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The oligopeptide (*opp*) gene cluster of *Streptococcus mutans*: identification, prevalence, and characterization

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Nepomuceno RSL, Tavares MB, Lemos JA, Griswold AR, Ribeiro JL, Balan A, Guimarães KS, Cai S, Burne RA, Ferreira LCS, Ferreira RCC. The oligopeptide (opp) *gene cluster of* Streptococcus mutans*: identification, prevalence, and characterization.*
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Introduction: The Opp system is an ATP-binding cassette-type transporter formed by membrane-associated proteins required for the uptake of oligopeptides in bacteria. In gram-positive bacteria, the Opp system, and particularly the oligopeptide-binding protein (OppA), has been shown to be involved in different aspects of cell physiology, including intercellular communication and binding to host proteins.
Methods: In the present study we began to investigate the Opp system of *Streptococcus mutans*, the main etiological agent of dental caries.
Results: Five *opp* genes (*oppABCDF*) organized in a single operon were identified in the genome of the *S. mutans* UA159 strain. Amino acid sequence analyses showed that the *S. mutans* OppA is closely related to an ortholog found in *Streptococcus agalactiae*. Incubation of *S. mutans* UA159 cells with an anti-OppA-specific serum did not inhibit biofilm formation on polystyrene plates. Moreover, *S. mutans* UA159 derivatives carrying deletions on the *oppA* or *oppB* genes did not show significant growth impairment, increased sensitivity to aminopterin, or defective capacity to form biofilms on polystyrene wells in the presence or not of saliva. Remarkably, only two out of three laboratory strains and one out of seven clinical strains recovered from tooth decay processes harbored a copy of the *oppA* gene and expressed the OppA protein.
Conclusion: Collectively, these results indicate that, in contrast to other *Streptococcus* species, the *S. mutans* Opp system, and particularly the OppA protein, does not represent an important trait required for growth and colonization.

Key words: biofilm; oligopeptide uptake; OppA; Opp system; *Streptococcus mutans*

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Bacterial oligopeptide transport systems (Opp) belong to a family of ATP-dependent transporters [ATP-binding cassette (ABC) transporters] that couple peptide transport with the energy released by ATP hydrolysis. The Opp transporters are multicomponent systems that comprise two transmembrane hydrophobic domains (OppB and OppC), delimiting a membrane pore in which the peptide is translocated to the cell cytoplasm, and two ATP-binding domains (OppD and OppF), attached to the inner wall of the cytoplasmic membrane components, regulating ATP hydrolysis and energy generation required for solute transport (20). In addition to these conserved components, the Opp systems employ a specific ligand-binding protein (OppA) that is responsible for recognizing, binding and ushering peptide substrates to the membrane-associated permease complex, thus, conferring specificity and affinity to the transport system (15, 20). The OppA of gram-negative bacteria are soluble periplasmic proteins, whereas in gram-positive bacteria OppA consists of a lipoprotein covalently linked to the cytoplasmic membrane via a thioether linkage between an N-terminal cysteine residue and diacylglycerol (31, 42).

In addition to the most obvious role in nutrient uptake, bacterial OppA orthologs are involved with various physiological functions. In *Escherichia coli* and *Salmonella typhimurium*, the recycling of up to 50% of cell wall muropeptides has been shown to rely on the Opp system (33). In several gram-positive bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation and transformation are peptide-mediated quorum-sensing responses that take place only in the presence of a functional oligopeptide uptake system, including at least one active OppA ortholog (3, 34, 38). The multiple effects of peptide pheromones on bacterial physiology also explain the reported roles of OppA on sporulation and modulation of gene expression among different gram-positive bacterial species (25, 36, 38). Expression of OppA has also been reported to contribute to the sensitivity to toxic peptides and aminoglycoside antibiotics in *E. coli* and *S. typhimurium* strains (1, 41). Moreover, several studies have established a link between the expression of virulence-associated traits, such as adhesions, and the presence of functional OppA orthologs (7, 23). More recently, the involvement between OppA expression and biofilm formation in *Vibrio fluvialis* has been reported (27).

Opp transporters are well represented among pathogenic and non-pathogenic *Streptococcus* species. Usually the *opp* genes are organized into a polycistronic operon with up to three copies of the *oppA* gene, suggesting that the higher OppA stoichiometry contributes both to a more efficient uptake of oligopeptide substrates and to adaptation to different environments. In *S. pneumoniae*, three OppA orthologs (AmiA, AliA, AliB) have been described and *amiA* and *aliA* mutants showed reduced adherence to lung epithelial cells and vascular endothelial cells, affecting *in vivo* colonization of the nasopharynx (12, 23). Mutations in *hppA*, one of three *oppA* orthologs of *Streptococcus gordonii*, negatively affect adhesin expression, leading to reduced aggregation to other oral bacteria and binding to human serum components (30). Two copies of OppA orthologs have been identified in *Streptococcus agalactiae* and both seem to be involved in the modulation of virulence traits, such as the adhesion to epithelial cells and fibronectin (40). OppA has also been shown to modulate the expression of several virulence-associated genes of *Streptococcus pyogenes* (45). Collectively these findings suggest that OppA, and the corresponding membrane permease, has a dual role in streptococcal physiology affecting both the lifestyle of the species and regulation of virulence-associated genes via the control of nutrient uptake and environmental sensing (10, 31).

Streptococcus mutans is considered the main etiological agent of human dental caries. Caries promotion requires adhesion of *S. mutans* strains to host proteins, enamel pellicle, and other oral bacterial colonizers followed by the development of a structurally complex three-dimensional biofilm (8, 9, 14). Survival in a stressful environment with periodic shifts in pH and nutrient availability also plays a pivotal role in the ability of *S. mutans* to thrive and cause tooth decay (28). Under such conditions the study of active transport systems dedicated to the acquisition of nutrients, specifically peptides, is particularly relevant for a better understanding of the physiology and virulence of *S. mutans*. In this report, we identified and characterized the Opp system of *S. mutans* with special emphasis on the oligopeptide binding-component, OppA, and its relation to biofilm formation.

Materials and methods

Bacterial strains and growth conditions

All bacterial strains used in the present study are listed in Table 1. The *S. mutans* strains were grown in tryptic soy broth supplemented with 2.5% yeast extract (TSBY) or in brain–heart infusion in a capnophilic atmosphere. *E. coli* strains were grown on Luria–Bertani broth or in M9 minimal medium at 37°C in an orbital shaker. When required, ampicillin (100 μg/ml) and/or kanamycin (50 μg/ml) were added to the growth media of recombinant *E. coli* strains. *S. mutans* clinical isolates were isolated according to previously described procedures (19). Saliva samples were collected from seven patients under treatment for tooth decay, plated on agar mitis salivarius medium and incubated for 48–72 h. Putative *S. mutans* colonies were screened for the presence of the *gtfB* gene using primers and amplification conditions that have been previously reported (32). The ability of cells to form stable biofilms in saliva-coated plates was assessed by growing the strains in Biofilm Medium (BM) broth (29) supplemented with glucose or sucrose at a final concentration of 20 and 10 mM, respectively.

Sequence analyses

Searches for OppA ortholog sequences were carried out using the KEGG2 program of the Bioinformatics Center Institute for Chemical Research Kyoto University (http://www.genome.ad.jp/keeg/keeg2.html). The sequences of different *opp* operons were also retrieved from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). The *S. mutans* OppA signal sequence and corresponding cleavage site were searched with the SIGNAL IP program (http://www.cbs.dtu.dk/services/SignalP/). The *opp* signature sequence motifs were searched with the motif sequence finder program (http://motif.genome.jp/). The alignments of multiple OppA sequences, including the signal peptides, were generated through the GENEDOC program (http://www.psc.edu/biomed/genedoc/), while un-rooted trees were generated with the CLUSTAL W program (http://align.genome.jp/) based on the neighbor-joining method with a bootstrap value of 100 replicas.

Construction of OppA-deficient and OppB-deficient strains

Streptococcus mutans oppA and *oppB* genes were mutated by allelic replacement with the Ω kanamycin-resistance gene cassette (Ω Km^R) harboring strong transcription/translation terminators (37) using

Table 1. Bacterial strains used in this study

Bacterial strains	Characteristics	Source or reference
S. mutans strains		
UA 159	Strain used in the genome sequencing	Laboratory strain
ATCC 25175	Reference strain isolated from a caries process	(11)
LT 11	Genetic modified strain used in transformation experiments	(43)
AR152	Δ*oppA*::Km^r derivative of the UA159 strain	This work
AR153	Δ*oppB*::Km^r derivative of the UA159 strain	This work
A1 to A7	Clinical isolates	This work
E. coli K12 strain		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F'proAB lacI^{q}ZΔ*M15 Tn10 (Tet^r)]	Stratagene

a polymerase chain reaction (PCR) ligation mutagenesis approach (24). Primers *oppA*-540S (5′-GAGTCGGGATAGATATGATCG-3′) and *oppA*-1340AS-*Sma*I (5′-CTTTTCTTTATTGCTATCAT*CCCGGG*TCCT-3′) were used to amplify an 800-base-pair (bp) region upstream of *oppA*. Primers *oppA*-2980S-*Sma*I (5′-CTTCACCTATGCTTAT*CCCGGG*TAACTTTT-3′) and *oppA*-3800AS (5′-CCATAGGACCCACTAAAGTCA-3′) were used to amplify an 800-bp region downstream of *oppA*. Primers *oppB*-2500S (5′-CAAGAGATTGGCGAAACATCAG-3′) and *oppB*-3100AS-*Sma*I (5′-GAATATATTTGAGCATAATG-*CCCGGG*TTTCTT-3′) were used to amplify a 600-bp region upstream of *oppB*. Primers *oppB*-4020S-*Sma*I (5′-CGTTTGCAGTAA*CCCGGG*TCACATGGC-3′) and *oppB*-4620AS (5′-GCAATAATCCCATTCCCCTAATAA-3′) were used to amplify a 600-bp region downstream of *oppB*. Restriction enzyme recognition sites are underlined. The PCR products were digested with *Sma*I and ligated to a *Sma*I-digested Ω Km^R fragment. The ligation mixtures were introduced into *S. mutans* UA159 by natural transformation, and bacteria were plated on BHI agar containing 1 mg/ml kanamycin. The target *oppA* and *oppB* genes were replaced by the kanamycin-resistance encoding cassette in a double-crossover recombination event. Positive transformants were confirmed by PCR using primers *oppA*-540S and *oppA*-3800AS to confirm *oppA* deletion (3260-bp product, wild-type; 4000-bp product, *oppA* mutant); and primers *oppB*-2500S and *oppB*-4620AS to confirm *oppB* deletion (2120-bp product, wild-type; 3600-bp product, *oppB* mutant).

Cloning, expression, and purification of the recombinant *S. mutans* OppA protein

The *S. mutans* UA159 *oppA* gene was amplified without the signal peptide and the first two initial amino acids of the mature protein sequence including the N-terminal cysteine, which is lipid acylated in the mature protein using primers cFaSB2FW (5′-GCGC*GGATCC*TCAGGAAGTAATCTGTTG-3′) and OppASS3rv (5′-GCGC*GAGCTC*TTATTTCTTATAAGCATAGGTGAAG-3′) according to the reported *oppA* gene sequence (GenBank accession number 1027847). *Bam*HI and *Sac*I restriction sites (underlined in the sequences) were introduced at the 5′ ends of the forward and reverse primers, respectively. PCR were carried out with Deep Vent® *Taq* high-fidelity polymerase (Invitrogen, Carlsbad, CA). Amplifications were performed with a PCT-100™ Programmable Thermal Controller (MJ Research, Waltham, MA) programmed for an initial incubation of 2 min at 96°C followed by 29 amplification cycles (96°C for 1 min and 30 s; 56.1°C for 2 min; 73°C for 1 min and 30 s) and a final extension step at 73°C for 8 min. The 1.6-kilobase amplified fragment was extracted from agarose gels and cloned into a blunt-end cleaved (*Sma*I) pBKSII$^+$ vector and, then, transferred to the cloned expression vector pQE-30 (Qiagen, Crawley, UK). One plasmid containing the right insert (pQRRJ) was confirmed by DNA sequencing and transferred to *E. coli* K12 XL1-Blue strain. Transformed cells were grown in M9 minimal medium containing ampicillin at 28°C until mid log phase (OD_{600} 0.5–0.6) and OppA-expression induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mmol/l. The cultures were incubated aerobically (200 r.p.m.) for 4 h at 28°C and sonically disrupted in a model Digital Sonifier® (Branson Ultrasonics, Danbury, CT). The cell lysates were centrifuged at 10,000 ***g*** for 5 min at 4°C and the soluble fractions were separated from the non-soluble material by centrifugation at 16,000 ***g*** for 20 min at 4°C. The purified recombinant OppA protein was obtained following binding to nickelated ProBond Resin (Invitrogen) equilibrated with buffer B (10 mmol/l Tris–HCl, 100 mmol/l NaH_2PO_4, 8 mol/l urea, pH 8.0). The charged resin was washed with 10 volumes of buffer B followed by step gradient elution with buffers containing increasing concentrations of imidazole (5–400 mmol/l). OppA was eluted as a sharp peak at 100 mmol/l imidazole. The eluted fractions protein composition was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% acrylamide gels stained with Coomassie Blue. Protein was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the supplier's instructions.

Growth sensitivity to aminopterin

To measure growth sensitivity in the presence of aminopterin (Sigma, Poole, UK), the chemically defined FMC medium (44) containing twofold serial dilutions of 10 μM aminopterin was inoculated with bacterial cells and incubated at 37°C for 24 h. Growth was monitored using a Bioscreen C growth monitor (Oy Growth Curves AB Ltd, New Jersey) with measurements taken every hour.

Biofilm assay

The *S. mutans* biofilm formation was assessed following cultivation of strains in 96-well flat-bottom microtiter plates (Nunc), as previously described but with minor modifications (5). The binding of *S. mutans* to saliva-coated plates was determined following incubation of microtiter plates with 50 μl of clarified saliva for 1 h at room temperature (6). Overnight cultures of *S. mutans* UA159, OppA-deficient (AR152) and OppB-deficient (AR153) strains were transferred to prewarmed BHI medium and grown at 37°C to an OD_{600} of ∼0.5. The cultures were diluted 1 : 100 in fresh BM broth supplemented with sucrose or glucose as a carbon source and 200-μl aliquots of the cell suspensions were transferred into the microtiter plate wells. Wells containing growth media were used as negative controls. Plates were incubated at 37°C in a capnophilic atmosphere for 24 h, followed by gentle washing in water to remove non-adherent bacteria. After drying the excess liquid on paper towels, adherent bacteria were stained with 100 μl of 0.1% crystal violet for 15 min at room temperature followed by two gentle rinses with water. The bound dye was solubilized by addition of 200 μl ethanol–acetone (8 : 2) and the absorbance of the solution was measured at 600 nm in a microtiter plate reader. To evaluate the antiadhesive properties of the anti-OppA serum, bacterial aliquots (10^7 colony-forming units) suspended in TSBY medium were incubated for 30 min with different mouse sera, transferred to polystyrene microplate wells and incubated for 24 h at 37°C. As a non-specific control serum, mouse serum raised with an unrelated protein (the maltose binding protein of *Xanthomonas axonopodis* pv. *citri*) was used at the same final dilutions.

Detection of *S. mutans oppA* gene and OppA protein

Chromosomal DNA from three laboratory strains and seven clinical *S. mutans* isolates were isolated and submitted to PCR amplification as described above. For Southern blot hybridizations, chromosomal DNA was digested with *Eco*RI, sorted in 0.7% agarose gels and transferred to Hybond nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK), as described by Sambrook et al. (39). A DNA fragment containing the full-length *oppA* gene was amplified from strain UA159 and labeled by nick

translation with [α^{32}P]dCTP (Amersham Bioscience, Buckinghamshire, UK). After hybridization, the membrane was washed with 1% sodium dodecyl sulfate in 1× sodium saline citrate and was exposed to radiography films (Kodak) for 24 h at –80°C. Detection of the *S. mutans* OppA protein was carried out with anti-OppA-specific sera generated in BALB/c mice injected with the purified recombinant OppA protein. Five 6- to 8-week-old female mice were immunized with four subcutaneous doses of the purified protein (37.5 μg/dose) suspended in complete (first dose) or incomplete (the remaining three doses) Freund's adjuvant at days 0, 15, 22, and 26. Sera were collected by retro-orbital puncture 1 week after the last immunization. Western blots were accomplished with whole cell extracts of different *S. mutans* strains sorted in 12.5% acrylamide gels and subsequently transferred to nitrocellulose filters blocked with phosphate-buffered saline (PBS) containing 2% skim milk for 1 h at room temperature. The anti-OppA serum was used at a final dilution of 1 : 500 in PBS–2% skim milk for 2 h and the peroxidase-conjugated goat antimouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Gove, PA) was used at a dilution of 1 : 3000 for a further 2 h. Protein bands reacting with the anti-OppA antibodies were detected with the SuperSignal chemiluminescent kit (Pierce) and MXG/Plus radiographic films (Kodak).

Results

Characterization of the *opp* operon of *S. mutans* UA159

Five structural genes (*oppA*, *oppB*, *oppC*, *oppD* and *oppF*) encoding components of the Opp system were identified in the *S. mutans* UA159 genome (2) as a single operon-like sequence encompassing a total length of 5692 bp (Fig. 1). The first cistron, *oppA*, encodes a 549-amino-acid (aa) lipoprotein with a molecular weight of 60.2 kDa corresponding to the peptide-binding domain. The OppA protein has a predicted signal peptide of 27 aa and an N-terminal cysteine residue acylated to membrane lipids after processing of the signal peptide. The next two genes of the operon, *oppB* and *oppC*, overlap by 6 bp and encode a 33.4-kDa (304-aa) protein and a 36.9-kDa (343-aa) protein, respectively, defining the membrane pore of the permease, as inferred by the presence of nine hydrophobic membrane-spanning domains. The ATP-binding components of the *S. mutans* Opp system, corresponding to a 39.1-kDa (350-aa) protein and a 34.8-kDa (308-aa) protein, were encoded by the two remaining genes, *oppD* and *oppF*, respectively. Both proteins carry the typical Walker A and B motifs common to nucleotide-binding proteins and a consensus signature sequence, LSGGQ, usually present among ABC superfamily members (46). No Opp paralogs with similarity threshold value higher than 10% for the respective mature amino acid sequences were detected in the genome of *S. mutans* UA159.

The *S. mutans opp* operon is identical to those previously described for several other bacterial species, including *Streptococcus* species in which the operon has been identified. An interesting feature of the *S. mutans* Opp system was the presence of a single copy of the *oppA* gene. The presence of paralogs with similar molecular weights and sharing at least 10% sequence identity was not found in the genome of the *S. mutans* UA159 strain. In contrast, two or three OppA paralogs were present in the genomes of other *Streptococcus* species such as *S. pneumoniae*, *Streptococcus thermophilus*, *S. pyogenes*, *Streptococcus uberis*, *S. agalactiae*, and *S. gordonii* (Fig. 1).

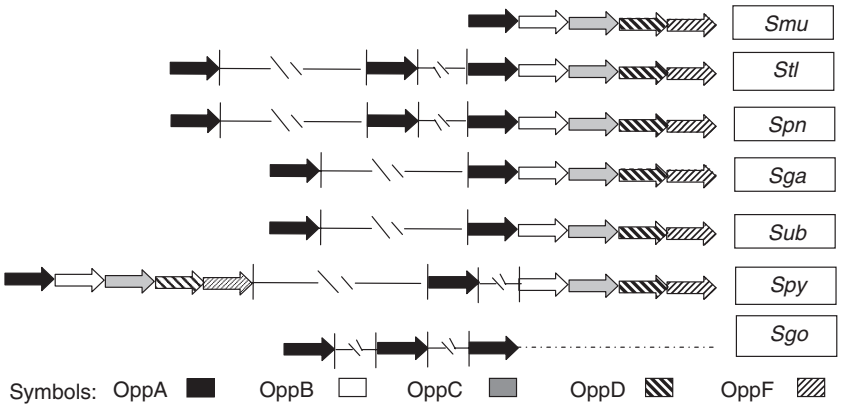

Fig. 1. Genetic organization of *Streptococcus opp* operons. The arrow shadings correspond to specific *opp* genes, as indicated in the figure. The bacterial species represented in the figure are: *Smu*, *S. mutans* UA 159; *Stl*, *S. thermophilus* LMG 18331; *Spn*, *S. pneumoniae* TIGR4; *Sga*, *S. agalactiae* 2603V/R; *Sub*, *S. uberis* 0140J; *Spy*, *S. pyogenes* SSI-1; *Sgo*, *S. gordonii* DL1 Challis.

Sequence similarity of streptococcal OppA proteins

Amino acid sequence analysis of OppA orthologs revealed that the *S. mutans* UA159 OppA is more closely related to the *S. agalactiae* and *S. uberis* orthologs than the protein expressed by other pathogenic *Streptococcus* species (Fig. 2). Overall, the unrooted phylogenetic tree of known streptococcal OppA revealed the presence of four distinct homology groups (Fig. 2). The first group encompasses the *S. mutans*, *S. agalactiae* (OppA2), and *S. uberis* (OppA1 and OppA2) orthologs with full-length identity values of 63% and 57% for the *S. agalactiae* and *S. uberis* OppA2 orthologs, respectively, with regard to the *S. mutans* ortholog. Group 2 encompasses the *Enterococcus faecalis* orthologs, sharing a maximum of 34% amino acid identity with the *S. mutans* OppA. Group 3 is composed of the *S. pyogenes* OppA1, the three *S. pneumoniae* proteins (AmiA, AliA, and AliB), the *S. thermophilus* orthologs (AmiA2 and Ami3), and the *S. gordonii* HppA. In this group, the *S. gordonii* HppA is the otholog most closely related to *S. mutans* OppA with an overall amino acid identity of 29% for the whole protein sequence. The fourth and last similarity group is formed by the *S. agalactiae* OppA1 and *S. pyogenes* OppA2 orthologs with identity values of 22% and 21%, respectively, with the *S. mutans* OppA ortholog.

Cloning, expression, and purification of the recombinant *S. mutans* OppA

The amplified 1600-bp fragment containing the gene encoding the mature OppA protein with the first two amino acids deleted, including the N-terminal lipid acylated cysteine, was amplified by PCR and subsequently cloned into the expression vector pQE-30. The recombinant protein (58 kDa) remained soluble and was recovered as a single protein band in Coomassie-blue-stained polyacrylamide gels (Fig. 3A). Under the tested purification conditions, the protein yield was approximately 2 mg/l IPTG-induced cultures. BALB/c mice were immunized with

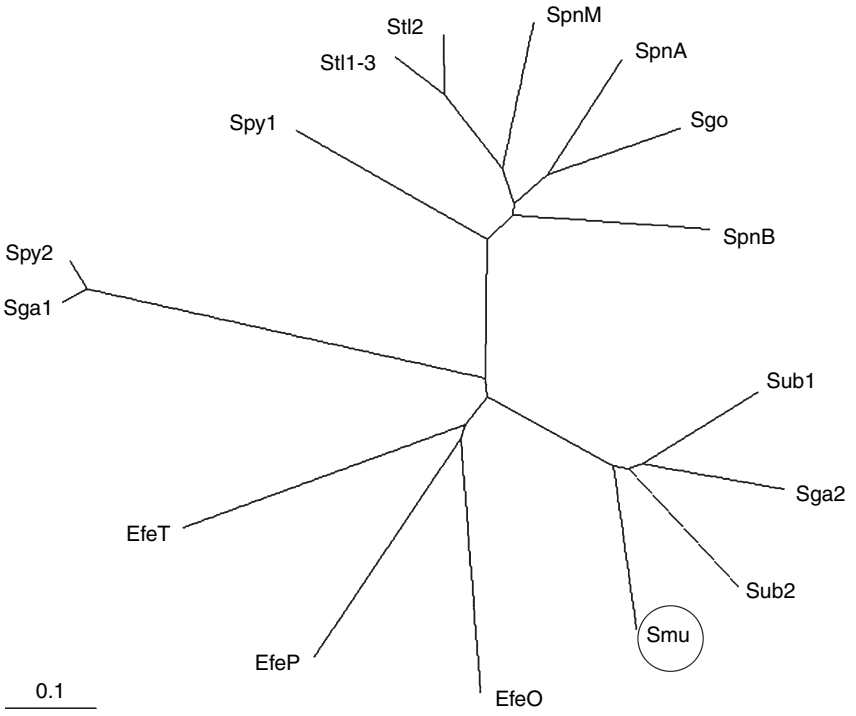

Fig. 2. An unrooted radial phylogenetic tree of OppA proteins expressed by different bacterial species. The represented bacterial species and corresponding OppA paralogs are: Smu, *S. mutans* (OppA); Sga1 and Sga2, *S. agalactiae* (OppA1, and OppA2, respectively); SpnM, SpnA, and SpnB, *S. pneumoniae* (AmiA, AliA, and AliB, respectively); Spy1 and Spy2, *S. pyogenes* (OppA1, OppA2, respectively); Stl1, Stl2, and Stl3, *S. thermophilus* (AmiA1, AmiA2, and AmiA3, respectively); Sgo, *S. gordonii* (HppA); Sub1 and Sub2, *S. uberis* (OppA1, and OppA2, respectively); EfeO, EfeP, and EfeT, *E. faecalis* (OppA, PrgZ, and TraC, respectively). Branch ramification confidential levels were based on 100 replica bootstraps. The similarity analyses were carried out with the GENEDOC and CLUSTAL W programs. Position of the *S. mutans* UA 159 OppA sequence is indicated on the tree. The bar represents 10% sequence divergence.

four subcutaneous doses of the purified protein and a serum pool enriched in anti-OppA antibodies (final titer of 18,829) was used in Western blot experiments to detect cross-reacting bands expressed by different *S. mutans* strains.

Characterization of OppA-deficient and OppB-deficient derivatives of S. mutans UA159

To further evaluate the role of the Opp system on the physiology and pathogenicity of *S. mutans*, we constructed derivatives of the UA159 strain defective in the expression of the *oppA* or *oppB* genes. The first strain, named AR152 strain, carries a deletion of the *oppA* gene while the *oppB* gene was deleted in strain AR153. Of note, the presence of strong transcription/translation terminators in the Ω Km^R gene cassette used to replace *oppA* and *oppB* implies that AR152 is defective in its expression of the entire operon, whereas the *oppB* mutant expresses only OppA. Western blot analysis using specific anti-OppA serum confirmed that OppA was absent in AR152 but was still expressed in strain AR153 (Fig. 3C). Both mutants showed no differences in growth rates when cultivated in the chemically defined FMC medium (Fig. 4) or in the nutritionally rich BHI medium (data not shown). Unexpectedly, when tested for sensitivity to the folate analog aminopterin, both the *oppA* and *oppB* mutant strains had the same minimum inhibitory concentration as the parent strain (2.5 μM) as well as comparable growth rates when grown in the presence of sub-inhibitory concentrations of aminopterin (Fig. 4).

The role of OppA on biofilm formation of the *S. mutans* UA159 strain was initially inferred using cells incubated with the anti-OppA serum. As indicated in Fig. 5, the amount of bacteria binding to the surface of polystyrene plates was not affected following incubation of the cells with the anti-OppA serum. Moreover, incubation of *S. mutans* UA159 cells with different anti-OppA serum concentrations did not affect the viability of the cells cultivated under different conditions (data not shown). The capacity of the *S. mutans* AR152 and AR153 strains to form stable biofilms was compared with that of the wild-type strain. The data obtained showed that inactivation of *oppA* or *oppB* did not affect biofilm formation in saliva-coated microtiter plates with either glucose or sucrose as the primary carbohydrate source (Fig. 6). Collectively, these results indicate that the Opp system, and particularly the OppA protein, does not affect growth and biofilm formation of *S. mutans* UA159.

Detection of OppA among S. mutans strains

A set of three laboratory *S. mutans* strains as well as seven bacterial strains recovered from patients with active dental tooth decay processes were screened for the presence of the *oppA* gene and the corresponding OppA protein. Among the laboratory strains, the *S. mutans* LT11 strain did not harbor the *oppA* gene, as evaluated by PCR and Southern blot (data not shown). This finding was supported by Western blots with the specific anti-OppA antibody (Fig. 3B). Moreover, the *oppA* gene was not detected in six out of seven *S. mutans* clinical isolates recovered from tooth decay processes (Fig. 3B) and only one of them expressed the OppA protein under the tested growth conditions. These results strongly suggest that, in contrast to other *Streptococcus* species, OppA is not required for survival and pathogenicity of *S. mutans* strains.

Discussion

The ABC-type transporters are one of the largest paralogous protein families, comprising as much as 5% of bacterial genomes and controlling the traffic of essential nutrients in and out of the cell (20, 46). The oligopeptide (Opp) permeases, one of the most comprehensively studied ABC transporter systems, comprise a set of membrane proteins responsible for the binding and translocation of peptides ranging from three to at least 18 amino acid residues (15, 20). Besides its primary role in peptide uptake, in many streptococcal species, the Opp system participates in physiological processes ranging from competence development to binding to mammalian proteins and tissues (10, 13, 23, 40).

The presence of multiple OppA copies expressing different affinities to peptide substrates represents a putative advantage to *Streptococcus* species thriving in different environments either for nutrition or intercellular signaling (3, 4, 18, 22, 35). In

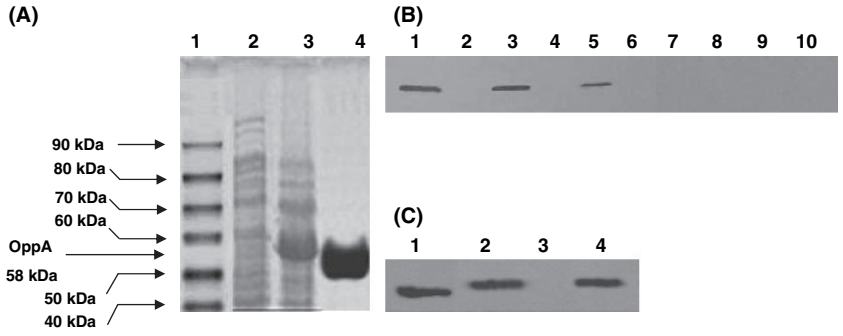

Fig. 3. Purification of OppA and detection of the protein expressed by wild type *S. mutans* strains. (A) Purification of the *S. mutans* OppA protein expressed by a recombinant *E. coli* K12 strain. Proteins extracted from pQRRJ-transformed *E. coli* XL1-Blue strain before (lane 2) and after induction (lane 3) with IPTG at 28°C. Single-step purification of the recombinant protein was achieved after eluting the protein bound to nickelated resin with buffer containing 100 mmol/l imidazole (lane 4). Molecular weight markers were added at lane 1 (BenchMark™ Protein Ladder; Invitrogen). (B) Detection of OppA expressed by different *S. mutans* strains. Immunological detection of OppA expressed in whole cell extracts of laboratory and clinically derived *S. mutans* strains. Proteins reactive with the OppA-specific serum raised in mice immunized with purified recombinant *S. mutans* OppA were detected by chemiluminescence. Samples: 1, UA159 strain; 2, LT11 strain; 3, ATCC 25175 strain; 4, A1 isolate, 5, A2 isolate; 6, A3 isolate; 7, A4 isolate; 8, A5 isolate; 9, A6 isolate; 10, A7 isolate. Proteins were sorted in a 12.5% polyacrylamide gel and blotted to nitrocellulose membranes. (C) Immunological detection of OppA in whole cell extracts of the AR152 (Δ*oppA*) and AR153 (Δ*oppB*) derivatives of the *S. mutans* UA159 strain. Samples: 1, purified *S. mutans* OppA; 2, whole cell extract of the AR152 strain; 3, whole cell extract of the AR152 strain; 4, whole cell extract of the AR153 strain.

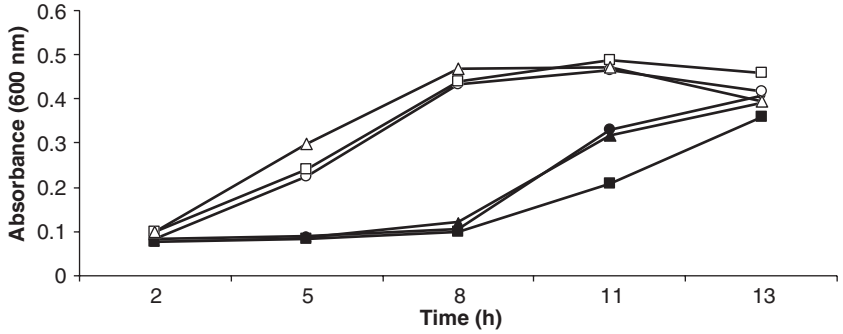

Fig. 4. Growth curves and sensitivity to aminopterin of *S. mutans* UA159 and Opp-defective mutants. The *S. mutans* UA159 (circles), AR152 (Δ*oppA*::Km^r) (squares), and AR153 (Δ*oppB*::Km^r) (triangles) were cultivated in the chemically-defined FMC medium in the absence (open symbols) or presence of 1.25 μM of aminopterin (closed symbols).

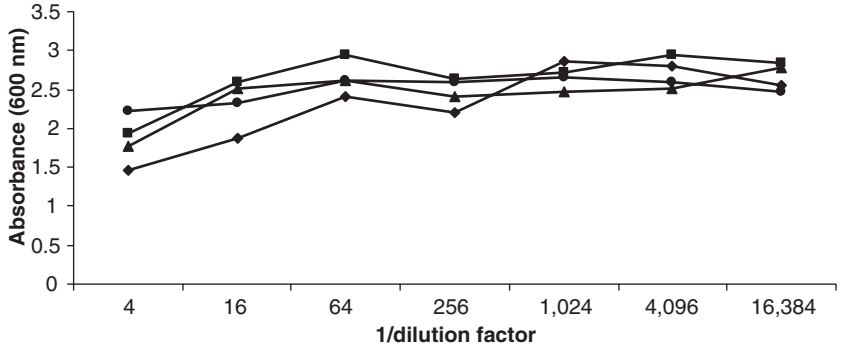

Fig. 5. Adhesion of the *S. mutans* UA159 strain to polystyrene microwell plates following incubation with anti-OppA serum. *S. mutans* cells were incubated with OppA-specific mouse serum (◆), non-specific serum raised in mice immunized with *X. citri* MalE protein (■), non-immune mouse serum (▲) or PBS (●). Adherent *S. mutans* cells were measured after staining adherent cells with crystal violet and then dye solubilization with ethanol. The final OD_{600} of the samples was measured in a microplate reader. Tested serum dilutions are indicated.

S. pyogenes, initially reported to harbor a single *oppA* gene (13), searches in the genome sequence of the SSI-1 strain revealed the presence of at least two copies of the gene (GenBank accession numbers NP801483 and NP802978). Extensive and detailed sequence searches of the *S. mutans* UA159 genome revealed a single copy of the *oppA* gene located at the first cistron of the *opp* operon. No other *opp* parologs could be found in the genome of the UA159 strain.

Based on the amino acid sequences, the OppA sequences found among *Streptococcus* species were organized into four similarity groups. No clear correlation could be drawn between these groups and the ecological and metabolic features of the corresponding *Streptococcus* species. OppA parologs found in some *Streptococcus* species, such as *S. agalactiae* and *S. pyogenes*, differ significantly, suggesting a divergent evolution in terms of substrate specificity. In the case of *S. mutans*, the lack of reminiscent transposase-encoding genes, absence of inverted sequences surrounding the genomic locus, and conserved codon usage indicate that the *oppA* gene, as well as the whole *opp* operon, were not horizontally transferred in the recent evolutionary history of the species (2). Moreover, based on the growth properties of *opp* mutants, the present data indicate that the *S. mutans* Opp system is not functional under standard *in vitro* conditions.

A recombinant *S. mutans* OppA was expressed and purified from *E. coli* K12 as a soluble cytosolic protein genetically fused at the N-terminal end with a His_6-Tag sequence. Attempts to express the recombinant protein in *E. coli* strains with an intact signal peptide and N-terminal cysteine indicated that the secretion apparatus of *E. coli* does not efficiently recognize the *S. mutans* OppA signal sequence (unpublished observations). Moreover, the expression levels achieved by the recombinant strains were low, suggesting that accumulation of the precursor protein was toxic to *E. coli* cells. Thus, removal of the signal peptide and subterminal amino acids represented an alternative for the successful expression and purification of recombinant *S. mutans* lipoproteins in *E. coli* hosts. Based on such cloning and expression strategies, we repeatedly obtained rather high amounts of the recombinant protein, employed in the generation of specific anti-OppA antibodies, that were adequate for biochemical and structural studies.

Based on the analyses with the *oppA* and *oppB* mutants, the Opp system does

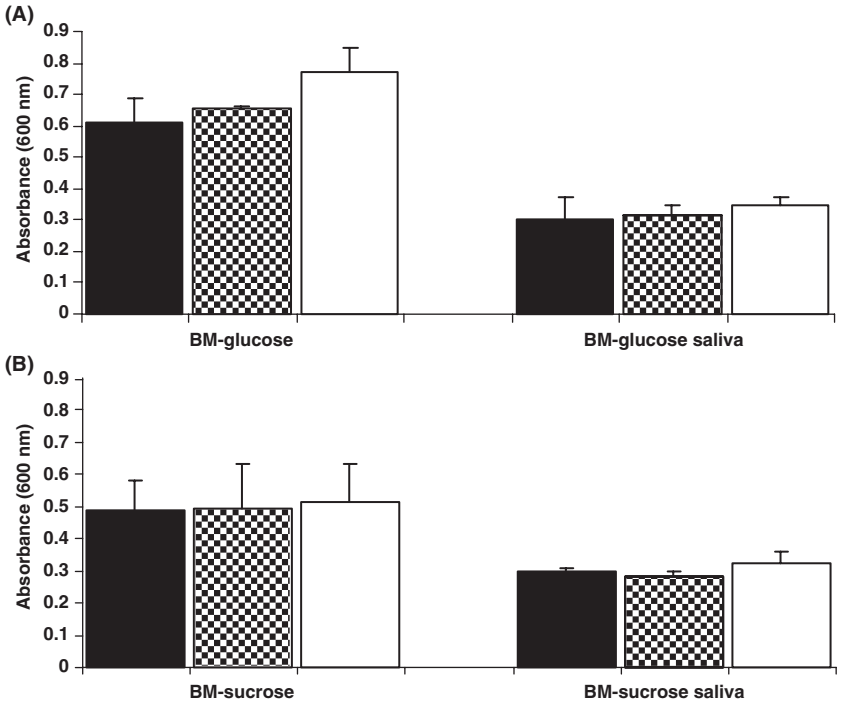

Fig. 6. Biofilm formation of *S. mutans* UA159, AR152 and AR153 strains. Adherence of *S. mutans* strains to polystyrene plates was measured following cultivation in BM medium with 20 mM glucose (BM-glucose) (A) or 10 mM sucrose (BM-sucrose) (B) added. Microtiter polystyrene plates were also treated with human saliva (BM-glucose saliva and BM-sucrose saliva) before the addition of the bacterial cells, as described in the MATERIALS AND METHODS section. *S. mutans* UA159 (black columns), *S. mutans* AR152 strains (gray columns), and *S. mutans* AR153 (white columns) were incubated for 24 h before staining of the biofilms formed.

not affect the viability and growth properties of *S. mutans* strains. OppA is known to participate in the regulation of the adhesive properties of several bacterial species, including different streptococcal species, such as *S. pneumoniae* and *S. gordonii* (12, 17, 21, 23, 30, 40, 45). Biofilm formation requires the production and secretion of insoluble polysaccharides derived from carbohydrate metabolism representing the second and most relevant step of the tooth surface adhesion (14). In *Vibrio fluvialis*, defective OppA expression resulted in increased biofilm formation, suggesting a negative role for the Opp system on biofilm biogenesis (27). Similarly, exposure of *S. gordonii*, another inhabitant of the oral environment, to saliva down-regulates expression of HppB, an OppA ortholog (16). In concert with these observations, our results show that deletion of *oppA*, as well as *oppB*, does not affect *in vitro* biofilm formation by *S. mutans* UA159. The recovery of OppA-defective strains from active caries processes further support the finding that the Opp system does not affect biofilm formation in *S. mutans*.

Interestingly, the laboratory strain LT11, which did not appear to harbor *opp* genes, is highly transformable and it was possible that the absence of the Opp system could account for this characteristic. We tested the transformation efficiencies of the UA159-derivative strains lacking *oppA* and *oppB* and the results did not indicate differences in transformability when compared with the parental strain (data not shown). Thus, it does not appear that lack of *opp* genes in LT11 accounts for the strain's high transformation frequencies.

The *oppA* gene is naturally unstable in *E. coli*, *S. typhimurium*, and *Lactococcus lactis* in both laboratory strains and clinical isolates (1, 20, 26). The present results clearly indicated that the *opp* locus is also unstable among *S. mutans* strains, which results in the absence of the *oppA* gene or defective expression of the encoded peptide in several laboratory or wild-type strains. Additionally the demonstration that the sensitivity to aminopterin is not altered following inactivation of *oppA* or *oppB* strongly indicates that the Opp system is either not functional or not involved in the uptake of this specific peptide. Therefore, uptake of oligopeptides in this bacterial species must occur through alternative uptake system(s). Further experiments aimed at elucidating the mechanisms involved in the uptake of oligopeptides and their role on the physiology and pathogenicity of *S. mutans* are warranted.

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