A Foreign Protein Incorporated on the Tip of T3 Pili in Lactococcus lactis Elicits Systemic and Mucosal Immunity

Bernard R. Quigley,1 Matthew Hatkoff,2 David G. Thanassi,2 Mahamoudou Ouattara,3 Zehava Eichenbaum,3 and June R. Scott1*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322; Center for Infectious Diseases, Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, New York 11794; and Department of Biology, College of Arts and Sciences, Georgia State University, Atlanta, Georgia 30303

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The use of Lactococcus lactis to deliver a chosen antigen to the mucosal surface has been shown to elicit an immune response in mice and is a possible method of vaccination in humans. The recent discovery on Gram-positive bacteria of pili that are covalently attached to the bacterial surface and the elucidation of the residues linking the major and minor subunits of such pili suggests that the presentation of an antigen on the tip of pili external to the surface of L. lactis might constitute a successful vaccine strategy. As a proof of principle, we have fused a foreign protein (the Escherichia coli maltose-binding protein) to the C-terminal region of the native tip protein (Cpa) of the T3 pilus derived from Streptococcus pyogenes and expressed this fusion protein (MBP*) in L. lactis. We find that MBP* is incorporated into pili in this foreign host, as shown by Western blot analyses of cell wall proteins and by immunogold electron microscopy. Furthermore, since the MBP* on these pili retains its native biological activity, it appears to retain its native structure. Mucosal immunization of mice with this L. lactis strain expressing pilus-linked MBP* results in production of both a systemic and a mucosal response (IgG and IgA antibodies) against the MBP antigen. We suggest that this type of mucosal vaccine delivery system, which we term UPTOP (for unhindered presentation on tips of pili), may provide an inexpensive and stable alternative to current mechanisms of immunization for many serious human pathogens.

Pili of Gram-positive bacteria are filamentous structures that extend outward from the bacterial surface and are covalently anchored to the bacterial cell wall. They are believed to be the primary means of attachment to the appropriate environmental receptor for the organism, which, for pathogens, is within the human host. The backbone of the pilus in Gram-positive bacteria is composed of multiple covalently linked identical subunits (major pilin), to which one or more minor pilin subunits are covalently attached. Pilin proteins are synthesized with an N-terminal Sec signal, which is cleaved during transit through the cytoplasmic membrane, and a C-terminal cell wall sorting signal (CWSS), which contains an LPXTG (or similar) amino acid motif, followed by a hydrophobic region and a positively charged C terminus. Pilus assembly is catalyzed by a pilus-specific sortase family transeptidase, which cleaves the CWSS motif between the threonine (T) and glycine (G) residues and forms a covalent bond between this T and a conserved lysine (K) residue of another major pilin subunit. As this process repeats, the pilus is polymerized until it is covalently linked to the cell wall by either the “housekeeping” sortase, which is responsible for anchoring most surface proteins of Gram-positive bacteria to the cell wall, or the pilus-specific sortase (for reviews, see references 21, 35, and 38).

We have been investigating assembly of T3 pili of Streptococcus pyogenes, an important human pathogen. In this organism, the T3 pilus locus (19) encodes the major pilin (T3) and the minor pilins Cpa and OrfB, the pilus-specific transpeptidase SrtC2, and SipA2, which is required for pilus polymerization by SrtC2 (44). Our investigations into the biogenesis of T3 pili have identified the residues of T3 and Cpa required for (i) polymerization of T3 and (ii) incorporation of Cpa into the pilus structure. We have demonstrated that lysine residue 173 (K173) (29) and the CWSS (QVPTG) of the T3 major pilin subunit (2, 29) are required for polymerization of T3. This indicates that individual T3 subunits are polymerized into the pilus structure by covalent bonds between K173 of T3 and the threonine of the CWSS (T315) of the adjacent T3 subunit. We have also demonstrated that K173 of T3, along with the CWSS (VPPTG) of the Cpa, are required for incorporation of the minor pilin, Cpa, into the pilus (29). Thus, the K173 residue of T3 is required for T3-T3 linkage and is also required for covalent linkage of Cpa to the T3 pilus, demonstrating that Cpa is located at the tip of T3 pili, a conclusion supported by immunogold electron microscopy (EM) (29).

Identification of the residues required for attachment of Cpa, the tip protein, to the T3 pilus suggested to us that genetic engineering could be used to produce a Gram-positive bacterial strain in which a foreign protein would be covalently linked by the bacterium to the pilus tip in place of Cpa. In the present study, we used the Escherichia coli maltose-binding protein...
(MBP) as a model protein to test this idea. We identified amino acid residues of the primary structure of Cpa that are sufficient for incorporation of a foreign protein into T3 pili in vivo by SrtC2. We propose that this approach constitutes a novel technology for presentation of foreign polypeptides external to the bacterial envelope, which we call UPTOP (for unhindered presentation of polypeptides on tips of pili). We suggest that any Gram-positive bacterium can be used as the host for UPTOP. We also propose that UPTOP can be used to present vaccine antigens to the immune system. As proof of this principle, we constructed a strain of the probiotic bacterium Lactococcus lactis engineered to produce T3 pili with the model protein MBP covalently linked at the pilus tips. We show in this study that mucosal administration to mice of this vaccine strain generates both an IgG and an IgA response to the model protein.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *E. coli* strains were cultured in LB media (34) supplemented with the appropriate antibiotic. Strains TOP10 (Invitrogen) and XL1-10 Gold (Stratagene) were grown at 30°C and strain BL21-CodonPlus (DE3)-RIL (Stratagene) at 37°C. *L. lactis* strain MG1363 was cultured without shaking at 30°C in M17 media (Oxoid) supplemented with 0.5% glucose (GM17). MG1363 was made competent by the method of Holo and Nes (14). Kanamycin and ampicillin were used at concentrations of 50 and 100 μg/ml, respectively, for *E. coli.* Spectinomycin was used at a concentration of 100 μg/ml for both *E. coli* and *L. lactis.*

**Construction of MBP**. Overlap PCR was performed according to the method of Ho et al. (12), using the primers shown in Table S1 in the supplemental material. In the first round of PCR, the template pJS9550 (29), encoding a portion of the FCT-3 pilus gene cluster (Fig. 1) from *S. pyogenes* strain AM3, was used with the primers CpaHA_F1_BamHI and MalE_Cpa_N_O_Anti to amplify a 198- bp 5′ region of *cpa* which extends from 30 nucleotides upstream of the Cpa initiation codon to 168 bases past the start of the Cpa open reading frame. This region includes the Cpa ribosomal binding site (RBS) and encodes the first 56 amino acid residues of Cpa (fragment 1). Plasmid pJS9550, along with the primers MalE_Cpa_C_O_Sense and SrtC2_R_XhoI, was used to amplify the 3′-2,800-bp region of the FCT-3 pilus gene cluster starting from the codon for amino acid 594 of Cpa, SipA2, T3, and SrtC2 (fragment 2). Similarly, plasmid pJS9550, along with the primers MalE_Cpa_C_O_Sense and Orf100_R_XhoI, was used to amplify the 3′-2,102-bp region of the FCT-3 pilus gene cluster starting from the codon for amino acid 594 of Cpa, SipA2, and T3 (fragment 3). Plasmid pMalE4E (New England Biolabs) was used, along with the primers MalE_Cpa_C_O_Sense and MalE_Cpa_C_O_Anti, to amplify a 196-bp sequence of *malE*, encoding residues 31 to 393 of the E. coli MBP (fragment 4).

**SDS-PAGE and Western blot analysis.** SDS-PAGE and Western blot analysis were performed as previously described (29). T3 typing serum was provided by B. Beall (CDC, Atlanta, GA). Mouse monoclonal anti-MBP antibody, used at a dilution of 1:2,000, was from New England Biolabs.

**Purification of anti-T3.** A PCR fragment generated by using primers SipA2_BamHI_Sense and T3_XhoI_Anti (see Table S1 in the supplemental material) with the template pEU7657 (Fig. 1) was ligated in frame with the C-terminal His tag coding sequence of pET21 (+) (Novagen), using BamHI and XhoI restriction endonucleases. The resulting plasmid, pEU7857 (Fig. 1), was transformed into BL21-CodonPlus (DE3)-RIL (Stratagene), and T3 was purified by using the B-PER His6 fusion protein purification kit (Pierce) according to the manufacturer’s instructions.

**Preparation of lysozyme/ml, 400 U of mutanolysin/ml, and Roche complete protease inhibitors (7) at 37°C for 3 h with gentle rotation. Cell wall and supernatant fractions were prepared for analysis as previously described (4, 44).

**FIG. 1.** Regions of the FCT-3 pilus cluster encoded by plasmids used in the present study. The position of the HA epitope tag in Cpa is indicated by a triangle. The region encoding MBP, inserted between the 5′ and 3′ regions of *cpa*, is shown as a rectangle (not to scale). The location of the His tag is shown as a square. Expression of the pilus gene cluster regions in pJS9550, pJS9556, and pJS9566 are under the control of the strong constitutive P23 promoter of pJS9508 (1).

**Analysis of protein surface presentation by dot blotting.** Overnight cultures of MG1363 were washed in saline and analyzed by dot blotting with the appropriate antibody as previously described (4).
and resuspended in PBS to give 50/H11003
80-kV accelerating voltage. Digital images were acquired with an AMT XR-60
acid (Ted Pella) for 35 s. The grids containing the negatively stained bacteria
PBS and twice with water and then negatively stained with 0.5% phosphotungstic
washed three times with PBS and then incubated for 1 h with a 1:50 dilution (in
PBS plus 1% BSA) of either the rabbit polyclonal anti-T3 antibody described above, a sheep
polyclonal anti-MBP antibody (Berkeley Antibody Company), or the mouse
monoclonal anti-HA (clone HA-7) antibody (Sigma-Aldrich). The grids were
washed three times with PBS and then incubated for 1 h with a 1:50 dilution (in
PBS plus 1% BSA) of either anti-rabbit IgG antibody, anti-sheep IgG antibody,
or anti-mouse IgG antibody conjugated to 12-nm-diameter colloidal gold parti-
cles (Jackson Immunoresearch Labs). The grids were washed three times with
PBS and twice with water and then negatively stained with 0.5% phosphotungstic acid (Ted Pella) for 35 s. The grids containing the negatively stained bacteria
were examined on an FEI Tecnai 12 BioTwin G20 microscope (FEI) at an
80-kV accelerating voltage. Digital images were acquired with an AMT XR-60
charge-coupled device digital camera system (Advanced Microscopy Tech-
niques).

Mouse immunization. Cells (MG1363/pJRS9545 or MG1363/pJRS9565)
grown at 30°C in GM17 containing 100 μg of spectinomycin/ml, were washed,
and resuspended in PBS to give 5 × 107 CFU/μl. Female CD1 mice were
vacccinated intranasally (i.n.) by administration of 20 μl of cell suspension (109
CFU) into the nostril. The mice were vaccinated every 10 days with a dose of 109
CFU for three consecutive days (i.e., the animals were vaccinated on days 1, 2, 3, 14, 15, and 16 and on days 27, 28, and 29). Blood samples collected on day 39
were analyzed. The mice were sacrificed on day 39, and lung lavage fluids were
obtained postmortem by inserting a nylon cannula into the exposed trachea,
which was tied in place. A 1.0-ml syringe was used to inject and withdraw 1 ml of
0.9% sodium chloride solution three times, the supernatants were then stored at
−80°C.

ELISA detection of antigen-specific antibodies in serum and lung lavage. A
96-well EIA/RIA microplate (Costar; Corning, Inc.) was coated overnight at 4°C
with 100 ng of MBP per well. The coated plate was blocked with 5% soy milk in
PBS-Tween to prevent nonspecific binding. Serum (1:50 dilution) or lung fluid
was reacted with the coated wells for 60 min. Antibody production was detected
by using anti-mouse IgG or anti-mouse IgA secondary antibodies coupled to
alkaline phosphatase (Sigma). Absorbance was measured at 405 nm after 45 min
after the addition of p-nitrophenyl phosphate hexahydrate disodium salt (pNPP)
tables dissolved in diethanolamine buffer solution (KPL). Endpoint (day 39) antibod-
tyititers were determined in pooled lung lavage and serum samples by using the
same enzyme-linked immunosorbent assay (ELISA), except that the
mouse samples were applied from a serial dilution to the plate. Antibody titers
were calculated as the dilution producing the same ODmax at two times the
background level (the reading obtained with sera or lung fluid of nonimmunized
mice).

ELISA reagents. Purified MBP and mouse anti-MBP (New England Biolabs),
alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG1 and anti-IgG2a
(Invitrogen), AP-conjugated anti-mouse IgG (Sigma), and purified Hs-tagged
T3 (described above) were used in the ELISA reactions.

IgG subclass determination. The IgG subclass profile in the mouse sera was
visualized by using a Rapid isotyping kit (Pierce) according to the manufacturer’s
instructions. In short, the serum samples were diluted (1:8,000) and applied into
the cassette well. The gold conjugates embedded in the cassette form specific subclass soluble complexes with the antibody in the sample. These complexes
travel the length of the membrane and are resolved on the membrane that is
impregnated with anti-isotype antibody. The results are then displayed as a red
band indicating the antibody isotype.

The endpoint (day 39) titers of MBP-specific IgG1 and IgG2a in pooled serum
samples were determined by using the ELISA with immobilized MBP and iso-
type-specific secondary antibodies. The serum samples were pooled and applied
from a serial dilution to the plate. Antibody titers were calculated as the dilution
producing the same ODmax at two times the background level (the reading
obtained with sera or lung fluid of nonimmunized mice).

RESULTS

The MBP* antigen is incorporated into T3 pili and exposed on the surface of L. lactis. Incorporation of a protein into the pilus structure requires the presence of an N-terminal Sec signal and a C-terminal CWSS (21, 35, 38). Therefore, to attach the E. coli MBP at the tip of T3 pili, the mature MBP protein (lacking its native Sec signal) was fused between the Sec signal of Cpa and the C terminus of Cpa, including its CWSS. Because mature Cpa is predicted to contain an intramolecular isopeptide bond (17), and the role of this bond in pilus morphogenesis is not known, the C-terminal region of
Cpa present in our construct (encoded by pJRS9565 [SrtC2*] and pJRS9566 [SrtC2*]; Fig. 1) includes both residues expected to form this bond (K599 and N704 of the unprocessed Cpa sequence). After processing by the leader peptidase and the pilus-specific sortase, the mature form of the resulting fusion protein, MBP*, should have the first 11 residues of the mature Cpa protein (N terminus), followed by the mature
MBP protein, followed by 119 amino acids from the C terminus
of mature processed Cpa. The predicted molecular mass of
the mature MBP* is 54 kDa.

To determine whether L. lactis expresses the MBP* protein on its surface, intact MG1363/pJRS9565 cells spotted on membranes were reacted with monoclonal anti-MBP antibody and, separately, with polyclonal anti-T3 antisera ("dot blots"). Strain MG1363/pJRS9566, which lacks SrtC2, was used as a negative control. The dot blots show that the intact cells re-
acted with both antibodies, indicating that both MBP* and T3 are
exposed on the bacterial surface (Fig. 2A and B).

To evaluate incorporation of MBP* into pili on the L. lactis
surface, cell wall fractions and concentrated culture supernatants of strains MG1363/pJRS9565 (MBP*), MG1363/pJRS9566 (−SrtC2), and MG1363/pJRS9545 (containing the empty vector)
were analyzed by Western blotting. Monomeric MBP* (apparent
molecular mass of 54 kDa) can be seen in the extracts from the
SrtC2 deletion mutant, MG1363/pJRS9566 (Fig. 2C, lanes 3 and
7), and it shows slight reactivity with the polyclonal anti-T3 anti-
serum (Fig. 2D), most likely due to the C-terminal residues
of mature processed Cpa in MBP* and/or to the known cross-reactivity of this anti-
serum with Cpa (20). A band that reacted with both monoclonal
anti-MBP antibody and polyclonal anti-T3 antisera was visible
at the location expected for the MBP*-T3 heterodimer (80 kDa)
in the strain expressing the T3 operon up to srtC2 (Fig. 2 lanes 1, 2, 5, and 6) and not in the SrtC2 deletion mutant derivative (Fig. 2, lanes 3 and
7), as expected. In extracts from two separate clones of the experimental strain, MG1363/pJRS9565, high-molecular-
weight (HMW) polymers, which are characteristic of pili in Gram-positive bacteria (21, 35, 38), were also visible, whereas these were absent from the srtC2 deletion control, as expected
(Fig. 2C and D). The reactivity of the HMW bands with anti-MBP
indicates that MBP* was incorporated into the pilus structure.
The higher-molecular-mass bands showed less reactivity with
anti-MBP, and the opposite was seen with anti-T3. This is ex-
pected since there are many T3 subunits per pilus but only one
MBP* subunit on the tip of each pilus (29). Similar to the cell wall
extracts, concentrated culture supernatants from MG1363/
pJRS9565 analyzed with anti-MBP and anti-T3 also showed the
HMW ladder characteristic of pili (Fig. 2C and D, lanes 5 and 6),

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whereas those of MG1363/pJRS9566 (lacking SrtC2) showed only monomeric forms of MBP* (Fig. 2C, lane 7).

**Immunogold EM confirms the incorporation of MBP** into T3 pili. We confirmed that the MBP protein was properly assembled into the T3 pili by using immunogold EM. L. lactis strains MG1363/pJRS9550 and MG1363/pJRS9565 (see Fig. 1), expressing T3 pili with HA-tagged Cpa and MBP*, respectively, assembled large amounts of pilus fibers that were abundantly labeled with the anti-T3 antiserum (Fig. 3B and C). This is in contrast to the vector control strain, MG1363/pJRS9545, which lacked pili and was not labeled by the anti-T3 antiserum (Fig. 3A).

Labeling of MG1363/pJRS9565 (MBP* T3 pili) with an anti-MBP antiserum confirmed that MBP* was incorporated into the pili (Fig. 3H and I). In the negative control, the anti-MBP antiserum did not label bacteria expressing pili lacking the MBP* fusion (MG1363/pJRS95950; Fig. 3G). A polyclonal anti-MBP antiserum was used for these experiments, as the monoclonal anti-MBP antibody did not react well with the bacteria under the immuno-EM conditions. Few MBP* subunits were present in the pili compared to the number of T3 subunits (compare Fig. 3H and I to Fig. 3C), and the MBP* was localized at what appeared to be the pilus tips (arrows in Fig. 3H and I), although the flexible and intertwined nature of the pili prevented definitive localization. The labeling pattern of MBP* matches the localization pattern of Cpa in T3 pili expressed in S. pyogenes (29), suggesting proper incorporation of MBP* in the L. lactis T3 pili. To confirm that MBP* localized similarly to Cpa in the T3 pili assembled by L. lactis, we labeled strain MG1363/pJRS9550, expressing HA-tagged Cpa, using an anti-HA antibody. As shown in Fig. 3E and F, labeling of the HA-tagged pili by the anti-HA antibody closely matched the appearance of pili labeled by the anti-MBP antiserum (arrows in Fig. 3). In the control, the anti-HA antibody did not label the MBP* pili, which lack the HA epitope (MG1363/pJRS9565; Fig. 3D).

**MBP** associated with T3 pili retains activity. Because it was possible that incorporation of MBP into pili would lead to its misfolding, we used the ability of pili containing MBP* to bind to an amylose resin to evaluate the activity of MBP. Lysates of MG1363/pJRS9565 (encoding MBP*), MG1363/pJRS9550 (the parental plasmid encoding intact Cpa), and MG1363/pJRS9566 (lacking SrtC2) were applied to an amylose resin, followed by analysis of the crude lysate, the flowthrough and the eluate fractions by Western blotting with anti-MBP antibody (Fig. 4A) and with purified anti-T3 antiserum (Fig. 4B). As expected, no signal was detected with anti-MBP in any of the fractions of strain MG1363/pJRS9550, which does not encode this protein (Fig. 4A, lanes 7 to 9). In the control strain (MG1363/pJRS9566), no polymeric forms of either MBP* (Fig. 4A) or T3 (Fig. 4B) were detected, a finding consistent with the requirement of SrtC2 for T3 pilus polymerization and incorporation of MBP*. HMW pilus forms were detected by both the anti-MBP antibody and the purified anti-T3 antiserum in the eluate fraction of MG1363/pJRS9565, which encodes MBP*. In the control extracts from the strain with native Cpa on the T3 pili (wild type), HMW pilus forms (detected with anti-T3) were present in the crude extract and in the flowthrough fraction but were not visible in the eluate fraction, indicating that these pili had not bound to the amylose resin (Fig. 4B lanes 7 to 9). This shows that the binding of pili containing MBP* to the amylose resin is due to the incorporation of a functional MBP molecule and is not a result of
interactions between the wild-type pilus subunits and the amylose bead matrix. Thus, we conclude that MBP* present in the T3 pili retains the activity of MBP, i.e., ability to bind amylose, which requires correct folding of the protein (36).

Elicitation of mucosal and systemic antibody responses in mice after mucosal immunization. The display of a correctly folded foreign antigen at the tip of a surface-exposed pilus by a probiotic bacterium may provide a new strategy for antigen delivery by mucosal vaccination. To examine the potential of *S. pyogenes* pili as vectors for antigen presentation, we investigated the immune response of mice to mucosal administration of live *L. lactis* expressing MBP* on their tips (strain MG1363/pJRS9565) compared to the same strain carrying the empty vector (MG1363/pJRS9545). An arbitrary dosage and protocol for vaccine administration were chosen (see Materials and Methods), and the response in mucosal secretions was determined in lung lavage, which was collected 10 days after the third antigen inoculation. The presence of MBP-specific IgA in the lavage was determined by using an ELISA with immobilized MBP (Fig. 5). A measurable and statistically significant reaction with immobilized MBP was demonstrated in all of the mice that were inoculated with bacteria expressing the recombinant pili (*P* < 0.001). The IgA response to MBP in undiluted serum was at least three times higher than the back-
ground in most of the animals, and the MBP-IgA endpoint titer (day 39) in pooled lavage samples was 1:10. Only background activity (the same as in uncoated wells) was found in lung lavage from untreated mice or mice inoculated i.n. with MG1363/pJRS9545 (empty vector; •, n = 8) or MG1363/pJRS9565 (MBP*; •, n = 10). The statistical significance between the experimental MG1363/pJRS9565 and the control group (MG1363/pJRS9545) determined by using the Student unpaired t test was P < 0.001.

The immune response to the T3 major pilus subunit was examined by using an ELISA with the immobilized T3 pilin protein. The T3-specific IgA antibody in undiluted lavage samples was at least 12 times higher than the background in all of the animals vaccinated with bacteria expressing the recombinant pili (MG1363/pJRS9565, P < 0.0001, Fig. 7). Little or no reaction was seen in samples from naive mice or mice immunized with bacteria carrying the control vector. The presence of T3-specific IgG antibody was also determined in pooled sera by using the same ELISA (Fig. 8). As with the lung lavage samples, a strong reaction with the T3 pilin was observed in serum from mice that were immunized with bacteria expressing the recombinant pili (MG1363/pJRS9565), whereas only low reactivity with the T3 protein was found in serum from the control

FIG. 4. MBP* incorporated into T3 pili in L. lactis retains activity. Whole-cell lysates of MG1363/pJRS9565 (MBP*) (lanes 1 to 3), MG1363/pJRS9566 (~SrtC2) (lanes 4 to 6), and MG1363/pJRS9550 (wild-type pilus cluster without MBP) (lanes 7 to 9) were purified with amylose resin and analyzed with monoclonal anti-MBP antibody (A) or purified anti-T3 antibody (B). Molecular masses (in kilodaltons) are indicated to the left of the figure. The locations of MBP* and the MBP*-T3 heterodimer are shown to the right of the figure. Fractions are indicated as follows: C, crude lysate; FT, flowthrough; and E, eluate.

FIG. 5. MBP-specific antibody response in mucosal secretions. MBP-specific IgA in lung lavage samples collected 10 days after the last immunization (day 39). The IgA response was determined in undiluted samples from individual mice by ELISA performed in quadruplicate. Each data point represents the average response in an individual animal, and horizontal lines represent the median response. Samples were taken from naive mice (▲, n = 2) or mice inoculated i.n. with MG1363/pJRS9545 (empty vector; ●, n = 8) or MG1363/pJRS9565 (MBP*; •, n = 10). The statistical significance between the experimental MG1363/pJRS9565 and the control group (MG1363/pJRS9545) determined by using the Student unpaired t test was P < 0.001.

FIG. 6. MBP-specific antibody response in serum. MBP-specific IgG in serum samples of experiment described in Fig. 5. The IgG response was determined in samples (diluted 1:50) from individual mice by ELISA performed in quadruplicate. Each data point represents the average response in an individual animal, and horizontal lines represent the median response. Samples were taken from naive mice (▲, n = 2) or mice inoculated i.n. with MG1363/pJRS9545 (empty vector; ●, n = 8) or MG1363/pJRS9565 (MBP*; •, n = 10). The statistical significance between the experimental MG1363/pJRS9565 and the control group (MG1363/pJRS9545) determined by using the Student unpaired t test was P < 0.0001.
animals (MG1363/pJRS9545, P < 0.0001). The endpoint titer in the pooled sera is 1:120,000. Together, these results show that i.n. vaccination with *L. lactis* expressing T3 pili with MBP* results in a significant T3-specific IgA response in secretion and a strong systemic IgG response. The T3 pilin immunity generated both in secretions and systemically was significantly stronger than the response to MBP, probably because while there is only one MBP subunit per pilus, there are hundreds of T3 subunits.

The profile of IgG isotypes in serum from vaccinated mice was compared to that found in pooled serum from untreated mice by using isotyping cassettes containing strips impregnated with anti-IgG1, -IgG2a, and -IgG2b antibodies. This analysis demonstrated that vaccination with *L. lactis* expressing the recombinant pilus resulted in a shift in the serum IgG profile to an increased prevalence of IgG2a (data not shown). The levels of MBP-specific IgG1 and IgG2a subclasses in the serum were determined by ELISA using isotype-specific secondary antibodies. The titer of IgG1 and IgG2a in the vaccinated mice was found to be 1:5,000 and 1:25,000, respectively. Therefore, i.n. administration of *L. lactis* displaying MBP* on the tip of the T3 pilus elicited a systemic IgG response that was dominated by IgG2a.

### DISCUSSION

This study had two main goals: (i) development of a system (UPTOP) for presentation of a polypeptide external to the envelope of a Gram-positive bacterium and (ii) demonstration that this system could be used to generate an immunogenic mucosal vaccine delivery system. These two objectives are discussed separately below.

**i) Bacterial surface display: UPTOP.** The use of bacteria as “nanoparticles” to present polypeptides is currently generating significant interest. Bacterial particles are being developed to present enzymes to improve bioremediation, to engineer better probiotic organisms, as additives to improve nutritional supplements for animals, and for many other uses. In addition, expression of antigens on the surface of bacteria is one approach being investigated for development of vaccine delivery vectors. Usually, these foreign proteins are attached directly to the bacterial surface: either the cytoplasmic membrane or the cell wall. However, antigens attached directly to the surface of live vaccine delivery vectors may be partially occluded by the bacterial envelope, since these proteins will not extend outwardly beyond any capsular material, S layers, or abundant protein on the cell surface and thus may have limited exposure to the environment. In agreement with this idea, a recent study using a live bacterial vector for vaccine antigen delivery determined that a stronger immune response resulted when the protective antigen was moved further from the bacterial surface by inserting a linker between it and the site attached to the bacterial cell wall (5). To overcome the limitation on exposure of the foreign protein that occurs when it is linked directly to the bacterial surface, we have developed the new technology UPTOP, which allows “unhindered presentation of a polypeptide on the tip of pilus.”

Pili, which are found on many bacteria, usually extend beyond the cell envelope of the organism and serve as the first contact between the bacterium and its environment. Thus, they often serve the role of adhesins that attach the bacterium to its specific niche. In Gram-positive bacteria, pili are covalently attached to the bacterial cell wall, which prevents their removal by washing even under extreme conditions. Polymerization of pili in *S. pyogenes* requires only one enzyme, the pilus-specific sortase, and one additional protein, SipA2 (44). In the T3 pilus of this organism, the protein Cpa is located on the pilus tip, and the threonine residue in its CWSS is linked to the major pilin subunit, T3 (29). In the present study, we capitalized on this finding to determine which residues of Cpa could be replaced by those of a model foreign protein, MBP. We showed that this model protein was covalently attached to the T3 pilus...
expressed in the foreign host L. lactis. At the C terminus of the chimeric protein MBP*, in addition to the Cpa CWSS, we included 119 amino acids of the Cpa protein so as to retain the predicted intramolecular isopeptide bond of Cpa (17). We found that deletions in this sequence prevented efficient incorporation of the chimeric MBP-Cpa protein into pili in L. lactis (data not shown), suggesting that residues N-terminal to the CWSS may be required for incorporation of the model protein into the T3 pilus.

Both for presentation of an antigen and of an enzyme, it is essential that the foreign protein on the pilus tip be correctly folded. The mechanism used to fold proteins on the exterior of the cell wall of Gram-positive bacteria is not completely understood. Because of this, and because covalent linkage to the T3 pilus might require a non-native conformation of the foreign protein, we felt it important to evaluate the structure of the foreign protein. The use of MBP as the model foreign protein allowed us to assess this, since the active site of MBP consists of amino acids located in a cleft formed between the two different domains in the correctly folded protein (36). We determined here that the MBP on the T3 pilus retains its activity, as measured by its ability to bind amylase. This indicates that MBP is most likely presented on the pilus tip in its native conformation.

(ii) Vaccine delivery system. L. lactis is an attractive delivery vehicle for mucosal vaccines. The majority of infections are initiated at mucosal surfaces, where some pathogens remain restricted to the mucosal membranes and others penetrate the epithelium and spread throughout the body. An effective mucosal immune response requires production of both secretory IgA and serum IgG. Mucosal IgA can form a barrier to pathogens at the mucosal surface by preventing the initial attachment of the pathogen and its infiltration of the surface layers or by binding to and neutralizing toxins that the pathogen produces. IgA defense is especially important for surfaces that cannot be reached effectively by serum IgG antibody (15, 27). Systemic IgG production supplements the mucosal defense provided by IgA and reduces the ability of the pathogen to cross mucosal membranes and spread within the body (3, 27, 43).

The properties of lactic acid bacteria (LAB) make them good candidates for live vaccine delivery vehicles. These bacteria are food-grade organisms, used in the production of fermented food products such as cheese and yogurt, and they have been safely consumed by humans for centuries. In addition, they are considered to be probiotics, i.e., live microorganisms believed to confer a health benefit on the host when administered in sufficient quantities (43). Thus, LAB, including L. lactis, have GRAS (i.e., generally regarded as safe) status and can be administered orally to people.

LAB are also attractive vaccine vectors because they have been found to have natural adjuvant activity (23, 41). In addition, L. lactis is a natural Toll-like receptor agonist (10, 18, 25, 39) that can stimulate the production of various interleukins, which can increase the antigen-specific immune response. Several mucosal immunization studies using live (7–9, 11, 22, 24, 28, 40, 42) or killed (32) LAB delivery systems expressing cytoplasmic, secreted, or cell wall-associated antigens have demonstrated both systemic and mucosal immune responses (for a review, see reference 43). Protection against challenge with pathogens was also demonstrated in a number of studies. Examples of pathogens against which protection was observed include the bacteria S. pyogenes (22), Streptococcus pneumoniae (11, 24, 28, 40), group B Streptococcus spp. (7), and enterotoxigenic E. coli (42).

UPTOP is a valuable technique for presentation of a vaccine antigen on L. lactis. LAB vaccines currently being investigated present the vaccine antigen to the mucosal surface as a cytoplasmic, cell wall anchored, or secreted protein (for a review, see reference 43). We describe here a novel method for the delivery of a vaccine antigen to the mucosal surface using UPTOP technology. We engineered the L. lactis delivery vector to produce the vaccine antigen covalently linked at the tips of pili, which are anchored covalently to the cell wall of the bacterium. Thus, although the antigen is covalently attached to the bacterium, since the pili produced are long, the antigen is exposed external to the bacterial envelope and thus is positioned to interact with maximal effectiveness with the environment, i.e., the immune system. This is particularly advantageous for B cells that can see the antigen directly, independent of processing and presentation by antigen-presenting cells. When the genes required for pilus production are expressed from a strong promoter, as we did in this work, each bacterium has hundreds of pili with the antigen on their tips, providing multiple copies of the antigen to which the immune system is exposed.

We have shown here that mucosal administration of this L. lactis strain expressing pilus-linked MBP* resulted in a detectable MBP-specific IgA response in the lung lavage of immunized mice. Therefore, this delivery system is effective in presenting protein antigens and triggering a mucosal response, at least in the upper respiratory tract. Since i.n. immunization can produce a significant IgA response in the cervicovaginal mucosa in addition to that in the upper airway mucosa (16, 26), it is possible that i.n. administration with this vaccine will induce an adaptive response in the genital tract as well.

In addition to the observed IgA response, a strong IgG response to MBP was detected in blood as a result of the course of i.n. immunization. The systemic response to MBP is very encouraging because serum antibody contributes significantly to the mucosal defense, especially in the lower respiratory and in the genitourinary pathways, where the epithelia are permeable to serum antibody (13). In addition, a serum humoral response can prevent the systemic spread of invasive pathogens. The observed mucosal and systemic response to the T3 pilin, in addition to the immunity mounted against MBP, suggests that the UPTOP system may allow the presentation of more than one antigen at a time. We have shown that insertion of the 9-amino-acid HA epitope within the T3 protein does not prevent pilus polymerization (2, 29, 44) and that mice inoculated with L. lactis expressing these HA tagged pili generate an HA-specific immune response (B. R. Quigley, Z. Eichenbaum, and J. R. Scott, unpublished results). Thus, it is possible that protective epitopes may be engineered into the T3 protein. In addition, it should be possible to use related but serologically different pili, if UPTOP is to be used for presentation of different vaccine antigens on successive occasions.

Analysis of pooled sera from our immunized mice for IgG subclass revealed a significant bias toward IgG2a (with a ratio of the titer of IgG2a to IgG1 of 5) (data not shown). The limited variation in the response of individual mice for both
MBP and T3 antigens (Fig. 5 to 7) suggests that the IgG1 and IgG2 titers in the pooled serum are an accurate representation of the population and are not likely to be driven by the response in one or two animals. The IgG2 dominant response suggests that the adaptive T-cell response to the pilus-linked MBP could be predominantly T helper 1 (Th1) type. Similarly, a predominant Th1 response was also seen when other live *L. lactis* vaccines were administrated at mucosal surfaces (11, 30). Th1 cells contribute to the humoral response by supporting the production of IgG2a, while inhibiting the formation of other IgG subclasses, such as IgG1. In addition, Th1 cells produce gamma interferon (IFN-γ) and interleukin-2 (IL-2) and therefore promote a cellular immune response, which includes macrophage activation, delayed-type hypersensitivity, and T-cell cytotoxicity. Based on recent work with *S. pneumoniae*, the relative elevation of the IgG2a subtype is likely to produce greater protection at the mucosal surface than a predominantly IgG1 response (11). Because the IgG2a/IgG1 ratio seen in our experiments seems high, we suggest that in addition to the effect of the live *L. lactis* bacteria in generating an effective immune response, the properties of the pili on their surface may also have contributed to the skewing of the immune response.

**Future improvements.** The model vaccine strain described here encodes the UPTOP expression system on a plasmid that also encodes an antibiotic resistance marker. However, because the genome sequence of *L. lactis* is available (6) and because this organism is amenable to genetic manipulation, the UPTOP pilus gene cluster can be stably integrated into the bacterial chromosome without an antibiotic resistance marker. This would minimize the number of foreign genes introduced and avoid possible problems of the environmental spread of an antibiotic resistance plasmid.

In addition, several opportunities exist for the possible improvement of immunogenicity of our *L. lactis*-based vaccine. For example, increasing the number of bacteria administered per dose and/or the number of pili expressed per bacterium should result in an increase in the immune response and should increase protection. For an *L. lactis*-based GBS vaccine, mice were immunized with 10^13 CFU/dose, i.e., 100 times the dose used in our study, without adverse effects (7). In addition, the frequency of administration of the vaccine and the fluid in which the vaccine strain is administered can be optimized, and the vaccine can also be delivered orally. It is also possible that the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen, the outcome might be improved by coexpression of IL-2 or IL-6 with the antigen.

**Conclusions.** In summary, in the present study we identified residues of the pilus tip protein that can be replaced by an antigen of choice using the *E. coli* MBP as a model. Replacement of these residues results in the stable attachment of the model protein to the bacterium, since the linkages are all covalent. We have also shown that a strain of *L. lactis* can be engineered to incorporate this model protein of 54 kDa (MBP*) into T3 pili. In addition, we demonstrated that mucosal administration of this vaccine strain resulted in a detectable MBP-specific IgG and IgA response. We conclude that UPTOP is a promising strategy for the presentation of polypeptides covalently attached to the surface of bacteria but external to the bacterial envelope and that it might be used to develop effective oral vaccines for protection against mucosal pathogens.

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