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Biofilm Growth and Detachment of *Actinobacillus actinomycetemcomitans*

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The gram-negative, oral bacterium *Actinobacillus actinomycetemcomitans* has been implicated as the causative agent of several forms of periodontal disease in humans. When cultured in broth, fresh clinical isolates of *A. actinomycetemcomitans* form tenacious biofilms on surfaces such as glass, plastic, and saliva-coated hydroxyapatite, a property that probably plays an important role in the ability of this bacterium to colonize the oral cavity and cause disease. We examined the morphology of *A. actinomycetemcomitans* biofilm colonies grown on glass slides and in polystyrene petri dishes by using light microscopy and scanning and transmission electron microscopy. We found that *A. actinomycetemcomitans* developed asymmetric, lobed biofilm colonies that displayed complex architectural features, including a layer of densely packed cells on the outside of the colony and nonaggregated cells and large, transparent cavities on the inside of the colony. Mature biofilm colonies released single cells or small clusters of cells into the medium. These released cells adhered to the surface of the culture vessel and formed new colonies, enabling the biofilm to spread. We isolated three transposon insertion mutants which produced biofilm colonies that lacked internal, nonaggregated cells and were unable to release cells into the medium. All three transposon insertions mapped to genes required for the synthesis of the O polysaccharide (O-PS) component of lipopolysaccharide. Plasmids carrying the complementary wild-type genes restored the ability of mutant strains to synthesize O-PS and release cells into the medium. Our findings suggest that *A. actinomycetemcomitans* biofilm growth and detachment are discrete processes and that biofilm cell detachment evidently involves the formation of nonaggregated cells inside the biofilm colony that are destined for release from the colony.

Actinobacillus actinomycetemcomitans is a gram-negative, nonmotile coccobacillus that colonizes the human oral cavity (20). *A. actinomycetemcomitans* has been implicated as the causative agent of several forms of severe periodontal disease, including localized juvenile periodontitis, early-onset periodontitis, and rapidly progressive periodontitis (37). Infrequently, *A. actinomycetemcomitans* can enter the submucosa and cause nonoral infections, including bacteremias, infective endocarditis, and localized abscesses (17). When cultured in broth, fresh clinical isolates of *A. actinomycetemcomitans* form extremely tenacious biofilms on surfaces such as glass, plastic, and saliva-coated hydroxyapatite (7, 8, 11, 13–16, 20, 29, 30). Nearly all of the cells grow attached to the surface, while the broth remains clear and is often sterile (7). The dense biofilm that forms on the surface is resistant to removal by agents such as detergents, proteases, heat, sonication, and vortex agitation (7) and exhibits increased resistance to antimicrobial agents compared with that exhibited by cells grown in planktonic form (6). Tight adherence has been shown to play an important role in the ability of *A. actinomycetemcomitans* to colonize the mouths of rats (9) and probably plays an equally important role in its ability to colonize humans. Tenacious surface attachment is dependent on the presence of long, bundled adhesive pili (fimbriae) that form on the surface of the cell (29). Mutations in *flp-1*, which encodes the major fimbrial protein subunit, result in cells that fail to produce fimbriae or attach to surfaces (15).

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Kaplan and Fine have recently shown that *A. actinomycetemcomitans* biofilm colonies are capable of releasing single cells or small clusters of cells into liquid medium and that these released cells can attach to the surface of the culture vessel and form new biofilm colonies, enabling the biofilm to spread (18). In the present report, we describe the morphology of *A. actinomycetemcomitans* biofilm colonies that were grown attached to glass and plastic surfaces and the dynamics of biofilm cell detachment. Our findings suggest that *A. actinomycetemcomitans* utilizes a novel mechanism of biofilm cell detachment that may involve the release of cells from inside the biofilm colony.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *A. actinomycetemcomitans* strains used in this study are listed in Table 1. Mutagenesis of strain CU1000N with transposon IS*903*φ*kan* was carried out as previously described (19). Bacteria were grown in Trypticase soy broth (BD Biosystems) supplemented with 6 g of yeast extract and 8 g of glucose/liter at 37°C in 10% CO_2. Single-cell suspensions were prepared as previously described (18). Culture vessels were 35- or 100-mm-diameter tissue culture-treated polystyrene petri dishes (Corning no. 340165 and Falcon no. 353003, respectively). Bacteria for scanning electron microscopy were grown on 20- by 25-mm pieces of acid-washed borosilicate glass slides (Fisher) placed in 35-mm-diameter petri dishes. Four milliliters of medium was used in 35-mm-diameter dishes, and 20 ml was used in 100-mm-diameter dishes.

Scanning electron microscopy. Biofilm colonies grown on glass slides were washed once with phosphate-buffered saline and fixed in 2% (vol/vol) cold (4°C) freshly prepared glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) overnight. Colonies were postfixed in 1% cold osmium tetroxide for 2 h. The glutaraldehyde was removed by vacuum aspiration, and the colonies were washed once with phosphate-buffered saline and then dehydrated through a graded acetone series. Samples were critical point dried, mounted on copper stubs, and vacuum coated in a Polaron sputter-coating unit (E5000) with approximately 10-nm-thick Au-Pd deposited from a circular target mounted 3 cm from the sample. Samples were examined in a JEOL 25S scanning electron microscope operating at 15 kV and photographed on type 665 positive-negative Land film.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference
A. actinomycetemcomitans strains		
CU1000	Wild-type clinical isolate	8
CU1000N	Spontaneous nalidixic acid-resistant mutant of CU1000	14
JK1002	CU1000N *wzt*::IS*903*φ*kan;* Km^r	19
JK1017	CU1000N *rmlA*::IS*903*φ*kan;* Km^r	This study
JK1022	CU1000N *dspA*::IS*903*φ*kan;* Km^r	19
Plasmids		
pJAK16	Broad-host-range, mobilizable IncQ vector; Cm^r; *tac* promoter, *lacI*^{q}	32
pJK595	pJAK16 with *wzt* of CU1000 expressed from *tac* promoter	This study
pJK596	pJAK16 with *dspA* of CU1000 expressed from *tac* promoter	This study
pJK597	pJAK16 with *rmlA* of CU1000 expressed from *tac* promoter	This study

[a] Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

Thin sectioning. Biofilm colonies grown in polystyrene petri dishes were washed and fixed as described above. After dehydration through graded ethanol, the cells were removed from the dishes by using propylene oxide. The floating biofilm layers were washed in several changes of propylene oxide, pelleted by low-speed centrifugation, and embedded in Epon 812. One-micron-thick and ultrathin sections were prepared on an LKB III ultramicrotome.

Light microscopy. One-micron-thick sections were stained in 1% toluidine blue-borax, coverslipped, and examined with an Olympus IMT inverted microscope. Light photomicrographs were taken with an Olympus DP10 digital camera or with a Polaroid MP4 system using type 667 film.

Transmission electron microscopy. Ultrathin sections were stained with uranyl acetate and lead acetate and examined in a Philips 300 transmission electron microscope operating at 60 kV. Photographs were made on Kodak electron imaging film with type 4463 photographic emulsion.

Ninety-six-well microtiter plate biofilm detachment assay. Biofilm colonies were grown on polystyrene rods suspended in broth in the wells of a 96-well microtiter plate. Cells that detached from the biofilms fell to the bottom of the well, where they attached to the surface and formed new biofilm colonies. The amount of biofilm growth on the bottom of the well, which was proportional to the number of cells that detached from biofilm colonies on the rods, was measured by staining with crystal violet. The detachment assay was carried out as follows.

(i) Construction of apparatus. The lid of a 96-well polystyrene flat-bottomed tissue culture plate (Falcon no. 353072) was modified as follows. First, 96 1.5-mm-diameter holes were drilled in the lid, with the position of each hole corresponding to the center of one of the 96 wells. Then, an 11-mm-long polystyrene rod (1.5-mm-diameter; Plastruct Corp., City of Industry, Calif.) was placed in each hole (with one end of the rod flush against the top of the lid) and secured with trichloromethane plastic solvent. When this modified lid was placed on a 96-well microtiter plate bottom, the rods were suspended in the wells, with the bottom of each rod being approximately 2 mm above the bottom of the well. The modified lid was sterilized by soaking in 70% ethanol for 30 min and air drying in a biological safety cabinet.

(ii) Inoculation and incubation of polystyrene rods. The microtiter plate bottom was filled with medium (100 μl per well), and each well was inoculated with a single 2- to 3-day-old colony from an agar plate by using a sterile toothpick. The modified lid was then placed on the inoculated plate to submerge the polystyrene rods in the inoculated medium, and the plate was incubated for 24 h to allow bacteria to adhere to the rods. The lid was then transferred to a fresh microtiter plate containing prewarmed medium and incubated for an additional 24 h to allow biofilm cells to detach from the rods.

(iii) Measuring detached cells. The lid was removed, and the plate was washed extensively under running tap water to remove loosely adherent cells. The wells were filled with 100 μl of Gram-staining reagent (2 g of crystal violet, 0.8 g of ammonium oxalate, 20 ml of ethanol per 100 ml), and the plate was incubated at room temperature for 10 min. The plate was rewashed extensively under running tap water to remove unbound dye. The wells were then filled with 100 μl of ethanol, and the plate was incubated at room temperature for 10 min to solubilize the dye. The optical density (at 590 nm) of the ethanol-dye solution in each well was measured by using a Bio-Rad Benchmark microplate reader.

Plasmids and DNA techniques. The plasmids utilized in this study are listed in Table 1. PCRs, plasmid DNA manipulations, and DNA sequence analyses were carried out as previously described (19). Plasmids containing wild-type *wzt*, *dspA* (dispersal; formerly ORFf1), and *rmlA* genes (pJK595, pJK596 and pJK597, respectively) for use in genetic complementation experiments were constructed as follows. First, the *wzt*, *dspA*, and *rmlA* genes from *A. actinomycetemcomitans* strain CU1000 (19) were amplified by PCR with primers that introduced a *Kpn*I restriction site 14 bp upstream from the ATG initiation codon of each gene and a *Bam*HI restriction site 1 bp downstream from the stop codon of each gene. PCR products were digested with *Kpn*I and *Bam*HI and ligated into the *Kpn*I and *Bam*HI restriction sites of the broad-host-range plasmid vector pJAK16 (14), which placed each gene under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter. The resulting plasmids were subjected to DNA sequence analysis to confirm the sequence of the insert. Complementing plasmids were mobilized into mutant strains by using the RK2 *oriT*-defective mutant plasmid pRK21761 as previously described (32). Plasmid-harboring strains were grown in broth supplemented with 3 μg of chloramphenicol/ml and 1 mM IPTG.

RESULTS

Microscopic analysis of *A. actinomycetemcomitans* biofilm colonies. Fig. 1 shows light and electron microscopic analyses of *A. actinomycetemcomitans* biofilm colonies grown from single-cell suspensions inoculated onto borosilicate glass slides and into tissue culture-treated polystyrene petri dishes. The *A. actinomycetemcomitans* biofilm colony morphologies (as determined by light microscopy at a magnification of ×40) and biofilm detachment phenotypes (see below) on these two surfaces, as well as those of colonies grown on untreated polystyrene and polycarbonate surfaces, were indistinguishable (data not shown). The photographs shown in Fig. 1 are representative of approximately 5 to 25 biofilm colonies examined for each sample. All of the colonies examined displayed morphologies that were indistinguishable from those shown in Fig. 1.

Figure 1A to D shows scanning electron micrographs of *A. actinomycetemcomitans* strain CU1000 biofilm colonies that were grown attached to glass slides. Very young, umbonate colonies (Fig. 1A) became domed or ovoid by day 1 (Fig. 1B) and distended and lobate by day 2 (Fig. 1C). By days 3 and 4, the surface of the colony was covered with a complex series of lobes and invaginations (Fig. 1D). Thin sections showed that day 1 colonies contained densely packed cells (Fig. 1E). By day 2, colonies had developed a dark-staining outer layer consisting of densely packed cells, a light-staining interior consisting of loosely packed, nonaggregated cells, and large, transparent internal cavities (lacunae) that formed near the outer surface of the colony (Fig. 1F). By days 3 and 4, the colonies were highly invaginated and contained lacunae both in the interior

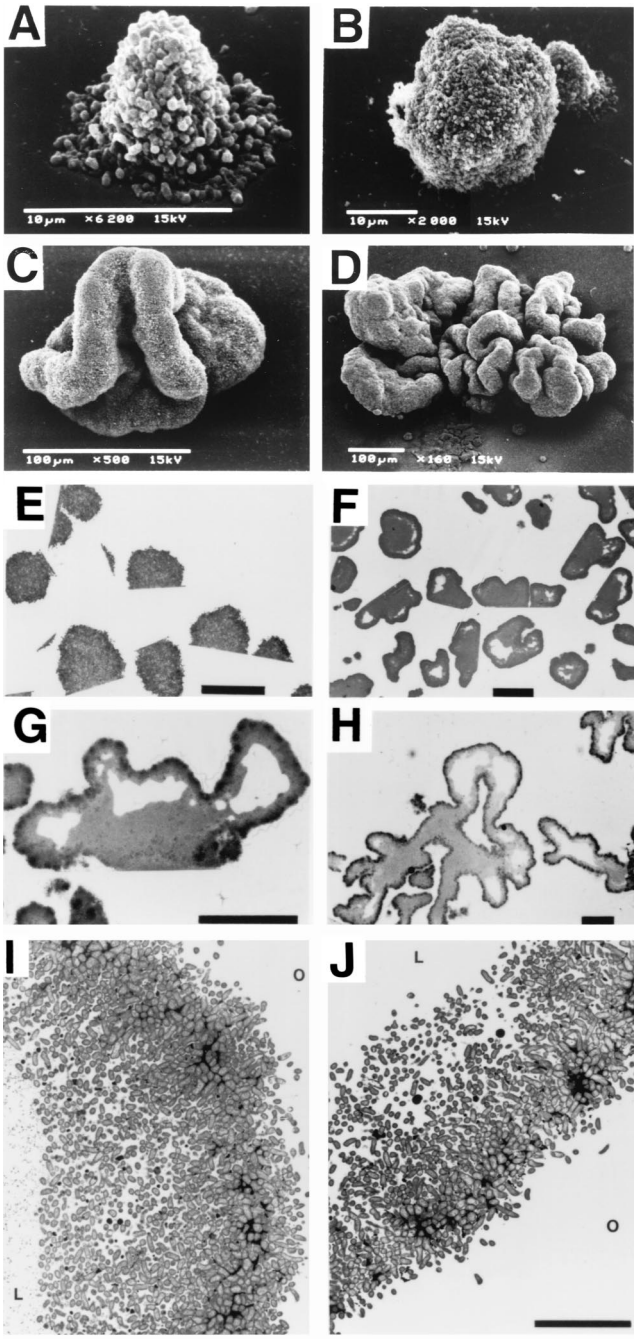

FIG. 1. Microscopic analyses of *A. actinomycetemcomitans* CU1000 biofilm colonies. (A to D) Scanning electron micrographs of day 0.5 (A), day 1 (B), day 2 (C), and day 4 (D) colonies grown on glass slides. (E to H) Thin sections of day 1 (E), day 2 (F), day 3 (G), and day 4 (H) colonies grown on polystyrene. Panels E and F show multiple biofilm colonies. (I and J) Transmission electron micrographs of the outer surface of day 3 (I) and day 4 (J) colonies grown on polystyrene. O, outside of the colony; L, the internal lacuna. Bars = 25 μm (E), 100 μm (F to H), and 10 μm (J).

of the colony and in the exterior lobes, and these lacunae occupied a large volume of the colony (Fig. 1G and H). Transmission electron micrographs confirmed that cells on the outer surface of the colony were densely packed, whereas cells located in the interior of the colony were loosely packed and nonaggregated (Fig. 1I and J). The cellular ultrastructure of strain CU1000 revealed by transmission electron microscopy was similar to that of other strains of *A. actinomycetemcomitans* (1, 12, 21).

Dispersal of *A. actinomycetemcomitans* biofilm colonies grown in polystyrene petri dishes. Figure 2A shows the growth over 3 days of a single *A. actinomycetemcomitans* biofilm colony in a polystyrene petri dish containing broth. By day 3, numerous small satellite colonies were growing on the surface of the dish. Satellite colonies covered the entire surface of the dish and were not visibly attached to mature biofilm colonies or to each other. The number of mature biofilm colonies growing on the surface of each dish was the same as the number of colonies that grew on an agar plate inoculated with the same inoculum, indicating that satellite colonies arose from cells released by mature biofilm colonies into the medium and not from slow settlers from the planktonic phase. Figure 2B shows a 100-mm-diameter petri dish inoculated with 1 CFU of strain CU1000 and incubated for 3 days. Approximately 10^5 satellite colonies grew in a localized area adjacent to the mature colony. Figure 2C shows an electron micrograph of the surface of a glass slide in an area approximately 5 mm away from a mature colony after 2 days of growth. The surface was covered with numerous single cells and small clusters of cells, suggesting that *A. actinomycetemcomitans* biofilm colonies released small units of adherent cells into the medium.

Isolation of *A. actinomycetemcomitans* biofilm dispersal mutants. Colonies of *A. actinomycetemcomitans* grown on nutrient agar plates exhibit a complex morphology characterized by a rough surface texture, an irregular edge, a star-shaped internal structure, and invasive growth and pitting of the agar surface (2, 8, 11, 13, 20) (Fig. 3A, left panel). We reasoned that some components of the *A. actinomycetemcomitans* colony morphology on agar might reflect components of the biofilm colony morphology observed in broth and that mutants that exhibit gross alterations in their colony morphology on agar may exhibit corresponding alterations in their biofilm colony morphology and may therefore be good candidates for biofilm developmental mutants. We used transposon IS*903*φ*kan* (32), which carries a cryptic kanamycin resistance (Km^r) gene that is expressed only after successful transposition into an actively transcribed gene, to mutagenize *A. actinomycetemcomitans* strain CU1000N (14), a spontaneous nalidixic acid-resistant variant of strain CU1000 that exhibits the same surface attachment, biofilm formation, and biofilm dispersal phenotypes as the parent strain (15) (see below). We selected three Km^r mutants (out of ≈1,000) that exhibited a colony morphology on agar that was rougher than the wild-type *A. actinomycetemcomitans* rough-colony phenotype (Fig. 3A, right panel). Figure 3B shows the biofilm colony morphology and dispersal phenotype in broth for strain CU1000N (left panel) and for one of these rough-colony mutants (JK1017; right panel). Strain JK1017 formed tightly adherent biofilm colonies that were similar in appearance to those of strain CU1000N but which failed to disperse in broth. Figure 3C shows 100-mm-diameter petri dishes inoculated with a small inoculum of strain CU1000N (left panel) and of mutant strain JK1017 (right panel). In the petri dish inoculated with strain CU1000N, satellite colonies appeared as a haze that covers the surface of the dish. This haze was absent from the dish inoculated with strain JK1017.

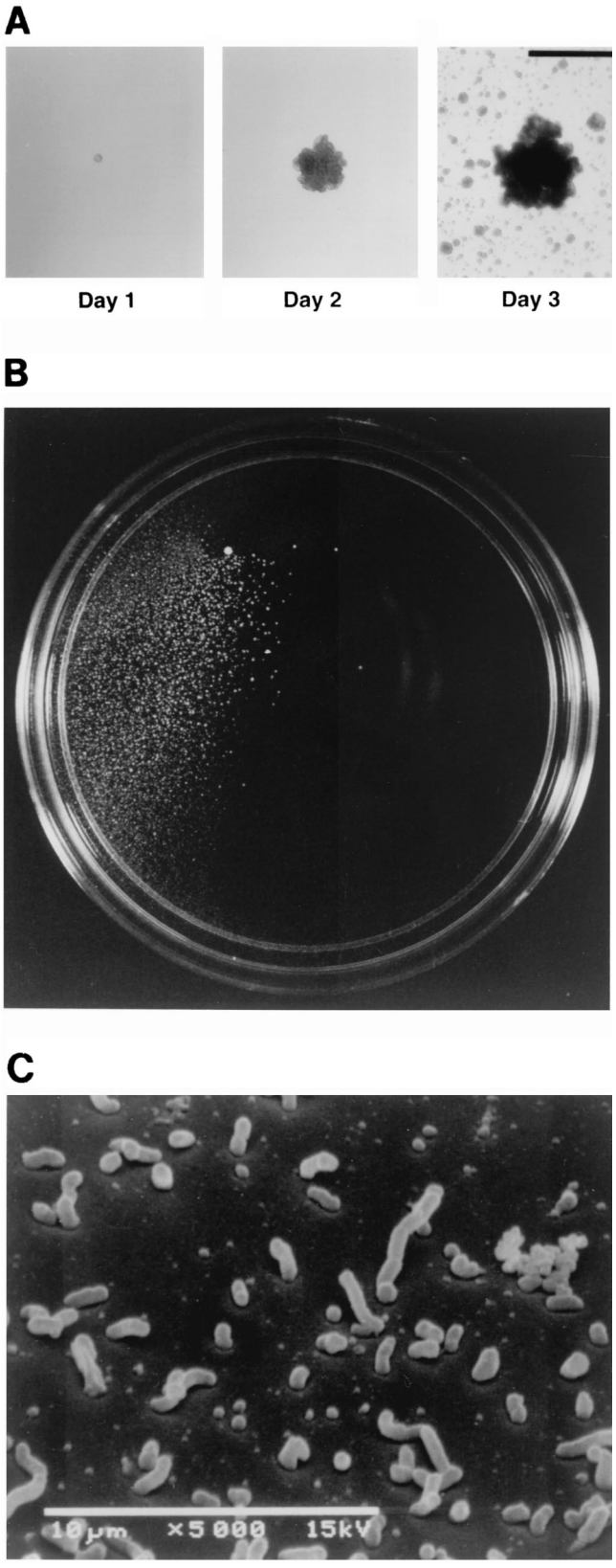

FIG. 2. Dispersal of *A. actinomycetemcomitans* CU1000 biofilm colonies in broth cultures. (A) Pictures, taken 1, 2, and 3 days after inoculation, of a single colony from a 35-mm-diameter petri dish inoculated with ≈25 CFU. Bar = 1 mm. (B) A 100-mm-diameter petri dish inoculated with 1 CFU of strain CU1000 and incubated for 3 days. Colonies appear as white spots on a dark background. (C) Scanning electron micrograph of the surface of a glass slide approximately 5 mm away from a mature biofilm colony after 2 days of growth. The glass slide contained five mature colonies.

Figure 3D shows thin sections of 2-day-old biofilm colonies of strains CU1000N (left panel) and JK1017 (right panel). Colonies of strain JK1017 were similar in size and shape to those of strain CU1000N but lacked internal, nonaggregated cells. The thin section shown in Fig. 3D (right panel) is representative of approximately 25 other JK1017 colonies examined, all of which displayed nearly identical morphologies. The agar and biofilm colony morphologies and biofilm dispersal phenotypes of the two other rough-colony mutants that we isolated (JK1002 and JK1022) were identical to those of strain JK1017 (data not shown).

Characterization of *A. actinomycetemcomitans* biofilm dispersal mutants. We mapped the transposon insertion sites in the three biofilm dispersal mutants by using inverse PCR (19, 33). The mapping of the transposon insertions in two of these mutants (JK1002 and JK1022) has been described in a previous study (19). All three insertions were located in genes necessary for the synthesis of the serotype f-specific O polysaccharide (O-PS) component of lipopolysaccharide in strain CU1000 (Fig. 4A) (19). These genes included *rmlA*, encoding glucose-1-phosphate thymidylyl transferase, which catalyzes the first step in the biosynthesis of dTDP-L-rhamnose, the activated nucleotide sugar precursor of *A. actinomycetemcomitans* O-PS carbohydrates (24, 36); *wzt*, encoding the hydrophilic ATPase component of an ATP-binding cassette (ABC) membrane transporter involved in the export and assembly of lipopolysaccharide components (5); and *dspA*, encoding a putative glycosyltransferase (19). All three biofilm dispersal mutants exhibited wild-type surface adherence properties in broth (Fig. 4B), reduced or absent O-PS antigenic side chains (Fig. 4C, filled bars) (19), and reduced biofilm cell detachment as measured by a 96-well biofilm detachment assay (Fig. 4D, filled bars).

To demonstrate that O-PS is directly involved in the detachment process, we cloned the wild-type *rmlA*, *wzt*, and *dspA* genes downstream from an inducible promoter on a broad-host-range plasmid and then introduced these recombinant plasmids into the mutant strains by conjugation with an *Escherichia coli* donor strain. Plasmids carrying complementing wild-type *rmlA*, *wzt*, and *dspA* genes (pJK597, pJK595, and pJK596, respectively) restored the ability of mutant strains to synthesize O-PS (Fig. 4C, open bars) and release cells into broth (Fig. 4D, open bars). These findings indicate that synthesis of serotype f-specific O-PS is necessary for detachment of cells from *A. actinomycetemcomitans* CU1000N biofilm colonies.

DISCUSSION

The morphology of *A. actinomycetemcomitans* biofilm colonies revealed in the present study was similar to that of the tower- and mushroom-shaped biofilm colonies commonly observed in other bacteria, including *Pseudomonas aeruginosa*,

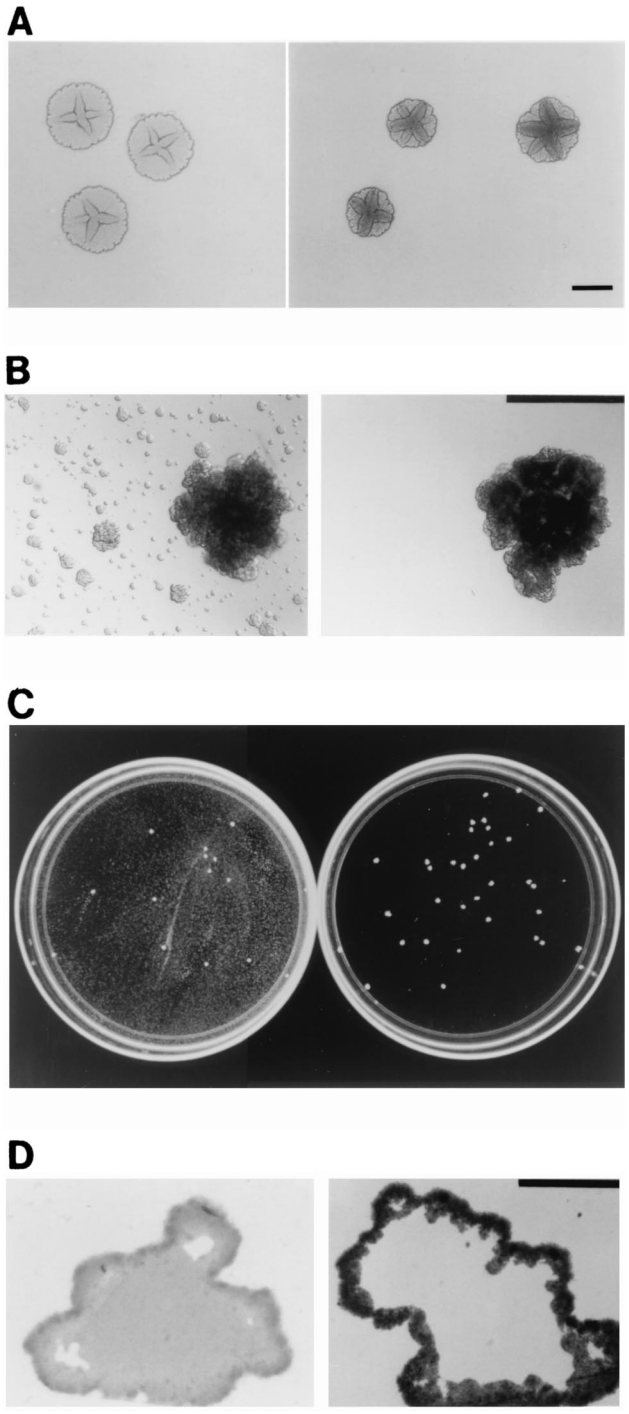

FIG. 3. Phenotypes of *A. actinomycetemcomitans* wild-type strain CU1000N (left side of panels) and biofilm dispersal mutant JK1017 (right side of panels). (A to C) Shown are colony morphology on agar (bar = 2 mm) (A), biofilm colony morphology in broth (bar = 1 mm) (B), and growth in 100-mm-diameter petri dishes (C), all after 3 days of growth. (D) Thin sections of 2-day-old biofilm colonies grown on polystyrene (bar = 100 μm).

P. fluorescens, *Vibrio parahaemolyticus* (22), *P. putida* (4), *V. cholerae* (34), and *Salmonella* spp. (28). In addition, our results indicate that *A. actinomycetemcomitans* biofilm colonies exhibit a distinct, phenotypic life cycle characterized by adherence of planktonic cells to a surface, the growth of asymmetric, lobed microcolonies that display complex morphological features, and the subsequent release of cells from the biofilm colony.

Our findings constitute the first report of defined bacterial mutants that are deficient in biofilm cell detachment. We

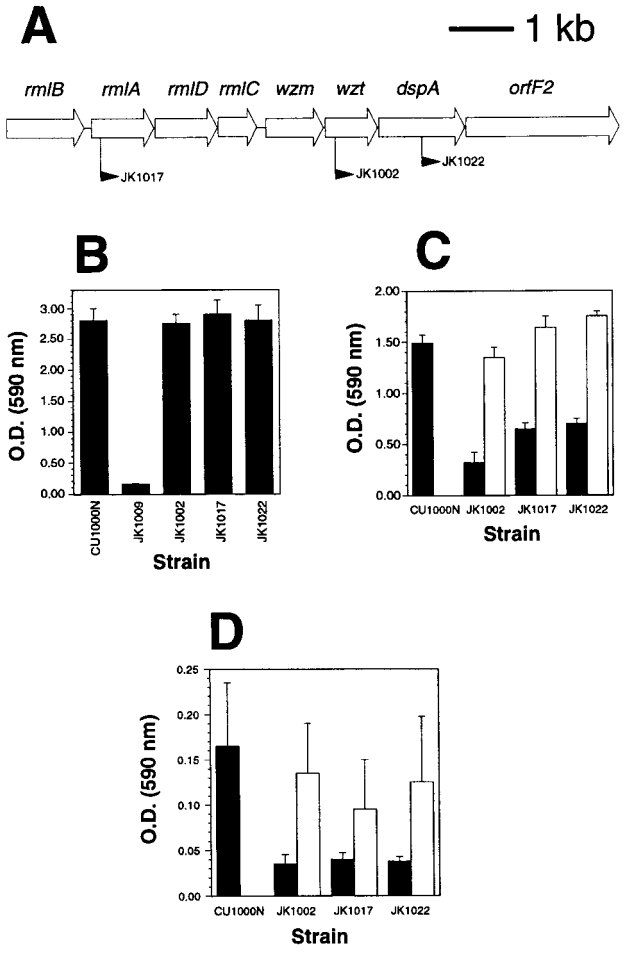

FIG. 4. Characterization of *A. actinomycetemcomitans* biofilm dispersal mutants. (A) Genetic map of an 8.7-kb region of the gene cluster responsible for the synthesis of serotype f-specific O-PS in strain CU1000 (19). Open arrows indicate open reading frames and direction of transcription. Gene names are shown above the arrows, and the filled arrowheads indicate the location and direction of transcription of IS*903*φ*kan* insertions in three different mutant strains. (B) Adherence to polystyrene was measured using a 96-well microtiter plate binding assay (15). Adherence is proportional to the optical density (O.D.) values; mean values plus standard errors for triplicate samples are shown. Strain JK1009 contains a transposon insertion in *flp-1* which results in complete loss of surface attachment (15). (C) Synthesis of O-PS in wild-type and mutant strains. O-PS was detected with an enzyme-linked immunosorbent assay using anti-CU1000 rabbit antiserum as previously described (19). The amount of O-PS is proportional to the O.D. values; mean values plus standard errors for triplicate samples are shown. Filled bars indicate the levels of O-PS from strains carrying vector plasmid pJAK16, and open bars indicate the levels from mutants JK1002, JK1017, and JK1022 carrying complementing plasmids pJK595, pJK597, and pJK596, respectively. (D) Detachment of cells from biofilm colonies grown on polystyrene rods as measured by a 96-well biofilm detachment assay. Cell detachment is proportional to the O.D. values; mean results plus standard errors for 8 to 15 wells for each strain are shown. Bars are as described for panel C.

showed that genes required for biofilm detachment in *A. actinomycetemcomitans* were not required for surface attachment or biofilm colony formation, indicating that biofilm detachment is a distinct process. Our findings suggest that this process involves the formation of nonaggregated cells inside the biofilm colony that are destined for release from the colony. This proposed detachment mechanism differs from the two previously proposed mechanisms of biofilm cell detachment, namely, erosion (the continuous release of single cells or small clusters of cells) and sloughing (the rapid detachment of large portions of the biofilm) (31).

Our results demonstrate that the synthesis of O-PS is required for *A. actinomycetemcomitans* biofilm cell detachment but not for surface attachment or biofilm colony formation. O-PS has been shown to play a role in biofilm development in other bacteria. Surface attachment and biofilm formation are decreased in O-PS mutants of *E. coli* (10), *V. cholerae* (25), and *Serratia marcescens* (27) but are increased in O-PS mutants of *P. aeruginosa* (23) and *P. fluorescens* (35). These latter findings suggest that biofilm cells of *A. actinomycetemcomitans* O-PS mutants may fail to detach because they are hyperattached to the inside of the colony. O-PS has also been shown to be necessary for multicellular development in *Myxococcus xanthus*, suggesting that O-PS could mediate cell-to-cell and cell-to-substratum interactions that are critical for cellular differentiation (3). These findings raise the possibility that biofilm growth and detachment in *A. actinomycetemcomitans* may be controlled by a genetically regulated developmental pathway (26). It is also possible that defects in O-PS may have pleiotropic effects on other extracellular structures, such as pili or fimbriae (10), which are required for biofilm development and biofilm cell detachment in *A. actinomycetemcomitans*.

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