Identification of the Genes Involved in the Biofilm-like Structures on *Actinomyces oris* K20, a Clinical Isolate from an Apical Lesion

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Abstract

Introduction: Although the production of biofilm is thought to be crucial in the pathogenesis of abscess formations caused by oral resident microorganisms, the particular mechanisms are still unknown. The aim of this study was to identify gene(s) responsible for maintaining the cell surface-associated meshwork-like structures, which are found in some biofilm-producing bacteria, in a clinical isolate of *Actinomyces oris* K20. **Methods:** Random insertional mutagenesis by using transposon EZ-Tn5 was performed against the strain K20. Transposon insertion mutants were screened by scanning electron microscopy for the absence of cell surface-associated meshwork-like structures. The disrupted genes by the transposon insertion were determined by direct genome sequencing with the transposon-end primers. **Results:** Five mutants without the meshwork-like structures were identified from 175 mutants. Sequencing of flanking regions of transposon insertion revealed that 3 mutants had a gene encoded polysaccharide deacetylase, Spo0J containing ParB-like nuclease domain, and hypothetical protein, respectively. The other 2 mutants had an insertion in a noncoding region and an unidentified region, respectively. **Conclusions:** Our findings indicated that these genes might be involved in the formation of meshwork-like structures on *Actinomyces oris* K20. *(J Endod 2013;39:44–48)*

Key Words

Actinomyces oris, apical abscess, biofilm, polysaccharide deacetylase, transposon mutagenesis

The biofilm infection is thought to be one of the most crucial factors for chronic or recurrent diseases. More than half of the infectious diseases that affect mildly compromised individuals involve bacteria species that are commensal in the human body or common in our environments (1, 2). Of importance with respect to medicines, sessile bacteria are inherently resistant to antimicrobial agents, showing that biofilm bacteria are much less susceptible than their planktonic counterparts (1–3). Thus, biofilm infections typically show recurring symptoms after cycles of antibiotic therapy until sessile population is surgically removed from the body.

In the apical part of the root canal, biofilm bacteria also cause many chronic, persistent, or recurrent infectious diseases. It is also recognized that the main reason for the lack of healing is the survival of embedded microorganisms within a biofilm matrix in the infected lesion (4–6). We previously reported that some clinical isolates of *Prevotella intermedia* from periodontitis lesions have the ability to form meshwork-like structures on their cell surfaces and produce biofilms (7, 8). The biofilm matrix on these cell surfaces consisted of mannose-rich polysaccharide as shown by chemical analyses, and the bacteria with this phenotype induced severe abscess lesions in mice with smaller number of cells than those of bacteria without meshwork-like structures (9, 10).

The genus *Actinomyces* contains a heterogeneous groups of anaerobic and facultatively anaerobic, gram-positive, pleomorphic or filamentous, non–spore-forming bacteria. *Actinomyces* species are known as early colonizers of oral cavities, involved in the development of oral biofilm (11–13), and in some occasions cause oral infections such as actinomycosis, apical abscess, root canal infection, dental implant–related infection (14–20), and non-oral infections in the human body (21). Kalfes et al (18) isolated *Actinomyces radicidentis*, which possesses the net-like cell surface structure, from the root canals of patients with failed endodontic treatments. Although they considered this structure as bundles of thick fimbriae-like appendices in a net arrangement, this cell surface structure has a similar appearance of the meshwork-like structure consisting of exopolysaccharides (EPS) in many biofilm-forming bacteria (8–10, 22, 23). Nair et al (24) also reported that *A. radicidentis* survived in the host tissue by building biofilms.

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We recently reported that *A. oris* K20 isolated from the apical abscess, which possessed unique phenotypic characteristics by dense meshwork-like structure on their cell surfaces (16). Viscous materials in culture supernatants of strain K20 consisted of mannose-rich EPS. Strain K20 induced abscesses in mice lasting at least for 5 days at a concentration of 10^8 cells/mL, whereas abscesses induced by ATCC 27044, a type strain for *A. oris* lacking biofilm-like structures, healed and disappeared at day 5, suggesting the biofilm phenotype might contribute to the pathogenicity of this organism (16).

Although biofilm bacteria play an important role in the pathogenicity of persistent infections (2), little is known about the mechanism of biofilm development of *A. oris* in the infectious lesions. In this study, we sought to determine the molecular basis of the development of biofilm by using transposon insertional mutagenesis in the clinical isolate of *A. oris* K20. Here we report the identification of genes that were predicted to be involved in the biofilm-like structures.

Methods

Bacterial Strains and Media

Strain K20 was isolated from a closed apical abscess lesion of a 39-year-old patient (generally healthy man) who presented with a body temperature of 36.8°C, anorexia, malaise, cheek swelling, redness, spontaneous pain, and regional lymphadenitis at initial visit. Strain K20 was presumptively identified as *A. viscosus* by 16S rRNA gene sequencing and catalase productivity and then re-identified as *A. oris* by means of whole genome sequencing and phylogenetic analysis by using a partial sequencing of housekeeping gene *atpA* (25) as described in our previous study (16).

This strain was routinely grown in trypticase soy broth (BBL Microbiology System, Cockeysville, MD) with 0.5% yeast extract (Difco Laboratories, Detroit, MI) (TSBY) without shaking or trypticase soy agar with 0.5% yeast extract (TSAY) at 37°C aerobically. When required, the antibiotic kanamycin (25 μg/mL) was added to the growth medium.

Competent Cell

Overnight culture of strain K20 was then diluted 100 times in fresh medium and incubated at 37°C until the culture reached an optical density at 600 nm of 0.2. Ten milliliters of culture was incubated on ice for 10 minutes and pelleted by centrifugation at 8000*g* for 10 minutes at 4°C. The cell pellet was washed twice in 10 mL cold distilled water and once in 10 mL of cold sterile 10% (v/v) glycerol. The pellet was resuspended in 100 μL of 10% (v/v) glycerol.

Mutagenesis

Electrocompetent cells (70 μL) of strain K20 were mixed with 20 ng EZ-Tn5<KAN-2> Tnp transposome (EZ-Tn5) (EZ-Tn5 <KAN-2>Tnp Transposome Kit; Epicentre, Madison, WI) on ice and then

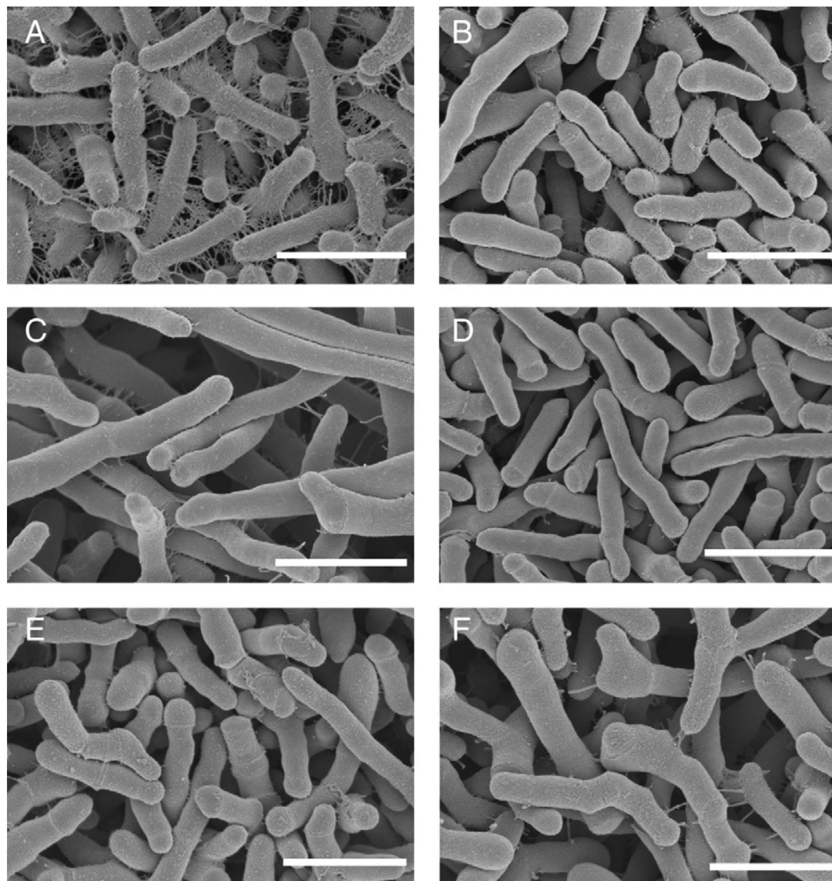

Figure 1. SEM analysis of the surface structure of *A. oris* K20 and its transposon-generated mutants. Parental strain K20 shows dense meshwork-like structures around the cells (*A*), whereas M154 (*B*), M206 (*C*), M7 (*D*), M23 (*E*), and M155 (*F*) do not display any meshwork-like structures on their cell surfaces. Scale bars = 2 μm.

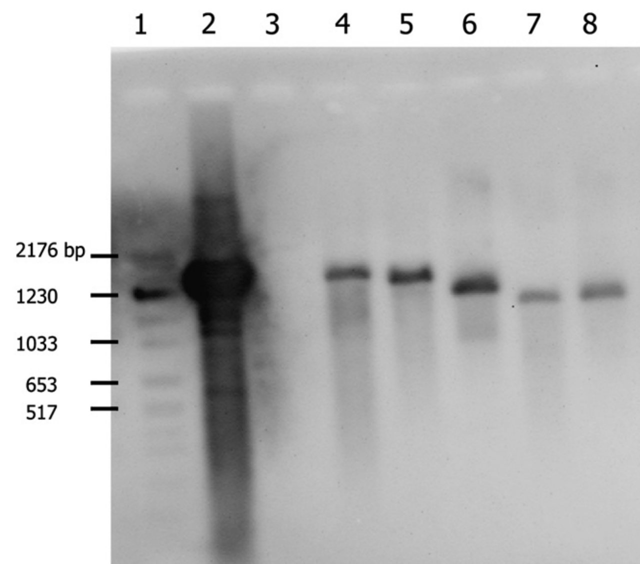

Figure 2. Southern hybridization of *Hin*cII-digested chromosomal DNAs from *A. oris* K20 and transposon mutants with a DIG-labeled EZ-Tn*5* probe. Lane 1, DNA size marker (*Bgl*I-digested pBR328); lane 2, the EZ-Tn*5* -carrying plasmid pCM190 as a positive control; lane 3, parental strain K20 as a negative control; lane 4, M154; lane 5, M155; lane 6, M205; lane 7, M7; lane 8, M23. DNA sizes are shown on the left. bp, base pair.

placed in a 0.56-mm flat-pack chamber (BTX, San Diego, CA). Electroporation was carried out at 800 V, 50 μF, 50 Ω. Cells were resuspended in 1 mL TSBY, incubated for 1 hour at 37°C without shaking, and plated onto TSAY with kanamycin (TSAY-Km) plates. Plates were incubated for 24–48 hours at 37°C aerobically. Growing colonies were harvested as transposon insertion mutants.

Morphologic Analysis by Scanning Electron Microscopy

Cell surface-associated meshwork-like structures were examined by scanning electron microscopy (SEM). Bacteria grown on TSAY for 48 hours were collected on a piece of filter paper (Glass fiber GA55; Toyo Roshi, Tochigi, Japan), fixed with 2% glutaraldehyde in 0.1 mol/L phosphate-buffered saline for 2 hours at 4°C and 1% OsO4 in 0.1 mol/L phosphate-buffered saline for 1 hour at 4°C, and dehydrated through an ethanol series and 2-methyl-2-propanol, followed by platinum ion coating (E-1030; Hitachi, Tokyo, Japan). Specimens were examined with an SEM (S-4800; Hitachi) at an accelerating voltage of 1 kV.

Southern Analysis

Southern analysis was performed to verify single insertion of the EZ-Tn5 into the genome of strain K20 by using a probe prepared from the internal sequence from EZ-Tn5. Briefly, the genomic DNA was extracted by using the MagExtractor (Toyobo, Osaka, Japan) and digested with *Hin*cII. The DNA fragments were electrophoresed on a 0.8% agarose gel (Nacalai tesque, Kyoto, Japan), transferred to a positive charged nylon membrane (Hybond-N$^+$ membrane; Amersham Bioscience UK, Bucks, England), and fixed on the membrane by ultraviolet light irradiation (HL-2000 HybriLinker; UVP, Upland, CA). To detect an insertion of EZ-Tn5, a digoxigenin (DIG)-labeled probe designed from the sequence of EZ-Tn5 was generated by using the Roche PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Manheim, Germany) with a primer pair for amplifying a kanamycin-resistant gene in EZ-Tn5 (EZ-Tn5 sequence is available at http://www.epibio.com/pdftechlit/138pl0611.pdf). The membrane was prehybridized (30 minutes, 50°C) in hybridization solution (DIG Easy Hyb Granules; Roche), subsequently hybridized overnight at 65°C with 2 μL/mL of DIG-labeled probe in hybridization solution, and then washed at 65°C by using the DIG Wash and Block Buffer Set (Roche Diagnostics). After hybridization, the membranes were developed by enzyme immunoassay by using the DIG Luminesent detection kit (Roche Diagnostics) according to the manufacturer's instruction. Signals were detected by the VersaDock Imaging System (Bio-Rad Laboratories, Hercules, CA).

Determination of EZ-Tn5 Integration Sites within the *A. oris* K20 Genome

The DNA sequences flanking EZ-Tn5 were determined by genome direct sequencing by using the Applied Biosystems DNA sequencer (Applied Biosystems 3130xl genetic analyzer; Applied Biosystems, Foster City, CA) with the transposon-specific outward primers KAN-2 RP-1 and KAN-2 FP-1 (EZ-Tn5 <KAN-2>Tnp Transposome Kit; Epicentre). Subsequently, the sequences that flanked EZ-Tn5 were used to conduct BLAST searches against the draft sequence of K20 (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi, accession number BABV01000001-BABV01000771) (16) and other nucleotides on the National Center for Biotechnology Information databases.

Results

Transposon Mutagenesis and Screening for Mutants without Meshwork-like Structures

Strain K20 that can form meshwork-like structures on the cell surface was subjected to mutagenesis with EZ-Tn5. The transposon insertion mutants were selected on TSAY-Km, because EZ-Tn5 has

TABLE 1. Description of Genes Identified in Each Meshwork-like Structure Defective Mutant of *Actinomyces oris* K20

Mutant	Locus tag (protein ID of K20 genome)*	Accession no.*	Description of gene
M154	AoriK_010100011036	ZP_08127025 AB540950 †	Polysaccharide deacetylase
M23	AoriK_010100000845	ZP_08125003	Spo0J containing ParB-like nuclease domain
M7	AoriK_010100014165	ZP_08127642	Hypothetical protein conserved in Actinobacteria
M206‡	AoriK_010100005764	ZP_08125978	Hypothetical protein
	AoriK_010100005769	ZP_08125979	TransRDD family protein
M155	ND§	ND§	—

*Locus tag and accession no. in National Center for Biotechnology Information databases.

†The sequence data of this gene determined in this study have been deposited in DNA Data Bank of Japan.

‡The transposon insertion was an intergenic region between ZP_08125978 and ZP_08125979.

§The transposon insertion site was inside a contig: AOKODU0745 of K20 draft genome information, but no open reading frame could be found.

the kanamycin-resistant gene. One hundred seventy-seven kanamycin-resistant colonies were harvested and screened by SEM. In total, 5 mutants without forming meshwork around their cells were obtained (Fig. 1).

Confirmation of EZ-Tn5 Insertion in Genome DNA

To confirm a single insertion of the transposon into the K20 genome, we performed the Southern hybridization with a DIG-labeled EZ-Tn5 probe for *Hinc*II-digested genomic DNA from each mutant. All 5 mutants had a single specific band, indicating the unique insertion of the transposon into each bacteria genome (Fig. 2).

Identification of the Transposon Insertion Sites in the Strain K20

To identify the EZ-Tn5 insertion site into the K20 genome, DNA sequences were determined by the genome direct sequencing analysis and compared with the draft genome sequence of K20 by using the genomic BLAST algorithm. In all 5 cases, a matching sequence was identified in the genome of K20 (Table 1). Mutant 1 (designated strain M154) had a transposon insertion into a gene encoding polysaccharide deacetylase. Mutant 2 (designated strain M23) had a mutation in a gene encoding Spo0J containing ParB-like nuclease domain. In mutant 3 (designated strain M7), transposon insertion was localized in a gene encoding hypothetical protein conserved in Actinobacteria. In mutant 4 (designated strain M206), we found that EZ-Tn5 was integrated into an intergenic region between 2 genes in divergent orientation, encoding hypothetical protein (ZP_08125978) and conserved transRDD family protein (ZP_08125979). In the last mutant (designated strain M155), no open reading frame could be found in the EZ-Tn5 flanking region.

Discussion

Previously, we isolated *A. oris* K20 from an apical abscess, which has the ability to produce biofilm-like structures, and characterized its pathogenicity to induce abscess in mice (16). In this study to look for the genes involved in biofilm development, we isolated 5 mutants without meshwork-like structures by EZ-Tn5 transposon mutagenesis, which is used in gram-positive and gram-negative bacteria successfully (http://www.epibio.com/transcite.asp). The transposon mutagenesis is a powerful tool for isolating biofilm-defective mutants and identifying genetic loci that are associated with biofilm formation. Several surveys demonstrated that many genes are associated with each stage of biofilm development in gram-positive bacteria, including *Staphylococcus epidermidis* (26), *Enterococcus faecalis* (27), *Listeria monocytogenes* (28), *Streptococcus gordonii* (29), and *Bacillus cereus* (30). To our knowledge, this is the first report to identify the genes involved in the formation of meshwork-like structures on *A. oris*.

One of the mutants without meshwork-like structure (strain M154) had a transposon insertion within the polysaccharide deacetylase gene. Several reports have demonstrated that polysaccharide deacetylase contributes to biofilm formation (23, 31). In *S. epidermidis*, the surface-attached protein IcaB is responsible for deacetylation of polysaccharide intercellular adhesin (PIA), which is an element of fibrous strands on the cell surface and an essential factor involved in biofilm formation (23). The *icaB* mutant of a biofilm-forming *S. epidermidis* clinical isolate had no fibrous strands on its cell surface, and PIA from *icaB* mutant is released into the culture supernatant, indicating deacetylation is essential for surface localization of PIA (23). The HmsF genes in *Yersinia pestis* encode a polysaccharide deacetylase domain, which is thought to be involved in the synthesis and modification of the EPS component of the biofilm (31). Our study showed that the viscous material of K20 was composed of mannose-rich polysaccharides, including glucose, ribose, galactose, xylose, arabinose, and rhamnose (16). The polysaccharide deacetylase gene mutant showed a more aggregated form of growth pattern in liquid culture as compared with the parent strain K20 (data not shown). We now hypothesize that polysaccharide deacetylase of *A. oris* might influence the cell surface electric charge. To understand the role of the polysaccharide deacetylase gene in the biofilm development, genetic approaches, such as a complementation study or construction of a non-polar deletion mutant, need to be used in further studies.

Mutant strain M23 had an insertion within the gene encoding Spo0J, which contains a ParB-like nuclease domain. Spo0J is a member of ParB family, which contributes to chromosome partitioning (32, 33). In *Bacillus subtilis*, Spo0A, a member of the response regulator family of transcription factors, is required for biofilm development (33, 34). Spo0A controls the gene expression of *spo0J* and directly regulates about 120 genes required for various stationary-phase phenomena including biofilm formation (35). Neither *spo0J* nor any other candidate determined in this study was known to be associated with biofilm formation of *Actinomyces* spp. Because these mutants could not form meshwork-like structures, we continue to work on whether these genes are responsible for the process of biofilm formation of *A. oris*.

In conclusion, we have identified 3 genes and 2 non-coding regions that might be associated with the biofilm formation in *A. oris* by transposon mutagenesis. Identification and characterization of biofilm-associated genes may facilitate the development of therapeutic agents and strategies to control biofilm-mediated infections by this organism. However, further investigations on these genes are needed to figure out a biofilm formation–gene expression relay system in *A. oris*.

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The authors deny any conflicts of interest related to this study.

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