INFECTION AND IMMUNITY, May 1999, p. 2464–2474 Vol. 67, No. 5
0019-9567/99/$04.00+0


# Insertional Inactivation of Genes Responsible for the D-Alanylation of Lipoteichoic Acid in *Streptococcus gordonii* DL1 (Challis) Affects Intrageneric Coaggregations

DANIEL L. CLEMANS,[1]† PAUL E. KOLENBRANDER,[1]* DMITRI V. DEBABOV,[2] QUNYING ZHANG,[2] R. DWAYNE LUNSFORD,[1]‡ HOLLY SAKONE,[2] CATHERINE J. WHITTAKER,[1]§ MICHAEL P. HEATON,[2]‖ AND FRANCIS C. NEUHAUS[2]

*Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892,[1] and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208[2]*

Received 10 November 1998/Returned for modification 6 January 1999/Accepted 25 February 1999

**Most human oral viridans streptococci participate in intrageneric coaggregations, the cell-to-cell adherence among genetically distinct streptococci. Two genes relevant to these intrageneric coaggregations were identified by transposon Tn*916* mutagenesis of *Streptococcus gordonii* DL1 (Challis). A 626-bp sequence flanking the left end of the transposon was homologous to *dltA* and *dltB* of *Lactobacillus rhamnosus* ATCC 7469 (formerly called *Lactobacillus casei*). A 60-kb probe based on this flanking sequence was used to identify the homologous DNA in a fosmid library of *S. gordonii* DL1. This DNA encoded D-alanine-D-alanyl carrier protein ligase that was expressed in *Escherichia coli* from the fosmid clone. The cloned streptococcal *dltA* was disrupted by inserting an *ermAM* cassette, and then it was linearized and transformed into *S. gordonii* DL1 for allelic replacement. Erythromycin-resistant transformants containing a single insertion in *dltA* exhibited a loss of D-alanyl esters in lipoteichoic acid (LTA) and a loss of intrageneric coaggregation. This phenotype was correlated with the loss of a 100-kDa surface protein reported previously to be involved in mediating intrageneric coaggregation (C. J. Whittaker, D. L. Clemans, and P. E. Kolenbrander, Infect. Immun. 64:4137–4142, 1996). The mutants retained the parental ability to participate in intergeneric coaggregation with human oral actinomyces, indicating the specificity of the mutation in altering intrageneric coaggregations. The mutants were altered morphologically and exhibited aberrant cell septa in a variety of pleomorphs. The natural DNA transformation frequency was reduced 10-fold in these mutants. Southern analysis of chromosomal DNAs from various streptococcal species with the *dltA* probe revealed the presence of this gene in most viridans streptococci. Thus, it is hypothesized that D-alanyl LTA may provide binding sites for the putative 100-kDa adhesin and scaffolding for the proper presentation of this adhesin to mediate intrageneric coaggregation.**

Coaggregation of human oral bacteria is thought to occur by specific interactions between complementary surface molecules on the partner cells (64). Cell-surface adhesins on one cell type may recognize and bind to complementary receptors on the partner cell type. Most of the interactions are among members of different genera, for example *Streptococcus* spp. and *Actinomyces* spp., and are termed intergeneric coaggregations. Streptococci also participate in intrageneric coaggregations, which occur among the human oral viridans streptococci, and these coaggregations are galactoside inhibitable (38). A 100-kDa putative adhesin on the surface of *Streptococcus gordonii* DL1 was proposed to mediate specifically these galactoside-inhibitable intrageneric coaggregations, because streptococcal insertional mutants as well as spontaneous coaggregation-defective mutants lost specifically the intrageneric coaggregation capability and lack this protein (9, 10, 63).

A potential role of lipoteichoic acid (LTA) in coaggregation is the proper presentation of adhesins and receptors on partner cells. LTAs are macroamphiphiles that contain alditolphosphates as integral parts of the hydrophilic chain (17). Most glycerol LTAs are substituted with D-alanyl ester residues (18). Because LTA is polyanionic, it binds $Ca^{++}$ ions (53) and may contribute to the proper environment for coaggregation, which requires divalent cations, especially $Ca^{++}$ (38, 46). Out of a total of 86 strains examined, LTA was found in all viridans streptococci except *Streptococcus mitis* and *Streptococcus oralis* (28). Since LTA inhibits the attachment of *S. gordonii* to substratum-located glucan polymer, the adhesion is thought to be mediated by LTA (60). LTA is reported to mediate binding of group A streptococci to fibronectin receptors on pharyngeal epithelial cells (4), and it inhibits binding of human oral viridans streptococci to fibronectin-coated spheroidal hydroxyapatite beads (27). LTA is thought to be important in the first of two steps of adhesion to human cells; the second step is postulated to occur by a specific adhesin(s) that determines tissue tropism (24). LTA exhibits properties of an enterococcal binding substance that is recognized by a mating cell-expressing aggregation substance (16), and the resulting union of the two cell types is part of the well-studied pheromone-inducible conjugation system in *Enterococcus faecalis* (12, 14).

The *dlt* operon involved in the synthesis of the D-alanyl

* Corresponding author. Mailing address: National Institutes of Health, Bldg. 30, Rm. 310, 30 Convent Dr., MSC 4350, Bethesda, MD 20892-4350. Phone: (301) 496-1497. Fax: (301) 402-0396. E-mail: pkolenbrander@dir.nidcr.nih.gov.

† Present address: University of Michigan, Department of Pediatrics and Communicable Diseases, Ann Arbor, MI 48109-2029.

‡ Present address: Antiinfectives Research, Smith-Kline Beecham Pharmaceuticals, Collegeville, PA 19426.

§ Present address: Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berks RG20 7NN, United Kingdom.

‖ Present address: USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center (MARC), Clay Center, NE 68933.

esters of LTA was discovered in *Lactobacillus rhamnosus* (25, 26, 47) and subsequently identified in *Bacillus subtilis* (21, 50). The operons encode, respectively, four and five genes in these organisms. The first gene, *dltA*, encodes the D-alanine–D-alanyl carrier protein ligase (Dcl), which catalyzes the D-alanylation of the D-alanyl carrier protein (Dcp) encoded by *dltC*. Dcp in turn transfers the D-alanine to a membrane acceptor for the D-alanylation of LTA. There is a coding sequence overlap between *dltA* and *-B* and between *dltC* and *-D* with putative ribosome binding sites preceding *dltA* and *dltC* (47). On the basis of the hydropathy profile of the putative DltB, it is hypothesized that DltB is located in the cytoplasmic membrane and displays 12 membrane-spanning domains. DltB is hypothesized to be involved in the efflux of activated D-alanine to the site of LTA acylation (47).

The function of the D-alanine esters has been a point of recent investigation for several genera of gram-positive bacteria. In *Lactobacillus rhamnosus* ATCC 7469 they appear to play a role in determining cell shape and cell septation (48), whereas in *B. subtilis*, the absence of D-alanine esters has no effect on ultrastructure or cell septation but does enhance autolytic and beta-lactam-induced cell lysis (62). D-Alanine esters provide limited protection to *B. subtilis* JH642 against methicillin but do not protect against phagocytosis and degradation of the bacterium in macrophages (61). In *Staphylococcus aureus* mutant strains defective in formation of D-alanine esters, the cells exhibited reduced autolysis and enhanced expression of methicillin resistance (49). Increased resistance to vancomycin in *Enterococcus faecium* D366 was accompanied by a doubling of the D-alanyl ester content of LTA (22). It was proposed that this event would reduce the ability of autolysins to bind to the heavily D-alanylated LTA, which may affect a later step in the pathway that triggers autolytic and beta-lactam-induced cell lysis. Insertion of IS*S*1 into *dltD* resulted in *Lactococcus lactis* MG1363 becoming UV-sensitive suggesting that cell envelope integrity and the ability to repair DNA are related (15). The *L. lactis dltD* mutant also grew more slowly and formed longer chains than the parent strain. Thus, the D-alanyl esters of LTA would appear to play a variety of roles in gram-positive organisms.

Here we report that inhibition of D-alanyl-LTA biosynthesis by specific insertional mutagenesis in *S. gordonii* DL1 resulted in undetectable levels of a 100-kDa putative adhesin on the cell surface, prevented intrageneric-galactoside-inhibitable coaggregation, contributed to altered cellular division and morphology, and reduced DNA transformation frequency. In contrast, intergeneric coaggregation with actinomyces was unchanged. We propose that the absence of D-alanyl esters disrupts one or more of the normal scaffolding functions of LTA in adhesin presentation on the streptococcal cell surface and in a variety of cellular processes including transformation, adherence, and cell division.

## MATERIALS AND METHODS

**Cultivation of bacteria.** All bacterial strains used in this study are listed in Table 1. Streptococci and actinomyces were cultured in CAMG medium (9) at 37°C under anaerobic conditions with the GasPak system (BBL Microbiology Systems, Cockeysville, Md.) (2). *S. gordonii* strains containing transposon Tn*916* were grown on CAMG medium supplemented with 10 μg of tetracycline (Sigma Chemical Co., St. Louis, Mo.) per ml of medium. All incubations for the generation of competent *S. gordonii* DL1 were carried out at 37°C. Transformants in the *dlt* operon were obtained by the method previously described (63) by using the transformation medium of LeBlanc and Hassel (39). The resultant *dlt* insertion mutants were tested for competence and transformation frequency by the method of Lunsford (42). All *Escherichia coli* strains were cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar (Gibco-BRL). LB medium supplemented with ampicillin (100 μg per ml), tetracycline (4 μg per ml), erythromycin (300 μg per ml), or chloramphenicol (20 μg per ml) was used to select for *E. coli* strains containing various plasmids (Table 2). *E. coli* CG120 was cultured in medium containing 100 μg of ampicillin and 4 μg of tetracycline per ml.

Bacterial cells used for coaggregation assays were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, washed three times in coaggregation buffer (1 mM Tris [pH 8.0], 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 mM NaCl, and 0.02% $NaN_3$), and stored in coaggregation buffer at 4°C. The visual assay for coaggregation has been described in detail elsewhere (63).

**Isolation of Tn*916*-insertion mutants.** The procedure of Behnke (5) was followed for the transformation of *S. gordonii* DL1 cultures with plasmid DNA as described previously (63). Tetracycline-resistant transformants were picked onto fresh CAMG agar medium containing tetracycline and incubated at 37°C under anaerobic conditions. The colonies were screened (63) for the absence of coaggregation with *Streptococcus oralis* 34 by the microtiter plate assay described previously by Kolenbrander (37). Cells of coaggregation-defective cultures were frozen in a dense suspension in CAMG broth and stored at −40°C.

**Recombinant DNA methods.** The procedures for preparing plasmid DNA from *E. coli* have been described previously (63), and genomic DNA from *S. gordonii* was prepared by the procedure described by Andersen et al. (2). To clone the region adjacent to the Tn*916* transposon, we used the single *Hin*dIII site located 6.5 kb from the left end (19). The 7.1-kb fragment from *Hin*dIII-digested (New England BioLabs, Beverly, Mass.) genomic DNA from the $Cog^-$ Tn*916* insertion mutant G7 was cloned into the *Hin*dIII site of pUC19 (Gibco-BRL, Gaithersburg, Md.) (63). The recombinant plasmid containing the 7.1-kb *Hin*dIII fragment was called pDC3 (Table 2), and the *E. coli* DH5α strain containing pDC3 was designated PK3322.

*S. gordonii* DL1 DNA flanking the left end of Tn*916* on the cloned *Hin*dIII fragment in pDC3 was sequenced by dideoxy sequencing by using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio) and [$^{35}$S]dATPαS (Dupont, NEN Research Products, Boston, Mass.). Oligonucleotide primers were prepared on a PCR-MATE model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). A radioactively labeled probe was generated by nick translation of the 7.1-kb cloned fragment or by end labeling the 60-base oligonucleotide for G7 (5′-AACATCCTCCAGCTCGATGCGGTACGCATTAAACTTAATTTGGAAATCCATTCGACCACC-3′).

A complete genomic library of *S. gordonii* DL1 was constructed in the fosmid pFOS1 by using *Sau*3A1 partially digested genomic DNA as described previously (36, 43). The library represented in excess of 2.5 genome equivalents.

Southern analysis of *dltA* homologs in genomic DNAs from representative oral streptococci with *S. gordonii dltA* as a probe was performed according to Whittaker et al. (63) and Andersen et al. (2). Hybridizations were performed at 50°C with Quick-Hyb hybridization solution (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. The strains tested were *S. gordonii* DL1, *S. oralis* H1, *S. oralis* 34, *S. oralis* J22, *Streptococcus* sp. strain SM PK509, *S. gordonii* PK488, *Streptococcus sanguis* 12, *Streptococcus parasanguis* FW213, *S. sanguis* ATCC 10556, *S. oralis* ATCC 10557, *S. gordonii* ATCC 10558, *Streptococcus milleri* K44Y, *Streptococcus sobrinus* 6715-10, *Enterococcus faecalis* GF590, *Streptococcus crista* PK1408, *Streptococcus mutans* LM7, *Streptococcus pyogenes* JRS4, *Streptococcus rattus* BHT, *Streptococcus cricetus* AHT, *Streptococcus pneumoniae* R6, *Streptococcus constellatus* PK2819, *Streptococcus salivarius* ATCC 25975, and *Streptococcus intermedius* PK2821.

**Insertional inactivation of the *S. gordonii* DL1 *dltA* gene with *ermAM*.** Restriction sites for *Kpn*I and *Bam*HI were incorporated into the DNA primers with the respective 5′ and 3′ sequences of the *dltA* gene and used to amplify a 1.4-kb fragment of the *dltA* gene from pDC5 as template. The two primer sequences were as follows: PCR1, 5′-CCGGATCCTGACCTCGCTGATTAAGCCC-3′; PCR2, 5′-GGGGGTACCTCTCCTGTCGTGGTCTATGGTGGGC-3′. PCR of the *S. gordonii* DL1 *dltA* homolog was performed with the GeneAmp PCR core reagents (Perkin-Elmer Cetus, Norwalk, Conn.). pDC5 DNA (5 ng) was mixed with 1 μM each primer, 2 mM $MgCl_2$, and 2.5 U of Ampli*Taq* DNA polymerase (Perkin-Elmer Cetus) in a final reaction volume of 50 μl. The thermocycler program (Perkin-Elmer Cetus) was 4 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and a final elongation of 5 min at 72°C. The 1.4-kb *dltA* fragment contained a single *Hin*cII site near the center and was used to clone a blunt-ended 922-bp *ermAM* cassette from pKSerm2 (43). The recombinant plasmid was linearized with *Bam*HI and used to transform *S. gordonii* DL1 (63) to disrupt the *dltA* gene with *ermAM*. Two transformants, PK3241 and PK3242, were chosen for study. Southern analyses of *Acc*I and *Sca*I restriction endonuclease-digested genomic DNAs from strains DL1, PK3241, and PK3242 were performed according to the procedure of Whittaker et al. (63) and were conducted in order to confirm insertion of *ermAM* into the *S. gordonii dltA* homolog. Both the 0.9-kb *ermAM* and the 1.4-kb *dltA* probes were end labeled with $^{32}$P by using T4-polynucleotide kinase (Lofstrand Labs Limited, Gaithersburg, Md.).

**Computer analyses.** Sequence assembly and analyses were performed with Assembly-LIGN and MacVector (International Biochemicals, Inc., New Haven, Conn.). Database searches were conducted with the BLAST algorithm (1) by using the current version of the nonredundant protein database at the National Center for Biotechnology Information (NCBI-NLM, Bethesda, Md.). Protein alignments were performed with PILEUP, GAP, and PRETTY programs (Genetics Computer Group, version 9.1, for VMS-VAX).

TABLE 1. List of strains used

| Organism and strain | Relevant characteristic(s) | Reference or source |
|---|---|---|
| *S. oralis* | | |
| 34 | Reference strain for streptococcal coaggregation group 3 | 38 |
| C104[a] | Reference strain for streptococcal coaggregation group 3 | 38 |
| *Streptococcus* sp. strain SM PK509 | Reference strain for streptococcal coaggregation group 5 | 38 |
| *A. naeslundii* ATCC 51655 (formerly called *A. naeslundii* PK606) | Reference strain for actinomyces coaggregation group D | 38 |
| *S. gordonii* | | |
| DL1 (Challis) | Reference strain for streptococcal coaggregation group 1 | 38 |
| PK1897[b] | $Cog^-$, selected with *S. oralis* C104 | 10 |
| G7[a] | DL1 ($Cog^-$ Tn*916* insertion mutant selected with *S. oralis* 34) | This study |
| PK3241[c] | DL1 ($Cog^-$ insertion mutant containing *dltA*::*ermAM* gene disruption in chromosome) | This study |
| PK3242[c] | DL1 ($Cog^-$ insertion mutant containing *dltA*::*ermAM* gene disruption in chromosome) | This study |
| *E. coli* | | |
| DH1 | $F^-$ *recA1 endA1 gyrA96 thi-1 hsdR17 supE44* | 23 |
| DH5α | $F^-$ f80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*($r_k^-$ $m_k^+$) *supE44* $\lambda^-$ *thi-1 gyrA96 relA1* | Gibco-BRL |
| XL1-Blue | *supE hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [*proAB*$^+$ *lacI*$^q$ *lacZ*Δ*M15* Tn*10*($Tc^r$)] | Stratagene |
| DH10B | $F^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) *endA recA* | Gibco-BRL |
| CG120 | DH1 containing plasmid pAM120 | D. Clewell; 19 |
| DH10B + pFOS1 | DH10B containing fosmid pFOS1 | 43 |
| XL1-Blue + pDAE1 | XL1-Blue containing plasmid pDAE1 | 25 |
| PK3322 | DH5α containing plasmid pDC3 | This study |
| PK3324 | DH10B containing plasmid pDC5 | This study |
| PK3327 | DH5α containing plasmid pDC8 | This study |
| PK3330 | DH5α containing pDC11 | This study |

[a] Resistant to tetracycline (10 μg/ml).
[b] Resistant to rifamycin (25 μg/ml), streptomycin (100 μg/ml), and spectinomycin (500 μg/ml).
[c] Resistant to erythromycin (10 μg/ml).

**Immunoblot analyses.** The proteins from streptococcal surface-sonicate extracts were separated on sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis gels (Novex, San Diego, Calif.) as described previously (63). Detection of the 100-kDa putative adhesin was done by previously published methods with anti-DL1 polyclonal serum that had been adsorbed with spontaneously occurring coaggregation-defective mutant strain PK1897 (9, 63).

For the determination of D-alanine esters, cells of *S. gordonii* were transferred to Whatman no. 42 filter paper. Cells were lysed by incubating the filter paper on Bio-Rad ultra-thick blotting paper saturated with lysis solution (10 mM EDTA [pH 8.0] and lysozyme [10 mg/ml]). The transfer of the lysate to Bio-Rad Trans-Blot polyvinylidene difluoride (PVDF) membrane was accomplished with the Bio-Rad Trans-Blot Semi-dry transfer cell. The PVDF membrane was treated with serum containing antibodies against D-alanine esters (40) and visualized with a secondary antibody (immunoglobulin G) conjugated with peroxidase. The blot was developed with the luminol reagent by using the ECL kit (Amersham, Arlington Heights, Ill.).

**Streptococcal surface protein preparations.** Streptococcal cells were washed three times with distilled water by centrifugation (10,000 × *g* for 10 min at 4°C) and resuspended to a concentration of 0.5 g (wet weight) cells per ml of distilled water up to a maximum of 1 ml and sonicated on ice for 1 min at maximum power (50 W) with a Micro Ultrasonic cell disrupter (Kontes, Vineland, N.J.). The sonicated suspension was centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatant fluid was stored frozen until used. Total protein was determined with the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.).

**Electron microscopy.** Exponential-phase cells were fixed overnight in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% (wt/vol) glutaraldehyde at 4°C. For scanning microscopy fixed samples were washed four times with 0.1 M cacodylate buffer. A drop of each sample on a glass coverslip was dehydrated in a graded

TABLE 2. List of plasmids used

| Plasmid | Relevant characteristic(s) | Reference or source |
|---|---|---|
| pAM120 | pGL101 carrying *Eco*RI F′ (F::Tn*916*) fragment of pAM211, $Tc^r$ | D. Clewell; 19 |
| pFOS1 | Fosmid, $Cm^r$ | 36 |
| pBS[a] | $Ap^r$ | Stratagene |
| pDAE1 | pBS KS+ with 1,568-bp *L. casei dltA* gene cloned in sense mRNA orientation | 25 |
| pKS*erm*2 | 922-bp *ermAM* cassette, $Ap^r$ $Em^r$ | 43 |
| pDC3 | pUC19 with a 7.1-kb *Hin*dIII containing the left end of Tn*916* and *S. gordonii* DL1 flanking DNA | This study |
| pDC5 | pFOS1 with 40-kb *S. gordonii* (Challis) insert containing the *dlt* operon | This study |
| pDC8 | pBS KS+ with 1.4-kb *Bam*HI-*Kpn*I PCR fragment of *S. gordonii* DL1 *dltA* gene | This study |
| pDC11 | pDC8 *Hin*cII (*ermAM*, blunt) | This study |

[a] pBS, pBluescript II KS.

series of ethanol concentrations, critical point dried, and sputter coated with gold-palladium. Specimens were examined with a JEOL JSM-1 or an ultra-high-resolution Hitachi S-4500 microscope. For transmission microscopy the fixed cells were washed four times with 0.1 M cacodylate buffer and postfixed for 1 h at 25°C with 0.5% (wt/vol) osmium tetroxide–0.8% (wt/vol) potassium ferricyanide in 0.1 M cacodylate (pH 7.4). After washing the samples three times with water, they were stained with 0.5% (wt/vol) uranyl acetate for 1 h at 25°C and washed three more times. The samples were pelleted in 2% (wt/vol) agar, cut into 1-mm cubes, and dehydrated in increasing concentrations of ethanol (34). The samples were infiltrated with Spurr's resin (56), embedded in BEEM capsules, and sectioned (80 nm, silver) on a Reichert Ultracut-E. The sections were poststained with 1% (wt/vol) uranyl acetate for 20 min and examined with a JEOL JEM-100CX 11 microscope at 80 kV.

**D-Alanine incorporation assay.** Incorporation of D-[$^{14}$C]alanine into toluene-treated cells was performed in a reaction mixture (20 μl) which contained 30 mM bis-Tris (pH 6.5), 10 mM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.11 mM D-[$^{14}$C]alanine (43 mCi/mmol), and 4 μl of permeabilized cell suspension. For the preparation of permeabilized *S. gordonii*, a modification of the procedure of St. Martin and Wittenberger (57) was used. The cells (0.65 gm/ml), suspended in 100 mM phosphate buffer (pH 7.0), were treated with 0.15 volumes of toluene-acetone (1:9 [vol/vol]) for 1 min of vigorous agitation on a Vortex mixer. The agitation was repeated three more times with intermittent cooling in an ice bath. The reaction mixture was incubated at 37°C, and the reaction was terminated by the addition of 0.9 ml of 30 mM bis-Tris (pH 6.5). The labeled cells were collected on a GN-6 Metricel filter and washed with three 1 ml and one 10 ml portion of 30 mM bis-Tris (pH 6.5). The filters were dissolved in ethyl acetate and assayed for radiolabel.

**Nucleotide sequence accession number.** The assigned GenBank accession number is AF059609.

## RESULTS

**Isolation of *S. gordonii* DL1 Cog$^-$ mutant.** Transposon mutagenesis was conducted (63) to isolate Cog$^-$ mutants that failed to coaggregate with streptococcal partners of the wild-type strain DL1. Transposon Tn*916* was inserted into the chromosome of *S. gordonii* DL1 by transformation of strain DL1 with the donor plasmid pAM120 (19, 63). This process yielded mutant strain G7, which lost specifically the ability to coaggregate with streptococcal partners while exhibiting wild-type levels of coaggregation with an actinomyces partner (Table 3). Intergeneric coaggregations of the mutant or wild type with *Actinomyces naeslundii* ATCC 51655 are not inhibited by lactose, whereas the intrageneric coaggregations between the wild-type DL1 with *S. oralis* 34, *S. oralis* C104, and *Streptococcus* sp. strain SM PK509 are inhibited by lactose (38). In these galactoside-inhibitable coaggregations, *S. gordonii* DL1 was sensitive to heat and protease treatment and was presumed to contain a putative adhesin which recognizes a carbohydrate-containing receptor on the surface of its partner strains (38). On the basis of the current work with Cog$^-$ mutant G7, it was discovered that Tn*916* inserted into a new coaggregation-relevant locus, which is different from the locus in the Cog$^-$ insertion mutant described previously by Whittaker et al. (63).

**Cloning and sequencing of the streptococcal DNA flanking the Tn*916* insert.** To obtain the nucleotide sequence of the coaggregation-relevant locus, the *Hin*dIII junction fragment (7.1 kb), which included the left end of Tn*916* and flanking *S. gordonii* DNA, was isolated and cloned into the *Hin*dIII site of plasmid pUC19 to yield pDC3. The 626-bp sequence of the streptococcal DNA flanking the left end of the *Hin*dIII fragment in strain G7 was obtained and is presented as a partial restriction map (Fig. 1A). Translation of the two partial open reading frames (ORFs) showed significant sequence identity to DltA (D-alanine–D-alanyl carrier protein ligase [Dcl]) and DltB (13, 25, 26). The two partial ORFs were 519 bp (ORF 1, *dltA* homolog) and 111 bp (ORF 2, *dltB* homolog) in length with the 3′ end of ORF 1 sharing four nucleotides with the 5′ end of ORF 2. A similar overlap of *dltA* and *dltB* in the *dlt* operon from *L. rhamnosus* was reported by Heaton and Neuhaus (25). Both ORFs appear to be transcribed towards the Tn*916* insertion with *dltB* being directly interrupted by the transposon insert. To examine the possibility that there was more than one streptococcal locus homologous to *dltA* from *L. rhamnosus*, a 60-bp probe (Fig. 1A) was used for Southern blot analysis of *Bam*HI-, *Cla*I- and *Hin*dIII-digested genomic DNA from *S. gordonii*. A single hybridizing band was observed in genomic digests with each of the three restriction enzymes (data not shown), suggesting a single locus for the *dlt* operon. The 60-bp probe (G7) hybridized with *L. rhamnosus dltA* cloned into pDAE1. These data confirmed the presence of a single homologous sequence in *S. gordonii*.

TABLE 3. Coaggregation properties of mutant and wild-type *S. gordonii* DL1 strains with representative oral bacteria

| Strain | Coaggregation score[a] with: | | | |
|---|---|---|---|---|
| | *S. oralis* 34 | *S. oralis* C104 | *Streptococcus* sp. strain SM PK509 | *A. naeslundii* ATCC 51655 |
| DL1 | $3^0$ | $3^0$ | $3^0$ | $4^4$ |
| G7 | 0 | 0 | 0 | $4^4$ |
| PK3241 | 0 | 0 | 0 | $4^4$ |
| PK3242 | 0 | 0 | 0 | $4^4$ |

[a] The method for assigning coaggregation scores has been described by Kolenbrander, Andersen, and Moore (38). The maximum score is 4; no coaggregation is given a zero score. Coaggregation scores are given in two parts: the first score is that given after mixing the two strains together, and the superscript is the score after adding lactose (final concentration, 60 mM) to the coaggregates.

The translated sequences of truncated *dltA* and *dltB* were aligned (Fig. 1B) with corresponding proteins from other bacteria by using PILEUP (20). DltA consensus regions II and III (Fig. 1B) described by Heaton and Neuhaus (25) are conserved. These regions are characteristic of nonribosomal-peptide condensation via a thiolester intermediate (25). Alignment of the truncated fragments of DltA and DltB of *S. gordonii* DL1 by using GAP (20) showed that DltA was 41, 44, 50, 75, and 79% identical to DltA of *S. aureus*, *B. subtilis*, *L. rhamnosus*, *S. pyogenes*, and *S. mutans*, respectively. Truncated DltB also shows significant similarities with the DltB of other organisms.

**Cloning and expression of the *S. gordonii* DL1 *dltA*.** To determine if a functional *dltA* gene product could be expressed from a cloned streptococcal *dltA*, we used the 60-bp G7 probe to locate the putative *dltA* locus in the fosmid-constructed genomic library of wild-type *S. gordonii* DL1 (Challis). Three fosmids hybridized with the 60-bp G7 probe; one was selected for further study and designated pDC5. Recombinant *E. coli* PK3324 containing the fosmid pDC5 exhibited Dcl activity (6.2 U/mg of protein) which was comparable to that of the cloned *L. rhamnosus dltA* in *E. coli* (11.2 U/mg of protein). These data indicated that the cloned streptococcal *dltA* in pDC5 encodes Dcl.

**Insertional inactivation of the *S. gordonii* DL1 *dltA* with *ermAM*.** To prepare plasmid for insertional inactivation, it was necessary to sequence *dltA*. Fosmid pDC5 was chosen as the template for sequencing the 5′-region flanking the 626-bp fragment described in Fig. 1A. From the sequence two PCR primers were constructed to amplify a fragment containing partial *dltA* (Fig. 1C). This product, which hybridized with the 60-bp G7 probe, was cloned into pBluescript to form pDC8. To insertionally inactivate *dltA*, the 0.9-kb streptococcal erythromycin resistance determinant (*ermAM*) was ligated into the unique *Hin*cII site (Fig. 1C, Hc) within the 1.4-kb fragment of cloned DNA in pDC8 (43, 45), and the resulting plasmid was designated pDC11. By digesting this plasmid with *Sca*I, which cuts the *ermAM* cassette asymmetrically (Fig. 1C), it was shown that the *ermAM* determinant was cloned in the opposite tran-

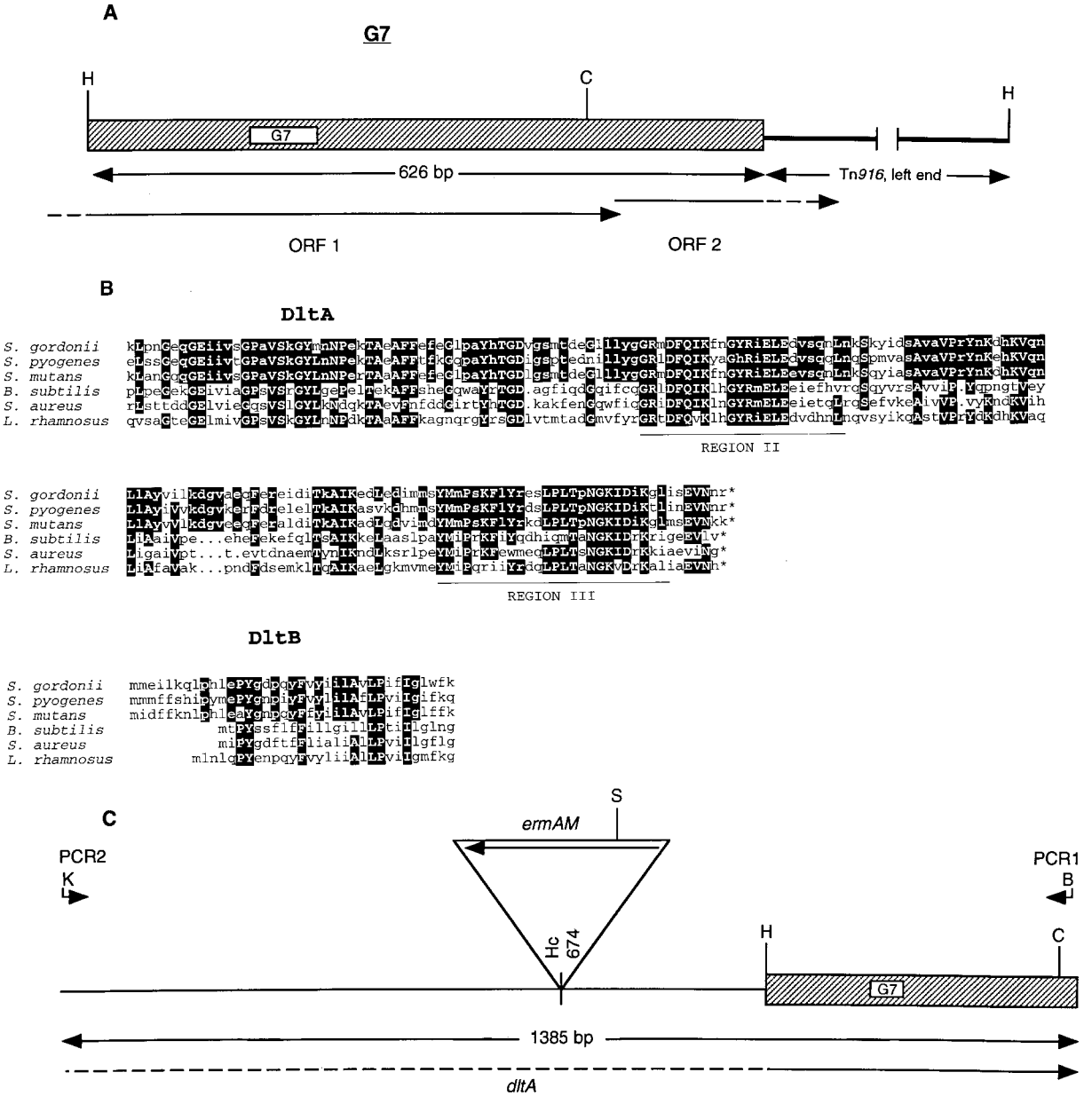


FIG. 1. (A) Partial restriction map of the streptococcal flanking region and the left end of Tn*916* from mutant G7. The location of the 60-bp G7 probe is indicated. Putative transcription orientation is from left to right. Abbreviations of restriction enzyme sites: C, *Cla*I; H, *Hin*dIII. (B) Comparison of deduced amino acid sequence of the 626-bp fragment from *S. gordonii* (panel A) with truncated sequences of DltA and DltB homologs from *L. rhamnosus*, *S. aureus*, *B. subtilis*, *S. mutans*, and *S. pyogenes*. Upper case reverse font indicates amino acid sequence identity in five or more proteins; lower case reverse font indicates identity among all three streptococcal strains. Regions II and III are indicated and are discussed in the text. The 60-bp G7 probe includes the sequence encoding the first 19 amino acids of region II. *S. pyogenes*, contig 320 (51a); *S. mutans*, accession no. AF049357 (55a) accession no. AF051356 (5a); *B. subtilis*, accession no. X73124 (21); *Staphylococcus aureus*, accession no. D86240 (46a) (nucleotide 3939 was changed from A to C to remove false stop codon); *L. rhamnosus*, accession no. U43894 (25). (C) Strategy for obtaining the *ermAM* insertion in the *dltA* gene of *S. gordonii* DL1. The 1,385-bp region was obtained by PCR with primers PCR1 and PCR2 (Materials and Methods). The location of the *Kpn*I (K) and *Bam*HI (B) restriction sites are shown. A portion of panel A is shown for orientation. The *Hinc*II site (Hc) of insertion of the 922-bp *ermAM* cassette is indicated. The transcriptional orientation of the cassette and the *Sca*I site (S) are indicated. The *dltA* fragment was cut with *Kpn*I and *Bam*HI and cloned into *Kpn*I- and *Bam*HI-digested pBluescript IIKS(+) (Stratagene).

scriptional orientation to the *dltA*. Linearized plasmid pDC11 DNA was transformed into *S. gordonii* DL1. Two $Em^r$ isolates, PK3241 and PK3242 (Table 3), unable to coaggregate with streptococcal partners but still able to coaggregate with *A. naeslundii* ATCC 51655, were identified and used for further study.

To confirm that *ermAM* had integrated into the chromosome, DNA was prepared from PK3241 and PK3242 and the wild-type strain DL1 and digested with either *Acc*I or *Sca*I. *Acc*I does not cut within *dltA* or *ermAM*, while *Sca*I yields a single asymmetrical cut within *ermAM* (Fig. 1C). Southern analysis using either the *ermAM* determinant or the 1.4-kb *S. gordonii* DL1 *dltA* homolog as probe revealed that both the *ermAM* and *dltA* probes recognized identical bands after di-

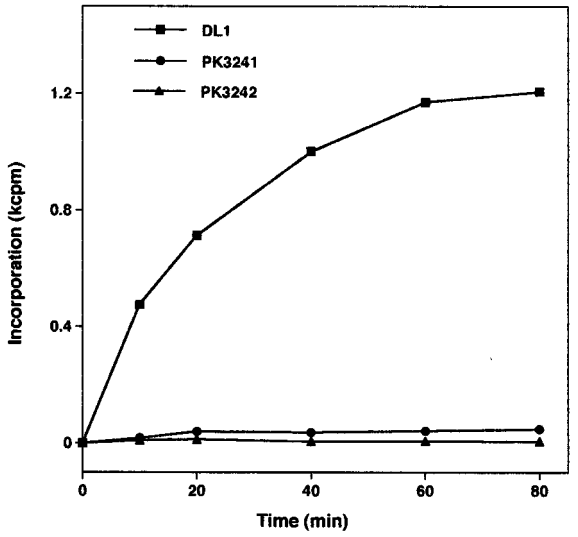


FIG. 2. Incorporation of D-[$^{14}$C]alanine into the parent and mutant strains. The D-alanine incorporation assay (described in Materials and Methods) was used with permeabilized cells of the indicated strains.

TABLE 4. Incorporation of D-alanine into LTA by in vitro combinations of membrane fragments and supernatant fractions from the parent and mutant strains[a]

| Strain | D-[$^{14}$C]alanine incorporation (cpm) | | | Dcl (U/mg)[c] |
|---|---|---|---|---|
| | M + S | $M_m + S_p$ | $M_p + S_m$ | |
| DL1 | 726 | NA[b] | NA | 0.530 |
| PK3241 | 6 | 47 | 52 | 0.065 |
| PK3242 | 21 | 29 | 33 | 0.015 |

[a] Data are averages of two experiments; membranes and supernatant fractions were prepared as described by Ntamere et al. (48). M (membrane fragment), 2.6 μg of protein/μl; $M_p$, membrane fragment preparation from parent strain DL1; $M_m$, membrane fragment preparation from *dltA* mutant strain; S (supernatant fraction), 2.6 μg of protein/μl; $S_p$, supernatant fraction from parent strain DL1; $S_m$, supernatant fraction from *dltA* mutant strain.

[b] NA, not applicable.

[c] Dcl was assayed according to the procedure described by Heaton and Neuhaus (25). One unit of enzyme activity is defined as that amount of enzyme that catalyzes the formation of 1 μmol of D-alanine hydroxamate per h. Specific activity is expressed as the number of units per milligram of protein.

gestion of the genomic DNA of PK3241 and PK3242 (data not shown). When the *dltA* probe was used, the *Sca*I digest of DL1 showed a higher-molecular-size band than found in the mutants, and the *Acc*I-digested DNA showed a lower-molecular-size band in the parent compared to those of the mutants. No hybridization was seen in *S. gordonii* DL1 wild-type genomic DNA blots probed with *ermAM*. These data indicated that *ermAM* integrated into *dltA* in transformants PK3241 and PK3242.

**Characterization of Em$^r$ *S. gordonii* DL1 *dltA* insertion mutants PK3241 and PK3242.** The insertion mutants exhibited an inability to participate in galactoside-inhibitable coaggregation with other streptococci while maintaining strong noninhibitable coaggregations with actinomyces, for example *A. naeslundii* ATCC 51655 (Table 3). The wild-type strain DL1 coaggregated strongly with the streptococcal partners, and these coaggregations were reversed by adding lactose. Also, the growth of the mutants was slower than that of the wild type, with doubling times at 37°C for *S. gordonii* DL1, PK3241, and PK3242 of 51, 96, and 117 min, respectively. Thus, insertional inactivation of *dltA* results both in a loss of galactoside-inhibitable coaggregation and in a slowing of the growth rate compared with that of the parent strain DL1.

The insertion mutants PK3241 and PK3242 were deficient for D-alanine incorporation into LTA (Fig. 2). To define further the defect(s) in the incorporation system of the mutants, we (i) assayed the activity of Dcl in each of the mutant supernatant fractions and (ii) assayed either the supernatant fraction or membrane fraction in the presence of its cognate fraction from DL1 for D-alanine incorporation. No significant D-alanine activation as measured by the hydroxamate assay was observed (Table 4) and thus, it was concluded that Dcl is absent in the mutants. However, further analysis of the membrane fractions revealed that membranes from the mutants were also not able to reconstitute D-alanine incorporation with the supernatant fraction from the parent. This result is most likely the consequence of translational coupling of *dltA* and *dltB* (47); the polar effect of the *dltA* insertion causes a lack of *dltB* expres-

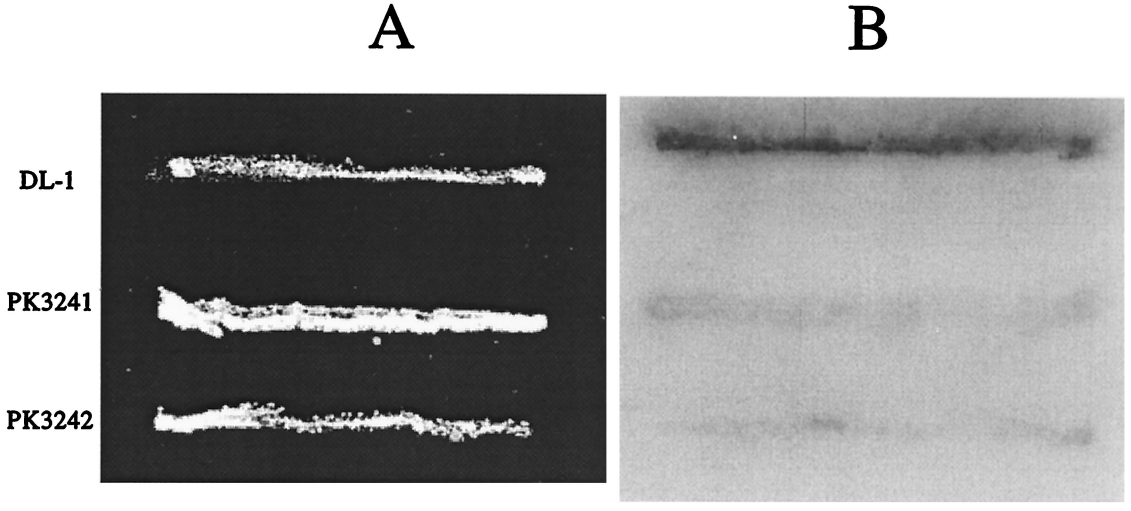


FIG. 3. D-Alanine ester content of *S. gordonii* wild-type DL1 and mutant strains PK3241 and PK3242. As described in the text, a Western blot analysis using serum with antibodies to D-alanyl esters (40) was used to detect the presence of D-alanyl esters. (A) Photograph of plate; (B) colony immunoblot.

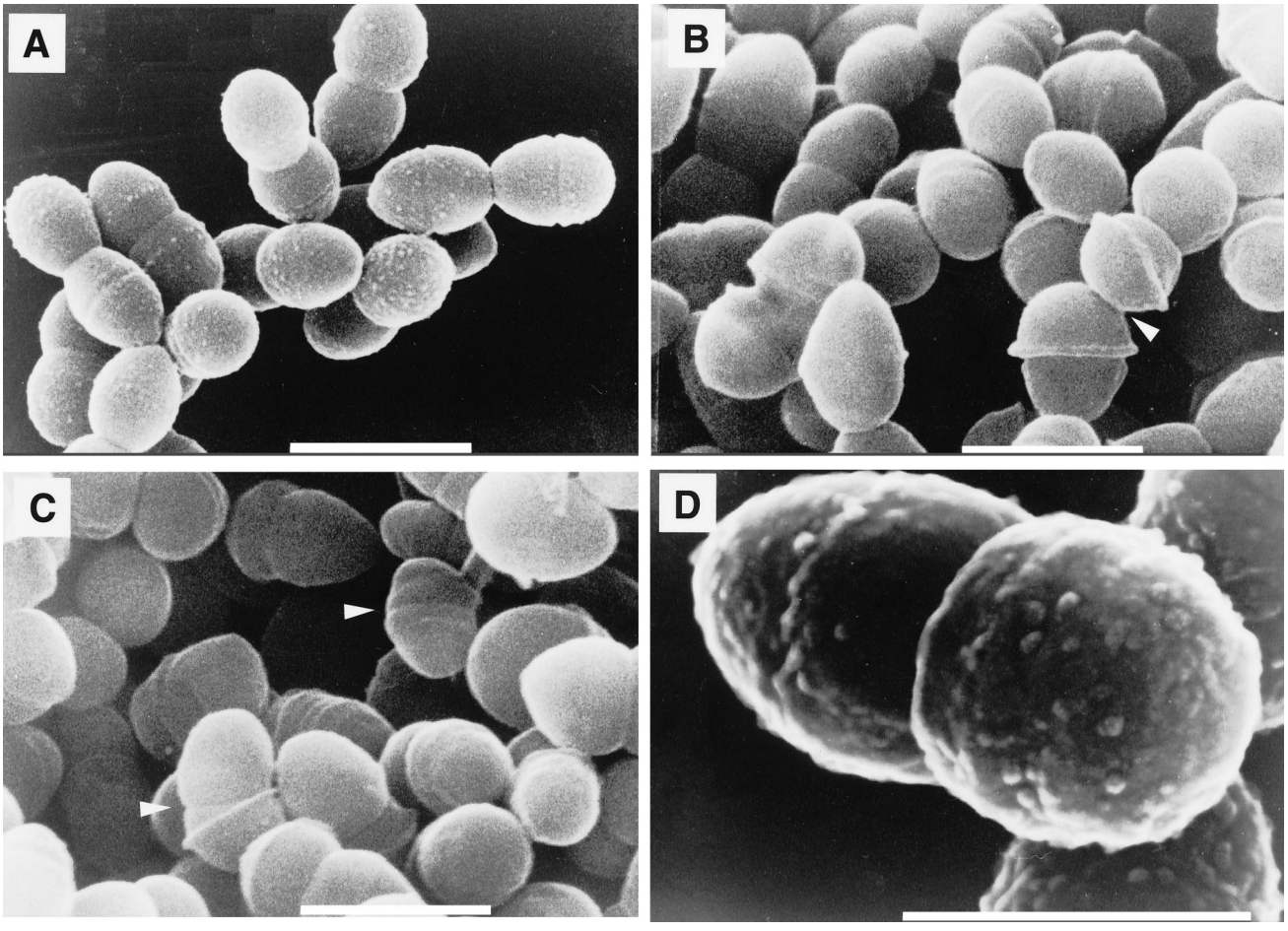


FIG. 4. Scanning electron micrographs of DL1 (A and D) and PK3241 (B and C). The specimens shown in panels A to C were examined with a JEOL JSM-1 microscope. Bar = 1.0 μm. The specimen shown in panel D was examined with a Hitachi S-4500 microscope after fixing and coating with gold-palladium (Materials and Methods). Bar = 0.5 μm. The arrow in panel B indicates nonlinear fission and the arrows in panel C indicate multiseptate pleomorphs.

sion. Since DltB is a putative membrane protein involved in D-alanine incorporation, insertional inactivation of *dltA* could also lead to inactive membranes for D-alanine incorporation.

In accord with the absence of Dcl activity, the mutants have no detectable D-alanyl LTA on their cell surface (Fig. 3). To monitor the presence of D-alanyl esters in the LTA of *S. gordonii* DL1 and the mutant strains, a serum preparation containing antibody to D-alanyl LTA (40) was used. As shown in the immunoblot in Fig. 3, mutants PK3241 and PK3242 are both deficient. The low background in the mutants may reflect the presence of antibody(s) for poly (glycerol phosphate) or other surface antigens.

The morphologies of DL1 and PK3241 were compared in scanning electron micrographs of exponential-phase cells (Fig. 4). The cell surface of the mutant PK3241 (Fig. 4B and C) appears smooth and nonstructured compared with that of the parent DL1 (Fig. 4A and D). In addition, a significant number of the cells appear to be multiseptated (Fig. 4C). In many cases, the septa are not parallel, leading to nonlinear binary fission (Fig. 4B). Multiseptation leads to "dumbbell-like" pleomorphs similar to those observed by Pucci et al. (51) in *Enterococcus hirae* ATCC 9790 (formerly called *Streptococcus faecalis* ATCC 9790) when treated with sublytic concentrations of beta-lactam antibiotics. For a more detailed morphological analysis of PK3241, transmission microscopy of sections was also used (Fig. 5). In the parent DL1, the new septal planes were formed parallel to the cell division plane and occurred at the midpoint of the elongating cells (Fig. 5A). A heavily stained layer was prominent in DL1 but was less obvious in the mutant PK3241 (Fig. 5B to D). The mutant exhibited a thick but uneven surface layer that was lightly stained. The reason for the difference in staining of this layer in the parent and mutant is unknown. In Fig. 5B the nascent divisional septa are not parallel to the completed septum, while the pleomorph in Fig. 5C has three septation sites at approximately the same stage of development. In Fig. 5D, the cells are poorly separated. This range of cellular morphologies as well as the related division-plane locations and geometry leads one to speculate that autolysins may not function properly. Thus, these results illustrate a number of pleiotropic defects resulting from insertional inactivation of *dltA*.

The two insertion mutants were tested for the presence of the putative 100-kDa adhesin that has been linked to mediating intrageneric coaggregations of the wild-type DL1 (9). This was done by immunoblot analysis as described previously (63) by examining the cell surface proteins obtained by mild sonication of cells (Fig. 6). This adhesin (Fig. 6) is present in the wild-type DL1 but is absent in the lanes containing cell surface proteins of the two insertion mutants PK3241 and PK3242. Some differences in the banding patterns of proteins with mo-

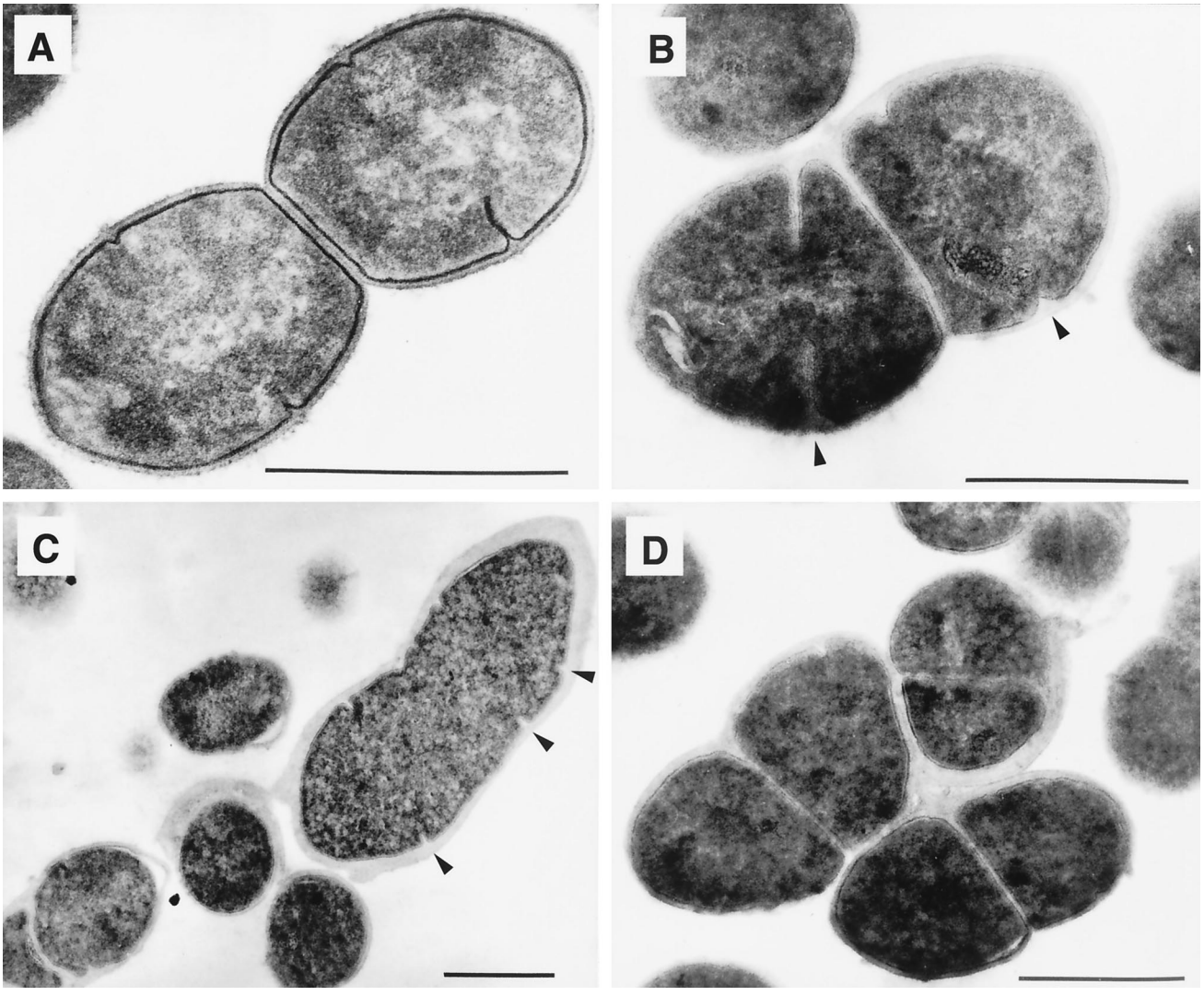


FIG. 5. Aberrant morphology of PK3241. Exponential-phase cells of DL1 (A) and PK3241 (B to D) were fixed, embedded, sectioned, and examined with a JEOL-100CX 11 microscope as described in Materials and Methods. Bars = 0.5 μm.

lecular sizes below 84 kDa are visible. However, only the 100-kDa protein is consistently absent (9, 63) in all mutants unable to exhibit intrageneric coaggregation, including PK3241 and PK3242 studied here. The data show that insertional inactivation of *dltA* results in the loss of this adhesin.

To test the possibility that the mutation in *dltA* may modulate the activity of other surface functions, we compared the ability of the parent and mutants to be transformed with homologous DNA from a spontaneous $Sm^r$ mutant of DL1 (44). The transformation frequency of the parent was $10^{-3}$ per viable cell as expected (41); the transformation frequency of the mutants was 10-fold lower. Given the noticeable difference in cell septation and morphology in the mutants, this reduction may represent changes in the segregation of transformants or possibly a global effect on surface function.

Southern blot analysis of streptococcal genomic DNA digested with *Pst*I and probed with the 1.4-kb *dltA* probe showed the presence of reactive fragments in all streptococci except *S. sanguis* ATCC 10556 and *S. oralis* ATCC 10557 (data not shown). Hybridizing bands of the same size were found for *S. gordonii* DL1, *S. oralis* H1, *S. oralis* 34, *S. milleri* K44Y, *S. rattus* BHT, and *S. salivarius* ATCC 25975. Another cluster of strains showing the same-size reactive fragment included *S. mutans* LM7, *S. pneumoniae* R6, *S. cricetus* AHT, and *S. constellatus* PK2819. Further studies will be necessary to characterize the *dlt* operon structure in these organisms.

## DISCUSSION

This is the first report of a mutation in the *dlt* operon resulting in altered adherence properties. The *dltA* mutants, PK3241 and PK3242, of *S. gordonii* DL1 specifically lost the ability to participate in intrageneric coaggregations while maintaining intergeneric coaggregations. The concomitant loss of the 100-kDa putative adhesin suggests that this protein binds to D-alanyl LTA. LTA has been implicated in the adhesion of a number of gram-positive bacteria to a variety of target cells and surfaces (6, 24, 58). In these examples, the participation of LTA as a direct mediator of adhesion has been postulated. In contrast, the present report describes the function of a putative adhesin which binds to D-alanyl-LTA and is presented to its partner cell. Thus, the D-alanyl esters would ap-

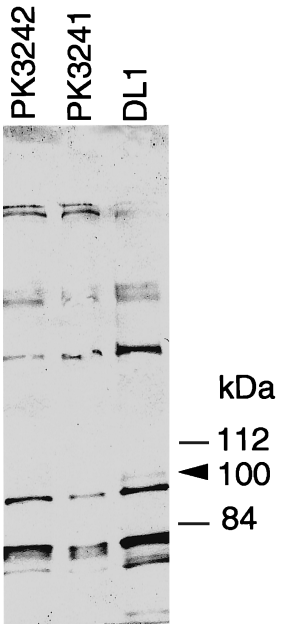


FIG. 6. Immunoblot analysis of sonic surface extracts of PK3241 and PK3242, *ermAM* insertion mutants in *dltA*, compared with wild-type *S. gordonii* DL1. The position of the putative adhesin (arrow) was determined by using prestained molecular weight standards (Bio-Rad). The standards of 112 and 84 kDa are indicated by dashes.

pear to play a role in determining the binding and presentation of this adhesin.

Some cell surface proteins of *S. gordonii* may bind to D-alanyl LTA in a way analogous to the binding of choline-binding proteins of *S. pneumoniae* (54, 65). Among the family of surface-located choline-binding proteins on *S. pneumoniae* is CbpA, a 75-kDa adhesin and virulence determinant. The family of proteins is noncovalently bound to the phosphorylcholine of the wall teichoic acid. By analogy, the 100-kDa putative adhesin that is lacking in the *S. gordonii* DL1 mutants studied here may normally bind to the D-alanine-substituted LTA. In support of a weakly bound protein is the observation that mild sonication of parent DL1 cells is sufficient to remove the 100-kDa protein and render them unable to participate in galactoside-inhibitable coaggregation with streptococci but still capable of other galactoside-noninhibitable coaggregations with actinomyces (data not shown). In this regard, the D-alanyl LTA may act as a scaffolding for presenting the bound 100-kDa protein on *S. gordonii* surface.

Another important class of protein ligands which bind to LTA are autolysins. These proteins are cell wall hydrolases, e.g. MurNAc L-ala amidase, which the bacterium must regulate for growth (11, 29). Gel-permeation chromatography demonstrated the binding of the amidase to LTA (30). In *B. subtilis*, insertional inactivation of the genes in the *dlt* operon results in an increased rate of autolysis (61, 62). The increased negative charge of LTA in the mutants resulting from a decrease in D-alanylation appeared to increase the amount of autolysin (s) bound. While the mechanism of adhesin binding would appear to be different from that of autolysin binding, the conclusion is made that D-alanylation of LTA provides a feature for regulating the ability of LTA to bind selected protein ligands.

Interestingly, many *S. gordonii* and *S. sanguis* strains in biovars that are positive for LTA (the Lancefield group H antigen [52]) also are positive for galactoside-sensitive adhesins detected by intrageneric coaggregation (32, 38). The group H antigen occurs in most strains of *S. gordonii* and *S. sanguis* and not at all in strains of *S. oralis* and *S. mitis* (35). These latter species include strains with GalNAc-containing cell wall polysaccharides, which are the receptors for the galactoside-sensitive adhesins, including the putative 100-kDa adhesin on *S. gordonii* DL1 (32, 38). Significantly, all streptococcal strains that are positive for the group H antigen are negative for GalNAc-containing cell wall polysaccharides and visa versa (7, 32). These findings implicate an association between the galactoside-sensitive 100-kDa putative adhesin and the group H antigen on the streptococcal cell surface. Moreover, the anti-DL1 polyclonal serum absorbed with spontaneous $Cog^-$ mutant PK1897 and used to detect the 100-kDa putative adhesin in DL1 (see Fig. 6) also identifies a 100-kDa protein in other streptococcal strains that also possess the group H antigen (9, 63). This absorbed antiserum does not react with *S. oralis* strains (9). The correlation of the reactivity of both anti-adhesin and anti-LTA sera with the same strains strongly supports the association of 100-kDa putative adhesin with LTA.

Previous results with D-alanine ester-deficient mutants of *L. rhamnosus* showed aberrant morphology and defective cell separation (48). However, these mutants may have resulted from multiple mutations or single mutations with pleiotropic effects. The results with the *S. gordonii* mutants show a correlation between D-alanine ester deficiency resulting from insertional inactivation of *dltA* and aberrant cell morphology, slower growth rate, and defective cell separation. Inactivation of the *dltD* of *L. lactis* also results in a mutant that grows more slowly and forms longer chains than the wild-type strain (15). In contrast to these results, mutations in *dltA-dltD* of *B. subtilis* did not result in changes in cellular morphology, cell growth, basic metabolism, and formation of flagella (61, 62). The only changes correlated with D-alanine ester deficiency in this organism were an enhanced rate of autolysis and a higher susceptibility to methicillin (61). Thus, at this time it cannot be concluded that D-alanine ester deficiency will have the same effect in each gram-positive organism.

A wide range of species exhibited *dltA*-reactive fragments in Southern blots (data not shown). Out of 23 strains tested, all but one strain of *S. sanguis* and one strain of *S. oralis* were positive for *dltA*. The surprising finding was that *S. pneumoniae*, *S. oralis*, and *S. mitis* possess *dltA* but do not have D-alanine esters of LTA. In fact, *S. pneumoniae* has the entire *dlt* operon (3). The expression of the *dlt* operon may be silent or may occur under environmental conditions that have not been tested in the laboratory. *S. oralis* and *S. mitis* do not express detectable amounts of glycerol LTA (28, 35), but *S. oralis* can incorporate radiolabelled choline into membrane components, suggesting that it may produce a choline-containing macroamphiphile similar to that of *S. pneumoniae* (31). Alternatively, these three species may use the *dlt* operon for D-alanylation of a different molecule or macroamphiphile than LTA (8). One possibility has been suggested for the DltA-DltD homologs of *S. mutans* (accession no. AF049357): mutants in genes encoding these proteins accumulate elevated levels of intracellular polysaccharide in the presence of fructose or sucrose (55). When grown in continuous culture, *S. mutans* Ingbritt produced two to six times as much LTA during growth on fructose compared to glucose, depending on the generation times of the cells (33). And, the LTA content of strain Ingbritt increased four- to fivefold when the pH of the culture medium was raised from 5.0 to 7.5, irrespective of the carbon source (33). Thus, environmental factors can greatly influence the amounts of LTA in streptococcal cells. A recent entry into GenBank, accession no. AF051356, reports a potential rela-

tionship of defects in the *dlt* operon to acid sensitivity in *S. mutans* (59). Taken collectively, the properties of adherence, intracellular polysaccharide accumulation, and acid sensitivity all being affected by mutations in the *dlt* operon suggest that the LTA macroamphiphile may act as a supporting matrix or scaffolding for binding of a family of proteins with specific functions for sensing the streptococcal environment.

## ACKNOWLEDGMENTS

D.L.C. was supported by a postdoctoral fellowship awarded by the Warner-Lambert Company. This study was supported by Public Health Service grant R01 GM51623 (F.C.N.) from the National Institute for General Medical Sciences.

We are indebted to E. W. Minner for his generous help in the Electron Microscopy Facility of the Department of Neurobiology and Physiology, Northwestern University. We thank V. Dravid, Material Sciences, for the specimen examined in the Hitachi S-4500 microscope. We thank M. Levine, University of Oklahoma at Oklahoma City Health Sciences Center, for serum samples containing antibody specific for the D-alanyl esters of LTA and for discussions of the use of these sera; H. F. Jenkinson for useful discussions on the preparation and properties of LTA from oral streptococci; E. J. St. Martin for suggesting the novel procedure for permeabilization of *S. gordonii*; J. London, N. Ganeshkumar, and J. Cisar for helpful comments in preparing the manuscript; and R. Andersen for technical assistance.

## REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Andersen, R. N., N. Ganeshkumar, and P. E. Kolenbrander.** 1993. Cloning of the *Streptococcus gordonii* PK488 gene, encoding an adhesin which mediates coaggregation with *Actinomyces naeslundii* PK606. Infect. Immun. **61:** 981–987.
3. **Baltz, R. H., F. H. Norris, P. Matsushima, B. S. DeHoff, P. Rockey, G. Porter, S. Burgett, R. Peery, J. Hoskins, L. Braverman, I. Jenkins, P. Solenberg, M. Young, M. A. McHenney, P. L. Skatrud, and P. R. Rosteck, Jr.** 1998. DNA sequence sampling of the *Streptococcus pneumoniae* genome to identify novel targets for antibiotic development. Microb. Drug Resist. **4:**1–9.
4. **Beachey, E. H., and W. A. Simpson.** 1982. The adherence of group A streptococci to oropharyngeal cells: the lipoteichoic acid adhesin and fibronectin receptor. Infection **10:**107–111.
5. **Behnke, D.** 1981. Plasmid transformation of *Streptococcus sanguis* (Challis) occurs by circular and linear molecules. Mol. Gen. Genet. **183:**490–497.

5a. **Boyd, et al.** Unpublished data.

6. **Ciardi, J. E., J. A. Reilly, R. H. Haller, W. H. Bowen, and G. Rolla.** 1981. The role of lipoteichoic acid in the adherence and colonization of oral streptococci, p. 353–364. *In* G. D. Shockman and A. J. Wicken (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, New York, N.Y.
7. **Cisar, J. O., A. L. Sandberg, G. P. Reddy, C. Abeygunawardana, and C. A. Bush.** 1997. Structural and antigenic types of cell wall polysaccharides from viridans group streptococci with receptors for actinomyces and streptococcal lectins. Infect. Immun. **65:**5035–5041.
8. **Clark, V. L., and F. E. Young.** 1978. D-Alanine incorporation into macromolecules and effects of D-alanine deprivation on active transport in *Bacillus subtilis*. J. Bacteriol. **133:**1339–1350.
9. **Clemans, D. L., and P. E. Kolenbrander.** 1995. Identification of a 100-kDa putative coaggregation-mediating adhesin of *Streptococcus gordonii* DL1 (Challis). Infect. Immun. **63:**4890–4893.
10. **Clemans, D. L., and P. E. Kolenbrander.** 1995. Isolation and characterization of coaggregation-defective ($Cog^-$) mutants of *Streptococcus gordonii* DL1 (Challis). J. Ind. Microbiol. **15:**193–197.
11. **Cleveland, R. F., J.-V. Höltje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman.** 1975. Inhibition of bacterial wall lysis by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. **67:**1128–1135.
12. **Clewell, D. B.** 1993. Sex pheromones and the plasmid-encoded mating response in *Enterococcus faecalis*, p. 349–368. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York, N.Y.
13. **Debabov, D. V., M. P. Heaton, Q. Zhang, K. D. Stewart, R. H. Lambalot, and F. C. Neuhaus.** 1996. The D-alanyl carrier protein in *Lactobacillus casei*: cloning, sequencing, and expression of *dltC*. J. Bacteriol. **178:**3869–3876.
14. **Dunny, G. M., B. A. B. Leonard, and P. J. Hedberg.** 1995. Pheromone-inducible conjugation in *Enterococcus faecalis*: interbacterial and host-parasite chemical communication. J Bacteriol. **177:**871–876.
15. **Duwat, P., A. Cochu, S. D. Ehrlich, and A. Gruss.** 1997. Characterization of *Lactococcus lactis* UV-sensitive mutants obtained by IS*S*1 transposition. J. Bacteriol. **179:**4473–4479.
16. **Ehrenfeld, E. E., R. E. Kessler, and D. B. Clewell.** 1986. Identification of pheromone-induced surface proteins in *Streptococcus faecalis* and evidence of a role for lipoteichoic acid in formation of mating aggregates. J. Bacteriol. **168:**6–12.
17. **Fischer, W.** 1994. Lipoteichoic acids and lipoglycans, p. 199–215. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science B. V., London, United Kingdom.
18. **Fischer, W., and H. U. Koch.** 1981. Alanine ester substitution and its effect on the biological properties of lipoteichoic acids, p. 181–194. *In* G. D. Shockman and A. J. Wicken (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, Inc., New York, N.Y.
19. **Gawron-Burke, C., and D. B. Clewell.** 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escherichia coli*: strategy for targeting and cloning of genes from gram-positive bacteria. J. Bacteriol. **159:**214–221.
20. **Genetics Computer Group.** 1991. Program manual for the GCG package, version 7. Madison, Wis.
21. **Glaser, P., F. Kunst, M. Arnaud, M.-P. Coudart, W. Gonzales, M.-F. Hullo, M. Ionescu, B. Lubochinsky, L. Marcelino, I. Moszer, E. Presecan, M. Santana, E. Schneider, J. Schweizer, A. Vertes, G. Rapoport, and A. Danchin.** 1993. *Bacillus subtilis* genome project: cloning and sequencing of the 97 kb region from 325° to 333°. Mol. Microbiol. **10:**371–384.
22. **Gutmann, L., S. Al-Obeid, D. Billot-Klein, E. Ebnet, and W. Fischer.** 1996. Penicillin tolerance and modification of lipoteichoic acid associated with expression of vancomycin resistance in VanB-type *Enterococcus faecium* D366. Antimicrob. Agents Chemother. **40:**257–259.
23. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
24. **Hasty, D. L., I. Ofek, H. S. Courtney, and R. J. Doyle.** 1992. Multiple adhesins of streptococci. Infect. Immun. **60:**2147–2152.
25. **Heaton, M. P., and F. C. Neuhaus.** 1992. Biosynthesis of D-alanyl lipoteichoic acid: cloning, nucleotide sequence, and gene expression of the *Lactobacillus casei* gene for D-alanine-activating enzyme. J. Bacteriol. **174:**4707–4717.
26. **Heaton, M. P., and F. C. Neuhaus.** 1994. Role of D-alanyl carrier protein in the biosynthesis of D-alanyl-lipoteichoic acid. J. Bacteriol. **176:**681–690.
27. **Hogg, S. D., and J. E. Manning.** 1988. Inhibition of adhesion of viridans streptococci to fibronectin-coated hydroxyapatite beads by lipoteichoic acid. J. Appl. Bacteriol. **65:**483–489.
28. **Hogg, S. D., R. A. Whiley, and J. J. DeSoet.** 1997. Occurrence of lipoteichoic acid in oral streptococci. Int. J. Syst. Bacteriol. **47:**62–66.
29. **Höltje, J.-V., and A. Tomasz.** 1975. Lipoteichoic acids: a specific inhibitor of autolysin activity in pneumococci. Proc. Natl. Acad. Sci. USA **72:**1690–1694.
30. **Horne, D., and A. Tomasz.** 1985. Pneumococcal Forssman antigen: enrichment in mesosomal membranes and specific binding to the autolytic enzyme of *Streptococcus pneumoniae*. J. Bacteriol. **161:**18–24.
31. **Horne, D. S., and A. Tomasz.** 1993. Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*. J. Bacteriol. **175:**1717–1722.
32. **Hsu, S. D., J. O. Cisar, A. L. Sandberg, and M. Kilian.** 1994. Adhesive properties of viridans streptococcal species. Microb. Ecol. Health Dis. **7:**125–137.
33. **Jacques, N. A., L. Hardy, L. K. Campbell, K. W. Knox, J. D. Evans, and A. J. Wicken.** 1979. Effect of carbohydrate source and growth conditions on the production of lipoteichoic acid by *Streptococcus mutans* Ingbritt. Infect. Immun. **26:**1079–1087.
34. **Kellenberger, E., J. Sechaud, and B. Blondel.** 1972. A micromethod for the embedding of small amounts of cells from liquid suspension. J. Ultrastruct. Res. **39:**606–607.
35. **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.
36. **Kim, U.-J., H. Shizuya, P. J. de Jong, B. Birren, and M. I. Simon.** 1992. Stable propagation of cosmid sized human DNA inserts in an F-factor based vector. Nucleic Acids Res. **20:**1083–1085.
37. **Kolenbrander, P. E.** 1982. Isolation and characterization of coaggregation-defective mutants of *Actinomyces viscosus*, *Actinomyces naeslundii*, and *Streptococcus sanguis*. Infect Immun. **37:**1200–1208.
38. **Kolenbrander, P. E., R. N. Andersen, and L. V. H. Moore.** 1990. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. Appl. Environ. Microbiol. **56:**3890–3894.
39. **LeBlanc, D. J., and F. P. Hassell.** 1976. Transformation of *Streptococcus sanguis* Challis by plasmid deoxyribonucleic acid from *Streptococcus faecalis*. J. Bacteriol. **128:**347–355.
40. **Levine, M.** 1982. Naturally occurring human serum precipitins specific for D-alanyl esters of glycerol teichoic acid. Mol. Immunol. **19:**133–142.
41. **Lunsford, R. D.** 1998. Streptococcal transformation: essential features and applications of a natural gene exchange system. Plasmid **39:**10–20.
42. **Lunsford, R. D.** 1995. A Tn*4001* delivery system for *Streptococcus gordonii* (Challis). Plasmid **33:**153–157.

43. **Lunsford, R. D., and J. London.** 1996. Natural genetic transformation in *Streptococcus gordonii*: *comX* imparts spontaneous competence on strain Wicky. J. Bacteriol. **178:**5831–5835.
44. **Lunsford, R. D., and A. G. Roble.** 1997. *comYA*, a gene similar to *comGA* of *Bacillus subtilis*, is essential for competence-factor-dependent DNA transformation in *Streptococcus gordonii*. J. Bacteriol. **179:**3122–3126.
45. **Martin, B., G. Alloing, V. Mejean, and J.-P. Claverys.** 1987. Constitutive expression of erythromycin resistance mediated by the *erm*AM determinant of pAMβ1 results from deletion of the 5′ leader peptide sequences. Plasmid **18:**250–253.
46. **McIntire, F. C., A. E. Vatter, J. Baros, and J. Arnold.** 1978. Mechanism of coaggregation between *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34. Infect. Immun. **21:**978–988.
46a. **Nakao, A., S. Imai, and T. Takano.** Unpublished data.
47. **Neuhaus, F. C., M. P. Heaton, D. V. Debabov, and Q. Zhang.** 1996. The *dlt* operon in the biosynthesis of D-alanyl lipoteichoic acid in *Lactobacillus casei*. Microb. Drug Resist. **2:**77–84.
48. **Ntamere, A. S., D. J. Taron, and F. C. Neuhaus.** 1987. Assembly of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: mutants deficient in the D-alanyl ester content of this amphiphile. J. Bacteriol. **169:**1702–1711.
49. **O'Brien, M. J., S. A. Kuhl, and M. J. Starzyk.** 1995. Correlation of teichoic acid D-alanyl esterification with the expression of methicillin resistance in *Staphylococcus aureus*. Microbios **83:**119–137.
50. **Perego, M., P. Glaser, A. Minutello, M. A. Strauch, K. Leopold, and W. Fischer.** 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. J. Biol. Chem. **270:**15598–15606.
51. **Pucci, M. J., E. T. Hinks, D. T. Dicker, M. L. Higgins, and L. Daneo-Moore.** 1986. Inhibition by β-lactam antibiotics at two different times in the cell cycle of *Streptococcus faecium* ATCC 9790. J. Bacteriol. **165:**682–688.
51a. **Roe, B. A.** Contig library. [Online.] http://dna1.chem.uokhnor.edu/strep.html. [2 December 1996, last date accessed.]
52. **Rosan, B., and L. Argenbright.** 1982. Antigenic determinant of the Lancefield group H antigen of *Streptococcus sanguis*. Infect. Immun. **38:**925–931.
53. **Rose, R. K., and S. D. Hogg.** 1995. Competitive binding of calcium and magnesium to streptococcal lipoteichoic acid. Biochim. Biophys Acta. **1245:** 94–98.
54. **Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortquist, and H. R. Masure.** 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol. Microbiol. **25:**819–829.
55. **Spatafora, G., K. Rohrer, D. Barnard, and S. Michalek.** 1995. A *Streptococcus mutans* mutant that synthesizes elevated levels of intracellular polysaccharide is hypercariogenic in vivo. Infect. Immun. **63:**2556–2563.
55a. **Spatafora, G. A., M. Sheets, R. June, D. Luyimbazi, K. Howard, R. Holbert, D. Barnard, M. El Janne, and M. C. Hudson.** 1999. Regulated expression of the *Streptococcus mutans dlt* genes correlates with intracellular polysaccharide accumulation. J. Bacteriol. **181:**2363–2372.
56. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31–43.
57. **St. Martin, E. J., and C. L. Wittenberger.** 1979. Characterization of a phosphoenolpyruvate-dependent sucrose phosphotransferase system in *Streptococcus mutans*. Infect. Immun. **24:**865–868.
58. **Sutcliffe, I. C., and N. Shaw.** 1991. Atypical lipoteichoic acids of gram-positive bacteria. J. Bacteriol. **173:**7065–7069.
59. **Svensäter, G., U.-B. Larsson, E. C. G. Greif, D. G. Cvitkovitch, and I. R. Hamilton.** 1997. Acid tolerance response and survival by oral bacteria. Oral Microbiol. Immunol. **12:**266–273.
60. **Vickerman, M. M., and G. W. Jones.** 1992. Adhesion of glucosyltransferase phase variants to *Streptococcus gordonii* bacterium-glucan substrata may involve lipoteichoic acid. Infect. Immun. **60:**4301–4308.
61. **Wecke, J., K. Madela, and W. Fischer.** 1997. The absence of D-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*. Microbiology **143:**2953–2960.
62. **Wecke, J., M. Perego, and W. Fischer.** 1996. D-Alanine deprivation of *Bacillus subtilis* teichoic acids is without effect on cell growth and morphology but affects the autolytic activity. Microb. Drug Resist. **2:**123–129.
63. **Whittaker, C. J., D. L. Clemans, and P. E. Kolenbrander.** 1996. Insertional inactivation of an intrageneric coaggregation-relevant adhesin locus from *Streptococcus gordonii* DL1 (Challis). Infect. Immun. **64:**4137–4142.
64. **Whittaker, C. J., C. M. Klier, and P. E. Kolenbrander.** 1996. Mechanisms of adhesion by oral bacteria. Annu. Rev. Microbiol. **50:**513–552.
65. **Yother, J., and J. M. White.** 1994. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. J. Bacteriol. **176:**2976–2985.

*Editor:* V. A. Fischetti