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# Construction and Use of Integration Plasmids To Generate Site-Specific Mutations in the *Actinomyces viscosus* T14V Chromosome

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**Stable transformants of *Actinomyces viscosus* T14V carrying heterologous DNA were obtained with the aid of integration plasmids. These plasmids contained a kanamycin resistance ($Km^r$) gene flanked by *A. viscosus* T14V genomic DNA, including parts of the type 1 structural fimbrial subunit gene (*fimP*) on one or both sides of the antibiotic marker. Significantly more $Km^r$ transformants were obtained with a plasmid carrying longer segments of homologous strain T14V DNA. Integration of this plasmid into the *A. viscosus* T14V genome affected the expression and function of type 1 fimbriae in the transformants. In the transformant strain designated *A. viscosus* MY50D, the inactivated *fimP* replaced the wild-type *fimP* via allelic replacement. *A. viscosus* MY51S and MY52S each contained a copy of the plasmid integrated into the genome by a Campbell-like insertion mechanism. *A. viscosus* MY50D and MY51S lacked type 1 fimbriae and did not bind to proline-rich proteins (the fimbrial receptors) immobilized on nitrocellulose. In contrast, strain MY52S synthesized the structural subunit protein, as detected by immunostaining with anti-*A. viscosus* T14V type 1 fimbria antibodies. However, the high-molecular-weight proteins observed in sodium dodecyl sulfate-polyacrylamide gels of fimbriae from the cell wall of the wild-type strain T14V were absent in cell wall preparations of this strain. Moreover, *A. viscosus* MY52S failed to bind, in vitro, to proline-rich proteins. Thus, these results demonstrate that insertion of heterologous DNA at specific sites of the *Actinomyces* genome can be facilitated with integratable plasmids and that the transformants and mutants generated will aid in the delineation of the roles and contributions of specific genes to the structure and function of any macromolecule produced by these organisms.**

The expression of fimbriae by members of the gram-positive bacterial genus *Actinomyces* has been documented, and the presence of these cell surface components has been correlated with adherence of these organisms to various host receptors (4, 7). The *Actinomyces* type 1 fimbriae are involved in bacterial colonization to teeth in the human oral cavity (4, 36). Results of previous studies also indicated that acidic proline-rich salivary proteins (PRPs) that coat the enamel surface are the receptors specific for this fimbrial type (13, 14). Whereas the structure of the *Actinomyces viscosus* T14V type 1 fimbria receptors has been identified, the receptor binding domain, whether localized on the structural fimbrial subunit or a fimbria-associated adhesin protein, has not been defined.

Little is known concerning the genetic basis of fimbria synthesis by these and other gram-positive bacteria (12, 15, 37). The identification and characterization of the *A. viscosus* T14V type 1 fimbrial structural subunit gene (*fimP*) (40) is an important first step in the genetic analysis of fimbriae from this organism. However, further attempts to isolate additional fimbria-associated genes have been difficult because of the lack of information concerning fimbria-associated proteins in this organism. The availability of mutants defective at various stages of fimbria synthesis would be ideal for these studies. Unfortunately, no genetically defined mutants of any kind have been described for *Actinomyces*. Attempts to construct genetically engineered *Actinomyces* mutants will depend largely on two essential tools: (i) a reproducible means of genetic transfer and (ii) vectors for the transfer of cloned genes or the inactivation of genes in the bacterial genome. In this regard, a transformation procedure for several *Actinomyces* spp. by the vector pJRD215 (8) was described recently (43). That study also resulted in the identification of two antibiotic resistance markers, kanamycin and streptomycin, that can be expressed by these bacteria. To assess the feasibility of generating mutations in the *Actinomyces* chromosome, it was necessary to investigate whether insertion of heterologous DNA into the genome of these bacteria could be accomplished and, if insertion was successful, whether the integrated foreign DNA could be maintained in the recombinants.

This study describes the construction of two integration plasmids that contained *A. viscosus* T14V genomic DNA, including portions of the type 1 structural fimbria subunit gene (40), and a kanamycin resistance ($Km^r$) gene that provided a means to select for transformants. The chimeric molecules were cloned into pUC13, which does not replicate in *Actinomyces* spp. Stable $Km^r$ transformants were obtained and characterized, and the expression of type 1 fimbriae by these strains was assessed. The results presented in this study provide the first evidence for integration and maintenance of foreign DNA in the genome of *Actinomyces* spp.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are described in Table 1. *Actinomyces* strains were incubated in a complex medium (6) or *Lactobacillus*-carrying medium (11), and *Escherichia coli* strains were incubated in Luria-Bertani (LB) (31) broth supplemented with antibiotics

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TABLE 1. Bacterial strains and plasmids used

| Bacterial strain or plasmid | Relevant characteristics[a] | Reference(s) or source |
|---|---|---|
| Bacterial strains | | |
| *A. viscosus* | | |
| T14V ($1^+$ $2^+$)[b] | Expresses types 1 and 2 fimbriae, $Km^s$ $Sm^r$ | 7, 36 |
| 5519 ($1^+$ $2^-$)[c] | Expresses only type 1 fimbriae, $Km^s$ $Sm^r$ | 7 |
| 147 ($1^-$ $2^-$)[d] | Lacks both types 1 and 2 fimbriae, $Km^s$ $Sm^r$ | 7 |
| MY50D | Lacks type 1 fimbriae, $Km^r$ $Sm^r$ Δ*fimP*::$Km^r$ | This study |
| MY51S | Lacks type 1 fimbriae, $Km^r$ $Sm^r$ Δ*fimP*::$Km^r$ | This study |
| MY52S | Expresses only the type 1 fimbrial structural subunit protein, $Km^r$ $Sm^r$ *fimP* Δ*fimP*::$Km^r$ | This study |
| *E. coli* DH5α | ϕ80d *lacZΔM15 endA1 recA1 hsdR17* ($r_K^-$ $m_K^-$) *supE44 thi-1* $\lambda^-$ *gyrA relA1* $F^-$ Δ(*lacZYA-argF*)U169 | GIBCO-BRL |
| Plasmids | | |
| pMY1001 | $Ap^r$, contains *fimP* | 40 |
| pMY1103 | $Ap^r$, contains *fimP* | This study |
| pMY301 | $Km^r$ $Ap^r$, contains Δ*fimP*::$Km^r$ | This study |
| pMY302 | $Km^r$ $Ap^r$, contains Δ*fimP*::$Km^r$ | This study |
| pJRD215 | $Sm^r$ $Km^r$ $Mob^+$ | 8 |
| pUC13 | $Ap^r$ | 33 |

[a] The presence of a complete or incomplete copy of the structural fimbrial subunit gene in the bacterial genome or plasmid is denoted by *fimP* or Δ*fimP*, respectively. $Km^r$, resistance to kanamycin; $Sm^r$, resistance to streptomycin; $Ap^r$, resistance to ampicillin; Mob, mobilization function.

[b] A spontaneous streptomycin-resistant wild-type strain was isolated by using standard techniques.

[c] Variant of *A. viscosus* T14V that lacks type 2 fimbriae.

[d] Variant of *A. viscosus* T14V that lacks both types 1 and 2 fimbriae.

(Sigma Chemical Co., St. Louis, Mo.) where appropriate. The antibiotics used in this study were kanamycin sulfate, streptomycin, and ampicillin, used at 40, 50, and 100 μg/ml, respectively.

**Subcloning and transformation.** CsCl-ethidium bromide density gradient-purified plasmid DNA (5 μg) was digested with restriction endonucleases (GIBCO-BRL Life Technologies, Gaithersburg, Md.) and then incubated with the Klenow fragment of DNA polymerase I (GIBCO-BRL) to prepare blunt-ended DNA fragments. The digested DNA fragments were extracted from agarose gels with reagents supplied with the Elu Quik kit (Schleicher & Schuell, Keene, N.H.). Ligation of blunt-ended DNA fragments was described previously (31), and transformation of *E. coli* DH5α by electroporation (10) was performed in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.). Transformants were plated on LB agar containing antibiotics, and plasmid DNA from *E. coli* transformants was isolated as described in Sambrook et al. (31). CsCl-ethidium bromide density gradient-purified covalently closed circular plasmid DNA (100 ng) was used for DNA transfer in *Actinomyces* spp. by electroporation (43). Transformants were selected on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with antibiotics. Procedures for the isolation of plasmid or chromosomal DNA from *Actinomyces* strains were described previously (43).

**DNA manipulations and PCR.** Southern blot hybridizations of digested chromosomal DNA on GeneScreen (DuPont New England Nuclear Research Products, Boston, Mass.) to [α-$^{32}$P (3,000 mCi/mmol, DuPont New England Nuclear)]dCTP-labeled DNA probes under conditions of high stringency were as described previously (39). The 83-bp *Stu*I-*Xho*I DNA fragment internal to *fimP* was obtained by PCR amplification in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, Mass.). Oligonucleotide primers flanking the 83-bp DNA fragment were prepared on a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Purified *A. viscosus* T14V genomic DNA (250 ng) was combined with 20 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide, and 2.5 U of *Taq* DNA polymerase (GIBCO-BRL). Genomic DNA was denatured at 94°C for 3 min, and amplification was completed with 35 cycles of denaturation (45 s at 94°C), annealing (30 s at 45°C), and extension (1 min 30 s at 72°C), followed by a final extension step of 10 min at 72°C. The PCR product was digested with *Stu*I and *Xho*I, and the digested mixture was applied onto a 1.5% agarose gel in Tris-borate-EDTA buffer (31). The 83-bp DNA fragment was extracted by using Whatman DE81 (DEAE-cellulose) paper (Whatman Corp., Hillsboro, Ore.) and labeled with [α-$^{32}$P]dCTP by random priming (31).

**Preparation and analysis of subcellular fractions.** Bacteria from a stationary-phase culture were washed with phosphate-buffered saline (PBS; pH 7.0), suspended in PBS containing 20% sucrose (grade I; Sigma), and incubated with lysozyme (1 mg/ml of original culture volume) at 37°C for 60 to 90 min. The supernatant fluid (designated solubilized cell wall fraction) was dialyzed (molecular weight cutoff of 12,000 to 14,000) against 0.5× PBS. The dialysate was brought to 20% saturation with solid ammonium sulfate, and the precipitated proteins were dissolved in Tris-borate-buffered saline (pH 7.8) (7). The protoplasts after lysozyme digestion were washed twice in 10% sucrose in PBS and then subjected to one freeze-thaw cycle at −20°C (overnight) and one freeze-thaw cycle at 37°C (within 15 min). The protoplasts were suspended in 50 mM Tris-HCl (pH 8.0)–5 mM EDTA (pH 8.0) and sonicated on ice for 4 min with a Branson Sonifier (Branson Ultrasonics Inc., Danbury, Conn.). The supernatant fluid (designated membrane-cytoplasm fraction containing proteins released from these subcellular fractions) was separated from the ruptured cells by centrifugation at 12,000 rpm for 20 min. The cell surface fimbriae also were obtained by incubation of bacteria in PBS at 60°C for 1 h followed by mild sonication (four 30-s bursts; 30% duty output) on ice. Greater than 90% of bacterial cells stained gram positive, indicating no gross disruption of cell wall under these conditions. The supernatant fluid (designated sheared cell surface fraction) was brought to 20% saturation with solid ammonium sulfate as described for the preparation of solubilized cell wall fractions. Proteins from each subcellular fraction were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (23) and transferred to nitrocellulose (0.45-μm pore size; Schleicher & Schuell) (1), and the filters were processed by using reagents from the Bio-Rad immunostaining kit (Bio-Rad Laboratories) as described previously (42).

**In vitro adherence and other assays.** The adherence of *Actinomyces* strains, in vitro, to PRPs was assessed by adopting a protocol developed by Ruhl et al. (30). Bacterial cells from stationary phase of growth were washed in PBS and suspended in PBS containing 2 mg of bovine serum albumin (fraction V; Sigma) per ml, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and the cell density was adjusted to $5 \times 10^9$/ml (optical density at 660 = 2.0). One milliliter of each adjusted cell suspension was incubated with NHS-LC-Biotin (100 μg/ml; Pierce, Rockford, Ill.). The labeled cells were washed in PBS, suspended in 1 ml of blocking buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5% bovine serum albumin, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% sodium azide), and incubated with a nitrocellulose square containing immobilized purified human PRPs (100 and 50 ng, applied as spots) (kindly provided by D. A. Johnson, The University of Texas Health Science Center at San Antonio) (39) and bovine serum albumin (2 and 1 μg). Unadsorbed bacteria were removed with a buffer containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.02% sodium azide. The nitrocellulose squares were incubated in the same buffer containing avidin D-alkaline phosphatase (0.2 U/ml; Vector, Burlingame, Calif.) and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (31).

The procedure of colony dot immunoassay with various antibodies was described previously (40). The presence of a lectin-like type 2 fimbrial activity in the *Actinomyces* strains was determined by a lactose-sensitive coaggregation assay with *Streptococcus oralis* (formerly *S. gordonii*) 34 (6). The expression of cell-associated sialidase was assessed by a rapid filter spot assay using a fluorogenic sialidase substrate (42).

## RESULTS

**Construction and characterization of integration plasmids.** The construction of integration plasmids was initiated with plasmid pMY1001 (40) (Fig. 1) carrying the *A. viscosus* T14V type 1 fimbrial structural subunit gene, *fimP* (39), obtained previously by cloning of an *A. viscosus* T14V *Sau*3AI DNA fragment into the *Bam*HI site of pUC13. Plasmid pMY1103 contained a complete *fimP* copy, whereas the integration plasmids pMY301 and pMY302 had deletions internal to the *fimP* gene. The $Km^r$ gene present on a 1.2-kb *Nhe*I-*Xho*I DNA fragment from pJRD215 (8) was cloned into the specific deletion region to yield pMY301 or pMY302 (Fig. 1). The direction of transcription of the $Km^r$ gene in these plasmids was opposite to that of *fimP*.

Results from a previous study showed that *E. coli* carrying pMY1001 directed the expression of the *A. viscosus* T14V type 1 fimbrial structural subunit that migrated on SDS-polyacrylamide gels as a 65-kDa protein (40). This protein was identified by immunostaining with polyclonal and monoclonal antibodies directed against the *A. viscosus* T14V type 1 fimbriae (5, 40). As expected, the same antibodies identified an immuno-

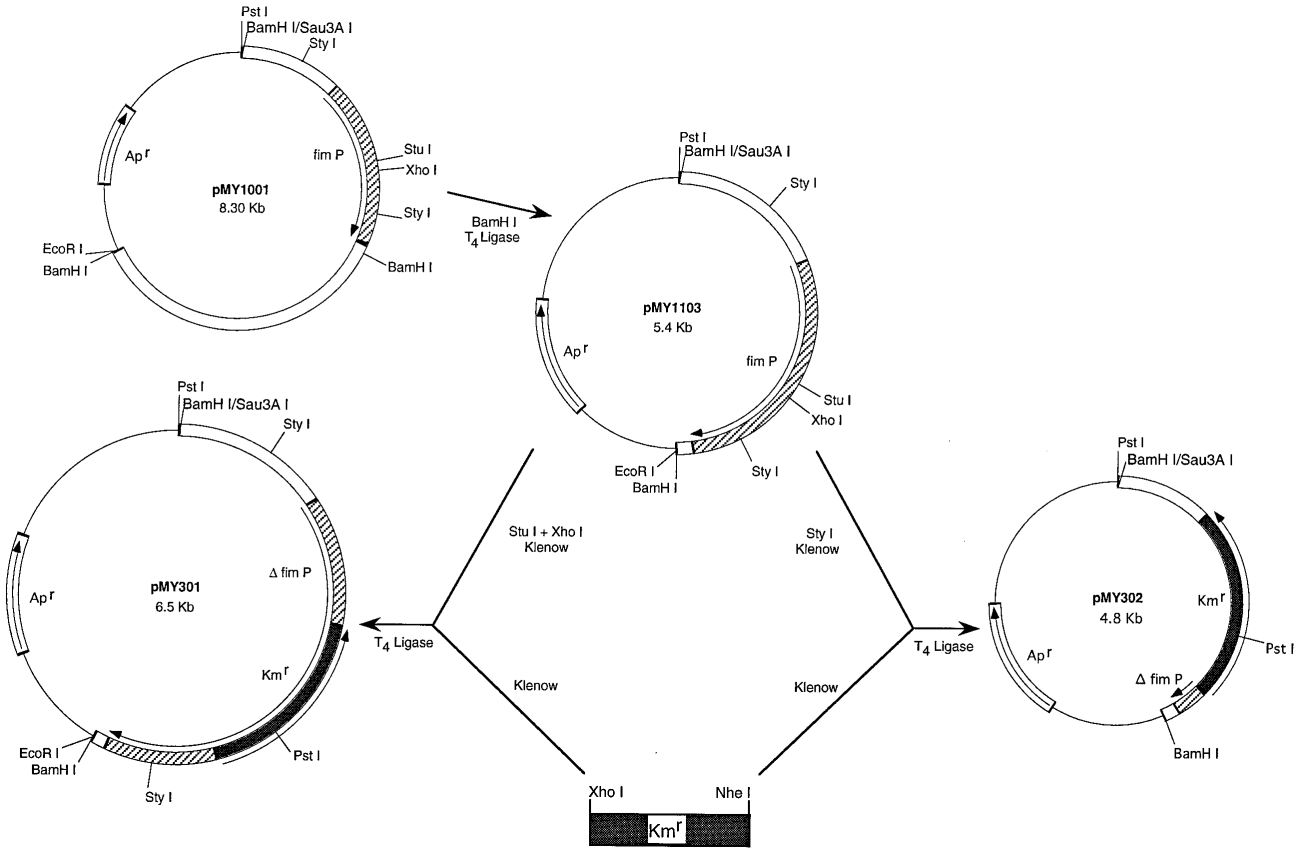


FIG. 1. Restriction endonuclease maps of derivatives of pMY001 and integration plasmids. Symbols: ——, pUC13 DNA; □▨□, *A. viscosus* T14V chromosomal DNA; ▨, *A. viscosus* T14V *fimP*; ■ Km$^r$ ■, DNA fragment derived from pJRD215 carrying the Km$^r$ gene. Ap$^r$, resistance to ampicillin. The directions of transcription of *fimP*, Km$^r$ and Ap$^r$ are indicated (arrows).

reactive protein of similar size in the cell lysate of *E. coli* carrying pMY1103 but not in those from *E. coli* carrying pMY301 or pMY302 (profile not shown). Thus, the *Stu*I-*Xho*I or *Sty*I DNA fragment internal to the *A. viscosus* T14V *fimP* was essential for the expression of immunoreactive proteins.

**Transformation of *A. viscosus* T14V with integration plasmids.** The average transformation efficiency of *A. viscosus* T14V with pMY301 was 48/μg of covalently closed circular plasmid DNA. A lower frequency of transfer (3/μg of covalently closed circular plasmid DNA) was observed with pMY302 under identical electrotransformation conditions. A total of seven transformants were generated with pMY302 in this study. None of the Km$^r$ transformants grew in a medium containing ampicillin. No extrachromosomal DNA was detected in each of 40 randomly selected Km$^r$ transformants generated with pMY301. Results of Southern blot analyses of undigested genomic DNA from these strains revealed the presence of a large DNA band that hybridized to $^{32}$P-labeled pMY301. The same DNA band in 29 of the 40 strains also hybridized to pUC13. Similarly, when undigested genomic DNA from the seven Km$^r$ transformants generated with pMY302 was hybridized to pMY302, a high-molecular-size DNA band was observed, and the same band in these strains also hybridized to pUC13. These results indicated that the transforming plasmids were not present as autonomous entities in the Km$^r$ transformants. Moreover, these plasmids had integrated into the *Actinomyces* genome by single or double crossovers.

Each Km$^r$ transformant examined by a colony dot immunoassay showed a strong reaction with the antibody prepared against *A. viscosus* T14V cells (5). None of the seven transformants generated with pMY302 reacted with the anti-*A. viscosus* T14V type 1 fimbria antibody. In contrast, 3 of 40 transformants generated with pMY301 reacted with a polyclonal and two monoclonal anti-type 1 fimbria antibodies (5). Thus, insertion of the integration plasmid DNA into *Actinomyces* strains also affected the expression of type 1 fimbriae among a majority of the transformants.

**Analysis of transformants obtained with pMY301.** Three predominant hybridization patterns were detected among the 40 Km$^r$ transformants generated with pMY301. A composite hybridization profile of restriction endonuclease-digested genomic DNA from *A. viscosus* MY50D, MY51S, and MY52S is illustrated in Fig. 2. The genomic organization of each strain was determined by analyses of the Southern blots and the restriction endonuclease map of a 9.2-kb region of *A. viscosus* T14V chromosomal DNA containing *fimP* (38). *A. viscosus* MY50D was generated by double crossover at two regions (arrows in Fig. 3A) flanking the Km$^r$ gene, since no hybridization signals were detected when pUC13 was the DNA probe (Fig. 2A). The lack of hybridization signal with the 83-bp *Stu*I-*Xho*I DNA probe indicated the replacement of this segment by the Km$^r$ gene from pMY301 (not shown). The presence of a 4.2-kb *Kpn*I-*Bam*HI DNA fragment that hybridized to *fimP* confirmed the insertion of the 1.2-kb Km$^r$ gene within the 3.1-kb *Kpn*I-*Bam*HI region (Fig. 2). In the wild-type strain,

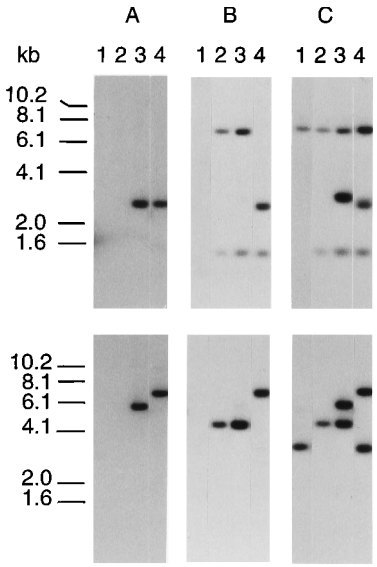


FIG. 2. Composite of Southern blot hybridization profiles of chromosomal DNA of *A. viscosus* T14V (lanes 1), MY50D (lanes 2), MY51S (lanes 3), and MY52S (lanes 4). Chromosomal DNA was digested with *Pst*I-*Bam*HI (top) or *Kpn*I-*Bam*HI (bottom), and the digested DNA was separated on a 0.7% agarose gel. The digested DNA was transferred to GeneScreen and hybridized to [$^{32}$P]dCTP-labeled pUC13 DNA (A), a 1.2-kb *Nhe*I-*Xho*I DNA fragment carrying the Km$^r$ gene (B), and a 1.85-kb DNA fragment carrying *fimP* (C) under conditions of high stringency. The sizes of selected molecular markers of a 1-kb ladder (GIBCO-BRL) are indicated on the left.

the 3.1-kb *Kpn*I-*Bam*HI DNA would be expected to hybridize to *fimP* (Fig. 3). To confirm allelic replacement in *A. viscosus* MY50D, two oligonucleotide primers (5′-CAGCTCACCGA GGAGGGCCG-3′ and 5′-CCCGCGGTGTTGGACAGGTC-3′) flanking the 83-bp *Stu*I-*Xho*I segment internal to *fimP* (41) were used in PCRs with chromosomal DNA from this strain as the template. A DNA fragment of approximately 1.2 kb that hybridized to the *Nhe*I-*Xho*I DNA fragment from pJRD215 was amplified (data not shown).

*A. viscosus* MY51S and MY52S were products of single crossovers generated by a Campbell-like insertion mechanism (2). The detection of a 2.7-kb DNA band by hybridization of genomic DNA digested with *Pst*I-*Bam*HI (Fig. 2) or *Pst*I-*Eco*RI (not shown) with $^{32}$P-labeled pUC13 indicated the acquisition of restriction endonuclease sites (e.g., *Eco*RI and *Pst*I) from the vector (Fig. 3B and C). *A. viscosus* MY51S was obtained by recombination of pMY301 at the region between *Sau*3AI and *Stu*I encompassing, in part, the 5′ coding sequence of *fimP* (Fig. 3B). The conclusion that duplication of this region had occurred was based on two observations: (i) three (6.8-, 2.8-, and 1.4-kb) *Pst*I-*Bam*HI and (ii) two (5.5- and 4.2-kb) *Kpn*I-*Bam*HI DNA fragments were detected in this strain with the *fimP* probe, while only the 6.8-kb *Pst*I-*Bam*HI or the 3.1-kb *Kpn*I-*Bam*HI DNA band would be expected in strain T14V. Duplication of this DNA region also resulted in the generation of a second *fimP* copy (designated *′fimP*) on the chromosome 3′ to the Km$^r$-interrupted Δ*fimP* (Δ*fimP*::Km$^r$) (Fig. 3B). In contrast, *A. viscosus* MY52S was generated by recombination of pMY301 at the region between the *Xho*I and *Bam*HI sites corresponding to the 3′ coding sequence of *fimP* (Fig. 3C). A complete *fimP* sequence was maintained in this strain, since a 3.1-kb *Kpn*I-*Bam*HI DNA fragment that hybridized to *fimP* was detected, as would be expected in the wild-type strain. Duplication of the DNA region of recombination resulted in the generation of a Km$^r$-interrupted Δ*fimP* 3′ to *fimP*, which accounted for the presence of a 6.6-kb *Kpn*I-*Bam*HI that hybridized to *fimP*. The detection of the same 6.6-kb DNA fragment with pUC13 or the Km$^r$ gene probe in *Kpn*I-*Bam*HI (Fig. 2)-digested DNA supported the predicted structure for this strain (Fig. 3C).

**Expression of type 1 fimbriae among Km$^r$ transformants.** A predominant protein of approximately 65 kDa that was immunostained by the anti-*A. viscosus* T14V type 1 fimbria antibody was observed in the solubilized cell wall fractions from strains T14V and 5519 (Fig. 4, lanes 2 and 3). Multiple protein bands of high molecular weight were present, consistent with a previous observation indicating the resistance of *Actinomyces* fimbriae to complete denaturation (40). No immunoreactive proteins were observed in strains 147, MY50D, and MY51S, while a protein of approximately 65 kDa was detected in MY52S (Fig. 4, lane 5) with the anti-type 1 fimbria antibody. Interestingly, the ladder of high-molecular-weight proteins, as seen in strain T14V or 5519, was not observed in this strain. Similar profiles were obtained with proteins from the cell surface and membrane-cytoplasm fractions (profiles not shown).

Intense signals that correlated with positive interactions between the biotin-labeled bacteria and immobilized PRPs were observed in strains T14V and 5519, which expressed type 1 fimbriae (Fig. 4). The reactions were specific, since no signals were detected with bovine serum albumin. No signals were observed with strain 147, which did not synthesize type 1 fimbriae (Fig. 4), or with strains MY50D, MY51S, and MY52S. All of the Km$^r$ strains formed lactose-reversible aggregates with *S. oralis* 34, indicating the presence of *Actinomyces* type 2 fimbriae (6). These strains also produced strong fluorescent signals, similar to those observed with strain T14V, in the in vitro rapid filter spot assay designed for the detection of cell-associated sialidase (42). Thus, these transformants had defects restricted only to properties associated with type 1 fimbriae.

**Stability of the *A. viscosus* transformants.** *A. viscosus* MY50D, MY51S, and MY52S were first incubated in a growth medium containing kanamycin. Diluted bacterial suspensions of stationary-phase cultures of each strain were serially transferred into a growth medium with or without kanamycin, and the genotypic and phenotypic characteristics of bacteria from cultures at the fifth, seventh, and tenth transfers were examined. The hybridization profiles of *Pst*I-*Bam*HI-digested genomic DNA from the various transfers were identical to those shown in Fig. 2 for each strain. Similarly, results of Western blot (immunoblot) analyses and in vitro adherence assays with bacterial cells from each transfer were comparable to those observed in each mutant strain before the serial transfer. Identical results were obtained with bacterial cultures prepared in the presence or absence of the antibiotic. Thus, the heterologous DNA, once integrated, was maintained stably in the *Actinomyces* genome.

## DISCUSSION

Integration plasmids are chimeras that contain DNA sequences homologous to the genome of a recipient host. Since these plasmids are incapable of autonomous replication in the recipient, the homologous DNA usually effects efficient integration into the host chromosome under selective conditions (22, 27). Plasmids of this nature have provided valuable tools for gene cloning, chromosome mapping, and insertional mutagenesis and for studies of gene regulation and function in various bacterial systems (22, 25, 27, 28, 35). The significance of the present study is that two predominant types of recombinants were generated by transformation of *Actinomyces*

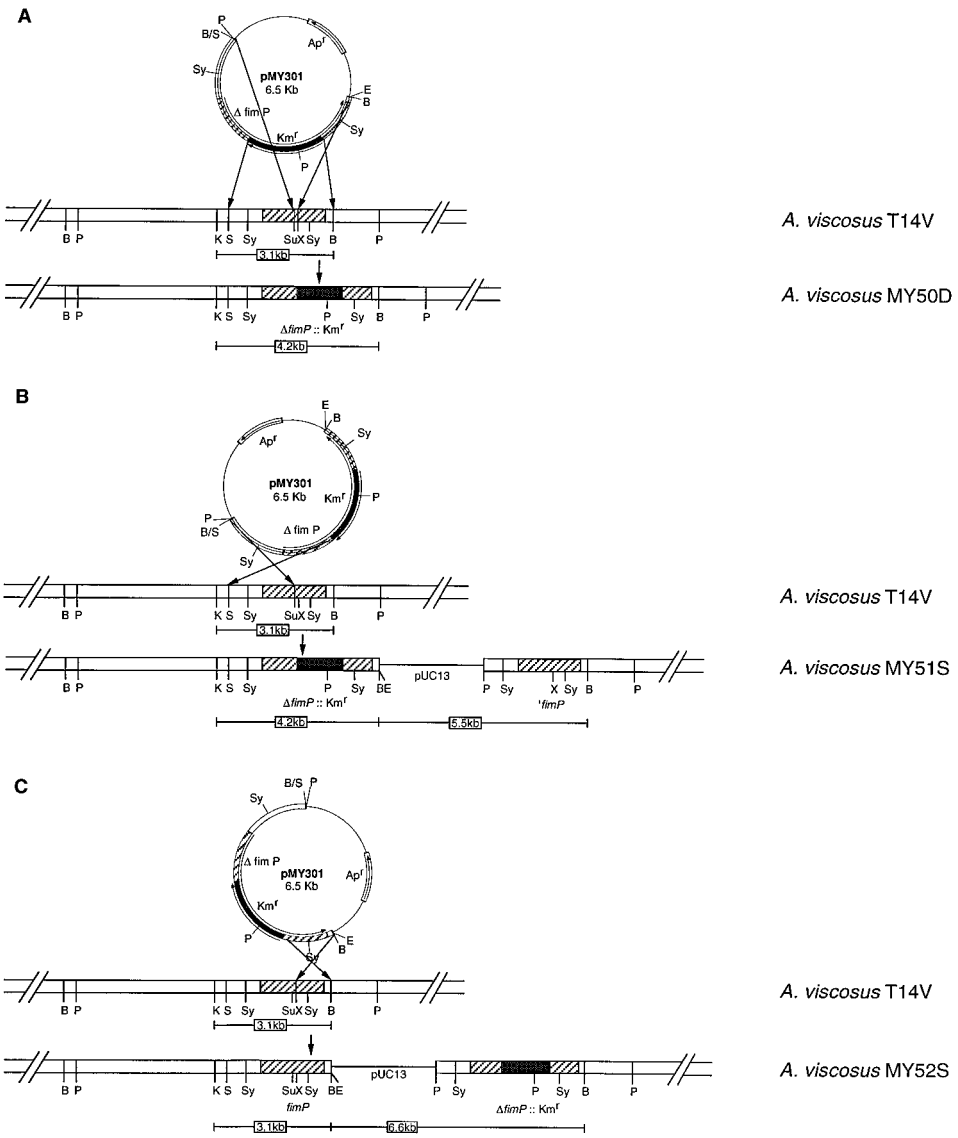


FIG. 3. Predicted mechanisms of heterologous DNA integration in *A. viscosus* T14V. (A) *A. viscosus* MY50D was obtained by replacement of the *Stu*I-*Xho*I DNA fragment from the *A. viscosus* T14V genome by the Km$^r$ gene-containing DNA fragment from pMY301 via double crossover. *A. viscosus* MY51S (B) and *A. viscosus* MY52S (C) were generated by insertion of pMY301 into the *A. viscosus* T14V chromosome by a Campbell-like integration mechanism. Only the chromosomal DNA region in close proximity to *fimP* and selected restriction endonuclease sites are illustrated. The regions encompassed by arrows are areas of homology at which crossover occurs. Symbols: ——, pUC13 DNA; □▨□, *A. viscosus* T14V chromosomal DNA; ▨, *A. viscosus* T14V *fimP*; ■ Km$^r$ ■, DNA derived from pJRD215 containing the Km$^r$ gene. Ap$^r$, resistance to ampicillin; B, *Bam*HI; P, *Pst*I; K, *Kpn*I; S, *Sau*3AI; Sy, *Sty*I; Su, *Stu*I; E, *Eco*RI; X, *Xho*I.

strains with integration plasmids; one type arose by double crossover and the other arose by single crossover mediated by a Campbell-like insertion mechanism (2). Moreover, the recombinants containing the inserted heterologous DNA were stably maintained. Genetic studies of members of this gram-positive bacterial genus have been scarce. A previous study described a reproducible procedure for genetic transfer to these bacteria (43). The present work has established a strategy that allowed the generation of mutations in these bacteria. With these findings taken together, it should now be possible to design and conduct genetic analyses of the type 1 fimbriae or any other cell surface components from *Actinomyces* spp.

The construction of the two integration plasmids for *Actinomyces* strains described in this study was facilitated by (i) the availability of a plasmid isolated from a previous study (40) that contains the *A. viscosus* T14V *fimP* and a pBR322-based replicon that does not function in *Actinomyces* spp., (ii) the expression of the Km$^r$ gene from pJRD215 in *Actinomyces* spp. (43), and (iii) the determined nucleotide sequence of *fimP* (41). The Km$^r$ gene derived from the transposon Tn*903* (26) also was considered during the early stages of plasmid construction. These studies were initiated on the basis of the finding that the Km$^r$ gene derived from either pJRD215 or Tn*903* was expressed in strains of *Mycobacterium* (16, 32). A plasmid that was similar to pMY301 except that the Km$^r$ gene was derived from Tn*903* was constructed. However, no transformants were obtained under optimal transformation conditions. Thus, *Actinomyces* spp. express the Km$^r$ gene that encodes type II aminoglycoside 3′-phosphotransferase (as present in pJRD215) but not type I aminoglycoside 3′-phosphotransferase (as present in Tn*903*) (26). Alternatively, the lack of transformants may be due to the presence on the Km$^r$

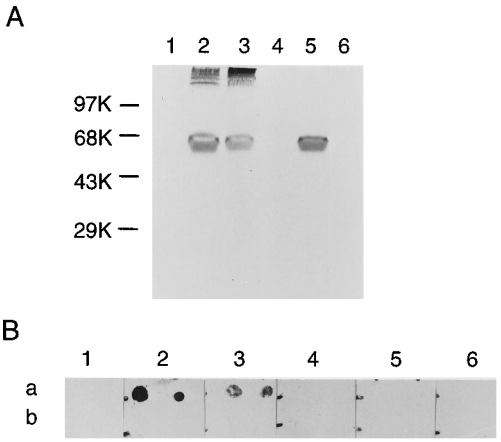


FIG. 4. Immunological and functional properties of *A. viscosus* strains. (A) Western blot analysis of proteins from solubilized cell wall fractions of *A. viscosus* 147, T14V, 5519, MY50D, MY52S, and MY51S (lanes 1 through 6, respectively). Protein samples were separated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose, and the filter was immunostained with a polyclonal anti-*A. viscosus* T14V type 1 fimbria antibody. The apparent molecular sizes in kilodaltons (K) of markers are indicated on the left. (B) Adherence of *A. viscosus* 147, T14V, 5519, MY50D, MY52S, and MY51S (lanes 1 through 6, respectively) to PRPs immobilized on a nitrocellulose membrane. Each filter square contained PRPs (a; 100 and 50 ng from left to right) and bovine serum albumin (b; 2 and 1 μg from left to right) that were applied as spots. Each square was incubated, in sequence, in a blocking buffer and then with a biotin-labeled bacterial suspension. The filters were developed with alkaline phosphatase color reagents.

gene of Tn*903* a site(s) sensitive to restriction in *Actinomyces* spp. Although both pMY301 and pMY302 had a Δ*fimP* that was interrupted by the Km$^r$ gene, greater than 95% of the *fimP* sequence was present in pMY301, while approximately 85% of the *fimP* and immediate upstream sequences were absent in pMY302 (Fig. 1). Both pMY301 and pMY302 had unequal amounts of *A. viscosus* T14V genomic DNA flanking the Km$^r$ gene. However, the length of homologous DNA flanking the Km$^r$ gene in pMY301 was longer than that in pMY302. Thus, the significantly higher frequency of transformation observed with pMY301 than with pMY302 was in agreement with the notion that the efficiency of recombination depends on the length of the transferred homologous DNA (3). By the same token, the fact that the higher percentage of recombinants generated with pMY301 exhibited the *A. viscosus* MY51S-like genomic structure was expected, since recombination at the region containing longer homologous sequences will be favored.

The integration plasmids constructed in this study also were used to transform *A. viscosus* MG1, which contains the *A. viscosus* T14V *fimP* homolog (39). The transformation efficiency ($1.1 \times 10^3$ or 10/μg of plasmid DNA) of strain MG1 with pMY301 or pMY302, respectively, was significantly higher than those observed with strain T14V. These results agreed with the previous observation that higher frequency of plasmid transfer was consistently obtained with strain MG1 (43). Significantly, the observation that plasmids carrying the *A. viscosus* T14V chromosomal DNA could be integrated into the genome of strain MG1 supported the potential of these types of plasmids in gene transfer between strains of *Actinomyces* spp. as long as enough homology is present to direct recombination.

In general, the percentage (25 or 75%) of *A. viscosus* recombinants generated with pMY301 by double or single crossovers, respectively, was consistent with the expected outcomes observed in other bacterial systems. Several transformants whose genomic structures did not agree with any one of the three predicted models also were obtained (Fig. 3). For example, one clone that appeared to be similar to strain MY51S was isolated, but results of Southern blot hybridization revealed a 2.5-kb (instead of a 2.7-kb) *Pst*I-*Bam*HI DNA fragment that hybridized to pUC13. Thus, deletions and/or rearrangements of the integrated DNA had occurred in *Actinomyces* strains, as has been observed in other bacterial systems (25, 35). Reports of studies with *Bacillus subtilis* (19, 28) or *Lactococcus lactis* subsp. *lactis* (25) have noted DNA amplification (presence of multiple copies of inserted DNA arranged in tandem) in recombinants generated by a Campbell insertion mechanism. However, no amplification of pMY301 was detected in strain MY51S or MY52S or a few other recombinants obtained by single crossover, even when they were incubated in the presence of elevated concentrations of kanamycin.

The recombinants obtained in the present study had defects only in the expression and function of type 1 fimbriae. These mutants otherwise resembled strain T14V, for example, with respect to the expression of type 2 fimbriae and cell-associated sialidase activity. Insertion of heterologous DNA in the middle of the wild-type *fimP* in strains MY50D and MY51S interrupted the reading frame of *fimP* and consequently inactivated the expression of the structural subunit. Deletions or truncations in the duplicated *fimP* copy (designated *'fimP*) in strain MY51S, which could occur during insertion-duplication, may account for the lack of expression of the structural subunit protein by this strain. Alternatively, integration of pMY301 at the region indicated may have interrupted expression of certain regulatory accessory genes that are required for *fimP* expression. In any event, the lack of fimbriae on these bacteria accounted for the failure to interact with PRPs. Interestingly, strain MY52S, which contained an uninterrupted copy of *fimP* and upstream sequences, although synthesized the structural subunit protein, also failed to adhere to PRPs. The detection of the subunit protein from a sheared cell wall fraction of MY52S supported the surface location of the protein. However, a close examination of the immunoblots revealed the absence of multiple protein bands (presumably multimers of the subunit) in strain MY52S that are frequently detected in fimbriae from strain T14V (5, 40). Thus, although insertion of heterologous DNA 3′ to *fimP* did not affect the expression of the *fimP* gene product or its export, such insertions might have interrupted the synthesis of a component necessary for the assembly and/or polymerization step in fimbria biogenesis. It is possible that the 5′ *fimP* copy has a small deletion that was too small to detect by Southern hybridization but was critical for protein function. Such a deletion also could result in the synthesis of an aberrant or modified subunit protein that reacted with the antifimbria antibodies but failed to undergo, or interfere with, the normal assembly process to produce the functional cell surface fimbriae. On the basis of the data from this study, the adherence-deficient phenotype exhibited by strain MY52S suggests a correlation between assembled fimbriae and the ability to interact with host receptors. The combined results obtained with the three mutants indicated that *fimP* is essential for fimbria synthesis and function, but synthesis of the *fimP* gene product alone is not sufficient to promote bacterial adherence. Thus, results from this study provided genetic evidence that *fimP* in the *Actinomyces* fimbrial system serves as a critical structural component. Whether the structural subunit protein also contains sites for interaction with the receptors, implying that *fimP* also serves a critical role in adherence, remains unclear. In this regard, the subunit of pili from *Neisseria* and *Pseudomonas* spp. serves as the structural and adhesive component (9, 24, 29, 34). Alternatively, insertion of the

heterologous DNA either in the middle of (in strains MY50D and MY51S) or 3′ to (in strain MY52S) *fimP* may have interrupted the expression of a component (adhesin) that is directly involved in binding to PRPs. The presence of an adhesin encoded by a gene distinct from the structural subunit gene has been described for several fimbrial systems in *E. coli* (17, 18, 20, 21). On the basis of the genetic map of MY52S, the putative adhesin gene, if it exists, would be present in close proximity 3′ to *fimP* on the *A. viscosus* T14V chromosome. Clearly, the mutants generated in this study have provided new insights into the role of *fimP* and its relationship to the functional properties associated with type 1 fimbriae. It should be possible to obtain additional mutants by a similar strategy, and they will be invaluable in the elucidation of genes involved in the biosynthesis and function of the *A. viscosus* T14V type 1 fimbriae.

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