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A 40-Kilodalton Cell Wall Protein-Coding Sequence Upstream of the *sr* Gene of *Streptococcus mutans* OMZ175 (Serotype f)

JOËLLE A. OGIER,* MARIE SCHÖLLER, YVES LEPOIVRE, SOPHIE GANGLOFF, RIDHA M'ZOUGHI, AND JEAN-PAUL KLEIN

Institut National de la Santé et de la Recherche Médicale Unité 157, Faculté de Chirurgie Dentaire, 1 Place de l'Hôpital, Strasbourg 67000, France

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Streptococcus mutans **surface proteins may be important in immunization against dental caries. We report the existence of an open reading frame of 1,005 bp that lies 1,162 bases upstream of the *S. mutans* OMZ175 *sr* gene and that encodes a cell wall-associated protein. This open reading frame codes for 335 amino acid residues. The first 18-amino acid region is predominantly hydrophobic and resembles a signal peptide, and the hydrophobic C-terminal region may function as an anchor to the bacterial cell wall. On the basis of the predicted antigenic determinants of the deduced amino acid sequence, a 16-residue synthetic peptide corresponding to the middle hydrophilic coiled region was synthesized. Antibodies raised against this synthetic peptide reacted with a protein with an apparent M_r of 40,000 that was identified by Western immunoblotting in a cell wall extract from *S. mutans* OMZ175. The high reactivity in an enzyme-linked immunosorbent assay of the antibodies with whole *S. mutans* OMZ175 cells showed that this protein was located on the bacterial cell surface. Furthermore, the antipeptide immunoglobulin G recognized an identical determinant on the cell surface of other members of the *S. mutans* group. However, the function of this protein is not yet known.**

Streptococcus mutans, the primary etiologic agent of the initiation of dental caries, possesses a number of cell surface and extracellular proteins which are implicated in the attachment of these bacteria to salivary glycoproteins in the tooth pellicle (15). These proteins are highly immunogenic and are protective in immunization studies (2). The virulence of *S. mutans* has been investigated by a genetic approach, and several genes specifying cell surface and extracellular proteins have been cloned. Extracellular proteins recently characterized are (i) glucosyltransferases (31), fructosyltransferases (27), and a glucan-binding protein (26), which are implicated in sucrose-dependent adherence; (ii) antigen I/II (12), a protein of M_r 185,000 present in all members of the *S. mutans* group except *S. rattus* and involved in sucrose-independent adherence; and (iii) antigen A (5), a protein of M_r 29,000 found in *S. mutans* and *S. rattus* and the role of which is not yet known. Animal studies have demonstrated that protection against dental caries could be obtained after immunization with purified antigen I/II or related proteins (13) and with antigen A (25). However, some side effects, such as the induction of heart-reactive antibodies (9), the production of rheumatoid factors (24), and antigen mimicry with human immunoglobulin G (IgG) (33), have hampered the development of a vaccine against *S. mutans*, although it was recently shown that the induction of heart-reactive antibodies was not due to antigenic similarity between antigen I/II and components of the human heart (34).

Therefore, the characterization of other *S. mutans* cell surface proteins is of particular importance in the development of a safe vaccine. Previously, we reported the cloning (19) and nucleotide sequence (20) of the *sr* gene of *S. mutans* OMZ175 (serotype f), which encodes the SR protein, an antigen I/II-related protein (12) implicated in the interactions of *S. mutans* with salivary glycoproteins. The present communication describes an open reading frame (ORF) lying 1,162 bases upstream of the *sr* gene. The results presented here indicate that this ORF encodes a cell wall protein of M_r 40,000 containing an antigenic determinant present in members of the *S. mutans* group. However, the role of this cell wall protein in *S. mutans* virulence is still not known.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. mutans* OMZ70 (serotype c), B-2 (serotype e), and OMZ175 (serotype f); *S. cricetus* E49 (serotype a); *S. rattus* OMZ51 (serotype b); *S. sobrinus* OMZ176 (serotype d) and 6715 (serotype g); and *S. downei* MFe 28 (serotype h) have been described elsewhere (1, 28). The strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). *Escherichia coli* NM522 (7) was used as the host for plasmids pTZ18R and pTZ19R (Pharmacia LKB, Uppsala, Sweden). Recombinant plasmid pHBsr-1 has been described elsewhere (19).

Antigens and CE. Cell wall extracted antigens (WEA) and extracellular soluble antigens (ESA) were prepared from exponential-phase cultures of *S. mutans* OMZ175 as previously described (28). Cellular extracts (CE) were prepared from *E. coli* HB101 cells transformed by plasmid pHBsr-1, and recombinant SR protein was prepared from CE as described by Ogier et al. (19).

DNA cloning and analysis procedures. Routine DNA manipulation procedures such as miniscale plasmid preparation, restriction endonuclease analysis, purification of DNA fragments from agarose gels, and subcloning were performed as described by Maniatis et al. (16). Restriction endonucleases and T4 DNA ligase were obtained from Appligene (Strasbourg, France).

DNA sequencing and computer analysis. Restriction fragments covering the 5′-flanking region of the *sr* gene were generated from pHBsr-1 and subcloned in plasmids pTZ18R and pTZ19R. Single-stranded templates were produced from the pTZ plasmids in accordance with the instructions of the manufacturer. Series of deletions extending from the *Bam*HI

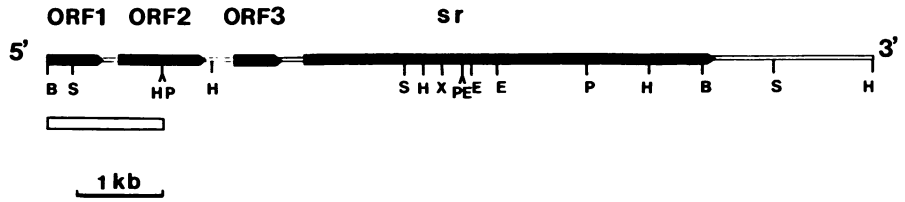

FIG. 1. Restriction map of the *sr* gene and its 5'-flanking region. The thick black bars indicate the ORFs. The open box represents the probe used in the hybridization experiment. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xba*I.

and *Pst*I restriction sites, respectively localized 2,983 bases and 1,692 bases upstream from the start of the *sr* gene, were produced by digestion with exonuclease III (8). The nucleotide sequence was determined on both strands by the dideoxy chain termination method with T7 DNA polymerase (Pharmacia), [^{35}S]dATP (>37 TBq/mmol; Amersham, Les Ulis, France), and the universal reverse primer or appropriate specific synthetic oligonucleotide primers (Appligene). The software package PC Gene (IntelliGenetics, Geneva, Switzerland) was used for the analysis of the sequence data and the prediction of the secondary structure of the protein and epitopes. The hydrophobicity and amphiphilicity properties of the deduced amino acid sequence were analyzed by the method of Kyte and Doolittle (10).

Southern blotting and hybridization procedure. Streptococcal DNA was recovered by the protocol of Robeson et al. (22). Each genomic DNA (20 μg) was *Bam*HI-*Pst*I digested to completion, and fragments were electrophoresed on an 0.8% agarose gel. The Southern blotting protocol (29) was slightly modified: DNA was transferred under vacuum onto nylon membranes (Amersham) and bound to the nylon under UV light at 254 nm. The *Bam*HI-*Pst*I restriction fragment covering the 5' part of ORF 2 (see below) was used to produce a probe labeled by nick translation with biotin-7-dATP (BRL, Cergy-Pontoise, France) in accordance with the instructions of the manufacturer. Hybridizations were performed under standard conditions (16) in the following solution: 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's reagent–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml. Detection was done with a streptavidin-alkaline phosphatase (AP) conjugate (BlueGene system; BRL).

Peptide synthesis and conjugation to carrier protein. On the basis of the predicted antigenic determinants of the deduced amino acid sequence, a 16-residue peptide (SNPQVNVETD SSEKDE) corresponding to the middle region (amino acids 167 to 182) of the protein was synthesized (Neosystem, Strasbourg, France) by a solid-phase method (17). The composition of the peptide was confirmed by amino acid analysis, and the purity (>85.9%) was checked by high-pressure liquid chromatography. Tyrosine was added to the carboxy terminus of the peptide to couple the peptide to ovalbumin through bisdiazobenzidine. The coupling ratio reached 12 mol of peptide per mol of ovalbumin. The peptide was also conjugated to bovine serum albumin (BSA) by glutaraldehyde, with a coupling ratio of 8 mol of peptide per mol of BSA.

Immunization and preparation of antisera. Antipeptide serum was raised in two rabbits by subcutaneous injections of equal mixtures of peptide conjugated to ovalbumin (100 μg of peptide in phosphate-buffered saline [PBS]) and Freund incomplete adjuvant at 15-day intervals. At least 400 μg of peptide was injected, and blood was collected 10 days after the last injection. Anti-SR protein serum was prepared as previously described (33). The IgG fraction of each antiserum was purified by chromatography on DEAE-Trisacryl M essentially by the method of Corthier et al. (3). Control preimmune sera were subjected to the same purification procedure. AP-antirabbit IgG was from Miles (Puteaux, France).

ELISA. The antibody recognition of the expressed protein was checked by an enzyme-linked immunosorbent assay (ELISA) as previously described (18, 33). In brief, microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μl of *S. mutans* whole cells (10^9 bacteria per ml), WEA, ESA, CE (10 μg/ml), or BSA-peptide (1 μg/ml). After the remaining binding sites were blocked with 0.5% gelatin in PBS containing 0.05% Tween 20, the plates were incubated with serial dilutions of antipeptide IgG (1 h, 37°C). Antibody binding was detected with AP-antirabbit IgG and by incubation with a substrate. The A_{405} was read with a Dynatech MR 5000 spectrophotometer.

Electrophoresis and immunoblotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 15% gels with the discontinuous buffer system of Laemmli (11). Proteins were detected by staining with Coomassie blue. In Western immunoblotting experiments, proteins were transferred from SDS-PAGE gels to nitrocellulose sheets by the procedure of Towbin et al. (30) and detected by incubation with antipeptide IgG and with AP-antirabbit IgG and an enzyme substrate.

RESULTS

Nucleotide sequence and analysis. We previously described the construction of plasmid pHBsr-1, which contains the *sr* gene of *S. mutans* OMZ175 on a 9-kbp *Bam*HI-*Hin*dIII insert (Fig. 1), as well as the sequencing strategy used to determine the 4,667 bp of the coding sequence of the *sr* gene (19, 20). We now determined the nucleotide sequence of the upstream region. Analysis of this nucleotide sequence revealed the existence of three ORFs (Fig. 1). The first ORF (ORF1), at the 5' end, extends from an undetermined initiation codon to the termination codon TAA (position 656) and is probably incomplete (Fig. 2). The second ORF (ORF2), starting at the ATG codon (position 816) which is preceded by possible promoter sequences (−35 and −10) and a potential ribosome-binding site, extends to the termination codon TAA (position 1821) (Fig. 2). The third ORF (ORF3) extends from base 1733 to base 2743, with a putative initiation codon ATG at position 2207. Analysis of ORF3 was done on the basis of this potential initiation codon. So defined, ORF3 has the potential to encode a peptide of 179 amino acids, mainly hydrophobic. Because of the poor probability of the existence of a secretory signal sequence in ORF3, we gave

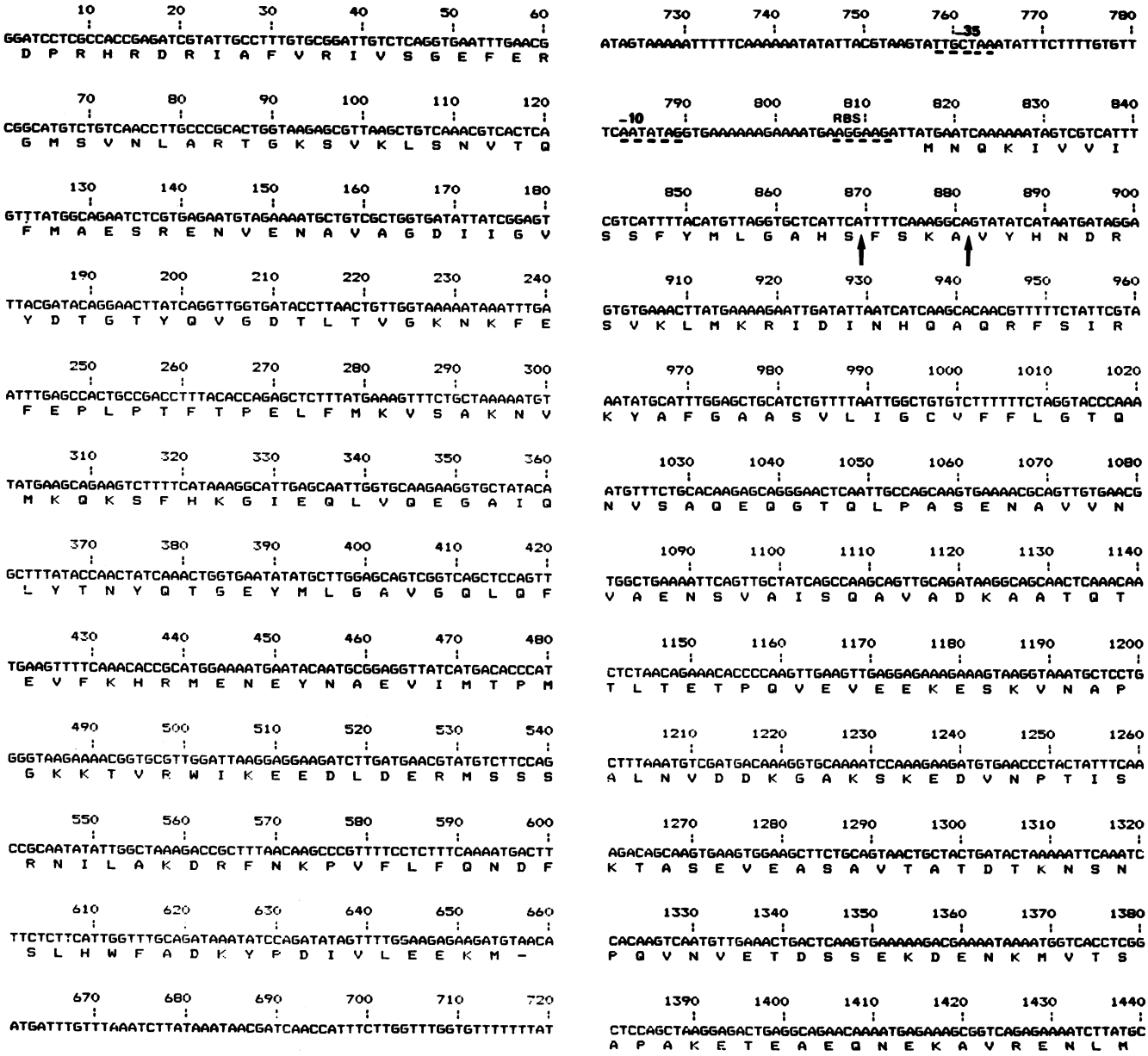

FIG. 2. Nucleotide sequence of the upstream region of the *sr* gene. Numbering starts at the *Bam*HI site (5′ end). The deduced amino acid sequence specified by the ORFs is given below the nucleotide sequence. Possible −35 and −10 promoter regions and a putative ribosome-binding site (RBS) are underlined. The vertical arrows show the putative cleavage sites for the removal of the signal peptide. The underlined ATG (position 2983) is the initiation codon of the gene *sr*.

priority to the study of ORF2, encoding a presumptive transmembrane exported protein.

Characteristics of ORF2. Computer analysis of the nucleotide sequence revealed the absence of internal repeats. ORF2 is transcribed with the same polarity as the *sr* gene and has the potential to encode a 335-amino-acid polypeptide (Fig. 2). To identify this putative gene product, we screened the EMBL Swiss-Prot protein sequence data bank for homologous sequences, but none was found. Examination of the deduced amino acid sequence revealed that the putative protein is hydrophilic, with hydrophobic regions at the N and C termini (Fig. 3). The hydrophobic N-terminal region, consistent with the −3,−1 rule (32), contains two potential signal peptide cleavage sites at amino acids 18 and 19, followed by a phenylalanine residue, and at amino acids 22 and 23. The hydrophobic C-terminal region does not contain any predicted antigenic determinant and may function as an anchor to the bacterial cell wall, although it does not display the features of a cell wall-spanning region described for other gram-positive organisms (21). The deduced molecular weight of the ORF2-encoded polypeptide is 37,141.

Characterization of the expression product. Our goal was to study the expression product of ORF2 by using the following three steps: (i) prediction analysis of the secondary structure of the putative protein, (ii) antibody production against a potential epitope by use of a synthetic peptide, and (iii) detection of the expression product in recombinant *E. coli* and *S. mutans* cells.

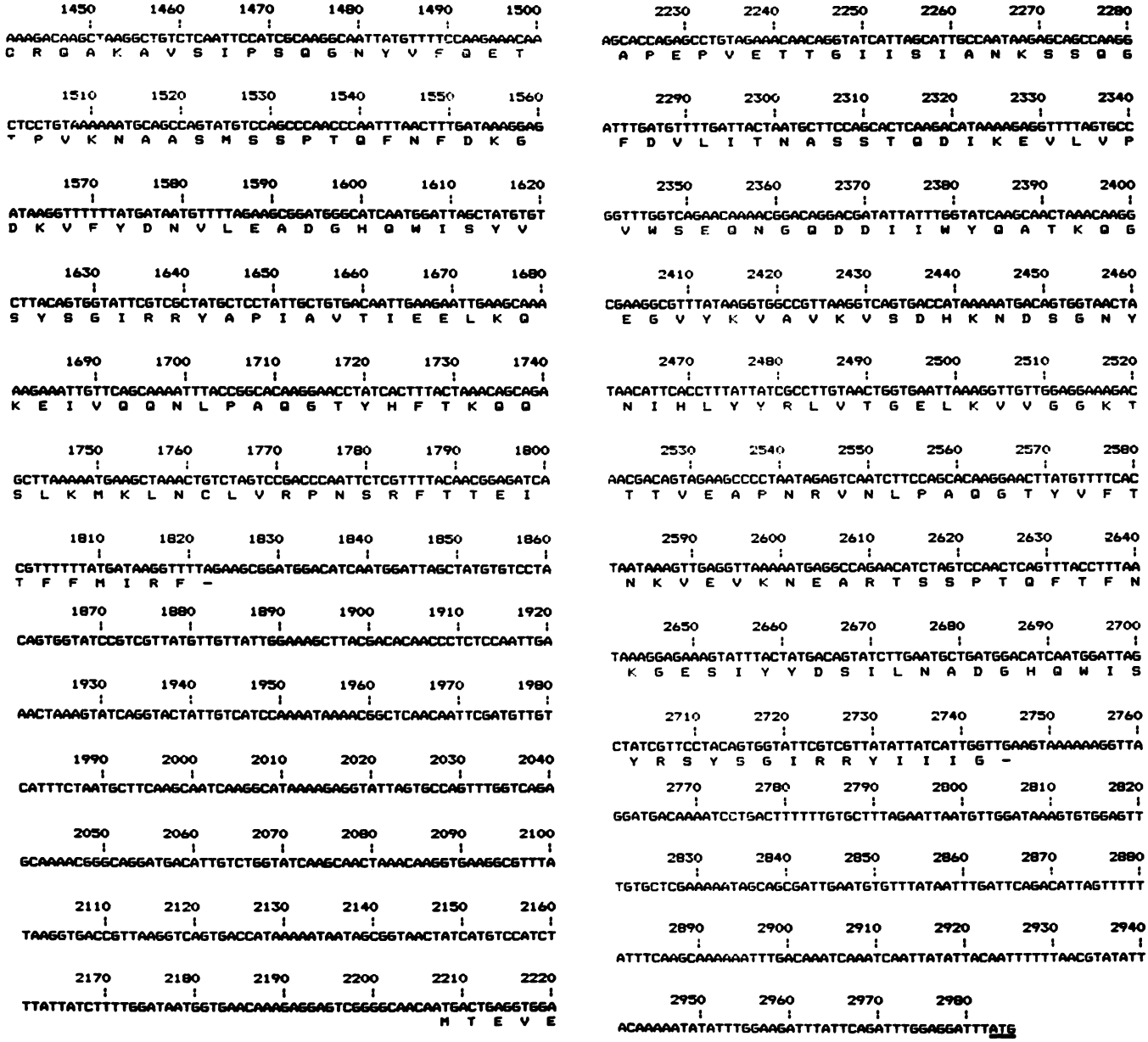

FIG. 2—*Continued.*

In accordance with the secondary structure prediction analysis, (14), we selected the middle hydrophilic coiled region ranging from residue 167 to residue 182 for modeling of the peptide. After synthesis, the 16-residue peptide was conjugated to ovalbumin and used for antibody production in two rabbits. The antipeptide response was measured in an ELISA with the peptide conjugated to a different carrier protein, BSA. The peptide elicited high titers of antibodies in both rabbits. Figure 4 shows the titration curve of antipeptide IgG. We used antipeptide IgG to detect the putative expression product of ORF2 by Western blotting and ELISA. Analysis of CE from *E. coli* HB101 cells carrying recombinant plasmid pHBsr-1 by Western blotting revealed that the expression product of ORF2 migrated as a single band with an apparent M_r of 40,000 (Fig. 5B). This M_r is close to that estimated from the deduced amino acid sequence. Antipeptide IgG showed strong reactivity in ELISA with CE from transformed *E. coli* (Fig. 4). To determine whether this protein was present in *S. mutans*, we analyzed various cellular fractions by ELISA. All of the activity was found associated with whole *S. mutans* cells and WEA but not with ESA, suggesting a cell surface localization of this protein (Fig. 4). Western blot analysis of *S. mutans* WEA and ESA revealed that antipeptide IgG reacted strongly with one protein present in WEA but not in ESA (Fig. 5B). This protein had a molecular weight of 40,000 and migrated like the protein detected in transformed *E. coli*. The presence of this protein in the other streptococci of the *S. mutans* group was determined by a whole-cell ELISA with antipeptide IgG. The results for heterologous binding revealed the presence of the antigenic determinant of the peptide in all of the streptococci (Table 1). On the other hand, we used a

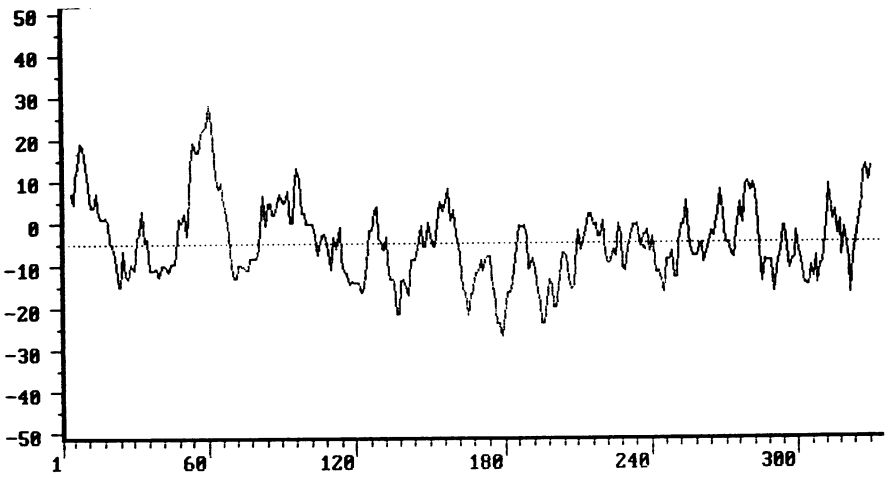

FIG. 3. Hydropathy plot of the ORF2 potential expression product. The *x* axis represents the amino acid number. Hydrophobic and hydrophilic domains are, respectively, above and below the dotted line.

DNA probe covering the 5′ portion of ORF2 (amino acids 1 to 157), with the exception of the peptide-encoding region, in the hybridization analysis. Under high-stringency conditions, the probe hybridized only with the homologous restriction fragment of *S. mutans* OMZ175 chromosomal DNA and with chromosomal fragments derived from *S. mutans* strains from serotypes c and e, indicating the absence of close homology with the other streptococcal genes (Fig. 6) or at least a weaker conservation of the 5′ portion of the gene.

DISCUSSION

Several approaches, including biochemical, immunological, and genetic methods, have been used to characterize *S. mutans* surface proteins because of their possible importance as potential antigens in immunization against dental caries. Here we report the existence of an ORF that lies upstream of the *S. mutans* OMZ175 *sr* gene and that encodes a cell-associated protein. From the DNA sequence analysis, we deduced that the encoded protein was composed of 335 amino acids and was possibly shortened by a signal peptide. To study the putative expression product of this ORF, we elicited antibodies against a synthetic 16-residue peptide derived from the deduced amino acid sequence and corresponding to the middle hydrophobic region of high predicted flexibility. Antipeptide IgG was used to determine, identify, and localize the authentic streptococcal protein. The antibodies reacted with a 40-kDa protein present in both CE from recombinant *E. coli* and WEA from *S. mutans*, showing that the expression of this *S. mutans* protein was efficient in *E. coli*. The M_r deduced from the ORF sequence is 37,100, similar to the M_r of the gene product in *E. coli* and to the M_r of the protein identified in *S. mutans*, suggesting the absence of any posttranslational maturation process. Antipeptide IgG reacted with the streptococcal protein not only in immunoblotting, possibly indicating a denatured state, but also in ELISA, in which the structure of the protein is likely to be more native. Furthermore, the high reactivity of the antibodies with whole *S. mutans* serotype f cells confirmed the fact that the protein is exported at the cell surface and that the hydrophobic C-terminal region probably anchors the protein to the cell wall. Antipeptide IgG recognized an identical determinant on the cell surface of other streptococci of the *S. mutans* group. However, that genetic diversity exists within these streptococci is evidenced by the hybridization

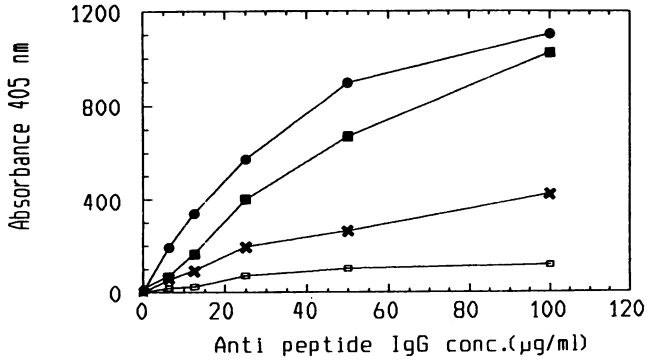

FIG. 4. Dose-dependent binding of antipeptide IgG to peptide conjugated to BSA (■), CE from *E. coli* HB101 (●), and WEA (×) and ESA (□) from *S. mutans* OMZ175. Values obtained with control preimmune IgG are deducted.

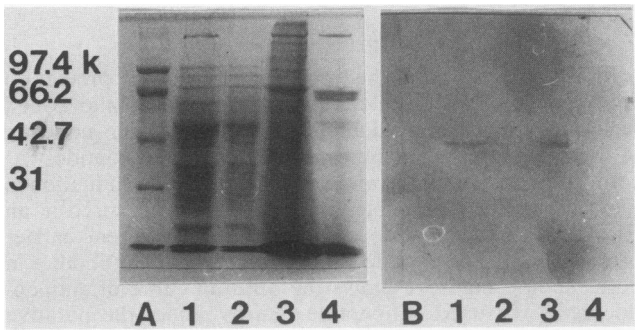

FIG. 5. (A) Coomassie blue-stained SDS-PAGE gel of CE from transformed (lane 1) and control (lane 2) *E. coli* HB101 and of *S. mutans* OMZ175 WEA (lane 3) and ESA (lane 4). Forty micrograms of protein per fraction was run. (B) Western blot of the gel incubated with antipeptide IgG and with AP-antirabbit IgG. Relative molecular mass markers are indicated at the left in kilodaltons (k).

TABLE 1. Binding of antipeptide IgG to streptococcal whole cells as measured by ELISA[a]

Strain (serotype)	A_{405}
S. cricetus E49 (a)	700
S. rattus OMZ51 (b)	1,140
S. mutans OMZ70 (c)	640
S. sobrinus OMZ176 (d)	1,080
S. mutans B-2 (e)	1,070
S. mutans OMZ175 (f)	1,100
S. sobrinus 6715 (g)	860
S. downei MFe 28 (h)	960

[a] Antipeptide IgG was used at a concentration of 50 μg/ml, and the results were read after 1 h at 405 nm. A_{405} values represent the read values minus values obtained with preimmune serum.

experiments, which revealed a poor degree of homology in DNAs from the different serotypes. The *S. mutans* OMZ175 DNA fragment used as a probe did not appear to have sequence homology with the DNAs of the strains of *S. cricetus*, *S. rattus*, *S. sobrinus*, and *S. downei* used in this study. A similar finding was recently reported by Goldschmidt and Curtiss concerning the antigen I component of the *S. sobrinus* SpaA protein (6). Although the antigen I-encoding fragment of *S. sobrinus* DNA did not appear to have sequence homology with the chromosomes of other members of the *S. mutans* group, the immunodeterminant of antigen I was shown to cross-react with cell surface proteins from *S. mutans* of various serotypes. Russell described a cell wall antigen (antigen III) present in *S. mutans* serotypes c, e, and f and *S. rattus* serotype b and having an M_r of 39,000, similar to the M_r of our protein (23). Recently, the gene specifying a precursor form of antigen A (*wapA*), a cell wall-associated antigen from *S. mutans* serotype c that is similar to antigen III, has been cloned (4) and sequenced (5). A comparison of the amino acid sequence reported here and the sequence of the *wapA*-encoded protein as well as those of other *S. mutans* proteins failed to reveal any homology, demonstrating that our 40-kDa protein is different from antigen III or A. In summary, by using antipeptide IgG, we identified a new *S. mutans* cell wall protein. The function of this 40-kDa protein remains unknown, and further studies will be necessary to define the role, if any, of this protein in the virulence of *S. mutans* and to determine whether interactions occur with other cell wall components.

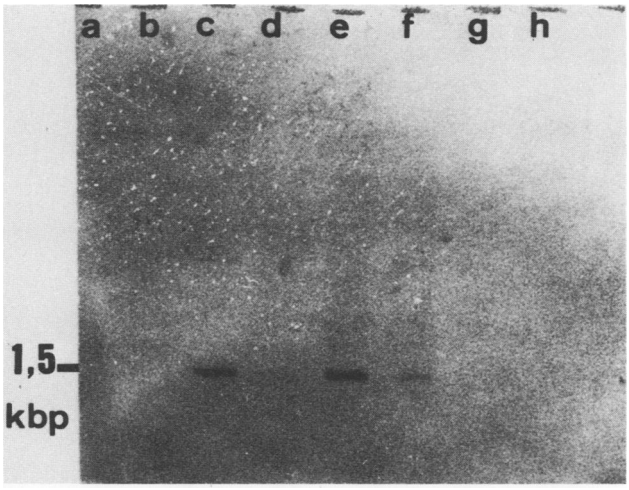

FIG. 6. Southern hybridization. Lanes a, b, c, d, e, f, g, and h indicate the patterns of hybridization of the biotin-7-dATP probe to the *Bam*HI-*Pst*I-restricted chromosomal DNAs (20 μg each) of *S. mutans* strains from the corresponding serotypes.

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