A recent approach to vaccine development is to stimulate secretion of mucosal immunoglobulins that inhibit colonization of specific bacterial pathogens at the mucosal site of invasion. Because colonization is required to initiate infection in many diseases, blocking of colonization should prevent disease.

Intranasal vaccines against Streptococcus pyogenes, which causes “strep throat,” exemplify this approach. M6 protein is a major surface component and virulence factor of S. pyogenes. Mucosal immunization with the conserved C repeat region of M6 protein (herein referred to as M6c) protects against streptococcal challenge and inhibits pharyngeal colonization by S. pyogenes in mice (3, 4, 11). Moreover, M6-specific secretory immunoglobulin A (IgA) inhibits adherence of S. pyogenes to cultured human pharyngeal cells (12).

Food-grade bacteria are emerging as possible alternatives to attenuated pathogens for the delivery and presentation of heterologous antigens to mucosal immune systems of animals and humans. Lactococcus lactis is a gram-positive bacterium that is used to make fermented dairy foods such as cheese and sour cream, and it has been safely consumed by humans and animals for millennia. L. lactis is designated GRAS (generally recognized as safe) by the Food and Drug Administration and is considered safe by the European Union for delivery of antigens to the mucosal immune system (7).

In this study, M6c was genetically fused to Pip and expressed in L. lactis. Expression was increased by genetic manipulations, and the fused protein was analyzed by Western blotting. The physiological function of Pip is unknown, and genetic deletion of Pip causes no phenotypic change in vitro except phage resistance (13, 22).

One of our labs has cloned and sequenced a chromosomal gene (pip) from L. lactis that is required for infection by one species of lactococcal bacteriophage (13). Pip is a membrane protein that serves as a receptor for bacteriophage (27, 42). The physiological function of Pip is unknown, and genetic deletion of Pip causes no phenotypic change in vitro except phage resistance (13, 22).

In this study, M6c was genetically fused to Pip and expressed in L. lactis. Expression was increased by genetic manipulations, and the fused protein was analyzed by Western blotting.

MATERIALS AND METHODS

Bacterial strains, phages, media, growth rates, and plaque assay. The strains and plasmids used are listed in Table 1. L. lactis subsp. lactis LM2301 and its isogenic pip-emm6c derivative BG301 were grown at 30°C and maintained on M17 medium (41) supplemented with 0.5% glucose (M17G). pGhost6-based plasmids (Apppligene, Pleasonton, Calif.) were maintained in lactococcal strains on M17G with 5 μg of erythromycin per ml. Escherichia coli DH5α (Life Technologies, Rockville, Md.) was grown at 37°C in Luria-Bertani (LB) medium supplemented with 0.5% glucose (LB) medium (35) with 20 μg of chloramphenicol per ml or 100 μg of ampicillin per ml for maintenance of pTRK81 or pUC19-derived vectors, respectively. pGhost6-derived vectors were maintained in DH5α on brain heart infusion (BHI) (Difco, Becton Dickinson Microbiology Systems, Sparks, Md.) with 250 μg of erythromycin per ml and 100 μg of ampicillin per ml. pTRK81-derived vectors were maintained in E. coli JM109 and DH5α on BHI with 250 μg of erythromycin per ml. E. coli CC181(pRB04) was grown on LB medium supplemented with chloramphenicol (20 μg/ml) or with kanamycin (30 μg/ml) and chloramphenicol...
(20 μg/ml) after transposition with TnphoA. Strepococcus gordonii GP1223 was grown at 37°C in BHI with 0.5 mg of streptomycin per ml.

Lactococcal bacteriophages were prepared from single plaques and plaque assayed as described previously (41). Phages were stored at −70°C in M17 medium containing 20% glycerol.

**Genetic constructions.** All recombinant DNA procedures were done as described by Sambrook et al. (35) or Ausubel et al. (2), except where noted. The C. repeat region of emm6 (17) from nucleotide 823 through 1131 (numbering begins at the start of translation) was copied by PCR from pSMB104 using primers 5’-GCTTCCGGAAAACAGTTTCAAGACAA-3’ and 5’-CGATCCGGGATA GCTACGTTTTTCTTTT-3’, which include BspEI sites (shown in boldface) at their 5’ ends. pSMB104 is a derivative of pSMB102 (30) that encodes M6 amino acids 1 to 7 and 222 to 441. PCR was done with primers

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<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Phenotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>lactis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM2301</td>
<td>Wild type pip, plasmid free</td>
<td>45 This work</td>
</tr>
<tr>
<td>BG301</td>
<td>LM2301 with single-copy, chromosomal pip-emm6c</td>
<td>45 This work</td>
</tr>
<tr>
<td><strong>S. gordonii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP1223</td>
<td>Expresses M6c on its surface</td>
<td>8</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td>DH5omcr</td>
<td>Cloning strain for screening TnphoA fusions</td>
<td>26 Life Technologies</td>
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<td>pSMB102</td>
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<td>Source of emm6 used for cloning</td>
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<td>Shuttle vector</td>
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<td>pRB04</td>
<td>pip cloned in shuttle vector pSA3</td>
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<td>pUC19</td>
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<td>High-copy-number shuttle vector</td>
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<td>pip with pip promoter in pTRKH2</td>
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<td>pTRK568</td>
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<td>T. R. Klaenhammer (gift)</td>
</tr>
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<td>pBG568</td>
<td>pTRKH568 with unique XbaI site</td>
<td>This work</td>
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<td>pip-emm6c with pip promoter in pTRKH2</td>
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</tr>
<tr>
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<td>pip with P6 promoter in pBG568</td>
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</tr>
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<td>pip-emm6c with P16 promoter in pBG568</td>
<td>This work</td>
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<tr>
<td>pΔPipM6c</td>
<td>PipM6c with large deletion in pip</td>
<td>This work</td>
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<tr>
<td>pΔPipM6c-r</td>
<td>Same as pΔPipM6c except reverse orientation of Δpip-emm6c in pTRKH2</td>
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<td>pGhost6</td>
<td>Integration vector used for allelic exchange</td>
<td>25 (Appligene, Pleasanton, Calif.)</td>
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<td>pGbpPipM6c</td>
<td>pip-emm6c cloned in pGhost6</td>
<td>This work</td>
</tr>
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<td>Source of promoter PLA16</td>
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FIG. 1. Diagram of membrane protein topology analysis. The membrane topology of Pip was analyzed by the alkaline phosphatase fusion method described in Materials and Methods. Pip is represented as a line. Fusion junctions from colonies that expressed high levels of alkaline phosphatase were mapped to the amino acid residues indicated by arrowheads. The residue code and number are shown below the corresponding arrowhead. The shaded boxes above the line indicate regions of hydrophobicity that are potential membrane-spanning segments. The diagram is drawn to scale.

**RESULTS**

Splicing site for M6c within Pip. An analysis of the deduced amino acid sequence of Pip predicts that the N-terminal two-thirds of the protein would face the external side of the plasma membrane (15). This region might be an ideal site into which M6c could be spliced, so that it would be exposed to the outside of the cell. However, the actual structure of Pip and its topology relative to the plasma membrane have never been tested.

Therefore, the molecular topology of Pip was analyzed by the phoA method of membrane protein mapping. Random genetic fusions between pip and phoA were constructed and expressed in *E. coli*. Fusion junctions from 10 pip-phoA isolates with high levels of alkaline phosphatase activity were mapped by restriction analysis and sequenced. All but three of the junctions were within the sequence of *pip* predicted to encode the large, hydrophilic, extracellular region of Pip (Fig. 1). Three junctions (V714, S717, and G751) were found within hydrophobic regions predicted to span the plasma membrane. These data confirm the external topology of the region of Pip encoded by bp 50 to 700 and suggest that this portion of Pip is a suitable site for the introduction of heterologous protein sequences.

**Construction of *L. lactis* with *pip-emplm6c***. The coding region of M6c (*emplm6c*) was copied by PCR and inserted in frame after bp 321 in *pip* (Fig. 2A and B). The genetic fusion (*pip-emplm6c*) included the putative native promoter and transcriptional terminator of *pip*.

*L. lactis* LM2301 was transformed with *pip-emplm6c*, and its single-copy, wild-type *pip* allele was replaced with the recombinant allele. The allele-exchanged strain (BG301) was isolated by selection on growth plates that contained phage c2 (data not shown). Analytical PCR using primers that flank the inserted *emplm6c* amplified one band of ~650 bp from chromosomal DNA of BG301 and one band of ~330 bp from chromosomal DNA of LM2301 (data not shown). The predicted sizes are 653 and 331 bp, respectively. DNA sequencing of the PCR products confirmed the exchange of alleles (data not shown). Neither the *ermAM* nor the *bla* marker of the recombinant plasmid was detected by analytical PCR using DNA from BG301, although control reactions with the same primers and recombinant plasmid yielded the predicted products (data not shown).
M6c expression in *L. lactis* BG301. M6c was expressed in exponential-phase cultures of *L. lactis* BG301. Equivalent amounts of washed cells and cell-free growth medium from the same cultures were analyzed by immunoblotting using a monoclonal antibody against M6c. The amount of M6c exposed to the surface of BG301 was 0.014 \( \mu g/OD_{600} \) unit, which is equivalent to 0.007 \( \mu g/10^8 \) cells or 0.015% of total cellular protein (Table 2). M6c was below the limit of detection in the culture medium. There was no detectable expression of M6c from control cultures of LM2301.

M6c expression from a multicopy plasmid. The *pip-emm6c* allele was subcloned to a high-copy-number vector (pPipM6c) and used to transform *L. lactis* LM2301. Surface expression of PipM6c from LM2301(pPipM6c) was 17-fold higher than that from BG301 (Table 2). The amount of PipM6c in the growth medium of LM2301(pPipM6c) was 1.0 \( \mu g/OD_{600} \) unit, which is fourfold higher than the amount on the cell surface. No M6c was detected from a control that expressed the wild-type *pip* allele from the same plasmid.

M6c expression from *S. gordonii*. M6c was measured on the surface and in the cell-free culture medium of *S. gordonii* GP1223, which is genetically engineered to express the C repeat region of M6 on its surface (8). Cells were harvested from cultures at exponential phase. Compared to LM2301(pPipM6c), *S. gordonii* GP1223 expressed 50 times more antigen on its cell surface (Table 2). However, M6c in the culture medium of GP1223 was only 28% of that in the medium of LM2301 (pPipM6c) (Table 2).

Effect of growth phase on expression of M6c. LM2301 (pPipM6c) was grown to stationary phase, and the expression of M6c was measured. The amounts of M6c on the cells and in the culture medium were nearly the same as those in the equivalent fractions from exponential-phase cultures when normalized for differences in cell densities (Table 2).

M6c expression from deletion allele of *pip*. Detection of M6c within the context of the chimeric protein may be limited by inaccessibility of the monoclonal antibody to its epitope. If the amount of Pip in the chimera was significantly reduced, the protein conformation might change, which might increase accessibility of the antibody to the M6c epitope. With this in mind, 451 aminoacyl residues of Pip adjacent to the M6c epitope were genetically deleted from PipM6c (Fig. 2C).

---

**TABLE 2. Expression of M6c**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean M6c level (( \mu g/OD_{600} ) unit/ml) ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell surface</td>
</tr>
<tr>
<td>LM2301</td>
<td>0* (5)</td>
</tr>
<tr>
<td>BG301</td>
<td>0.014 ± 0.007 (3)</td>
</tr>
<tr>
<td>LM2301(pPipM6c)</td>
<td>0.24 ± 0.1 (5)</td>
</tr>
<tr>
<td>LM2301(pPipM6c)(^b)</td>
<td>0.23 ± 0.05 (3)</td>
</tr>
<tr>
<td>LM2301(p(\Delta)PipM6c)</td>
<td>0.21 ± 0.06 (2)</td>
</tr>
<tr>
<td>LM2301(p(\Delta)PipM6c-(r))</td>
<td>0.047 ± 0.05 (2)</td>
</tr>
<tr>
<td>LM2301(pP6pipM6c)</td>
<td>3.3 ± 0.2 (2)</td>
</tr>
<tr>
<td>LM2301(pP6pipM6c)</td>
<td>6.6 ± 0.4 (2)</td>
</tr>
<tr>
<td>GP1223</td>
<td>12 ± 2.6 (6)</td>
</tr>
</tbody>
</table>

\^a Below the limit of detection, which was about 0.002 \( \mu g/OD_{600} \) unit/ml.
\(^b\) Stationary phase.
The deletion allele (Δpip-emm6c) was expressed in L. lactis from the same multicopy plasmid used to construct pPipM6c. Immunoblot analysis of the resulting strain, LM2301(pΔPipM6c), showed that the amount of M6c on the cell surface was similar to that on LM2301(pPipM6c) (Table 2). The cell-free culture medium of LM2301(pPipM6c) contained 26% of that in the medium of LM2301(pPipM6c).

The Δpip-emm6c allele was subcloned in the opposite orientation on the same plasmid and expressed in L. lactis. Cultures of the resulting strain, LM2301(pΔPipM6c-r), expressed about 22 and 12% of the amounts of PipM6c on the cell surface and in the cell-free medium, respectively, expressed by LM2301(pΔPipM6c) (Table 2).

Replacement of pip promoter. The level of expression of PipM6c was increased by replacing the pip promoter with promoters P6 and P16, which were previously isolated from Lactobacillus (1, 10). In L. lactis, the expression of the cat-86 gene was about 1.5-fold higher from P16 than from P6 (1, 10). Immunoblot analysis showed that in L. lactis(pP6pipM6c), P6 increased surface expression of PipM6c about 14-fold compared to that from the native pip promoter (Table 2). P16 increased cell surface expression of PipM6c another twofold above that from P6. The level of surface expression of PipM6c from promoter P16 in L. lactis was approximately half of that from S. gordonii GP1223.

The greatest increase in expression of M6c occurred in the culture medium. When expressed from promoters P6 and P16, the amounts of M6c in the culture medium increased 57- and 113-fold, respectively (Table 2). This was 201- and 403-fold higher, respectively, than the levels expressed in the culture medium. When expressed from promoters P6 and P16, the amounts of M6c in the culture medium increased 57- and 113-fold, respectively (Table 2). This was 201- and 403-fold higher, respectively, than the levels expressed in the culture from S. gordonii GP1223.

M6c was not detected on the surface or in the medium of cultures of LM2301(pP6pip) or LM2301(pP16pip).

Molecular size of M6c on cell surface and in culture medium. Washed cells and cell-free culture media were analyzed by Western blotting with anti-M6c monoclonal antibody. The results (Fig. 3) showed seven intensely stained bands associated with the cell, which had mean molecular masses (+ standard deviations) of 111 (+2), 106 (+4), 100 (+8), 52 (+1), 49 (+1), 32 (+0), and 29 (+1) kDa. This agrees closely with the hypothetical size of the chimeric protein (106 kDa), considering that coiled-coil proteins like M6 and Pip (B. Geller, unpublished analysis) are notorious for running at aberrant sizes on denaturing gels (17). The relative intensities of the three largest bands varied significantly among blots, and often the top band was most intense. There were numerous bands of lesser intensity that varied in number depending on the amount of time that the blot was left in the chromogenic reagent, suggesting that PipM6c may have been proteolytically degraded during sample preparation, despite the inclusion of eight protease inhibitors. It appears unlikely that the fragments were produced in vivo, because many of the fragments are too small to include both M6c and the cell membrane anchoring region (15) at the carboxy terminus.

Only one band was detected in the culture medium, and its apparent size was 79 (+2) kDa. The patterns from LM2301(pPipM6c) and LM2301(pP16pipM6c) were qualitatively the same (data not shown).

**DISCUSSION**

We have shown that Pip can be used to direct surface expression of a heterologous protein in L. lactis. The results of the pip-phoA fusions suggested that the region within Pip between residues 64 and 684 is external to the plasma membrane. This guided our selection of the site at residue 108 for fusing Pip to M6c. Indeed, PipM6c was detected on the surface of intact cells, which shows that the region of Pip at residue 108 is outside the plasma membrane. Further analysis, including lacZ fusions, will be necessary to map the topology of Pip in more detail.

Insertion of M6c disrupted the phage receptor activity of Pip. This phenotypic change was exploited in this study to select strains that had undergone pip-emm6c allelic exchange. A rapid, direct selection for exchange of pip alleles has been previously demonstrated using a pUC-based integration vector.
that lacks a gram-positive origin of replication but encodes a selectable marker for \textit{L. lactis} (22). The \textit{pip} locus may be an ideal location for inserting recombinant alleles into the chromosome of \textit{L. lactis}, because \textit{pip} is not required for viability or rapid growth in vitro (13), and allelic replacement can be selected directly on plates that contain phage.

Expression of cell surface-associated M6c was increased 17-fold by moving \textit{pip-emmtoc} from the chromosome to a multi-copy plasmid. Replacing the native promotor increased total (cell-associated plus cell-free medium) expression of PipM6c up to about 100-fold, of which cell-associated PipM6c was about 7% of total cellular protein. Most of the increase was found in the cell-free culture medium. The reason for the appearance of much of the additional PipM6c in the cell-free culture medium is unknown. One possibility is that overexpression of PipM6c exceeded the capacity of the cell to sort or attach PipM6c to the surface of the cell. In any case, finding a 79-kDa fragment of PipM6c in the culture medium shows that a large fragment of PipM6c is not anchored to the cell.

The amount of antigen delivered by \textit{L. lactis} to the mucosal immune system may be limited to that expressed in vitro before vaccination (46). This is because \textit{L. lactis} does not colonize the gastrointestinal tract (16, 21), and its metabolic activity in the gastrointestinal tract is not required for effectiveness as a mucosal vaccine (29, 34). The relatively high level (7% of total cellular protein) of surface-expressed antigen (M6c) that we report here is similar to levels of expression reported for other antigens in effective lactococcal vaccines (28, 29, 34, 46, 47) and may be sufficient for eliciting a mucosal immune response.

Surface expression of immunogens may be the most effective way of presenting lactococcal-based vaccines to the immune system (28, 46). Particulate-associated proteins evoke a stronger response and are less likely to produce tolerance in the mucosal immune system than soluble proteins (5, 6, 48). One report showed that subcutaneous injection of tetanus toxin fragment C elicited a stronger systemic immune response than an equivalent dose of \textit{L. lactis} (3). Another report showed that subcutaneous injection of tetanus toxin fragment C elicited a stronger systemic immune response when compared to intranasal administration of the same antigen (5, 6, 48). One possibility is that proteolytic enzymes or the acidic conditions of the lactococcal culture released the protein from the cell surface. In any case, finding a 79-kDa fragment of PipM6c in the culture medium shows that a large fragment of PipM6c is not anchored to the cell.

Another factor that may be important for lactococcal-based vaccines is access of surface-attached antigens to the immune system. One report has shown that a heterologous antigen can be attached to the surface of \textit{L. lactis} and is accessible on intact cells (39). Another report showed that although surface-attached tetanus toxin fragment C was immunogenic, it was not accessible on intact lactococcal cells by using immunolabeling techniques (28). Clearly, we have shown in this report that M6c is accessible on the surface of \textit{L. lactis}. It remains to be learned if this accessibility translates to higher immunogenicity or if this could create a liability for exposure to proteases or stomach acid.

\section*{ACKNOWLEDGMENTS}
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