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Target cell specificity of a bacteriocin molecule: a C-terminal signal directs lysostaphin to the cell wall of *Staphylococcus aureus*

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Microbial organisms secrete antibiotics that cause the selective destruction of specific target cells. Although the mode of action is known for many antibiotics, the mechanisms by which these molecules are directed specifically to their target cells hitherto have not been described. *Staphylococcus simulans* secretes lysostaphin, a bacteriolytic enzyme that cleaves staphylococcal peptidoglycans in general but that is directed specifically to *Staphylococcus aureus* target cells. The sequence element sufficient for the binding of the bacteriocin as well as of hybrid indicator proteins to the cell wall of *S.aureus* consisted of 92 C-terminal lysostaphin residues. Targeting to the cell wall of *S.aureus* occurred either when the hybrid indicator molecules were added externally to the bacteria or when they were synthesized and exported from their cytoplasm by an N-terminal leader peptide. A lysostaphin molecule lacking the C-terminal targeting signal was enzymatically active but had lost its ability to distinguish between *S.aureus* and *S.simulans* cells, indicating that this domain functions to confer target cell specificity to the bacteriolytic molecule.

Keywords: bacteriocin/lysostaphin/peptidoglycan/protein targeting/staphylococci

Introduction

Bacteriocin or antibiotic molecules cause the selective killing of sensitive bacteria and are thought to provide an advantage for the growth and survival of the producing microorganism (Kolter, 1992). Investigations into the mode of action of bacteriocins led to the discovery and elucidation of fundamental biological processes such as DNA replication (Garrido *et al.*, 1988), membrane transport (Pattus *et al.*, 1990) and cell wall biosynthesis (Strominger *et al.*, 1967). These studies also provided tangible results in that they supplied therapeutics for the treatment of human infections by bacterial pathogens. One important question in understanding the mode of action of antibiotics is the mechanism of target cell specificity that allows the bacteriocin to distinguish between the host and the target cell. Some colicins, for example, kill sensitive *Escherichia coli* cells by forming voltage-dependent ion channels after their insertion into the cytoplasmic membrane (Pattus *et al.*, 1990). Colicin-sensitive cells harbor a specific receptor protein in the outer membrane that allows the binding and specific import of the bacteriocin across the outer envelope of these strains (Benedetti *et al.*, 1991). Colicin-producing *E.coli* cells require an immunity determinant for protection against the secreted bacteriocin (Pilsl and Braun, 1995).

In contrast to the well-studied bacteriocins from Gram-negative bacteria, very little is known about the target cell specificity of antibiotic molecules directed against Gram-positive organisms. Most bacteriocins directed against Gram-positive cells are in fact small peptides, or microcins, that diffuse through the bacterial cell wall and exert their bactericidal action by inserting into the cytoplasmic membrane (Jack *et al.*, 1995). Because many of these molecules display broad killing activity for a wide variety of bacterial species, it is questionable whether or not these microcins display target cell specificity. Microcin-secreting organisms, therefore, require specific immunity proteins for protection from the bactericidal activity, but the molecular mechanisms of immunity have not been resolved yet (Jack *et al.*, 1995).

The Gram-positive bacterium *Staphylococcus simulans* secretes lysostaphin, a bacteriolytic enzyme that cleaves the pentaglycine cross-bridges of staphylococcal peptidoglycans, specifically that of the target organism *Staphylococcus aureus* (Schindler and Schuhardt, 1964). Pre-prolysostaphin is exported from the bacterial cytoplasm by an N-terminal leader peptide which is cleaved during membrane translocation (Heinrich *et al.*, 1987; Recsei *et al.*, 1987). Subsequently, 14 tandem repeats of a 13 residue peptide are proteolytically removed from the N-terminus of the secreted pro-enzyme, thereby generating mature lysostaphin in the culture medium (Heinrich *et al.*, 1987; Recsei *et al.*, 1987). The pentaglycine cross-bridges, the substrates for the bactericidal activity of lysostaphin (Browder *et al.*, 1965; Sloan *et al.*, 1977), are present in both *S.simulans* and *S.aureus* (Schleifer and Kandler, 1972), yet *S.simulans* host cells are not killed by the secreted bacteriocin. Thus, we wondered how lysostaphin could distinguish between the host and target cells.

Our results show that the information for target cell specificity is encoded within the lysostaphin molecule. A C-terminal domain of lysostaphin is both necessary and sufficient for the targeting of indicator molecules to the cell wall of *S.aureus*. Sequences homologous to this cell wall targeting signal were found in other proteins of Gram-positive bacteria, and we demonstrate that one of these elements, i.e. that of staphylococcal amidase (Wang *et al.*, 1991a), also functions to direct fusion proteins to the cell wall of *S.aureus*. Deletion of the targeting signal did not interfere with endopeptidase activity but abolished the bacteriolytic killing of *S.aureus* cells, indicating that this domain functions to address specifically the bacteriocin molecule to its target cells.

Results

Expression of lysostaphin in S.simulans and S.aureus cells

To identify the parameters responsible for bacteriocin targeting to *S.aureus* cells, we investigated the export, processing and cellular location of lysostaphin expressed in both its natural host and target cell organisms. Because the constitutively expressed bacteriocin would be expected to cause the bacteriolytic killing of *S.aureus*, we cloned the lysostaphin gene under control of the *bla*ZRI regulon (Wang *et al.*, 1991b) and induced its expression with the addition of methicillin. Staphylococcal cultures were pulse-labeled with [^{35}S]methionine and chased by adding non-radioactive methionine. During the pulse or at timed intervals of the chase, aliquots of the culture were precipitated with ice-cold trichloroacetic acid (TCA). The staphylococcal peptidoglycan was digested with *Chalaropsis* muramidase (Hash, 1963) and the samples were re-precipitated with TCA followed by immunoprecipitation with anti-lysostaphin prior to SDS–PAGE.

In its natural host *S.simulans*, pulse-labeled pre-pro-lysostaphin (LST) was exported and cleaved to the pro-form within 2 min of its synthesis. The processing of pro-lysostaphin to the mature enzyme occurred much more slowly, requiring >60 min (Figure 1). *S.simulans* culture supernatants were analyzed for the presence of pro- and mature lysostaphin during logarithmic growth and stationary phase. We found that the processing of pro-lysostaphin occurred almost exclusively during stationary phase. When expressed in *S.aureus*, pre-pro-lysostaphin was converted very rapidly to the mature species ($t_{1/2}$ <30 s) without a detectable pro-lysostaphin intermediate. Pulse-labeled lysostaphin could only be immunoprecipitated within 5 min of the pulse, indicating that when synthesized by *S.aureus* cells the bacteriocin was quickly degraded. The addition of *S.simulans* culture medium containing pulse-labeled pro-lysostaphin to *S.aureus* cells did not increase the processing of the pro-form to the mature species (data not shown).

To distinguish secreted from cell-associated lysostaphin, staphylococci were centrifuged and the medium (MD) was separated from the cells (Φ). The cell pellet was subjected to peptidoglycan degradation with muramidase, and both medium and pellet fractions were precipitated with TCA. In pulse-labeled *S.simulans* cultures, lysostaphin was found exclusively in the culture medium, whereas all lysostaphin immunoprecipitated from *S.aureus* was located in the cell pellet fraction (Figure 1). TCA precipitation of staphylococcal cultures followed by suspension in hot SDS causes the solubilization of only those proteins that are secreted into the medium, whereas all cellular proteins remain insoluble unless the thick peptidoglycan layer of these organisms has been degraded (Schneewind *et al.*, 1992). We took advantage of this and measured the secretion of lysostaphin by comparing its solubility in hot SDS either with (H) or without prior peptidoglycan degradation (CH). Lysostaphin secreted into the medium of *S.simulans* cultures was directly soluble in hot SDS. In contrast, all lysostaphin synthesized in *S.aureus* cells required prior peptidoglycan degradation for solubility in hot SDS, indicating that lysostaphin remained cell associated and trapped within the staphylococcal cell wall.

The cell wall targeting signal of lysostaphin

The primary structure of lysostaphin displays two notable features: 14 N-terminal repeats (NTR) of a 13 residue peptide that are absent from mature lysostaphin, and 92 C-terminal amino acids with homology to the C-terminus of staphylococcal amidase (LYTA). We asked if these elements contribute to the targeting of lysostaphin to *S.aureus* cells (Figure 1). Deletion of the 14 N-terminal repeats of lysostaphin ($LST_{\Delta NTR}$) did not alter the cellular location of the mutant molecule. We were surprised to find that a mutant lysostaphin lacking its C-terminal 92 residues ($LST_{\Delta CWT}$) was secreted into the culture medium of *S.aureus*. Cleavage of the mutant precursor to the pro-form was slower ($t_{1/2}$ = 2 min) and processing of its 14 tandem repeats required >60 min; rates similar to those measured for wild-type lysostaphin secreted into the culture medium of *S.simulans*. These results indicated that the C-terminal 92 residues of lysostaphin were required for bacteriocin targeting to the envelope of *S.aureus* cells as well as for the rapid processing of the pro-form.

Targeting of hybrid proteins to the cell wall of S.aureus

To test whether the C-terminal domain of lysostaphin was sufficient for the targeting of indicator molecules to *S.aureus* cells, we employed enterotoxin B (SEB), a protein normally secreted into the staphylococcal medium (Tweten and Iandolo, 1983). The cellular location of hybrid SEB molecules was analyzed by fractionating pulse-labeled staphylococcal cultures into the medium, cell wall, cytoplasm and membrane compartments. A chimeric protein with a fusion of the C-terminal domain of lysostaphin to the C-terminus of enterotoxin B (SEB–LST) was located in the cell wall compartment of *S.aureus* and was only soluble in hot SDS after the peptidoglycan had been degraded (Figure 2). As a control for the correct fractionation of staphylococci, our studies included indicator molecules that were either secreted into the medium (SEB), covalently linked to the peptidoglycan (SEB–$SPA_{490-524}$) or membrane anchored (SEB–ACTA) (Schneewind *et al.*, 1993).

Surface proteins of *S.aureus* are covalently linked to the bacterial peptidoglycan via a C-terminal sorting signal. The covalent linkage of proteins to the cell wall can be measured by specifically cleaving the staphylococcal peptidoglycan with two different enzymes (Ghuysen, 1968), thereby releasing anchored protein with different amounts of linked cell wall (Schneewind *et al.*, 1993). To test for a peptidoglycan linkage of SEB–LST, we digested the cell wall of pulse-labeled staphylococci with either lysostaphin (L) or muramidase (H, Hash-enzyme) and compared the mass of immunoprecipitated proteins on SDS–PAGE (Figure 2C). Peptidoglycan digestion with either lysostaphin or muramidase did not alter the migration of SEB–LST on SDS–PAGE, indicating that the hybrid molecule was not covalently linked to the staphylococcal cell wall. As a control for a cell wall-linked protein, cleavage of the peptidoglycan with muramidase released SEB–$SPA_{490-524}$ as a spectrum of fragments with increasing mass, all of which migrated more slowly on SDS–PAGE than the lysostaphin-released counterpart (Schneewind *et al.*, 1993).

Taken together with the results presented in the previous

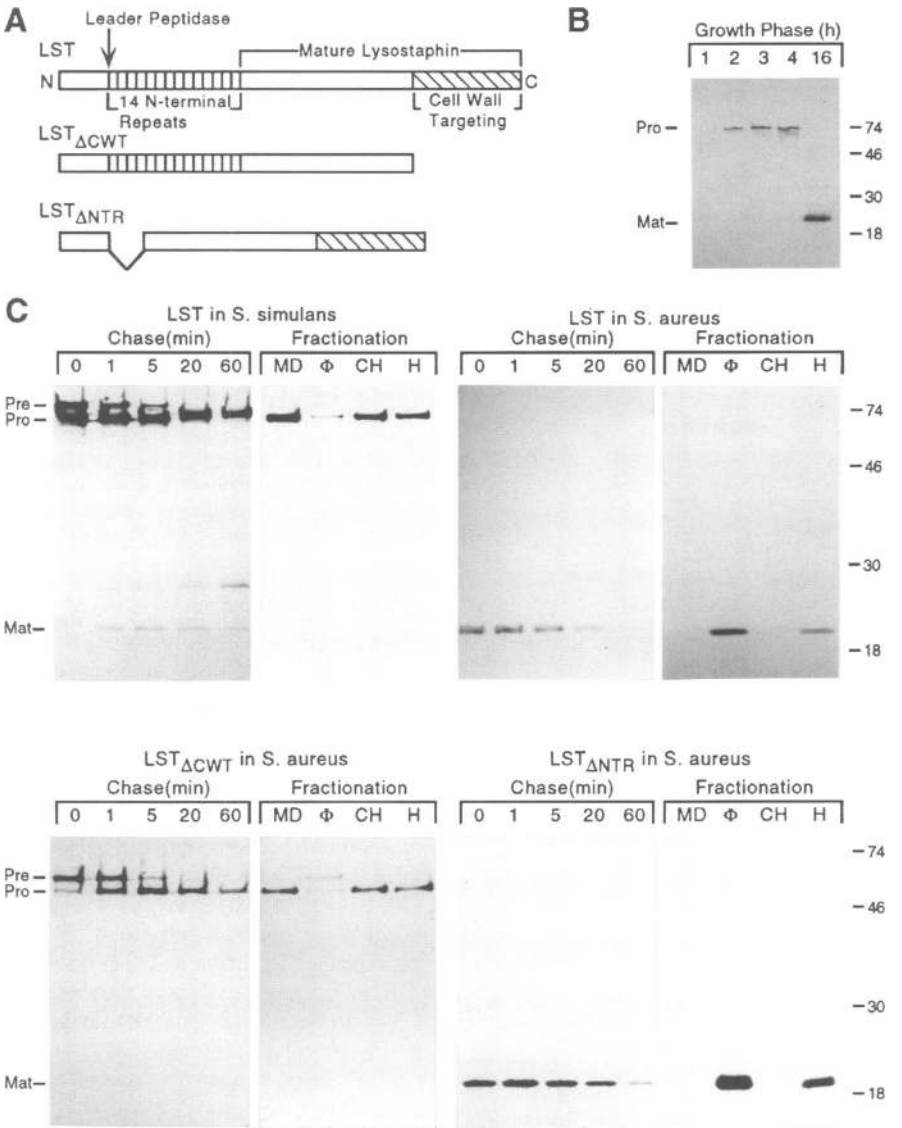

Fig. 1. Export, processing and cellular location of lysostaphin. (**A**) The drawing shows the structures of wild-type lysostaphin (LST) and its mutants with a deletion of either the C-terminal cell wall targeting domain ($LST_{\Delta CWT}$) or the 14 N-terminal repeats ($LST_{\Delta NTR}$). Pre-pro-lysostaphin consists of an N-terminal leader peptide (open bar) followed by 14 N-terminal tandem repeats (NTR) of a 13 residue peptide, which are cleaved from pro-lysostaphin to generate the mature enzyme. (**B**) *S.simulans* cells were inoculated into fresh media and grown at 37°C. At timed intervals, aliquots of the culture were withdrawn, the cells pelleted by centrifugation and the supernatant precipitated with TCA. The precipitates were boiled in sample buffer, separated on SDS–PAGE and immunoblotted with a lysostaphin-specific antiserum. (**C**) Staphylococci were pulse-labeled with [^{35}S]methionine for 2 min followed by a chase of non-radioactive methionine. Aliquots of the pulse-labeled culture were precipitated with TCA during the pulse (0) and 1, 5, 20 or 60 min after the addition of the chase. Pulse-labeled lysostaphin was immunoprecipitated, separated on 12% SDS–PAGE and fluorographed. To separate secreted from cell-associated lysostaphin, pulse-labeled cultures were fractionated into medium (MD) and cell pellet (Φ) and precipitated with TCA. Proteins in the cell pellet (Φ) were released from the peptidoglycan by treatment with muramidase followed by TCA precipitation. In parallel, pulse-labeled cultures were precipitated with TCA in duplicate. Proteins secreted into the staphylococcal medium were solubilized by boiling in SDS (CH), whereas all cellular proteins of staphylococci were solubilized in hot SDS after the peptidoglycan had been digested with muramidase (H). Molecular weight markers are indicated (kDa).

section, this demonstrates that the C-terminal 92 residues of lysostaphin were necessary and sufficient for protein targeting to the cell wall of *S.aureus*. We therefore named this element the cell wall targeting (CWT) signal. Previous work reported homology between the C-terminal sequences of staphylococcal amidase and lysostaphin (56% sequence identity) (Wang *et al.*, 1991a). To ask whether the C-terminal domain of amidase (LYTA) has a function similar to that of the lysostaphin targeting signal, we fused C-terminal amino acids of LYTA to SEB and found that this hybrid molecule was also targeted to the cell wall of *S.aureus* (Figure 2).

To investigate if proteins targeted to the wall of *S.aureus* were displayed on the cell surface, we measured the binding of FITC-labeled immunoglobulin to protein A located on the staphylococcal surface via UV light microscopy. The C-terminal end of wild-type protein A (SPA) is linked to and buried within the bacterial cell wall, whereas its N-terminal immunoglobulin binding domains bind FITC-labeled IgG on the cell surface as indicated by a strong fluorescent halo surrounding staphylococci (Figure 3). When the C-terminal sorting signal of protein A was replaced with the targeting signal of lysostaphin (SPA_{CWT}), the binding of FITC-labeled IgG to the staphylo-

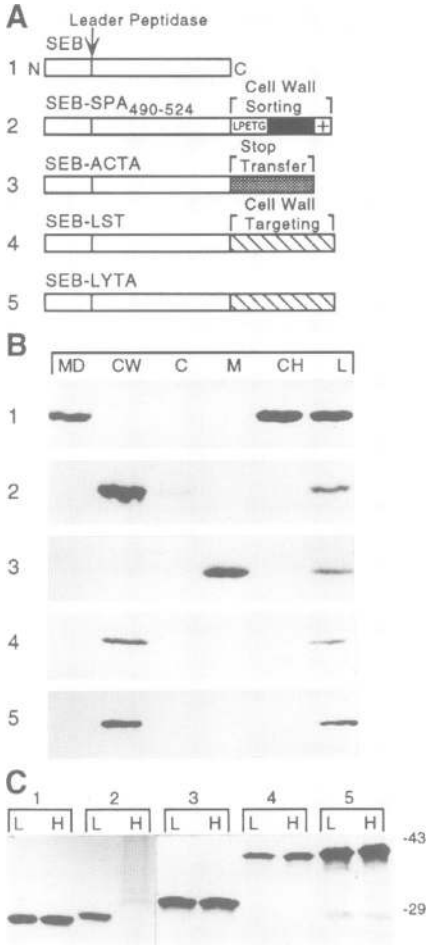

Fig. 2. Targeting of enterotoxin B to the cell wall of *S.aureus*. (**A**) The drawing shows the structures of enterotoxin B (SEB, 1) and its hybrid proteins with a C-terminal fusion of: (2) the cell wall sorting signal of protein A which consists of the LPXTG motif (LPETG), the C-terminal hydrophobic domain (black bar) and the charged tail (+); (3) the membrane anchor segment of *Listeria monocytogenes* ActA (stippled bar); (4) the cell wall targeting domain of lysostaphin (hatched bar); (5) the cell wall targeting domain of staphylococcal amidase (LYTA) (**B**) *S.aureus* OS2 expressing the genes of various chimeras was pulse labeled with [^{35}S]methionine for 1 min and chased with non-radioactive amino acids for 5 min. The culture was fractionated into medium (MD), cell wall (CW), cytoplasm (C) and membrane (M) compartments. In parallel, 1 ml of culture was pulse labeled and chased, and two 500 µl aliquots were each precipitated with TCA. One of these samples was lysostaphin digested (L) prior to boiling in hot SDS, whereas the other sample (CH) was boiled directly in hot SDS. Proteins were immunoprecipitated with anti-SEB and analyzed by SDS–PAGE and fluorography. (**C**) Pulse-labeled staphylococci were precipitated with TCA in duplicate. The two samples were digested with either lysostaphin (L) or *Chalaropsis* muramidase (H, Hash-enzyme). Proteins were precipitated with TCA, immunoprecipitated with anti-SEB and analyzed on 12% SDS–PAGE.

coccal surface occurred with an intensity similar to that measured for wild-type protein A. No binding of FITC-labeled IgG to the cell surface was observed for cells that produce a protein A mutant (SPA_{1-519}) that is secreted into the culture medium (Schneewind *et al.*, 1992) (Figure 3).

If the targeting signal of lysostaphin was sufficient to direct bacteriocin molecules to *S.aureus* cells, the addition of exogeneous hybrid proteins to staphylococcal cultures should allow their targeting to *S.aureus* but not to *S.simulans* cells. This prediction was tested by purifying a

Table I. Target cell specificity and bacteriolytic activity of lysostaphin

	Substrate			Target specificity
	S.simulans	*S.aureus*	Acetyl-Gly_6	
Mock	3.8×10^5	4.0×10^5	0	–
Mature LST	2.1×10^5	4	3.86×10^4	5.3×10^4
Pro-LST	3.1×10^5	5.5×10^2	3.58×10^4	5.6×10^2
Pro-$LST_{\Delta CWT}$	3.1×10^5	2.4×10^5	3.23×10^4	1.3

Purified mature lysostaphin (LST), pro-lysostaphin (Pro-LST) or a mutant pro-lysostaphin lacking the C-terminal targeting signal (Pro-$LST_{\Delta CWT}$) were incubated with a suspension of *S.simulans* and *S.aureus* cells and the surviving bacteria were counted by plating on agar plates. The target specificity was calculated as the number of surviving host cells (*S.simulans*) divided by the number of surviving target cells (*S.aureus*). The amount of added enzyme [60 ng (LST) or 60 µg (Pro-LST and Pro-$LST_{\Delta CWT}$)] was adjusted to ~3.5×10^4 endopeptidase units employing acetyl-Gly_6 as a substrate. The results shown are representative of three independent experiments.

hybrid glutathione *S*-transferase (GST; Smith and Johnson, 1988) with a fused targeting signal (GST–CWT) and measuring its binding to *S.aureus* and *S.simulans* cells (Figure 4). We quantitated the binding of GST–CWT to staphylococci by collecting cells with bound protein via centrifugation and measuring the decrease of GST activity in the supernatant. *S.aureus* precipitated GST–CWT from the supernatant whereas *S.simulans* did not. Increasing amounts of *S.aureus* cells depleted increasing amounts of GST–CWT from the supernatant in a near linear manner (2×10^7 up to 1×10^8 c.f.u.). Nevertheless, increasing the number of *S.aureus* cells to $>1\times10^8$ c.f.u. did not result in a further depletion of GST–CWT from the supernatant. This effect may be caused either by a population of GST–CWT molecules that is unable to bind *S.aureus* cells or by a receptor molecule that is partially soluble and hence does not allow the complete precipitation of bound GST–CWT.

In another assay, staphylococci were incubated with GST–CWT, collected by centrifugation, boiled in SDS and soluble components were finally analyzed by SDS–PAGE. Since staphylococci do not lyse in hot SDS, this treatment results in the selective solubilization of GST–CWT bound to the cell surface. Unbound GST–CWT left in the supernatant was precipitated with TCA, separated on SDS–PAGE and all hybrid protein was identified by Coomassie staining. Increasing the concentration of *S.aureus* cells caused greater depletion of GST–CWT from the supernatant and accumulation in the cell pellet similar to that observed by measuring GST activity in cell supernatants. By dividing the molar amount of added fusion protein by the number of cells required for 50% precipitation, we calculated the number of binding sites for the targeting signal to be 10^6/c.f.u. No significant precipitation of GST–CWT fusion protein occurred when it was added to *S.simulans* cells.

The targeting signal confers killing specificity onto lysostaphin

We developed an assay to measure specifically the bacteriolytic activity and target cell specificity of lysostaphin (Table I). Lysostaphin or its mutants were incubated with a mixture of *S.simulans* and *S.aureus* cells, and

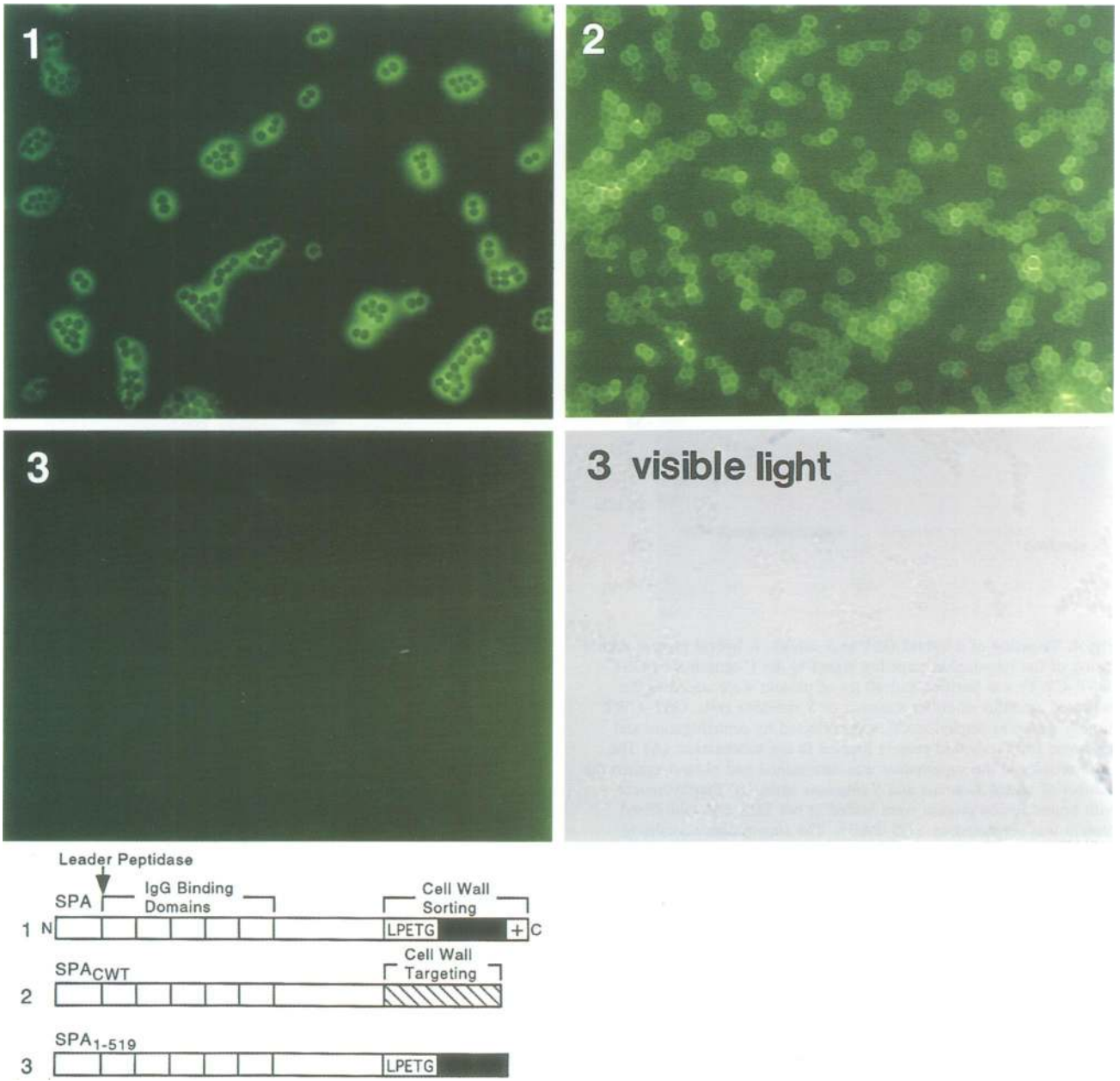

Fig. 3. Surface display of proteins targeted to the cell wall of *S.aureus*. Staphylococci expressing wild-type or mutant protein A were harvested by centrifugation, washed and incubated with FITC-labeled rabbit immunoglobulin (FITC-IgG). Binding of FITC-IgG to protein A located on the staphylococcal surface was visualized by photomicrography under UV light (1000-fold magnification). *S.aureus* OS2 expressing either wild-type protein A (SPA) (1), protein A with a C-terminal targeting signal replacing the wild-type sorting signal (SPA_{CWT}) (2) or a mutant protein A (SPA_{1-519}) that is secreted into the culture medium (3). The presence of staphylococci in panel (3) is demonstrated by light microscopy (3, visible light).

aliquots were plated on tryptic soy agar containing either erythromycin or streptomycin, thereby allowing the selective growth of either *S.aureus* (eythromycin resistant) or *S.simulans* (streptomycin resistant) survivors. Purified mature lysostaphin (3.86×10^4 units) caused the selective killing of all but four out of 4×10^5 *S.aureus* target cells, whereas only few *S.simulans* cells were killed. We calculated the target cell specificity of mature lysostaphin as the number of surviving host cells (*S.simulans*) divided by the number of surviving target cells (*S.aureus*) and obtained a factor of 5.3×10^4.

To purify mutant bacteriocins, recombinant pro- and mature lysostaphin were expressed in *E.coli*. Measurements of lysostaphin activity in *E.coli* cell extracts revealed that recombinant pro-lysostaphin was active whereas recombinant mature lysostaphin was not (data not shown), suggesting that the pro-region (14 N-terminal repeats) may function as an intramolecular chaperone for folding (Zhu *et al.*, 1989). When tested with an acetylated hexaglycine substrate (Kline *et al.*, 1994), the purified pro-lysostaphin (Pro-LST) was significantly less active than mature lysostaphin isolated from *S.simulans*. This reduction is probably caused by the inefficient re-folding of pro-lysostaphin, because the recombinant molecule had been purified under denaturing conditions. Pro-LST (3.58×10^4 units) was tested for its bacteriolytic activity,

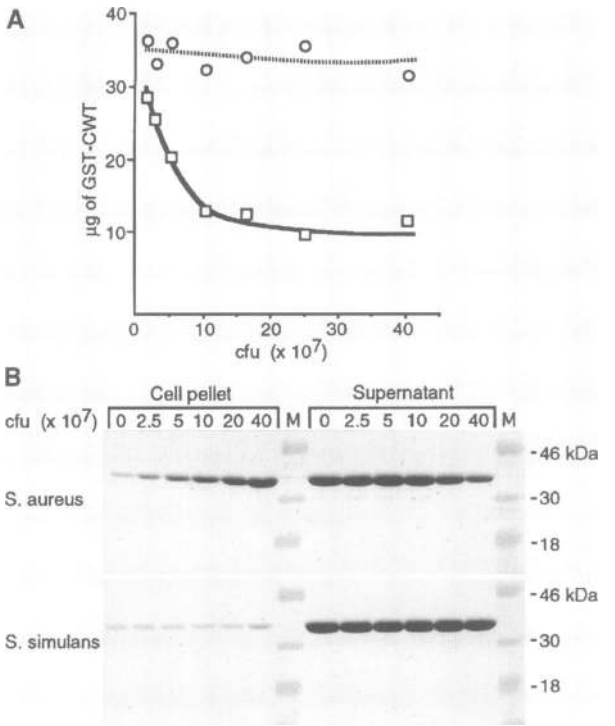

Fig. 4. Targeting of a hybrid GST to *S.aureus*. A hybrid protein with a fusion of the lysostaphin targeting signal to the C-terminus of GST (GST–CWT) was purified and 40 µg of protein were added to the indicated amounts of either *S.aureus* or *S.simulans* cells. GST–CWT protein bound to staphylococci was collected by centrifugation and separated from unbound protein located in the supernatant. (**A**) The GST activity in the supernatant was determined and plotted against the number of added *S.aureus* and *S.simulans* cells. (**B**) Staphylococci with bound fusion protein were boiled in hot SDS and solubilized protein was separated on SDS–PAGE. The supernatant containing unbound GST–CWT protein was precipitated with TCA and subjected to SDS–PAGE followed by Coomassie staining.

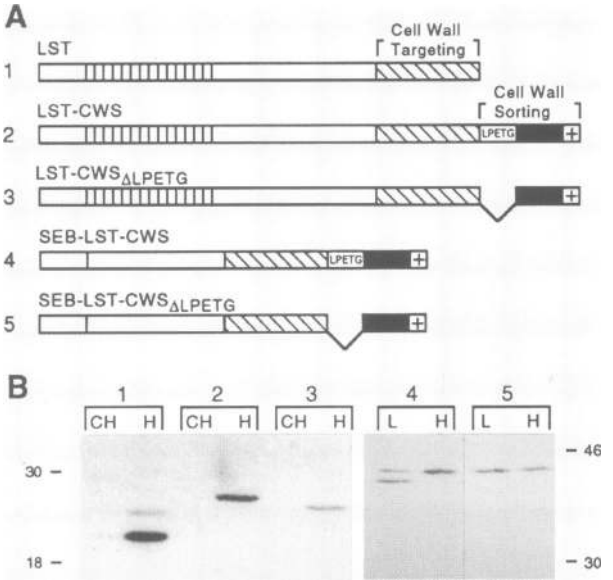

Fig. 5. The targeting signal of lysostaphin interferes with the sorting of surface proteins. (**A**) The drawing displays the structures of wild-type lysostaphin (LST, 1) and hybrid proteins with a C-terminal fusion of: (2) the cell wall sorting signal of protein A; (3) a sorting signal devoid of its LPXTG motif. In addition, we constructed hybrid enterotoxin B molecules with a C-terminal fusion of both the lysostaphin targeting signal and the protein A sorting signal (4) or a similar hybrid molecule without an LPXTG motif (5). When fused to exported proteins, the sorting signal causes cleavage at the LPXTG motif and the linkage of the polypeptide chain to the bacterial peptidoglycan. (**B**) The cell wall linkage of the hybrid proteins was analyzed by digesting pulse-labeled staphylococci with muramidase (H), which solubilizes anchored proteins as a spectrum of fragments with increasing mass due to linked peptidoglycan fragments (see Figure 2C, sample 2). As a control, TCA-precipitated samples were either boiled in hot SDS (CH) or subjected to lysostaphin digestion (L). Hybrid proteins were immunoprecipitated with either anti-lysostaphin (1–3) or anti-enterotoxin B (4–5), separated on 12% SDS–PAGE and fluorographed.

and we found that the targeting specificity of the pro-enzyme was somewhat reduced (target cell specificity 5.6×10^2). In contrast, a mutant pro-lysostaphin molecule lacking the C-terminal targeting signal (Pro-LST$_{\Delta CWT}$, 3.23×10^4 units) had lost its ability specifically to destroy *S.aureus* cells (target cell specificity 1.3). This result indicated that the enzymatic endopeptidase activity of lysostaphin is not sufficient for the specific killing of *S.aureus* cells, but that the target specificity of this bacteriocin is dependent on its C-terminal targeting signal.

The targeting signal interferes with the sorting of surface proteins

Cell wall sorting signals, consisting of an LPXTG motif, hydrophobic domain and charged tail, cause the covalent anchoring of surface proteins to the Gram-positive cell wall (Schneewind *et al.*, 1992). During anchoring, the sorting signal is cleaved between the threonine and the glycine of the LPXTG motif (Navarre and Schneewind, 1994), and the liberated carboxyl of threonine subsequently is amide linked to a free amino in the pentaglycine cross-bridge of the staphylococcal peptidoglycan (Schneewind *et al.*, 1995). We tried to covalently link lysostaphin to its normal substrate, the pentaglycine cross-bridge, by fusing the sorting signal of protein A to the C-terminal end of the polypeptide chain (LST–CWS) (Figure 5). The covalent linkage of the LST–CWS molecule was measured by digesting the peptidoglycan of *S.aureus* with muramidase. We were surprised to find that the hybrid LST–CWS molecule migrated uniformly on SDS–PAGE, suggesting that either the chimeric molecule was not linked to the peptidoglycan or its own enzymatic activity caused its uniform solubilization from the peptidoglycan, a feature characteristically observed after lysostaphin digestion of cell wall-linked proteins.

To distinguish between these two possibilities, we exploited the fact that the cleavage of the sorting signal at the LPXTG motif causes cell wall-linked proteins to migrate faster on SDS–PAGE than their uncleaved and unanchored mutant counterparts. We constructed a hybrid lysostaphin molecule with a fused sorting signal devoid of its LPETG sequence (LST–CWS$_{\Delta LPETG}$) and found that this molecule migrated faster on SDS–PAGE than the LST–CWS protein harboring a wild-type sorting signal. This result indicated that the sorting signal of the LST–CWS protein had not been cleaved and that the molecule had therefore not been linked to the peptidoglycan of *S.aureus*.

To determine whether the sorting failure of LST–CWS was due to a general property of lysostaphin, we tested the cell wall linkage of another protein harboring both

targeting and sorting signals (Figure 5). The sorting signal of protein A was fused to the C-terminus of SEB–LST. When solubilized with lysostaphin, two different SEB–LST–CWS species were observed on SDS–PAGE. The slower migrating species was also present in muramidase-solubilized samples and it was therefore not anchored to the staphylococcal cell wall. In contrast, the faster migrating species of lysostaphin-digested samples was solubilized as a spectrum of fragments with increasing mass, indicating that part of SEB–LST–CWS protein had been linked to the peptidoglycan (data not shown). Furthermore, the unanchored species of SEB–LST–CWS migrated more slowly on SDS–PAGE than a control molecule with a defective LPXTG motif, which suggests that its sorting signal had not been cleaved. Thus, the targeting signal of lysostaphin was, at least in part, responsible for the failure of the sorting signal to link lysostaphin to the staphylococcal cell wall.

Discussion

Our results provide evidence that lysostaphin is addressed specifically to its target organism *S.aureus*. The information for target cell specificity is encoded within the C-terminal 92 residues of lysostaphin. Because *S.simulans* and *S.aureus* cells have a similar peptidoglycan structure (Schleifer and Kandler, 1972), it is likely that the targeting domain of lysostaphin recognizes a non-peptidoglycan component of the *S.aureus* cell wall. The targeting mechanism is not required for the enzymatic activity of lysostaphin because a mutant bacteriocin truncated for its targeting signal retained activity, although it had lost the ability to specifically kill *S.aureus* cells. These results also suggest that the targeting mechanism may be responsible, at least in part, for the relative immunity of *S.simulans* to secreted lysostaphin. Only when incubated in the presence of large amounts of enzyme can the peptidoglycan of *S.simulans* be digested completely with lysostaphin (Heinrich *et al.*, 1987), indicating that the cell wall of the host organism does serve as a substrate of bacteriocin activity.

Recently, Sloan and co-workers proposed a hypothesis for the immunity of *S.simulans* to the secreted lysostaphin (Dehart *et al.*, 1995). When *S.simulans* is cured of the plasmid encoding the lysostaphin gene, it displays an increased sensitivity to lysostaphin, similar to that of *S.aureus* cells (Heath *et al.*, 1989). Cloning of the lysostaphin gene and its flanking DNA sequences into *S.aureus* resulted in viable cells that were less sensitive to lysostaphin digestion, suggesting that at least two genes specifying for lysostaphin and a proposed immunity determinant (*epr*) were present. Compositional peptidoglycan analysis suggested that both wild-type *S.simulans* and *epr*-transformed *S.aureus* cells contain an increased amount of serine over glycine residues (Dehart *et al.*, 1995). The authors proposed that an altered peptidoglycan structure, for example a replacement of glycine with serine in the wall cross-bridge, may be responsible for the relative immunity of *S.simulans* to secreted lysostaphin. Another interpretation of these data would be that the *epr* determinant functions to alter the bacterial envelope so that it cannot be recognized by the targeting signal of lysostaphin. This hypothesis would certainly be consistent with the well-established fact that the cell wall of *S.simulans* can be digested with lysostaphin.

Jayaswal and co-workers reported the homology of C-terminal sequences between lysostaphin and staphylococcal phage amidase (Wang *et al.*, 1991a). We demonstrate here that the C-terminal sequence of staphylococcal amidase also functions as a targeting signal to direct proteins to the staphylococcal cell wall. A database search identified homologies between the targeting signal of lysostaphin and C-terminal sequences of cell wall-associated proteins of other Gram-positive bacteria (data not shown). These findings are reminiscent of previously reported work on the modular organization of peptidoglycan hydrolases from pneumococci (Díaz *et al.*, 1989). The C-terminal repeat domains of *Streptococcus pneumoniae* amidase, for example, promote its binding to choline within the pneumococcal cell wall (Sanz *et al.*, 1992), a compound that is not present in staphylococcal peptidoglycans. Currently, we are purifying the lysostaphin receptor from the cell wall of *S.aureus*. Our measurements of 10^6 lysostaphin binding sites per colony-forming unit suggest that the lysostaphin receptor may not be a protein molecule.

Because the bacterial cell wall is a complex organelle requiring both concerted assembly and turnover at specific sites, it is plausible that targeting domains of enzymes responsible for such processes direct the molecules to their specific locations within the cell wall. For example, the localized hydrolysis and synthesis of peptidoglycan during cell division at the newly forming septum requires enzymes to be properly addressed to this site (Oshida *et al.*, 1995; Yamada *et al.*, 1996). The molecular information for these events will probably be encoded in both the targeted polypeptide chains as well as the specific chemical nature of a distinct site within the cell wall. To understand these targeting mechanisms in detail, we will pursue our initial observation and characterize the molecular substrate of lysostaphin binding to the cell wall of *S.aureus*. By studying several such targeting events, a detailed chemical map of distinct topographical sites within the bacterial cell wall may perhaps be obtained and we may thus arrive at a better understanding of this complex cellular organelle. Furthermore, this information may also provide us with novel targets for anti-microbial therapy, which is particularly urgent at a time when many Gram-positive pathogens have developed resistance mechanisms to almost all known antibiotics.

Materials and methods

Plasmids

In order to express lysostaphin (*lst*) in *S.aureus*, we cloned the *lst* gene under the control of the β-lactamase regulon (*bla*ZRI), thereby allowing its inducible expression via the addition of methicillin to staphylococcal cultures. The plasmid pLST was assembled from three components: (i) the lysostaphin gene, (ii) *bla*ZRI sequences encoding the repressor (*bla*I), the signal transducer (*bla*R) as well as the promoter of the β-lactamase regulon and (iii) the *E.coli–S.aureus* shuttle vector pOS1 (Schneewind *et al.*, 1993). The *lst* gene was amplified with PCR using *S.simulans* ATCC1362 DNA as a template with the primers LS-Nde (5′-AACATATGAAGAA AACAAAAAACAATTATTATA-3′) and LS-Bam (5′-AAGGATCCT-CACTTTATAGTTCCCCAAA-3′) and digested with *Nde*I and *Bam*HI. The *bla*ZRI sequences were amplified from pI258 (Novick and Richmond, 1965) with the primers Bla-Pro-Nde (5′-TCCCCCGGGCATATGA-AAACCCTCCGATATTACAGTT-3′) and Bla-18 (5′-AAGAATTCC-ATATCTCTAATTACTTTTTACTAA-3′) and digested with *Nde*I and

*Eco*RI. Finally, pOS1 was digested with *Eco*RI and *Bam*HI and all three fragments were ligated and electroporated into *S.aureus* OS2. The *Nde*I (CATATG) and *Bam*HI sites of pLST allowed the precise insertion of the *lst* gene at the start codon and stop codons of *bla*Z. A mutant lysostaphin ($LST_{\Delta CWT}$) truncated for residues 389–480 of lysostaphin was amplified with the primers LS-Nde and LS-BamΔC (5′-AAGGATCCTCAACCTGTATTCGGCGTTG-3′) and cloned as described for pLST. To generate $LST_{\Delta NTR}$, sequences encoding the leader peptide of enterotoxin B (SEB) were amplified from pSEB-SPA with the primers Seb-Nde (5′-AACATATGTATAAGAGATTATTTCACAT-3′) and Seb6 (5′-AAGGTACCGGTTGACTCTCTGCTAAAA-3′) and digested with *Nde*I and *Kpn*I. The *lst* sequences were amplified with the primers LS-KpnΔN (5′-AAGGTACCCGCAACACATGAACATTCAGCA-3′) and LS-Bam. The $LST_{\Delta NTR}$ protein is lacking the leader peptide and 14 N-terminal repeats of lysostaphin (residues 1–235) and is exported by the N-terminal leader peptide of SEB (Jones and Khan, 1986). Fusions of either C-terminal lysostaphin (LST) or amidase (LYTA) sequences to the C-terminus of enterotoxin B employed previously constructed plasmids (Schneewind *et al.*, 1993). Lysostaphin sequences coding for residues 384–480 were amplified with LS-Fus (5′-AAGGTACCACGCCGAATACAGGTTGGAA-3′) and LS-Bam, cut with *Kpn*I and *Bam*HI and cloned into the corresponding sites of pSEB-SPA to generate pSEB-LST. The amidase sequences were amplified from purified φ11 phage DNA (Novick, 1991) with the primers LA-Kpn (5′-AAGGTACCGTTAAACCAGTTGCAAGTGCA-3′) and LA-Bam (5′-AAGGATCCTAACTGATTTCTCCCCATAA-3′) and cloned to yield plasmid pSEB-LYTA. The targeting signal of lysostaphin was released from pSEB-LST with *Kpn*I and *Bam*HI digestion and cloned into the corresponding sites of $pSPA_{Kpn}$ to generate a fusion protein with staphylococcal protein A (SPA). In order to fuse the targeting signal of lysostaphin to the C-terminal end of GST, the *lst* sequences were amplified with GST-Bam (5′-AAGGATCCACGCCCAATACAGGTTGGAA-3′) and LS-Bam, digested with *Bam*HI and cloned into pGEX2T (Pharmacia). The recombinant Pro-LST and Pro-$LST_{\Delta CWT}$ proteins were expressed as N-terminal His_6 fusion proteins by cloning PCR amplified sequences in the expression vector pQE30 (QIAGEN). The Pro-Bam (5′-AAGGATCCATGAAGAAAACAAAAAAACAATTATTA-3′) primer specified a 5′ *Bam*HI site starting at residue 37 of pre-pro-lysostaphin whereas the LS-Hind (5′-AAAAGCTTTTATAGTTCCCCAAAGAACA-3′) (Pro-LST) and LST-Hind (5′-AAAAGCTTTACCTGTATTCGGCGTTGGA-3′) (Pro-$LST_{\Delta CWT}$), specified a 3′ *Hin*dIII site.

Protein targeting assays

Staphylococcal cultures were grown overnight in chemically defined medium (van de Rijn and Kessler, 1980), diluted 1:20 into minimal medium and the cultures were pulse labeled when they reached OD_{600} 0.5. The expression of lysostaphin in *S.aureus* was induced with 1 μM methicillin 15 min prior to pulse labeling. After labeling with 100 μCi [^{35}S]methionine for 2 min, the pulse was chased with 50 μl of chase solution (100 mg of casamino acids, 10 mg of methionine/ml) for up to 60 min.

Pulse chase experiments. At timed intervals during either the pulse or the chase, 200 μl aliquots of the culture were precipitated with 200 μl of ice-cold 10% TCA. The TCA precipitate was washed in acetone, dried, digested with muramidase for 2 h at 37°C [1 ml of 0.05 M sodium acetate (pH 5.7), 100 μg/ml muramidase] and the samples were again precipitated with TCA.

Secreted and cell-associated lysostaphin. Cells were recovered by centrifugation for 4 min at 15 000 *g* and the supernatant was removed and precipitated with TCA (secreted fraction). The cell pellet was digested with muramidase for 10 min at 37°C in 1 ml of STM buffer [0.5 M sucrose, 0.02 M Tris–HCl, 0.02 M $MgCl_2$ (pH 7.5), 100 μg/ml muramidase] and precipitated by the addition of TCA (cell-associated fraction).

Cell fractionations. One ml of staphylococcal culture expressing SEB fusions was pulse labeled and the cells were recovered by centrifugation for 4 min at 15 000 *g*. The supernatant was removed and precipitated with TCA (medium) and the cells were lysostaphin digested for 10 min at 37°C in 500 μl of SMM buffer [0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ (pH 6.5), 100 μg/ml lysostaphin]. The protoplasts were collected by centrifugation for 4 min at 15 000 *g* and the supernatant was removed and precipitated with TCA (cell wall fraction). The protoplasts were lysed in 250 μl of membrane buffer [0.1 M NaCl, 0.1 M Tris–HCl, 0.01 M $MgCl_2$ (pH 7.5)] with five cycles of freeze–thawing in a dry ice–ethanol bath. The membranes were pelleted by ultracentrifugation in a Beckman TL-100 instrument at 100 000 *g* for 30 min. The supernatant (cytoplasm) and the pellet (membranes) were separated and precipitated with TCA.

Solubility in hot SDS. One ml of culture was pulse labeled and chased for 5 min, split into two 500 μl aliquots and precipitated with TCA. After TCA precipitation and an acetone wash, one of the two chase samples was lysostaphin digested (500 μl of 0.5 M Tris–HCl, 100 μg/ml lysostaphin) for 30 min at 37°C and again precipitated with TCA prior to boiling in hot SDS. The other sample was boiled directly in hot SDS. All TCA-precipitated samples were washed in acetone, dried and boiled in 50 μl of 0.5 M Tris–HCl pH 8.0, 4% SDS. Soluble material was immunoprecipitated prior to SDS–PAGE.

Immunofluoresence. One ml of mid-log staphylococcal culture (OD_{600} 0.5) grown in tryptic soy broth was centrifuged (5 min 15 000 *g*) and the cells were washed twice in ice-cold water and finally suspended in 1 ml of phosphate-buffered saline (PBS, 0.02% sodium azide). Five μl of cell suspension were applied to a glass slide, air dried and heat fixated. The cells were incubated with 50 μl of FITC-labeled rabbit immunoglobulin (Sigma) diluted 1:20 in PBS. The glass slides were washed several times in PBS and finally evaluated by photomicrography under UV light at 1000-fold magnification.

Targeting of GST–CWT

Escherichia coli strain XL-1 Blue harboring pGST-CWT was grown to mid-log phase in 500 ml of LB medium at 37°C, induced with 1 mM IPTG and incubated for another hour. Cells were harvested by centrifugation, suspended in 8 ml of F buffer [20% sucrose, 50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 10 mM EDTA and 0.1 mg/ml of lysozyme] and incubated for 30 min at room temperature. The cells were disrupted by ultrasonication (W-220F instrument, Heat Systems-Ultrasonic Inc.). Unbroken cells were removed (15 min, 15 000 *g*) and the supernatant was subjected to affinity chromatography on glutathione–Sepharose 4G (Pharmacia), 1 ml column volume pre-equilibrated with PBS. The column was washed with 20 ml of PBS and eluted with 2 ml of 10 mM glutathione, 50 mM Tris–HCl pH 8.0 yielding 200 μg/ml protein (approximate purity 98%). *S.aureus* OS2 or *S.simulans* ATCC1362 cells were grown to mid-log phase (OD_{600} 0.5) and washed several times with 50 mM Tris–HCl, pH 8.0. Twenty μg of purified GST–CWT protein were added to various amounts of staphylococci and incubated for 20 min at room temperature. The cells were collected by centrifugation (5 min, 15 000 *g*) and the supernatant was removed from the pellet. The GST–CWT protein present in the supernatant was precipitated with 5% TCA, washed in acetone and solubilized in 20 μl of sample buffer. The GST–CWT protein bound to staphylococci was eluted by adding 20 μl of sample buffer. All samples were boiled for 5 min and analyzed on 12% SDS–PAGE.

Purification of Pro-LST and Pro-$LST_{\Delta CWT}$

Escherichia coli strain XL-1 Blue harboring either pPro-LST or pPro-$LST_{\Delta CWT}$ was grown to mid-log phase in 1 l of LB, induced (1 mM IPTG) and incubated for another 3 h. Cells were harvested by centrifugation, resuspended in buffer A (6 M guanidine–HCl, 0.1 M sodium phosphate, 0.01 M Tris–HCl (pH 8.0)] and incubated for 1 h at room temperature. After removal of cell debris (15 min, 10 000 *g*), the lysate was applied onto 1 ml of Ni^{2+}-NTA–Sepharose (QIAGEN) pre-equilibrated with buffer A. The column was washed with 20 ml of buffer A, 20 ml of buffer B [8 M urea, 0.1 M sodium phosphate, 0.01 M Tris–HCl (pH 8.0)] and 20 ml of buffer C (buffer B, but pH 6.3). Proteins were eluted with 5 ml of buffer E (buffer B, pH 4.5). For renaturation and folding, the purified bacteriocins were dialyzed against 1 l of 0.05 M Tris–HCl pH 7.5, 1 M urea, 0.005% Tween 80 for 24 h at 4°C without stirring, followed by another dialysis against the same buffer without urea for 16 h at 4°C with slow stirring.

Endopeptidase activity measurements

Lysostaphin activity was measured as the release of free amino groups from *N*-acetylated hexaglycine (acetyl-Gly_6) (Kline *et al.*, 1994). Briefly, 0.5 ml of substrate solution (5 mM trisodium citrate, 1 mM disodium EDTA, 100 mM sodium borate, 10 mM acetyl-Gly_6) and 0.5 ml of purified bacteriocin were mixed and incubated for 1 h at 37°C. Sixty μl of 2,4,6,-trinitrobenzenesulfonic acid in 0.1 M sodium bicarbonate were added and incubated for 20 min at room temperature. The reactions were finally quenched with 100 μl of 3 M sodium acetate, and the absorbance at 405 nm was measured against a negative control with no added enzyme.

Target cell specificity of lysostaphin

Staphylococcus aureus OS2 (*spa*$^-$, *ermC*) (Schneewind *et al.*, 1992) and *S.simulans* TNK1 (spontaneous streptomycin derivative of strain AT-

CC1362) were grown in TSB medium to mid-log phase. The cells were collected by centrifugation, washed and finally diluted to 1.0×10^6 c.f.u./ml with fresh TSB. One ml of a 1:1 mixture of both diluted cultures was incubated with 0.5 ml of purified bacteriocin [60 ng of mature LST, 60 μg of Pro-LST or 60 μg of Pro-$LST_{\Delta CWT}$ in 50 mM Tris–HCl (pH 7.5), 0.005% Tween 80] and incubated for 3 h at 37°C. Aliquots of the reaction mixture were plated either directly or in a series of 10-fold dilutions on TSB agar containing the appropriate antibiotic (10 μg/ml of erythromycin or 250 μg/ml of streptomycin).

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