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Sequence Homology between the Subunits of Two Immunologically and Functionally Distinct Types of Fimbriae of *Actinomyces* spp.

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Nucleotide sequencing of the type 1 fimbrial subunit gene of *Actinomyces viscosus* T14V revealed a consensus ribosome-binding site followed by an open reading frame of 1,599 nucleotides. The encoded protein of 533 amino acids (M_r = 56,899) was predominately hydrophilic except for an amino-terminal signal peptide and a carboxy-terminal region identified as a potential membrane-spanning segment. Edman degradation of the cloned protein expressed in *Escherichia coli* and the type 1 fimbriae of *A. viscosus* T14V showed that both began with alanine at position 31 of the deduced amino acid sequence. The amino acid compositions of the cloned protein and fimbriae also were comparable and in close agreement with the composition of the deduced protein. The amino acid sequence of the *A. viscosus* T14V type 1 fimbrial subunit showed no significant global homology with various other proteins, including the pilins of gram-negative bacteria. However, 34% amino acid sequence identity was noted between the type 1 fimbrial subunit of strain T14V and the type 2 fimbrial subunit of *Actinomyces naeslundii* WVU45 (M. K. Yeung and J. O. Cisar, J. Bacteriol. 170:3803–3809, 1988). This homology included several different conserved sequences of up to eight identical amino acids that were distributed in both the amino- and carboxy-terminal thirds of each *Actinomyces* fimbrial subunit. These findings indicate that the different types of fimbriae on these gram-positive bacteria share a common ancestry.

Fimbriae or pili have been identified on a number of different microorganisms that inhabit host tissue surfaces, and the role of these structures in bacterial adherence has been firmly established (1, 26). The fimbriae of many gram-negative bacteria are composed of structural subunits, referred to as pilins, that range in size from approximately 15 to 30 kilodaltons (32). With certain microorganisms, such as *Escherichia coli*, additional minor proteins have been identified and associated with fimbria biogenesis and adherence (33, 34). Common evolutionary pathways for different pili have been inferred from comparisons of amino acid sequences. For instance, significant homology was noted between the type 1, PAP, and K99 pilins of *E. coli* (26) and also between pilins from other members of the family *Enterobacteriaceae* (17, 31, 35). These proteins are, however, unrelated to pilins from certain species of *Neisseria*, *Moraxella*, *Pseudomonas*, *Bacteroides*, and *Vibrio*, each of which has a similar amino-terminal sequence beginning with *N*-methylphenylalanine (12, 34). In contrast to the wealth of structural and genetic information on the fimbriae of gram-negative bacteria, little is known about the fimbriae of gram-positive bacteria. Indeed, the presence of these structures has been observed on only a limited number of organisms, including certain species of *Actinomyces* (6, 19), *Streptococcus* (15, 21, 23), and *Corynebacterium* (43).

Two antigenically distinct types of fimbriae have been identified on strains of *Actinomyces viscosus* and *Actinomyces naeslundii* (4). The specific receptors for *Actinomyces* type 1 fimbriae include the acidic proline-rich proteins that coat the tooth surface (18), whereas those for the type 2 fimbriae are certain galactose- and *N*-acetylgalactosamine-containing glycoconjugates of mammalian cells (2, 36) and polysaccharides of certain other bacteria (29). Type 1 and type 2 *Actinomyces* fimbriae were not dissociated to subunits by reduction and boiling in the presence of sodium dodecyl sulfate (4). However, cloning and expression of *A. viscosus* T14V or *A. naeslundii* WVU45 genes in *E. coli* resulted in the identification of proteins, each with apparent molecular weight near 60,000, that reacted with antibodies specific for either the type 1 (45) or type 2 (10) fimbriae of strain T14V or the type 2 fimbriae of strain WVU45 (46). The protein detected by the latter reaction was identified as the structural subunit of *A. naeslundii* WVU45 type 2 fimbriae by the finding that these fimbriae and the mature form of the protein encoded by the cloned gene have identical amino-terminal sequences (46).

This study was designed to determine the nucleotide sequence of the cloned type 1 fimbrial subunit gene of *A. viscosus* T14V as well as the amino-terminal sequences of the encoded protein expressed in *E. coli* and the fimbriae of *A. viscosus*. The amino acid sequence of the mature fimbrial subunit predicted from the gene was compared with those of various other proteins, including pilins of gram-negative bacteria and the type 2 fimbrial subunit of *A. naeslundii* WVU45 (46).

MATERIALS AND METHODS

DNA sequencing. Plasmid pMY3833 carrying the *A. viscosus* T14V type 1 fimbrial subunit gene (45) was purified by CsCl-ethidium bromide gradient centrifugation (28) and digested with restriction enzymes *Pst*I and *Bam*HI (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The 1.83-kilobase-pair *Pst*I-*Bam*HI insert containing the subunit gene was separated from the vector by agarose gel electrophoresis, electroeluted in 0.5× Tris-acetate buffer (28), and cloned into M13mp18 and M13mp19 phage vectors (Bethesda Research Laboratories, Inc.), using *E. coli* JM109 (44) as the host strain. A series of deletion clones was generated by using exonuclease III and S1 nuclease (Bethesda Research Laboratories, Inc.) according to the protocol of Henikoff (22). Nucleotide sequencing was performed by the dideoxy-

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chain termination method (37), using a 17-mer primer (New England BioLabs, Inc., Beverly, Mass.), [α-^{35}S]dATP (500 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.), and the large fragment (Klenow) of *E. coli* DNA polymerase I (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). DNA regions with a high G+C content were also analyzed by using the modified T7 polymerase protocol (Sequenase; United States Biochemical Corp., Cleveland, Ohio) as described previously (46). The sequence reactions were analyzed on 6% polyacrylamide–8 M urea linear and buffer gradient gels. The nucleotide sequence was determined for both strands and assembled by using the computer program developed by Staden (39). DNA sequence analyses were performed on a VAX computer (Digital, Inc., Maynard, Mass.), using the software of the Genetics Computer Group (GCG; University of Wisconsin Biotechnology Center, Madison) (9), Protein Identification Resource (PIR; National Biomedical Research Foundation [NBRF], Georgetown University Medical Center, Washington, D.C.), and Integrated Database and Extended Analysis System for Nucleic Acids and Proteins (IDEAS; August 1986 revision; M. Kinehisa, National Cancer Institute, Frederick, Md.). Searches for nucleotide and amino acid sequence homologies were performed by using the GenBank (release 60, June 1989; IntelliGenetics, Inc., Mountain View, Calif.) and NBRF (release 21, June 1989) data base libraries.

Amino-terminal sequence and amino acid analyses. The cloned *A. viscosus* type 1 fimbrial subunit was isolated from an extract of *E. coli* MY3833 (45) by immunoaffinity chromatography using an immobilized monoclonal antibody against type 1 fimbriae and further purified by Sephacryl S200 (Pharmacia Inc., Piscataway, N.J.) column chromatography (J. O. Cisar, E. L. Barsumian, R. P. Siraganian, W. B. Clark, M. K. Yeung, S. D. Hsu, S. H. Curl, A. E. Vatter, and A. L. Sandberg, submitted for publication). Type 1 fimbriae were obtained by sonication of *A. viscosus* PK455, a mutant of *A. viscosus* T14V that lacks type 2 fimbriae (5). Purification of these fimbriae by fractional ammonium sulfate precipitation, gel filtration, and immunoaffinity column chromatography has been described elsewhere (Cisar et al., submitted). Fimbrial proteins were dissolved in 1% trifluoroacetic acid and analyzed by automated Edman degradation in a 470A gas-phase sequencer (Applied Biosystems, Inc., Foster City, Calif.) as described previously (46). Amino acid compositions were determined by using the Pico-Tag system (Waters Associates, Inc., Milford, Mass.) after the hydrolysis of proteins in 6 M HCl at 115°C for 24 h (46).

RESULTS

Nucleotide sequence of the *A. viscosus* T14V type 1 fimbrial subunit gene. The DNA sequence of the *Pst*I-*Bam*HI fragment from pMY3833 carrying the type 1 fimbrial subunit gene (45) was determined in both orientations according to the strategy illustrated in Fig. 1. The sequence of the nontranscribed strand is shown in Fig. 2. Examination of this sequence revealed an open reading frame of 1,599 nucleotides (nucleotides 94 to 1,692) preceded by a putative *E. coli* ribosome-binding site (38) nine bases upstream from the initiation codon, ATG. The reading frame encoded a protein of 533 amino acid residues with a calculated molecular weight of 56,899. The amino-terminal sequences determined by Edman degradation of the cloned protein isolated from *E. coli* and the type 1 fimbriae of *A. viscosus* T14V were identical through the first 26 residues and matched the sequence encoded by the gene beginning with alanine,

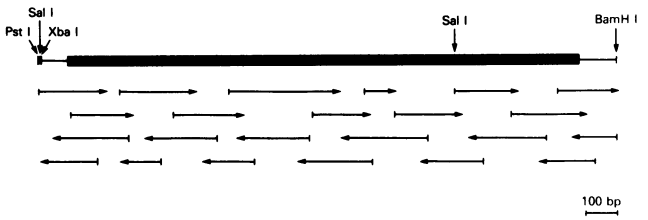

FIG. 1. Restriction map and nucleotide sequencing strategy of the 1.83-kilobase-pair *Pst*I-*Bam*HI fragment from pMY3833 containing the *A. viscosus* T14V type 1 fimbrial subunit gene. The amino acid-coding region of the gene is indicated by the thickened line. Arrows indicate the direction and extent of the nucleotide sequence determined with different subclones. bp, Base pairs.

residue 31 of the predicted protein sequence (Fig. 3). The percent recoveries of N-terminal alanine for these determinations were similar, 40 and 46% for the cloned protein and fimbriae, respectively. The amino acid compositions of these proteins also were comparable and in close agreement with that expected from the amino acid sequence of the deduced protein (Table 1).

Sequence comparison between the *A. viscosus* T14V type 1 and *A. naeslundii* WVU45 type 2 fimbrial subunits. No significant global homologies were noted when the nucleotide and encoded amino acid sequences of the *A. viscosus* T14V type 1 fimbrial subunit gene were compared with those of other proteins included in the GenBank and NBRF data base libraries. However, the type 1 fimbrial subunit gene showed obvious homology with the type 2 fimbrial subunit gene of *A. naeslundii* WVU45 (46). When compared by the program BestFit (GCG), 53% identity was noted between the nucleotide sequences of these genes (data not shown). The encoded proteins contained 48% identical and conserved amino acid residues (Fig. 4). Similarly, a global homology of 34% amino acid sequence identity was noted by the program ALIGN (PIR [8, 16]), using a mutational data matrix of 250 percent accepted mutations, a bias of 6, and a gap penalty of 6. When another 100 alignments were computed by using these parameters, an alignment score of 22.53 was obtained. Values greater than 3 indicate highly significant structural similarity (8, 42). Several conserved sequences containing as many as 8 identical or 13 identical plus conserved amino acid residues were located within the amino- and carboxy-terminal thirds of each protein. When the distribution of different conserved amino acids was examined, each of the seven conserved proline residues was found within a different highly conserved sequence (Fig. 4 and 5; amino acid residues 46 to 53, 110 to 120, 128 to 133, 145 to 153, 375 to 387, 420 to 429, and 466 to 478 of the *A. viscosus* type 1 fimbrial subunit and residues 45 to 52, 121 to 131, 140 to 145, 159 to 167, 370 to 382, 417 to 426 and 460 to 472 of the *A. naeslundii* type 2 fimbrial subunit, respectively). No internal repeating sequences were noted in either *Actinomyces* fimbrial subunit as shown by dot matrix plots for self homology (DotPlot; GCG).

Analysis of each protein sequence for predicted secondary structures (3) (Fig. 5B) gave a slightly greater content of beta sheet than alpha helix: 41 versus 33% for the *A. viscosus* T14V type 1 subunit and 35 versus 24% for the *A. naeslundii* WVU45 type 2 subunit. The hydropathy profiles displayed by the method of Kyte and Doolittle (27) revealed hydrophobic regions at the amino- and carboxy-terminal ends of each predicted protein sequence (Fig. 5C). The former was associated with the presence of a signal peptide, and the latter was associated with a possible membrane-spanning

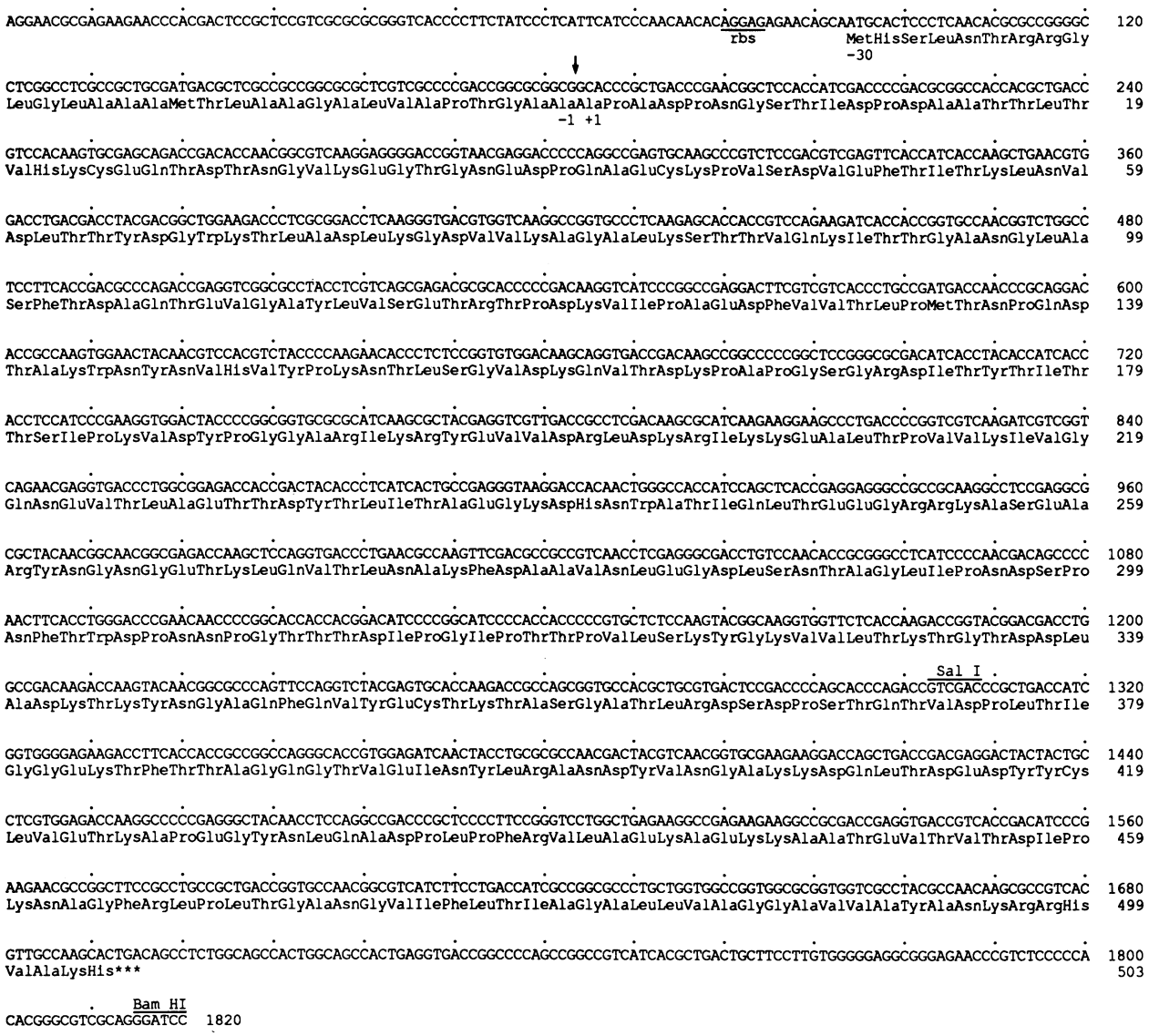

FIG. 2. Nucleotide and predicted amino acid sequences of the *A. viscosus* T14V type 1 fimbrial subunit gene (GenBank/EMBL/DDBJ accession no. M32067). The consensus ribosome-binding site (rbs) is underlined. The signal peptide is designated by negative numbers (−30 to −1), and the mature protein is indicated by positive numbers beginning with amino acid residue +1. The signal peptide processing site is indicated by an arrow.

region. The predicted boundaries of the membrane-spanning region were defined by the program ALOM (IDEAS [25]) as amino acid residues 471 (−3, +4) and 493 (−2, +2) of the *A. viscosus* type 1 fimbrial subunit and residues 466 (−1, +1) and 487 (−4, +4) of the *A. naeslundii* type 2 fimbrial subunit. Except for these regions, each protein was predominantly hydrophilic.

Codon usages in the *A. viscosus* T14V type 1 and *A. naeslundii* WVU45 type 2 fimbrial subunit genes were similar and showed a bias for C or G at the third position of each codon (results not shown). This bias was consistent with the relatively high G+C content present in the type 1 and type 2 subunit genes (67 and 63 mol%, respectively) as well as in the chromosomal DNA (7) of *A. viscosus* T14V and *A. naeslundii* WVU45 (66.7 ± 0.51 and 67.4 ± 0.47 mol%, respectively).

DISCUSSION

Sequence analysis of the *A. viscosus* T14V type 1 fimbrial subunit gene has provided a structural basis for comparing the fimbriae of this gram-positive microorganism with those of other bacteria. Whereas this gene encoded a protein of 533 amino acid residues, the removal of a 30-amino-acid signal peptide would be expected to yield a mature fimbrial subunit of 503 residues with a molecular weight of 54,053. This protein is comparable in size to the type 2 fimbrial subunits of *A. naeslundii* WVU45 (46) and *A. viscosus* T14V (J. A. Donkersloot, personal communication) and approximately two to three times larger than the pilins from various gram-negative bacteria (26, 32). The amino acid sequence of the *A. viscosus* T14V type 1 fimbrial subunit showed no significant global homology with those of pilins or other

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          -30                 -25                 -20
GENE:     M H S L N T R R G L G L A A A
          -15                 -10                  -5                  -1
          M T L A A G A L V A P T G A A
          +1                   5                  10                  15
GENE:     A P A D P N G S T I D P D A A
SUBUNIT:  A P A D P N G S T I D P D A A
FIMBRIAE: A P A D P N G S T I D P D A A
                    20                  25
GENE:     T T L T V H K C E Q T
SUBUNIT:  T (T) L - V (H) - - E (Q) T
FIMBRIAE: T T L - V (H) - - E Q T
```

FIG. 3. Amino-terminal sequence of the *A. viscosus* T14V type 1 fimbrial subunit. The amino-terminal sequence of the subunit predicted from the DNA sequence (GENE) is aligned with those determined by Edman degradation of the cloned protein expressed in *E. coli* (SUBUNIT) and the fimbriae from *A. viscosus* T14V (FIMBRIAE). Symbols: -, positions where an individual amino acid was not identified; (), the most likely amino acid. Negative numbers denote the signal peptide; position +1 indicates the amino-terminal end of the mature protein.

TABLE 1. Comparison of the amino acid composition of the deduced *A. viscosus* T14V type 1 fimbrial subunit with those determined for the purified protein from *E. coli* or the isolated fimbriae

Amino acid	No./100 residues		
	Deduced protein[a]	Purified protein[b]	Fimbriae[c]
Asx	13.4	13.4	14.1
Thr	13.3	13.2	13.1
Glx	8.4	9.4	9.6
Ala	9.5	8.7	9.1
Lys	8.0	9.2	8.5
Gly	8.0	7.9	8.0
Pro	5.8	7.4	7.9
Val	8.4	6.8	7.2
Leu	7.2	7.7	7.2
Ile	4.0	3.3	3.7
Arg	3.0	3.3	3.4
Ser	3.0	3.8	3.1
Tyr	3.6	2.7	2.8
Phe	2.0	2.2	2.0
His	1.0	0.7	0.4
Met	0.2	0.5	0.3
Cys	0.8	—[d]	—
Trp	0.8	—	—

[a] Mature protein deduced from the nucleotide sequence of the cloned gene.
[b] Protein purified from *E. coli* MY3833.
[c] Type 1 fimbriae from *A. viscosus* PK455.
[d] —, Not determined.

bacterial surface proteins included in the NBRF data base. However, 34% amino acid sequence identity was seen between the subunits of the *A. viscosus* T14V type 1 and the *A. naeslundii* WVU45 type 2 fimbriae (Fig. 4). Similarly, 33% sequence identity has recently been noted between the type 1 and type 2 fimbrial subunits of *A. viscosus* T14V (Donkersloot, personal communication). Taken together, these findings suggest that the structural subunits of *Actinomyces* fimbriae are members of a distinct superfamily. The occurrence of related proteins on other gram-positive microorganisms is not yet known.

The signal peptides encoded by the *A. viscosus* T14V type 1 and *A. naeslundii* type 2 fimbrial subunit genes were somewhat shorter than those of streptococcal M proteins (about 40 amino acid residues [24, 30]), similar in length to those of various other gram-positive bacteria (about 30 amino acid residues [11, 24]), and longer than those of gram-negative bacteria (about 20 amino acid residues [11]). Features of the *Actinomyces* signal peptides that were typi-

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A. viscosus   T14V  Type 1:    MHSLNTRRGLGLAAAMTLAAGALVAPTGAA--   -1
A. naeslundii WVU45 Type 2:    MKYNTSTLGRRAAAAAGVLTLAVLGLAPMAQA   -1

APADPNGSTIDPDAATTLTVHKCEQTDTNGVKEGTGNE-DPQAECKPVSDVEFTITKLN-VDLTTY   64
--ENANHGDINTEALGSLTIHKHLNGDGNPIGAPDGTASNDDGKGAPVSGVQFTAYEINGIDLKTS   64

DGWKTLADLKGDVVKAGALKSTTVQKITTGANGLASFTDAQTE----------VGAYLVSETRTP   119
EGWAKVNALTNTGAIPDNACANPGQPTLPNYTFRSSRVSGDTDRDGEAKIESLPVKAYLVCETKTP   130

DKVIP-AEDFVVTLPMTNPQDTAK--WNYNVHVYPKNTLSGVDKQVTDKPAPGSGRDITYTITTSI   182
GNIVQKAKPFVVTIPHPNTAAKADGTWLYDVHVYPKNEKIEVAKTIEDQRNNGYIVGSKVRFPVSS   196

PKVDYPGGARIKRYEVVDRLDKRIKKEALTPVVKIVGQNEVTLAETTDYTLITAEGKDHNWATIQL   248
TLPKLDDNSYYKYYQFKDTLDNRLKQVTATD----VTLGGTRLDEGTDYTL----GTDGQTVTVTF   254

TEEGRRKASEARYNGNGETKLQVTLNAKFDAAVNLEGDLSNTAGLIPNDSPNFTWDPNNPGTTTDI   314
NQNGLSK-----LKGNPGQKLQAVFEGVVSEVG--DGSINNTAQLISDTTYAEQPPAPETPPANPD   313

PGIPTTPVLSKYGKVVLTKTGTDD-LADKTKYNGAQFQVYECTKTASGATLRDSDPSTQTVDPLTI   379
NPPTTEQVTSKWGDLTIKKVDGNDRSGDKDGLKGAEFQIYKAKDAYADTCSPEADG-----QPLTI   374

GGEKTFTTAGQGTVEINYLRANDYVNGAKKDQLTDED--YYCLVETKAPEGYNLQADPLPFRVLAE   443
NGESTFTTGEGGTINFKALFVSDSVQDTGRDNRVDAPHRCYVLVETKAPAGYVLPADASRAITVEP   440

KAEKKAATEVTVTDIPKNAGFRLPLTGANGVIFLTIAGA--LLVAGGAVVAYANKRRHVAKH      503
GA---GVTQQVVIDNVKQSVPGLPLTGANGMLILTASGAALLMIAVGSVLVARYRERKRNRDLAA   502
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FIG. 4. Sequence homology of *A. viscosus* T14V type 1 (upper sequence) and *A. naeslundii* WVU45 type 2 (lower sequence) fimbrial subunits. The predicted amino acid sequences of the two proteins were aligned by using the program BestFit (GCG) (gap weight = 5.0; gap length weight = 0.3). Identical (|) and conserved (:) amino acid residues are indicated.

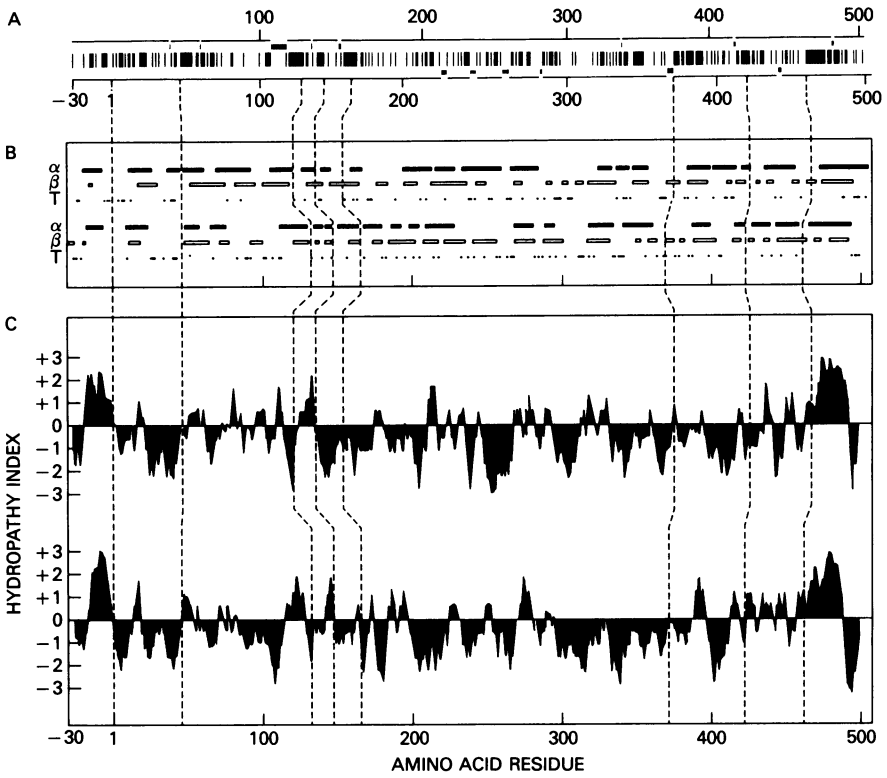

FIG. 5. Protein sequence analyses of the *A. viscosus* T14V type 1 (upper profile) and *A. naeslundii* WVU45 type 2 (lower profile) fimbrial subunits. (A) Protein sequence homology from Fig. 4 displayed by the program GapShow (GCG). Locations of identical and conserved amino acids are indicated by dashes; gaps within each sequence are marked by horizontal bars. (B) Possible secondary structures predicted by the program CHOFAS (IDEAS). α, alpha helix; β, beta sheet; T, beta turn; unmarked area, random coil. (C) Hydropathy profiles determined by the program HPLOT (IDEAS), using the Kyte-Doolittle scale (27) and a sliding window of seven amino acids; positive and negative indices represent hydrophobic and hydrophilic regions, respectively. Vertical dashed lines are positioned at the amino-terminal end of each mature protein (amino acid residue 1) and at each of the seven conserved proline residues.

cal (11) included a hydrophilic N-terminal region with two or three basic amino acids followed by a hydrophobic domain rich in alanine and leucine. The common presence of proline at position −5, glycine or alanine at position −3, and alanine at position −1 presumably contributes to the formation of a specific cleavage site (11, 40) recognized in *A. viscosus* as well as *E. coli* MY3833 (Fig. 3).

Whereas amino acid sequence homology between the signal peptides of the *A. viscosus* T14V type 1 and *A. naeslundii* WVU45 type 2 fimbrial subunits was not striking, several different conserved sequences of up to eight identical residues were present in the mature proteins. The occurrence of these conserved sequences in both the N- and C-terminal regions of each protein and their association with each of seven conserved proline residues (Fig. 5) suggests a common pattern of protein folding for the different fimbrial subunits. The absence of cross-reactivity between type 1 and type 2 fimbriae of *Actinomyces* spp. implies that the homologous sequences probably are not antigenic determinants of the fimbriae but instead may be buried, either within each fimbrial subunit or by the interaction between adjacent subunits.

The conserved sequence near the carboxy-terminal end of the type 1 and type 2 fimbrial subunits was associated with a potential membrane-spanning region. This region was similar to the putative membrane anchors present in certain nonfimbrial cell surface proteins of gram-positive origin, including *Staphylococcus aureus* protein A (20), streptococcal M proteins (24, 30), and *Streptococcus* protein G (13). With each of these proteins, a carboxy-terminal hydrophobic region was preceded by the conserved sequence Leu-Pro-X-Thr-Gly and followed by a cluster of basic amino acids near the C-terminal end. Based on the expected orientation of integral membrane proteins (41) and the predicted boundaries (25) of the membrane-spanning segments, the conserved sequence, Leu-Pro-Leu-Thr-Gly, of each *Actinomyces* fimbrial subunit (Fig. 4) may be situated on the cell surface side of the membrane and the basic residues on the cytoplasmic side. That the C-terminal end of the *A. viscosus* T14V type 1 fimbrial subunit may function as a membrane anchor is suggested by the previous observation that a 47-kilodalton N-terminal truncated protein, expressed in *E. coli* from the *PstI-SalI* fragment of the type 1 fimbrial subunit gene, was secreted into the periplasm whereas the intact protein was associated with the inner membrane fraction (45). A transient association between the carboxy-terminal region of the type 1 subunit and the *A. viscosus* membrane may also occur during fimbria biogenesis and be coordinated with polymerization of fimbrial subunits. If so, the putative membrane-spanning region may be integrated into the developing fimbria or cleaved from each fimbrial subunit as it is translocated across the membrane.

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ADDENDUM

The authors of a recent report (14) found no homology between the predicted amino acid sequences of the *A. viscosus* T14V type 1 and *Streptococcus sanguis* FW213 type 1 fimbrial subunits but have suggested functional homology between these proteins on the basis of similarities of predicted secondary structure and patterns of hydrophobicity-hydrophilicity. The validity of this proposal remains to be established, since the structural features of *Actinomyces* as well as *Streptococcus* fimbriae responsible for functional activity have not been defined.

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