The Inhibitory Spectrum of Thermophilin 9 from *Streptococcus thermophilus* LMD-9 Depends on the Production of Multiple Peptides and the Activity of BpG_{St}, a Thiol-Disulfide Oxidase[†]

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The *blp*_{St} cluster of *Streptococcus thermophilus* LMD-9 was recently shown to contain all the genetic information required for the production of bacteriocins active against other *S. thermophilus* strains. In this study, we further investigated the antimicrobial activity of *S. thermophilus* LMD-9 by testing the susceptibility of 31 bacterial species (87 strains). We showed that LMD-9 displays an inhibitory spectrum targeted toward related gram-positive bacteria, including pathogens such as *Listeria monocytogenes*. Using deletion mutants, we investigated the contribution of the three putative bacteriocin-encoding operons *blpD*_{St}-*orf2*, *blpU*_{St}-*orf3*, and *blpE*_{St}-*blpF*_{St} (*bac*_{St} operons) and of the *blpG*_{St} gene, which encodes a putative modification protein, to the inhibitory spectrum and immunity of strain LMD-9. Our results present evidence that the *blp*_{St} locus encodes a multi-peptide bacteriocin system called thermophilin 9. Among the four class II bacteriocin-like peptides encoded within the *bac*_{St} operons, BpD_{St} alone was sufficient to inhibit the growth of most thermophilin 9-sensitive species. The *blpD*_{St} gene forms an operon with its associated immunity gene(s), and this functional bacteriocin/immunity module could easily be transferred to *Lactococcus lactis*. The remaining three *Bac*_{St} peptides, BpU_{St}, BpE_{St}, and BpF_{St}, confer poor antimicrobial activity but act as enhancers of the antagonistic activity of thermophilin 9 by an unknown mechanism. The *blpG*_{St} gene was also shown to be specifically required for the antilisteria activity of thermophilin 9, since its deletion abolished the sensitivities of most *Listeria* species. By complementation of the motility deficiency of *Escherichia coli dsbA*, we showed that *blpG*_{St} encodes a functional thiol-disulfide oxidase, suggesting an important role for disulfide bridges within thermophilin 9.

Bacteriocins are small (20- to 60-residue) ribosomally synthesized and extracellularly released peptides displaying antimicrobial activity against species that may or may not be closely related to the producer bacteria (24). Generally, bacteriocin producer strains secrete multiple antimicrobial peptides, each exhibiting a specific inhibitory spectrum, which provides the producer strain with a competitive advantage by eliminating concurrent strains (or species) sharing the same ecological niche. Well-known examples are the production by *Streptococcus mutans* of mutacins (five mutacins have been identified so far), which are active against streptococcal species present in the human oral cavity (19, 23), and the production by *Lactobacillus sakei* LTH673 of sakacin P, which has the greatest activity against *Carnobacterium* and *Listeria* species (39), and sakacin Q, mainly active against *Lactobacillus coryniformis* (31). The last decade has seen a growing interest in the bacteriocins of lactic acid bacteria (LAB), particularly those active against clostridia, listeria, and enteropathogen species, because of their potential use as natural biopreservatives to protect food products against bacterial contamination.

Streptococcus thermophilus is of major importance for the food industry, since it is widely used for the manufacture of dairy products (with an annual market of around \$40 billion),

and it is considered the second most important industrial dairy starter after *Lactococcus lactis* (22). In *S. thermophilus* LMD-9, the production of bacteriocin-like inhibitory substances active against other strains of *S. thermophilus* has recently been shown to depend on the chromosomally encoded class II *blp*_{St} locus (15). The class II bacteriocins of LAB include non-post-translationally modified peptides (36) and are further subdivided into three main subcategories: IIa, the pediocin-like bacteriocins with strong antilisterial effects, which contain a conserved N-terminal YGNGVXC sequence (12), and IIb, bacteriocins whose activities depend on the complementary activities of two peptides (α and β peptides) (18). All other nonmodified bacteriocins are classified as class IIc (36). Class II bacteriocins of LAB permeabilize the target cell membrane by the formation of poration complexes, which leads to the release of essential molecules and the dissipation of the proton motive force (20).

As is the case for the production of many bacteriocins by LAB, the Bp_{St}-related antimicrobial activity of *S. thermophilus* is regulated at the transcriptional level by a cell-density mechanism (quorum sensing) (15, 25). The pheromone precursor BpC_{St} is processed downstream of a double-glycine (two-Gly) motif and secreted through a specific transport apparatus consisting of the ABC transporter BpA_{St} and the accessory protein BpB_{St} (15). The processed forms of BpC_{St}, (D9C-30 [30 amino acids {aa}] and D9C-19 [19 aa]) activate a signaling cascade involving the BpH_{St}/BpR_{St} two-component system, which triggers the transcription of the bacteriocin and immunity structural genes (15). These genes are organized into three operons (*blpD*_{St}-*orf2*, *blpU*_{St}-*orf3*, and *blpE*_{St}-*blpF*_{St}), each

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TABLE 1. Primary sequence analysis of the Bac_{St} and Orf peptides encoded in the *blpD_{St}-blpF_{St}* region

Gene product	Size (aa)	Sequence ^a	% Hydrophobic amino acid residues ^b	No. of TM segments ^c	pI
BlpD _{St} *	35	LSCDEGMLAVGGLGAVGGPWGAVGGVLVGAALYCF	48.6	1	3.7
BlpU _{St} *	53	GCSWGGFAKQGVATGVGNGLRLGIKTRTWQGAAGAAGGAIIVGGVGYGATCWW	41.5	2	9.9
BlpE _{St} *	56	RVNWERWGMCGASVAVGASEGFSAAAGGTAFFIGPYAIGTGAVGAAIGGGVALLGC	48.2	1	6.1
BlpF _{St} *	43	AVCLMPNVNNKEGDPLKDGWVSPPRYRSGEAYPMVYLPVCAIM	30.2	0	6.2
Orf1	66		36.4	1	8.8
Orf2	51		47.0	2	10.0
Orf3	57		43.9	2	9.4
Orf4	55		50.9	2	10.1
Orf5	56		50.0	2	8.9
Orf6	133		51.9	4	10.1
Orf7	130		56.1	4	9.7

^a Only given for Bac_{St} peptides. The cysteine residues (boldface, underlined) and the GG, AG, and GA repeats (boldface) are indicated.

^b Residues taken into account are A, I, L, F, W, and V.

^c Number of transmembrane segments, as predicted with TMHMM 2.0 (27).

comprising putative bacteriocin genes (collectively named *bac_{St}* genes) and *orf* genes (15). The four Bac_{St} peptides BlpD_{St}, BlpU_{St}, BlpE_{St}, and BlpF_{St} contain a two-Gly leader, which is likely cleaved off during secretion by the BlpAB_{St} transport system. However, their individual functionalities and their putative interactions as a multi-peptide bacteriocin remained hypothetical (15). The Orf peptides and the predicted mature Bac_{St} peptides, except for the mature peptide BlpF_{St}* (the asterisk represents the putative mature part of the peptide, i.e., after cleavage downstream of the first two-Gly motif), contain stretches of highly hydrophobic residues that are predicted to form transmembrane segments (Table 1). The predicted mature peptides BlpD_{St}*, BlpU_{St}*, and BlpE_{St}* contain several Gly-Gly, Ala-Gly, and Gly-Ala repeats, which is typical of two-component class IIb bacteriocins (Table 1). Additionally, BlpE_{St}*, BlpU_{St}*, and BlpD_{St}* share 28, 28, and 29% identity (44, 36, and 51% similarity) with the β peptide of lactococcin M, the α peptide of thermophilin 13, and the β peptide of brochocin C, respectively (30, 33, 35).

The predicted processed forms of the four Bac_{St} peptides contain two cysteine residues at their N- and C-terminal ends. Recent studies have highlighted a strong correlation between the toxicity of class IIa bacteriocins and the number of disulfide bridges formed, which might play a role in their structural stability (11, 37). Intriguingly, the *blpG_{St}-blpX_{St}* operon, which is also regulated by the BlpC_{St} pheromone, encodes a putative modification protein (BlpG_{St}) with a thioredoxin fold domain containing the conserved thioredoxin catalytic motif CXXC. In *Bacillus subtilis*, the BdbB thiol-disulfide oxidase was shown to be required for the production of active sublancin 168, a bacteriocin that contains two disulfide bridges (9). Although the *blpG_{St}-blpX_{St}* operon of *S. thermophilus* does not seem to be required for the intraspecies antimicrobial activity of strain LMD-9 (15), its potential role in the inhibition of other species has not been investigated.

The aim of this study was to identify the genetic determinants involved in the Blp_{St}-related antimicrobial activity and immunity of *S. thermophilus* LMD-9 by genetic dissection of the *blp_{St}* locus and heterologous expression. We first determined the inhibitory spectrum of strain LMD-9 against a large set of indicator species. Appropriate *blp_{St}* mutants were then

constructed, and their antimicrobial activities and immunity phenotypes were investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study, except for the indicator bacteria used to determine the antimicrobial spectrum of *S. thermophilus* LMD-9, are listed in Table 2. *Escherichia coli* was grown with shaking at 37°C in Luria-Bertani (LB) broth (38). *E. coli* motility was assayed on swarm plates containing 1% Bacto tryptone (wt/vol), 0.5% NaCl (wt/vol), and 0.3% agar (wt/vol), as previously described (3). Swarm plates were inoculated with 5 μ l of exponentially growing cultures (optical density at 600 nm [OD₆₀₀], 1.0) of *E. coli* and incubated at 37°C for 20 h. *S. thermophilus* was grown anaerobically (BBL GasPak systems; Becton Dickinson, Franklin Lakes, NJ) in M17 broth (Difco Laboratories Inc., Detroit, MI) with 1% (wt/vol) glucose (M17G broth) at 42°C. *L. lactis* was grown in M17 broth with 0.5% (wt/vol) glucose at 29°C without shaking. When appropriate, erythromycin was added to the media at the following concentrations: 250 μ g/ml for *E. coli*, 2.5 μ g/ml for *S. thermophilus*, and 5 μ g/ml for *L. lactis*. Solid agar plates were prepared by adding 2% (wt/vol) agar to the media.

DNA techniques and transformation. General molecular biology techniques were performed according to the instructions given by Sambrook et al. (38). Plasmids derived from pMG36e (40) and pGhost9 (28) were constructed in *E. coli* strains TG1 (38) and EC1000 (26), respectively. Electrotransformation of *E. coli*, *S. thermophilus*, and *L. lactis* was performed as described by Dower et al. (10), Blomqvist et al. (4), and Leenhouts et al. (27), respectively. The transformed *S. thermophilus* cells were immediately resuspended in 1 ml of M17G and were incubated anaerobically for 6 h at 37°C (pMG36e derivatives) or 29°C (pGhost9 derivatives). Chromosomal DNAs of *S. thermophilus* and *Lactobacillus plantarum* were prepared as described previously (13). PCRs were performed with *Pfu* DNA polymerase (Promega, Madison, WI) in a GeneAmp PCR system 2400 (Applied Biosystems, Lennik, Belgium).

Construction of the *blpG_{St}* expression vector. The entire open reading frame (ORF) of *blpG_{St}* was amplified by PCR with primers BlpG1 (5'-AAACCATG GTGAAAAAAGGACATTAACGC-3') and BlpG2 (5'-CGGGTACCTTCTC TTTCTACTCTCTTCG-3'), yielding a 0.69-kb fragment that was then digested with *Nco*I and *Kpn*I (the restriction sites are underlined in the primer sequences) and translationally fused with the P32 expression signals into the similarly digested pGIBG001 plasmid (15). The resulting plasmid was designated pGILF003.

Construction of the vectors for heterologous expression of BlpAB_{St} and *blpD_{St}-orf2* in *L. lactis*. First, the *ldhL* transcriptional terminator from *L. plantarum* NCIMB8826 was PCR amplified using primers LdhTER1 (5'-AAAGCATGCACATCATCAA CTTGAAGGG-3') and LdhTER2 (5'-ATACTGCCCCCAATCATAAGTCCAC G-3'), digested with *Sph*I (the restriction site is underlined in the LdhTER1 sequence) and *Kpn*I (the restriction site present in the sequence of *ldhL*), and cloned into the similarly digested pMG36e vector (40), yielding pMG36eT. In a second step, a fragment encompassing the *blpD_{St}* ribosome binding site and the *blpD_{St}-orf2* region was PCR amplified with primers pMGopD1 (5'-AAAGTCGACAAATTTT

TABLE 2. Bacterial strains and plasmids used in this study

Strain, plasmid, or primer	Characteristic(s) ^a	Source or reference
Strains		
<i>S. thermophilus</i>		
LMD-9	Wild type	15
LF105 ($\Delta 1$)	LMD-9 $\Delta(bl pD_{St}-bl pF_{St})$	15
LF106 ($\Delta 7$)	LMD-9 $\Delta(bl pG_{St}-bl pX_{St})$	15
LF109 ($\Delta 2$)	LMD-9 $\Delta(bl pU_{St}-bl pF_{St})$	This study
LF110 ($\Delta 3$)	LMD-9 $\Delta(bl pD_{St}-orf6)$	This study
LF109.1 ($\Delta 9$)	LMD-9 $\Delta(bl pU_{St}-bl pF_{St}) \Delta bl pD_{St}$	This study
LF109.2 ($\Delta 10$)	LMD-9 $\Delta(bl pU_{St}-bl pF_{St}) \Delta(bl pD_{St}-orf1)$	This study
LF111 ($\Delta 4$)	LMD-9 $\Delta(bl pD_{St}-orf2)$	This study
LF111.1 ($\Delta 4/\Delta 5$)	LMD-9 $\Delta(bl pD_{St}-orf2) \Delta(bl pE_{St}-bl pF_{St})$	This study
LF112 ($\Delta 5$)	LMD-9 $\Delta(bl pE_{St}-bl pF_{St})$	This study
LF113 ($\Delta 6$)	LMD-9 $\Delta(bl pU_{St}-orf6)$	This study
LF114 ($\Delta 8$)	LMD-9 $\Delta bl pG_{St}$	This study
<i>L. plantarum</i>		
NCIMB8826	Wild type	NCIMB ^b
<i>L. lactis</i>		
NZ3900	MG1363 derivative	7
<i>E. coli</i>		
TG1	<i>supE hsd$\Delta 5$ thi $\Delta(lac-proAB)$ F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	38
EC1000	Km ^r RepA ⁺	26
AH50	MC1000 <i>phoR $\Delta ara714 leu^{+} phoA68$</i>	21
AH55	AH50 <i>dsbA::kan-1</i>	21
Plasmids		
pGIBG001	Em ^r ; pMG36e in which an NcoI restriction site has been introduced to allow the cloning of genes in translational fusion with the P32 expression cassette	15
pGILF003	Em ^r ; pGIBG001 with a 0.69-kb insert containing the <i>bl pG_{St}</i> ORF of <i>S. thermophilus</i> LMD-9 in translational fusion with the P32 expression cassette	This study
pMG36e	Em ^r ; <i>E. coli-S. thermophilus</i> shuttle vector; contains the P32 promoter	40
pMG36eT	Em ^r ; pMG36e with a 0.36-kb insert containing the 3' end of the <i>ldhL</i> ORF and the transcriptional terminator of <i>ldhL</i> from <i>L. plantarum</i> WCFS1	This study
pGILF004	Em ^r ; pMG36eT with a 0.6-kb insert containing the <i>bl pD_{St}-orf2</i> operon of <i>S. thermophilus</i> LMD-9 in transcriptional fusion with the P32 promoter	This study
pGILF005	Em ^r ; pMG36eT with a 0.6-kb insert containing the <i>bl pD_{St}-orf2</i> operon and a 3.55-kb insert containing the <i>bl pAB_{St}</i> ORFs of <i>S. thermophilus</i> LMD-9 in transcriptional fusion with the P32 promoter	This study
pGhost9	Em ^r Ts	28
pGILF109	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pU_{St}-bl pF_{St}</i>	This study
pGILF109.1	Em ^r Ts; pGhost9 derivative for the in-frame deletion of <i>bl pD_{St}</i> in strain LF109	This study
pGILF109.2	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pD_{St}-orf1</i> in strain LF109	This study
pGILF110	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pD_{St}-orf6</i>	This study
pGILF111	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pD_{St}-orf2</i>	This study
pGILF112	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pE_{St}-bl pF_{St}</i>	This study
pGILF113	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pU_{St}-orf6</i>	This study
pGILF114	Em ^r Ts; pGhost9 derivative for the deletion of <i>bl pG_{St}</i>	This study

^a Km^r, Em^r, and Ts indicate resistance to kanamycin, resistance to erythromycin, and temperature-sensitive mutations in the RepA replication protein, respectively.

^b NCIMB, The National Collections of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland.

AGGAGGTAGTTGC-3') and pMGopD2 (5'-AACCTGCAGGCTAATTCCTTC TACTAGGCC-3'), digested with SalI and PstI (the restriction sites are underlined in the primer sequences), and transcriptionally fused with the P32 promoter into the similarly digested pMG36eT vector, yielding plasmid pGILF004. Finally, a PCR fragment encompassing the *bl pA_{St}* ribosome binding site and the *bl pAB_{St}* genes, obtained with primers BlpAB1 (5'-AACCTGCAGGATAATTTGTGATGAAAG GG-3') and BlpAB2 (5'-AAAGCATGCTTAGCCATCAGTAATTCTCC-3'), was digested with SbfI and SphI (the restriction sites are underlined in the primer sequences) and cloned between the corresponding sites of plasmid pGILF004, downstream of the *bl pD_{St}-orf2* operon. In the resulting plasmid, pGILF005, the *bl pD_{St}-orf2* operon and the *bl pAB_{St}* genes formed an artificial operon structure whose transcription was controlled by the P32 promoter.

Construction of deletion mutants in the *bl p_{St}* locus. The deletion vectors were constructed in the thermosensitive pGhost9 vector by successively cloning two

fragments of approximately 1 kb, corresponding to the upstream and downstream regions of the target gene(s), respectively. Deletions were performed by a two-step homologous-recombination process, as previously described (29). Both recombination steps (plasmid integration and excision) were confirmed by PCR using primers located upstream and downstream of the recombination regions. Table S1 in the supplemental material gives an overview of the strategy used for the construction of the different deletion vectors and the corresponding *S. thermophilus* mutant strains.

Analysis of the antimicrobial activities and immunities of LMD-9 derivatives. The synthetic mature form of BlpC_{St}, named D9C-30 (H₂N-SGWMDYINGFL KGFGGQRTLPTKDYNIPQA-COOH) (purity > 95%), was purchased from Sigma-Genosys (Sigma-Genosys Ltd., Haverhill, United Kingdom). Two alternative methods were used to assay antimicrobial activity, as previously described (15). For the spot-on-lawn method, overnight cultures of the producer strains

TABLE 3. Inhibitory spectrum of *S. thermophilus* LMD-9 and derivatives

Indicator species or strain	No. of strains tested	No. of indicator strains inhibited by the induced producer strains ^a										
		LMD-9 (D U E F) +	$\Delta 1$ (-) +	$\Delta 2$ (D) +	$\Delta 3$ (E F) +	$\Delta 4/\Delta 5$ (U) +	$\Delta 4$ (U E) (F) +	$\Delta 9$ (-) +	$\Delta 5$ (D U) +	$\Delta 6$ (D E) (F) +	$\Delta 7$ (D U E) (F) - ^b	$\Delta 8$ (D U E) (F) - ^b
<i>Streptococcus</i>												
<i>S. thermophilus</i>	8	7	0	7	0	0	0	0	7	7	7	7
<i>S. thermophilus</i> SFi16	1	1	0	0	0	0	0	0	0	0	1	1
<i>S. thermophilus</i> LMG18311	1	1	0	1	1 ^d	0	1 ^d	0	1	1	1	1
<i>S. salivarius</i>	3	3	0	3	0	0	0	0	3	3	3	3
<i>Enterococcus</i>												
<i>E. faecalis</i> OG1X	1	1	0	1	0	0	0	0	1	1	1	1
<i>E. faecalis</i> JH2-SS	1	1	0	1 ^d	0	0	0	0	1 ^d	1	1 ^d	1 ^d
<i>E. faecalis</i> JH2-2	1	1	0	1	0	0	0	0	1	1	1 ^d	1 ^d
<i>Lactococcus</i>												
<i>L. lactis</i>	3	3	0	3	0	0	0	0	3	3	3	3
<i>L. lactis</i> IL-1403	1	1	0	0	0	0	0	0	1	0	1	1
<i>L. lactis</i> MG1363	1	1	0	1 ^d	0	0	0	0	1 ^d	1 ^d	1 ^d	1 ^d
<i>Bacillus</i>												
<i>B. coagulans</i>	4	3	0	3	0	0	0	0	3	3	3	3
<i>B. coagulans</i> NCIB8523	1	1	0	1	0	0	0	0	1	1	1 ^d	1 ^d
<i>B. fusiformis</i> S10056	1	1	0	1	0	0	0	0	1	1	1	1
<i>Listeria</i>												
<i>L. monocytogenes</i>	3	3	0	3	0	0	0	0	3	3	0	0
<i>L. monocytogenes</i> IHE-92	1	1	0	1	0	0	0	0	1	1	1 ^d	1 ^d
<i>L. ivanovii</i> IHE-3	1	1	0	1	0	0	0	0	1	1	1	1
<i>L. innocua</i> IHE-2	1	1	0	1	0	0	0	0	1	1	0	0
<i>L. seeligeri</i>	2	2	0	2	0	0	0	0	2	2	0	0
<i>L. welshimeri</i>	3	0	NT ^c	NT	NT	NT	NT	NT	NT	NT	NT	NT
<i>Lactobacillus</i>												
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	2	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
<i>L. helveticus</i>	3	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

^a Antimicrobial activity was assayed using the overlay protocol in the presence of 400 ng/ml D9C-30. Growth of the indicator strain was considered inhibited when the radius of the inhibitory zone was at least 1.5 mm. Each experiment was repeated at least three times. The letters in parentheses indicate the content in *bac*_{St} genes of the producer strain: D, *blpD*_{St}; U, *blpU*_{St}; E, *blpE*_{St}; F, *blpF*_{St}. “-” denotes the absence of *bac*_{St} genes. The presence (+) or absence (-) of *blpG*_{St}, encoding the putative disulfide oxidase, is also indicated.

^b In strain $\Delta 7$, the *blpG*_{St}-*blpX*_{St} operon is deleted, while *blpG*_{St} alone is deleted in strain $\Delta 8$.

^c NT, not tested.

^d Highly significant difference in the size of the inhibitory zone produced by the LMD-9 derivative compared to the wild-type LMD-9 strain (Student's *t* test with a *P* value of <0.01).

were diluted 100-fold in fresh M17G broth and incubated anaerobically at 42°C. At an OD₆₀₀ of 0.1, the synthetic D9C-30 peptide was added at a final concentration of 400 ng/ml, and the cultures were then reincubated for 2 h (final OD₆₀₀, 1.6). Small volumes (5 µl) of the induced cultures were then spotted directly on a 6-ml soft M17G layer (0.8% agar) containing 10⁸ CFU of indicator strains. The plates were incubated anaerobically at 42°C overnight before analysis of the inhibition zones surrounding the producer cells. For the overlay method (multilayer method), overnight cultures of *S. thermophilus* or *L. lactis* producer strains were diluted 100-fold in fresh M17G broth and incubated anaerobically at 42°C (*S. thermophilus*) or at 29°C (*L. lactis*). At an OD₆₀₀ of 1.0, 100 µl of the cultures was diluted 10⁶-fold in 6 ml of prewarmed soft M17G medium (0.8% agar) and uniformly poured on a petri dish containing a supporting layer of 25 ml solid M17G medium (2% agar). A second 6-ml soft M17G layer containing 400 ng/ml D9C-30 was poured on the layer of producer cells. Following growth of the producer strain (10 h of incubation), a third 6-ml layer of soft medium containing 10⁸ CFU of the indicator strain was added. The plates were incubated for 10 h before analysis of the inhibition zones surrounding the producer colonies. The composition of the growth medium used for the layer of indicator cells, as well as the incubation conditions, was adapted according to the indicator species: strains of *Streptococcus salivarius* were grown anaerobically in M17G broth at 42°C, strains of *Enterococcus faecalis* were grown in M17 with 0.5% (wt/vol)

glucose at 37°C, strains of *Lactobacillus* (29°C, aerobically) and *Clostridium* (37°C, anaerobically) were grown in MRS broth (Difco), strains of *Bacillus* (29°C, aerobically, for *Bacillus fusiformis*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus mycoides*, and *Bacillus thuringiensis*; 42°C, anaerobically, for *Bacillus coagulans*) were grown in LB broth, and strains of *Staphylococcus*, *Enterobacter*, *Micrococcus luteus*, *Salmonella*, and *Listeria* were grown in LB broth in the presence of oxygen at 37°C. The strains of *Staphylococcus*, *Enterobacter*, *Salmonella*, *Listeria*, *B. fusiformis*, *B. cereus*, *B. licheniformis*, *B. mycoides*, *B. thuringiensis*, *Micrococcus luteus*, *Enterobacter gergoviae*, *Escherichia hermannii*, *Pseudomonas aeruginosa*, and *Salmonella enterica* subsp. *enterica* serovar Bredeney were from the bacterial collection of the Unité de Microbiologie de l'Université catholique de Louvain (Louvain-la-Neuve, Belgium).

RESULTS

Inhibitory spectrum of *S. thermophilus* LMD-9. To date, Blp_{St}-associated antimicrobial activity of *S. thermophilus* LMD-9 has been reported to inhibit the growth of other *S. thermophilus* strains (15). Here, we further investigated the inhibitory spectrum

FIG. 1. Schematic representation of the genetic deletions performed in this study. Complete strain names and the corresponding genotypes are listed in Table 2. Genes encoding peptides with known or predicted functions are represented by patterned arrows: ABC transporter (small squares), accessory transport protein (large squares), induction factor (dark gray), response regulator (vertical lines), histidine kinase (horizontal lines), hydrophobic peptides of unknown function (black), hydrophilic peptides of unknown function (white), peptide similar to immunity proteins of class IIa bacteriocins (checkerboard squares), and modification protein (dots). Genes encoding the putative bacteriocins with a two-Gly leader are represented by light-gray arrows. The flags represent promoters, black flags for BlpC_{St}-induced promoters and gray flags for vegetative promoters. The hairpin structures indicate potential transcription terminators. The letters and numbers in italics refer to the corresponding *blp*_{St} and *orf* genes, respectively. (A) Deletions performed in the *blp*_{St} locus of strain LMD-9. (B) Deletions performed in the *blp*_{St} locus of strain LF109 [LMD-9 Δ (*blpU*_{St}-*blpF*_{St})].

of strain LMD-9 upon expression of the *blp*_{St} locus (400 ng/ml of the BlpC_{St}* pheromone, D9C-30); the susceptibilities of a range of bacterial strains were assayed using a multilayer protocol, as described previously (15). In total, 87 strains belonging to 31 bacterial species were tested as indicator strains. Among these, growth inhibition was observed for 33 strains, including all strains of *S. thermophilus* except LMG7952 and strains belonging to the closely related species *S. salivarius*, *E. faecalis*, and *L. lactis* (Table 3). Other species of LAB involved in food fermentations, such as *L. plantarum*, *L. sakei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus casei*, and *Pediococcus pentosaceus* were found to be resistant. Interestingly, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus*, used in cofermentations with *S. thermophilus* for the production of yogurt and cheese, respectively, were also not inhibited (Table 3). None of the gram-negative species tested (*E. gergovia*, *E. coli*, *E. hermannii*, *P. aeruginosa*, and *S. enterica* subsp. *enterica* serovar Bredeney) displayed sensitivity to the antimicrobial compounds produced by *S. thermophilus* LMD-9 (data not shown). Similarly, no antimicrobial activity was detected against the more distantly related gram-positive species *Staphylococcus aureus*, *Staphylococcus hominis*, *Clostridium acetobutylicum*, *B. cereus*, *B. thuringiensis*, *B. licheniformis*, *B. mycoides*, and *M. luteus* (data not shown). Notably, a number of exceptions were observed: *S. thermophilus* LMD-9 was found to inhibit the growth of the gram-positive species *B. coagulans*, *B. fusiformis*, and a number of *Listeria* species, including all strains of *Listeria innocua* and *Listeria seeligeri*, and of the pathogenic species *Listeria monocytogenes* and *Listeria ivanovii* (Table 3).

Thermophilin 9 is a multipetide bacteriocin. The *blpD*_{St}-*blpF*_{St} region is organized into three operons encoding a total of four

Bac_{St} peptides (BlpD_{St}, BlpU_{St}, BlpE_{St}, and BlpF_{St}) that show characteristics of class IIb bacteriocins (15). To confirm the implications of the three operons in the antimicrobial activity of strain LMD-9, the inhibitory spectrum of the Δ (*blpD*_{St}-*blpF*_{St}) mutant (Δ 1) (Fig. 1A and Table 3) was investigated. As expected, none of the 33 LMD-9-sensitive strains was inhibited. This shows that the region encompassing the three operons is responsible not only for the intraspecies inhibition, as previously shown for *S. thermophilus* LMG18311 and CNRZ1066 (15), but also for the interspecies antimicrobial activity of LMD-9. The relative contributions of the different *bac*_{St} genes to this antimicrobial activity was then investigated by analyzing the inhibitory spectra of three mutant strains (Δ 2, *blpD*_{St}⁺; Δ 3, *blpE*_{St}⁺ *blpF*_{St}⁺; Δ 4/ Δ 5, *blpU*_{St}⁺) (Fig. 1A), each carrying only one of the three *bac*_{St} operons, under conditions of induction of the *blp*_{St} locus (400 ng/ml of D9C-30). This assay was performed exclusively with the 33 indicator strains that showed sensitivity to the wild-type LMD-9 strain (Table 3).

Strain Δ 2, which contains only the *blpD*_{St}-*orf*2 operon, retained most of its antimicrobial activity; the cell target range and activity were only slightly attenuated compared to those of the wild-type strain (31/33 strains were inhibited). Growth inhibition of *S. thermophilus* SF16 and *L. lactis* IL-1403 was lost, while the sizes of the inhibitory zones for the indicator strains *L. lactis* MG1363 and *E. faecalis* JH2-SS were significantly reduced (Tables 3 and 4). In contrast, the sole expression of the *blpU*_{St}-*orf*3 operon (strain Δ 4/ Δ 5) failed to inhibit the growth of any of the 33 indicator strains (Table 3). The *blpE*_{St}-*blpF*_{St} operon alone (strain Δ 3) conferred a poor antimicrobial activity against *S. thermophilus* LMG18311, while growth of the

TABLE 4. Antimicrobial activities of *S. thermophilus* LMD-9 and derivatives against a defined set of indicator strains

Indicator strain	Inhibitory zone produced by the induced producer strains ^a							
	LMD-9 (<i>D U E F</i>) +	$\Delta 2$ (<i>D</i>) +	$\Delta 3$ (<i>E F</i>) +	$\Delta 4$ (<i>U E F</i>) +	$\Delta 5$ (<i>D U</i>) +	$\Delta 6$ (<i>D E F</i>) +	$\Delta 7$ (<i>D U E F</i>) –	$\Delta 8$ (<i>D U E F</i>) –
<i>S. thermophilus</i> LMG18311	6.5 $\pm$ 0.5	6.5 $\pm$ 0.5	3.5 $\pm$ 0.5 ^c	3.6 $\pm$ 0.4 ^c	6.7 $\pm$ 0.4	6.5 $\pm$ 0.5	6.5 $\pm$ 0.5	6.5 $\pm$ 0.5
<i>E. faecalis</i> JH2-2	3.5 $\pm$ 0.4	3.4 $\pm$ 0.3	ND ^b	ND	3.4 $\pm$ 0.4	3.5 $\pm$ 0.4	1.8 $\pm$ 0.2 ^c	1.9 $\pm$ 0.2 ^c
<i>E. faecalis</i> JH2-SS	3.5 $\pm$ 0.5	2.0 $\pm$ 0.1 ^c	ND	ND	2.0 $\pm$ 0.1 ^c	3.5 $\pm$ 0.5	1.9 $\pm$ 0.2 ^c	1.8 $\pm$ 0.2 ^c
<i>L. lactis</i> subsp. <i>cremoris</i> MG1363	3.0 $\pm$ 0.2	2.0 $\pm$ 0.1 ^c	ND	ND	2.0 $\pm$ 0.1 ^c	2.0 $\pm$ 0.1 ^c	1.9 $\pm$ 0.2 ^c	1.9 $\pm$ 0.2 ^c
<i>B. coagulans</i> NCIMB8523	3.0 $\pm$ 0.1	3.0 $\pm$ 0.2	ND	ND	3.0 $\pm$ 0.1	3.1 $\pm$ 0.2	2.0 $\pm$ 0.2 ^c	2.0 $\pm$ 0.1 ^c
<i>L. monocytogenes</i> IHE-92	3.3 $\pm$ 0.4	3.3 $\pm$ 0.3	ND	ND	3.4 $\pm$ 0.4	3.3 $\pm$ 0.4	2.4 $\pm$ 0.3 ^c	2.2 $\pm$ 0.2 ^c

^a Antimicrobial activity was assayed using the overlay protocol, in the presence of 400 ng/ml D9C-30. The numbers represent the radii of the inhibitory zones around the producer colonies measured around at least 60 colonies (20 colonies from three independent experiments). The results are given as mean values $\pm$ standard deviations. The letters in parentheses indicate the content in *bac*_{St} genes of the producer strain: *D*, *blpD*_{St}; *U*, *blpU*_{St}; *E*, *blpE*_{St}; *F*, *blpF*_{St}. The presence (+) or absence (–) of *blpG*_{St}, encoding the putative disulfide oxidase, is also indicated.

^b ND, not detected.

^c Highly significant difference in the size of the inhibitory zone produced by the LMD-9 derivative compared to the wild-type LMD-9 strain (Student's *t* test with a *P* value of <0.01) (Table 3).

remaining 32 indicator strains was not affected (Tables 3 and 4). These results strongly suggest that *blpD*_{St} encodes the key determinant of the antagonistic activity of strain LMD-9. To further validate this hypothesis, we determined the inhibitory spectra of strains Δ (*blpD*_{St}-*orf2*) ($\Delta 4$, *blpU*_{St}⁺ *blpE*_{St}⁺ *blpF*_{St}⁺) and Δ *blpD*_{St} Δ (*blpU*_{St}-*blpF*_{St}) ($\Delta 9$, *bac*_{St} mutant and *orf1*⁺ *orf2*⁺) (Fig. 1A and B). As expected, neither of these two mutant strains inhibited growth of any of the LMD-9-sensitive strains, with the exception of *S. thermophilus* LMG18311, which displayed sensitivity to strain $\Delta 4$ (Table 3 and 4).

The attenuated inhibitory spectrum and activity of strain $\Delta 2$ (*blpD*_{St}⁺) suggest that the growth inhibition of four LMD-9-sensitive strains (*S. thermophilus* SFi16, *L. lactis* IL-1403, *L. lactis* MG1363, and *E. faecalis* JH2-SS) relies on the coexpression of *blpD*_{St} together with *blpU*_{St} and/or *blpEF*_{St}. Strains Δ (*blpE*_{St}-*blpF*_{St}) ($\Delta 5$, *blpD*_{St}⁺ *blpU*_{St}⁺) and Δ (*blpU*_{St}-*orf6*) ($\Delta 6$, *blpD*_{St}⁺ *blpE*_{St}⁺ *blpF*_{St}⁺) were thus constructed in order to compare their inhibitory spectra to those of strains LMD-9 and $\Delta 2$ (*blpD*_{St}⁺). The results showed that the growth inhibition of *L. lactis* IL-1403 could occur only when both the *blpD*_{St}-*orf2* and *blpU*_{St}-*orf6* operons were expressed ($\Delta 5$) (Table 3), while the inhibition of *E. faecalis* JH2-SS required the coexpression of the *blpD*_{St}-*orf2* and *blpE*_{St}-*blpF*_{St} operons ($\Delta 6$) (Tables 3 and 4). Interestingly, the complete inhibition of *S. thermophilus* SFi16 and *L. lactis* MG1363 required the expression of all three *bac*_{St} operons (Tables 3 and 4).

Taken collectively, our results demonstrate that *blpD*_{St} is the main determinant of antimicrobial activity in *S. thermophilus* LMD-9 and that the coexpression of *blpU*_{St} and *blpEF*_{St} with *blpD*_{St} enhances the activity and cell target range of BlpD_{St}^{*}. The Bac_{St} peptides of *S. thermophilus* LMD-9 thus seem to be part of a multipetide bacteriocin, which was named thermophilin 9.

BlpG_{St} is a functional disulfide oxidase required for the antilisterial activity of thermophilin 9. We have shown previously that the *blpG*_{St}-*blpX*_{St} operon is not associated with intraspecies inhibition (15). Here, we investigated a possible role of these genes in the inhibition of indicator strains belonging to

other species. Deletion of this operon [strain Δ (*blpG*_{St}-*blpX*_{St}), $\Delta 7$] (Fig. 1A) completely abolished the growth inhibition of six of the eight LMD-9-sensitive *Listeria* strains (Table 3). In addition, this mutant strain displayed decreased antimicrobial activity against five indicator strains (*E. faecalis* JH2-SS and JH2-2, *L. monocytogenes* IHE-92, *B. coagulans* NCIB8523, and *L. lactis* MG1363), as measured by the size of the inhibitory zone (Table 4). The role of the putative modification protein BlpG_{St} in the attenuated interspecies inhibitory activity of the mutant strain $\Delta 7$ was further investigated by constructing an in-frame deletion of *blpG*_{St} alone ($\Delta 8$) (Fig. 1A). As shown in Tables 3 and 4, the two mutant strains exhibited identical inhibitory spectra, thus revealing the specific role of BlpG_{St} in interspecies inhibition.

Besides the CYYC thioredoxin motif (Fig. 2A), BlpG_{St} (230 aa) contains a putative 27-aa signal sequence with a predicted cleavage probability of 1.0 (prediction by signalP). This suggests that the protein is released at the cell surface, where it might promote the formation of disulfide bonds in the antimicrobial compounds secreted by *S. thermophilus* LMD-9. To validate this hypothesis, we tested the ability of *blpG*_{St} to complement the motility deficiency of a *dsbA*-deficient mutant of *E. coli* AH50 (21), as previously described (3). DsbA is a periplasmic disulfide oxidase that catalyzes the formation of disulfide bridges in proteins of the flagellar machinery of *E. coli* (6). A *blpG*_{St} expression vector was constructed by cloning the *blpG*_{St} ORF downstream of the P32 expression cassette carried by an autoreplicative plasmid. As shown in Fig. 2B, expression of *blpG*_{St} was able to restore motility in the *dsbA*-deficient background, showing that BlpG_{St} is exported in the periplasmic space and is a functional thiol-disulfide oxidase.

The functional role of *orf* genes in the innate immunity of *S. thermophilus* LMD-9. In a previous study, we demonstrated that the genetic determinants of immunity against thermophilin 9 are located within the *blpD*_{St}-*blpF*_{St} region (15). Each *bac*_{St} gene was shown to be cotranscribed with one or more *orf* genes (Fig. 1A). These *orf* genes encode small peptides that display structural similarities (high pI and the presence of one

FIG. 2. Functional role of BlpG_{St}. (A) Primary sequence alignment of the putative thioredoxin domains of BlpG_{St} from *S. thermophilus* LMD-9 and similar proteins (accession numbers: *S. mutans* UA159 Smu.1904c, NP722210; *Brochothrix campestris* ATCC 43754 BrcD, AAC95141; *Lactobacillus salivarius* UCC118 Orf1, AAM61773; *B. subtilis* 168 BdbB, CAB14062; *E. coli* K-12 DsbA, AAB02995). Blocks of conserved identical residues are shown on a black background. Amino acids showing conservation among all proteins are shown on a gray background. (B) Complementation of the motility deficiency of *E. coli* AH50 *dsbA* by expression of BlpG_{St}. Motility assays were performed on 0.3% agar-containing plates. The plates were incubated at 37°C for 20 h.

to four predicted transmembrane segments) (Table 1) to the immunity peptides of the class IIb bacteriocins brochocin C and lacticin F (2, 33), supporting their potential roles in thermophilin 9 immunity. The absence of a signal peptide and a two-Gly leader sequence suggests that the products of the *orf* genes are located in the cell membrane. To investigate which of these *orf* genes is required for immunity, we first determined the impact of deleting each *bac*_{St} operon individually: the immunity phenotypes of mutants Δ4 (*orf3*⁺ to *orf7*⁺), Δ5 (*orf1*⁺ to *orf6*⁺), and Δ6 (*orf1*⁺ *orf2*⁺ *orf7*⁺) were determined against the induced thermophilin 9-producing strain LMD-9 using the spot-on-lawn method. Among the three indicator strains tested, only strain Δ6 displayed resistance against LMD-9 (Table 5), suggesting that *orf7*, together with *orf1* and/or *orf2*, encodes the immunity determinants of thermophilin 9.

TABLE 5. Immunities of *S. thermophilus* LMD-9 and derivatives

Indicator strain	No. of <i>orf</i> genes ^b	Producer strain ^a				
		LMD-9 (D U E F)	Δ4 (U E F)	Δ6 (D E F)	Δ5 (D U)	Δ2 (D)
LMD-9 ^c	1–7	+	+	+	+	+
Δ1	—	—	+	—	—	—
Δ6	1, 2, 7	+	+	+	+	+
Δ4	3–7	—	+	—	—	—
Δ5	1–6	—	+	+	+	+
Δ3	7	—	+	—	—	—
Δ2	1, 2	—	+	+	+	+
Δ9	1, 2	—	+	+	+	+
Δ10	2	—	+	—	—	—

^a The immunity phenotype of the strains was determined by the spot-on-lawn method. “+” denotes the ability of the indicator strain to grow in the presence of the producer strain. The letters in parentheses indicate the content in *bac*_{St} genes of the producer strain: D, *blpD*_{St}; U, *blpU*_{St}; E, *blpE*_{St}; F, *blpF*_{St}.

^b Content in *orf* genes of the producer strain. “—” denotes the absence of *orf* genes.

FIG. 3. Antimicrobial activity of *L. lactis* NZ3900 containing the *blpD*_{St}-*orf2* (pGILF004) or the *blpD*_{St}-*orf2* and *blpAB*_{St} expression vector (pGILF005) against the indicator strain *S. thermophilus* LMG18311 (overlay method).

We further investigated the immunity specificities of the *orf1*, *orf2*, and *orf7* genes by comparing the resistances of strains Δ1 (no *orf* gene), Δ2 (*orf1*⁺ *orf2*⁺), and Δ3 (*orf7*⁺) against a set of LMD-9 derivatives producing only a subset of *Bac*_{St} peptides: Δ4 (*blpU*_{St}⁺ *blpE*_{St}⁺ *blpF*_{St}⁺), Δ6 (*blpD*_{St}⁺ *blpE*_{St}⁺ *blpF*_{St}⁺), Δ5 (*blpD*_{St}⁺ *blpU*_{St}⁺), and Δ2 (*blpD*_{St}⁺). As shown in Table 5, all strains were able to grow when tested against strain Δ4, with the *blpD*_{St}-*orf2* operon deleted, which supports the previous observation that BlpU_{St}^{*} and BlpE_{St}^{*} BlpF_{St}^{*} have no or poor antimicrobial activity, respectively. Reciprocally, expression of the *blpD*_{St}-*orf2* operon alone (indicator strain Δ2) was sufficient to confer immunity to all producer strains tested (Δ2, Δ4, Δ5, and Δ6), provided that all four *bac*_{St} genes were not expressed together (producer strain LMD-9; Table 5). Since BlpD_{St}^{*} is the main component of thermophilin 9 activity, these results suggest that *orf1* and/or *orf2* is specifically required for immunity against BlpD_{St}^{*}, while the concomitant expression of *orf7* is required against the antimicrobial activity resulting from a combination of BlpD_{St}^{*} with BlpU_{St}^{*} and BlpE_{St}^{*} BlpF_{St}^{*}.

To specifically assess the function of *orf1* in immunity to BlpD_{St}^{*}, the resistance spectra of strains bearing either both *orf1* and *orf2* genes [*ΔblpD*_{St} Δ(*blpU*_{St}-*blpF*_{St}), Δ9] (Fig. 1B) or *orf2* alone [*ΔblpD*_{St}-*orf1* Δ(*blpU*_{St}-*blpF*_{St}), Δ10] (Fig. 1B) were compared. As expected, strain Δ9 displayed a resistance spectrum identical to that of strain Δ2 (Table 5), showing that the BlpD_{St}^{*} immunity determinants were efficiently expressed in a background devoid of *blpD*_{St}. The absence of *orf1* (strain Δ10) resulted in the complete loss of immunity against all strains producing BlpD_{St}^{*} (Table 5), confirming that at least *orf1* is required in this process.

Heterologous production of Blp_{St} bacteriocins. The main determinants of the antimicrobial activity and immunity of thermophilin 9 were shown to be encoded within the *blpD*_{St}-*orf2* operon. In order to confirm these results, we tested the possibility of conferring BlpD_{St}-dependent antimicrobial activity to *L. lactis* by constitutive expression of the *blpD*_{St}-*orf2* operon. The operon was cloned in transcriptional fusion with the vegetative promoter P32 carried by the multicopy plasmid pMG36eT (40). Introduction of this construct (plasmid pGILF004) into *L. lactis* NZ3900 did not result in the production of antimicrobial compounds active against the indicator strain *S. thermophilus* LMG18311, as assayed with the overlay protocol (Fig. 3). Growth inhibition of *S. thermophilus* LMG18311 could be achieved only through the co-expression of the *blpD*_{St}-*orf2* operon with the transporter genes

blpAB_{St} in an artificial operon structure (plasmid pGILF005). *L. lactis* NZ3900(pGILF005) was active against the same strain carrying the cloning vector pMG36eT and against all LMD-9-sensitive *S. thermophilus* strains (data are shown only for LMG18311) (Fig. 3), except SFi16. This is consistent with the previous observation that all three *bac_{St}* operons are required for inhibition of strain SFi16 (Table 3). As for immunity, plasmid pGILF004 conferred resistance to the LMD-9 derivatives $\Delta 5$ (*blpD_{St}⁺ blpU_{St}⁺*) and $\Delta 6$ (*blpD_{St}⁺ blpE_{St}⁺ blpF_{St}⁺*), but not against LMD-9 itself (*blpD_{St}⁺ blpU_{St}⁺ blpE_{St}⁺ blpF_{St}⁺*) (data not shown). The immunity spectrum of NZ3900(pGILF005) was thus identical to that of the LMD-9 mutant bearing the *blpD_{St}-orf2* operon alone ($\Delta 2$), confirming the finding that *orf1* and *orf2* are sufficient to protect against BlpD_{St}^{*}.

These results demonstrate that the *blpD_{St}-orf2* operon is a bacteriocin/immunity-encoding module able to fulfill its functions in the absence of other genes from *S. thermophilus*. However, efficient secretion and maturation of BlpD_{St} require a specific transport system encoded by *blpAB_{St}*, which seems to have no functional counterpart in *L. lactis*.

DISCUSSION

S. thermophilus LMD-9 displays an inhibitory spectrum against gram-positive bacteria (10 sensitive species among 26 gram-positive species tested) and is mainly active against closely related species. Its cell target range was shown to depend on the secretion of at least three Bac_{St} peptides and the activity of BlpG_{St}, a thiol-disulfide oxidase specifically required for the inhibition of *Listeria* species.

Among the *bac_{St}* genes, the sole expression of *blpD_{St}* was sufficient to inhibit most indicator strains. Its coexpression with the *blpU_{St}*- and *blpE_{St}-blpF_{St}*-containing operons further increased the antimicrobial activity and cell target range of *S. thermophilus* LMD-9. The results obtained from our deletion analysis suggest that thermophilin 9 is a multicomponent bacteriocin whose activity results from the combination of BlpD_{St}^{*} with BlpU_{St}^{*}, BlpE_{St}^{*}, and/or BlpF_{St}^{*}. In this context, the BlpD_{St}^{*} and BlpU_{St}^{*} BlpE_{St}^{*} BlpF_{St}^{*} peptides, respectively, could be compared to the active (α) and nonactive (β) subunits of thermophilin 13 (30), brochocin C (33), or lactacin F (1), all belonging to the class IIb bacteriocins. Interestingly, coexpression of the genes encoding the α and β peptides of lactacin F from *Lactobacillus johnsonii* was also shown to expand its cell target range (1). For thermophilin 9, the combination of Bac_{St} peptides required for optimal growth inhibition appears to depend on the indicator species and strain. The efficient antimicrobial activity of thermophilin 9 could result from the formation of pores consisting of the α peptide BlpD_{St}^{*} and one or more of the β peptides BlpU_{St}^{*}, BlpE_{St}^{*}, or BlpF_{St}^{*}, as proposed for most two-component bacteriocins (18). Alternatively, the different Bac_{St} peptides could act synergistically to form pores of complementary specificities, resulting in increased efficiency, similar to what has been hypothesized for the one-component lactococcins A and B (class IIc) and the two-component lactococcin M (class IIb) of *L. lactis* (35).

To our knowledge, BlpG_{St} from *S. thermophilus* LMD-9 and BdbB from *B. subtilis* 168 (9) are the only examples of disulfide oxidases whose involvement in bacteriocin production has been demonstrated. However, their direct implication in the

formation of disulfide bridges in the associated bacteriocin has never been shown. With the exception of the conserved thio-redoxin motif (CXXC), the catalytic domain of BlpG_{St} differs significantly from those of BdbB (15% identity) and DsbA (30% identity). Higher similarities were observed to the catalytic domains of the putative thioredoxins BrcD (45% identity), Smu.1904c (64% identity), and Orf1 (60% identity), encoded in the class IIb bacteriocin loci involved in the production of brochocin C (33), mutacin V (19), and ABP-118 (14), respectively (Fig. 2A). Interestingly, these bacteriocins share structural features with BlpD_{St}^{*}, BlpU_{St}^{*}, and BlpE_{St}^{*}: these GA-rich peptides are predicted to form amphiphatic/hydrophobic α helices and to contain N- and C-terminal cysteine residues. Recently, the presence of one disulfide bridge was reported for BrcA (an α peptide of brochocin C) (17). In the present study, a correlation was observed between the presence of *blpG_{St}* in the producer strain and the antilisterial activity of thermophilin 9 (Table 3). Since only BlpD_{St}^{*} seems to be required for the inhibition of *Listeria* strains, it can be speculated that BlpG_{St} is required for the formation of intra- and/or intermolecular disulfide bridges between the cysteine residues of BlpD_{St}^{*}. These disulfide bridges might be important for the interaction of BlpD_{St}^{*} with the membrane in *Listeria* species, whose lipid composition is known to differ significantly from that of LAB membranes (16).

Different mechanisms of immunity against bacteriocins have been reported. The immunity factors can either interact with the bacteriocin itself (e.g., NisI), thus preventing its interaction with the membrane, or actively export the intracellularly accumulated bacteriocins (e.g., NisFEG or Smu1913 in *S. mutans*) (5, 32). Recently, a novel mechanism was reported for class IIa and class IIc bacteriocins, in which the immunity peptides bind to the complex involving the bacteriocin and its membrane receptor, the mannose phosphotransferase (PTS) system (8). However, this PTS system does not seem to be involved in binding of thermophilin 9 to the target membrane, since a mutant of the indicator strain *S. thermophilus* LMG18311 in which the mannose PTS system was inactivated displayed the same level of sensitivity to the LMD-9 producer strain (data not shown). The structural similarities between Orf7 from *S. thermophilus* LMD-9 and Smu1913 from *S. mutans* (four predicted transmembrane domains and a large number of basic residues at the C-terminal end) might suggest a similar mode of action. Immunity against BlpD_{St}^{*} could also occur through direct interactions of the Orf1, Orf2, and/or Orf7 peptide with BlpD_{St}^{*}, which displays opposite charge properties (Table 1).

In conclusion, our results show that the *blpD_{St}-orf2* operon encodes a functional bacteriocin/immunity module. A similar functional organization could be hypothesized for the *blpU_{St}-orf3* and *blpE_{St}-blpF_{St}* operons, although the cell target range of BlpU_{St}^{*} and BlpE_{St}^{*}/BlpF_{St}^{*} remains to be identified. The *S. thermophilus* strains used as indicator strains in this study all carry *blp_{St}* loci, which contain the *blpABC_{St}* and *blpRH_{St}* operons, as well as *blpG_{St}* (data not shown). The region located between *blpR_{St}* and *blpG_{St}*, which contains the bacteriocin/immunity determinants in strain LMD-9, is highly variable in size, between 2.5 kb (SFi16) and 4.5 kb (LMG7952). These strains also differ in the number and nature of the bacteriocin/immunity modules present in their *blp_{St}* loci: most strains carry only one bacteriocin operon (similar to *blpD_{St}-orf2* or *blpU_{St}-*

orf3), while LMG7952 bears two of these modules (similar to *blpD_{St}-orf2* and *blpE_{St}-blpF_{St}*). The presence of highly similar sequences upstream (*blpRH_{St}*) and downstream (*blpG_{St}*) of the bacteriocin/immunity modules, as well as the high sequence conservation between the promoter regions of all *bac_{St}* operons, suggests a plasticity mechanism for *blp_{St}* loci by the acquisition/loss of modules through homologous recombination, as proposed for the sakacin P cluster of *L. sakei* (34). The insertion element present in all sequenced clusters except that of LMD-9 might also play a role in this process. Finally, the modular nature of the *blp_{St}* locus offers interesting perspectives for the engineering of industrial LAB with the specific aim of inhibiting the growth of nonstarter strains or food-borne pathogen species, such as listeria.

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