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Synthesis and Function of *Actinomyces naeslundii* T14V Type 1 Fimbriae Require the Expression of Additional Fimbria-Associated Genes

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The nucleotide sequence of the chromosomal DNA flanking the *Actinomyces naeslundii* (formerly *A. viscosus*) T14V type 1 fimbrial structural subunit gene (*fimP*) was determined. Six open reading frames (ORFs), in the order 5′ ORF3, ORF2, ORF1, *fimP*, ORF4, ORF5, ORF6 3′, were identified. ORF1 encoded a protein of 408 amino acid residues (M_r = 39,270) and had significant sequence homology with the *A. naeslundii* T14V type 1 and *A. naeslundii* WVU45 type 2 fimbrial structural subunits. An in-frame fusion of ORF1 to the *malE* gene of the expression vector, pMAL-c2, yielded a protein that was immunostained with antibodies raised against the maltose binding protein and *A. naeslundii* T14V whole bacteria. Digestion of the fusion protein with factor Xa released a protein (apparent molecular mass of 34 kDa) that was immunostained only with the antibody directed against *A. naeslundii* T14V whole bacterial cells. Integration plasmids carrying a kanamycin resistance gene (*kan*) that was used to substitute for ORF1 or for DNA fragments internal to the coding region of the other five ORFs were used to transform *A. naeslundii* T14V. Neither type 1 fimbriae nor the 65-kDa fimbrial structural subunit was detected in mutants obtained by allelic replacement of ORF1 or ORF2. Mutants obtained by allelic replacement of ORF3 or ORF4 expressed only the 65-kDa fimbrial structural subunit. These mutants did not bind, in vitro, to proline-rich proteins that serve as the receptors for *Actinomyces* type 1 fimbriae. In contrast, a mutant in which the integration plasmid DNA had been inserted at a site close to the carboxyl terminus of ORF6 expressed type 1 fimbriae and had adherence properties similar to those observed in the wild-type strain. These results demonstrate the existence of additional genes near *fimP* that are likely to be involved in the synthesis and function of cell surface fimbriae of *A. naeslundii* T14V.

Type 1 fimbriae of *Actinomyces* spp. are major cell surface components that bind to salivary proline-rich proteins (PRPs) that coat the tooth enamel (5, 16). This specific interaction is one of several mechanisms that lead to a firm attachment of primary colonizers, such as members of *Actinomyces* spp. and others (24, 30), to the tooth surface. The initial adherence of *Actinomyces* spp. in the oral cavity contributes, in part, to the eventual presence of various other bacterial species in the oral cavity, since they coaggregate with a high degree of specificity with various plaque bacteria, including certain putative periodontal pathogens (24, 27). While it is clear that *Actinomyces* fimbriae serve as major adherence factors, the mechanism by which these cell surface molecules interact with host receptors and other plaque bacteria is still poorly understood.

Fimbriae are ubiquitous cell surface components of numerous gram-negative bacteria, and a wealth of information is available concerning the organization of genes involved in their synthesis, assembly, and function (9, 14, 18, 22). In contrast, little is known regarding the genetics of fimbriae produced by *Actinomyces* spp. or other gram-positive bacteria (13, 19, 41). Results of earlier studies that focused on the cloning and sequencing of the structural subunit gene of the *Actinomyces naeslundii* T14V type 1 and type 2 fimbriae (11, 44) and the *A. naeslundii* WVU45 type 2 fimbriae (45) indicate that, like fimbriae of a majority of gram-negative bacteria, *Actinomyces* fimbriae are composed of a major subunit. No amino acid homologies have been noted between the sequence of *A. naeslundii* T14V type 1 or *A. naeslundii* WVU45 type 2 fimbrial subunit and those of other bacterial fimbriae. However, significant homology was observed between the subunits of these two functionally and immunologically distinct fimbrial types (46). Results of a recent study further demonstrate that the *A. naeslundii* T14V type 1 fimbrial subunit gene (*fimP*) is highly conserved among various strains of *Actinomyces* spp. of human or nonhuman origin (42). Thus, the *Actinomyces* fimbriae very likely evolved from an ancestral gene distinct from those associated with other bacterial fimbriae.

This study describes the results of further efforts aimed at a molecular characterization of the *A. naeslundii* T14V type 1 fimbriae. Since genes involved in the biosynthesis of macromolecules in prokaryotes are usually clustered within a chromosomal DNA region, studies were initiated to isolate DNA fragments flanking the *A. naeslundii* T14V *fimP* gene. A 9.3-kb DNA fragment including the *fimP* locus was mapped, and six putative open reading frames (ORFs) were identified based on the nucleotide sequence. Expression of the putative genes in *Escherichia coli* was investigated with several in vitro protein expression systems. A series of integration plasmids that consisted of the kanamycin resistance gene (*kan*) from pJRD215 (8) substituting for either all or a segment of the coding region of each ORF was constructed. These plasmids were used to transform *A. naeslundii* T14V by a previously established transformation procedure (48). Mutants generated by homologous recombination were examined for type 1 fimbria expression and for the ability to adhere to PRPs. The results suggest that some of the putative ORFs flanking the *A. naeslundii* T14V *fimP* gene are likely to be involved in the expression and

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Relevant phenotype and genotype[a]	Reference or source
Bacterial strains		
A. naeslundii		
T14V (1^+ 2^+)[b]	Wild-type strain, expresses type 1 and 2 fimbriae, Km^s Sm^r	7
147 (1^- 2^-)[c]	Lacks both types 1 and 2 fimbriae, Km^s Sm^r	7
MY50D	Lacks type 1 fimbriae, nonadherent, Km^r Sm^r Δ*fimP*::*kan*	43
MY306-16	Lacks type 1 fimbriae, nonadherent, Km^r Sm^r ΔORF1::*kan*	This study
MY308-22	Lacks type 1 fimbriae, nonadherent, Km^r Sm^r ΔORF2::*kan*	This study
MY202-6	Nonadherent, expresses only the fimbrial structural subunit, Km^r Sm^r ΔORF3::*kan*	This study
MY304-24	Nonadherent, expresses only the fimbrial structural subunit, Km^r Sm^r, ΔORF4::*kan*	This study
MY304-2	Expresses type 1 fimbriae, adheres to PRPs, contains pMY304, interruption of chromosome occurs at nucleotide 8665, Km^r Sm^r	This study
E. coli		
DH5α	ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^-) *supE44 thi1* λ^- *gyrA relA1* F^- Δ(*lacZYA-argF*)U169	GIBCO-BRL
CSR603	F^- *recA1 uvrA6 phr-1 thi-1 leuB6 lacY1 galK2 argE3 rpsL31 tsx-33 supE44 gyrA98* λ^-	35
LE329	*hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 mcrA*	Promega
JM109(DE3)	Δ(*lac-pro*)*endA1 gyrA96 thi hsdR17 supE44 relAT recA*/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15 λ(DE3)	Promega
Plasmids		
pMY1012	Contains a 0.7-kb *Hin*dIII/*Sst*I and a 1.2-kb *Bam*HI/*Pst*I DNA fragment flanking the 5′ and 3′ regions of *fimP*	42
pMY261	λGEM-11 recombinant clone that contains DNA sequences extended to the left of *fimP*	This study
pMY263	λGEM-11 recombinant clone that contains DNA sequences extended to the right of *fimP*	This study
pMY261A	Contains the 6.8-kb DNA from pMY261 subcloned into pHSG575	This study
pMY261A-61	DNA fragment containing ORF3 subcloned into pGEM5Zf(+)	This study
pMY202	Derivative of pMY216A-61, ΔORF3::*kan*; deletion of a 1.0-kb *Sty*I DNA fragment internal to ORF3 and substituted with *kan*	This study
pMY261A-63	DNA fragment containing ORF1 and ORF2 subcloned into pGEM7Zf(+)	This study
pMY308	ΔORF2::*kan*, a derivative of pMY261A-63, obtained by the deletion of an 80-bp *Stu*I-*Stu*I DNA fragment internal to ORF2 and substitution of the deletion with *kan*	This study
pMY261A-65	DNA fragment containing ORF1 and the 5′ end of *fimP* subcloned into pGEM7Z(+)	This study
pMY261A-65D	ORF1-containing DNA fragment subcloned into pGEM7Zf(+)	This study
pMY306	ΔORF1::*kan*, a derivative of pMY261A-65D obtained by the deletion of a 1.1-kb *Nru*I-*Nru*I DNA fragment internal to ORF1 and substitution of the deletion with *kan*	This study
pMY261A-72	ORF2 and ORF3 containing DNA fragment subcloned into pGEM7Zf(+)	This study
pMY261A-68	ORF2 containing DNA fragment subcloned into pGEM7Zf(+)	This study
pMY261A-100	DNA fragment containing the complete coding sequences of *fimP*, ORF1, ORF2, and ORF3	This study
pMY1113	Contains the 2.7-kb DNA derived from pMY263 subcloned into pUC13	This study
pMY1113E	Contains the 2.1-kb *Bam*HI DNA fragment derived from pMY1012 subcloned into pGEM7Zf(+). This DNA fragment starts at the *Bam*HI site 3′ to *fimP* and ends at a site upstream of the *Sst*I site located at the end of the 9.3-kb chromosomal region	This study
pMY304	ΔORF4::*kan*, a derivative of pMY1113E obtained by deletion of a 97-bp *Hin*cII DNA fragment internal to ORF4 and substitution with *kan*	This study
pMAL-c2	*malE* fusion plasmid, Ap^r	New England BioLabs
pMAL-c2-ORF1	Contains the coding sequence of ORF1 cloned into *Pst*I-*Eco*RI of pMAL-c2, Ap^r	This study
λGEM-11	Derivative of EMBL3	Promega
pJRD215	Sm^r Km^r Mob^+	8
pHSG575	Cm^r	40
pGEM5Zf(+)	Ap^r	Promega
pGEM7Zf(+)	Ap^r	Promega
pUC13	Ap^r	Gibco-BRL

[a] Km, kanamycin; Sm, streptomycin; Ap, ampicillin; Cm, chloramphenicol; Mob, mobilization function.
[b] The wild-type strain was made resistant to streptomycin by incubation in a growth medium containing streptomycin.
[c] Variant of *A. naeslundii* T14V that lacks both types 1 and 2 fimbriae.

function of type 1 fimbriae. To our knowledge, this study is the first report that multiple genes are required in the biogenesis of fimbriae in gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are summarized in Table 1.

Construction and screening of *A. naeslundii* T14V genomic libraries. *A. naeslundii* T14V genomic DNA (50 μg) was partially digested with *Sau*3AI (Life Technologies, Inc., Gaithersburg, Md.), and DNA fragments of 9 to 18 kb were obtained by sucrose density gradient centrifugation (34). Aliquots of the *Sau*3AI DNA fragments were mixed in the presence of T4 DNA ligase with the *Bam*HI arms of the bacteriophage vector λGEM-11 (Promega Corp., Madison, Wis.), a derivative of the replacement vector, EMBL3 (15). The procedures for in vitro packaging of phage and transfection of *E. coli* LE392 with the packaged DNA were those recommended by the manufacturer. Plaques from Luria-Bertani (LB) (34) agar plates were transferred to sterile nitrocellulose circles (Schleicher & Schuell, Inc., Keene, N.H.) and hybridized under conditions of high stringency (42) to [^{32}P]dCTP (3,000 mCi/mol; Du Pont New England Nuclear, Boston, Mass.)-labeled DNA probes. Plaques with strong hybridization signals were purified at least three times, and phage DNA was isolated as described previously (34).

Subcloning and DNA manipulations. Purified phage DNA from recombinant clones was digested with various restriction endonucleases (Life Technologies) and separated by agarose gel electrophoresis in Tris-acetate-EDTA buffer (34),

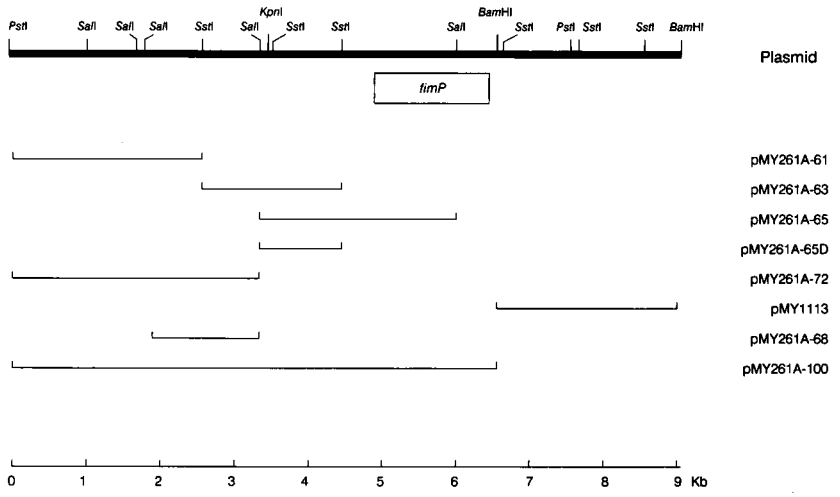

FIG. 1. Physical map of a 9.3-kb region of the *A. naeslundii* T14V chromosome containing the type 1 fimbrial structural subunit gene (*fimP*). The relative locations of *A. naeslundii* DNA (⌊____⌋) carried in each plasmid are illustrated. Only those restriction endonuclease recognition sites used for the construction of the plasmids are indicated.

and isolated DNA fragments were eluted from agarose by using reagents from an Elu Quik kit (Schleicher & Schuell). These DNA fragments were subcloned onto pHSG575 (40) and transformed into *E. coli* DH5α, and the transformants were selected on LB plates containing chloramphenicol (30 μg/ml). For DNA sequencing and expression studies, selected DNA fragments were subcloned onto pGEM5Zf(+) or pGEM7Zf(+) (Promega) in both orientations with respect to the *lacZ* promoter of the vector and transformed into *E. coli* DH5α. Transformants were selected on LB plates supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis, Mo.), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Life Technologies), and ampicillin (100 μg/ml; Sigma). Purified plasmid DNA was prepared by CsCl-ethidium bromide (EtBr) density gradient centrifugation. Nested deletions of purified plasmid DNA were obtained by the method of Henikoff (21), and double-stranded plasmid DNA sequencing was conducted by the dideoxy method of Sanger et al. (36). Primers for DNA sequencing included the M13 forward and reverse primers (Life Technologies) and specific oligonucleotides based on the determined sequence. The custom primers were prepared on a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).

In vitro protein expression studies. Plasmid-encoded proteins were examined by an in vitro transcription-translation system described previously (1, 50) (Amersham Corp., Arlington Heights, Ill.). Briefly, CsCl-EtBr gradient-purified plasmid DNA (15 μg) was incubated with the 30S cell extract in a medium containing [^{35}S]methionine (1,050 Ci/mmol, 5 μCi/ml; New England Nuclear) for 30 min at 37°C. Methionine (final concentration, 40 mg/ml) was then added, and the mixture was incubated for 5 min. The labeled cells were suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (26) and boiled for 5 min. Aliquots (10^6 dpm) were analyzed by PAGE and autoradiography as described previously (47). Plasmid-encoded proteins also were assessed with a bacteriophage T7 RNA polymerase expression system as described by Studier and Moffatt (39), with modifications. Purified plasmid DNA was transferred to *E. coli* JM109(DE3) (Promega). A mid-exponential-phase culture (optical density at 590 nm = 0.5) in M9 medium (34) supplemented with 10 mM $MgSO_4$, 0.4% glucose, 0.001% thiamine, and ampicillin (100 μg/ml) was induced by the addition of IPTG (2 mM) for 1 h at 37°C. Rifampin (200 μg/ml; Sigma) was then added, and the culture was incubated for another 30 min. The induced culture was incubated with ^{14}C-amino acid mix (2,000 μCi/mmol; final concentration, 8 μCi/ml; Amersham) or [^{35}S]methionine (1,050 mCi/mmol, 8 μCi/ml; Du Pont New England Nuclear) at 37°C for 1 h. Radiolabeled cells were washed with phosphate-buffered saline (pH 7.2) and suspended in SDS-PAGE sample buffer, and aliquots were denatured at 100°C for 5 min prior to PAGE and autoradiography. Purified plasmid DNA also was transferred to *E. coli* CSR603, and protein expression was examined by maxicell analysis (35) in a sulfate-free medium supplemented with [^{35}S]methionine (final concentration, 5 μCi/ml).

Expression of maltose binding protein-ORF1 fusion protein. The ORF1 coding sequence was amplified by PCR in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Mass.). Two primers (5′ GCCGTGAA TTCACGATGC 3′ and 5′ GTTGGGCTGCAGGAGGG 3′) with recognition sequences for *Eco*RI and *Pst*I, respectively, were designed to ensure in-frame fusion of ORF1 3′ to the *malE* gene of pMAL-c2 (New England BioLabs, Inc., Beverly, Mass.) (28, 33). The PCR product was digested with *Eco*RI and *Pst*I and purified by agarose gel electrophoresis, and the eluted DNA was mixed with pMAL-c2 digested previously with the same enzymes. The ligation mixtures were used to transform *E. coli* DH5α, and transformants were selected on LB agar supplemented with ampicillin (100 μg/ml), IPTG (0.3 mM), and X-Gal (80 μg/ml). *E. coli* carrying the recombinant plasmid (pMAL-c2-ORF1) was induced with IPTG (final concentration of 0.25 mM) for 2 h at 30°C. Bacterial cells were suspended in a buffer solution composed of 100 mM Tris (pH 8.0), 10 mM EDTA, and 200 μg of lysozyme per ml. Cells were disrupted (Branson Ultrasonics, Danbury, Conn.), and the sonicated lysate was applied onto an amylose resin affinity column (New England BioLabs). The bound protein was eluted with 10 mM maltose under conditions recommended by the manufacturer, with modifications. Aliquots of the eluted protein were digested with factor Xa (1.5 to 2.5% by weight; New England BioLabs) at 23°C for 8 or 20 h. The efficiency of protein purification was monitored by SDS-PAGE followed by Coomassie blue staining and Western blot analysis using antibodies against *A. naeslundii* T14V fimbriae or whole bacteria (6) or an antiserum against purified maltose binding protein (New England BioLabs).

Construction and characterization of *A. naeslundii* T14V mutants with deletions in various ORFs. Recombinant plasmid carrying various ORFs (Fig. 1) was digested with restriction endonucleases that resulted in the deletion of a DNA fragment internal to a specific ORF. The DNA fragment-containing vector was treated with the Klenow large fragment, mixed with the *Nhe*I-*Xho*I DNA fragment carrying the *kan* gene from pJRD215 (8) that had been treated with the Klenow large fragment. The ligation mixtures were used to transform *E. coli* DH5α, and transformants were selected on LB plates containing kanamycin and ampicillin (50 and 100 μg/ml, respectively). CsCl-EtBr density gradient-purified integration plasmid was used to transform *A. naeslundii* T14V by electroporation (48), and transformants were selected on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) containing kanamycin (40 μg/ml). Chromosomal DNA from transformants was isolated (43), and the predicted restriction endonuclease map of each mutant was determined based on Southern blot analyses as described previously (42). Proteins released from the bacterial cell surface, solubilized cell walls, and cytoplasmic membranes from *Actinomyces* strains were prepared as described previously (43). Aliquots of the various protein fractions were examined by Western blot analysis with the immunoglobulin G (IgG) fraction of a monospecific polyclonal, a monoclonal anti-*A. naeslundii* T14V type 1 fimbria, or a polyclonal anti-*A. naeslundii* T14V whole bacterium antibody (6). The ability of each of the mutants to bind to PRPs, the receptors for *Actinomyces* type 1 fimbriae (16), was assessed by the in vitro adherence assay described previously (43).

Sequence analysis. Nucleotide and deduced amino acid sequences were analyzed by software programs from the University of Wisconsin Genetic Computer Group (GCG) (version 7.0) (10). Sequence similarities were assessed by the program BESTFIT with a gap weight and gap length of 3.0 and 0.1, respectively, and homology searches for similarities to other bacterial proteins in published databases was performed with the program BLASTP (2, 32).

Nucleotide sequence accession numbers. The DNA sequences described in this report corresponding to regions 5′ and 3′, respectively, of *fimP* (46) have been assigned GenBank accession no. U85708 and U85709.

RESULTS

Analysis of DNA sequences flanking the *A. naeslundii* T14V type 1 fimbrial subunit gene, *fimP*. Approximately 10^3 plaques

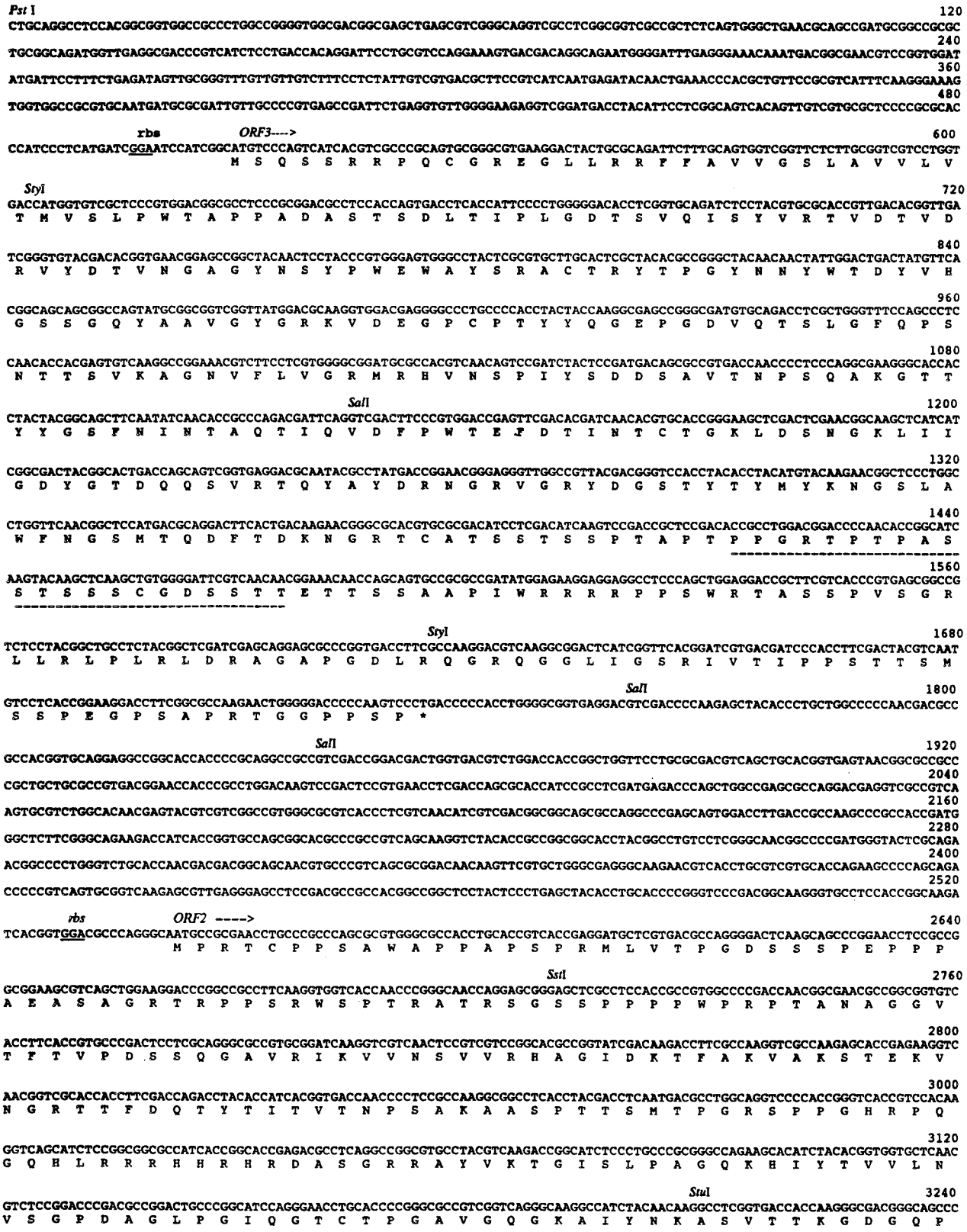

FIG. 2. Complete nucleotide sequence of the DNA regions 5′ and 3′ of the *A. naeslundii* T14V *fimP* gene. Only a portion of the *fimP* (nucleotides 4825 to 6424) sequence published previously (46) is included for reference. The deduced amino acid sequences of six putative ORFs (ORF1 to ORF6), along with the putative ribosomal binding sequences (*rbs*) (underlined) and the termination codon (*), are indicated. The direction of transcription of each ORF and *fimP* is indicated by a horizontal arrow. The prokaryotic lipoprotein attachment motif identified in ORF3 is highlighted by a dotted underline.

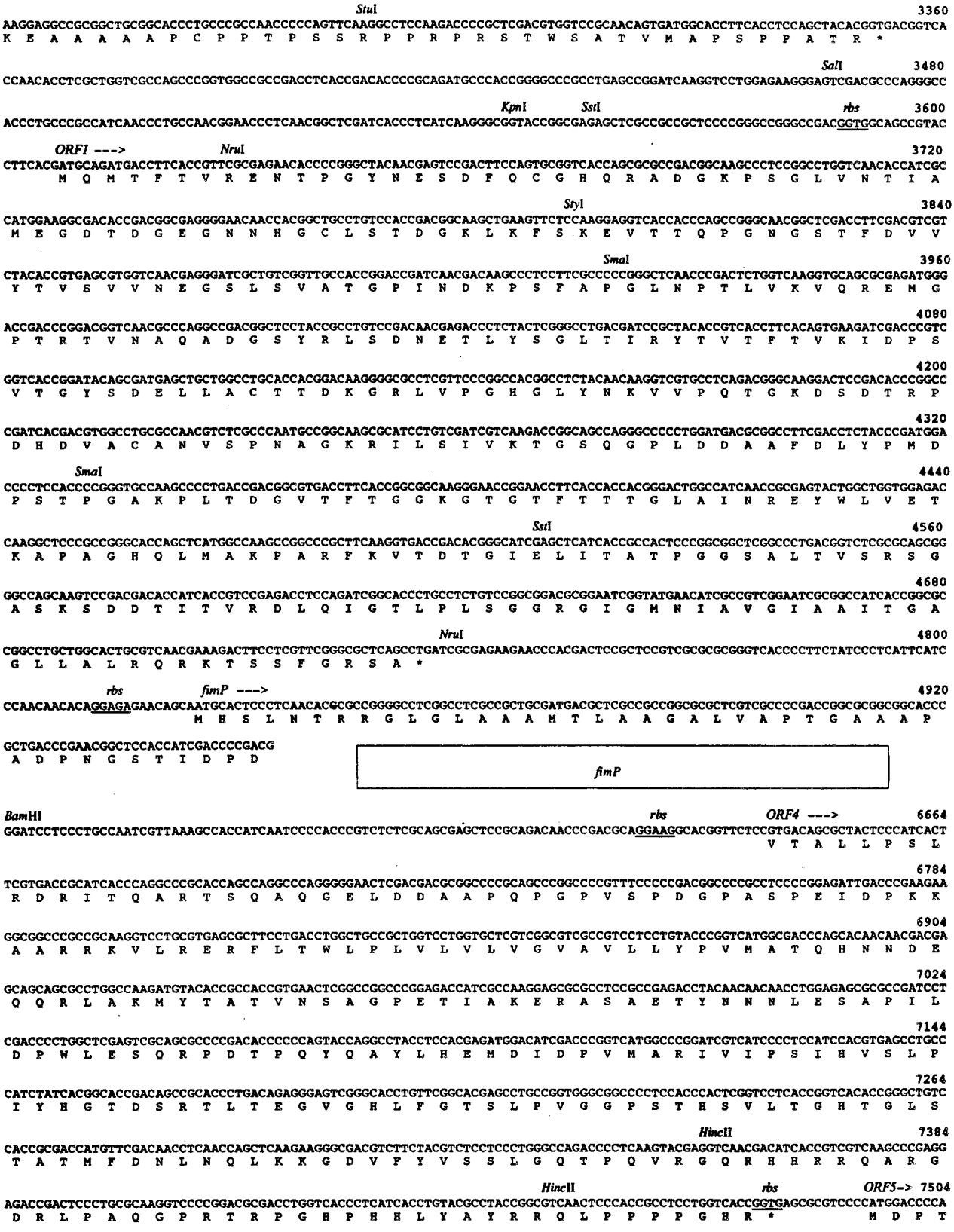

FIG. 2—*Continued.*

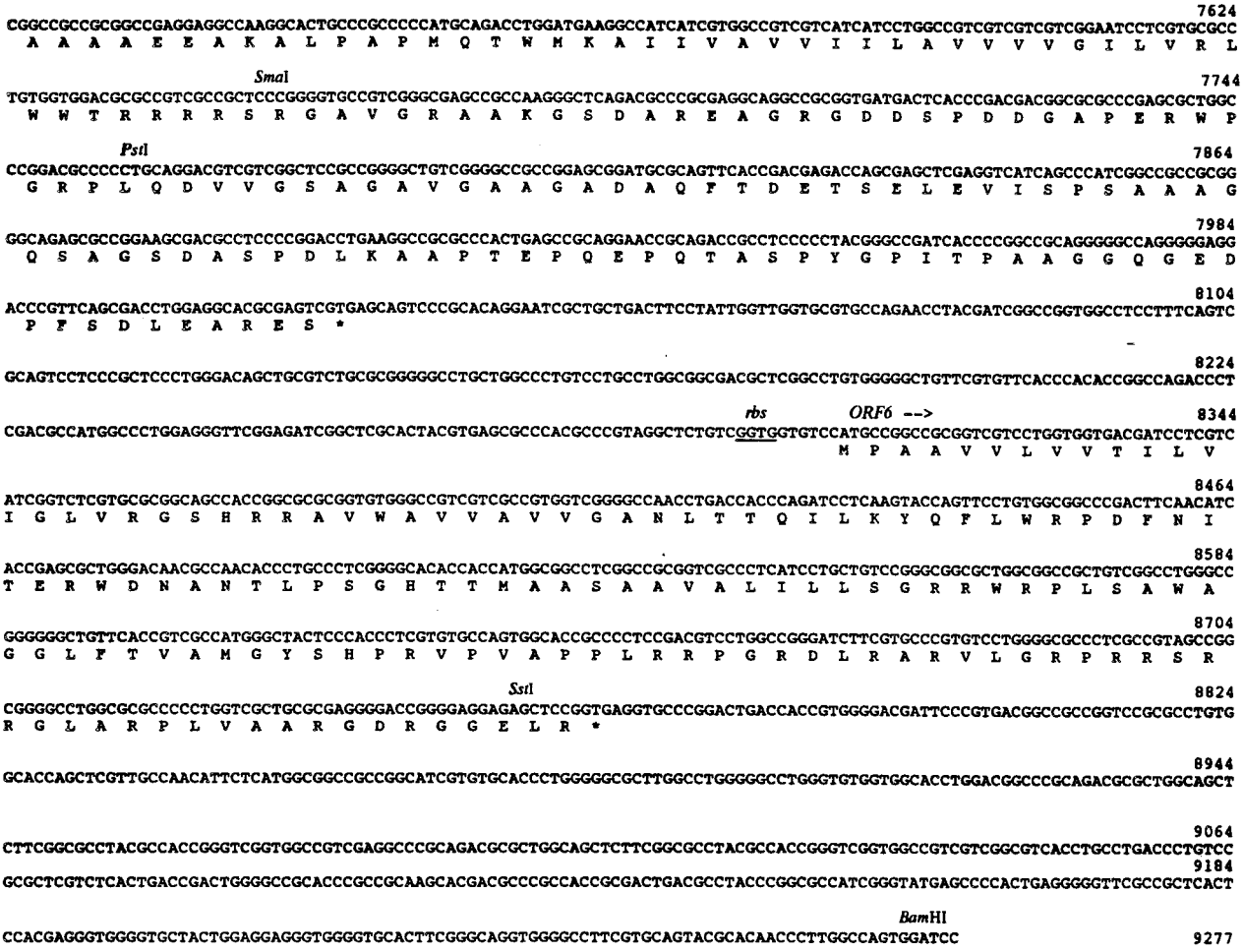

FIG. 2—*Continued.*

from the library of *A. naeslundii* T14V chromosomal DNA cloned into λGEM-11 were screened by hybridization to three DNA probes. The probes included a 1.9-kb DNA fragment consisting of the *A. naeslundii* T14V type 1 fimbrial subunit gene, *fimP*, and 0.7- and 2.1-kb DNA fragments located immediately 5′ and 3′, respectively, of *fimP* (42). Strong hybridization signals were detected in 12 plaques with the *fimP* DNA probe. Of these, two, designated pMY261 and pMY262, hybridized to the 2.1-kb but not the 0.7-kb DNA probe, and three, designated pMY263, pMY264, and pMY265, hybridized to the 0.7-kb but not the 2.1-kb DNA probe. Partial restriction endonuclease maps of the inserted *A. naeslundii* T14V DNA in pMY261 and pMY263 were determined. A 6.8-kb *Pst*I DNA fragment derived from pMY261 contained DNA extended to the left and overlapped only a small portion of *fimP*. A 2.7-kb DNA fragment derived from pMY263 extended beyond the 3′ end of *fimP*. These DNA fragments were subcloned onto pHSG575 and pUC13 for the construction of pMY261A and pMY1113, respectively (Table 1). A map of the contiguous 9.3-kb segment of chromosomal DNA containing the *A. naeslundii* T14V *fimP* is shown in Fig. 1. Additional plasmids carrying various DNA fragments (indicated in Fig. 1 as single horizontal lines) derived from this 9.3-kb region were used to determine the sequence of the DNA flanking *fimP*. Analysis of the nucleotide sequence revealed the presence of six putative ORFs, in the same orientation as *fimP* (Fig. 2). No ORFs of significant size were detected on the reverse strand. Based on the locations of potential ribosomal binding sites (25, 38) (Fig. 2, underlined sequences), the putative initiation codons of ORF1, -2, -3, -5, and -6 were AUG, whereas that of ORF4 was GTG. Analysis of the codon usage pattern by these ORFs was conducted with the GCG program Codonpreference (3, 17) and compared to a calculated codon usage frequency table prepared from the deduced amino acid sequence of *A. naeslundii* T14V *fimP* (46). The results indicated that each ORF had a statistical expression probability of at least 1.5 over a threshold of 0.1. These values suggested that these putative genes would be expressed.

No significant sequence homology was observed between other bacterial proteins and the deduced amino acid sequence of the product of ORF2 (nucleotides 2542 to 3351, encoding a protein of 270 amino acids with a predicted molecular weight of 28,174) or ORF6 (nucleotides 8306 to 8761, encoding a peptide of 152 residues with a predicted molecular weight of 16,668). Some degree of sequence homology was observed between the predicted protein (280 amino acids with a calculated molecular weight of 30,877) encoded by ORF4 (nucleotides 6642 to 7481) and several precursors of PRPs. A motif search revealed that the gene product (408 amino acid residues with a molecular weight of 44,040) of ORF3 (nucleotides 509 to 1732) contained a consensus prokaryotic lipoprotein attachment binding site (Fig. 2, dotted underline) (20), suggesting

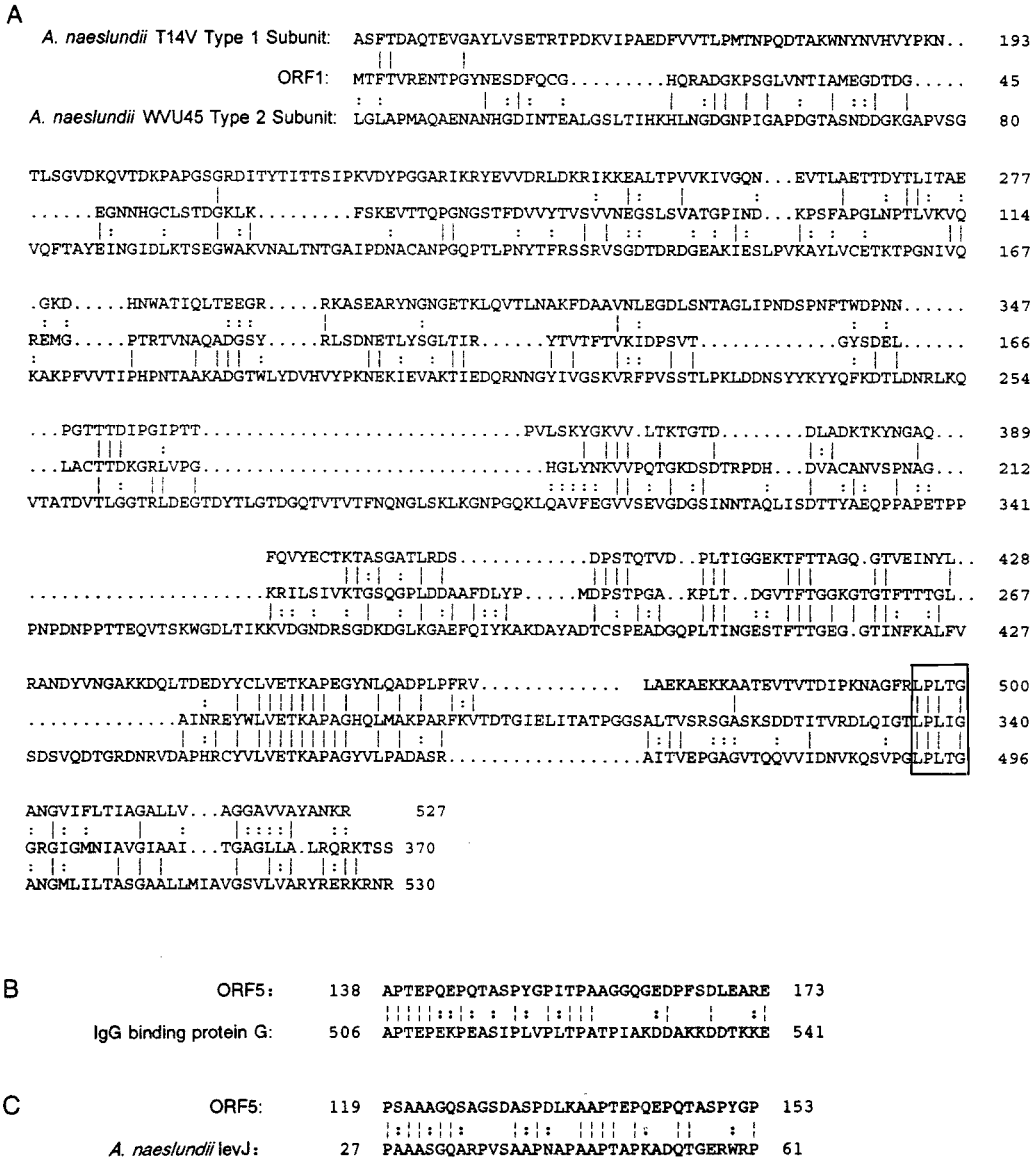

FIG. 3. (A) Alignment of the deduced amino acid sequences encoded by ORF1 (middle sequence), *A. naeslundii* T14V type 1 fimbrial subunit (top sequence), and *A. naeslundii* WVU45 type 2 fimbrial subunit (bottom sequence). The protein sequences were aligned by the GCG program BESTFIT (gap weight = 3.0, gap length = 0.1). The typical gram-positive membrane-anchoring sequence (LPXTG) is boxed. (B) Sequence homology of a 36-amino-acid sequence of the ORF5 gene product and the membrane-spanning region of IgG binding protein G of *Streptococcus* strain G148 (31). (C) Sequence homology of a 35-amino-acid sequence of ORF5-encoded protein and the *A. naeslundii* T14V levanase (29). Identical (|) and conserved substituted (:) amino acid residues are indicated.

that it might be a membrane protein. The carboxyl-terminal end of the deduced amino acid sequence of ORF5 (nucleotides 7493 to 8016 encoding a protein of 174 amino acids with a predicted molecular weight of 17,796) shared significant homology (38% sequence identity and 58% similarity over a range of 36 residues) with streptococcal IgG binding proteins (23, 31) (Fig. 3B; sequence shown is that of *Streptococcus* strain G148 IgG binding protein). Interestingly, significant homology (42% identity and 62% similarity for a peptide of 35 amino acid residues) also was noted between ORF5 and *A. naeslundii* T14V levanase (29) (Fig. 3C). Significantly, the gene product of ORF1 (nucleotides 3608 to 4732, encoding a protein of 375 residues with a calculated molecular weight of 39,270) had overall 46 and 49% sequence similarities with the amino acid sequences of the fimbrial structural subunits of *A. naeslundii* T14V type 1 and *A. naeslundii* WVU45 type 2 (Fig. 3C). A high degree of homology was observed at the carboxyl-terminal end that included a conserved proline-containing peptide with up to 12 identical amino acid residues (amino acids 273 to 294) (46) and a typical membrane-anchoring sequence (LPXTG) (Fig. 3A, boxed sequence) that is common among several gram-positive bacterial surface proteins (12, 37), including the IgG binding proteins (23, 31).

In vitro protein expression of ORFs. The expression of the identified ORFs in *E. coli* was assessed by using three different in vitro protein expression systems. A series of plasmids containing DNA fragments encoding one or more ORFs was generated in pGEM5Zf(+) or pGEM7Zf(+) (Fig. 1 and Table 1). In addition, we constructed a chimeric clone that contained the 6.8-kb *Pst*I DNA fragment subcloned into pGEMEX-2 to yield

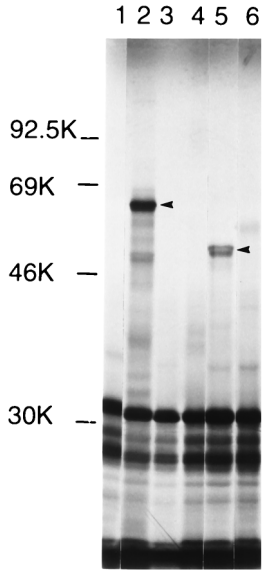

FIG. 4. SDS-PAGE of [^{35}S]methionine-labeled proteins obtained from in vitro transcription-translation assays. Approximately 10^6 dpm from each assay mixture containing pGEM7Zf(+), pMY261A-100, pMY261A-61, pMY261A-63, pMY261A-65, and pMY1113 (lanes 1 through 6, respectively) was analyzed on an SDS–10% polyacrylamide gel followed by autoradiography. The molecular masses of ^{14}C-labeled protein markers are indicated on the left. Arrowheads denote the 65- and 47-kDa proteins expressed by pMY261A-100 and pMY261A-65, respectively, that also reacted with anti-*A. naeslundii* T14V type 1 fimbriae when these proteins were subsequently transferred to nitrocellulose and immunostained with the antibody.

pMY261A-100. Results from in vitro transcription-translation analyses showed that two plasmids, pMY261A-100 and pMY261A-65, directed the expression of a 65- and a 47-kDa protein, respectively (Fig. 4, lanes 2 and 5). These were the expected fimbrial subunit protein and the truncated amino-terminal fimbrial subunit protein described previously (44). No proteins with molecular sizes predicted by each ORF were detected in the assay mixtures with plasmids containing the different ORFs (Fig. 4, lanes 3, 4, and 6). Both the 65- and 47-kDa proteins were immunostained by a polyclonal or a monoclonal antibody raised against the *A. naeslundii* T14V type 1 fimbriae (6) on a Western blot; the profiles (not shown) were similar to those published previously (44). Results obtained with maxicells and the T7 RNA polymerase expression plasmid system also indicated expression plasmid-encoded proteins only in pMY261A-100 and pMY261A-65, as shown in Fig. 4.

Expression and preliminary characterization of ORF1 and its gene product. A predominant protein band of approximately 80 kDa was observed in a Coomassie blue-stained SDS-polyacrylamide gel containing cell lysates from a recombinant *E. coli* strain carrying pMAL-c2-ORF1 (Table 1). This protein was observed only in *E. coli* cell lysates derived from cultures that had been induced with IPTG and was not detected in the cell lysate of *E. coli* carrying pMAL-c2 (profile not shown). The size of the presumed fusion protein was in agreement with the combined molecular masses of maltose binding protein (42 kDa) and the predicted ORF1 protein (39 kDa). The cell lysate was subject to a one-step purification on an affinity column which consisted of amylose resin; the two fractions obtained by elution of the fusion product with 10 mM maltose were digested with factor Xa. A protein of approximately 34 kDa that was immunostained with the IgG fraction of an antibody prepared for *A. naeslundii* T14V whole bacteria was detected (Fig. 5A, lanes 2 and 3). Based on the relative amounts of 34-kDa protein detected, it seemed that most of the fusion protein was present in the first fraction. However, more than 50% of the fusion product remained undigested even with a high factor Xa concentration (2.5% by weight) (Fig. 5A, lane 3). The 34-kDa protein did not cross-react with anti-maltose binding protein antibody (Fig. 5B) or with anti-*A. naeslundii* T14V type 1 fimbria antibodies (profile not shown). Thus, although the ORF1 gene product contained epitopes shared by certain *A. naeslundii* T14V cell surface antigens, it was immunologically distinct from the fimbrial structural subunit.

Potential role of the putative ORFs in fimbria synthesis and function. The potential role of ORF1 and other putative ORFs in fimbria synthesis was evaluated with the aid of several integration plasmids, including pMY202, -304, -306, and -308 (Table 1). Those kanamycin-resistant transformants that did not hybridize to pGEM5Zf(+) or pUC plasmid DNA were presumed to be mutants that had arisen by homologous recombination. Genomic DNA from each mutant was digested with various restriction endonucleases and hybridized to a panel of DNA probes which consisted of the *kan* gene, *fimP*, and DNA fragments corresponding to the various ORFs. Figure 6 summarizes the predicted physical maps of isogenic mutant strains from this study. We generated allelic exchange mutants in which a DNA fragment internal to the wild-type alleles of ORF1, -2, -3, and -4 was replaced by the *kan* gene, but no allelic exchange mutants were obtained for ORF5 or ORF6. However, a mutant strain with an interrupted ORF6, designated MY304-2, was obtained by transformation of *A. naeslundii* T14V with pMY304. Southern blot hybridization analyses revealed that pMY304 was inserted into the *A. naeslundii* 14V chromosome by a single crossover recombinational event, via the Campbell insertion-duplication mechanism (4), and the crossover region spanned nucleotides 7452 to 8665. The insertion of heterologous DNA derived from pMY304-2 was mapped to nucleotide 8665, near the 3′ end of ORF6 (Fig. 2 and 6).

No type 1 fimbrial proteins were detected by Western blot analyses in the solubilized cell wall fraction obtained from mutant strains that lacked the wild-type ORF1 (MY306-16) or ORF2 (MY308-22) (Fig. 7A, lanes 5 and 6). Mutant strains MY202-6 and MY304-24, generated by allelic replacement of ORF3 and ORF4, respectively, expressed only the 65-kDa type 1 structural subunit protein that was immunostained with the *A. naeslundii* T14V type 1 fimbrial antibody (Fig. 7A, lanes 4 and 7). However, proteins of higher molecular masses were not observed in these strains. In contrast, the electrophoretic protein profile of fimbriae from strain MY304-2 was similar to that of the wild-type strain (Fig. 7A, lanes 2 and 8). A profile similar to that shown in Fig. 7A was obtained when proteins released from cytoplasmic membranes or from bacterial cell surfaces were analyzed. Moreover, a similar protein profile was observed when the immunoblot was developed with a monoclonal antibody (A8 [8]) prepared against the purified *A. naeslundii* T14V type 1 fimbriae (not shown). Only the wild-type strain and mutant MY304-2 were able to bind, in vitro, to PRPs immobilized previously onto nitrocellulose (Fig. 7B). Under the experimental conditions used, binding of biotinylated bacteria to PRPs was detected in less than 5 min, while no binding was observed even after 3 h in the other mutant strains. Thus, interruption of some of the ORFs in close proximity to *fimP* has affected the expression and function of the *Actinomyces* type 1 fimbriae.

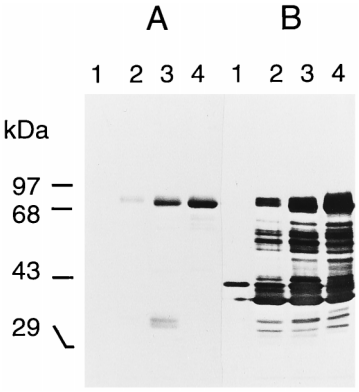

FIG. 5. Expression of ORF1-encoded protein as a maltose binding protein fusion product. Crude extract (15 μg) from *E. coli* carrying pMAL-c2-ORF1 (lane 4) was applied onto an amylose resin affinity column. The bound proteins eluted with 10 mM maltose were collected as individual fractions (lanes 2 and 3). Protein (7.5 μg) from each fraction was digested with factor Xa (final concentration, 1% by weight) at 23°C for 20 h. The protein samples were analyzed on an SDS–10% polyacrylamide gel. One hundred nanograms of purified maltose binding protein also was included as a control (lane 1). Proteins were transferred to nitrocellulose and immunostained with anti-*A. naeslundii* T14V whole bacterium antibodies (IgG fraction at 2.5 μg/ml) (A) or an antiserum against purified maltose binding protein (1:10,000 dilution) (B). Apparent molecular sizes are indicated on the left.

DISCUSSION

Little is known concerning the organization of genes involved in the biogenesis of fimbriae by gram-positive bacteria in general. Data obtained from this study have provided evidence that chromosomal DNA in close proximity to, and located 5′ and 3′ of, the *A. naeslundii* T14V subunit gene, *fimP*, encodes fimbria-associated genes that affect the synthesis and function of type 1 fimbriae in this organism. Of six putative ORFs identified on a 9.3-kb fragment of an *A. naeslundii* T14V chromosomal fragment, the product of ORF1 not only had significant sequence homology with the fimbrial structural subunit but also exhibited cross-immunoreactivity with a monospecific polyclonal antibody prepared against *A. naeslundii* T14V bacterial cells. Like the ORF1 protein, the ORF5 gene product contained a typical membrane-spanning domain at the carboxyl terminus, suggesting that it too is likely to be cell surface associated. Moreover, results obtained with isogenic strains with mutations in ORF1 or other ORFs suggest strongly that the expression of at least some of the putative genes flanking *fimP* may play a role in the expression of type 1 fimbriae and also in the ability of the bacteria to adhere to PRPs which serve as the receptors for *Actinomyces* type 1 fimbriae. In gram-negative bacteria, multiple genes in clusters are involved in fimbria synthesis, assembly, and function (9, 14, 18, 22). To our knowledge, the results presented in this study suggest a similar genetic arrangement for the fimbria and/or fimbria-associated genes in a gram-positive bacterial species.

The lack of expression of the putative ORFs in *E. coli*, using three different expression strategies (1, 35, 39, 50), suggests that not all *Actinomyces* genes can be expressed in *E. coli* even though results from this study and from previous studies (11, 44, 45) clearly demonstrated the expression of the *Actinomyces* fimbrial subunit genes in this host. Interestingly, results from studies on *Porphyromonas gingivalis* fimbriae (49) showed that while expression of fimbrial genes from this organism in the pUC vectors was poor, overexpression of the same genes was possible when they were subcloned into vectors suitable for protein expression in the T7 RNA polymerase system. Clearly, the mechanism(s) that governs expression of heterologous genes in *E. coli* is more complicated than once thought. The use of low-copy-number plasmids may be an alternative strategy in future studies to express these ORFs. Alternatively, expression of the putative genes as fusion products would seem highly feasible, based on the results obtained in this study with ORF1. In the attempt to isolate ORF1 gene product in this study, more than 50% of the maltose binding protein-ORF1 fusion protein appeared to be insensitive to digestion with a relatively high concentration (2.5% by weight) of factor Xa (Fig. 5A). Thus, modifications of the purification procedure will be needed to provide sufficient amounts of ORF1 protein for further biochemical characterization and the generation of antibodies.

Results from studies with the allelic exchange mutants have provided the evidence that multiple genes are likely to be involved in *Actinomyces* type 1 fimbria biogenesis. However, any speculations on the specific role of the putative ORFs described in this study would be premature. Clearly, isolation and characterization of in-frame deletions in the respective ORFs or the use of *trans*-complementation analysis would have

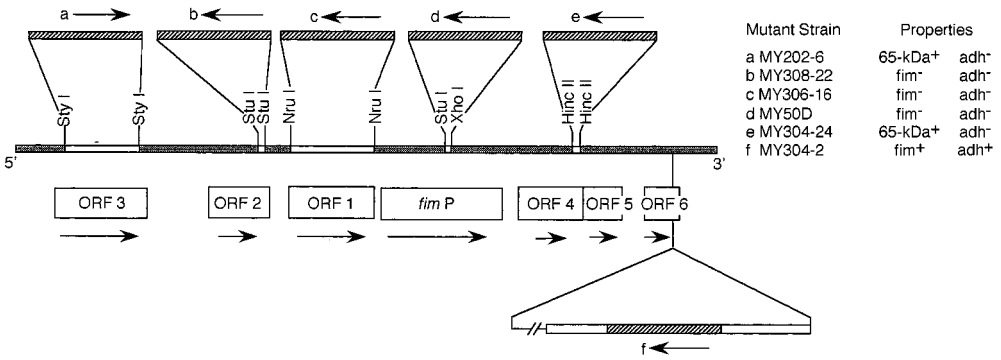

FIG. 6. Physical maps of *A. naeslundii* mutant strains (a through e) obtained by replacement of a DNA fragment internal to a specific ORF by a *kan* gene (▨). The restriction endonucleases used to create a deletion within each ORF are indicated. The mutant strain MY304-2 (f) was obtained by integration of plasmid pMY304 mediated by the Campbell insertion-duplication mechanism. The location of the inserted heterologous DNA into the *A. naeslundii* T14V chromosome is marked by a solid line and is located at the carboxyl-terminal end of ORF6. The direction (arrows) of transcription of the *kan* gene and that of *fimP* and the other ORFs are indicated. The relative locations of the various ORFs (boxed) are illustrated below the 9.3-kb *A. naeslundii* T14V chromosomal DNA fragment (▩). The phenotypic properties of the mutants with regard to the expression of type 1 fimbriae (fim) or the structural subunit protein (65-kDa) as determined by Western blot analyses and the ability of these strains to adhere (adh) to PRPs are summarized on the right. The superscript + or − indicates the presence or absence, respectively, of each phenotype.

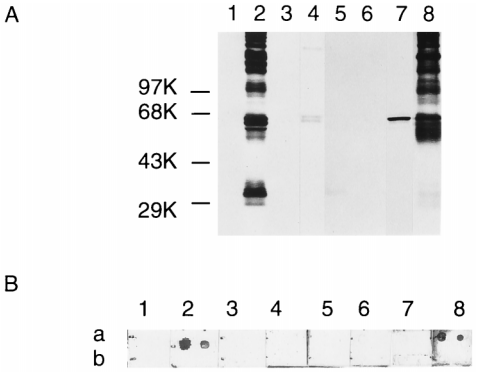

FIG. 7. Characteristics of *A. naeslundii* T14V mutants. (A) Western blot analysis of proteins from solubilized cell wall fractions isolated from *A. naeslundii* 147, T14V, MY50D, MY202-16, MY308-22, MY304-24, and MY304-2 (lanes 1 through 8, respectively). Proteins were separated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose, and the filter was immunostained with anti-*A. naeslundii* T14V type 1 fimbria antibody (IgG fraction at 1 μg/ml). Apparent molecular sizes are indicated on the left. (B) Adherence of *Actinomyces* strains to purified human salivary PRPs. Bacterial cells (5×10^9) washed previously with Tris-buffered saline were incubated with NHS-LC-biotin. The labeled cells were washed in Tris-buffered saline and added to a nitrocellulose square containing immobilized PRPs (a) (100 and 50 ng from left to right) and bovine serum albumin (b) (2 and 1 μg from left to right). Unadsorbed bacteria were removed, and the squares were developed with alkaline phosphatase color reagents.

provided a confirmation of the role of each putative gene in fimbria synthesis and function. Unfortunately, such genetic approaches are not available for the *Actinomyces* system. On the other hand, the insertion-substitution approach using the *kan* gene cassette as described in this study has proven feasible for these bacteria (44). However, this approach suffers from an inherent weakness in that polar effects might affect the interpretation of data. Thus, although the lack of type 1 fimbriae in mutant strain MY306-16 or MY308-22 might be due to a potential polar effect created by insertion of the *kan* gene at ORF1 or ORF2, respectively, the possibility that no expression of ORF1 or ORF2 might be directly responsible for the observed defect must also be considered. On the other hand, the observation that only the fimbrial structural subunit was expressed in mutant strain MY202-6 or MY304-24, which contained allelic replacement of ORF3 or ORF4, respectively, would suggest strongly a potential role of ORF3 and ORF4 in fimbria synthesis and/or assembly in *A. naeslundii* T14V. The lack of assembled fimbriae in strains MY202-6 and MY304-24 was correlated with their failure to adhere to PRPs. Thus, the phenotypic characteristics of these strains are similar to those of mutant strain MY52S of *A. naeslundii* T14V (43) isolated previously. This latter strain contains DNA from the integration plasmid inserted at the *Bam*HI site (nucleotide 6544) located immediately 3′ to *fimP* (43). It was of interest to examine whether strain MY304-24 or MY202-6 secreted unassembled subunits into the growth medium. Studies were conducted to obtain ammonium sulfate-precipitable proteins from the culture supernatant fluids. However, little or no subunit proteins were recovered, suggesting that synthesis and assembly of the subunit precursors in this organism must be tightly regulated.

It should be noted that the expression of *A. naeslundii* T14V fimbriae may require genes located elsewhere on the chromosome, in addition to the six putative ORFs identified in this study. If multiple gene clusters are involved, results obtained from this study with the mutant strain MY304-2 suggest that additional genes directly 3′ to ORF6 may not be involved in fimbria biogenesis. This is based on the observation that interruption of the *Actinomyces* chromosomal DNA with heterologous DNA at a site 3′ to ORF6 in the genome of strain MY304-2 had no effect on the synthesis of type 1 fimbriae or its ability to bind to PRPs. Clearly, information generated from this study provides the basis for further studies on the roles of the various ORFs flanking *fimP*, and additional genes, in fimbria synthesis and function of these organisms.

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