INFECTION AND IMMUNITY, May 1992, p. 1902–1907
0019-9567/92/051902-06$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 60, No. 5

Delivery and Expression of a Heterologous Antigen on the Surface of Streptococci

GIANNI POZZI,[1,2]* MARIO CONTORNI,[3] MARCO R. OGGIONI,[1] RICCARDO MANGANELLI,[1] MASSIMO TOMMASINO,[3] FILIPPO CAVALIERI,[3] AND VINCENT A. FISCHETTI[4]

Sezione di Microbiologia, Dipartimento di Biologia Molecolare, Università di Siena, Via Laterina,[1] and Centro Ricerche Sclavo,[3] 53100 Siena, and Istituto di Microbiologia, Università di Verona, 37134 Verona,[2] Italy, and The Rockefeller University, New York, New York 10021[4]

Received 2 December 1991/Accepted 3 February 1992

We have developed a system in which a foreign antigen replaces nearly all of the surface-exposed region of the fibrillar M protein from *Streptococcus pyogenes* and is fused to the C-terminal attachment motif of the M molecule. The fusion protein is thus expressed on the surface of *Streptococcus gordonii*, a commensal organism of the oral cavity. The antigen chosen to be expressed within the context of the M6 molecule was the E7 protein (98 amino acids) of human papillomavirus type 16. Stable recombinant streptococci were obtained by integrating genetic constructs into the chromosome, exploiting in vivo homologous recombination. The M6-E7 fusion protein expressed on the *S. gordonii* surface was shown to be immunogenic in mice. This is the first step in the construction of recombinant live vaccines in which nonpathogenic streptococci as well as other gram-positive bacteria may be used as vectors to deliver heterologous antigens to the immune system.

Gram-negative bacteria, cloned to express foreign antigens either on the surface or within the cell, have been used to deliver these molecules to mammalian hosts for the induction of an immune response (2, 21, 35). With the exception of mycobacteria (34), gram-positive bacteria have not as yet been exploited for this purpose. In this report, we describe the expression of a heterologous antigen on the surface of a streptococcus. The fibrillar M6 protein, a surface protein from *Streptococcus pyogenes* (4, 5, 8), was modified to deliver the E7 protein (98 amino acids) of human papillomavirus type 16 (HPV16) to the surface of *Streptococcus gordonii*. E7 is an oncoprotein (1, 3, 15, 37), antibodies to which are found in patients with cervical cancer (13); it is considered a major candidate antigen for vaccines against HPV-induced malignant neoplasias (19). Its use in this construct emphasizes the ability to deliver a molecule far removed from a bacterial protein to the bacterial cell surface.

S. gordonii Challis, formerly classified as *Streptococcus sanguis* (16), was isolated from the human oral cavity and found to be naturally competent for genetic transformation (23). This strain, in which DNA can be efficiently introduced (17, 28, 30), was chosen as a model host for these experiments. Our approach to genetic manipulation of streptococci is based on the integration of recombinant DNA molecules into the bacterial chromosome, for both transformable and nontransformable species (22, 27, 29, 32). To obtain surface expression of heterologous antigens in streptococci, we developed a host-vector system that allows the construction, chromosomal integration, and expression of translational fusions with the M6 protein gene (*emm*-6.1) (10).

MATERIALS AND METHODS

Recombinant DNA techniques. Gene fusions in *Escherichia coli* vectors were obtained and controlled by standard procedures (18).

Streptococcal transformation. Frozen cells of naturally competent *S. gordonii* Challis were prepared and transformed as already described in detail (28). Plating and scoring of transformants on multilayered plates were also done as previously described (30); erythromycin was added at 5 μg/ml to the overlay.

Genetic analysis of transformants. Transformants were streaked on the surfaces of three blood agar plates by toothpick transfer of colonies from the selection plate. The first plate contained erythromycin (5 μg/ml), the second contained chloramphenicol (5 μg/ml), and the third contained no drugs. The plates were incubated for 36 h at 37°C. After incubation, a nitrocellulose membrane was applied to the surface of the plate that contained no drugs and kept there for 20 min at room temperature. The membrane was then incubated for 30 min at 37°C and for 15 min at 80°C in a vacuum oven. The presence of E7 protein bound to the nitrocellulose was detected as already described (6) with anti-E7 polyclonal antibodies (1:5,000 dilution) obtained from rabbits immunized with an MS2-E7 fusion protein produced in *E. coli* (36).

Immunofluorescence. Bacteria grown in Todd-Hewitt broth (Difco) were harvested in the late exponential phase, applied to a clean glass slide, air dried, and fixed with methanol. The dried bacterial spot was flooded with 50 μl of M6- or E7-specific antibodies (1:50 dilution) and incubated for 30 min in a moist chamber. After being washed, the bacteria were reacted with goat anti-rabbit (or anti-mouse) immunoglobulin G antiserum (1:100 dilution) conjugated with rhodamine (Cappel) and were reincubated for 30 min. The washed and dried slides were observed and photographed with a Con-focal fluorescence imaging system MRC-500 Bio-Rad microscope.

Western blot (immunoblot) analysis of cell extracts. Streptococci were grown to the late stationary phase in Todd-Hewitt broth. Cells were harvested and resuspended in 50 mM Tris (pH 8.0)–50 mM $MgCl_2$–30% sucrose. Protoplasts were obtained by treating the cell suspension with lysozyme (100 μg/ml) for 30 min at 0°C. Protoplasts were then centrifuged and resuspended in 50 mM Tris (pH 8.0). Thorough lysis was achieved by five cycles of quick freezing and

* Corresponding author.

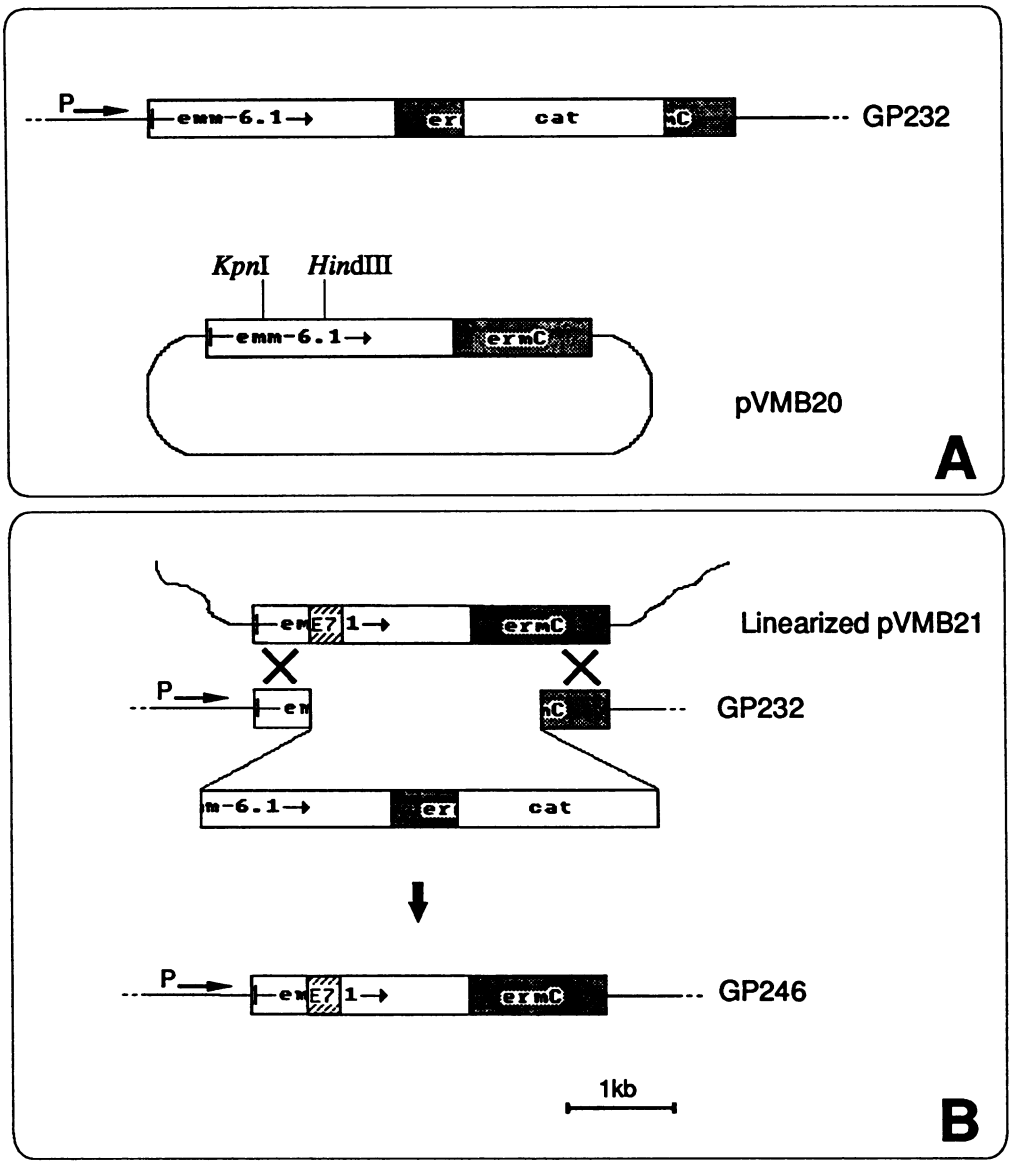

FIG. 1. Expression of the E7 protein of HPV16 in *S. gordonii*. (A) Host-vector system for heterologous gene expression. In the chromosome of host strain *S. gordonii* GP232, a copy of the M6 protein gene (*emm*-6.1) (10), promoterless but with its own ribosome binding site, is integrated downstream of a strong chromosomal promoter (P). Adjacent to *emm*-6.1 is *ermC* (11), whose coding sequence is interrupted by insertion in its *BclI* site of the 1.8-kb *MboI* fragment of pC221 containing a *cat* gene (38). GP232 expresses M6 on its surface and is resistant to chloramphenicol and sensitive to erythromycin; it was obtained by use of transformation to integrate heterologous DNA into the streptococcal chromosome. The structure (depicted here) and the size (5.2 kb) of the heterologous DNA integrated into the chromosome of GP232 were determined by Southern blot analysis (data not shown). Integration vector pVMB20 is a 6.3-kb *E. coli* plasmid that does not replicate in *Streptococcus* strains. It was obtained by subcloning in pBLUESCRIPT (Stratagene, La Jolla, Calif.) a 3.4-kb *ClaI* fragment of plasmid pVMB3 (31) containing *emm*-6.1 and *ermC*. This is the same *ClaI* fragment that is integrated into the chromosome of GP232, the only difference being that in GP232 *ermC* is interrupted by *cat*. When pVMB20 is used as donor DNA in the transformation of competent cells of *S. gordonii* GP232, erythromycin-resistant transformants are obtained by recombination between the integration vector and the homologous chromosomal sequences. The DNA fragment containing the *cat* gene is deleted in the chromosome of these transformants, whereas an intact *ermC* gene is restored. (B) Chromosomal integration of the M6-E7 translational fusion. The E7 protein gene of HPV16 (33) was cloned into the *emm*-6.1 sequence of pVMB20 to yield pVMB21. pVMB20 was digested with *KpnI* and *HindIII* and ligated with a *KpnI-HindIII* segment containing the E7 sequences obtained by in vitro DNA amplification (polymerase chain reaction) performed on plasmid pMBS21L/E7 (36). Amplification primers were designed to obtain in-frame insertion of the 294 bp encoding E7 into *emm*-6.1. Nucleotide sequence analysis of pVMB21 confirmed the expected structure of the M6-E7 translational fusion (data not shown). pVMB21 was linearized and used to transform GP232. E7 was found to be expressed in 6% of the erythromycin-resistant transformants. In these transformants, integration of the pVMB21 sequences produced a deletion involving the *cat* gene. The structure of GP246, a representative transformant, was confirmed by Southern blot analysis. The nucleotide sequence of the junction fragments of the M6-E7 gene fusion present on the chromosome of GP246 was also determined after cloning in pBLUESCRIPT of the *ClaI* fragment containing the M6-E7 gene fusion (data not shown).

thawing of the suspension. Unlysed cells and gross debris were discarded by low-speed centrifugation (1,000 × *g*) for 15 min, whereas the supernatant, containing membranes and cytoplasm, was used for Western blot analysis. Extracts obtained from about 5×10^8 streptococcal cells were processed in this way, and Western blotting was performed as already described (6).

Immunization of mice. BALB/c mice were immunized subcutaneously with 5×10^8 live GP246 streptococcal cells (5×10^7 CFU) emulsified in complete Freund's adjuvant. Two and 3 weeks after the primary immunization, animals were given subcutaneous boosters of the same bacterial dose emulsified in incomplete Freund's adjuvant. Animals were bled 7 days after the last boost.

RESULTS

Host-vector system. In a previous study, we constructed strains of *S. gordonii* Challis in which a promoterless *emm*-6.1 gene was integrated into the chromosome downstream of a strong promoter (31). A strain that expressed large quantities of surface M6 protein was selected and used to create strain GP232, the recipient host for transformation experiments. Integration vector pVMB20 was constructed to allow the insertion of heterologous DNA sequences into the *emm*-6.1 gene present on the chromosome of GP232. pVMB20 is an *E. coli* plasmid that does not replicate in *Streptococcus* strains and carries *emm*-6.1 and the erythromycin resistance marker *ermC* (11). Host-vector system GP232-pVMB20 is described in detail in Fig. 1A.

M6-E7 gene fusion. Insertion vector pVMB20 was used to construct a translational fusion between *emm*-6.1 and the E7 protein gene of HPV16. The coding sequence of *emm*-6.1, between sites *Kpn*I and *Hin*dIII (538 bp), was substituted for by 294 bp encoding E7. The region of the M6 protein that was excised resulted in the placement of the E7 molecule at the cell surface (24), with a 122-amino-acid segment of the M6 N terminus fused to the N terminus of E7. The construct was made in *E. coli*, and the recombinant plasmid was linearized and used to transform *S. gordonii* GP232. Transformants selected for erythromycin resistance were also sensitive to chloramphenicol, and genetic analysis revealed that 6.0% of these also expressed E7. A representative E7-positive transformant was named GP246 and further characterized. Southern blotting and nucleotide sequence analysis of GP246 (data not shown) confirmed the expected structure of the recombinant DNA integrated into the streptococcal chromosome; the E7 coding sequence replaced the 538-bp *Kpn*I-*Hin*dIII fragment of *emm*-6.1, and the 1.8-kb fragment containing *cat* was deleted. As expected from results previously discussed (27, 29), GP246 proved to be a stable recombinant; the two phenotypes (E7 surface expression and erythromycin resistance) were not lost by the strain after growth without selection for 50 generations as previously described (31, 32). The construction and chromosomal integration of the M6-E7 translational fusion are described in detail in Fig. 1B.

Surface expression of the E7 protein. Expression of the E7 protein of HPV16 on the surface of *S. gordonii* GP246 was verified by immunofluorescence with antibodies specific for either the M6 protein carrier or the E7 insert. GP246, containing the M6-E7 gene fusion (Fig. 1B), exhibited positive fluorescence when reacted with either M6-specific or E7-specific polyclonal antibodies (Fig. 2), confirming the surface location of the E7 protein and the M6 protein on *S. gordonii*. No fluorescence was observed when GP246 was reacted with monoclonal antibody 10A11, which is specific for an epitope of M6 whose coding region was contained in the *Kpn*I-*Hin*dIII fragment deleted in the construction of the M6-E7 gene fusion (Fig. 2) (14).

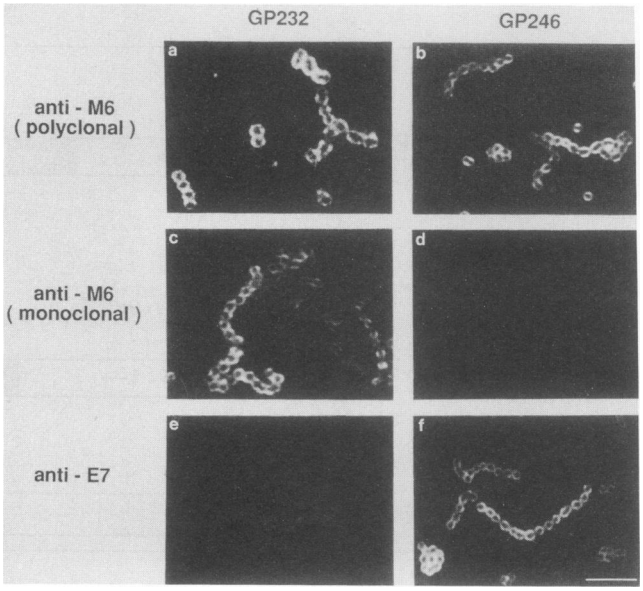

FIG. 2. Immunofluorescence of recombinant *S. gordonii* expressing the M6 protein (GP232) and the M6-E7 fusion protein (GP246). (a and b) GP232 and GP246, respectively, treated with an M6-specific rabbit serum. (c and d) GP232 and GP246, respectively, treated with monoclonal antibody 10A11, which reacts with the portion of M6 deleted in the M6-E7 fusion (14). (e and f) GP232 and GP246, respectively, treated with an E7-specific rabbit serum (36). Bar, 5 μm.

To demonstrate that the E7-expressing recombinant streptococci did in fact produce an M6-E7 fusion protein, we analyzed *S. gordonii* cell extracts by Western blotting (Fig. 3). In cell extracts of GP246, the same bands reacted with E7- and M6-specific antibodies, whereas no E7-specific reactivity was found in recipient GP232, whose extracts showed M6-specific reactivity (Fig. 3). It should be noted that, similar to what has been observed for the M6 protein produced in *E. coli* (10), the molecular size estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the recombinant proteins produced by *S. gordonii* was 20% larger than that deduced from the DNA sequence (i.e., 59 kDa compared with 49 kDa for the M6 protein and 54 kDa compared with 45 kDa for the M6-E7 fusion) (Fig. 3).

Immune response to the fusion protein on the streptococcal surface. The immunogenicity of the M6-E7 fusion protein was examined by immunizing mice with recombinant *S. gordonii* GP246 expressing the M6-E7 fusion protein. Control mice were immunized with isogenic strain GP232, which expresses only the M6 protein. Sera from three animals immunized with each strain were pooled and tested by Western blotting for their reactivity with purified E7 protein produced in *Schizosaccharomyces pombe* (36). Figure 4 shows that animals immunized with strain GP246 containing surface M6-E7 produced E7-reactive antibodies, indicating that the E7 protein is immunogenic when expressed as a fusion protein on the streptococcal surface. No antibodies to the E7 protein were seen in the sera of mice immunized with strain GP232, containing only M6.

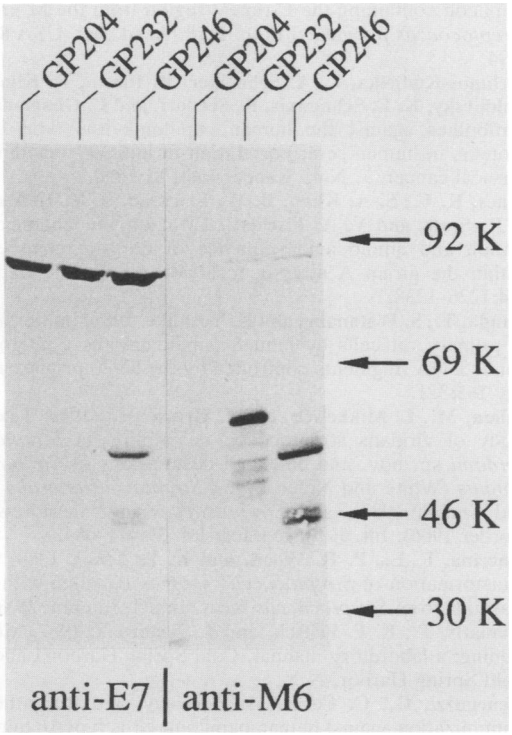

FIG. 3. Western blot analysis of cell extracts of recombinant *S. gordonii*. Cell extracts of wild-type *S. gordonii* GP204 (28) and derivatives GP232 (expressing the M6 protein) and GP246 (expressing the M6-E7 fusion protein) were analyzed for protein content by Western blotting. Anti-E7 polyclonal antibodies (1:5,000 dilution) obtained from rabbits immunized with an MS2-E7 fusion protein produced in *E. coli* (36) reacted with GP246 cell extracts, yielding a major band of 54 kDa and two minor bands of 46.5 and 44.5 kDa. The same three bands also reacted with anti-M6 polyclonal antibodies. As expected, GP232 cell extracts showed M6-specific reactivity, yielding a major band of 59 kDa and multiple bands of smaller sizes. The positions of the Rainbow molecular weight standards (Amersham) are indicated on the right (in thousands [K]).

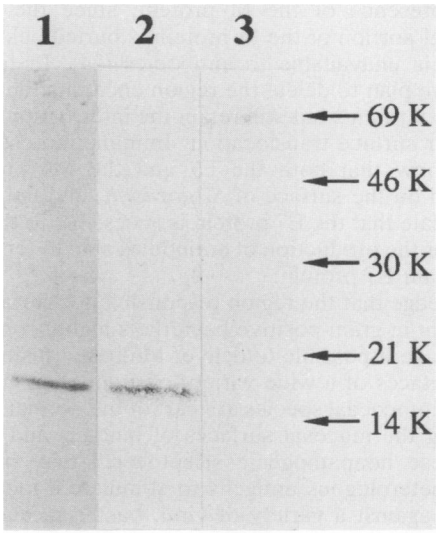

FIG. 4. Western blotting for the detection of E7-specific antibodies in pooled sera from mice immunized with recombinant *S. gordonii*. Purified E7 protein produced in *S. pombe* (36) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose filters. Filter strips were blocked with 3% bovine serum albumin, cut, and reacted with the following sera: (1) positive control, E7-specific polyclonal serum obtained from rabbits immunized with an MS2-E7 fusion protein produced in *E. coli* (1:5,000 dilution); (2) pooled sera from three mice immunized with *S. gordonii* GP246, expressing the M6-E7 fusion protein (1:100 dilution); (3) negative control, pooled sera from three mice immunized with *S. gordonii* GP232, expressing the M6 protein (1:100 dilution). The locations of antibodies on the nitrocellulose were visualized with peroxidase-conjugated anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G serum (Cappel). The positions of the Rainbow molecular weight standards (Amersham) are indicated on the right (in thousands [K]).

DISCUSSION

Live vectors have been used to deliver antigens to the immune system to enhance the immune response to the delivered molecules. In most instances, viruses, such as vaccinia virus (12, 25), or salmonellae (9, 26) have been successfully used for this purpose. In these delivery schemes, the presentation of the foreign antigen is not defined. For instance, in the vaccinia virus system, the antigen is a product of the replicating virus in infected mammalian cells which is randomly produced and delivered in an imprecise way to the immune system (25). In the salmonella vector, cloned antigens are either sequestered in the cytoplasm or translocated into the periplasmic space to be delivered to the immune system after cell lysis (20). In some cases, the antigen has been inserted into the *E. coli* outer membrane LamB protein (2) or into the salmonella flagellin (21).

The M protein molecule is an α-helical coiled-coil structure extending nearly 60 nm from the streptococcal cell surface. A previous study has identified the surface-exposed portion of the M protein to extend from the N terminus to about residue 300 of this 441-residue molecule (24). The *Hin*dIII site (10) used for the construct described in this paper corresponds to residue 302 of the M6 sequence. Thus, fusion proteins obtained by cloning at this *Hin*dIII site would be predicted to be localized at the cell surface. The M protein is tightly anchored to the bacterial cell by a process that is not completely understood (7, 24). An analysis of the C-terminal regions of surface proteins from gram-positive bacteria revealed that they all exhibit the common characteristics of a C-terminal charged tail, a hydrophobic membrane-spanning region, and a cell wall-spanning region rich in proline, glycine, threonine, and serine amino acids (7). All these proteins also have a nearly 100% conserved hexapeptide with the consensus sequence LPSTGE, located about three residues N-terminal from the hydrophobic region (7). Collectively, these data suggest that a common mechanism is used by gram-positive bacteria to attach surface proteins within the bacterial cell wall.

With this realization, a system was devised by which proteins may be effectively delivered to the surfaces of gram-positive bacterial cells. We elected to express the E7 protein of HPV16 because of its distinction from a bacterial protein and chose to express it on the surface of a commensal gram-positive bacterium, *S. gordonii*. A segment of the *emm*-6.1 gene responsible for encoding most of the surface-exposed portion of the molecule was excised and replaced with the E7 gene. The N terminus of the M6 protein, including the leader sequence through residue 122, was fused to the N terminus of the E7 protein to both enable the proper translocation of the fusion molecule and serve as a marker

for the presence of the M protein, since the remaining C-terminal portion of the M protein is buried below the cell wall and is unavailable to antibodies (24). In future constructs, we plan to delete the region encoding the mature M protein N terminus but still retain the information necessary for proper surface translocation. Immunofluorescence data clearly show that both the E7 and the M6 proteins are expressed on the surface of *S. gordonii*, and immunization data indicate that the E7 protein is accessible to the immune system for the production of antibodies able to recognize the recombinant E7 protein.

Knowledge that the region responsible for surface protein attachment in gram-positive bacteria is highly conserved (7) should make it possible to deliver M6-based fusion proteins to the surfaces of a wide variety of gram-positive bacteria. Since streptococcal species are part of the normal microflora colonizing the mucosal surfaces of humans and other animals, these nonpathogenic streptococci may be used to express heterologous antigens to stimulate a local immune response against a variety of viral, bacterial, and parasitic diseases.

ACKNOWLEDGMENTS

This research was supported by grants from Consiglio Nazionale delle Ricerche (Progetto Finalizzato Ingegneria Genetica, grant 91.00016.90) and Instituto Superiore della Sanità (IV Progetto AIDS, grant 6207-011) to G.P. and by Public Health Service grant AI11822 and a grant from the Mallinckrodt Foundation to V.A.F.

REFERENCES

1. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61:**962–971.
2. **Charbit, A., E. Sobczak, M.-L. Michel, A. Molla, P. Tiollais, and M. Hofnung.** 1987. Presentation of two epitopes of the *preS2* region of hepatitis B virus on live recombinant bacteria. J. Immunol. **139:**1658–1664.
3. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934.
4. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2:**285–314.
5. **Fischetti, V. A.** 1991. Streptococcal M protein. Sci. Am. **264:** 57–65.
6. **Fischetti, V. A., K. F. Jones, and J. R. Scott.** 1985. Size variation of the M protein in group A streptococci. J. Exp. Med. **161:** 1384–1401.
7. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins of gram-positive cocci. Mol. Microbiol. **4:**1603–1605.
8. **Fischetti, V. A., D. A. D. Parry, B. L. Trus, S. K. Hollingshead, J. R. Scott, and B. N. Manjula.** 1988. Conformational characteristics of the complete sequence of group A streptococcal M6 protein. Proteins Struct. Funct. Genet. **3:**60–69.
9. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
10. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1986. Complete nucleotide sequence of type 6 M protein of the group A streptococcus: repetitive structure and membrane anchor. J. Biol. Chem. **261:**1677–1686.
11. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150:**804–814.
12. **Hruby, D. E., O. Schneewind, E. M. Wilson, and V. A. Fischetti.** 1991. Assembly and analysis of a functional vaccinia virus amplicon containing the C-repeat region from the M protein of *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **88:**3190–3194.
13. **Jochmus-Kudielka, I., A. Schneider, R. Braun, R. Kimmig, U. Koldovsky, K. E. Schneweis, K. Seedorf, and L. Gissmann.** 1989. Antibodies against the human papillomavirus type 16 early proteins in human sera: correlation of anti E-7 reactivity with cervical cancer. J. Natl. Cancer Inst. **81:**1698.
14. **Jones, K. F., S. A. Khan, B. W. Erickson, S. K. Hollingshead, J. R. Scott, and V. A. Fischetti.** 1986. Immunochemical localization and amino acid sequence of cross-reactive epitopes within the group A streptococcal M6 protein. J. Exp. Med. **164:**1226–1238.
15. **Kanda, T., S. Watanabe, and K. Yoshiike.** 1988. Immortalization of primary rat cells by human papillomavirus type 16 subgenomic DNA fragments controlled by the SV40 promoter. Virology **165:**321.
16. **Kilian, M., L. Mikkelsen, and J. Henrichsen.** 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrewes and Horder 1906). Int. J. Syst. Bacteriol. **39:**471–484.
17. **Macrina, F. L., P. H. Wood, and K. R. Jones.** 1980. Genetic transformation of *Streptococcus sanguis* (Challis) with cryptic plasmids from *Streptococcus ferus*. Infect. Immun. **28:**692–699.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Meneguzzi, G., C. Cerni, M. P. Kieny, and R. Lathe.** 1991. Immunization against human papillomavirus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. Virology **181:**62.
20. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an *asd*$^+$ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6:**693–697.
21. **Newton, S. M. C., C. O. Jacob, and B. A. D. Stocker.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70–72.
22. **Oggioni, M. R., and G. Pozzi.** 1990. Conjugative mobilization of the cloned M6 protein gene from *Streptococcus pneumoniae* to *Streptococcus pyogenes*. Microbiologica **13:**273–281.
23. **Pakula, R., Z. Fluder, E. Hulanicka, and W. Walczak.** 1958. Studies on transformation of streptococci. Bull. Acad. Pol. Sci. Ser. Sci. Biol. **6:**319–323.
24. **Pancholi, V., and V. A. Fischetti.** 1988. Isolation and characterization of the cell-associated region of group A streptococcal M6 protein. J. Bacteriol. **170:**2618–2624.
25. **Perkins, M. E., A. Piccini, B. R. Lipinskas, and E. Paoletti.** 1985. Recombinant vaccinia virus: immunization against multiple pathogens. Science **229:**981–984.
26. **Poirier, T. P., M. A. Kehoe, and E. H. Beachey.** 1988. Protective immunity against group A streptococci evoked by attenuated *aroA Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168:**25–32.
27. **Pozzi, G., and W. R. Guild.** 1985. Modes of integration of heterologous plasmid DNA into the chromosome of *Streptococcus pneumoniae*. J. Bacteriol. **161:**909–912.
28. **Pozzi, G., R. A. Musmanno, P. M. Lievens, M. R. Oggioni, P. Plevani, and R. Manganelli.** 1990. Methods and parameters for genetic transformation of *Streptococcus sanguis* Challis. Res. Microbiol. **141:**659–670.
29. **Pozzi, G., R. A. Musmanno, E. A. Renzoni, M. R. Oggioni, and M. G. Cusi.** 1988. Host-vector system for integration of recombinant DNA into chromosomes of transformable and nontransformable streptococci. J. Bacteriol. **170:**1969–1972.
30. **Pozzi, G., R. A. Musmanno, M. Stellini, and A. M. Molina.** 1987. Transformation of *Streptococcus sanguis* Challis with a plasmid of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **48:**189.
31. **Pozzi, G., M. R. Oggioni, R. Manganelli, and V. A. Fischetti.** Expression of M6 protein gene of *Streptococcus pyogenes* in *Streptococcus gordonii* after chromosomal integration and tran-

scriptional fusion. Res. Microbiol., in press.
32. **Pozzi, G., M. R. Oggioni, R. Manganelli, and P. Plevani.** 1991. Genetic manipulation of streptococci by chromosomal integration of recombinant DNA, p. 59–61. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
33. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
34. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351**:456.
35. **Strugnell, R. A., D. Maskell, N. Fairweather, D. Pickard, A. Cockayne, C. Penn, and G. Dougan.** 1990. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium*. Gene **88**:57.
36. **Tommasino, M., M. Contorni, V. Scarlato, M. Bugnoli, K. Maundell, and F. Cavalieri.** 1990. Synthesis, phosphorylation, and nuclear localization of human papillomavirus E7 protein in *Schizosaccharomyces pombe*. Gene **93**:265.
37. **Watanabe, S., T. Kanda, and K. Yoshiike.** 1989. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J. Virol. **63**:965–969.
38. **Wilson, C. R., S. E. Skinner, and W. V. Shaw.** 1981. Analysis of two chloramphenicol resistance plasmids from *Staphylococcus aureus*: insertional inactivation of Cm resistance, mapping of restriction sites, and construction of cloning vehicles. Plasmid **5**:245–258.