**Title:** TARGETING OF PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA

**Abstract**

A method of stable noncovalent display of proteins, peptides, or compounds covalently linked to proteins or peptides on the surface of Gram-positive bacteria provides advantages over phage display. One embodiment of the present invention comprises a method for noncovalent protein targeting, comprising the steps of: (1) cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal; (2) growing the bacterium into which the nucleic acid segment has been cloned to express the chimeric protein to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal; and (3) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand. Alternatively, the chimeric protein can be produced by expression in another expression system and contacted with the Gram-positive bacterium.
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TARGETING OF PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA

GOVERNMENT RIGHTS

This invention was supported by grants from the United States Government, namely grants from the National Institutes of Health. Accordingly, the government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention is directed to methods for targeting of proteins to the cell wall of Gram-positive bacteria, particularly Staphylococcus, and the use of such targeted proteins.

For many purposes, it is desirable to display proteins or peptides on the surfaces of bacteria so that the proteins or peptides are accessible to the surrounding solution and can, for example, be bound by a ligand that is bound specifically by the protein or peptide. In particular, the display of proteins on the surface of bacteria is desirable for the preparation of vaccines, the linkage of molecules such as antibiotic molecules or diagnostic reagents to cells, in screening reagents such as monoclonal antibodies, and in the selection of cloned proteins by displaying the cloned proteins and then observing their reaction with specific reagents such as antibodies. One way of doing this has been with phage display (G.P. Smith, "Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface," Science 228:1315-1316 (1985)). However, phage display is limited in its
practicality, because it requires that the protein to be displayed be inserted into a coat protein of filamentous phage and retain its activity while not distorting the conformation of the coat protein, allowing functional virions to be formed. In general, this technique is therefore limited only to small peptides and proteins.

Therefore, there is a need for a more general method of peptide and protein display. Preferably such a method is capable of displaying proteins that have been expressed not only in Gram-positive bacteria such as Staphylococcus aureus, but in Gram-negative bacteria such as Escherichia coli, Salmonella typhimurium and other Gram-negative bacteria. Preferably, such a method is also capable of binding proteins that have been expressed in eukaryotic cells such as insect or animal cells.

**SUMMARY**

We have developed a method of stable noncovalent display of proteins, peptides, or compounds covalently linked to proteins or peptides on the surface of Gram-positive bacteria, particularly Staphylococcus aureus and other species of Staphylococcus.

One embodiment of the present invention comprises a method for protein targeting and display employing expression of a chimeric protein in a Gram-positive bacterium and subsequent targeting of the chimeric protein to the cell wall and display of that protein. In general, such a method comprises the steps of:

1. cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to
generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal;

(2) growing the bacterium into which the nucleic acid segment has been cloned to express the cloned chimeric protein in the Gram-positive bacterium to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal;

(3) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

The carboxyl-terminal cell-wall targeting signal can be a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

The carboxyl-terminal cell-wall targeting signal can have the sequence W-K-X₁₁-N-K-T-G-T-X₁₂-Y-X₁₃-X₁₄-
X₃₅-P-X₃₇-X₃₈-X₃₉-X₄₀-X₄₁-X₄₂-X₄₃-G-X₄₅-X₄₆-X₄₇-X₄₈-Y-X₅₀-E-V-M-

wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or amidase targeting signals.

In this sequence, X₁ can be R or T, X₉ is Y or L, X₁₁ is M or K, X₁₂ is E or S, X₁₅ is R or S, X₁₉ is N or P, X₂₀ is G or N, X₂₁ is N or T, X₂₂ is Q or D, X₂₃ is P or I, X₂₆ is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₂₈ is K or T, X₂₉ is V or T, X₃₃ is L or R, X₃₅ is G or M, X₃₇ is V or Q, X₃₈ is G or S, X₃₉ is Y or G, X₄₀ is Q or V, X₄₁ is P or L, X₄₂ is Q or K, X₄₃ is P or A, X₄₅ is G or Q, X₄₆ is Y or T, X₄₇ is C or L, X₄₈ is D or H, X₅₀ is T or D, X₅₄ is L or K, X₆₅ is W or G, X₆₆ is E or N, X₆₇ is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X₇₁ is Y or I, X₈₀ is G or K, X₈₂ is A or T, X₉₂ is P or N, X₈₄ is P or T, X₈₅ is N or an absent amino acid with an amide bond between the preceding and following amino acids in
the chain, $X_{86}$ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{87}$ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{94}$ is E or T, and $X_{95}$ is S or K.

The conservative amino acid substitutions in highly conserved regions can be the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa. The conservative amino acid substitutions in less highly conserved regions can be the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

As an alternative to expression in a Gram-positive bacterium and stable noncovalent binding to the cells in which the chimeric protein are expressed, the targeting method can comprise:

(1) generating a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal by expression of a nucleotide sequence encoding the chimeric protein in an expression system other than an expression system employing a Gram-positive bacterium as host;

(2) adding the chimeric protein to a culture medium in which a Gram-positive bacterium is growing; and

(3) allowing the chimeric protein to bind stably and noncovalently to the cell wall of the Gram-positive bacterium so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand..

Another aspect of the present invention is a method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a chimeric protein including therein: (1) a protein antigen and (2) a carboxyl-terminal cell wall-targeting signal bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the chimeric protein is accessible to the antigen-processing system of the antibody-producing animal.

The antigen can be a polypeptide antigen produced by Candida albicans, Aspergillus fumigatus,
Histoplasma capsulatum, Microsporum canis, Plasmodium falciparum, Trypanosoma cruzi, Borrelia burgdorferi, Treponema pallidum, Borrelia recurrentis, Leptospira icterohaemorrhagiae, Neisseria gonorrhoeae, Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhosa, Hemophilus influenzae, Bordetella pertussis, Actinomyces israelii, Streptococcus mutans, Streptococcus equi, Streptococcus agalactiae, Streptococcus anginosus, human immunodeficiency virus (HIV), poliovirus, influenza virus, rabies virus, herpes virus, foot and mouth disease virus, psittacosis virus, paramyxovirus, myxovirus, or coronavirus.

Alternatively, the antibody-producing animal can be immunized with a complex comprising a non-protein antigen or hapten covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal for production of vaccines. The antigen or hapten can be a drug, an alkaloid, a steroid, a carbohydrate, or an aromatic compound. The same method can be used for vaccination with a protein antigen, by immunizing an antibody-producing animal with a complex comprising a protein antigen covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal.

Another aspect of the present invention is a method for screening a cloned protein for reactivity with a specific binding partner comprising:

1. incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal cell wall-targeting signal;
(2) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(3) reacting the noncovalent complex with a labeled specific binding partner to screen the cloned protein for reactivity with the specific binding partner. The labeled specific binding partner is typically an antibody.

Another aspect of the present invention is a method for treating an infection caused by a Gram-positive bacterium comprising:

(1) conjugating an antibiotic to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate; and

(2) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat the infection.

An analogous method can be used to detect the presence of a Gram-positive bacterium and thereby diagnose an infection by conjugating a diagnostic reagent to the protein instead of the antibiotic and binding the conjugate to the cell walls of the bacterium.

Another aspect of the present invention is a chimeric protein comprising:

(1) a protein to be targeted to the cell wall of a Gram-positive bacterium; and

(2) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting
signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Additional aspects of the present invention include DNA or other nucleic acid segments encoding such chimeric proteins; vectors incorporating the DNA or other nucleic acid segments encoding the chimeric proteins; stable noncovalent complexes of bacterial cells and cell-wall targeted proteins; covalent conjugates of protein or non-protein antigens and cell-wall targeted peptides, optionally including a spacer; and noncovalent complexes of these conjugates and Gram-positive bacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and accompanying drawings where:

Figure 1A shows the structures of wild-type lysostaphin (LST) and its mutants;

Figure 1B shows pulse-chase experiments to determine the localization of lysostaphin in subcellular fractions after pulse-labeling with $^{35}$S methionine followed by a chase of non-radioactive methionine; pulse-labeled lysostaphin was immunoprecipitated with a specific antiserum, separated on a polyacrylamide gel and fluorographed;

Figure 1C is a similar experiment showing that secreted LST from Staphylococcus simulans cells was not cleaved rapidly even in the presence of S. aureus cells;
Figure 2A shows the structures of staphylococcal enterotoxin B (SEB) and its hybrid proteins with: (1) a C-terminal fusion of the cell wall sorting signal of staphylococcal protein A which consists of the LPXTG motif, the C-terminal hydrophobic domain and the charged tail; (2) the membrane anchor segment of *Listeria monocytogenes* ActA; (3) the cell-wall targeting domain (C-terminal 92 amino acids) of lysostaphin; (4) and the cell-wall targeting domain (C-terminal 99 amino acids) of staphylococcal amidase;

Figure 2B shows a pulse-chase experiment in *S. aureus* OS2 expressing genes of various hybrid proteins, showing the localization of the resulting proteins in various cell components or in the culture medium;

Figure 2C shows a similar experiment in which the samples were digested with either lysostaphin (L) or Chalaropsis B muramidase (Hash-enzyme) (H) and precipitated; proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB, and subjected to 12% SDS-polyacrylamide gel electrophoresis;

Figure 3 shows the 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 located on the staphylococcal surface was visualized by microscopy under ultraviolet light; panel (1) is cells expressing wild-type protein A; panel (2) is cells expressing protein A with a C-terminal cell-wall targeting signal; and panel (3) is cells expressing a mutant protein A (SPA_{1-519}) known to be secreted into the
culture medium; the presence of staphylococci in this panel is demonstrated by light microscopy (3 visible light);

Figure 4 is a diagram showing the homology between the amidase and lysostaphin amino acid sequences;

Figure 5A demonstrates the targeting of a hybrid glutathione S-transferase to S. aureus; the glutathione S-transferase activity remaining in the supernatant after reaction with added S. aureus (squares) or S. simulans (circles) cells is shown;

Figure 5B shows the same result by polyacrylamide gel electrophoresis, showing increasing binding of the hybrid glutathions S-transferase to S. aureus, but not to S. simulans, as shown by increasing presence of the hybrid protein in the cell pellet for S. aureus but not for S. simulans;

Figure 6 is a table showing the target cell specificity and bacteriolytic activity of mature wild-type lysostaphin, pro-lysostaphin, and a mutant of lysostaphin in which a portion of the cell wall targeting signal is deleted;

Figure 7A shows the structures of wild-type lysostaphin (LST, 1) and hybrid lysostaphins with a C-terminal fusion of (2) the cell wall sorting signal of protein A; (3) a sorting signal devoid of its LPXTG (SEQ ID NO: 10) motif, as well as hybrid enterotoxin B molecules with: (4) a C-terminal fusion of both the lysostaphin targeting signal and the protein A targeting signal or (5) a similar molecule without a LPXTG (SEQ ID NO: 10) motif; and
Figure 7B shows the results of polyacrylamide gel electrophoresis and fluorography to analyze the cell wall linkage of the hybrid proteins.

**DESCRIPTION**

We have developed a method for the stable and noncovalent targeting of proteins to the cell wall of Gram-positive bacteria, particularly *Staphylococcus aureus*.

Proteins targeted to the cell wall are accessible to the surrounding medium and retain their ability to bind ligands, such as substrates, inhibitors, allosteric modulators, hormones, ligands, antigens, haptons, or small molecules such as metal ions.

I. **PROTEIN TARGETING BY EXPRESSION IN GRAM-POSITIVE BACTERIA**

One aspect of the present invention is a method for protein targeting and display employing expression of a chimeric protein in a Gram-positive bacterium and subsequent targeting of the chimeric protein to the cell wall and display of that protein. In general, such a method comprises the steps of:

1. Cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal that is any of: (a) the lysostaphin signal; (b) the amidase signal; (c) a signal related to the lysostaphin signal or the amidase signal by one or more conservative amino acid substitutions that preserve the existence of consensus
sequence regions occurring in both the lysostaphin signal and the amidase signal, including signals from other Gram-positive bacteria; and (d) a signal related to these signals by truncation.

(2) Growing the bacterium into which the nucleic acid segment has been cloned to express the cloned chimeric protein to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal.

(3) Stable noncovalent binding of the expressed chimeric protein to the cell wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface on the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

As used herein, the terms "noncovalent" and "noncovalently" refer to the initial binding between the expressed chimeric protein and the bacterial cell wall and do not exclude subsequent covalent fixation of the chimeric protein to the cell wall by the use of crosslinking reagents such as glutaraldehyde, the formation of Schiff bases that are stabilized by reduction with borohydride, or other methods known in the art.

Typically, the Gram-positive bacterium is a species of Staphylococcus. A particularly preferred species of Staphylococcus is Staphylococcus aureus. However, other Gram-positive bacteria such as Streptococcus pyogenes, other Streptococcus species, and Gram-positive bacteria of other genera can also be used.
A. Cloning the Nucleic Acid Segment Into the Gram-Positive Bacterium

Cloning the nucleic acid segment encoding the chimeric protein into the Gram-positive bacterium is performed by standard methods. In general, such cloning involves: (1) isolation of a nucleic acid segment encoding the protein to be targeted; (2) joining to the cell-wall targeting signal to produce a chimeric nucleic acid segment encoding both the protein to be targeted and the cell-wall targeting signal; (3) cloning by insertion into a vector compatible with the Gram-positive bacterium in which expression is to take place; and (4) incorporation of the vector including the chimeric nucleic acid segment into the bacterium.

Typically, the nucleic acid segment encoding the protein to be targeted is DNA; however, the use of RNA in certain cloning steps is within the scope of the present invention. As used herein, the term "nucleic acid segment" includes both DNA and RNA unless otherwise specified, and, unless otherwise specified, includes both double-stranded and single-stranded nucleic acids. Also included are hybrids such as DNA-RNA hybrids. In particular, a reference to DNA includes RNA that has either the equivalent base sequence except for the substitution of uracil in RNA for thymine in DNA, or has a complementary base sequence except for the substitution of uracil for thymine, complementarity being determined according to the Watson-Crick base pairing rules. Reference to a nucleic acid sequence can also include modified bases as long as the modification does not significantly interfere either with binding of a ligand
such as a protein by the nucleic acid or Watson-Crick base pairing.

When dealing with genes from eukaryotic organisms, it is preferred to use cDNA, because the natural gene typically contains intervening sequences or introns that are not translated. Alternatively, if the amino acid sequence is known, a synthetic gene encoding the protein to be targeted can be constructed by standard solid-phase oligodeoxyribonucleotide synthesis methods, such as the phosphotriester or phosphite triester methods. The sequence of the synthetic gene is determined by the genetic code, by which each naturally-occurring amino acid is specified by one or more triplet codons. Additionally, if a portion of the protein sequence is known, but the gene or messenger RNA has not been isolated, the amino acid sequence can be used to construct a degenerate set of probes according to the known degeneracy of the genetic code. General aspects of cloning are described, for example, in J. Sambrook et al., "Molecular Cloning: A Laboratory Manual" (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989); in B. Perbal, "A Practical Guide to Molecular Cloning" (2d ed., John Wiley & Sons, New York 1988), in S.L. Berger & A.R. Kimmel, "Guide to Molecular Cloning Techniques" (Methods in Enzymology, vol. 152, Academic Press, Inc., San Diego, 1987), and in D.V. Goeddel, ed., "Gene Expression Technology" (Methods in Enzymology, vol. 185, Academic Press, Inc., San Diego, 1991).

Once isolated, DNA encoding the protein to be targeted is then joined to the cell-wall targeting
signal. This is typically accomplished through ligation, such as using *Escherichia coli* or bacteriophage T4 ligase. Conditions for the use of these enzymes are well known and are described, for example, in the above general references.

The ligation is done in such a way so that the targeted protein and the cell-wall targeting signal are joined in a single contiguous reading frame so that a single protein is produced. This may, in some cases, involve addition or deletion of bases to the cloned DNA segment to maintain a single reading frame. This can be done using standard techniques.

Cloning is typically performed by inserting the cloned DNA into a vector containing control elements to allow expression of the cloned DNA. The vector is then incorporated into the bacterium in which expression is to occur, using standard techniques of transformation or other techniques for introducing nucleic acids into bacteria.


If the chimeric protein is cloned under control of the BlaZRI regulon, expression can be induced by the addition of the β-lactam antibiotic methicillin.

The cell-wall targeting signal that can be used can be any of: (1) the lysostaphin signal; (2) the
amidase signal; (3) a signal related to the lysostaphin signal or the amidase signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal, including signals from other Gram-positive bacteria; and (4) a signal related to the lysostaphin signal or the amidase signal by truncation.

1. The Lysostaphin Signal

The lysostaphin signal comprises the 92 carboxyl-terminal residues in lysostaphin, a bacteriolytic enzyme that cleaves the pentaglycine crossbridges of staphylococcal peptidoglycans (C.A. Schindler & V.T. Schuhardt, Proc. Natl. Acad. Sci. USA 51:414 (1964)).


Identical signals are known to exist in other Gram-positive organisms. For example, the bacterium Staphylococcus staphylolyticus has a carboxyl-terminal region of its lysostaphin protein, residues 389-480, that is identical with the carboxyl-terminal residues of the
lysostaphin protein in \textit{S. simulans} that include the C-terminal cell-wall targeting signal.

2. \textbf{The Amidase Signal}


3. \textbf{Signals Related to the Lysostaphin Signal or the Amidase Signal by One or More Conservative Amino Acid Substitutions}

Other C-terminal cell-wall targeting sequences exist that are suitable for use in chimeric proteins for processes according to the present invention.

For example, there is a substantial consensus between the C-terminal cell-wall targeting sequences of the amidase and lysostaphin gene. If the conserved residues are taken into account, most generally, the consensus sequence is: W-K-X_1-N-K-T-G-T-X_9-Y-X_{11}-X_{12}-E-S-A-X_{16}-F-T-X_{19}-X_{20}-X_{21}-X_{22}-X_{23}-I-T-X_{26}-R-X_{28}-X_{29}-G-P-F-X_{33}-S-X_{35}-P-X_{37}-X_{39}-X_{40}-X_{42}-X_{43}-G-X_{45}-X_{46}-X_{47}-X_{48}-Y-X_{50}-E-V-M-X_{54}-Q-D-G-H-V-W-V-G-Y-T-X_{65}-X_{66}-X_{67}-G-Q-R-X_{71}-Y-L-P-X_{75}-R-T-W-N-

X_{80}-S-X_{82}-X_{83}-X_{84}-X_{85}-X_{86}-X_{87}-L-G-V-L-W-G-X_{94}-I-X_{96}.
Although the residues referred to by subscripts can be varied broadly, as discussed below, as long as the conformation of the resulting sequence is not altered significantly, preferably, within this consensus sequence, $X_3$ is selected from the group consisting of R and T, $X_9$ is selected from the group consisting of Y or L, $X_{11}$ is selected from the group consisting of M and K, $X_{12}$ is selected from the group consisting of E and S, $X_{16}$ is selected from the group consisting of R and S, $X_{19}$ is selected from the group consisting of N and P, $X_{20}$ is selected from the group consisting of G and N, $X_{21}$ is selected from the group consisting of N or T, $X_{22}$ is selected from the group consisting of Q and D, $X_{23}$ is selected from the group consisting of P and I, $X_{26}$ is selected from the group consisting of V and an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{28}$ is selected from the group consisting of K and T, $X_{29}$ is selected from the group consisting of V and T, $X_{31}$ is selected from the group consisting of L and R, $X_{35}$ is selected from the group consisting of G and M, $X_{37}$ is selected from the group consisting of V and Q, $X_{38}$ is selected from the group consisting of G and S, $X_{39}$ is selected from the group consisting of Y and G, $X_{40}$ is selected from the group consisting of Q and V, $X_{41}$ is selected from the group consisting of F and L, $X_{42}$ is selected from the group consisting of Q and K, $X_{43}$ is selected from the group consisting of P and A, $X_{45}$ is selected from the group consisting of G and Q, $X_{46}$ is selected from the group consisting of Y and T, $X_{47}$ is selected from the group consisting of C and I, $X_{48}$ is selected from the
group consisting of D and H, \( x_{40} \) is selected from the
group consisting of T and D, \( x_{44} \) is selected from the
group consisting of L and K, \( x_{45} \) is selected from the
group consisting of W and G, \( x_{66} \) is selected from the
group consisting of E and N, \( x_{67} \) is selected from the
group consisting of an absent amino acid with an amide
bond between the preceding and following amino acids in
the chain and S, \( x_{71} \) is selected from the group consisting
of Y and I, \( x_{80} \) is selected from the group consisting of G
and K, \( x_{82} \) is selected from the group consisting of A and
T, \( x_{83} \) is selected from the group consisting of P and N,
\( x_{84} \) is selected from the group consisting of P and T, \( x_{85} \)
is selected from the group consisting of N and an absent
amino acid with an amide bond between the preceding and
following amino acids in the chain, \( x_{86} \) is selected from
the group consisting of Q and an absent amino acid with
an amide bond between the preceding and following amino
acids in the chain, \( x_{87} \) is selected from the group
consisting of I and an absent amino acid with an amide
bond between the preceding and following amino acids in
the chain, \( x_{94} \) is selected from the group consisting of E
and T, and \( x_{96} \) is selected from the group consisting of S
and K.

This sequence can be extended by additional
residues at the amino-terminus, that is the terminus
immediately adjacent to the carboxyl-terminus of the
targeted protein in the fusion construct.

For the purposes of this discussion, this
consensus sequence can be divided into completely
conserved regions, in which the sequences for lysostaphin
and amidase agree in best alignment, and less highly
conserved regions, in which the sequences diverge. This distinction, as used here, is not intended to imply a functional difference.

In the less highly conserved portions of the consensus sequence, conservative amino acid substitutions can be made according to general principles of protein chemistry. Such conservative amino acid substitutions include, but are not limited to, substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa, and serine (S) for threonine (T) and vice versa. The above-mentioned substitutions are not the only amino acid substitutions that can be considered "conservative." Other substitutions can also be considered conservative, depending upon the environment of the particular amino acid. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can be alanine and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the different pK's of these two amino acid residues or their different sizes are not significant. Still other changes can be considered "conservative" in particular environments. For example, if an amino acid on the surface of a protein is not involved in a hydrogen bond or salt bridge interaction with another molecule, such as another protein subunit or a ligand bound by the protein,
negatively charged amino acids such as glutamic acid and aspartic acid can be substituted for positively charged amino acids such as lysine or arginine and vice versa. Histidine (H), which is more weakly basic than arginine or lysine, and is partially charged at neutral pH, can sometimes be substituted for these more basic amino acids. Additionally, the amides glutamine and asparagine can sometimes be substituted for their carboxylic acid homologues, glutamic acid and aspartic acid.

In the completely conserved regions of the consensus sequence, it is likely that highly conservative amino acid substitutions, such as any of isoleucine, valine, and leucine for any of these other amino acids, aspartic acid for glutamic acid and vice versa, glutamine for asparagine and vice versa, and serine for threonine and vice versa are allowable substitutions; other substitutions may be allowable if the conformation of the protein remains substantially unchanged.

The fact that certain amino acids can be omitted in this consensus sequence means that the total length of the consensus sequence can vary. In fact, a total of five amino acids can be omitted from the sequence, leaving a total of 91. Clearly, any intermediate number of amino acids between these limits, i.e., between 91 and 96, can be tolerated within the consensus sequence. Thus, any sequence of between 91 and 96 amino acid residues that has only highly conservative amino acid substitutions in the completely conserved regions as determined by a comparison of the LST and LytA sequences, and has the somewhat less highly conservative amino substitutions described above in the less highly
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conservative regions, is suitable for cell-wall targeting according to the methods of the present invention.


Another protein expected to have cell-wall targeting activity is a cell-wall protein precursor in *Staphylococcus mutans*, strain OMZ175, serotype f. This sequence includes a sequence of S-S-P-T-Q-P-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4). This sequence has 37% exact matches with the lysostaphin sequence, and 55% exact matches or homologous amino acids. Cell-wall targeting sequences useful in the methods of the present invention can therefore include amino acid segments including these sequences, with a total length of about 80 to 100 amino acids.

Because signals according to the present invention are intended to be operable as part of longer chimeric fusion proteins, it is believed that these signals remain functional even when additional amino acid residues are appended to their carboxyl termini, or when
additional amino acid residues are appended to the amino termini of the targeted proteins.

4. Truncated Signals

Comparison of the signals recited above indicates that conserved regions exist throughout the signal sequence and that no single conserved region is required to obtain a functional signal. This suggests that some portions of the signal sequence can be omitted without abolishing the binding to the cell surface. Therefore, signals that are related to the above-described signals by truncation in such a way that the total length of the signal sequence is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity are also useful in processes according to the present invention.

B. Expression in the Gram-Positive Bacterium to Generate a Chimeric Protein Including Therein a C-Terminal Cell-Wall Targeting Signal

The next step is expression in a Gram-positive bacterium to generate a chimeric protein including therein a C-terminal cell-wall targeting signal. Expression is typically under the control of various control elements associated with the vector incorporating the DNA encoding the chimeric protein; such elements can include promoters and operators, which can be regulated by proteins such as repressors. The conditions required for expression of cloned proteins in Gram-positive
bacteria, particularly *S. aureus*, are well known in the art and need not be further recited here. An example is the induction of expression of the lysostaphin gene under control of the BlaZRI regulon induced by the addition of methicillin.

When expressed in *Staphylococcus aureus*, the chimeric protein is first exported with an amino-terminal leader peptide, such as the hydrophobic region of Figure 1 or the hydrophobic signal peptide at the amino-terminal region of the cloned lysostaphin of Recsei et al. (P. Recsei et al., “Cloning, Sequence, and Expression of the Lysostaphin Gene from *Staphylococcus simulans,*" *Proc. Natl. Acad. Sci. USA* 84: 1127-1131 (1987)).

**C. Stable Noncovalent Binding of the Chimeric Proteins to the Cell Wall**

The third step is stable noncovalent binding of the chimeric protein to the cell wall via the C-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that it is accessible to a ligand. This binding is noncovalent, as shown in Example 1. However, it is stable under conditions in which noncovalent complexes such as antigen-antibody or hapten-antibody complexes are stable, allowing the use of a complex such as in immunization for the production of vaccines.

**II. CLONING AND EXPRESSION IN CELLS OTHER THAN GRAM-POSITIVE BACTERIA AND SUBSEQUENT TARGETING OF CHIMERIC PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA**
As an alternative to the cloning and expression of a chimeric protein containing the cell-wall targeting signal in Gram-positive bacteria such as *S. aureus* and the subsequent binding of the chimeric protein to the cell wall of the bacteria in which the chimeric protein was expressed, the chimeric protein can be generated by expression in another expression system, added to the culture medium of the Gram-positive bacteria such as *S. aureus*, and allowed to bind noncovalently to the cell wall. In other words, even if the chimeric protein is produced from another source, the cell-wall targeting signal of the chimeric protein directs the molecules to the cell wall of Gram-positive bacteria such as *S. aureus* to form a stable noncovalent association.

For this embodiment of a method according to the present invention, the assembly of the chimeric protein by genetic engineering techniques is basically same as discussed above in section I(A), and the cell-wall targeting signals used can be the same. In this system, expression can be in any expression system commonly used in the art such as described in the references by Sambrook et al., Perbal, Berger & Kimmel, and Goeddel. In general, these methods require the insertion of the DNA encoding the chimeric protein including the C-terminal cell-wall targeting signal into a suitable vector. This vector is then introduced into the cell. Suitable vectors and methods for incorporating the DNA encoding the chimeric protein into the vectors and introducing the vectors into the cells used for expression are described in the above-identified references and elsewhere. Among the expression systems
that can be used are Gram-negative bacteria such as E. coli and S. typhimurium, Bacillus subtilis, mammalian cells, and insect cells. The vectors required for these different cell types are well known in the art and need not be further described here; an example is the use of baculovirus vectors for insect cells.

The proteins resulting from expression, which typically are secreted into the culture medium in the cells producing them, are then isolated, purified if desired, and contacted with the Gram-positive bacteria such as S. aureus to allow the chimeric proteins containing the C-terminal cell-wall targeting signal to bind to the cell wall of the Gram-positive bacteria. This contact normally occurs in conditions compatible with the growth of the Gram-positive bacteria.

This embodiment greatly expands the utility of the method by allowing cloning and expression in any organism that will support an expression system.

III. USE OF CELL-WALL TARGETED PROTEINS AND COMPLEXES BETWEEN CELL-WALL TARGETED PROTEINS AND GRAM-POSITIVE BACTERIA

Cell-wall targeted proteins and complexes between cell-wall targeted proteins and Gram-positive bacteria according to the present invention have a number of uses.

One use is use in the production of vaccines that can be used to generate immunity against infectious diseases affecting mammals, including both humans and non-human mammals, such as cattle, sheep, goats, poultry, and fish. This invention is of special importance to
mammals. The usefulness of these complexes for vaccine production lies in the fact that the proteins are on the surface of the cell wall and are accessible to the medium surrounding the bacterial cells, so that the antigenic part of the chimeric protein is accessible to the antigen processing system. It is well known that presenting antigens in particulate form greatly enhances the immune response. In effect, bacteria containing antigenic peptides on their surfaces linked to the bacteria by these noncovalent but stable interactions function as natural adjuvants. Here follows a representative list of typical microorganisms that express polypeptide antigens against which useful antibodies can be prepared by the methods of the present invention:

(1) Fungi: Candida albicans, Aspergillus fumigatus, Histoplasma capsulatum (all cause disseminating disease), Microsporum canis (animal ringworm).

(2) Parasitic protozoa: (1) Plasmodium falciparum (malaria), Trypanosoma cruzei (sleeping sickness).

(3) Spirochetes: (1) Borrelia burgdorferi (Lyme disease), Treponema pallidum (syphilis), Borrelia recurrentis (relapsing fever), Leptospira icterohaemorrhagiae (leptospirosis).

(4) Bacteria: Neisseria gonorrhoeae (gonorrhea), Staphylococcus aureus (endocarditis), Streptococcus pyogenes (rheumatic fever), Salmonella typhosa (salmonellosis), Hemophilus influenzae (influenza), Bordetella pertussis (whooping cough), Actinomyces israelii (actinomycosis), Streptococcus
mutans (dental caries), Streptococcus equi (strangles in horses), Streptococcus agalactiae (bovine mastitis),
Streptococcus anginosus (canine genital infections).
(5) Viruses: Human immunodeficiency virus
(HIV), poliovirus, influenza virus, rabies virus, herpes virus, foot and mouth disease virus, psittacosis virus,
paramyxovirus, myxovirus, coronavirus.

Typically, the resulting immunological response occurs by both humoral and cell mediated pathways. One possible immunological response is the production of antibodies, thereby effecting protection against infection by the pathogen.

This method is not limited to protein antigens.

As discussed below, non-protein antigens or haptens can be covalently linked to the C-terminal cell-wall targeting segment, which can be produced as an independently expressed polypeptide, either alone, or with a spacer at its amino-terminal end. If a spacer at the amino-terminal end is used, typically the spacer will have a conformation allowing the efficient interaction of the non-protein antigen or hapten with the immune system, most typically a random coil or α-helical form. The spacer can be of any suitable length; typically, it is in the range of about 5 to about 30 amino acids; most typically, about 10 to about 20 amino acids. In this version of the embodiment, the independently expressed polypeptide, once expressed, can then be covalently linked to the hapten or non-protein antigen. Typical non-protein antigens or haptens include drugs, including both drugs of abuse and therapeutic drugs, alkaloids, steroids, carbohydrates, aromatic compounds, including
many pollutants, and other compounds that can be covalently linked to protein and against which an immune response can be raised.

Alternatively, a protein antigen can be covalently linked to the independently expressed cell-wall targeting segment or a cell-wall targeting segment including a spacer.

Many methods for covalent linkage of both protein and non-protein compounds to proteins are well known in the art and are described, for example, in P. Tijssen, "Practice and Theory of Enzyme Immunoassays" (Elsevier, Amsterdam, 1985), pp. 221-295, and in S.S. Wong, "Chemistry of Protein Conjugation and Cross-Linking" (CRC Press, Inc., Boca Raton, FL 1993).

Many reactive groups on both protein and non-protein compounds are available for conjugation.

For example, organic moieties containing carboxyl groups or that can be carboxylated can be conjugated to proteins via the mixed anhydride method, the carbodiimide method, using dicyclohexylcarbodiimide, and the N-hydroxysuccinimide ester method.

If the organic moiety contains amino groups or reducible nitro groups or can be substituted with such groups, conjugation can be achieved by one of several techniques. Aromatic amines can be converted to diazonium salts by the slow addition of nitrous acid and then reacted with proteins at a pH of about 9. If the organic moiety contains aliphatic amines, such groups can be conjugated to proteins by various methods, including carbodiimide, tolylene-2,4-diisocyanate, or maleimide compounds, particularly the N-hydroxysuccinimide esters.
of maleimide derivatives. An example of such a compound is 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid. Another example is m-maleimidobenzoyl-N-hydroxysuccinimide ester. Still another reagent that can be used is N-succinimidyl-3-(2-pyridyldithio) propionate. Also, bifunctional esters, such as dimethylpimelimidate, dimethyladipimidate, or dimethyldermaidate, can be used to couple amino-group-containing moieties to proteins.

Additionally, aliphatic amines can also be converted to aromatic amines by reaction with p-nitrobenzoylchloride and subsequent reduction to a p-aminobenzoylamide, which can then be coupled to proteins after diazotization.

Organic moieties containing hydroxyl groups can be cross-linked by a number of indirect procedures. For example, the conversion of an alcohol moiety to the half ester of succinic acid (hemisuccinate) introduces a carboxyl group available for conjugation. The bifunctional reagent sebacyldichloride converts alcohol to acid chloride which, at pH 8.5, reacts readily with proteins. Hydroxyl-containing organic moieties can also be conjugated through the highly reactive chlorocarbonates, prepared with an equal molar amount of phosgene.

For organic moieties containing ketones or aldehydes, such carbonyl-containing groups can be derivatized into carboxyl groups through the formation of O-(carboxymethyl) oximes. Ketone groups can also be derivatized with p-hydrazinobenzoic acid to produce carboxyl groups that can be conjugated to the specific binding partner as described above. Organic moieties
containing aldehyde groups can be directly conjugated through the formation of Schiff bases which are then stabilized by a reduction with sodium borohydride.

One particularly useful cross-linking agent for hydroxyl-containing organic moieties is a photosensitive noncleavable heterobifunctional cross-linking reagent, sulfosuccinimidyl 6-[(4'-azido-2'-nitrophenylamino) hexanoate. Other similar reagents are described in S.S. Wong, "Chemistry of Protein Conjugation and Cross-Linking," supra.

Other cross-linking reagents can be used that introduce spacers between the organic moiety and the specific binding partner.

These methods need not be described further here.

Another use of the chimeric proteins and complexes of the present invention is in the biotechnological field of bacterial cell surface display. If these molecules are associated with cell surfaces, they can be used in various ways for screening. For example, samples of expressed proteins from an expression library containing the expressed proteins on the surfaces of the cells can be used to screen for clones that express a particular desired protein when a labeled antibody or other labeled specific binding partner for that protein is available.

In general, such a method comprises:

1) incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal cell wall-targeting signal;
(2) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(3) reacting the noncovalent complex with a labeled specific binding partner to screen the chimeric protein for reactivity with the specific binding partner.

As used herein, the term "incorporating" means to include in a single molecule, typically a fusion protein. The fusion protein is formed by methods well known in the art and can include a spacer.

As used herein, the term "antibody" includes both intact antibody molecules of the appropriate specificity and antibody fragments (including Fab, F(ab'), Fv, and F(ab')₂ fragments) as well as chemically modified intact antibody molecules and antibody fragments, including hybrid antibodies assembled by in vitro reassocation of subunits. Also included are single-chain antibody molecules generally denoted by the term sFv and humanized antibodies in which some or all of the originally non-human constant regions are replaced with constant regions originally derived from human antibody sequences. Both polyclonal and monoclonal antibodies are included unless otherwise specified.

In the last step, the cells are merely exposed to the labeled antibody or other labeled specific binding partner, unreacted antibody is removed as by a wash, and label associated with the cells is detected by conventional techniques such as fluorescence, chemiluminescence, or autoradiography. As described above, this is an improvement on phage display.
Cell-wall targeted molecules can also be used for the diagnosis and treatment of bacterial infections caused by Gram-positive bacteria. Such infections continue to present a serious medical challenge, particularly because of the spread of antibiotic resistance strains of Gram-positive bacteria. Antibiotic molecules or fluorescent or any other diagnostic molecules can be chemically linked to a cell-wall targeting peptide segment, which may include a spacer as described above, and then can be injected into animals or humans. The cell-wall targeted molecules are specifically directed to the bacterial target, thereby avoiding all host cells and fulfilling the specificity requirements of a "magic bullet."

In general, these methods comprise:

(1) conjugating an antibiotic or a detection reagent to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate;

(2) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat or diagnose the infection.

The antibiotic used can be, but is not limited to, a penicillin, ampicillin, vancomycin, gentamicin, streptomycin, a cephalosporin, amikacin, kanamycin, neomycin, paromomycin, tobramycin, ciprofloxacin, clindamycin, rifampin, chloramphenicol, or norfloxacin.

The detection reagent is typically an antibody or other specific binding partner labeled with a detectable label, such as a radiolabel. Such methods are
well known in the art and need not be described further here.

In this context, the conjugates can be administered using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral, or intralymphatic. Other routes of injection can alternatively be used. Oral or intraperitoneal administration is generally preferred. The composition can be administered in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends on the mode of administration and a quantity administered.

The compositions for administration preferably also include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate. The most effective mode of administration and dosage regimen for the conjugates as used in the methods of the present invention depend on the severity and course of the disease, the patient's health, the response to treatment, the particular strain of bacteria infecting the patient, other drugs being administered and the development of resistance to them, the accessibility of the site of infection to blood flow, pharmacokinetic considerations such as the condition of the patient's liver and/or kidneys that can affect the
metabolism and/or excretion of the administered conjugates, and the judgment of the treating physician. Accordingly, the dosages should be titrated to the individual patient.

IV. NOVEL COMPOUNDS ACCORDING TO THE PRESENT INVENTION

Another aspect of the present invention is novel compounds embodying the concept of the present invention. These novel compounds include: (1) chimeric proteins including a protein to be targeted and a cell-wall targeting signal; (2) DNA or other nucleic acid segments encoding such chimeric proteins; (3) vectors incorporating the DNA or other nucleic acid segments encoding the chimeric proteins; (4) stable noncovalent complexes of bacterial cells and cell-wall targeted proteins; (5) covalent conjugates of protein or non-protein antigens and cell-wall targeted proteins, optionally including a spacer; and (6) noncovalent complexes of these conjugates and Gram-positive bacteria.

The production of these novel compounds according to the present invention is as described above.

In general, chimeric proteins according to the present invention comprise:

(1) a protein to be targeted to the cell wall of a Gram-positive bacterium; and

(2) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the
surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Nucleic acid segments encoding the chimeric proteins of the present invention can be either DNA or RNA; typically, they are DNA. The term "nucleic acid" as used herein includes both DNA and RNA as well as DNA-RNA hybrids unless otherwise indicated, and can include both single and double-stranded nucleic acid sequences. If a DNA sequence is referred to, reference is generally to both strands of a DNA sequence, either individually or as a Watson-Crick double helix. If only one strand is specified, the complementary strand whose antiparallel sequence is determined by Watson-Crick based pairing rules is also included unless the complementary sequence is specifically excluded. If only one strand is specified in double-stranded DNA, the strand specified is the sense strand whose strand would be equivalent to the sequence of any RNA transcribed from the double-stranded DNA, except for the replacement of thymidine (T) in the DNA by uridine (U) in the RNA. Reference to a nucleic acid sequence also includes modified bases as long as the modification does not significantly interfere with Watson-Crick base pairing or other specified functions of the nucleic acid, and can, for example, include substitution of uridine for thymidine in DNA as well as methylation of bases or modification of sugars.

Construction of nucleic acid segments according to the present invention can be accomplished by techniques well known in the art including solid-phase nucleotide synthesis, the polymerase chain reaction (PCR) technique, reverse transcription of DNA from RNA, the use of DNA
polymerases and ligases, and other techniques. If an amino acid sequence is known, the corresponding nucleic acid segment can be constructed according to the genetic code.

Another aspect of the invention is a vector comprising the nucleic acid segment operatively linked to at least one control sequence that controls the expression or regulation of the nucleic acid segment. Such control sequences are well known in the art and include operators, promoters, enhancers, promoter-proximal elements, and replication origins. The techniques of vector construction, including cloning, ligation, gap-filling, the use of the polymerase chain reaction (PCR) procedure, solid-state oligonucleotide synthesis, and other techniques, are also well known in the art and need not be described further here.

Another aspect of the present invention is noncovalent complexes comprising chimeric proteins according to the present invention and a Gram-positive bacterium. In the complex, the chimeric protein is bound noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Yet another aspect of the present invention is a covalent conjugate comprising:

(1) a cell-wall targeting segment including a carboxyl-terminal cell-wall targeting signal therein that can be bound noncovalently and stably to a cell wall of a
Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal; and

(2) an antigen or hapten conjugated covalently to the cell-wall targeting segment.

In these conjugates, the antigen or hapten can be a protein antigen; alternatively, it can be a nonprotein antigen or hapten. The cell-wall targeting segment can further include an amino-terminal spacer.

Yet another aspect of the present invention is a noncovalent complex comprising the covalent conjugate and a Gram-positive bacterium stably and noncovalently binding the covalent conjugate.

The invention is further described by means of the following Example. This Example is for illustrative purposes only and is not to be construed as limiting the scope of the invention in any manner.

EXAMPLE

20 Targeting of Proteins to the Staphylococcal Cell Wall by Cell-Wall Targeting Segments Derived from Lysostaphin and Amidase

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 (R. Kolter, "Genetics of Ribosomally Synthesized Peptide Antibiotics," *Annu. Rev. Microbiol.*, 46: 141-163 (1992). Investigations into the mode of action of bacteriocins led to the discovery and elucidation of fundamental biological processes such as DNA replication (M.C. Garrido et al., "The Export of the
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 (R.W. Jack et al., "Bacteriocins of Gram-Positive Bacteria," Microbiol. Rev. 59: 171-200 (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 (R.W. Jack et al. (1995), supra).


It is demonstrated here that the information for target cell specificity is encoded within the lysostaphin molecule. A C-terminal sequence element of lysostaphin is both necessary and sufficient for targeting to the cell wall of S. aureus; this sequence element is the C-terminal cell-wall targeting signal described above.

Sequence elements homologous to the cell-wall targeting signal were found in several other cell-wall proteins of gram-positive bacteria (A. Wang et al. (1991), supra; V.C. Neuman et al., FEMS Microbiol. Lett. 110:205 (1993)). One of these elements, the targeting signal of staphylococcal amidase (LytA), also functions to direct chimeric 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 specifically address the bacteriocin molecule to its target cells.
Results

To understand the mechanism for both targeting of lysostaphin to the cell wall of S. aureus and the immunity of S. simulans cells to secreted lysostaphin, the export, processing, and cell location of lysostaphin expressed in both of these organisms was analyzed.

To prevent the bacteriolytic killing of S. aureus, the lysostaphin gene (lst) was cloned under control of the BlaZRI regulon (P.-Z. Wang et al., Nucl. Acids Res. 19:4000 (1991)) and expression was induced by the addition of the β-lactam antibiotic methicillin to staphylococcal cultures. The plasmid pLST was assembled from three components: (1) the lysostaphin gene; (2) blaZRI sequences encoding the repressor (blaI), the signal transducer (blaR), as well as the promoter of the β-lactamase regulon; and (3) the E. coli-S. aureus shuttle vector pOS1 (O. Schneewind et al., “Cell Wall Sorting Signals in Surface Protein of Gram-Negative Bacteria,” EMBO J. 12: 4803-4811 (1993)). The lst gene was amplified by the polymerase chain reaction (PCR) using S. simulans ATCC1362 DNA as a template with the primers LS-Nde (5'-AACATATGAAGAAAAACAAAAACAATTATTATA-3') (SEQ ID NO: 5) and LS-Bam (5'-AAGGATCCTCCTTTTATAGTTCCCCAA-3') (SEQ ID NO: 6) and digested with NdeI and BamHI. The blaZRI sequences were amplified from pI258 (R.P. Novick & M.H. Richmond, "Nature and Interactions of the Genetic Elements Governing Penicillinase Synthesis in Staphylococcus aureus," J. Bacteriol. 90: 467-480 (1965)) with the
primers Bla-Pro-Nde (5'-
TCCCCGGGCGATATGAAACCCTCCGATATTACAGTT-3') (SEQ ID NO: 7) and Bla-18 (5'-AAGAATTCCATATCTCTCTAATTCAATTTTTACTAA-3') (SEQ ID NO: 8) and digested with NdeI and EcoRI. Finally, pOS1 was digested with EcoRI and BamHI and all three fragments were ligated and electroporated into S. aureus OS2. The NdeI (CATATG) and BamHI sites of pLST allowed the precise insertion of the 1st gene at the start codon and stop codons of blaZ.

Staphylococcal cultures were pulse-labeled with [35S]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 (J.H. Hash, "Purification and Properties of Staphyloolytic Enzymes from Chalaropsis sp.," Arch. Biochem. Biophys. 102: 379-388 (1963)) and the samples were re-precipitated with TCA followed by immunoprecipitation with anti-lysostaphin prior to SDS polyacrylamide gel electrophoresis.

The following procedures were used in the experiments whose results are shown in Figures 1-3. Staphylococcal cultures were grown overnight in chemically defined medium (L. van de Rijn & R.E. Kessler, "Growth Characteristics of Group A Streptococci in a New Chemically Defined Medium," Infect. Immun. 27: 444-448 (1980)), diluted 1:20 into minimal medium. The cultures were pulse-labeled when they reached OD600 0.5. The expression of lysostaphin in S. aureus was induced with 1
μ methicillin 15 min prior to pulse-labeling. After labeling with 100 μCi [35S] methionine for 2 min, the pulse was chased with 50 μl chase solution (100 mg casamino acids, 10 mg methionine/ml) for up to 60 min.

For pulse chase experiments, at timed intervals during either the pulse or the chase, 200 μl aliquots of the culture were precipitated with 200 μl ice-cold 10% TCA. The TCA precipitate was washed in acetone, dried, digested with muramidase for 2 h at 37°C (1 ml 0.05 M sodium acetate, pH 5.7, 100 μl muramidase) and the samples were again precipitated with TCA. For analysis of secreted and cell associated lysostaphin, cells were recovered by centrifugation for 4 min at 15,000 x 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 STM buffer (0.5 M sucrose, 0.02 M Tris-HCl, 0.02 M MgCl2, pH 7.5, 100 μg muramidase) and precipitated by the addition of TCA (cell associated fraction). For cell fractionations, 1 ml of staphylococcal culture expressing SEB fusions was pulse labeled and the cells were recovered by centrifugation for 4 min at 15,000 x 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 SMM buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M MgCl2, pH 6.5, 100 μg lysostaphin). The protoplasts were collected by centrifugation for 4 min at 15,000 x g and the supernatant was removed and precipitated with TCA (cell wall fraction). The protoplasts were lysed in 250 μl membrane buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.01 M MgCl2,
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 ultracentrifuge at 100,000 × g for 30 min. The supernatant (cytoplasm) and the pellet (membranes) were separated and precipitated with TCA. To determine solubility in hot SDS, 1 ml of culture was pulse-labeled and chased for 5 min, split into two aliquots of 500 μl each, and precipitated with TCA. After TCA precipitation and an acetone wash, one of the two chase samples was lysostaphin digested (500 μl 0.5 M Tris-HCl, 100 μg lysostaphin) for 30 min at 37°C and again precipitated with TCA prior to boiling in hot SDS. The other of the two aliquots was directly boiled in hot SDS. All TCA precipitated samples were washed in acetone, dried, and boiled in 50 μl 0.5 M Tris-HCl, pH 8.0, 4% SDS. Soluble material was immunoprecipitated prior to SDS-PAGE. For immunofluorescence, 1 ml of midlog staphylococcal culture (OD_{600} 0.5) grown in tryptic soy broth was centrifuged (5 min at 15,000 × g) and the cells were washed twice in ice-cold water and finally suspended in 1 ml phosphate buffered saline (PBS, 0.02% sodium azide). The cell suspension (5 μl) was applied to a glass slide, air dried and heat fixated. The cells were incubated with 50 μl of FITC-labeled rabbit immunoglobulin (Sigma, St. Louis, MO) diluted 1:20 in PBS. The glass slides were washed several times in PBS and finally evaluated by photomicrography under ultraviolet light at 1000-fold magnification.
In its natural host, *S. simulans*, pulse-labeled pre-pro-lysostaphin was rapidly secreted into the culture medium; the enzyme 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 more than 60 min (Fig. 1); the half-time for processing was 60 min. *S. simulans* culture supernatants were analyzed for the presence of pro- and mature lysostaphin during logarithmic growth and stationary phase. It was 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$ sec) 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 rapidly 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.

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 (O. Schneewind et al., "Sorting of Protein A to the Staphylococcal Cell Wall," *Cell* 70: 267-281 (1992)). This property was exploited and the secretion of lysostaphin was measured 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.

In Fig. 1, panel (A), the drawing shows the structures of wild-type lysostaphin (LST) and its mutants. Pre-pro-lysostaphin consists of a N-terminal leader peptide (open bar) that is thought to be responsible for protein export from the cytoplasm. Fourteen N-terminal tandem repeats (NTR) of a 13-residue peptide (NH$_2$-AEVETSAPVNT-COOH) (SEQ ID NO: 9) are cleaved from pro-lysostaphin to generate the mature enzyme, which consists of an enzyme domain (central open bar) and a C-terminal cell-wall targeting signal (hatched bar, CWT). To identify a specific cell-wall targeting signal, mutant lysostaphin molecules with a deletion of either the C-terminal domain (LST$_{CWT}$) or the 14 N-terminal repeats (LST$_{NTR}$) were constructed.
In panel (B), pulse-chase experiments are shown. During pulse-chase experiments, staphylococci were pulse-labeled with $[^{35}S]$ methionine for 2 min followed a chase of non-radioactive methionine. Aliquots of the pulse-labeled culture were precipitated with trichloroacetic acid (TCA) during the pulse (0) and 1, 5, 20, or 60 min after the addition of the chase. Pulse-labeled lysostaphin was immunoprecipitated with a specific antiserum, separated on 12% SDS-polyacrylamide gel electrophoresis and fluorographed. In S. aureus cells, expression of LST and its truncated forms, genes which were under the control of the BlaZRI regulon, were induced by 1 μM of methicillin 15 min prior to pulse labeling (right panel). In cell-fractionation experiments, pulse-labeled cultures were fractionated into medium (MD) and whole cell pellet ($\Phi$) and precipitated with TCA. Proteins in the cell pellet ($\Phi$) were released from the peptidoglycan by treatment with 100 μg/ml of Chalaropsis B muramidase (Hash-enzyme) and again precipitated with TCA. In parallel, pulse-labeled cultures were precipitated with TCA in duplicate. To solubilize proteins secreted into the staphylococcal medium, one of the samples was directly boiled in hot SDS (CH) while the other sample was digested with hash-muramidase prior to TCA precipitation (H), a protocol that solubilizes the cellular proteins of staphylococci.

Figure 1(C) shows the results of an experiment indicating that secreted LST from S. simulans cells was not cleaved rapidly even in the presence of S. aureus cells. A mid-log culture of S. simulans was pulse-
labeled for 2 min and the culture supernatant containing secreted LST was immediately removed after collecting the cells by centrifugation. The supernatant was then added to the unradiolabeled cell pellet of *S. aureus* (left) or *S. simulans* (right) which were separated from mid-log cultures. After suspending the cells in the radiolabeled LST-containing supernatant, samples were incubated for indicated periods (in min) at 37°C. At each period, reaction was stopped by adding TCA. Precipitated materials were solubilized in hot SDS and then subjected to immunoprecipitation in SDS-polyacrylamide gel electrophoresis as in (B).

The C-terminal 92 residues of lysostaphin display sequence homology to the C-terminal domains of staphylococcal amidase (LytA) and cell-wall proteins of *Staphylococcus mutans* (A. Wang et al. (1991), *supra*). A mutant lysostaphin was therefore constructed with a C-terminal truncation of 92 residues (LST\textsubscript{92\textsuperscript{C}}). It was surprising that this mutant lysostaphin 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 fourteen tandem repeats required more than 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.

The 14 N-terminal repeats of pro-lysostaphin were not required for cell-wall targeting, because a
-51-
mutant lacking the sequences (LST\textsubscript{ANTR}) was located in the cell-wall compartment of \textit{S. aureus} similar to the targeting of wild-type lysostaphin. Therefore, deletion of the 14 N-terminal repeats did not alter the cellular location of the mutant molecule.

To see whether the C-terminal domain of lysostaphin was sufficient for the targeting of indicator molecules to \textit{S. aureus} cells, enterotoxin B (SEB) was employed. This is a protein normally secreted into the staphylococcal medium (R.K. Tweten & J.J. Iandolo, "Transport and Processing of Staphylococcal Enterotoxin B," \textit{J. Bacteriol.} 153: 297-303 (1983)) (Figure 2). The cellular location of hybrid SEB molecules was analyzed by fractionating pulse-labeled staphylococcal cultures into the medium, cell wall, cytoplasm, and membrane components. 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 \textit{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, the studies included indicator molecules that were either secreted into the medium (SEB), covalently linked to the peptidoglycan (SEB-SPA\textsubscript{490-524}) or membrane anchored (SEB-ACTA) (O. Schneewind et al., "Cell Wall Sorting Signals in Surface Protein of Gram-Positive Bacteria," \textit{EMBO J.} 12: 4803-4811 (1993)).

Surface proteins of \textit{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 (J.-M. Ghuysen, "Use of Bacteriolytic Enzymes in Determination of Wall Structure and Their Role in Cell Metabolism," *Bacteriol. Rev.* 32: 425-464 (1968)), thereby releasing anchored protein with different amounts of linked cell wall (Schneewind et al. (1993), *supra*). To test for a peptidoglycan linkage of SEB-LST, the cell wall of pulse-labeled was digested with either lysostaphin (L) or muramidase (H, Hash-enzyme) and compared the mass of immunoprecipitated proteins on SDS-PAGE (Fig. 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), *supra*).

Taken together with the results above, this demonstrates that the C-terminal 92 residues of lysostaphin were necessary and sufficient for protein targeting to the cell wall of *S. aureus*. This element was therefore named the cell wall targeting (CWT) signal.

Previous work reported homology between the C-terminal sequences of staphylococcal amidase and lysostaphin (56% sequence identity) (A. Wang et al., "Sequence Analysis of a *Staphylococcus aureus* Gene Encoding a Peptidoglycan Hydrolase Activity," *Gene* 102: 105-109 (1991)). To ask whether the C-terminal domain of amidase (LytA) has a
function similar to that of the lysostaphin targeting signal, the C-terminal amino acid region of LytA was fused to SEB; it was found that this hybrid molecule was also targeted to the cell wall of S. aureus (Fig. 2).

In Figure 2(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 (SEQ ID NO: 10) motif (LPETG) (SEQ ID NO: 11), 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) and (5) the cell-wall targeting domain (C-terminal 99 amino acids) of Staphylococcal amidase (LytA) (hatched bar).

In Figure 2(B), S. aureus OS2 expressing genes of various hybrid proteins 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 500 µl each precipitated with TCA. One of these samples was lysostaphin digested (L) prior to boiling in hot SDS, whereas the other sample, (CH), was directly boiled in hot SDS. Proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB antibody, and subjected to 12% SDS-polyacrylamide gel electrophoresis.

In Figure 2(C), 1 ml of S. aureus OS2 culture expressing genes of SEB proteins was pulse-labeled with \(^{[35]S}\) methionine for 1 min, chased with non-radioactive
amino acids, and 500 µl each precipitated with TCA. The two samples were digested with either lysostaphin (L), or Chalaropsis B muramidase (Hash-enzyme) (H), and precipitated with TCA. Proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB, and subjected to 12% SDS-polyacrylamide gel electrophoresis. Molecular weight markers are indicated (kDa).

Upon cell fractionation, the hybrid SEB-LST was found mostly in the cell-wall compartment of S. aureus.

This hybrid molecule was soluble in SDS and did not meet other criteria established for the covalent linkage of surface proteins for the peptidoglycan (O. Schneewind et al., (1993), supra), indicating that it was bound but not covalently linked to the staphylococcal cell wall. As a control for the correct fractionation of staphylococcal cells, hybrid SEB molecules known to be either covalently linked to the cell wall (SEB-SPA<sub>490-524</sub>) or membrane anchored (SEB-ActA) were included (O. Schneewind et al. (1993), supra).

The database was searched for sequence elements homologous to the cell-wall targeting signal of lysostaphin, and five cell-wall proteins harboring similar sequences were found. To analyze the function of one of these sequence elements, the cell-wall targeting signal of LytA was fused to the C-terminal end of SEB; it was found that this hybrid molecule was also targeted to the cell wall of S. aureus.

The hybrid molecules were bound but not covalently linked to the cell wall because the SEB-LST and SEB-LytA proteins do not display a size difference when solubilized from the staphylococcal cell wall with
two different bacteriolytic enzymes (lysostaphin and Hash-enzyme). Because these enzymes cleave the cell wall at different points, if the chimeric proteins were covalently bound to the cell wall, they would be expected to display different molecular weights after cleavage because different portions of the cell wall would remain covalently attached. However, if the molecules are released from the cell wall by the digestion conditions without the necessity of cleaving any covalent bonds, the molecular weight would be expected to be identical. See panel (C), number 2 of Figure 2 as a control for a covalently linked molecule targeted by the protein A segment, which is known to result in covalent binding to the peptidoglycan layer of the cell wall (O. Schneewind et al., "Sorting of Protein A to the Staphylococcal Cell Wall," Cell 70:267-281 (1992)).

To investigate the cell surface display of molecules targeted to the cell wall of S. aureus, staphylococcal protein A was employed as an indicator molecule. Wild-type protein A is covalently linked to the staphylococcal cell wall by a mechanism requiring a C-terminal sorting signal (O. Schneewind et al. (1993), supra; O. Schneewind et al. (1992), supra). During cell-wall anchoring, the sorting signal is cleaved between the threonine (T) and the glycine (G) of an LPXTG (SEQ ID NO: 10) sequence motif (W.W. Navarre & O. Schneewind, Mol. Microbiol. 14:115 (1994)). The carboxyl of threonine is subsequently amide linked to the peptidoglycan crossbridge thereby anchoring the C-terminal end of the polypeptide chain to the peptidoglycan while the N-terminal immunoglobulin binding domains are displayed on
the staphylococcal cell surface (O. Schneewind et al., 268:103 (1995)).

This comparison is shown in Figure 3, comparing the surface display of proteins targeted to the cell wall of S. aureus by covalent or noncovalent mechanisms. Specifically, to investigate if proteins targeted to the wall of S. aureus were displayed on the cell surface, 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 (Fig. 3). Staphylococci expressing wild-type or mutant protein A were harvested by centrifugation, washed and incubated with fluorescein isothiocyanate labeled rabbit immunoglobulin (FITC-IgG).

Binding of FITC protein A located on the staphylococcal surface was visualized by microscopy under ultraviolet light (1000x).

Figure 3 shows the results with S. aureus OS2 expressing either wild-type protein A (1), protein A harboring a C-terminal cell-wall targeting signal (SPA\textsubscript{CWM}) (2) or a mutant protein A (SPA\textsubscript{1-519}) known to be secreted into the culture medium (3). The presence of staphylococci in panel (3) was demonstrated by light microscopy (3 visible light).

Wild-type protein A (SPA) bound FITC-labeled IgG on the staphylococcal surface as indicated by a strong fluorescent halo surrounding the staphylococci.
When the C-terminal sorting signal of protein A, which generates covalent binding, was replaced with the cell-wall targeting signal of lysostaphin (SPA<sub>cm7</sub>), it was observed that the binding of FITC-labeled IgG to the staphylococcal surface occurred with intensity similar to that of wild-type protein A. This shows not only that the noncovalent binding induced by the cell-wall targeting signal of lysostaphin causes the targeted protein present in the chimeric protein to be displayed to the medium to allow the binding of specific binding partners such as antibodies, but that the strength of the noncovalent binding of the chimeric protein to the staphylococcal surface is sufficiently great to allow labeling comparable to that observed with a covalently bound protein A molecule.

As a negative control, no binding of FITC-labeled IgG to surface displayed protein A was observed for truncated protein A molecule (SPA<sub>1-513</sub>) that was secreted into the Staphylococcal culture medium (O. Schneewind et al. (1992), supra).

These results suggest that lysostaphin is specifically bound to the cell wall of Staphylococcus aureus by a C-terminal cell-wall targeting domain. This binding is noncovalent, but is sufficiently tight to allow use of the targeted protein for labeling. The targeted protein is accessible to the medium surrounding the cells for reaction with ligands, including specific binding partners such as antibodies.

Similar cell-wall targeting domains were found in several other cell-wall proteins, suggesting that
these molecules may be targeted to the cell wall by a mechanism similar to that of lysostaphin.

A comparison of the sequences for LST and LytA, showing substantial homology between the C-terminal sequences of lysostaphin (LST) and amidase (LytA), is shown in Figure 4.

If the targeting signal of lysostaphin were sufficient to direct bacteriocin molecules to *S. aureus* cells, the addition of exogenous 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 hybrid glutathione S-transferase (D.B. Smith & K.S. Johnson, "Single-Step Purification of Polypeptides Expressed in Escherichia coli as Fusions with Glutathione S-Transferase," *Gene* 67: 31-40 (1988)) with a fused targeting signal (GST-CWT) and measuring its binding to *S. aureus* and *S. simulans* cells (Fig. 5). The binding of GST-CWT to staphylococci was quantitated by collecting cells with bound protein via centrifugation and measuring the decrease of glutathione S-transferase activity in the supernatant. *S. aureus* precipitated GST-CWT from the supernatant while *S. simulans* did not. Increasing amounts of *S. aureus* cells 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 x 10^7 up to 1 x 10^8 cfu). Nevertheless, increasing the number of *S. aureus* cells to more than 1 x 10^8 cfu 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, the number of binding sites for the targeting signal was calculated to be $10^6$ per colony forming unit. No significant precipitation of GST-CWT fusion protein occurred when it was added to S. simulans cells.

For these experiments, E. 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 of Tris-HCl, pH 8.0, 1 mM DTT, 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-200F instruments, Heat Systems-
Ultrasonic Inc.). Unbroken cells were removed by centrifugation for 15 min at 15,000 x g and the supernatant was subjected to affinity chromatography on glutathione Sepharose 4G (Pharmacia), with a 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 midlog phase (OD<sub>600</sub> 0.5) and washed several times with 50 mM Tris-HCl, pH 8.0. Purified GST-CWT protein (20 µg) was added to various amounts of staphylococci and incubated for 20 min at room temperature. The cells were collected by centrifugation (5 min at 15,000 x 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.

For the results shown in Figure 5, the hybrid protein with a fusion of the lysostaphin targeting signal to the C-terminus of GST-CWT was purified and 40 µg protein was added to 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. In panel (A), the glutathione S-transferase activity in the supernatant was determined and plotted against the number of *S. aureus* (circles) and *S. simulans*
(squares) cells. In panel (B), staphylococci with bound fusion protein were boiled in hot SDS and solubilized protein was precipitated on SDS-PAGE. The supernatant containing unbound GST-CWT protein was precipitated with TCA and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining.

Additionally, the targeting signal confers killing specificity onto lysostaphin. An assay was developed to specifically measure the bacteriolytic activity and target cell specificity of lysostaphin (Figure 6). Lysostaphin or its mutants were incubated with a mixture of S. simulans and S. aureus cells, and aliquots were plated on tryptic soy agar containing either erythromycin or streptomycin, thereby allowing the selective growth of either S. aureus (erythromycin resistant) or S. simulans (streptomycin resistant) survivors. For the experiments for which data is given in Figure 6, purified mature lysostaphin (LST), pro-lysostaphin (Pro-LST) or a mutant pro-lysostaphin lacking the C-terminal targeting signal (Pro-LST\textsubscript{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\textsubscript{CWT}) was adjusted to approximately $3.5 \times 10^4$ units employing Acetyl-Gly\textsubscript{6} as a substrate. The results shown in Figure 6 are representative of three independent experiments.
Purified mature lysostaphin (3.86 x 10^4 units) caused the selective killing of all but 4 out of 10^5 S. aureus target cells whereas only a few S. simulans cells were killed. The target cell specificity of mature lysostaphin was calculated 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 x 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, suggesting that the pro-region (14 N-terminal repeats) may function as an intramolecular chaperone for folding (X.L. Zhu et al., "Pro-Sequence of Subtilisin Can Guide the Refolding of Denatured Subtilisin in an Intermolecular Process," *Nature* 339: 483 (1989)). When tested with an acetylated hexaglycine residue (S.A. Kline et al., "A Colorimetric Microtiter Plate Assay for Lysostaphin Using a Hexaglycine Substrate," *Anal. Biochem.* 217: 329-331 (1994), the purified pro-lysostaphin (Pro-LST) was significantly less active than mature lysostaphin isolated from S. simulans. This reduction is likely caused by the inefficient re-folding of pro-lysostaphin, because the recombinant molecule had been purified under denaturing conditions. Pro-LST (3.53 x 10^4 units) was tested for its bacteriolytic activity, and it was found that the targeting activity of the pro-enzyme was somewhat reduced (target cell specificity 5.6 x 10^2). In contrast, a mutant pro-lysostaphin molecule
lacking the C-terminal targeting signal (Pro-LST\textsubscript{ACNT}, 3.23 x 10\(^4\) units) had lost its ability to specifically 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.

In these experiments, E. coli strain XL-1 Blue harboring either pPro-LST or pPro-LST\textsubscript{ACNT} was grown to midlog phase in 1 l of LB, induced with 1 mM IPTG, and incubated for another 3 hr. 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 hour at room temperature. After removal of cell debris by centrifugation for 15 min at 10,000 g, the lysate was applied onto 1 ml 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 (same composition as buffer B, but pH 6.3). Proteins were eluted with 5 ml of buffer E (same composition as buffer B, but 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% of Tween 80 for 24 hours at 4°C without stirring, followed by another dialysis against the same buffer without urea for 16 hours at 4°C with slow stirring.
Lysostaphin activity was measured as the release of free amino groups from N-acetylated hexaglycine (acetyl-Gly₆) (S.A. Kline et al. (1994), supra). Briefly, 0.5 ml substrate solution (5 mM trisodium citrate, 1 mM disodium EDTA, 100 mM sodium borate, 10 mM acetyl-Gly₆) and 0.5 ml of purified bacteriocin were mixed and incubated for 1 hour at 37°C. The colorimetric reagent 2,4,6-trinitrobenzenesulfonic acid (60 µl) in 0.1 M sodium bicarbonate was added and the reactions were incubated for 20 min at room temperature. The reactions were finally quenched with 100 µl of 3 M sodium acetate and absorbance at 405 nm was measured against a negative control with no added enzyme.

To determine the target cell specificity of lysostaphin, S. aureus OS2 (spa⁺, ermC) (O. Schneewind et al. (1992), supra), and S. simulans TNK1 (simultaneous streptomycin-resistant derivative of ATCC1362) were grown in TSB medium to mid-log phase. The cells were collected by centrifugation, washed and finally diluted to 1.0 x 10⁶ cfu/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-LST₄CMT in 50 mM Tris-HCl, pH 7.5, 0.005% of Tween 80) and incubated for 3 hours 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).

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 (O. Schneewind et al. (1992), supra). During anchoring, the sorting signal is cleaved between the threonine and the glycine of the LPXTG (SEQ ID NO: 10) motif (W.W. Navarre & O. Schneewind, "Proteolytic Cleavage and Cell Wall Anchoring at the LPXTG Motif of Surface Proteins in Gram-Positive Bacteria," Mol. Microbiol. 14: 115-121 (1994)), and the liberated carboxyl of threonine is subsequently amide linked to a free amino in the pentaglycine crossbridge of the staphylococcal peptidoglycan (O. Schneewind et al., "Structure of the Cell Wall Anchor of Surface Proteins in Staphylococcus aureus," Science 268: 103-106 (1995)). An attempt was made to covalently link lysostaphin to its normal substrate, the pentaglycine crossbridge, by fusing the sorting signal of protein A to the C-terminal end of the polypeptide chain (LST-CWS (Figure 7)). The covalent linkage of the LST-CWS molecule was measured by digesting the peptidoglycan of S. aureus with muramidase. However, the hybrid LST-CWS molecule migrated uniformly on SDS-PAGE, suggesting that either the chimeric molecule was not linked to the peptidoglycan or that 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, the fact that the cleavage of the sorting signal at the LPXTG (SEQ ID NO: 10) motif causes cell wall-linked proteins to migrate faster on SDS-PAGE than their uncleaved and unanchored mutant counterparts was
exploited. A hybrid lysostaphin molecule was constructed, with a fused sorting signal devoid of its LPETG (SEQ ID NO: 11) sequence (LST-CWS_{LPETG}); it was 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, the cell wall linkage of another protein harboring both targeting and sorting signals was tested (Figure 7). 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. Furthermore, the unanchored species of SEB-LST-CWS protein migrated more slowly on SDS-PAGE than a control molecule with a defective LPXTG (SEQ ID NO: 10) 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.
In the experiments shown in Figure 7, in panel (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 (SEQ ID NO: 10) motif. In addition, hybrid enterotoxin B molecules were constructed 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 (SEQ ID NO: 10) motif (5). When fused to exported proteins, the sorting signal causes cleavage at the LPXTG (SEQ ID NO: 10) motif and the linkage of the polypeptide chain to the bacterial peptidoglycan. In panel (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 Fig. 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.

These results provide evidence that lysostaphin is specifically addressed 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 (K.H. Schleifer & O. Kandler (1972), supra) 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 lysostaphin even though the pentaglycine crossbridges in the peptidoglycan of *S. simulans* can be cleaved by lysostaphin. Only when incubated in the presence of large amounts of enzyme can the peptidoglycan of *S. simulans* be completely digested with lysostaphin (P. Heinrich et al., "The Molecular Organization of the Lysostaphin Gene and Its Sequences Repeated in Tandem," *Mol. Gen. Genet.* 209: 563-569 (1987)), indicating that the cell wall of the host organism does serve as a substrate of bacteriocin activity.

1109 (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 as well as epr transformed S. aureus contain an increased amount of serine over glycine residues (Dehart et al. (1995), supra). The authors proposed that an altered peptidoglycan structure, for example a replacement of glycine with serine in the wall crossbridge, may be responsible for the relative immunity of S. simulans to secreted lysostaphin. Another interpretation of this 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 (A. Wang et al. (1991), supra). The results here demonstrate 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. Other cell wall-associated proteins, such as peptidoglycan hydrolase from
pneumococci (E. Diaz et al., "Subcellular Localization of the Major Pneumococcal Autolysin: A Peculiar Mechanism of Secretion in Escherichia coli," J. Biol. Chem. 264: 1238-1244 (1989)), have similar modular organization. The C-terminal repeat domains of Streptococcus pneumoniae amidase, for example, promote its binding to choline within the pneumococcal cell wall (J.M. Sanz et al., "Studies on the Structure and Function of the N-Terminal Domain of the Pneumococcal Murein Hydrolases," Mol. Microbiol. 6: 921-931 (1992)), a compound that is not present in staphylococcal peptidoglycans. The lysostaphin receptor from the cell wall of S. aureus is currently being purified. The reported 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 (T. Oshida et al., "A Staphylococcus aureus Autolysin That Has an N-Acetylmuramoyl-L-Alanine Amidase Domain and an Endo-β-N-Acetylglucosaminidase Domain: Cloning, Sequence Analysis, and Characterization," Proc. Natl. Acad. Sci. USA 92: 285-289 (1995); S. Yamada et al., "An Autolysin Ring Associated with Cell Separation of Staphylococcus..."
"aureus," *J. Bacteriol.* 178: 1565-1571 (1996)). The molecular information for these events is likely encoded in both the targeted polypeptide chains as well as the specific chemical nature of a distinct site within the cell wall.

ADVANTAGES OF THE PRESENT INVENTION

The processes and compositions of the present invention allow the specific noncovalent targeting of a wide variety of molecules to the cell surfaces of Gram-positive bacteria, particularly *Staphylococcus aureus*. The molecules targeted can be used for the generation of vaccines by immunization, the screening of cloned genes in a manner similar to that for phage display, and for the targeting of antibiotics and labeling substances to pathogenic bacteria. Large protein molecules can be accommodated as long as the chimeric protein can be formed. In addition, non-protein molecules such as alkaloids, steroids, and carbohydrates can be bound to the cell surface by covalent linkage to a peptide containing the cell-wall targeting signal. The cell-wall targeting produced by the process of the present invention is stable, and the resulting targeted molecules are accessible to the medium for reaction with ligands, such as molecules of the immune system that recognize antigens. A wider variety of molecules can be targeted then is the case for phage display, which is limited to relatively small molecules. This method provides great versatility, and has utility in the preparation of vaccines, the detection of cloned proteins in surface display in biotechnology, and in the specific targeting
of antibiotics and labeling molecules to pathogenic gram-positive bacteria.

Although the present invention has been described with considerable detail, with reference to certain preferred versions thereof, other versions and embodiments are possible.
SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANTS: Schneewind, Olaf
    Baba, Tadashi

(ii) TITLE OF THE INVENTION: TARGETING OF PROTEINS TO THE CELL
    WALL OF GRAM-POSITIVE BACTERIA

(iii) NUMBER OF SEQUENCES: 11

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(v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Diskette
    (B) COMPUTER: IBM Compatible
    (C) OPERATING SYSTEM: DOS
    (D) SOFTWARE: FastSEQ Version 1.5

(vi) CURRENT APPLICATION DATA:
    (A) INTERNATIONAL APPLICATION NUMBER:
    (B) FILING DATE: 22-AUG-1996
    (C) CLASSIFICATION:

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    (A) APPLICATION NUMBER: __________________
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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 92 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: C-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Trp Lys Thr Asn Lys Tyr Gly Thr Leu Tyr Lys Ser Glu Ser Ala Ser
1     5    10    15
Phe Thr Pro Asn Thr Asp Ile Ile Thr Arg Thr Gly Pro Phe Arg
20   25   30
Ser Met Pro Gln Ser Gly Val Leu Lys Ala Gly Gln Thr Ile His Tyr
35   40   45
Asp Glu Val Met Lys Gln Asp Gly His Val Trp Val Gly Tyr Tyr Gly
50   55   60
Asn Ser Gly Gln Arg Ile Tyr Leu Pro Val Arg Thr Trp Asn Lys Ser
65   70   75   80
Thr Asn Thr Leu Gly Val Leu Trp Gly Thr Ile Lys
85   90
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: C-terminal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Ala Ser Ala Trp Lys Arg Asn Lys Tyr Gly Thr Tyr Tyr Met Glu
1      5     10     15
Glu Ser Ala Arg Phe Thr Asn Gly Asn Gln Pro Ile Thr Val Arg Lys
20     25     30
Val Gly Pro Phe Leu Ser Cys Pro Val Gly Tyr Gln Phe Gln Pro Gly
35     40     45
Gly Tyr Cys Asp Tyr Thr Glu Val Met Leu Asn Asp Gly His Val Trp
50     55     60
Val Gly Tyr Thr Trp Glu Gly Gln Arg Tyr Tyr Leu Pro Ile Arg Thr
65     70     75     80
Trp Asn Gly Ser Ala Pro Pro Asn Gln Ile Leu Gly Asp Leu Trp Gly
85     90     95
Glu Ile Ser

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr Gly Val Leu Pro Asn Asn Ala Thr Ile Lys Tyr Asp Gly Ala Tyr
1      5      10     15
Cys Ile Asn Gly Tyr Arg Trp Ile Thr Tyr Ile Ala Asn Ser Gly Gln
20     25     30
Arg Arg Tyr Ile Ala Thr Gly Glu Val Asp Lys Ala Gly Asn Arg Ile
35     40     45

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ser Pro Thr Gln Phe Thr Phe Asn Lys Gly Glu Ser Ile Tyr Tyr
1 5 10 15
Asp Ser Ile Leu Asn Ala Asp Gly His Gln Trp Ile Ser Tyr Arg Ser
20 25 30
Tyr Ser Gly Ile Arg Arg Tyr Ile
35 40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AACATATGAA GAAAAACAAA AAACAATTATA

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AAGGATCTTC ACTTTATAGT TCCCCAAA

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCCCCCGGGC ATATGAACC CTCCGATATT ACAGTT

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGAATTCCA TATCTCTAA TCA TTTTGC TAA

33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Glu Val Glu Thr Ser Lys Ala Pro Val Glu Asn Thr

1     5     10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Pro Xaa Thr Gly
1  5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Pro Glu Thr Gly
1  5
What is claimed is:

1. A method for targeting a protein to the cell wall of a Gram-positive bacterium comprising:
   (a) cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal;
   (b) growing the bacterium into which the nucleic acid segment has been cloned to express the cloned chimeric protein in the Gram-positive bacterium to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal; and
   (c) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

2. The method of claim 1 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of Staphylococcus simulans; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of Staphylococcus aureus; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions.
occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.


5. The method of claim 2 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.


7. The method of claim 6 wherein X₃ is R or T, X₉ is Y or L, X₁₁ is M or K, X₁₂ is E or S, X₁₆ is R or S, X₁₉ is N or P, X₂₀ is G or N, X₂₁ is N or T, X₂₂ is Q or D, X₂₃ is P or I, X₂₆ is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₂₈ is K or T, X₂₉ is V or T, X₃₃ is L or R, X₃₅ is G or M, X₃₇ is V or Q, X₃₈ is G or S, X₃₉ is Y or G, X₄₀ is Q or V, X₄₁ is F or L, X₄₂ is Q or K, X₄₃ is P or A, X₄₅ is G or Q, X₄₆ is Y or T, X₄₇ is C or I, X₄₈ is D or H, X₅₀ is T or D, X₅₄ is L or K, X₆₅ is W or G, X₆₆ is E or N, X₆₇ is an absent amino acid with an amide bond between the preceding and following amino acids in the
chain or S, $X_{21}$ is Y or I, $X_{80}$ is G or K, $X_{82}$ is A or T, $X_{83}$ is P or N, $X_{84}$ is P or T, $X_{85}$ is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{86}$ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{87}$ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{94}$ is E or T, and $X_{96}$ is S or K.

8. The method of claim 5 wherein:

(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and

(b) the conservative amino acid substitutions in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

10. The method of claim 1 wherein the Gram-positive bacterium is a Staphylococcus species.

11. The method of claim 10 wherein the Staphylococcus species is Staphylococcus aureus.

12. A method for targeting a protein to the cell wall of a Gram-positive bacterium comprising:
   (a) generating a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal by expression of a nucleotide sequence encoding the chimeric protein in an expression system other than an expression system employing a Gram-positive bacterium as host;
   (b) adding the chimeric protein to a culture medium in which a Gram-positive bacterium is growing; and
   (c) allowing the chimeric protein to bind stably and noncovalently to the cell wall of the Gram-positive bacterium so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.
13. The method of claim 12 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of Staphylococcus simulans; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of Staphylococcus aureus; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.


16. The method of claim 13 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

18. The method of claim 17 wherein X₃ is R or T, X₉ is Y or L, X₁₁ is M or K, X₁₂ is E or S, X₁₆ is R or S, X₁₉ is N or P, X₂₀ is G or N, X₂₁ is N or T, X₂₂ is Q or D, X₂₃ is P or I, X₂₆ is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₂₈ is K or T, X₃₀ is V or T, X₃₀ is L or R, X₃₁ is G or M, X₃₇ is V or Q, X₃₈ is G or S, X₃₉ is Y or G, X₄₀ is Q or V, X₄₁ is F or L, X₄₂ is Q or K, X₄₃ is P or A, X₄₅ is G or Q, X₄₆ is Y or T, X₄₇ is C or I, X₄₈ is D or H, X₅₀ is T or D, X₅₄ is L or K, X₆₅ is W or G, X₆₆ is E or N, X₆₇ is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X₇₁ is Y or I, X₈₀ is G or K, X₈₂ is A or T, X₈₃ is P or N, X₈₄ is P or T, X₈₅ is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₈₆ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₈₇ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₉₄ is E or T, and X₉₆ is S or K.

19. The method of claim 16 wherein:
(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and
(b) the conservative amino acid substitutions in less highly conserved regions are the following: any
of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.


21. The method of claim 12 wherein the Gram-positive bacterium is a Staphylococcus species.

22. The method of claim 21 wherein the Staphylococcus species is Staphylococcus aureus.

23. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a chimeric protein including therein: (1) a protein antigen and (2) a
carboxyl-terminal cell wall-targeting signal bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the chimeric protein is accessible to the antigen-processing system of the antibody-producing animal.

24. The method of claim 23 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-P-T-F-N-K-G-E-S-I-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal
results in at least about 80% homology and at least about 70% identity.


27. The method of claim 23 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

28. The method of claim 27 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-X_{1}-N-K-T-G-T-X_{9}-Y-X_{11}-X_{12}-E-S-A-X_{16}-F-T-X_{19}-X_{20}-X_{21}-X_{22}-X_{23}-I-T-X_{26}-R-X_{28}-X_{29}-G-P-F-X_{33}-S-X_{35}-P-X_{37}-X_{38}-X_{39}-X_{40}.
X_{41}\cdot X_{42}\cdot X_{43}\cdot G\cdot X_{45}\cdot X_{46}\cdot X_{47}\cdot X_{48}\cdot Y\cdot X_{50}\cdot E\cdot V\cdot M\cdot X_{54}\cdot Q\cdot D\cdot G\cdot H\cdot V\cdot W\cdot V\cdot G\cdot 
Y\cdot T\cdot X_{65}\cdot X_{66}\cdot X_{67}\cdot G\cdot Q\cdot R\cdot X_{71}\cdot Y\cdot L\cdot P\cdot X_{75}\cdot R\cdot T\cdot W\cdot N\cdot X_{90}\cdot S\cdot X_{82}\cdot X_{83}\cdot X_{84}\cdot 
X_{85}\cdot X_{86}\cdot X_{87}\cdot L\cdot G\cdot V\cdot L\cdot W\cdot G\cdot X_{94}\cdot I\cdot X_{96}, \text{ wherein the residues designated by subscripts are chosen so that the}
conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or amidase targeting signals.

29. The method of claim 28 wherein X_{3} is R or T, X_{9} is Y or L, X_{11} is M or K, X_{12} is E or S, X_{16} is R or S, X_{19} is N or P, X_{20} is G or N, X_{21} is N or T, X_{22} is Q or D, X_{23} is P or I, X_{26} is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X_{28} is K or T, X_{29} is V or T, X_{33} is L or R, X_{35} is G or M, X_{37} is V or Q, X_{38} is G or S, X_{39} is Y or G, X_{40} is Q or V, X_{41} is F or L, X_{42} is Q or K, X_{43} is P or A, X_{45} is G or Q, X_{46} is Y or T, X_{47} is C or I, X_{48} is D or H, X_{50} is T or D, X_{54} is L or K, X_{65} is W or G, X_{66} is E or N, X_{67} is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X_{71} is Y or I, X_{80} is G or K, X_{82} is A or T, X_{83} is P or N, X_{84} is P or T, X_{95} is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X_{86} is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X_{87} is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X_{94} is E or T, and X_{96} is S or K.
30. The method of claim 27 wherein:

(a) the conservative amino acid substitutions
in highly conserved regions are the following: any of
isoleucine, valine, and leucine for any of these other
amino acids; aspartic acid for glutamic acid and vice
versa; glutamine for asparagine and vice versa; and
serine for threonine and vice versa; and

(b) the conservative amino acid substitutions
in less highly conserved regions are the following: any
of isoleucine, valine, and leucine for any of these other
amino acids; aspartic acid for glutamic acid and vice
versa; glutamine for asparagine and vice versa; serine
for threonine and vice versa; glycine for alanine and
vice versa; alanine for valine and vice versa; methionine
for any of leucine, isoleucine, or valine; lysine for
arginine or vice versa; one of aspartic acid or glutamic
acid for one of arginine or lysine; histidine for one of
arginine or lysine; glutamine for glutamic acid and vice
versa; and asparagine for aspartic acid and vice versa.

31. The method of claim 23 wherein the
carboxyl-terminal cell wall targeting signal has from
about 80 amino acids to about 100 amino acids, and
includes therein an amino acid sequence that is T-G-V-L-
R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-
32. The method of claim 23 wherein the Gram-positive bacterium is a Staphylococcus species.

33. The method of claim 32 wherein the Staphylococcus species is Staphylococcus aureus.

34. The method of claim 23 wherein the antigen is a polypeptide antigen produced by Candida albicans, Aspergillus fumigatus, Histoplasma capsulatum, Microsporum canis, Plasmodium falciparum, Trypanosoma cruzi, Borrelia burgdorferi, Treponema pallidum, Borrelia recurrentis, Leptospira icterohaemorrhagiae, Neisseria gonorrhoeae, Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhosa, Hemophilus influenzae, Bordetella pertussis, Actinomyces israelii, Streptococcus mutans, Streptococcus equi, Streptococcus agalactiae, Streptococcus anginosus, human immunodeficiency virus (HIV), poliovirus, influenza virus, rabies virus, herpes virus, foot and mouth disease virus, psittacosis virus, paramyxovirus, myxovirus, or coronavirus.

35. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a non-protein antigen or hapten covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal, the complex bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the complex is displayed on
the surface of the Gram-positive bacterium in such a way that the complex is accessible to the antigen-processing system of the antibody-producing animal.

36. The method of claim 35 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of Staphylococcus simulans; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of Staphylococcus aureus; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.

37. The method of claim 36 wherein the carboxyl-terminal cell-wall targeting signal is the
L-W-G-T-I-K (SEQ ID NO: 1).

Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

39. The method of claim 35 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

40. The method of claim 39 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-X₁-N-K-T-G-T-X₉-Y-X₁₁-X₁₂-E-S-A-X₁₆-F-T-X₁₉-X₂₀-
X₂₁-X₂₂-X₂₃-I-T-X₂₆-R-X₂₈-X₂₉-G-P-F-X₃₃-S-X₃₅-P-X₃₇-X₃₈-X₃₉-X₄₀-
Y-T-X₆₅-X₆₆-X₆₇-G-Q-R-X₇₁-Y-L-P-X₇₅-R-T-W-N-X₈₀-S-X₈₂-X₈₃-X₈₄-
X₈₅-X₈₆-X₈₇-L-G-V-L-W-G-X₉₄-I-X₉₆, wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains
substantially equivalent to that of the lysostaphin or amidase targeting signals.

41. The method of claim 40 wherein X₁ is R or T, X₉ is Y or L, X₁₁ is M or K, X₁₂ is E or S, X₁₆ is R or S, X₁₉ is N or P, X₂₀ is G or N, X₂₁ is N or T, X₂₂ is Q or D, X₂₃ is P or I, X₂₆ is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₂₈ is K or T, X₂₉ is V or T, X₃₃ is L or R, X₃₅ is G or M, X₃₇ is V or Q, X₃₈ is G or S, X₃₉ is Y or G, X₄₀ is Q or V, X₄₁ is F or L, X₄₂ is Q or K, X₄₃ is P or A, X₄₅ is G or Q, X₄₆ is Y or T, X₄₇ is C or I, X₄₈ is D or H, X₅₀ is T or D, X₅₄ is L or K, X₆₅ is W or G, X₆₆ is E or N, X₆₇ is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X₇₁ is Y or I, X₈₀ is G or K, X₈₂ is A or T, X₈₃ is P or N, X₈₄ is P or T, X₈₅ is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₈₆ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₈₇ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X₉₄ is E or T, and X₉₆ is S or K.

42. The method of claim 39 wherein:
(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice
versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and
(b) the conservative amino acid substitutions
in less highly conserved regions are the following: any
of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

43. The method of claim 35 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-

44. The method of claim 35 wherein the Gram-positive bacterium is a Staphylococcus species.

45. The method of claim 44 wherein the Staphylococcus species is Staphylococcus aureus.
46. The method of claim 35 wherein the antigen or hapten is a drug, an alkaloid, a steroid, a carbohydrate, or an aromatic compound.

47. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a protein antigen covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal, the complex bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the complex is displayed on the surface of the Gram-positive bacterium in such a way that the complex is accessible to the antigen-processing system of the antibody-producing animal.

48. The method of claim 47 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-
T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-P-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.


51. The method of claim 47 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the
existence of consensus sequence regions occurring in both
the lysostaphin signal and the amidase signal.

52. The method of claim 51 wherein the
carboxyl-terminal cell-wall targeting signal has the
Y-T-X65-X66-X67-G-Q-R-X71-Y-L-P-X75-R-T-W-N-X85-S-X82-X83-X84-
X85-X86-X87-L-G-V-L-W-G-X94-I-X96, wherein the residues
designated by subscripts are chosen so that the
conformation of the targeting signal remains
substantially equivalent to that of the lysostaphin or
amidase targeting signals.

53. The method of claim 52 wherein X3 is R or
T, X9 is Y or L, X11 is M or K, X12 is E or S, X16 is R or
S, X19 is N or P, X20 is G or N, X21 is N or T, X22 is Q or
D, X23 is P or I, X26 is V or an absent amino acid with an
amide bond between the preceding and following amino
acids in the chain, X28 is K or T, X29 is V or T, X33 is L
or R, X35 is G or M, X37 is V or Q, X38 is G or S, X39 is Y
or G, X40 is Q or V, X41 is F or L, X42 is Q or K, X43 is P
or A, X45 is G or Q, X46 is Y or T, X47 is C or I, X48 is D
or H, X50 is T or D, X54 is L or K, X55 is W or G, X65 is E
or N, X67 is an absent amino acid with an amide bond
between the preceding and following amino acids in the
chain or S, X71 is Y or I, X80 is G or K, X82 is A or T, X83
is P or N, X84 is P or T, X85 is N or an absent amino acid
with an amide bond between the preceding and following
amino acids in the chain, $X_{96}$ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{97}$ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{94}$ is E or T, and $X_{96}$ is S or K.

54. The method of claim 51 wherein:
(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and
(b) the conservative amino acid substitutions in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

55. The method of claim 47 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-
56. A method for screening a cloned protein for reactivity with a specific binding partner comprising:

(a) incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal cell wall-targeting signal;

(b) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(c) reacting the noncovalent complex with a labeled specific binding partner to screen the cloned protein for reactivity with the specific binding partner.

57. The method of claim 56 wherein the labeled specific binding partner is an antibody.

58. A method for treating an infection caused by a Gram-positive bacterium comprising:

(a) conjugating an antibiotic to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate; and

(b) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat the infection.
59. The method of claim 58 wherein the antibiotic is a penicillin, ampicillin, vancomycin, gentamicin, streptomycin, a cephalosporin, amikacin, kanamycin, neomycin, paromomycin, tobramycin, ciprofloxacin, clindamycin, rifampin, chloramphenicol, or norfloxacin.

60. The method of claim 58 wherein the Gram-positive bacterium is a Staphylococcus species.

61. The method of claim 60 wherein the Staphylococcus species is Staphylococcus aureus.

62. A method for detecting the presence of a Gram-positive bacterium so as to diagnose an infection caused by a Gram-positive bacterium comprising:
   (a) conjugating a diagnostic reagent to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate; and
   (b) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the diagnostic reagent noncovalently to the cell walls of the bacterium, thereby detecting the Gram-positive bacterium and diagnosing the infection.

63. The method of claim 62 wherein the diagnostic reagent is an antibody labeled with a detectable label.
64. The method of claim 62 wherein the Gram-positive bacterium is a *Staphylococcus* species.

65. The method of claim 64 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

66. A chimeric protein comprising:
   (a) a protein to be targeted to the cell wall of a Gram-positive bacterium; and
   (b) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

67. The chimeric protein of claim 66 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence
NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-
G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e)
a signal related to the signals of (a), (b), (c), or (d)
by truncation such that the total length of the signal is
reduced to no less than about 80 amino acids and the best
alignment of the truncated signal and the original signal
results in at least about 80% homology and at least about
70% identity.

68. The chimeric protein of claim 67 wherein
the carboxyl-terminal cell-wall targeting signal is the
lysostaphin signal having an amino acid sequence of W-K-
L-W-G-T-I-K (SEQ ID NO: 1).

69. The chimeric protein of claim 67 wherein
the carboxyl-terminal cell-wall targeting signal is the
amidase signal having an amino acid sequence of V-A-S-A-
V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-
Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

70. The chimeric protein of claim 67 wherein
the carboxyl-terminal cell-wall targeting signal is a
signal related to the lysostaphin signal by one or more
conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

71. The chimeric protein of claim 70 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-X_{12}-N-K-T-G-T-X_{9}-Y-X_{11}-E-S-A-X_{16}-F-T-X_{19}-X_{20}-X_{21}-X_{22}-X_{23}-I-T-X_{26}-R-X_{28}-X_{29}-G-P-F-X_{33}-S-X_{35}-P-X_{37}-X_{38}-X_{39}-X_{40}-X_{41}-X_{42}-X_{43}-G-X_{45}-X_{46}-X_{47}-X_{48}-Y-X_{50}-E-V-M-X_{54}-Q-D-G-H-V-W-V-G-Y-T-X_{65}-X_{66}-X_{67}-G-Q-R-X_{71}-Y-L-P-X_{75}-R-T-W-N-X_{80}-S-X_{82}-X_{83}-X_{84}-X_{85}-X_{86}-X_{87}-L-G-V-L-W-G-X_{94}-I-X_{96}, wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or amidase targeting signals.

72. The chimeric protein of claim 71 wherein X_{3} is R or T, X_{5} is Y or L, X_{11} is M or K, X_{12} is E or S, X_{16} is R or S, X_{19} is N or P, X_{20} is G or N, X_{21} is N or T, X_{22} is Q or D, X_{23} is P or I, X_{26} is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X_{28} is K or T, X_{29} is V or T, X_{33} is L or R, X_{35} is G or M, X_{37} is V or Q, X_{38} is G or S, X_{39} is Y or G, X_{40} is Q or V, X_{41} is F or L, X_{42} is Q or K, X_{43} is P or A, X_{45} is G or Q, X_{46} is Y or T, X_{47} is C or I, X_{48} is D or H, X_{50} is T or D, X_{54} is L or K, X_{55} is W or G, X_{65} is E or N, X_{67} is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X_{71} is Y or I, X_{80} is G or K, X_{82} is A or T, X_{83} is P or N, X_{84} is P or T, X_{85} is N or an absent amino acid
with an amide bond between the preceding and following amino acids in the chain, $X_{85}$ is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{87}$ is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, $X_{94}$ is E or T, and $X_{96}$ is S or K.

73. The chimeric protein of claim 70 wherein:
   (a) the conservative amino acid substitutions
   in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and
   (b) the conservative amino acid substitutions
   in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

74. The chimeric protein of claim 66 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and
includes therein an amino acid sequence that is T-G-V-L-
R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-

75. A nucleic acid segment encoding the chimeric protein of claim 66.

76. The nucleic acid segment of claim 75 wherein the nucleic acid segment is DNA.

77. A vector comprising the nucleic acid segment of claim 75 operatively linked to at least one control sequence that controls the expression or regulation of the nucleic acid segment.

78. A noncovalent complex comprising:
(a) the chimeric protein of claim 66; and
(b) a Gram-positive bacterium, in which the chimeric protein is bound noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

79. The noncovalent complex of claim 78 wherein the Gram-positive bacterium is a Staphylococcus species.
80. The noncovalent complex of claim 79 wherein the Staphylococcus species is Staphylococcus aureus.

81. A covalent conjugate comprising:
   (a) a cell-wall targeting segment including a carboxyl-terminal cell-wall targeting signal therein that can be bound noncovalently and stably to a cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal; and
   (b) an antigen or hapten conjugated covalently to the cell-wall targeting segment.

82. The covalent conjugate of claim 81 wherein the antigen or hapten is a protein antigen.

83. The covalent conjugate of claim 81 wherein the antigen or hapten is a nonprotein antigen or hapten.

84. The covalent conjugate of claim 81 wherein the cell-wall targeting segment further includes an amino-terminal spacer.

85. A noncovalent complex comprising:
   (a) the covalent conjugate of claim 81; and
   (b) a Gram-positive bacterium stably and noncovalently binding the covalent conjugate.
86. The noncovalent complex of claim 85 wherein the Gram-positive bacterium is a *Staphylococcus* species.

87. The noncovalent complex of claim 86 wherein the *Staphylococcus* species is *Staphylococcus aureus*. 
Fig. 1
Fig. 5

A

B

Cell pellet Supematant

cfu (x 10^7) 0 2.5 5 10 20 40 0 2.5 5 10 20 40

S. aureus S. simulans

-46 kDa -30

-18

-46 kDa

-30

-18
Target Cell Specificity and Bacteriolytic Activity of Lysostaphin

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FIGURE 6
# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

| U.S. | 530/300, 350, 333, 334, 387.3; 435/320.1, 254.2, 7.1, 7.33, 7.6, 252.3; 436/536, 518, 501, 543, 532; 536/25.3 |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

T document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A document member of the same patent family

Date of the actual completion of the international search: 24 OCTOBER 1996

Date of mailing of the international search report: 13 NOV 1996

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

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[Signature]

TelephoneNumber (703) 308-0197

Authorized officer

NITA M. MINNIFIELD

TelephoneNumber (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)*
<table>
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<td>Y</td>
<td>WO 87/06264 A1 (PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK) 22 October 1987, see entire document.</td>
<td>1-11</td>
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INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found uns searchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ✒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11

Remark on Protest ☐ The additional search fees were accompanied by the applicant’s protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*
A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
G01N 33/53, 33/569, 33/536, 33/543, 33/566, 33/549, 33/531; C12N 1/20, 15/00, 15/63, 1/14, 15/09, 15/70, 15/74;
A61K 38/00; C07K 2/00, 4/00, 5/00, 7/00, 16/00, 17/00; C12P 21/08; C07H 21/00, 21/02, 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :
530/300, 350, 333, 334, 387.3; 435/320.1, 254.2, 7.1, 7.33, 7.6, 252.3; 436/536, 518, 501, 543, 532; 536/25.3

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):
IntelliGenetics, MaasPar, CaPlus, WPIDS, Derwent Biotechnology Abs, Medline, CAB Abstracts, Life Sciences
Collection, Dissertation Abstracts Online, FEDRIP, AGRICOLA, Oceanic Abst, EMBASE, BIOSIS PREVIEWS,
PASCAL, CRIS/USDA, Wilson Appl. Sci & Tech Abs

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single
inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional
examination fees must be paid.

Group I, claim(s)1-11, drawn to a method for targeting a protein to the cell wall of a Gram-positive bacterium.
Group II, claim(s) 12-22, drawn to a method for targeting a protein to the cell wall of a Gram-positive
bacterium.
Group III, claim(s) 23-34, drawn to method for producing a vaccine to an antigen.
Group IV, claim(s)35-46, drawn to method for producing a vaccine to an antigen.
Group V, claim(s) 47-55, drawn to method for producing a vaccine to an antigen.
Group VI, claim(s) 56-57, drawn to method for screening a cloned protein.
Group VII, claim(s) 58-61, drawn to method for treating infection caused by a Gram-positive bacterium.
Group VIII, claim(s)62-65, drawn to method for detecting the presence of a Gram-positive bacterium.
Group IX, claim(s) 66-80, drawn to chimeric protein.
Group X, claim(s) 81-87, drawn to covalent conjugates.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under
PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods
set forth in Groups I and II are both drawn to a method for targeting a protein to the cell wall of a Gram-positive
bacterium, however the specific steps involved in each method are different. Groups III-V are drawn to method for
producing a vaccine to an antigen, however the specific steps involved in each method are different. The methods steps
for Group I, II, III, IV, V, VI, VII, and VIII are all different and therefore lack the same technical feature. The
methods (Groups I-VIII), chimeric proteins (Group IX), and covalent conjugates (Group X) do not share the same
technical feature. The conjugates comprise cell wall proteins conjugated to an antigen or hapten or spacers, which is
not the same as the chimeric protein.