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Nucleotide Sequence Analysis of a Type 1 Fimbrial Gene of *Streptococcus sanguis* FW213

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A structural gene for type 1 fimbriae of *Streptococcus sanguis* FW213 was located within a 6-kilobase fragment cloned in *Escherichia coli*. A 1.6-kilobase internal fragment contains an open reading frame of 927 bases coding for an immunoreactive peptide of 34,349 daltons, which corresponds in size with an observed cytoplasmic form of fimbrial peptide (P. M. Fives-Taylor, F. L. Macrina, T. J. Pritchard, and S. J. Peene, Infect. Immun. 55:123–128, 1987). Disruption of the reading frame by insertional mutagenesis results in loss of immunoreactivity. Consensus sequences for initiation of transcription and translation were identified 5′ to the coding region. Transcription terminator-like sequences were found downstream of the coding region. The deduced amino acid sequence of the cloned fimbrial peptide shows a strongly hydrophobic signal sequence at the amino terminus. The carboxyl-terminal region does not include a hydrophobic membrane anchor sequence such as has been reported for other gram-positive surface structures. A hydrophobic region of 12 to 14 amino acids downstream from the putative signal sequence cleavage site exhibits homology with the *Streptococcus pyogenes* type 6 M protein repetitive region A (S. K. Hollingshead, V. A. Fischetti, and J. R. Scott, J. Biol. Chem., 261:1677–1686, 1986). Functional homology at the level of protein secondary structure with *Actinomyces viscosus* T14V type 1 fimbriae (M. K. Yeung, B. M. Chassy, and J. O. Cisar, J. Bacteriol., 169:1678–1683, 1987) is proposed.

Dental caries and periodontal disease both result from the pathogenic effects of several species of bacteria associated with dental plaque. Adhesion of *Streptococcus sanguis* to the salivary pellicle of dental surfaces is a primary event in plaque formation, which may mediate the later development of the complex dental microenvironment.

Studies of *S. sanguis* have demonstrated that specific surface proteins are involved in its adhesion to the in vitro model of the tooth surface (6, 14, 17, 25). Immunological characterization of surface proteins (5, 6), adhesion assays (10, 12), and electron microscopy (5, 12) have provided evidence for the role of peritrichous fimbriae in adhesion.

Initial analyses of *S. sanguis* adhesins at the molecular level have recently been reported. Fives-Taylor et al. (11) have cloned a fimbrial structural gene of *S. sanguis* FW213 in an *Escherichia coli* expression vector. The cloned fimbrial gene encodes a protein with a M_r of 30,000 which is antigenically related to several proteins of higher molecular weight found in fimbrial extracts and which corresponds in size with a fimbrial protein found in cytoplasmic extracts of FW213. Ganeshkumar et al. (13) have cloned a 36,000-M_r adhesin (designated SsaB) of *S. sanguis* 12 which is one of two distinct adhesins associated with this organism. Rosan et al. (28) have cloned an 84,000-M_r adhesin of *S. sanguis* G9B, which is the major antigen associated with the binding of G9B to saliva-coated hydroxyapatite. These two adhesins appear to be distinct in cellular location from the fimbrial adhesin of *S. sanguis* FW213. These studies are the first attempts reported to apply molecular cloning techniques to the study of *S. sanguis* adhesins.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* VT644, which carries plasmid pVT618 (11), was the source of *S. sanguis* DNA coding for a fimbrial structural gene. *E. coli* JM101 (33) was used as the host strain for subcloning and preparation of sequencing templates. *E. coli* GM2929 (19) was used as a host strain for preparation of plasmid DNA for certain enzymatic manipulations. Plasmid vectors pTZ18R and pTZ19R (23) were used for subcloning, preparation of deletion mutants, and preparation of single-stranded DNA. Helper phage M13K07 (33) was used to package single-stranded DNA. Tetracycline (10 μg/ml), ampicillin (50 μg/ml), or kanamycin (70 μg/ml) was added as appropriate to select for plasmid or bacteriophage. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indoyl-β-D-galactoside were added to plate media to detect clones expressing the *lacZ* gene.

DNA manipulations. DNA isolation, endonuclease restriction, ligation, and transformation of competent *E. coli* cells were carried out as previously described (11).

Immunoassays. Lysates of recombinant clones containing all or part of the 6-kilobase (kb) fimbrial fragment were screened for expression of the cloned fimbrial protein by probing with polyclonal antisera to *S. sanguis* FW213 or with fimbrial-specific antisera (6).

Nucleotide sequence determination and analysis. Nested sets of deletion mutants were constructed by the method of Henikoff (15). A series of clones, each differing by approximately 200 base pairs, was chosen for sequencing. Single-stranded plasmid DNA produced from the f1 origin of replication contained on the plasmid vector was recovered from culture supernatants (4). A 17-mer reverse sequencing primer (Pharmacia, Inc., Piscataway, N.J.), Klenow fragment DNA polymerase 1 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and [α-^{35}S]dATP (Dupont, NEN

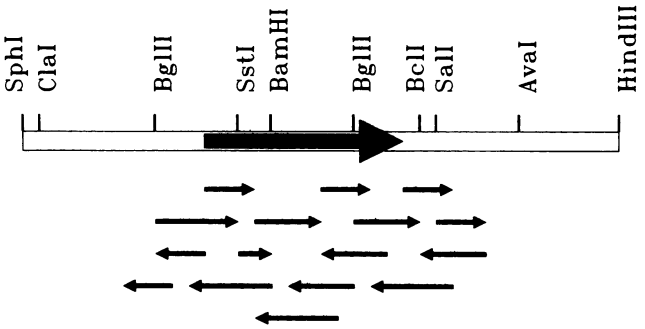

FIG. 1. A restriction map of a 3.1-kb *Sph*I-*Hin*dIII DNA fragment containing the *S. sanguis* fimbrial gene is shown as an open bar. The fimbrial reading frame is represented by a heavy arrow superimposed on the restriction map, with the direction of the arrow representing the 5'-3' orientation of the fimbrial open reading frame. The smaller arrows below represent DNA sequences determined from individual deletion mutants. The direction of the arrows indicates the strand of DNA sequenced from each clone.

Research Products, Boston, Mass.) were used for dideoxy chain termination sequencing reactions (29). Nucleotide sequences were analyzed with the University of Wisconsin Genetics Computer Group software package on a VAX 8600 computer.

RESULTS

Subcloning the fimbrial gene. The plasmid pVT618 contains a fimbrial gene on a 6-kb *Eco*RI fragment of *S. sanguis* FW213 DNA (11). The 6-kb fragment was isolated and ligated into the multiple cloning sites of pTZ18R and pTZ19R (23, 33). These vectors permit screening for inducible expression of cloned genes under transcriptional control of an *E. coli lac* promoter located adjacent to the multiple cloning site. The streptococcal DNA fragment was aligned in the same orientation with respect to the *lac* promoter of the pTZ vectors as it had been in pVT618. *E. coli* clones carrying this plasmid construct express a single immunoreactive protein with a M_r of 30,000 when probed with either adhesion-blocking fimbrial-specific antisera (6, 11) or polyclonal antisera to FW213 (data not shown). Fimbrial gene expression was evident without induction but was increased by growth in the presence of IPTG. Clones containing segments of the 6-kb fragment were similarly screened for fimbrial gene expression, with anti-FW213 sera to probe for the cloned fimbrial antigen. Two immunoreactive clones (VT292 and VT290), each containing a 3.1-kb *Sph*I-*Hin*dIII streptococcal DNA fragment cloned in opposite orientation, were chosen as substrates for construction of deletion mutants and further characterization of fimbrial gene expression. The upper portion of Fig. 1 shows a restriction map of the 3.1-kb *Sph*I-*Hin*dIII fragment.

Construction and screening of deletion mutants. Series of deletion mutants were constructed by unidirectional digestion of the cloned *Sph*I-*Hin*dIII fragment from the *Sph*I end of VT292 and the *Hin*dIII end of VT290. The fimbrial gene was further localized by screening deletion mutants for fimbrial gene expression. VT290 and its derivatives showed no increase in expression with induction by IPTG. VT292 and its immunoreactive derivatives were inducible with IPTG, indicating that the fimbrial gene is transcribed in the same direction as the disrupted *lacZ* gene in VT292. The smallest immunoreactive derivative of VT290 has a deletion of approximately 900 nucleotides from the *Hin*dIII site. The smallest antibody-positive derivative of VT292 is VT579, which has a deletion of approximately 700 nucleotides from the *Sph*I site. The clones chosen for sequencing were approximately 300 base pairs larger than the smallest expressing clone in either direction.

Nucleotide sequence analysis of the fimbrial gene. The strategy employed for Sanger sequencing of overlapping deletion mutants is shown in the lower portion of Fig. 1. More than 95% of the fimbrial gene sequence was determined by sequencing both strands in opposite directions. The remaining sequence was determined by reading the same strand in two independently isolated clones.

The nucleotide sequence of the cloned fimbrial gene and flanking regions is shown in Fig. 2. The DNA sequence contains several open reading frames, including one which could code for a protein of the size observed. A possible coding region of 927 base pairs begins with an ATG (methionine) codon at base 322 and ends at a TAA stop codon at base 1249. This open reading frame is aligned in the same direction as transcription from the *lac* promoter in VT292 and is aligned opposite to transcription from the *lac* promoter in VT290. This corresponds with the observation that expression of the cloned gene is inducible in VT292. The GC content of the fimbrial coding region is 44%, which is within the range determined for *S. sanguis* strains (8). The proposed translation start codon is located six bases downstream from a typical gram-positive ribosome-binding site labeled RBS in Fig. 2. The proposed ribosome-binding site is similar to those reported for a number of other streptococcal genes (9). The underlined sequence, AAAGGAG, is complementary to 7 of the 10 bases of the 3'-OH terminus of the *Bacillus subtilis* 16S ribosomal subunit (22, 24).

Two promoter-like sequences (21), labeled P1 and P2 in Fig. 2, have been identified in the upstream flanking region by probing with consensus sequences for −10 and −35 regions identified as RNA polymerase-binding sites in other streptococcal systems (9). Neither of these sequences, located 44 and 121 nucleotides upstream from the ribosome binding site, exhibits strong homology with other putative streptococcal promoter sequences.

Rho-independent transcription termination sequences may be identified by the probability of stable intrachain binding resulting in stem and loop or hairpin structures in mRNA (27). These structures are commonly followed by a string of uracil residues, which may enhance the instability of the DNA-RNA heteroduplex (20). A secondary structural analysis of the sequence downstream of the TAA stop codon yields two closely linked terminator-like sequences (labeled with arrows in Fig. 2) within 100 nucleotides of the TAA stop codon (1). Inferred RNA stem and loop structures with free energies of −5.4 kcal and −2.0 kcal begin at nucleotides 1286 and 1315, respectively. Both of these are followed within 10 to 20 bases by strings of five uracil residues in the predicted transcript.

Insertional inactivation of the fimbrial gene. VT579 is an immunoreactive deletion derivative of VT292. The streptococcal DNA fragment carried on pVT579 begins at position 319, 3 base pairs upstream from the start codon of the fimbrial gene and in frame with vector-coded *lacZ* transcription. A mutation created at position 760 by duplication of the four-base overhang of the *Bam*HI site (i) disrupts the *Bam*HI site, (ii) creates a unique *Cla*I site, and (iii) creates a frameshift mutation which disrupts the portion of the reading frame downstream of the insertion. There is only one open reading frame in this region of the DNA sequence. The left

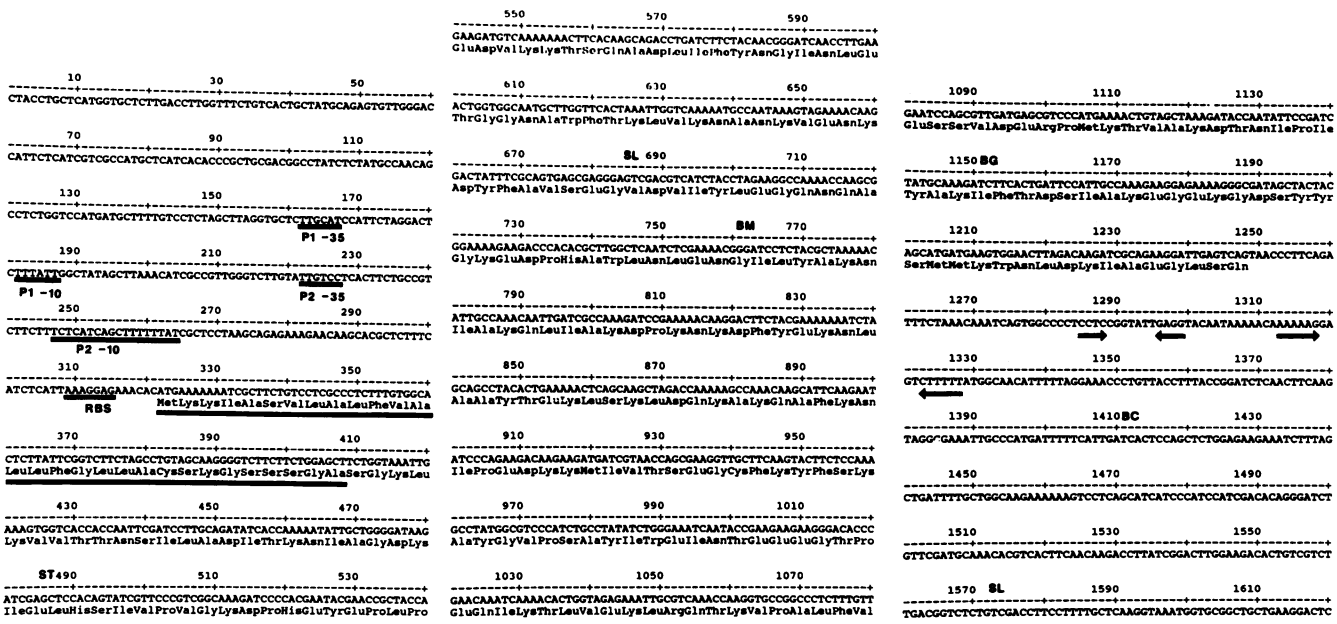

FIG. 2. DNA sequence of the *S. sanguis* type 1 fimbrial gene. Putative promoter sites are labeled P1 and P2. RBS, Ribosome-binding site. The deduced amino acid sequence is indicated below the DNA sequence beginning with the ATG codon at base 322 and continuing through the TAA stop codon at residue 1249. The putative signal sequence is underlined. Cleavage is proposed at the alanine residue at position 29. Proposed factor-independent terminators are indicated by arrows centered at bases 1292 and 1321. Restriction enzyme recognition sites are labeled as follows: BG (*Bgl*II), BM (*Bam*HI), BC (*Bcl*I), St (*Sst*I), and SL (*Sal*I).

side of Fig. 3 shows restriction fragments of pVT579 and the mutant pVT287 which demonstrate the destruction of the *Bam*HI site and its replacement by a *Cla*I site. Lanes A and B show *Bam*HI-*Eco*RI double digests of pVT579 and pVT287, respectively. Lanes C and D show *Cla*I-*Eco*RI double digests of pVT579 and pVT287, respectively. Immunoassay of lysates of VT579 and VT287 (Fig. 3, right side) show that, as predicted, VT287 does not produce the cloned fimbrial protein.

Characteristics of the fimbrial peptide. The deduced sequence of the 309 residue fimbrial peptide is shown under the nucleotide sequence in Fig. 2, beginning at nucleotide position 322. This peptide has a molecular mass of 34,349 daltons, which corresponds in size with the observed cloned fimbrial protein with a M_r of 30,000 (11).

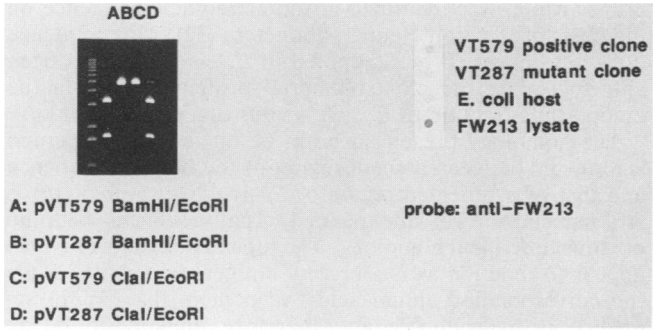

FIG. 3. Insertional inactivation of the fimbrial gene. On the left is an agarose gel of restricted plasmid DNA containing the fimbrial gene (lanes A and C) and the mutated fimbrial gene (lanes B and D) demonstrating the loss of the *Bam*HI site and acquisition of a *Cla*I site by the mutant pVT287. On the right is an immunoblot of lysates of *E. coli* clones carrying the fimbrial gene (VT579) or the mutated fimbrial gene (VT287). A lysate of *E. coli* containing no streptococcal DNA insert serves as a negative control. A lysate of *S. sanguis* FW213 is included as a positive control. The blot is probed with anti-FW213 sera.

The amino-terminal region of the fimbrial peptide contains a sequence typical of a signal peptide (30). Two hydrophilic residues immediately following the start codon are followed in turn by a string of hydrophobic residues with an internal glycine residue. The terminal point of this signal sequence has not been determined by amino acid sequencing. The most likely cleavage point, based on the Von Heijne parameters for signal peptides (32), is at the alanine at amino acid 29.

The amino acid composition of the fimbrial peptide with and without its proposed signal sequence is shown in Table 1. The mature peptide contains only one cysteine residue (one is also found in the signal sequence). The peptide is very rich in lysine (13.2 mol%). Also present in high concentrations are leucine and isoleucine (14.6% combined), glutamic acid and aspartic acid (15.0% combined), and alanine (7.9%).

Comparisons with other functionally related genes. The fimbrial nucleotide and amino acid sequences were compared with published sequences of other genes encoding adhesin, fimbrial, or other surface structure proteins. Some functional homology at the amino acid level is found between the fimbrial protein and a number of unrelated exported proteins. This appears to be due to the presence of a signal sequence at the amino terminus of the fimbrial protein. No significant homology was found between the fimbrial gene and gram-negative fimbrial sequences available through GenBank.

The fimbrial gene was then compared with those of other gram-positive surface proteins with which some homology might be expected. The fimbrial sequence contains a region of 12 amino acids (40 to 51) which exhibit partial homology with the repeated sequence A1-A3 found in *Streptococcus*

TABLE 1. Amino acid composition of fimbrial peptide with and without proposed signal sequence[a]

Residue	Entire sequence		Sequence without proposed signal peptide	
	No.	Mol%	No.	Mol%
Ala	27	8.74	22	7.86
Cys	2	0.65	1	0.36
Asp	17	5.50	17	6.07
Glu	25	8.09	25	8.93
Phe	11	3.56	9	3.21
Gly	19	6.15	16	5.71
His	3	0.97	3	1.07
Ile	22	7.12	21	7.50
Lys	40	12.95	37	13.21
Leu	26	8.41	20	7.14
Met	5	1.62	4	1.43
Asn	18	5.83	18	6.43
Pro	12	3.88	12	4.29
Gln	9	2.91	9	3.21
Arg	2	0.65	2	0.71
Ser	20	6.47	15	5.36
Thr	15	4.85	15	5.36
Val	19	6.15	17	6.07
Trp	4	1.29	4	1.43
Tyr	13	4.21	13	4.64

[a] The entire sequence had a M_r of 34,349 and 309 residues. The sequence without the proposed signal peptide had a M_r of 31,483 and 280 residues.

pyogenes type 6 M protein (*emm6*) (16). The sequence is not repeated in the fimbrial peptide. Within the string of 12 amino acids, four are identical and three are functionally homologous. Four of the five unpaired residues are hydrophobic in the fimbrial peptide. Comparable leucine residues at fimbrial residue 42 and *emm6* residues 42, 56, and 70 are coded by CTT and TTA codons, respectively. Of 36 nucleotides, 16 are identical between these two fragments, although the M6 fragment is nearly 60% adenine alone, compared with 60% A+T in the fimbrial fragment. Comparison of this fragment of *emm6* nucleotide sequence with randomly shuffled versions of the fimbrial fragment demonstrates statistically significant homology between the *emm6* and fimbrial nucleotide sequence fragments (data not shown). The observed occurrence of 16 matches was >2 standard deviations above the mean number of matches between the M6 fragment and sequence generated randomly from the nucleotides present in the fimbrial sequence fragment.

The *S. sanguis* fimbrial sequence was compared with that of the type 1 fimbrial subunit of *Actinomyces viscosus* T14V (34, and M. Yeung, personal communication). *A. viscosus* and *S. sanguis* are both primary colonizers of the tooth surface. Adhesion to the tooth surface is mediated by fimbriae in both systems. No significant homology was evident between the *S. sanguis* FW213 and *A. viscosus* T14V fimbrial sequences at either the nucleotide or amino acid level. However, overall secondary structure predictions for the fimbrial amino acid sequences (3) combined with patterns of hydrophobicity-hydrophilicity suggest functional homology between the two peptides. Figure 4A and B show Chou-Fasman plots of secondary structure of the *S. sanguis* and *A. viscosus* fimbrial peptides overlaid with symbols representing hydrophilic and hydrophobic regions. Analysis of the first 309 residues of the 533-residue *A. viscosus* fimbrial peptide reveals an overall structure similar to that of the *S. sanguis* fimbrial peptide in terms of predicted locations of turns, alpha-helices, and beta-sheets. Both peptides contain hydrophobic signal sequences. Most significantly, both peptides exhibit similar patterns of alternating hydrophilic and hydrophobic regions throughout the amino acid sequence. In comparison, the predicted secondary structure and hydrophobicity profiles of the *S. pyogenes emm6* peptide (Fig. 4C) are quite distinct from those of the fimbrial peptides.

DISCUSSION

Insertional mutagenesis causing a frameshift within the fimbrial reading frame resulted in loss of immunoreactivity of the cloned protein. The frameshift mutation is located in a region of the DNA sequence which contains only one open reading frame, thus confirming the location and identity of the fimbrial gene.

The ribosome-binding site at nucleotides 309 to 315 is complementary to the 3′ binding sequence of a typical gram-positive 16S ribosomal subunit (22, 24). This sequence also appears to be recognized by the somewhat less stringent *E. coli* ribosome as a binding site for the initiation of translation from the ATG start codon 6 bases downstream.

It is clear from the expression studies reported here and by Fives-Taylor et al. (11) that *E. coli* recognizes a cloned streptococcal promoter. The level of expression of fimbrial protein is quite low from clones in which transcription of the fimbrial gene is not induced from the vector-coded *lac* promoter. The lack of strong consensus among published streptococcal promoter sequences (9) suggests that other factors (nucleotide or peptide) may typically be involved in regulation of transcription of streptococcal genes.

The transcription termination signal identified adjacent to the fimbrial gene sequence is typical of factor-independent procaryotic termination sequences (20, 27). The free binding energy of the putative fimbrial terminator is rather low. It has been observed that streptococcal genes often have weak terminators (2). The overall structure of the proposed terminator, consisting of two rather weak stem and loop structures, resembles the terminator of the *tetO* gene of *Streptococcus mutans* (18). Determination of the in vivo activity of the proposed terminator awaits isolation and analysis of the fimbrial transcript.

The cloned fimbrial protein contains a putative signal peptide sequence for translocation through the cell membrane, which was identified in the amino acid sequence on the basis of the Von Heijne parameters (32). This sequence does not appear to be functional in *E. coli*. We have been able to isolate the cloned fimbrial protein only from the cytoplasmic fraction of *E. coli* lysates (data not shown).

The homology present at both the nucleotide and amino acid levels between a small region of the fimbrial sequence and that of a repeated region of *S. pyogenes emm6* (16) is striking and as yet unexplained. The sequences bear no obvious functional homology. The repeated regions of *emm6* appear to function as a source of antigenic diversity, while the corresponding amino acid fragment in the FW213 sequence is hydrophobic and therefore unlikely to be an antigenic site. The sequence homology may be most usefully viewed as a conserved evolutionary artifact.

The apparent functional homology between *S. sanguis* fimbrial peptide and *A. viscosus* type 1 fimbrial peptide is intriguing. Secondary structures derived from the Chou-Fasman algorithm have limited value in predicting native protein structure; however, the similarities in structure predicted between these two peptides coupled with the similarity in hydrophobicity patterns suggest a level of

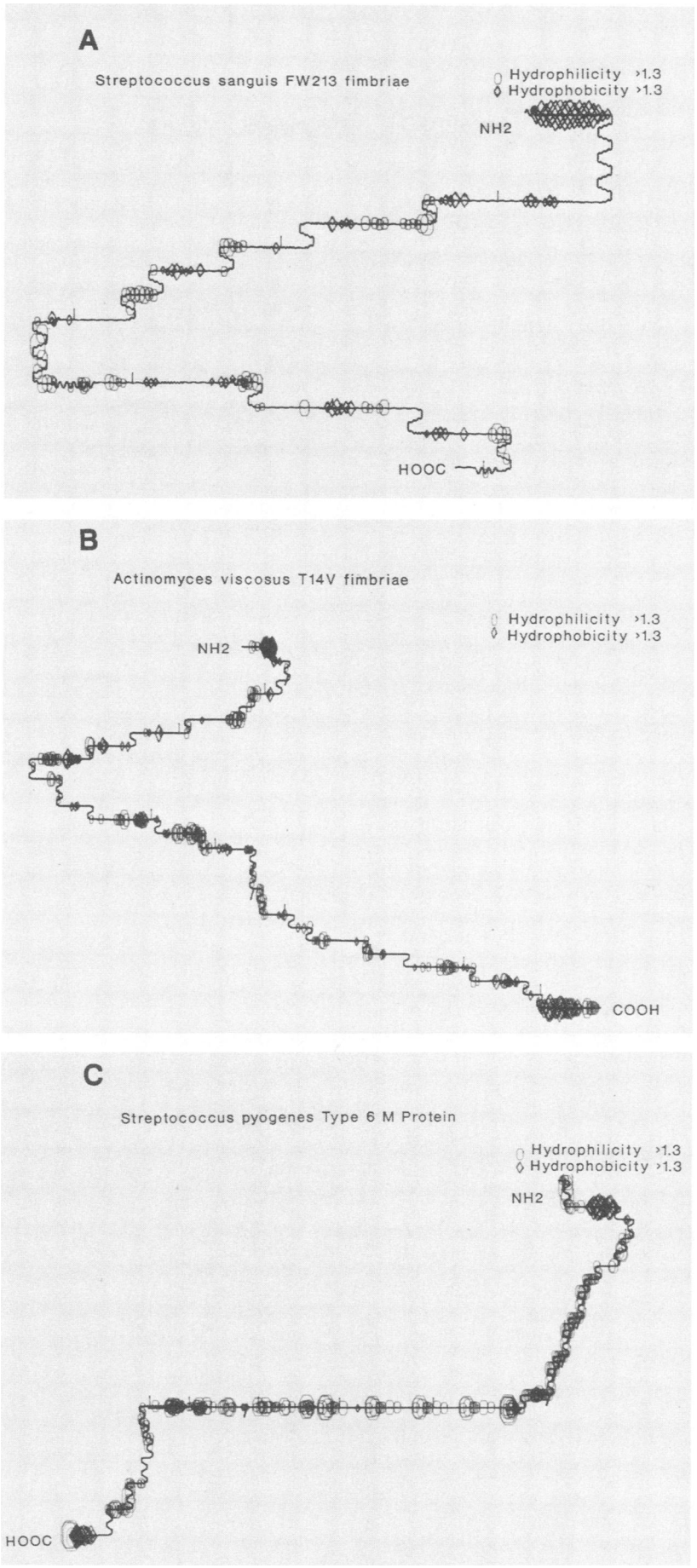

FIG. 4. Protein secondary structure predictions. Predicted secondary structures are shown as templates overlaid with symbols indicating regions of hydrophobicity and hydrophilicity. Plots were prepared by using University of Wisconsin Genetics Computer Group sequence analysis programs and the Chou-Fasman method of secondary structure prediction (3). (A) *S. sanguis* FW213 type 1 fimbriae. (B) *A. viscosus* T14V type 1 fimbriae. (C) *S. pyogenes* type 6 M protein.

structural and functional homology. While these organisms are not closely related, they share an ecological niche as early colonizers of dental surfaces and express adhesion fimbriae. The observation that their fimbrial subunits exhibit functional homology which is reflected at neither the nucleotide nor the amino acid level suggests that this may be an example of convergent evolution.

We have identified and characterized a structural gene of *S. sanguis* adhesion fimbriae. Questions remain as to the location and identity of the adhesin on the fimbriae, as well as to the location of the cloned protein within the fimbrial structure. Further studies are planned to locate specific immunoreactive sites on the fimbrial peptide and to determine the ability of the cloned fimbrial protein to block adhesion of *S. sanguis*. This laboratory has developed several monoclonal antibodies which recognize fimbrial epitopes in extracts made from FW213, one of which specifically inhibits adhesion of FW213 to saliva-coated hydroxyapatite (5). We are currently purifying the cloned fimbrial protein for use in adhesion blocking assays.

The mechanisms of gram-positive fimbrial assembly have not yet been characterized. At present, one may speculate on the possibility of similarities with fimbrial assembly in gram-negative organisms. The assembly of fimbriae of *S. sanguis* is not well understood, in part because of the difficulty in dissociating the native fimbriae. This resistance to dissociation has also been observed for fimbriae of *A. viscosus* (34). The cloned fimbrial peptide with a M_r of 30,000 is not found in surface extracts of FW213 but is found in cytoplasmic extracts (11). The smallest extracellular native fimbrial peptide detected has a M_r of 43,000. The cloned fimbrial peptide does not contain a hydrophobic carboxyl-terminal membrane anchor as has been reported for several gram-positive surface proteins (7, 16, 26, 31). These data suggest the involvement of at least one other structural protein in fimbrial assembly. If that is the case, then there are likely to be several levels of regulation of expression and assembly of these adhesion fimbriae. Further studies of fimbrial gene expression are planned to identify any closely linked structural or regulatory genes.

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