**impA, a Gene Coding for an Inner Membrane Protein, Influences Colonial Morphology of Actinobacillus actinomycetemcomitans**

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Directed mutagenesis of a gene coding for a membrane protein of the periodontopathogen Actinobacillus actinomycetemcomitans was achieved by conjugation. The gene was disrupted by insertion of an antibiotic cassette into a unique endonuclease restriction sequence engineered by inverse PCR. The disrupted gene was cloned into a conjugative plasmid and transferred from Escherichia coli to *A. actinomycetemcomitans*. The allelic replacement mutation resulted in the loss of a 22-kDa inner membrane protein. The loss of this protein (ImpA) resulted in changes in the outer membrane protein composition of the bacterium. Concurrent with the mutation in *impA* was a change in the pattern of growth of the mutant bacteria in broth cultures. The progenitor bacteria grew as a homogeneous suspension of cells compared to a granular, autoaggregating adherent cell population described for the mutant bacteria. These data suggest that ImpA may play a regulatory role or be directly involved in protein(s) that are exported and associated with colony variations in *A. actinomycetemcomitans*.

**Actinobacillus actinomycetemcomitans** is widely recognized as a major pathogen in the etiology of localized juvenile periodontal disease and cases of refractory adult periodontal disease (32, 33, 41). The bacteria initiate a cascade of events that involve both a cellular and a humoral immune response which results in chronic inflammation (14, 40). The contribution of both bacterial and host factors may lead to the loss of tissue-matrix components and ultimately the loss of teeth. Multiple virulence determinants have been described for this organism which may participate in the infection of the host and protection of the host’s immune system (8). To define the mechanisms of bacterial factors in the infection process, molecular strategies need to be developed.

Recently, genes coding for exported proteins in *A. actinomycetemcomitans* have been identified using translational fusions to alkaline phosphatase (22). Based on the deduced amino acid sequence, several of these sequences were found to be homologous to bacterial membrane proteins but with no known associated function(s). A powerful method to deduce protein function is the generation of defined isogenic mutants. Therefore, one of the above gene sequences that has homology to a hypothetical 22-kDa transmembrane protein of *Haemophilus influenzae* was used as the prototypic gene to demonstrate the utility of conjugation for directed mutagenesis in *A. actinomycetemcomitans*.

Fresh clinical isolates of *A. actinomycetemcomitans* express a rough colony phenotype which grows in broth as granular, autoaggregate adherent cells that leave a clear broth (7, 11, 13). Upon successive rounds of in vitro subculturing on solid media, the colonies convert to a smooth phenotype that grows as a homogeneous suspension with no adherent cells (7, 11, 13). Allelic replacement mutagenesis of the gene coding for the 22-kDa protein of a smooth phenotype strain of *A. actinomycetemcomitans* resulted in a reversion of the growth, in broth, from a homogeneous suspension to an aggregated growth morphology. We describe here a method for directed mutagenesis in *A. actinomycetemcomitans* by conjugation and characterization of a strain mutant for the 22-kDa protein.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** *A. actinomycetemcomitans* strains in this study were grown statically in Trypticase soy broth supplemented with 0.6% yeast extract (TSBYE) at 37°C. *E. coli* strains DH5α (araD139, endA1, hsdR17, lacZΔM15, proA6, proC, recA1, strA398, supE44, thyA1, xyl5, yamE, Δ(lac-proAB)158) and the conjugative plasmid pGPF04 were obtained from the laboratory of Murry Stein (University of Vermont, Burlington). The *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C with aeration. The plasmid was propagated and purified from *E. coli* strain CC118a grown in LB medium containing 50 μg of ampicillin per ml. Mu phage was obtained from the laboratory of Howard A. Shuman (Columbia University, New York, N.Y.). Phage DNA was purified as described previously (22).

A nalidixic acid-rifampin-resistant strain of *A. actinomycetemcomitans* was derived from strain SUNY 465 (smooth phenotype). A rifampin-resistant spontaneous mutant was selected by plating 10⁸ logaritmically growing bacteria on TSBYE agar plates containing 60 μg of rifampin per ml followed by incubation for 3 days in a humidified, 10% CO₂ atmosphere at 37°C. The Rif+ colonies were replated on fresh TSBYE agar plates containing antibiotic. A single colony was propagated and frozen in 10% dimethyl sulfoxide. This strain was shown to be resistant to rifampin at 200 μg/ml. The Rif+ Nal⁻ strain of *A. actinomycetemcomitans* was generated by plating 3 × 10⁶ Rif+ cells (grown in rifampin-containing medium) on TSBYE agar plates containing 0.1 to 10 μg of nalidixic acid per ml and incubated as described above. The colonies that grew on the highest concentration were replated on plates containing incremental increasing concentrations of nalidixic acid. The Rif+ Nal⁻ strain (VT1169) was routinely passaged using 100 and 50 μg of rifampin and nalidixic acid per ml, respectively.

**Plasmid construction for allelic replacement mutagenesis.** The complete *impA* sequence is presented in Fig. 1 and can be obtained from GenBank (accession no. AF04561). *impA* was amplified by PCR using primers corresponding to sequences starting 67 bp 5′ of the start of the signal sequence (5′-ACA TAG CGA ACA AGT GGT GGG-3′, residues 1 to 20; Fig. 1) and 5′-CAT ACG GAG CTT TGA AGC G-3′, residues 729 to 741; Fig. 1) and cloned into pT7-Blue (Promega, Inc., Madison, Wis.). A unique *SalI* restriction site was engineered by inverse PCR (25) using primers corresponding to nucleotides 384 to 402 (5′-AAA GAC TTC CTA CAG GGA AAG-3′; Fig. 1) and 356 to 335 (5′-AGC AAG GGT AGG AGC GTA GGT-5′; Fig. 1). The gene was disrupted by insertion of the spectinomycin gene isolated from plasmid pDL269 (20). The *aadB* was released from pDL269 by incubation with *NdeI* and *HindIII* and gel purified. The DNA fragment was ligated with *Klenow* to blunt-end ligation with the inverse PCR product restricted with *SalI*. The ligation mixture was transformed into *E. coli* strain 31079 cells by electroporation and plated onto LB agar plates containing 50 μg of spectinomycin per ml. Spec+ colonies were selected, and the plasmids were isolated using a rapid plasmid purification scheme (1). The constructs were confirmed by restriction mapping and PCR. The disrupted gene was released from the plasmid by digestion with *HindIII/EcoRI* and treated with *Klenow*. The fragment was ligated with pGFP04 previously cleaved with *EcoRV*. Electroproduct DHH5α (araD) cells were transformed with the ligation mixture, and transformants were selected on LB agar containing 50 μg of spectinomycin per ml.
HEPES (pH 7.4). Based on bacterial membrane protein solubility in sarcosinate (6), inner membrane proteins are defined as proteins soluble in the detergent and outer membrane proteins are insoluble in the detergent. Protein concentra-
tions were determined by absorbance at 280 nm. The insoluble proteins were
solubilized in 1% SDS before determination of the protein concentration. Equal
concentration of proteins were boiled for 10 min in sample buffer, before appli-
cation to 5 to 15% polyacrylamide-SDS gels, and electrophoresis was performed
according to the method of Laemmli (18).

Hydropophicity assay. Relative surface hydrophobicity was determined using a
rapid method (organic phase partitioning) adapted from Rosenberg et al. (20).

RESULTS

Inactivation of the gene coding for ImpA of A. actinomycetemcomitans. A PCR product (737 bp) comprised of impA
including 67 bp 5' of the start of the signal sequence and 55 bp
of the stop codon was cloned into pT7Blue (Fig. 2, lane B). A unique Stul site was engineered into impA by inverse PCR,
and the gene was disrupted by ligation of a spectinomycin gene
(1.1 kb) by blunt-end ligation (Fig. 2, lane C). The primers
selected for inverse PCR resulted in a 49-bp deletion in impA.
The final construct was composed of ~350 bp of impA (flank-
ing and coding sequence) juxtaposed next to the spectinomycin
gene, which was contiguous with the remaining ~350 bp of the
gene and flanking sequence.

Allelic replacement mutagenesis in A. actinomycetemcomitans. Transformation of A. actinomycetemcomitans by electro-
poration (35) with the above construct resulted in few transformants, all of which contained the entire plasmid integrated
into the chromosome (data not shown). However, the fre-
cuency of recombinatorial events was increased using bacterial
mating or conjugation. Successful gene transfer was achieved
following conjugation of E. coli SM10(krip) containing the dis-
rupted impA on the conjugative plasmid pGPF04 with a spon-
taneous mutant of A. actinomycetemcomitans SUNY 465 resis-
tant to nalidixic acid and rifampin. Transconjugants were
recovered from this mating experiment after counterselection
on TSBYE agar plates containing spectinomycin. Transconju-
gants containing an allelic replacement of impA were selected
by replica plating on TSBYE agar plates containing ampicillin
(plasmid marker) or spectinomycin (gene marker). Transconju-
gants that grew on the spectinomycin plates but not on the
plates containing ampicillin indicated that the disrupted gene
sequence but not the vector sequence integrated into the ge-
nome of these transconjugants.

Confirmation of the integration event was determined by
PCR using primers that hybridized to the flanking sequences

1 acatagcgaacctggtttgtagcatcatttaaggcactattacattataatagtt
21 taaggggaaagaaaatgcgt
31 cagttataccctgctattgcaggaagcatgcttgagttttagctttgaga
41 gagcattcctagcaacctaggtatctacttcctcgactgtttacggttgctccqg
51 acaattcgtagggccaatctttggcttcctcggcttgaaagcttaaaacctgcagaacag
61 aggcattggaacgacgccaaaacctcggttcagcggcagcatcaatttagccaagacgcaga
71 ttttaagggctttgtttcagccctttgaggtctactaggt

FIG. 1. Nucleotide sequence of impA and flanking sequence from A. actinomycetemcomitans SUNY 465. Potential prokaryotic
−35 −10
−9
−9
−9
−9
−6
−3

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μg of spectinomycin per ml. Plasmids were isolated and the construct
was confirmed by PCR. Plasmid containing the disrupted gene was purified using
Qiagen spin columns (Qiagen, Inc., Valencia, Calif.) and transformed by elec-
}
of impA. The antibiotic resistance profile of the transconjugants indicated that a double crossover event had occurred. However, PCR of the chromosomal DNA indicated that two products were generated for most of the transconjugants: one product corresponding to the intact gene and the other corresponding to the disrupted gene (data not shown). However, one of the transconjugants was observed to have a single PCR product. The gel mobility of this product (Fig. 2, lane D) corresponded to the mobility of the disrupted gene contained on the conjugal plasmid (Fig. 2, lane C). Both of these products migrated more slowly than the product generated using the parent or wild-type chromosomal DNA as the template. This difference in mobility corresponded to the size of the spectinomycin gene.

The allelic replacement was confirmed by Southern analysis (Fig. 3). DNA extracted from wild-type and impA mutant strains and probed with labeled impA hybridized to fragments which differed in size that corresponded to the size of the spectinomycin gene (Fig. 3A). The presence of the spectinomycin gene in this fragment was demonstrated by hybridization of a labeled spectinomycin gene exclusively with the ~9-kb DNA fragment (Fig. 3B). Southern analysis using vector sequence as the probe did not hybridize with chromosomal DNA extracted from the impA mutant strain (data not shown). These data indicate that the disrupted gene replaced the wild-type gene in a site-directed and specific manner into the A. actinomyctetcomitans genome.

E. coli SM10(pspir) has an integrated form of a conjugal plasmid that is used to mobilize the suicide vector (31). This plasmid has functional copies of bacteriophage Mu, a temperate phage that integrates by random transposition. The RP-4-based system used to transfer plasmids has been successfully used in a number of bacteria (4, 15, 36). However, this system has proven to be problematic in Legionella pneumophila (37). In the L. pneumophila system, mutants resulting from matings with an RP-4-based plasmid containing TnphoA were found to be introduced by random insertions of Mu into the L. pneumophila genome. To determine the presence or absence of random integration of Mu into the impA mutant chromosome, DNA was restricted with EcoRI and transferred to nylon membrane for Southern blot analysis. The blot was probed a 1.1-kb HindIII fragment of Mu phage DNA conjugated with horse-radish peroxidase. The blot did not reveal any detectable signal in the lane corresponding to the impA mutant DNA (data not shown). These data indicate that Mu sequence is not present in the genome of the impA mutant.

Characterization of the impA mutant strain of A. actinomycetcomitans. Broth cultures of primary derived impA mutant grew as aggregates on the side and bottom of the tube leaving a clear broth as compared to a turbid, homogeneous suspension with no aggregates, as observed with the parent strain (Fig. 4). These aggregates could be partially dissociated by sonication. When grown on agar for the same period of time, the impA mutant strain colonies were larger in size than the wild-type colonies and demonstrated an alteration in the colony morphology. Both strains were observed to be circular and did not show any differences in surface elevation. However, the edges of the colonies were observed to be slightly different. The edge of the wild-type colonies displayed an entire morphology, whereas the edges of impA mutant strain colonies had a more erose morphology (data not shown).

The differences in growth characteristics and colony morphology suggested that there may be differences in the expression of membrane proteins. These possible differences were investigated by analysis of the membrane proteins by SDS-polyacrylamide gel electrophoresis (PAGE). Inner and outer membrane proteins were separated by differential solubilization in the detergent sodium lauryl sarcosinate. Comparison of the outer membrane proteins of impA mutant and wild-type strains revealed a number of differences in the protein profiles.

FIG. 2. Identification of a chromosomal mutation by PCR. Chromosomal DNA was isolated from transconjugants or wild-type cells and was used as a template for PCR with primers corresponding to the 5' and 3' ends of impA. Products were analyzed by agarose gel electrophoresis. Lane A, 1-kb DNA ladder; lane B, wild-type chromosomal DNA; lane C, cloned impA disrupted with a spectinomycin cassette; lane D, impA mutant chromosomal DNA.

FIG. 3. Southern blot analysis of chromosomal DNA. Chromosomal DNA was isolated from wild-type and impA mutant strains and incubated with EcoRI. Products were separated by agarose gel electrophoresis and transferred to nylon membranes. Duplicate membranes were probed with labeled impA (A) or spc (B). Lanes: +, wild-type DNA; −, mutant DNA.
Two proteins (~67 and ~60 kDa) were observed to be absent in the impA mutant outer membrane as compared to the parent strain. Conversely, an additional ~44-kDa protein was present in the impA mutant profile compared to the parent strain. In addition, two proteins (~42 and ~26 kDa) stained with less intensity in the mutant strain compared to the wild-type strain following staining with Coomassie brilliant blue.

The change in the outer membrane protein profile may be correlated with the absence of the 22-kDa inner membrane protein of the impA mutant strain (Fig. 5). This protein molecular mass corresponds to the molecular mass deduced from the impA sequence. Only minor differences were observed in the electrophoretic mobilities and staining intensities of the remaining inner membrane proteins isolated from impA wild-type and mutant strains of A. actinomycetemcomitans.

Associated with the changes in the outer membrane protein profile of the mutant, an increase in the hydrophobicity of the organism was also observed. Hydrophobicity, as determined by the percentage of organisms that partition into the organic phase (hexadecane) was increased 13% for the mutant compared to the parent strain (58.9 ± 5.5 versus 45.8 ± 5.2, respectively; P < 0.05).

Northern analysis and reverse transcription-PCR (RT-PCR) of impA. The inactivation of impA leads to a change in the protein profile of the outer membrane fraction (Fig. 5). These data suggest that impA is part of an operon and that the disruption of this gene may lead to polar effects of the downstream genes. To address this possibility, total RNA was isolated from the parent strain (VT1169) and used in Northern analysis with impA as the probe (Fig. 6). A 0.6-kb transcript was detected that corresponds to the size of the coding region of impA (612 bp). The hybridization signal in the region of the 1.35-kb marker corresponds to the ribosomal subunits of A. actinomycetemcomitans (D. Galli, personal communication).

Based on information obtained from the A. actinomycetemcomitans genome sequencing project being conducted at the University of Oklahoma, primers were designed for RT-PCR. A putative open reading frame transcribed in the same orientation as impA was indicated 500 bp downstream of the trans-
lational stop codon of impA. This protein shares homology with a putative protein of *H. influenzae* (H10149). This primer was used as the RNA primer for the RT reaction using the SUPERSCRIPT One-Step RT-PCR System according to the manufacturer’s instruction (Gibco-BRL). The resulting cDNA was used as the template in PCR with the above primer and a primer corresponding to the 5' end of impA (nucleotides 1 to 20; Fig. 1). Analysis of this reaction did not result in any product (data not shown). The RT reaction was verified using the same RNA with the 3' primer of impA as the RNA primer (nucleotides 729 to 741; Fig. 1). Analysis of the PCR product of the resulting cDNA with the 5' and 3' primers of impA resulted in the expected PCR product. Only one other open reading frame was found that was approximately 1 kb downstream of the translational stop codon of impA. However, this gene is transcribed in the opposite orientation of impA. This protein shares homology with DnaA, a replication initiator protein of *E. coli* (39). The primers used in the RT-PCR experiments were tested with *A. actinomycetemcomitans* DNA as the template and generated the expected PCR products (data not shown).

In addition, analysis of the impA sequence suggests the presence of a 7-bp inverted repeat (ΔG = −10.9) 7 bp from the stop codon TAG (Fig. 1). Collectively, the data indicate that impA is transcribed as a monocistronic mRNA. These data further suggest that the disruption of impA should not interfere with downstream gene expression and the phenotypes observed were associated with disruption of impA.

**DISCUSSION**

The molecular analysis of virulence factors of *A. actinomycetemcomitans* has been enhanced by the development of an efficient electroporation system and shuttle plasmids (2, 9, 19, 23, 24, 34, 35). This system is sufficient for the transformation of *A. actinomycetemcomitans* by replicating plasmids, but we found that the frequency for integration events mediated by homologous recombination is greatly reduced. An alternative approach to electrotropination is conjugative methods which have been used to transfer broad-host-range group P and Q plasmids from *E. coli* to *A. actinomycetemcomitans* (10) and to transfer a Tn5 minitransposon into the Y4 strain of *A. actinomycetemcomitans* (16). However, conjugative transfer methods have not been used for the development of defined mutants by homologous recombination in *A. actinomycetemcomitans*.

The prototypic gene used in this study was identified to code for an exported protein in *A. actinomycetemcomitans* using translational gene fusions to alkaline phosphatase (22). The gene codes for a 22-kDa inner membrane protein (ImpA). Due to the lack of a suitable endonuclease restriction site, an engineered site was generated by inverse PCR to disrupt the gene by insertion of a spectinomycin cassette. The disrupted gene was cloned into the suicide vector plasmid pGP704, which contained in *E. coli* SM10(λpir) for conjugation with a strain of *A. actinomycetemcomitans* developed to be resistant to both nalidixic acid and rifampin. The use of a double antibiotic selection was used to reduce the number of spontaneous mutations of the donor cells based on the high numbers of bacteria used in plating the conjugation mixture.

Transconjugants that were sensitive to ampicillin and resistant to spectinomycin, indicating the absence of the vector marker, were screened for the double-crossover event by PCR. Interestingly, only one of the transconjugants displaying this antibiotic profile contained a single copy of impA disrupted with the spectinomycin cassette. The remaining transconjugants contained two genes corresponding to an intact and a disrupted impA as determined by PCR. The presence of these transconjugants suggests that either the vector inserted into the chromosome by nonhomologous or illegitimate recombination that leads to inactivation of bla or by homologous recombination of the suicide vector. Based on our studies, transconjugants were not obtained after transfer of the suicide plasmid, pGP704, into *A. actinomycetemcomitans* VT1169. This is in contrast to side-by-side conjugation experiments using the identical plasmid containing homologous *A. actinomycetemcomitans* DNA sequence which resulted in thousands of transconjugants (data not shown). In addition, Southern analysis of wild-type chromosomal DNA using the conjugation vector as the probe under high-stringency conditions did not generate any signal (data not shown). These data indicate that the suicide plasmid alone does not integrate into the chromosome when introduced into *A. actinomycetemcomitans* by conjugation.

Prior to this study, there has been only one report of directed mutagenesis in *A. actinomycetemcomitans* (17). The successful mutation in the leukotoxin gene was generated by electroporation of a nonreplicating plasmid containing a construct in which the disrupted gene was flanked by 1 kb of homologous DNA sequence. In the majority of transformants, the plasmid integrated into the genome via a single crossover event. Only one transformant appeared to be generated by a reciprocal double crossover. In the present study, only 0.35 kb of homologous DNA flanking sequence was present for directed mutagenesis and one transconjugate was obtained that was generated by a reciprocal double crossover. Based on studies in *Streptococcus parasanguinis* by electroporation using a nonreplicating plasmid, the number of reciprocal double crossover transformants increased with increasing size of homologous flanking sequence in the target DNA (5). Therefore, increasing the amount of homologous flanking sequence in our construct should increase the number of transconjugants and, therefore, the number of reciprocal double crossover events. These studies are currently under way in our laboratory.

Allelic replacement of the wild-type impA with a disrupted gene leads to the deletion of a 22-kDa protein localized to the inner membrane. Concurrent with the deletion of impA was a change in the phenotype of the cells when grown in broth culture and on agar. Fresh clinical isolates of *A. actinomycetemcomitans* display a rough colony phenotype characterized by a transparent, dull, circular colony with irregular borders when grown on agar that express fimbriae (13, 26, 27, 30). The bacteria change to a smooth colony phenotype that has reduced or lack of fimbriation (a more opaque, glistening, circular colony with regular borders) following extensive subculturing (13). An intermediate colonial variant of *A. actinomycetemcomitans* has also been characterized that grows as transparent colonies but lack the fimbriae of the rough phenotype (13). The morphology of the mutant colonies grown on agar was more closely related to the smooth phenotype than to the rough morphology. However, the outer edges of the mutant colonies did appear to be more irregular than the edges of the parent or smooth phenotype.

The rough and intermediate phenotype grow in aggregates in broth that adhere to the walls of the tube (13). This is the same pattern of growth that was demonstrated for the impA mutant strain compared with the parent strain that grows as a turbid, homogeneous solution. Based on these data, the impA mutant strain appeared to display an intermediate phenotype compared to the smooth phenotype of the parent strain when grown in broth cultures. To determine if the phenotype is directly correlated with the inactivation of impA, complemen-
tation studies should be performed. Currently, complementation in A. actinomycetemcomitans has not been demonstrated. However, studies in this laboratory are under way to develop a complementation system for A. actinomycetemcomitans. Further studies will be required to determine if these pleiotropic effects are primary or secondary events of the mutation.

The changes in colony morphology suggested a change in the surface composition of the individual bacteria within the colony. These morphological changes maybe related to changes in the outer membrane proteins of the impA mutant bacteria. Based on SDS-PAGE analysis, several outer membrane proteins were either absent or reduced in staining intensity of the mutant strain compared to the parent strain. In contrast, a number of allelic replacement mutagenesis in A. actinomycetemcomitans has not been demonstrated. These morphological changes maybe related to changes in the outer membrane proteins of the impA mutant bacteria. Based on SDS-PAGE analysis, several outer membrane proteins were either absent or reduced in staining intensity of the mutant strain compared to the parent strain. In contrast, a number of allelic replacement mutagenesis in A. actinomycetemcomitans has not been demonstrated.

The differences in the protein composition may be related to the increase in hydrophobicity observed for the impA mutant strain. Differences in cell surface hydrophobicity are found between laboratory strains and fresh isolates. Fresh isolates are more hydrophobic than laboratory strains. Taken together, the data suggest that the impA mutant strain may represent a transitional stage in the rough-to-smooth phenotype conversion.

The inactivation of impA, presented here, is the first example of allelic replacement mutagenesis in A. actinomycetemcomitans achieved by conjugative transfer. The number of genes identified from A. actinomycetemcomitans will likely increase due to the sequencing of the entire genome. This genetic transfer system has significant potential for the elucidation of the function of gene products identified from the A. actinomycetemcomitans genome.

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REFERENCES


