

## Immunity to the Bacteriocin Sublancin 168 Is Determined by the SunI (YolF) Protein of *Bacillus subtilis*<sup>∇</sup>

Jean-Yves F. Dubois,<sup>†</sup> Thijs R. H. M. Kouwen,<sup>†</sup> Anna K. C. Schurich, Carlos R. Reis, Hendrik T. Ensing, Erik N. Trip, Jessica C. Zweers, and Jan Maarten van Dijk\*

Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, Hanzplein 1, P.O. Box 30001, 9700 RB Groningen, The Netherlands

Received 6 September 2008/Returned for modification 14 November 2008/Accepted 24 November 2008

***Bacillus subtilis* strain 168 produces the extremely stable lantibiotic sublancin 168, which has a broad spectrum of bactericidal activity. Both sublancin 168 production and producer immunity are determined by the SP $\beta$  prophage. While the *sunA* and *sunT* genes for sublancin 168 production have been known for several years, the genetic basis for sublancin 168 producer immunity has remained elusive. Therefore, the present studies were aimed at identifying an SP $\beta$  gene(s) for sublancin 168 immunity. By systematic deletion analysis, we were able to pinpoint one gene, named *yolF*, as the sublancin 168 producer immunity gene. Growth inhibition assays performed using plates and liquid cultures revealed that YolF is both required and sufficient for sublancin 168 immunity even when heterologously produced in the sublancin-sensitive bacterium *Staphylococcus aureus*. Accordingly, we propose to rename *yolF* to *sunI* (for sublancin immunity). Subcellular localization studies indicate that the SunI protein is anchored to the membrane with a single N-terminal membrane-spanning domain that has an N<sub>out</sub>-C<sub>in</sub> topology. Thus, the bulk of the protein faces the cytoplasm of *B. subtilis*. This topology has not yet been reported for known bacteriocin producer immunity proteins, which implies that SunI belongs to a novel class of bacteriocin antagonists.**

Lantibiotics are small posttranslationally modified peptides with antimicrobial activity and are produced by gram-positive bacteria (7, 31, 44). In general, this class of bacteriocins is characterized by the presence of the unusual dehydrated amino acids 2,3-didehydroalanine (Dha) and/or 2,3-didehydrobutyryne (Dhb). With neighboring cysteine residues, Dha and Dhb can form thioether-linked lanthionine and 3-methyl-lanthionine bridges, respectively (15, 35).

Two major types of lantibiotics have been previously identified (21). Type A lantibiotics such as nisin (26, 47), epidermin (40), and Pep5 (34) are flexible, elongated, amphipathic molecules with a positive charge. They usually act by forming pores in the cytoplasmic membrane of a sensitive target organism in processes that may involve other molecules such as the cell wall precursor lipid II (3, 55). In contrast, type B lantibiotics such as cinnamycin (14) and mersacidin (8) are globular, conformationally defined peptides that inhibit enzyme functions. Type A lantibiotics are further subdivided into type AI and AII lantibiotics on the basis of their structures; type AI lantibiotics are linear whereas type AII lantibiotics are globular at the C-terminal region. Type A lantibiotics are usually synthesized with an N-terminal leader peptide. Subsequently, they are translocated across the membrane by an ABC transporter. During membrane translocation, the leader peptide is cleaved either by a protease domain of the ABC transporter or by a separate protease (15). The leader sequences are thought to

prevent lantibiotic activation prior to membrane translocation (6, 53).

The sequenced *Bacillus subtilis* 168 strain is known to produce an extremely stable lantibiotic, named sublancin 168, which exhibits bactericidal activity against other gram-positive bacteria, including important pathogens such as *Bacillus cereus*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (38, 49). Sublancin 168 has been classified as a type AII lantibiotic, although it displayed the, for lantibiotics, extraordinary characteristic of having two disulfide bonds in addition to a  $\beta$ -methyl-lanthionine bridge (38). The gene encoding sublancin 168, named *sunA*, was identified by sequencing the SP $\beta$  prophage region of the *B. subtilis* 168 chromosome (29). *SunA* is transcribed into a monocistronic mRNA (46). An operon of four successive genes (*sunT*, *bdbA*, *yolJ*, and *bdbB*) was found to be located downstream of *sunA* (46). The *sunT* gene, immediately downstream of *sunA*, encodes a bifunctional ABC transporter with an ATP-binding cassette domain and a proteolytic domain (31). SunT is indispensable for sublancin 168 production. This ABC transporter is therefore thought to be required for sublancin 168 export from the cytoplasm and concomitant removal of the leader peptide (10). The *bdbA* and *bdbB* genes encode thiol-disulfide oxidoreductases. Whereas BdbA is dispensable for sublancin 168 production, BdbB is of major importance for this process (2, 10, 24). A possible role of the *yolJ* gene in sublancin 168 production has not yet been documented.

Any bacterium producing a bacteriocin must be immune to its bactericidal activity. To date, two general mechanisms for bacteriocin producer immunity have been reported. Firstly, dedicated ABC transporters of the LanFEG type can actively pump bacteriocins out of the membrane, thereby preventing their accumulation to toxic levels (32, 40, 43). Secondly, the bacterial cell can employ dedicated small producer immunity

\* Corresponding author. Mailing address: Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, Hanzplein 1, P.O. Box 30001, 9700 RB Groningen, The Netherlands. Phone: 31-50-3615187. Fax: 31-50-3619105. E-mail: J.M.van.Dijk@med.umcg.nl.

<sup>†</sup> J.-Y.F.D. and T.R.H.M.K. contributed equally to the work.

<sup>∇</sup> Published ahead of print on 1 December 2008.

proteins of the LanI type that are usually weakly associated with the extracytoplasmic membrane surface. Such immunity proteins bind specific lantibiotics to intercept them before they can cause cell damage (20, 50, 51). An alternative type of producer immunity protein, NukH, was more recently described (36, 37). Although the function of NukH resembles that of LanI, its topology is very different, since NukH is a membrane protein with three transmembrane domains. In addition to these active immunity mechanisms, cells can also achieve resistance to lantibiotics by modifying the charge of the cell wall or cytoplasmic membrane. For example, the D-alanylation of teichoic acids or the lysinylation of phospholipids will make the cell wall or membrane, respectively, more positively charged (39, 41). As a consequence, bacterial cells with such modifications will be more resistant to cationic bacteriocins than cells lacking these modifications.

Recent studies by Butcher and Helmann have shown that the *yqeZ* and *yqfAB* genes of the  $\sigma^W$  regulon confer resistance to sublancin 168 (5). However, full producer immunity to sublancin 168 is known to require gene functions of the SP $\beta$  prophage (18), while none of the  $\sigma^W$  regulon genes implicated in sublancin 168 resistance are located on this prophage. Thus, it has remained unclear which SP $\beta$  gene or genes are required for sublancin 168 producer immunity. Notably, our previous studies have shown that the ABC transporter SunT, the thiol-disulfide oxidoreductases BdbA and BdbB, and the YoIJ protein of unknown function are fully dispensable for sublancin 168 producer immunity (10). Moreover, none of the 187 SP $\beta$  genes show homology to known bacteriocin producer immunity genes (29).

In the present studies, we have addressed the issue of which SP $\beta$  gene or genes are required for sublancin 168 producer immunity. Our results show that only 1 of the 187 genes of the SP $\beta$  prophage, *yolF*, is both required and sufficient for immunity of *B. subtilis* to sublancin 168. We therefore propose changing the name of this gene to *sunI*. Interestingly, SunI (YoIF) seems to belong to a new class of bacteriocin producer immunity proteins.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth media.** The bacterial strains and plasmids used in this study are listed in Table 1. The LB medium consisted of 1% tryptone, 0.5% yeast extract, and 1.0% NaCl (pH 7.4). Where necessary, media were supplemented with antibiotics at the following concentrations: ampicillin (Ap), 100  $\mu$ g/ml (*Escherichia coli*); kanamycin (Km), 20  $\mu$ g/ml (*E. coli*, *B. subtilis*, and *S. aureus*); chloramphenicol (Cm), 5  $\mu$ g/ml (*E. coli* and *B. subtilis*); tetracycline (Tc), 10  $\mu$ g/ml (*E. coli* and *B. subtilis*); and erythromycin (Em), 100  $\mu$ g/ml (*E. coli*), 2  $\mu$ g/ml (*B. subtilis*), or 5  $\mu$ g/ml (*S. aureus*). To visualize  $\alpha$ -amylase activity (specified by the *amyE* gene), LB plates were supplemented with 1% starch.

**DNA techniques.** Procedures for DNA amplification, restriction, ligation, and transformation of *E. coli* DH5 $\alpha$  and TG90 were carried out according to standard laboratory procedures (45). Chromosomal DNA of *B. subtilis* was isolated according to the procedures of Bron and Venema (4). *B. subtilis* was transformed as described by Kunst and Rapoport (28). All primers used for PCR are listed in Table 2. PCR products were purified using a High Pure PCR purification kit (Roche Applied Science).

**Construction of mutant strains.** To construct *B. subtilis* mutants with deletions in the SP $\beta$  prophage, a "three-PCR protocol" was followed. First, the flanking regions of the deleted sequences were amplified by PCR. The primers representing the beginning and end points of the deleted sequences contained extensions that were complementary to the Km resistance cassette (~900 bp) from plasmid pDG783. This cassette was amplified by PCR with the primers Kana1 and Kana2. Importantly, these primers were also complementary to extensions in

the primers that represent the beginning and end points of deleted genomic sequences. In a subsequent PCR, the amplified flanking regions and the amplified Km resistance cassette were fused. For this purpose, the three PCR-amplified fragments were purified and mixed in equal amounts (100 ng) in a PCR mixture that also contained the distal primers of the amplified flanking regions. After 10 cycles with an optimal annealing temperature for Kana1 and Kana2, the annealing temperature was increased to the optimum for the distal primers and the PCR was continued for 20 cycles. Next, *B. subtilis* was transformed with the Km cassette fused on both sides to the amplified flanking regions, and Km-resistant transformants were selected on plates. The replacement of genomic sequences by the Km resistance cassette in these transformants was verified by PCR.

To construct the ANC1 mutant, an approximately 1,200-bp fragment downstream of the *bdbB* gene was amplified using primers ASP1 and CRP2b. Next, an approximately 900-bp fragment downstream of the SP $\beta$  prophage was amplified using primers CRP3 and ASP4. Both fragments were fused by PCR to the Km resistance cassette, and the resulting product (approximately 3,000 bp) was used to transform competent *B. subtilis* 168 cells, resulting in the ANC1 mutant.

To construct the ANC2 mutant, an approximately 900-bp fragment downstream of the *yolC* gene was amplified using the CRP5 and ASP6 primers. The 5' sequence of the CRP5 primer was complementary to the Kana2 primer. After purification, this PCR fragment was mixed with the fragment obtained with primers ASP1 and CRP2b (see above) and the amplified Km resistance cassette. The three fragments were merged in a single PCR, and the resulting fragment (approximately 3,000 bp) was purified and used to transform *B. subtilis* 168, resulting in the ANC2 mutant.

To construct the ANC3 mutant, a 900-bp fragment upstream of the *yolF* gene was amplified using the pYF1 and pYF2 primers. The 5' sequence of the pYF1 primer was complementary to the Kana2 primer sequence. The amplified fragment was purified and mixed with the fragment obtained by PCR with the ASP1 and CRP2b primers and the amplified Km resistance cassette. The three fragments were merged by PCR and, after purification, ligated to plasmid pUC18 cleaved with HincII. The resulting plasmid, pUC-anc3-Km<sup>r</sup>-pYF, was used to transform competent *B. subtilis* cells, resulting in *B. subtilis* ANC3. The double-crossover integration of the Km resistance cassette into the chromosome was verified by PCR using the Kana1 and ASP6 primers.

To construct *B. subtilis*  $\Delta$ *sunA*- $\Delta$ *yolF*, an approximately 900-bp fragment downstream of the *sunA* gene was amplified using the pSU1 and pSU2 primers. The 5' sequence of the pSU2 primer was complementary to the Kana1 primer. After purification, this PCR fragment was mixed with the PCR fragment obtained by PCR with the pYF1 and pYF2 primers and the PCR-amplified Km resistance cassette. The resulting fragment (approximately 3,000 bp) was then ligated to HincII-cleaved pUC18. The resulting plasmid, pUC-pSu-Km<sup>r</sup>-pYF, was used to transform competent *B. subtilis* cells, resulting in the  $\Delta$ *sunA*- $\Delta$ *yolF* strain. Double-crossover chromosomal integration of the Km cassette was verified by PCR using the Kana1 and ASP6 primers.

*B. subtilis*  $\Delta$ *sunA* was constructed by transforming *B. subtilis* 168 with genomic DNA of the *B. subtilis* JH642 *sunA::Km* strain (laboratory strain HB61664) (5) and selection of Km-resistant transformants.

The plasmids pGDL-*yolF* and pGDL-*yolF*<sup>C</sup> were constructed as follows. The *yolF* gene, including its ribosomal binding site but lacking the promoter sequences, was amplified using the Rel1fw and Re2rvb primers. Both primers contained EcoRI restriction sites. The amplified fragment was ligated into the pCR-BluntII TOPO plasmid (Invitrogen, Inc.), resulting in the pCR-*yolF* plasmid. After EcoRI excision of *yolF* from pCR-*yolF* plasmid, the gene was ligated to the EcoRI-cleaved plasmid pGDL48. Thus, two plasmids were obtained that contained *yolF* in opposite orientations. In plasmid pDGL-*yolF*, the *yolF* gene is under the transcriptional control of the constitutive promoter of the truncated Em resistance gene of pGDL48. Plasmid pGDL-*yolF*<sup>C</sup> contains *yolF* in the opposite orientation; consequently, the promoterless *yolF* gene of this plasmid cannot be transcribed. The orientation and sequences of the inserts were checked by sequencing.

The *B. subtilis* 168 Cm strain was constructed by transformation of *B. subtilis* 168 with plasmid pX and subsequent selection of transformants in which the Cm marker was integrated into the *amyE* gene via a double-crossover recombination event. The amylase-negative phenotype of the Cm-resistant transformants was checked using starch-containing plates. The *B. subtilis*  $\Delta$ SP $\beta$  Tc and *B. subtilis*  $\Delta$ SP $\beta$  Tc pGDL-*yolF* strains were constructed by transformation of *B. subtilis*  $\Delta$ SP $\beta$  or *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF* with the plasmid pXTC. Subsequently, transformants were selected in which the Tc marker was integrated into the *amyE* gene via a double-crossover recombination event. The amylase-negative phenotype of Tc-resistant transformants was checked on starch-containing plates.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	F <sup>-</sup> , $\phi$ 80d <i>lacZ</i> $\Delta$ <i>M15 endA1 recA1 gyrA96 thi-1 hsdR17</i> ( <i>r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup></i> ) <i>supE44 relA1 deoR</i> $\Delta$ ( <i>lacZYA-argF</i> ) U169	Invitrogen Life Technologies, Inc.
TG90	Derivative of the TG1 strain which carries the <i>pcnB80</i> mutation and replicates plasmids at a low copy no.	30
<i>B. subtilis</i>		
168	<i>trpC2</i>	27
168 Cm	<i>trpC2</i> ; <i>amyE</i> ::pX; Cm <sup>r</sup>	This work
$\Delta$ SP $\beta$	<i>trpC2</i> ; $\Delta$ SP $\beta$ ; sublancin 168 sensitive	10
$\Delta$ SP $\beta$ Tc	<i>trpC2</i> ; $\Delta$ SP $\beta$ ; <i>amyE</i> ::pXTC; sublancin 168 sensitive; Tc <sup>r</sup>	This work
ANC1	<i>trpC2</i> ; $\Delta$ <i>yokA</i> ; $\Delta$ <i>yokB</i> ; $\Delta$ <i>yokC</i> ; $\Delta$ <i>yokD</i> ; $\Delta$ <i>yokE</i> ; $\Delta$ <i>yokF</i> ; $\Delta$ <i>yokG</i> ; $\Delta$ <i>yokH</i> ; $\Delta$ <i>yokI</i> ; $\Delta$ <i>yokJ</i> ; $\Delta$ <i>yokK</i> ; $\Delta$ <i>yokL</i> ; $\Delta$ <i>yolA</i> ; $\Delta$ <i>yolB</i> ; $\Delta$ <i>yolC</i> ; $\Delta$ <i>yolD</i> ; $\Delta$ <i>uvrX</i> ; $\Delta$ <i>yolF</i> ; $\Delta$ <i>sunA</i> ; $\Delta$ <i>sunT</i> ; $\Delta$ <i>bdbA</i> ; $\Delta$ <i>yolJ</i> ; $\Delta$ <i>bdbB</i> ; Km <sup>r</sup>	This work
ANC2	<i>trpC2</i> ; $\Delta$ <i>yolC</i> ; $\Delta$ <i>yolD</i> ; $\Delta$ <i>uvrX</i> ; $\Delta$ <i>yolF</i> ; $\Delta$ <i>sunA</i> ; $\Delta$ <i>sunT</i> ; $\Delta$ <i>bdbA</i> ; $\Delta$ <i>yolJ</i> ; $\Delta$ <i>bdbB</i> ; Km <sup>r</sup>	This work
ANC3	<i>trpC2</i> ; $\Delta$ <i>yolF</i> ; $\Delta$ <i>sunA</i> ; $\Delta$ <i>sunT</i> ; $\Delta$ <i>bdbA</i> ; $\Delta$ <i>yolJ</i> ; $\Delta$ <i>bdbB</i> ; Km <sup>r</sup>	This work
$\Delta$ <i>sunA</i> - $\Delta$ <i>yolF</i>	<i>trpC2</i> ; $\Delta$ <i>yolF</i> ; $\Delta$ <i>sunA</i> ; Km <sup>r</sup>	This work
$\Delta$ <i>sunA</i>	<i>trpC2</i> ; $\Delta$ <i>sunA</i> ; Km <sup>r</sup>	This work
$\Delta$ SP $\beta$ pGDL- <i>yolF</i>	<i>trpC2</i> ; $\Delta$ SP $\beta$ ; contains pDGL- <i>yolF</i> , which allows for constitutive expression and translation of <i>yolF</i> ; Km <sup>r</sup>	This work
$\Delta$ SP $\beta$ Tc pGDL- <i>yolF</i>	<i>trpC2</i> ; $\Delta$ SP $\beta$ ; <i>amyE</i> ::pXTC; contains pDGL- <i>yolF</i> , which allows for constitutive expression and translation of <i>yolF</i> ; Km <sup>r</sup> ; Tc <sup>r</sup>	This work
$\Delta$ SP $\beta$ pGDL- <i>yolF</i> <sup>C</sup>	<i>trpC2</i> ; $\Delta$ SP $\beta$ ; contains pDGL- <i>yolF</i> <sup>C</sup> which does not express <i>yolF</i> ; Km <sup>r</sup>	This work
<i>S. aureus</i>		
RN4220	Restriction-deficient derivative of NCTC 8325; cured of all known prophages	25
RN4220 pGDL- <i>yolF</i>	RN4220 that contains pDGL- <i>yolF</i> for constitutive expression of <i>yolF</i> ; Km <sup>r</sup>	This work
RN4220 Em	RN4220 that contains the pMAD vector for Em resistance; Em <sup>r</sup>	This work
<b>Plasmids</b>		
pDG783	pSB118 derivative; contains the Km resistance marker from <i>Streptococcus faecalis</i> ; Ap <sup>r</sup> ; Km <sup>r</sup>	16
pUC18	Ap <sup>r</sup> ; ColE1; $\phi$ 80 <i>lacZ</i> ; <i>lac</i> promoter	45
pUC-anc3-Km <sup>r</sup> -pYF	pUC18 derivative; contains the fragment anc3-Km <sup>r</sup> -pYF in the multiple cloning site of the plasmid	This work
pUC-pSU-Km <sup>r</sup> -pYF	pUC18 derivative; contains the fragment pSU-Km <sup>r</sup> -pYF in the multiple cloning site of the plasmid	This work
pCR-BluntII TOPO	Km <sup>r</sup> ; Zeocin <sup>r</sup> ; <i>lac</i> promoter; <i>ccdB</i> lethal gene for <i>E. coli</i> is disrupted after blunt ligation of insert	Invitrogen Life Technologies, Inc.
pCR- <i>yolF</i>	pCR-BluntII TOPO derivative; contains the <i>yolF</i> gene	This work
pGDL48	pSB118 derivative; contains the Km resistance marker from <i>Streptococcus faecalis</i> ; Ap <sup>r</sup> ; Km <sup>r</sup>	33
pGDL- <i>yolF</i>	pGDL48 derivative; contains an expressed copy of the <i>yolF</i> gene	This work
pGDL- <i>yolF</i> <sup>C</sup>	pGDL48 derivative; contains a nonexpressed copy of the <i>yolF</i> gene	This work
pX	Vector for the integration of genes in the <i>amyE</i> locus of <i>B. subtilis</i> ; integrated genes are transcribed from the xylose-inducible <i>xyIA</i> promoter; carries the <i>xyIR</i> gene; Ap <sup>r</sup> ; Cm <sup>r</sup>	22
pXTC	pX derivative in which the Cm resistance marker has been replaced with a Tc resistance marker; Ap <sup>r</sup> ; Tc <sup>r</sup>	9
pMAD	Shuttle vector for <i>E. coli</i> and <i>S. aureus</i> ; contains the <i>bgaB</i> gene; Em <sup>r</sup> ; Ap <sup>r</sup>	1

**Sublancin 168 activity assay.** A sublancin 168-induced *B. subtilis* growth inhibition assay was performed on plates essentially as described by Dorenbos et al. (10). Briefly, indicator strains and strains to be tested for sublancin 168 production were grown overnight in LB broth containing the appropriate antibiotic(s). Overnight cultures of the indicator strains were then diluted 100-fold in LB, and 100- $\mu$ l aliquots of the diluted cultures were plated on LB agar. After drying of the plates, 2- $\mu$ l aliquots of undiluted overnight cultures of strains to be tested for sublancin 168 production were spotted onto the plates. The plates were then incubated overnight at 37°C, and growth inhibition of the indicator strain was analyzed the next day.

**Spent-medium growth experiments.** *B. subtilis* 168 was grown in LB medium overnight. Cells were removed by centrifugation (4,000  $\times$  g for 10 min), and the supernatant was filtered with a 0.45- $\mu$ m-pore-size filter. The obtained spent medium was supplemented with 10 $\times$  LB medium and sterile demineralized water to reach the desired spent-medium percentage. Supplementation of the spent media with 10 $\times$  LB medium was necessary to achieve growth of *B. subtilis* in the spent media.

**Coculturing of *B. subtilis* and *S. aureus* strains.** *B. subtilis* 168 Cm, *B. subtilis*  $\Delta$ SP $\beta$  Tc, and *B. subtilis*  $\Delta$ SP $\beta$  Tc pGDL-*yolF* were grown overnight as separate cultures in LB medium. In the morning, cultures were diluted to an optical density at 600 nanometers (OD<sub>600</sub>) of 0.05 in fresh LB medium and mixed in a 1:1 ratio, resulting in cocultures consisting of 50% *B. subtilis* 168 Cm and 50% of either *B. subtilis*  $\Delta$ SP $\beta$  Tc or *B. subtilis*  $\Delta$ SP $\beta$  Tc pGDL-*yolF*. Upon mixing, growth was continued for 8 h. Samples for plating were taken at hourly intervals during growth. The samples thus obtained were diluted 10<sup>4</sup>- or 10<sup>6</sup>-fold and plated on LB agar containing either Cm or Tc. After overnight incubation at 37°C, Cm- and Tc-resistant colonies were counted, and numbers of CFU per milliliter of culture of each strain at the time of sampling were calculated.

The same procedure was applied for coculturing of *B. subtilis* 168 Cm with either *S. aureus* RN4220 Em or *S. aureus* RN4220 pGDL-*yolF*. In this case, the coculture samples were plated on LB agar containing Cm, Em, or Km.

**SDS-PAGE and Western blotting analyses.** The presence of YolF, LipA, thioredoxin A (TrxA), SipS, and BdbD in cell lysates or subcellular fractions was assayed by Western blotting analysis using specific polyclonal antibodies. For this



TABLE 2. Primers used in this study

Primer	Sequence (5'-3') <sup>a</sup>
Kana1	.....GTGAATTGGAGTTCGCTCTTG
Kana2	.....TATGGACAGTTGCCGATGTA
ASP1	.....GCTTCTCCAATAAACCAAC
CRP2b	.....CAAGACGAACTCCAATTCACATAAAGAAGTAACCCG CCTTG
CRP3	.....TACATCCGCAACTGTCCATAAAGCCTGTCTATCCA TTAGG
ASP4	.....TACTGAAAACCTACGTACG
CRP5	.....TACATCCGCAACTGTCCATAGCCCAGCTCTTTATTT AAGC
ASP6	.....ACTTGTACCAAGGAGGATTTAG
pYF1	.....TACATCCGCAACTGTCCATAGATTATCATAACTAC ATATTCAT
pYF2	.....GCTACTCAGTAAGCTTGCAC
pSU1	.....ATATATACCATCATTGAATCGAGA
pSU2	.....CAAGACGAACTCCAATTCACAAAACATATCGTCAAT TCTGCAGA
Re1fw	.....GGGGGGAATTCACATAAGAAAGAGTGATTATAT GG
Re2rvb	.....GGGGGGAATTCACCTTCTATTGTAAGAAGGTACT

<sup>a</sup> The 5' sequences of primers CRP2b and pSU2 (italics) are complementary to the Kana1 primer. The 5' sequences of primers CRP3, CRP5, and pYF1 (italics) are complementary to the Kana2 primer. Primers Re1fw and Re2rvb contain EcoRI restriction sites (underlined).

purpose, cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (using precast NuPAGE gels from Invitrogen) and then semidry blotted (1.25 h at 100 mA per gel) onto nitrocellulose membranes (Roche Molecular Biochemicals). Specific antibodies against YoIF, BdbD, or TrxA of *B. subtilis* were raised by immunization of rabbits (Eurogentec, Belgium) with C-terminally His<sub>6</sub>-tagged variants of these proteins, overproduced in *E. coli*. The overproduction and metal-affinity purification of these proteins were done essentially as described previously (24). The detection of bound antibodies was performed with fluorescent immunoglobulin G secondary antibodies (IRDye 800 CW-conjugated goat anti-rabbit from LiCor Biosciences) in combination with the Odyssey infrared imaging system (LiCor Biosciences). Fluorescence was recorded at 800 nm.

**Subcellular localization of YoIF.** Fractionation experiments were performed to localize the YoIF protein and the control proteins LipA, TrxA, SipS, and BdbD in *B. subtilis*. Cells were grown overnight in LB medium, collected by centrifugation, and resuspended in protoplast buffer (100 mM Tris-HCl [pH 8.2], 20 mM MgCl<sub>2</sub>, 20% sucrose, 1 mg/ml lysozyme, 0.01% DNase, and Complete protease inhibitors). After 30 min of incubation at 37°C, proteins released from the cells by protoplasting (i.e., the cell wall fraction) were separated from the protoplasts by centrifugation (10 min at 4,000 × *g* and 4°C). The protoplasts were resuspended in disruption buffer (50 mM Tris-HCl [pH 8.2], 2.5 mM EDTA) and disrupted using glass beads and a bead beater. Cellular debris and unbroken protoplasts were removed by centrifugation (10 min at 4,000 × *g* and 4°C), and the supernatant was ultracentrifuged (30 min at 200,000 × *g* and 4°C). Next, the supernatant fraction with the cytosolic proteins was collected. The pellet was resuspended in solubilization buffer (20 mM Tris [pH 8.0], 10% glycerol, 50 mM NaCl, 0.03% DDM [*n*-dodecyl-β-D-maltoside]) and incubated overnight at 4°C. Nonsolubilized membranes and solubilized membrane proteins were subsequently separated by centrifugation (15 min at 100,000 × *g* and 4°C), and the supernatant fraction with the solubilized membrane proteins was collected. The subcellular fractions thus obtained were analyzed by SDS-PAGE, Western blotting, and immunodetection with specific antibodies.

**Membrane topology of YoIF.** To determine the subcellular location of free thiols in the cysteine-containing proteins YoIF, BdbD, and TrxA in *B. subtilis*, the non-membrane-permeative thiol-specific cross-linking agent 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate (AMS; Molecular Probes) was used. Cells were grown overnight in LB medium, collected by centrifugation, and resuspended in protoplast buffer (20 mM potassium phosphate [pH 7.5], 15 mM MgCl<sub>2</sub>, 20% sucrose, 1 mg/ml lysozyme) with or without 15 mM AMS. Protoplasting and AMS labeling were performed for 20 min at room temperature. Next, the protoplasts were washed twice in protoplast buffer to remove unbound AMS. In parallel, protoplasting was carried out in the presence of 15 mM AMS and 1% Triton X-100, resulting in protoplast lysis and labeling of all proteins

containing reduced cysteine residues. To separate proteins with and without bound AMS, all samples were analyzed by nonreducing SDS-PAGE, Western blotting, and immunodetection with specific antibodies. AMS binding resulted in apparent mass increases of 0.5 kDa per bound AMS molecule.

## RESULTS

### YoIF is indispensable for sublancin 168 producer immunity.

To identify which gene(s) on the SPβ prophage would confer sublancin 168 producer immunity, growth inhibition assays were performed in which strains potentially producing sublancin 168 were spotted onto a lawn of sensitive or immune indicator cells (Fig. 1). The applicability of this assay was demonstrated in the following series of baseline experiments. First, *B. subtilis* 168 was used both as an indicator strain and as a producing strain. No zone of growth inhibition was formed around the spotted *B. subtilis* 168 cells, confirming that this strain is resistant to the sublancin 168 it produces (Fig. 1A). Next, we confirmed that the *B. subtilis* ΔSPβ strain was not able to grow in the vicinity of the sublancin 168-producing parental 168 strain (Fig. 1A). In this case, a clear zone of growth inhibition was visible around the spot of *B. subtilis* 168 cells. Additionally, using a Δ*sunA* strain, we confirmed with that growth inhibition of the plated ΔSPβ strain was strictly dependent on the presence of an intact copy of the *sunA* gene for sublancin 168 in the spotted cells (Fig. 1A). Conversely, the producer immunity to sublancin 168 did not depend on the presence of the *sunA* gene, as no zone of growth inhibition was visible when *B. subtilis* Δ*sunA* was used as an indicator strain and the parental strain 168 was used as a sublancin 168-producing strain (Fig. 1A). Taken together, these findings demonstrate that the *sunA* gene is responsible for the observed growth inhibition of cells lacking the SPβ prophage and that this gene does not play a role in sublancin 168 producer immunity. Furthermore, these findings also imply that a mutant strain lacking the gene(s) responsible for sublancin 168 immunity will be viable only in a Δ*sunA* background. For this reason, all mutants that were constructed to identify determinants for sublancin producer immunity also lacked the *sunA* gene.

As a first approach for identification of the sublancin 168 immunity gene(s), two deletion mutants named ANC1 and ANC2 were constructed. These strains lacked, respectively, 23 and 9 SPβ genes, including the sublancin 168 locus. The sublancin 168 sensitivity of the ANC1 and ANC2 strains was tested by using them as indicator strains and the parental *B. subtilis* 168 strain as the sublancin 168-producing strain. As shown in Fig. 1B, neither the ANC1 strain nor the ANC2 strain was able to grow in the vicinity of *B. subtilis* 168, showing that both strains were sensitive to sublancin 168. This suggested that at least one of the nine genes deleted in the ANC2 strain was required for sublancin 168 producer immunity. As the five genes in the sublancin 168 locus (*sunA*, *sunT*, *bdbA*, *yoIJ*, and *bdbB*) were already known to be dispensable for sublancin 168 producer immunity (reference 10 and this study), we focused attention on the possible roles of the four remaining deleted genes of *B. subtilis* ANC2 (i.e., *yoIC*, *yoID*, *uvrX*, and *yoIF*) in immunity to sublancin 168. To narrow down the possibilities, BlastP analyses using the four respective amino acid sequences were performed to identify proteins of *B. subtilis* with potentially similar or overlapping functions. This revealed that YoIC

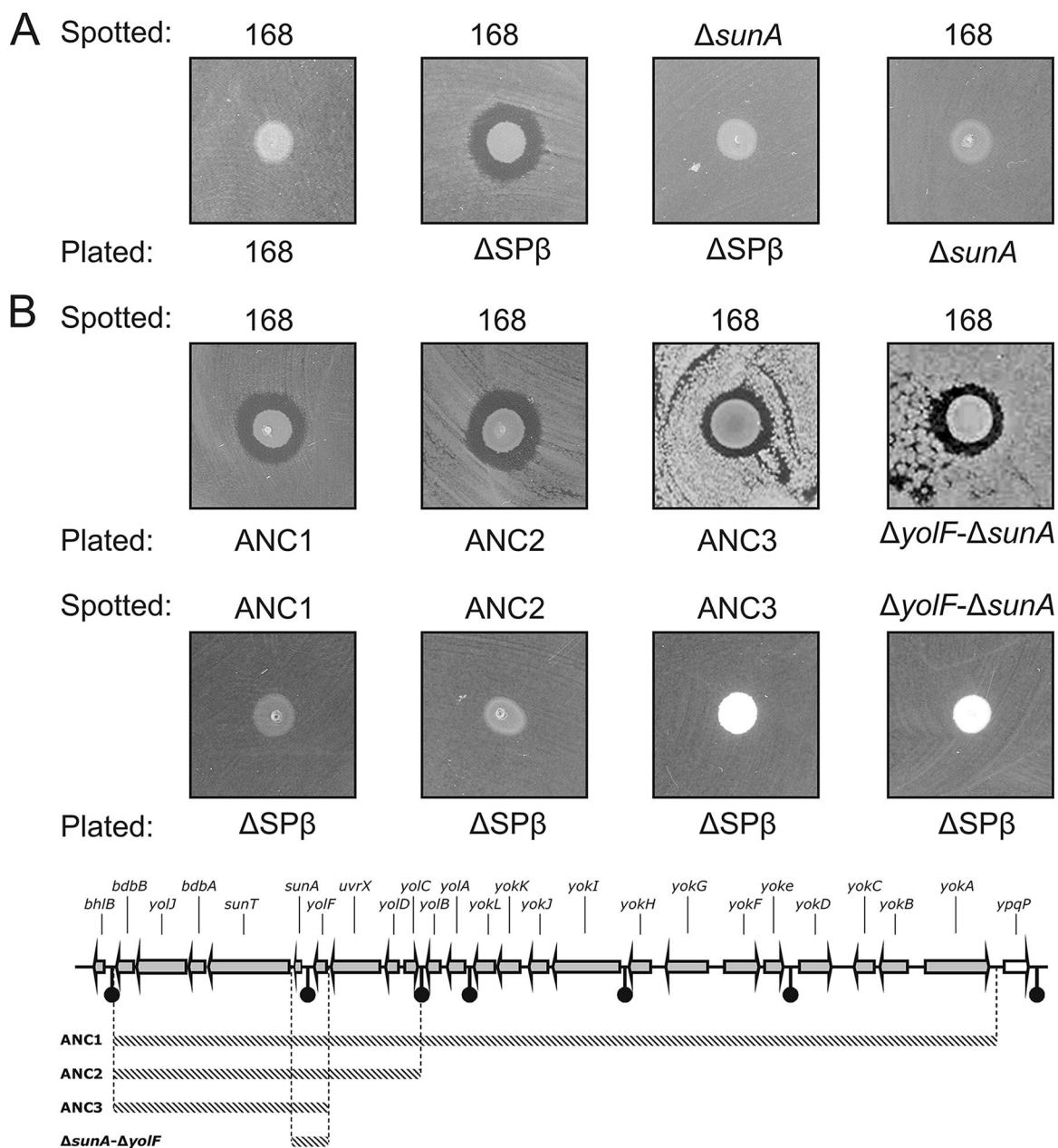


FIG. 1. Identification of the subblancin 168 immunity gene. (A) Subblancin 168 growth inhibition assay. Strains to be tested for subblancin 168 production were spotted on a lawn of indicator cells. The names of strains that were spotted to test for subblancin production are listed above the plate images. The names of the strains that were plated as indicators for subblancin sensitivity/immunity are listed below the plate images. (B) Subblancin immunity assays and schematic representation of deleted SPβ prophage genes. The names of strains that were spotted to test for subblancin production are listed above the plate images. The names of the strains that were plated as indicators for subblancin sensitivity/immunity are listed below the plate images. SPβ genes are indicated by arrows. The dashed lines indicate the respective parts of the SPβ region that were deleted to construct the ANC1, ANC2, ANC3, and  $\Delta sunA-\Delta yoIF$  strains. Potential transcriptional terminators are indicated as “balls on sticks” (46).

has a *B. subtilis* homologue, namely, YozM (91% identical residues and conservative replacements in a stretch of 111 residues), that YoID has a *B. subtilis* homologue, namely, YozL (92% identical residues and conservative replacements in 97 residues), and that UvrX has three *B. subtilis* homologues, namely, YobH (98% identical residues and conservative replacements in 201 residues), YqjW (59% identical residues and conservative replacements in 414 residues), and YozK (98%

identical residues and conservative replacements in 115 residues). In contrast, no protein with a high degree of similarity to YoIF was identified. The protein with the highest similarity to the YoIF sequence is YnzG, with both proteins sharing merely 51% identical residues and conservative replacements in a stretch of 68 residues. It is, however, noteworthy that the *ynzG* gene lies in an operon containing a gene for a delta endotoxin homologue, suggesting a potential role in the handling of this

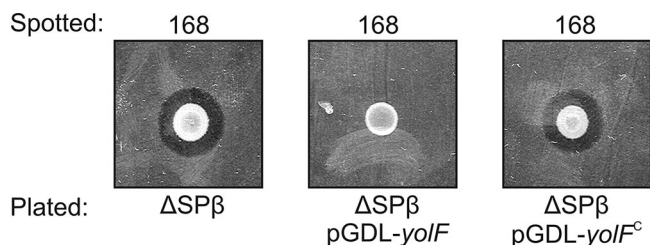


FIG. 2. YoIF confers subclancin 168 immunity to an SP $\beta$ -free *B. subtilis* strain. *B. subtilis* 168 was spotted on the plates for subclancin 168 production. The names under the pictures refer to the indicator strains plated to monitor subclancin 168 immunity.

toxin. We therefore focused attention on a possible role of *yolF* in subclancin 168 producer immunity. For this purpose, we constructed the *B. subtilis* ANC3 strain by deletion of the five genes of the subclancin locus plus *yolF* as well as the *B. subtilis*  $\Delta$ *sunA*- $\Delta$ *yolF* strain. As shown in Fig. 1B, neither the ANC3 strain nor the  $\Delta$ *sunA*- $\Delta$ *yolF* strain was able to grow in the vicinity of the *B. subtilis* 168 strain (Fig. 1B), showing that both strains are sensitive to the presence of subclancin 168. Furthermore, and consistent with the applied approach of nested gene deletions, the ANC1, ANC2, ANC3, and  $\Delta$ *sunA*- $\Delta$ *yolF* strains did not produce active subclancin 168 (Fig. 1B). Taken together, these results demonstrate that *yolF* is indispensable for the producer immunity of *B. subtilis* 168 to subclancin 168.

**YoIF is sufficient to confer subclancin 168 immunity.** Since *yolF* was identified as being necessary for subclancin 168 producer immunity, we addressed the issue of whether it is also sufficient to confer immunity to the subclancin 168-sensitive  $\Delta$ SP $\beta$  strain. Notably, the  $\Delta$ SP $\beta$  strain does not contain any genes of the SP $\beta$  prophage that could encode a YoIF partner protein involved in subclancin 168 producer immunity. Therefore, we expressed the *yolF* gene ectopically in *B. subtilis*  $\Delta$ SP $\beta$  by use of the promoter of the Em resistance gene on the *B. subtilis* pGDL48 expression vector. Genes placed under the control of this promoter are usually expressed constitutively and at moderate levels, which precludes excessive overproduction of the respective gene product. The resulting plasmid was named pGDL-*yolF*. A negative-control plasmid, containing the *yolF* gene in the opposite orientation, was named pGDL-*yolF*<sup>C</sup>. Interestingly, *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF* used as an indicator strain was fully resistant to the subclancin 168-producing 168 strain (Fig. 2). In contrast, *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF*<sup>C</sup> was as sensitive to the subclancin 168 produced by strain 168 as the *B. subtilis*  $\Delta$ SP $\beta$  strain (Fig. 2). Taken together, these results show that the *yolF* gene is not only necessary but also sufficient to confer immunity to subclancin 168.

Next, we verified these findings by growing the *B. subtilis*  $\Delta$ SP $\beta$  and  $\Delta$ SP $\beta$  pGDL-*yolF* strains and the parental 168 strain in liquid medium containing 90% of spent LB medium that was derived from an overnight culture with *B. subtilis* 168 (Fig. 3A). In this spent medium, *B. subtilis* 168 grew slightly slower than in fresh LB medium (data not shown). Importantly, the *B. subtilis*  $\Delta$ SP $\beta$  strain was unable to grow in the spent medium of *B. subtilis* 168, whereas the *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF* strain did grow in this medium. This shows that the pGDL-*yolF* plasmid confers subclancin 168 immunity to *B. subtilis*  $\Delta$ SP $\beta$ . Nevertheless, growth of the *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF* strain

on spent medium of strain 168 was slightly slower than that of the parental 168 strain. As demonstrated by Western blotting with specific antibodies against YoIF, this reduced growth rate in subclancin 168-containing medium might be due to the fact that the pGDL-*yolF* plasmid directs a slightly lower level of YoIF production than the chromosomal *yolF* gene of the parental 168 strain, especially in the exponential growth phase (Fig. 3B).

As an ultimate test for the subclancin 168 immunity function of YoIF, we performed coculturing and competition experiments in liquid medium. Firstly, the subclancin 168-producing *B. subtilis* 168 *amyE*::pX (Cm<sup>r</sup>) strain was used to inoculate growth medium in a 1:1 ratio with the nonproducing *B. subtilis*  $\Delta$ SP $\beta$  *amyE*::pXTC (Tc<sup>r</sup>) strain with or without pGDL-*yolF*. The results of cocultivation and subsequent transfer of samples to plates containing either Cm or Tc showed that the  $\Delta$ SP $\beta$  strain, which does not produce YoIF, was able to survive for only a few hours in the presence of the subclancin 168-producing strain (Fig. 4A). In contrast, the  $\Delta$ SP $\beta$  strain producing YoIF from the pGDL-*yolF* plasmid was not inhibited by the deleterious effects of the strain producing subclancin 168 (Fig. 4B). Notably, the observed growth of the *B. subtilis*  $\Delta$ SP $\beta$  pGDL-*yolF* strain was slightly slower than that of the parental 168 strain, as was observed in the experiment represented in Fig. 3. Secondly, to rule out the possibility that YoIF might require other *B. subtilis* proteins to fulfill its function in subclancin 168 immunity, we introduced the *yolF* gene into a bacterium that is naturally sensitive to subclancin 168, namely, *Staphylococcus aureus*. For this purpose, the *B. subtilis* 168 *amyE*::pX (Cm<sup>r</sup>) strain was used to inoculate growth medium in a 1:1 ratio with the *S. aureus* strain RN4220 containing either the pGDL-*yolF* plasmid (Km<sup>r</sup>) or the pMAD control plasmid (Em<sup>r</sup>). The results of these cocultivation experiments confirmed that *S. aureus* RN4220 lacking the *yolF* gene was able to survive for only a few hours in the presence of the subclancin 168-producing *B. subtilis* strain (Fig. 4C). In contrast, the introduction of pGDL-*yolF* allowed *S. aureus* RN4220 to grow in the presence of *B. subtilis* 168 (Fig. 4D). Taken together, these findings show that *yolF* is both essential and sufficient for subclancin 168 immunity.

**Localization of YoIF.** YoIF is a small basic protein (pI 9.2) of 105 amino acids (12.1 kDa). To predict the localization of YoIF, we first employed the SignalP 3.0 algorithm that identifies potential signal peptides for protein export from the cytoplasm (<http://www.cbs.dtu.dk/services/>). Although use of both neural network (NN) and hidden Markov model (HMM) algorithms with SignalP 3.0 software resulted in a positive signal peptide prediction, the signal peptidase cleavage sites predicted by these algorithms differed (NN, cleavage between amino acids 21 and 22; HMM, cleavage between amino acids 26 and 27). Furthermore, the predicted signal peptidase cleavage sites in YoIF (NN, VFL-N; HMM, RYS-F) differed considerably from the consensus signal peptidase cleavage sites in known signal peptides of *B. subtilis* (AXA-A) (52). It therefore appeared more likely that YoIF would be synthesized with an N-terminal transmembrane domain. Accordingly, procedures to produce predictions for transmembrane domains were conducted using the Topcons server (<http://topcons.net>). The SCAMPI, PRODIV, and PRO algorithms identified one potential N-terminal transmembrane domain between amino



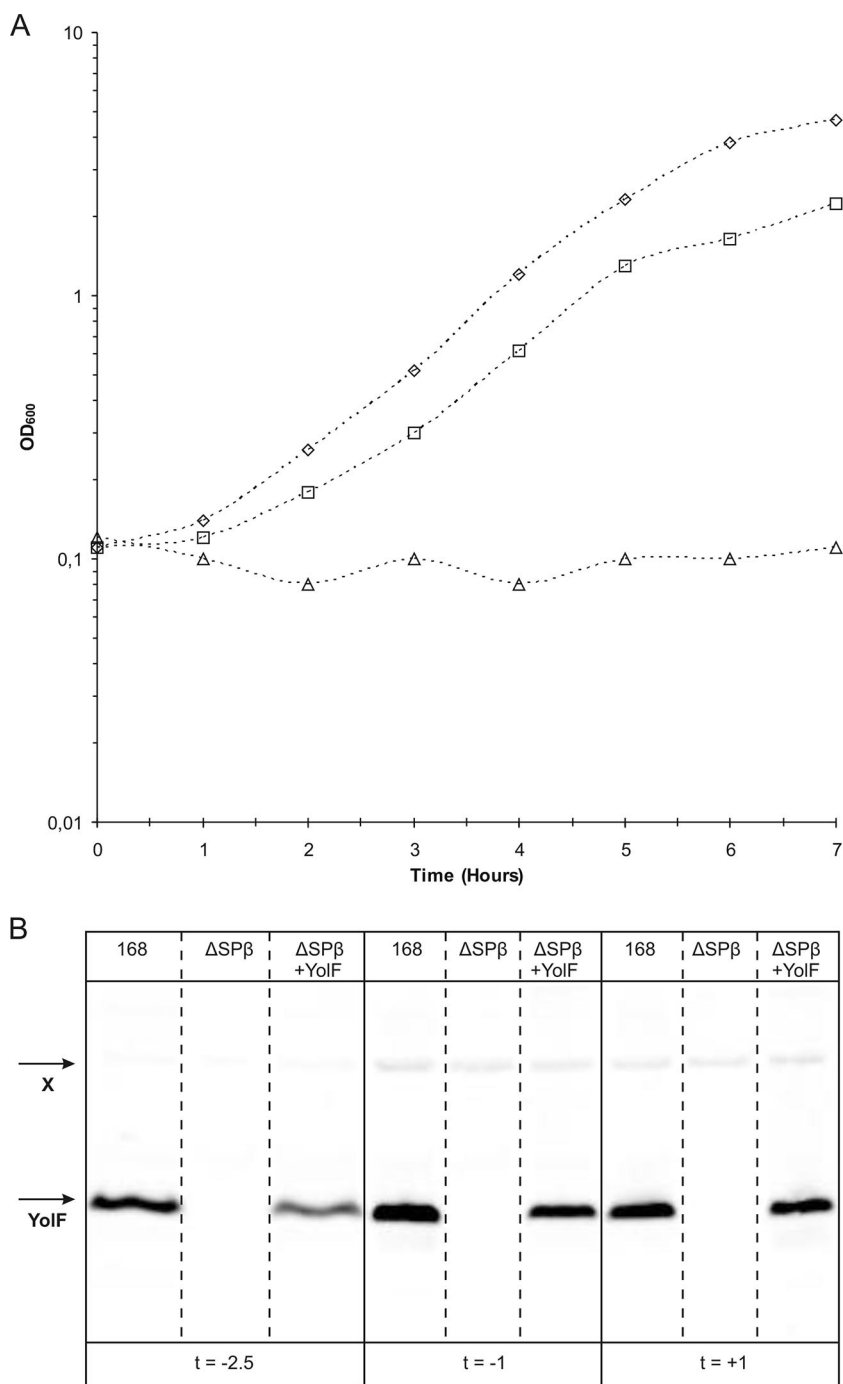


FIG. 3. YoIF production is required for growth of *B. subtilis* on spent medium containing sublancin 168. (A) Growth of *B. subtilis* strains 168 (diamonds),  $\Delta$ SP $\beta$  (triangles), and  $\Delta$ SP $\beta$  pGDL-yolF (squares) in spent medium of *B. subtilis* 168. Cells grown overnight were diluted to an OD<sub>600</sub> of 0.1 in spent medium of *B. subtilis* 168 cells grown overnight and supplemented with 10 $\times$  LB medium. Next, growth was continued for 7 h and the OD<sub>600</sub> was measured at hourly intervals. (B) Expression of YoIF in cells of *B. subtilis* 168, *B. subtilis*  $\Delta$ SP $\beta$ , and *B. subtilis*  $\Delta$ SP $\beta$  carrying the pGDL-yolF plasmid. Samples were taken at 2.5 h (t = -2.5) and 1 h (t = -1) prior the transition point between exponential and postexponential growth or 1 h after the transition point (t = +1). Cell lysates were prepared, and equal amounts of each lysate were separated by SDS-PAGE. YoIF was detected by immunoblotting with specific antibodies against YoIF. The position of YoIF is indicated by an arrow. An additional band that cross-reacted with the YoIF antibody is marked (X) and can be regarded as an internal standard for sample loading.

acids 3 and 23. The OCTOPUS algorithm identified an N-terminal transmembrane domain from residues 2 to 22. Furthermore, the MEMSAT3 algorithm (<http://bioinf.cs.ucl.ac.uk/memsat/>) predicted an N-terminal domain from residues 3 to

21. All these algorithms predicted that the YoIF protein would have an N<sub>out</sub>-C<sub>in</sub> topology. To verify this prediction, we also applied two other algorithms, namely, PrediSi (<http://www.predisi.de/>), which predicts signal peptides, and Phobius ([Downloaded from <http://aac.asm.org/> on June 20, 2019 by guest](http://</a></p>
</div>
<div data-bbox=)

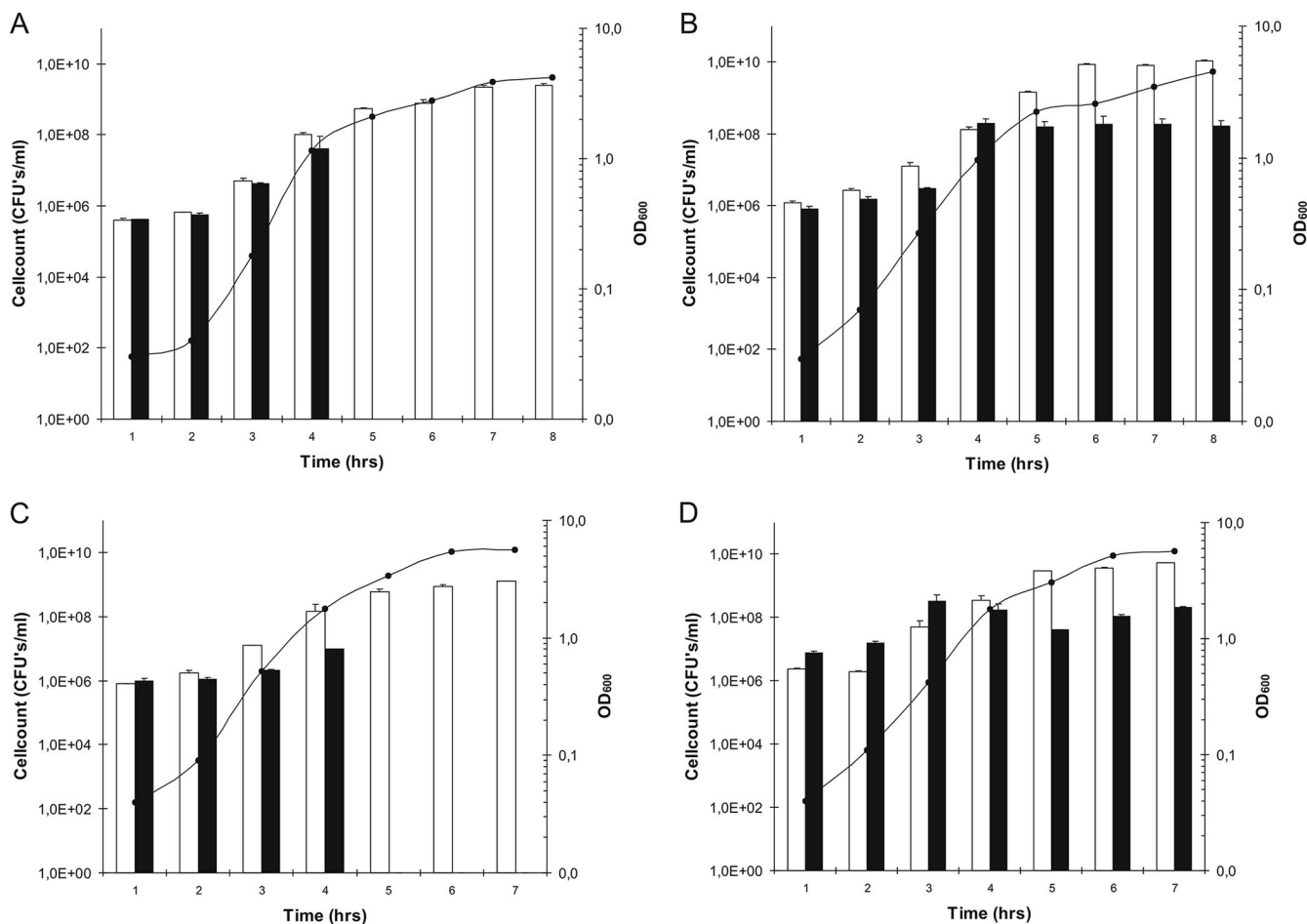


FIG. 4. Assessment of sublancin 168 immunity in *B. subtilis* or *S. aureus* by coculturing with the sublancin 168-producing *B. subtilis* 168 Cm strain. *B. subtilis* 168 Cm (white bars) was cocultured together with *B. subtilis*  $\Delta$ SP $\beta$  Tc (black bars) (A), *B. subtilis*  $\Delta$ SP $\beta$  Tc pGDL-*yolF* (black bars) (B), *S. aureus* RN4220 Em (black bars) (C), or *S. aureus* RN4220 pGDL-*yolF* (black bars) (D). The tested *B. subtilis* and *S. aureus* strains were grown overnight as separate precultures. Upon dilution of the overnight cultures to an OD<sub>600</sub> of 0.05 in fresh LB medium, the cells were mixed in a 1:1 ratio, resulting in cocultures consisting of 50% *B. subtilis* 168 Cm and 50% of *B. subtilis*  $\Delta$ SP $\beta$  Tc, *B. subtilis*  $\Delta$ SP $\beta$  Tc pGDL-*yolF*, *S. aureus* RN4220 Em, or *S. aureus* RN4220 pGDL-*yolF*. Growth was continued, and samples were plated at hourly intervals. Cm-, Tc-, Em-, or Km-resistant colonies were counted and used to calculate the number of CFU per milliliter of culture for each strain at each time point of sampling.

//phobius.cgb.ki.se), which discriminates between signal peptides and transmembrane domains. The predictions thus obtained supported the view that YolF does not have a cleavable signal peptide. Furthermore, Phobius indicated an N-terminal transmembrane domain between amino acids 6 and 25 with an N<sub>out</sub>-C<sub>in</sub> topology. Taken together, these predictions strongly suggested that YolF is a membrane-associated protein with one N-terminal transmembrane domain and with the bulk of the protein facing the cytoplasm.

As is consistent with our predictions, YolF was recently identified as a membrane-associated protein in a study of the composition of the *B. subtilis* membrane proteome (11). To verify the localization of YolF in the membrane, we separated the proteins in the growth medium, cytoplasm, membrane, and cell wall of *B. subtilis* 168 and subsequently analyzed the presence of YolF by SDS-PAGE and immunoblotting with specific antibodies. The results clearly show that YolF is predominantly located in the membrane of *B. subtilis* (Fig. 5). Additionally, a small portion of the YolF protein was found in the cell wall

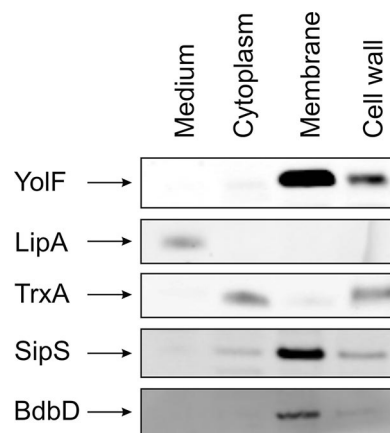


FIG. 5. Subcellular localization of YolF. Wild-type *B. subtilis* 168 cells were grown overnight and separated from the growth medium by centrifugation. Next, the collected cells were fractionated into cell wall, cytoplasmic, and membrane fractions as indicated in Materials and Methods. The proteins in each of these fractions were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and used for immunodetection with specific antibodies against YolF or the control proteins LipA, TrxA, SipS, and BdbD.



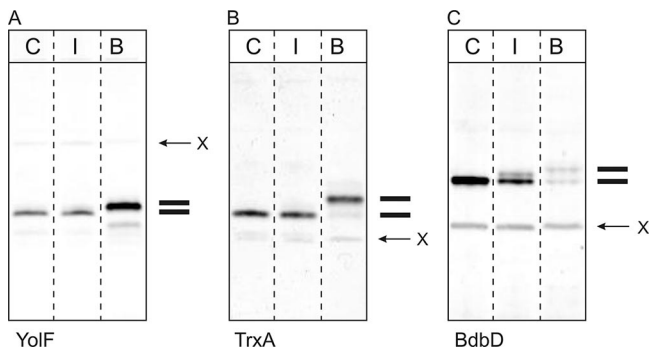


FIG. 6. Topology of YoIF. Wild-type *B. subtilis* 168 cells were grown overnight and collected by centrifugation. Next, equal amounts of cells were subjected to a protoplast procedure in the absence of the thiol-specific cross-linking reagent AMS, in the presence of AMS, or in the presence of AMS and 1% Triton X-100. Notably, addition of Triton X-100 results in protoplast lysis and, consequently, AMS labeling of all proteins containing reduced cysteine residues. After washing, protoplasts were lysed and subjected to nonreducing SDS-PAGE to separate proteins with and without bound AMS. Subsequently, proteins were transferred onto nitrocellulose membranes and used for immunodetection with specific antibodies raised against YoIF, TrxA, or BdbD. The two horizontal stripes mark fast-migrating protein species without bound AMS and slow-migrating protein species with bound AMS. Additional bands that cross-reacted with the TrxA, BdbD, or YoIF antibodies are indicated (X). C, control protoplasts without AMS labeling; I, AMS-labeled intact protoplasts; B, AMS-labeled protoplasts broken with Triton X-100.

fraction. However, this was most likely the result of some protoplast lysis during the fractionation procedure, since a similar observation was made for the control membrane proteins BdbD and SipS and the cytoplasmic protein TrxA. By contrast, the secreted control protein LipA was exclusively detected in the growth medium fraction (Fig. 5).

To investigate the orientation of YoIF in the membrane, we used a recently developed procedure based on the membrane impermeability of the thiol-specific cross-linking reagent AMS (13). Since AMS is unable to cross the membrane, it can only be cross-linked to reduced cysteine residues on the extracytoplasmic side of the cell. The YoIF protein possesses one cysteine residue, predicted to be located in the cytoplasm just behind the transmembrane domain at amino acid position 31. Therefore, we incubated cells from an overnight culture with AMS and monitored AMS binding to YoIF via nonreducing SDS-PAGE. The results showed that the cysteine residue of YoIF was accessible to AMS only when the cells were disrupted (Fig. 6). In protoplasts with an intact membrane, no YoIF labeling with AMS could be observed. Similar AMS labeling results were obtained for the cytoplasmic control protein TrxA, which has two cysteine residues that are mainly present in a reduced state (19). In contrast, a fraction of the extracytoplasmic control protein BdbD was labeled with AMS even when the cytoplasmic membrane was intact. It should be noted that no complete AMS labeling of BdbD is possible due to the fact that this protein is an oxidase with two cysteine residues that are disulfide bonded in the majority of molecules; only a fraction of BdbD becomes reduced as part of the catalytic cycle (24). Taken together, these observations show that the cysteine residue of YoIF behaves like a cytoplasmic cysteine residue. It thus seems that YoIF is indeed a membrane

protein with an  $N_{out}-C_{in}$  topology, with the bulk of the protein facing the cytoplasmic compartment.

## DISCUSSION

The present studies were aimed at identifying which *B. subtilis* 168 genes are responsible for producer immunity against the lantibiotic sublancin 168. By systematically narrowing down the chromosomal region that was known to contain the respective gene(s), we were able to pinpoint one gene, named *yoIF*, as the sublancin 168 producer immunity gene. Furthermore, we were able to show using plates and liquid cultures that *yoIF* is both required and sufficient to confer immunity against active sublancin 168, even in a heterologous host. We therefore propose to rename *yoIF* to *sunI* for sublancin immunity. In silico analyses, subcellular fractionation, and AMS cross-linking studies revealed that the SunI protein is anchored to the membrane with a single N-terminal membrane-spanning domain that has an  $N_{out}-C_{in}$  topology. Thus, the bulk of the protein faces the cytoplasmic compartment of the cell. Such properties have not yet been reported for known bacteriocin immunity proteins. This implies that SunI belongs to a novel class of bacteriocin antagonists.

Sublancin 168 has a broad spectrum of bactericidal activity against gram-positive bacteria, including staphylococci, streptococci, and even other *B. subtilis* strains. Since its first discovery in 1980, it has been known that the genes for sublancin 168 synthesis and producer immunity are located on the SP $\beta$  prophage (10, 18). However, it was 18 years before sublancin 168 and the gene encoding this bacteriocin were identified (38), and it remained unclear until now which of the 187 genes on the SP $\beta$  prophage would be required for sublancin 168 immunity. This old issue has now been resolved by the identification of *sunI* as the sublancin 168 immunity gene. Recently, it was reported that genes in the  $\sigma^W$  regulon serve important functions in the protection of *B. subtilis* against sublancin 168 (5). Butcher and Helmann reported that the *yqeZ* and *yqfAB* genes, which are part of the  $\sigma^W$  regulon, confer sublancin 168 resistance to SP $\beta$ -deficient *B. subtilis* strains. Consistent with its protective function, the *yqeZyqfAB* operon is induced by sublancin 168, as is the case for the entire  $\sigma^W$  regulon. Nevertheless, our present observation that the  $\Delta$ SP $\beta$  strain is not able to grow in a coculture with the parental strain 168 shows that this natural  $\sigma^W$ -dependent resistance mechanism provides insufficient protection for growth and survival in the presence of a sublancin 168-producing strain. In contrast, ectopic expression of *sunI* in the  $\Delta$ SP $\beta$  strain is fully sufficient to allow for growth of *B. subtilis* in the presence of sublancin 168, at least at the levels produced by the 168 strain. Additionally, sublancin 168 resistance was observed even in the naturally sublancin-sensitive *S. aureus* RN4220 strain when *sunI* was heterologously expressed in this bacterium. The latter finding supports the view that SunI is the only *B. subtilis* protein required for sublancin 168 producer immunity.

So far, no studies of the biological function of SunI have been documented in the publicly available literature. Moreover, SunI does not show any significant sequence similarity to other proteins of a known function, and even small conserved-sequence signatures such as a proteolytic triad appear to be absent. This makes it difficult to speculate exactly how SunI

confers resistance to sublancin 168. Our topological analyses show that the SunI protein has an  $N_{\text{out}}\text{-}C_{\text{in}}$  orientation in the membrane, with the bulk of the protein facing the cytoplasm. Strikingly, this topology has not yet been reported for known bacteriocin immunity proteins. All dedicated small bacteriocin immunity proteins have so far been detected in association with the extracytoplasmic membrane surface (17, 42, 48) or are embedded in the membrane (36, 37). Indeed, ABC transporters involved in bacteriocin immunity do have cytoplasmic domains, but the SunI sequence does not display any similarity to those of known ABC transporters (12). In fact, the topology of SunI also makes it unlikely that it functions as a transporter that removes sublancin 168 from the membrane. Another possibility would be that SunI could function by modifying the cytoplasmic membrane to prevent entrance of sublancin 168, but this type of immunity is usually provided by larger lipoproteins at the extracytoplasmic side of the membrane (23, 26). Thus, only a few possible modes of action are still conceivable for SunI, all of which differ from the known bacteriocin immunity mechanisms. For example, SunI could cooperate with a transporter in the extrusion of sublancin 168 from the membrane or cytoplasm. This putative transporter would not be SunT, however, as SunI confers sublancin resistance to *B. subtilis* and *S. aureus* strains that lack the SunT transporter. Alternatively, SunI might block the entrance of sublancin 168 into the membrane or cytoplasm, or SunI might even protect a dedicated target of sublancin 168.

The production of sublancin 168 from a prophage (i.e., SP $\beta$ ) is interesting from an evolutionary perspective, since the presence of the *sunA* gene in the phage genome ensures the maintenance of this lysogenic phage in a *B. subtilis* population from the moment that phage infection and chromosomal insertion occurs. This generates a necessity for the phage also to carry the *sunI* gene, because otherwise, infected host cells would pass away, which would clearly be disadvantageous from the phage's perspective. From a host cell perspective, acquisition of the SP $\beta$  prophage is also advantageous, since the production of the potent bacteriocin sublancin 168 provides this cell with a clear competitive advantage over other cells, as illustrated in our cocultivation experiments. The chromosomal localization of *sunI* directly next to *sunA* also seems to underscore the importance of SunI for immunity. It seems likely that evolutionary selective pressure has linked these two genes closely together, which is consistent with the notion that a spontaneously occurring loss of *sunI* would result in a nonviable situation for strains maintaining an intact *sunA* gene. Nevertheless, *sunI* and *sunA* are not located in the same operon but are transcribed from different promoters (46). Interestingly, sublancin 168, unlike many other bacteriocins, was recently reported to be produced under conditions of exponential growth (54). This is in agreement with our present data showing that SunI is continuously produced. This ensures that sublancin 168-producing cells are immune to their own bacteriocin during all stages of growth.

The reason SunI differs from other known bacteriocin immunity determinants, especially with respect to its topology, most likely relates to the fact that it protects against a unique type of bacteriocin. Sublancin 168 was originally classified as a type AII lantibiotic because of the presence of a methyllanthionine bridge and a leader peptide with the characteristic "dou-

ble glycine" cleavage site motif of type AII lantibiotics (38). Nevertheless, it is quite a special member of this bacteriocin subgroup (7). The two unique disulfide bonds are not encountered in other lantibiotics and give sublancin a structure that is clearly distinct from that of any other known type of lantibiotic. It has therefore been proposed to classify sublancin 168 to a completely different group of lantibiotics (35). Unfortunately, the mechanism of sublancin's bactericidal activity is presently unknown and, thus, provides no clues for a possible mechanism by which SunI provides immunity to sublancin 168. Our continuing efforts are therefore aimed at elucidating the mode of action of sublancin 168, which should also allow us to resolve the seemingly unique mechanism of producer immunity conferred by SunI.

#### ACKNOWLEDGMENTS

We thank Wim Quax for support and Bronwyn Butcher and John Helmann for kindly providing *B. subtilis* strain JH642 prior to publication.

Funding for this project was provided by CEU projects LSHG-CT-2004-503468, LSHG-CT-2004-005257, LSHM-CT-2006-019064, and LSHG-CT-2006-037469, the transnational SysMO initiative through project BACELL SysMO, the European Science Foundation under the EUROCORES Programme EuroSCOPE, and grant 04-EScope 01-011 from the Research Council for Earth and Life Sciences of The Netherlands Organization for Scientific Research.

#### REFERENCES

1. Arnaud, M., A. Chastanet, and M. Debarbouille. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl. Environ. Microbiol.* **70**:6887–6891.
2. Bolhuis, A., G. Venema, W. J. Quax, S. Bron, and J. M. van Dijk. 1999. Functional analysis of paralogous thiol-disulfide oxidoreductases in *Bacillus subtilis*. *J. Biol. Chem.* **274**:24531–24538.
3. Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl, and B. de Kruijff. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**:2361–2364.
4. Bron, S., and G. Venema. 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.
5. Butcher, B. G., and J. D. Helmann. 2006. Identification of *Bacillus subtilis* sigma-dependent genes that provide intrinsic resistance to antimicrobial compounds produced by bacilli. *Mol. Microbiol.* **60**:765–782.
6. Chakicherla, A., and J. N. Hansen. 1995. Role of the leader and structural regions of prelantibiotic peptides as assessed by expressing nisin-subtilin chimeras in *Bacillus subtilis* 168, and characterization of their physical, chemical, and antimicrobial properties. *J. Biol. Chem.* **270**:23533–23539.
7. Chatterjee, C., M. Paul, L. Xie, and W. A. van der Donk. 2005. Biosynthesis and mode of action of lantibiotics. *Chem. Rev.* **105**:633–684.
8. Chatterjee, S., S. Chatterjee, S. J. Lad, M. S. Phansalkar, R. H. Rupp, B. N. Ganguli, H. W. Fehlhaber, and H. Kogler. 1992. Mersacidin, a new antibiotic from *Bacillus*. Fermentation, isolation, purification and chemical characterization. *J. Antibiot. (Tokyo)* **45**:832–838.
9. Darmon, E., R. Dorenbos, J. Meens, R. Freudl, H. Antelmann, M. Hecker, O. P. Kuipers, S. Bron, W. J. Quax, J. Y. Dubois, and J. M. van Dijk. 2006. A disulfide bond-containing alkaline phosphatase triggers a BdbC-dependent secretion stress response in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **72**:6876–6885.
10. Dorenbos, R., T. Stein, J. Kabel, C. Bruand, A. Bolhuis, S. Bron, W. J. Quax, and J. M. van Dijk. 2002. Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. *J. Biol. Chem.* **277**:16682–16688.
11. Dreisbach, A., A. Otto, D. Becher, E. Hammer, A. Teumer, J. W. Gouw, M. Hecker, and U. Volker. 2008. Monitoring of changes in the membrane proteome during stationary phase adaptation of *Bacillus subtilis* using in vivo labeling techniques. *Proteomics* **8**:2062–2076.
12. Duan, K., M. L. Harvey, C. Q. Liu, and N. W. Dunn. 1996. Identification and characterization of a mobilizing plasmid, pND300, in *Lactococcus lactis* M189 and its encoded nisin resistance determinant. *J. Appl. Bacteriol.* **81**:493–500.
13. Facey, S. J., S. A. Neugebauer, S. Krauss, and A. Kuhn. 2007. The mechanosensitive channel protein MscL is targeted by the SRP to the novel YidC membrane insertion pathway of *Escherichia coli*. *J. Mol. Biol.* **365**:995–1004.
14. Fredenhagen, A., G. Fendrich, F. Marki, W. Marki, J. Gruner, F. Raschdorf,

- and H. H. Peter. 1990. Duramycins B and C, two new lanthionine containing antibiotics as inhibitors of phospholipase A2. Structural revision of duramycin and cinnamycin. *J. Antibiot. (Tokyo)* **43**:1403–1412.
15. Guder, A., I. Wiedemann, and H. G. Sahl. 2000. Posttranslationally modified bacteriocins—the lantibiotics. *Biopolymers* **55**:62–73.
  16. Guéroul-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier. 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**:335–336.
  17. Heidrich, C., U. Pag, M. Josten, J. Metzger, R. W. Jack, G. Bierbaum, G. Jung, and H. G. Sahl. 1998. Isolation, characterization, and heterologous expression of the novel lantibiotic epicidin 280 and analysis of its biosynthetic gene cluster. *Appl. Environ. Microbiol.* **64**:3140–3146.
  18. Hemphill, H. E., I. Gage, S. A. Zahler, and R. Z. Korman. 1980. Prophage-mediated production of a bacteriocinlike substance by SP beta lysogens of *Bacillus subtilis*. *Can. J. Microbiol.* **26**:1328–1333.
  19. Hochgräfe, F., J. Mostertz, D. Albrecht, and M. Hecker. 2005. Fluorescence thiol modification assay: oxidatively modified proteins in *Bacillus subtilis*. *Mol. Microbiol.* **58**:409–425.
  20. Hoffmann, A., T. Schneider, U. Pag, and H.-G. Sahl. 2004. Localization and functional analysis of PepI, the immunity peptide of Pep5-producing *Staphylococcus epidermidis* strain 5. *Appl. Environ. Microbiol.* **70**:3263–3271.
  21. Jung, G. 1991. Lantibiotics: a survey, p. 1–34. *In* G. Jung and H. Sahl (ed.), *Nisin and novel lantibiotics*. Escm, Leiden, The Netherlands.
  22. Kim, L., A. Mogk, and W. Schumann. 1996. A xylose-inducible *Bacillus subtilis* integration vector and its application. *Gene* **181**:71–76.
  23. Klein, C., and K. D. Entian. 1994. Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 6633. *Appl. Environ. Microbiol.* **60**:2793–2801.
  24. Kouwen, T. R., A. van der Goot, R. Dorenbos, T. Winter, H. Antelmann, M. C. Plaisier, W. J. Quax, J. M. van Dijk, and J. Y. Dubois. 2007. Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. *Mol. Microbiol.* **64**:984–999.
  25. Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick. 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**:709–712.
  26. Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos. 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. *Eur. J. Biochem.* **216**:281–291.
  27. Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Conner-ton, A. Danchin, et al. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**:249–256.
  28. Kunst, F., and G. Rapoport. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* **177**:2403–2407.
  29. Lazarevic, V., A. Dusterhoft, B. Soldo, H. Hilbert, C. Mauel, and D. Karamata. 1999. Nucleotide sequence of the *Bacillus subtilis* temperate bacteriophage SPbetac2. *Microbiology* **145**(Pt. 5):1055–1067.
  30. Lopilato, J., S. Bortner, and J. Beckwith. 1986. Mutations in a new chromosomal gene of *Escherichia coli* K-12, *penB*, reduce plasmid copy number of pBR322 and its derivatives. *Mol. Gen. Genet.* **205**:285–290.
  31. McAuliffe, O., R. P. Ross, and C. Hill. 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* **25**:285–308.
  32. McLaughlin, R. E., J. J. Ferretti, and W. L. Hynes. 1999. Nucleotide sequence of the streptococcal A-FF22 lantibiotic regulon: model for production of the lantibiotic SA-FF22 by strains of *Streptococcus pyogenes*. *FEMS Microbiol. Lett.* **175**:171–177.
  33. Meijer, W. J., A. de Jong, G. Bea, A. Wisman, H. Tjalsma, G. Venema, S. Bron, and J. M. van Dijk. 1995. The endogenous *Bacillus subtilis* (natto) plasmids pTA1015 and pTA1040 contain signal peptidase-encoding genes: identification of a new structural module on cryptic plasmids. *Mol. Microbiol.* **17**:621–631.
  34. Meyer, C., G. Bierbaum, C. Heidrich, M. Reis, J. Suling, M. I. Iglesias-Wind, C. Kemper, E. Molitor, and H. G. Sahl. 1995. Nucleotide sequence of the lantibiotic Pep5 biosynthetic gene cluster and functional analysis of PepP and PepC. Evidence for a role of PepC in thioether formation. *Eur. J. Biochem.* **232**:478–489.
  35. Nagao, J., S. M. Asaduzzaman, Y. Aso, K. Okuda, J. Nakayama, and K. Sonomoto. 2006. Lantibiotics: insight and foresight for new paradigm. *J. BioSci. Bioeng.* **102**:139–149.
  36. Okuda, K., Y. Aso, J. Nagao, K. Shioya, Y. Kanemasa, J. Nakayama, and K. Sonomoto. 2005. Characterization of functional domains of lantibiotic-binding immunity protein, NukH, from *Staphylococcus warneri* ISK-1. *FEMS Microbiol. Lett.* **250**:19–25.
  37. Okuda, K., Y. Aso, J. Nakayama, and K. Sonomoto. 2008. Cooperative transport between NukFEG and NukH in immunity against the lantibiotic nukacin ISK-1 produced by *Staphylococcus warneri* ISK-1. *J. Bacteriol.* **190**:356–362.
  38. Paik, S. H., A. Chakicherla, and J. N. Hansen. 1998. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *J. Biol. Chem.* **273**:23134–23142.
  39. Peschel, A., and L. V. Collins. 2001. Staphylococcal resistance to antimicrobial peptides of mammalian and bacterial origin. *Peptides* **22**:1651–1659.
  40. Peschel, A., and F. Gotz. 1996. Analysis of the *Staphylococcus epidermidis* genes *epiF*, *-E*, and *-G* involved in epidermin immunity. *J. Bacteriol.* **178**:531–536.
  41. Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung, and F. Gotz. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* **274**:8405–8410.
  42. Reis, M., M. Eschbach-Bludau, M. I. Iglesias-Wind, T. Kupke, and H. G. Sahl. 1994. Producer immunity towards the lantibiotic Pep5: identification of the immunity gene *pepI* and localization and functional analysis of its gene product. *Appl. Environ. Microbiol.* **60**:2876–2883.
  43. Rincé, A., A. Dufour, P. Uguen, J. P. Le Penec, and D. Haras. 1997. Characterization of the lactacin 481 operon: the *Lactococcus lactis* genes *lctF*, *lctE*, and *lctG* encode a putative ABC transporter involved in bacteriocin immunity. *Appl. Environ. Microbiol.* **63**:4252–4260.
  44. Sahl, H. G., and G. Bierbaum. 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol.* **52**:41–79.
  45. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
  46. Serizawa, M., K. Kodama, H. Yamamoto, K. Kobayashi, N. Ogasawara, and J. Sekiguchi. 2005. Functional analysis of the YvrGHb two-component system of *Bacillus subtilis*: identification of the regulated genes by DNA microarray and Northern blot analyses. *Biosci. Biotechnol. Biochem.* **69**:2155–2169.
  47. Siegers, K., S. Heinzmann, and K. D. Entian. 1996. Biosynthesis of lantibiotic nisin. Posttranslational modification of its prepeptide occurs at a multimeric membrane-associated lanthionine synthetase complex. *J. Biol. Chem.* **271**:12294–12301.
  48. Skaugen, M., C. I. Abildgaard, and I. F. Nes. 1997. Organization and expression of a gene cluster involved in the biosynthesis of the lantibiotic lactocin S. *Mol. Gen. Genet.* **253**:674–686.
  49. Stein, T. 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* **56**:845–857.
  50. Stein, T., S. Heinzmann, S. Dusterhus, S. Borchert, and K. D. Entian. 2005. Expression and functional analysis of the subtilin immunity genes *spaIFEG* in the subtilin-sensitive host *Bacillus subtilis* MO1099. *J. Bacteriol.* **187**:822–828.
  51. Stein, T., S. Heinzmann, I. Solovieva, and K. D. Entian. 2003. Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*. *J. Biol. Chem.* **278**:89–94.
  52. Tjalsma, H., and J. M. van Dijk. 2005. Proteomics-based consensus prediction of protein retention in a bacterial membrane. *Proteomics* **5**:4472–4482.
  53. van der Meer, J. R., H. S. Rollema, R. J. Siezen, M. M. Beerthuyzen, O. P. Kuipers, and W. M. de Vos. 1994. Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. *J. Biol. Chem.* **269**:3555–3562.
  54. Veening, J. W., O. A. Igoshin, R. T. Eijlander, R. Nijland, L. W. Hamoen, and O. P. Kuipers. 2008. Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. *Mol. Syst. Biol.* **4**:184.
  55. Wiedemann, I., E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff, and H. G. Sahl. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J. Biol. Chem.* **276**:1772–1779.