\Delta$ SU), SipS, SipU, and SipV ( $\Delta$ SUV), SipT ( $\Delta$ T), SipT, and SipU ( $\Delta$ TU), or SipT, SipU, and SipV ( $\Delta$ TUV) was analyzed by SDS-PAGE and Western blotting. Samples were taken from overnight cultures in TY medium. (p) Precursor; (m) mature. Relative amounts of precursor and mature forms of AmyQ were determined by densitometer scanning of films. The average values of three independent experiments are shown, and the standard deviation is indicated by error bars. (B) The accumulation of pre-AmyQ in pKTH10-transformed cells of the parental strain *B. subtilis* 8G5 (WT), and pKTH10-transformed cells lacking SipW ( $\Delta$ W), SipS, SipU, and SipV ( $\Delta$ SUV), SipS, SipU, SipV, and SipW ( $\Delta$ SUVW), SipT, SipU, and SipV ( $\Delta$ TUV), or SipT, SipU, SipV, and SipW ( $\Delta$ TUVW), was analyzed by SDS-PAGE and Western blotting. Samples were prepared from cells grown in TY medium until the early postexponential growth phase ( $t = 4$ ). Relative amounts of precursor and mature forms of AmyQ are indicated as in A.



**Figure 7.** Analysis of the transcription of the *sip* genes of *B. subtilis*. (A) Schematic presentation of the *sipW* locus on the chromosome of *B. subtilis* 8G5::pLGW201. A transcriptional *sipW-lacZ* gene fusion was constructed with plasmid pLGW200 (van Sinderen et al. 1990), a chromosomal integration plasmid for *B. subtilis* containing a promoterless *spoVG-lacZ* gene fusion. For this purpose, a fragment comprising the *yqxM* gene and the 5' end of *sipW* was amplified by PCR with the primers lbw-3 (5'-CATCTAGATACAGGAGTAAGATATG-3') and lbw-5 (5'-AGTTCTAGAGTATTTCATCCTGC-3'). Next, the amplified fragment was cloned into the *XbaI*-site of the multiple cloning site upstream of the *spoVG-lacZ* gene fusion on pLGW200, resulting in pLGW201. Finally, the *sipW-lacZ* gene fusion was introduced in the chromosome of *B. subtilis* 8G5 by a Campbell-type integration of pLGW201, resulting in *B. subtilis* 8G5::pLGW201. In the latter strain, the transcription of *lacZ* is directed by the promoter(s) of *sipW*. Only restriction sites relevant for the constructions are shown: (Hi) *HindIII*; (Nd) *NdeI*; (Sc) *SacI*; (Xb) *XbaI*. (ori pBR322) Replication origins of pBR322. (B) Time courses of the expression of *sip-lacZ* gene fusions were determined in cells growing in minimal medium at 37°C.  $\beta$ -Galactosidase activities (in units per OD<sub>600</sub>) were determined for *B. subtilis* 8G5::pGDE22 ( $\square$ ; *sipS-lacZ*), *B. subtilis* 8G5::pLGT207 ( $\blacksquare$ ; *sipT-lacZ*), *B. subtilis* 8G5::pLGU202 ( $\circ$ ; *sipU-lacZ*), *B. subtilis* 8G5::pLGV201 ( $\bullet$ ; *sipV-lacZ*), and *B. subtilis* 8G5::pLGW201 ( $\blacktriangle$ ; *sipW-lacZ*). Zero time ( $t = 0$ ) indicates the transition point between the exponential and postexponential growth phases. (C) Effects of the *degU32(Hy)* mutation on the expression of *sip-lacZ* gene fusions were determined as in B. To this purpose, strains containing the *sip-lacZ* gene fusions indicated in B were transformed with chromosomal DNA of *B. subtilis* 8G5 *degU32(Hy)*. (D) Detection of *sipT*-specific transcripts by Northern hybridization. Equal amounts of total RNA from cells of *B. subtilis* 8G5, 8G5 *degU32(Hy)*, and 8G5 *degU-degS* in the transition state were loaded in each lane. A [<sup>32</sup>P]dCTP-labeled DNA fragment, amplified by PCR with the primers lbt-9 (5'-ATGAATTCAGCCCGTTATCTCC-3') and lbt-10 (5'-GGAAGTCGACATACGTACCTGGAATGG-3'), was used as a probe.

tions, this may be of particular importance for the fitness of *B. subtilis* and other Gram-positive eubacteria, which can secrete large amounts of proteins into the medium as an adaptive response to changes in the environment.

Our results show that SipS and SipT play a major role in the processing of secretory preproteins and that, under the conditions tested, the three other chromosomally encoded SPases (SipU, SipV, and SipW) contribute only to a minor extent to this process. Interestingly, the transcription of *sipS* (Bolhuis et al. 1996) and *sipT* (this study) is temporally controlled via the DegS-DegU two-component regulatory system, in concert with the transcription

of the genes for most secretory proteins. Thus, *B. subtilis* can increase its preprotein processing capacity under conditions of high-level protein secretion in the postexponential growth phase. Our observation that cells lacking SipS and SipT stop growing, and die in the transition phase between exponential and postexponential growth, indicates that the potential to increase the processing capacity is important for cell growth and viability. Nevertheless, the production of very small amounts of SipS-D146A, a temperature-sensitive variant of SipS, is sufficient for growth and cell viability. This suggests that the increased processing capacity provided by SipS and SipT

relates to, as yet undefined, differences between the specificities of these two SPases and those of SipU, SipV, and/or SipW. This view is supported by the observation that SipU, SipV, and SipW cannot complement for the absence of SipS and SipT, even if the corresponding genes are placed on multi-copy plasmids (H. Tjalsma et al., unpubl.). In this respect, it is important to note that we have shown previously that SipS, SipT, SipU, SipV, and SipW have overlapping substrate specificities (Tjalsma et al. 1997). At present, we do not know the precise function of each of the SPases of *B. subtilis*, but it seems likely that SipS and SipT are required, in particular, for the processing of preproteins that are synthesized in the postexponential growth phase. In contrast, as the *sipU*, *sipV*, and *sipW* genes are transcribed constitutively, the corresponding SPases are most likely involved in the processing of preproteins which are synthesized during all growth phases.

The previously reported presence of SPase-encoding genes on endogenous plasmids (Meijer et al. 1995) seems to give certain strains of *B. subtilis* an additional possibility to increase their capacity for preprotein processing. The latter hypothesis is supported by our observation that the SPase SipP, specified by plasmid pTA1015, can complement for the absence of SipS and SipT (H. Tjalsma et al., unpubl.). Thus, it seems that SipS, SipT, and SipP have similar substrate specificities.

Dalbey and Wickner (1985) have shown that *E. coli* cells depleted of their type I SPase (i.e., leader peptidase) accumulate preproteins on the outer surface of the cytoplasmic membrane. Thus, processing by SPase was shown to be a prerequisite for the release of translocated proteins from the membrane. However, the question whether the accumulation of secretory preproteins in cells depleted of SPase would affect protein translocation across the membrane remained unresolved. Our observation that biotinylated pre-AmyQ-PSBT accumulated in *B. subtilis* cells lacking functional SipS and SipT shows that this is the case. Thus, the decreased viability of these cells may be due to impaired protein translocation across the membrane. However, the alternative explanation that certain proteins, which are essential for cell growth and viability, do not reach their correct cellular localization (e.g., the cell wall) cannot be excluded. Interestingly, no biotinylated mature AmyQ-PSBT was produced by cells lacking functional SipS and SipT, suggesting that biotinylated pre-AmyQ-PSBT is not translocated across the membrane of *B. subtilis*. As biotinylated proteins can be transported by the preprotein translocase of *E. coli* (Jander et al. 1996), it seems that the preprotein translocase of *B. subtilis* is more restrictive with respect to (partially) folded, biotinylated preproteins than that of *E. coli*.

An intriguing result of the present studies is that *B. subtilis* contains an ER-type SPase (SipW). Thus, *B. subtilis* is the first known eubacterium containing SPases of both the P- and the ER-type. This may not be a peculiarity of *B. subtilis*, as we identified the partial sequence of a SipW/SPC21-like protein from *Clostridium perfringens* by similarity searches in GenBank (accession no.

X86488). Furthermore, the identification of SipW of *B. subtilis* has at least three interesting implications. First, the presence of ER-type SPases in all three domains of life suggests that the P-type SPases, which are only present in eubacteria and the organelles of eukaryotes, have evolved from the ER-type SPases. However, the alternative possibilities of convergent evolution of P- and ER-type SPases and/or horizontal transfer of ER-type SPases from archaea or eukaryotes to eubacteria cannot be excluded. Second, recent studies indicate that the P-type SPases make use of a serine-lysine catalytic dyad (see Dalbey et al. 1997). In contrast, the catalytic mechanism of the ER-type SPases is presently unclear, because the conserved lysine residue in domain D, which is invoked in the catalytic mechanism of the P-type SPases, is not conserved in the ER-type SPases; instead the ER-type SPases contain a conserved histidine residue at this position (Fig. 1B). This could mean that the ER-type SPases use the latter histidine residue for catalysis, for example in a serine-histidine-aspartic acid catalytic triad, like in the classical serine proteases. In addition to the conserved histidine residue in domain D, such a catalytic triad could involve the strictly conserved serine residue in domain B, which is essential for the activity of the P-type SPases, and the strictly conserved aspartic acid residue in domain E. Alternatively, the ER-type SPases could make use of a serine-lysine catalytic dyad involving the strictly conserved serine and lysine residues in domains B and E, respectively. Third, the ER-type SPase of yeast and higher eukaryotes are part of larger complexes, containing three additional subunits of which the SPC3 or SPC22/23 subunits, respectively, are essential for SPase activity (Newsome et al. 1992; Fang et al. 1997; Meyer and Hartmann 1997). None of these additional subunits is conserved in eubacteria or archaea (H. Tjalsma et al., unpubl.), suggesting that the ER-type SPases of the latter groups of organisms require no, or very different additional subunits for activity. The identification of the catalytic mechanism of the ER-type SPases, and the identification of their putative partner proteins in eubacteria and archaea, are major challenges for future research.

Finally, the present studies on the *sip* gene family of *B. subtilis* have important implications for projects on the functional analysis of the genomes of *B. subtilis* and other organisms, such as *E. coli* and yeast, because the presence of paralogous gene families is a common feature of these microbial genomes. As exemplified by the lack of effect of the single disruption of the *sipW* gene, strategies based on single gene disruptions will probably be inadequate to assign functions to many multiplied genes with thus far unidentified functions.

## Materials and methods

### Plasmids, bacterial strains, and media

Table 1 lists the plasmids and bacterial strains used. TY medium (tryptone/yeast extract) contained Bacto-tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). Minimal medium for *B. subtilis* contained: 100 mM potassium phosphate buffer at pH

7.0, glucose (1%), trisodium citrate (3 mM), MgSO<sub>4</sub> (3 mM), casamino acids (0.1%), potassium glutamate (0.2%), tryptophan (20 µg/ml), histidine (20 µg/ml), methionine (20 µg/ml), tyrosine (20 µg/ml), adenine (20 µg/ml), uracil (20 µg/ml), nicotinic acid (0.4 µg/ml), riboflavin (0.4 µg/ml), and Fe-ammonium citrate (1.1 µg/ml). S7 media 1 and 3, used for labeling of *B. subtilis* proteins with [<sup>35</sup>S]methionine (Amersham), were prepared as described by van Dijl et al. (1990a, b). If required, medium for *E. coli* was supplemented with ampicillin (Ap, 50 µg/ml), erythromycin (Em, 100 µg/ml), or kanamycin (Km, 40 µg/ml); media for *B. subtilis* were supplemented with Cm (5 µg/ml), Em (2 µg/ml), Km (10 µg/ml), or spectinomycin (Sp, 100 µg/ml).

#### DNA and RNA techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook et al. (1989). Enzymes were from Boehringer Mannheim. *B. subtilis* was transformed by growth in minimal medium until an OD<sub>600</sub> of ±1, the subsequent addition of plasmid or chromosomal DNA to the culture, and continued incubation for at least 4 hr. Multiple *sip* mutants were constructed by transforming single mutants with chromosomal or linearized plasmid DNA containing a disrupted *sip* gene. To minimize the number of antibiotic resistance markers in the cells, triple *sip*-mutants were prepared from double *sip*-mutants containing no, or only one, antibiotic resistance marker. Correct integration of linearized DNA fragments, or plasmids in the chromosome of *B. subtilis* was verified by Southern hybridization. PCR was carried out with Vent DNA polymerase (New England Biolabs) as described by van Dijl et al. (1995). DNA and protein sequences were analyzed by use of version 6.7 of the PCGene Analysis Program (Intelligenetics Inc.). The BLAST algorithm (Altschul et al. 1997) was used for protein comparisons in GenBank. RNA was isolated with the RNeasy total RNA kit from Qiagen. RNA reference markers were from GIBCO BRL Life Technologies Inc. Northern hybridizations were performed as described by Bolhuis et al. (1996).

Plasmid pKTH10-BT was constructed in three steps. First, the *amyQ* gene was amplified by PCR with the primers amyQ5' (5'-GGCCTAGGATCCTAAGAGAGGGAGAGGAAACATG-AT-3') and amyQ3' (5'-GGCCATGTCGACAGTACTGCATGCTTATCTAGATTTCTGAACATAAATGGAGAC-3') using pKTH10 as a template. The amplified fragment was cleaved with *Bam*HI and *Sal*I, and cloned into the corresponding sites of pBR322, resulting in pBR322*amyQ*. Second, the sequences specifying the biotinylation domain (PSBT) of the *P. shermanii* transcarboxylase 1.3 S subunit were amplified by PCR with the primers PSBT1 (5'-GGCCATGAGCTCTCTAGAGTCCGG-GTAAGGCCCGGAGAG-3') and PSBT2 (5'-GAATTCGCATGCGCGTCAGCCGATCTTGATGAGACC-3') using plasmid pCY66 (Cronan 1990) as a template. The amplified fragment was cleaved with *Xba*I and *Sph*I, and cloned into the corresponding sites of pBR322*amyQ*, resulting in pBR322*amyQ*-BT. Third, the *amyQ*-PSBT gene fusion was amplified by PCR with the primers amyQ-BT5' (5'-GGCCATAAGCTTTAATCAA-TCCGTGTTTGTATGTTCC-3') and amyQ-BT3' (5'-GGCCA-TGGATCCGCGTCAGCCGATCTTGATGAGACC-3'), using pBR322*amyQ*-BT as a template. The amplified fragment was cleaved with *Hind*III and *Bam*HI, and ligated into the corresponding sites of pKTH10, resulting in pKTH10-BT.

#### Plate assay for the processing of the (A13i)-β-lactamase precursor

The plate assay for the processing of the hybrid precursor

pre(A13i)-β-lactamase was carried out as described by van Dijl et al. (1992).

#### Pulse-chase protein labeling, immunoprecipitation, SDS-PAGE, and fluorography

Pulse-chase labeling of *B. subtilis*, immunoprecipitation, SDS-PAGE, and fluorography were performed as described previously (van Dijl et al. 1991a, b). <sup>14</sup>C-Methylated molecular weight markers were from Amersham International. Relative amounts of precursor and mature forms of secreted proteins were estimated by film scanning with an LKB ultrascan XL laser densitometer.

#### Western blot analysis

Western blotting was performed as described by Kyhse-Andersen (1984). After separation by SDS-PAGE, proteins were transferred to Immobilon-PVDF membranes (Millipore Corporation). To detect the α-amylase AmyQ, (A13i)-β-lactamase, or SipS, *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 by van Dijl et al. (1991a). AmyQ, (A13i)-β-lactamase, or SipS were visualized with specific antibodies and HRP anti-rabbit IgG conjugates (Amersham International). A streptavidin-HRP conjugate (Amersham International) was used to detect biotinylated AmyQ-PSBT.

#### β-Galactosidase activity assay

Overnight cultures were diluted 100-fold in fresh medium and samples were taken at hourly intervals for OD<sub>600</sub> readings and β-galactosidase activity determinations. The assay and the calculation of β-galactosidase units (expressed as units per OD<sub>600</sub>) were carried out as described by Miller et al. (1982).

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