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The Pst system of *Streptococcus mutans* is important for phosphate transport and adhesion to abiotic surfaces

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Keywords: ATP-binding cassette transporter; cell binding; phosphate uptake; Pst system
Accepted 29 December 2011
DOI: 10.1111/j.2041-1014.2012.00641.x

SUMMARY

The Pst system is a high-affinity inorganic phosphate transporter found in many bacterial species. *Streptococcus mutans*, the etiological agent of tooth decay, carries a single copy of the *pst* operon composed of six cistrons (*pstS*, *pstC1*, *pstC*, *pstB*, *smu.1134* and *phoU*). Here, we show that deletion of *pstS*, encoding the phosphate-binding protein, reduces phosphate uptake and impairs cell growth, which can be restored upon enrichment of the medium with high concentrations of inorganic phosphate. The relevance of Pst for growth was also demonstrated in the wild-type strain treated with an anti-PstS antibody. Nevertheless, a reduced ability to bind to saliva-coated surfaces was observed, along with the reduction of extracellular polysaccharide production, although no difference on pH acidification was observed between mutant and wild-type strains. Taken together, the present data indicate that the *S. mutans* Pst system participates in phosphate uptake, cell growth and expression of virulence-associated traits.

INTRODUCTION

Acquisition of inorganic phosphate (Pi) in most studied bacterial species occurs primarily through two independent uptake systems, Pit and Pst. Pit is a low-affinity transporter formed by a single integral protein. The Pst system is a typical ATP-binding cassette (ABC) transporter composed of three main domains: (i) the ATP-binding domain; (ii) the transmembrane domain; and (iii) the substrate-binding domain (Dassa, 2000). In *Escherichia coli* the Pst system is encoded by four genes organized in a single operon; *pstS*, *pstC*, *pstA* and *pstB.* A fifth gene, *phoU*, encodes a protein of unknown function that does not play a role in Pi transport (Steed & Wanner, 1993; Wanner, 1996). PstS is a periplasmic/surface-exposed protein that carries a phosphate-binding domain, PstC and PstA are pore-forming proteins located at the cytoplasmic membrane, whereas PstB is an ATPase that generates the energy required for transport. The *pst* operon belongs to the phosphate (Pho) regulon, a global regulatory circuit that controls phosphate homeostasis based upon the activation or repression of hundreds of genes. These include genes not necessarily involved in Pi uptake, such as virulence-associated factors encoded by different pathogenic species (Wanner, 1993; Van Veen, 1997; Ferreira & Spira, 2008; Lamarche *et al.*, 2008).

Previous studies have linked the Pst system with regulation of biofilm formation by pathogenic bacteria species such as *Proteus mirabilis* and *Pseudomonas*

spp. (Monds *et al.*, 2001; Jacobsen *et al.*, 2008; O'May *et al.*, 2009). In the gram-positive pathogen *Streptococcus pneumoniae*, inactivation of the Pst system resulted in lower *in vivo*, virulence as demonstrated by the development of septicemia in challenged animals (Polissi *et al.*, 1998; Novak *et al.*, 1999).

Streptococcus mutans is the major etiological agent of human dental caries (Bowen *et al.*, 1991). Tooth decay caused by *S. mutans* is the result of two sets of bacterial traits: adhesion to the tooth surface, followed by the subsequent formation of structurally complex biofilms and production of acid (acidogenicity) concomitant with the ability to survive and replicate in acidic environments (aciduricity) (Burne, 1998). Biofilm formation by *S. mutans* is a limiting factor in the process of tooth cavity formation and involves an initial reversible sucrose-independent stage and a subsequent irreversible sucrose-dependent stage (Hamada *et al.*, 1984; Bowen *et al.*, 1991; Burne, 1998; Crowley *et al.*, 1999).

In this paper, we provide evidence that the Pst system of *S. mutans* plays a role in Pi uptake and expression of virulence-associated traits. Our results demonstrate that a PstS-deleted strain shows growth deficiency and reduced adhesion to saliva-coated surfaces and secretion of polysaccharides. The results suggest that the Pst system is involved in phosphate uptake and pathogenicity of *S. mutans*.

METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. The *S. mutans* strains were cultivated in brain–heart infusion (BHI) broth at 37°C and 5% CO_2. When required, kanamycin (1 mg ml^{-1}) or chloramphenicol (10 μg ml^{-1}) were added to the medium.

Sequence analyses

DNA sequences were retrieved from the *S. mutans* UA159 genome sequence available at the GenBank. Orthologous PstS amino acid sequences were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the Bioinformatics Center Institute for Chemical Research Kyoto University (http://www.genome.ad.jp/keeg). The *pst* signature motifs were searched using the motif sequence finder program (http://www.genome.jp/tools/motif/). The PstS signal sequence and corresponding cleavage site were searched with the SignalP program (http://www.cbs.dtu.dk/services/SignalP).

Table 1 Bacterial strains and plasmids used in this study

Strains and vectors	Characteristics	Source
Streptococcus mutans strains		
UA159	Reference strain	Ajdíc *et al.*, 2002
DR1	Δ*pstS*:NPKm^r	This work
Vectors or plasmids		
pMSP3535	Replicative *S. mutans* vector (Erm^r)	Bryan *et al.*, 2000
pALH124	Kanamycin-resistance plasmid	Ahn *et al.*, 2005
pHT08	Expression shuttle vector (cm^{-1})	Nguyen *et al.*, 2007

Cloning and expression of *pstS* and antibody production

The *S. mutans* UA159 *pstS* gene was amplified without the putative signal peptide and the first two initial amino acid residues of the mature protein sequence using primers FwpstSBamHI (5′-ggCAgTCggATCC CAgTgTTgg-3′) and RvpstSSacI (5′-CTTCTCTgAgC TCTTTACT-TAgCA-3′) according to the reported *pstS* gene sequence (Accession number AE014133). *Bam*HI and *Sma*I restriction sites were introduced at the 5′ ends of the forward and reverse primers, respectively. The 0.8-kb amplified fragment was cloned into pHT08 vector, and a plasmid containing the correct insert (pHTpstS) was confirmed by DNA sequencing and transferred into *Bacillus subtilis* WB800 (Nguyen *et al.*, 2007). Transformed cells were grown in Luria–Bertani medium containing chloramphenicol at 37°C until mid log phase [optical density at 600 nm (OD_{600}) 0.5–0.6] and PstS expression was achieved following addition of isopropyl β-D-1-thiogalactopyranoside at final concentration 0.1 mM. The cultures were incubated aerobically (200 r.p.m.) for 4 h at 37°C, cells were collected by centrifugation and then 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

insoluble material by centrifugation at 16,000 ***g*** for 20 min at 4°C. The purified recombinant PstS protein was obtained following binding to nickelated ProBond Resin (Invitrogen, Grand Island, NY) equilibrated with buffer A (100 mM Tris–HCl, 0.5 M NaCl, pH 7.2). The recombinant PstS protein was eluted with 150 mM imidazole, and an additional electrodialysis purification step was carried out using polyacrylamide gels (Keen & Findlay, 1995). Protein concentrations were measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the supplier's instructions. For antibody production, we subcutaneously inoculated BALB/c mice four times with 50 μg PstS protein, and one final bleeding was performed to obtain the serum.

Construction of an *Streptococcus mutans pstS* knockout strain

The *S. mutans pstS* gene was mutated by allelic replacement with a non-polar kanamycin-resistance gene cassette (NP:Km^r) using a polymerase chain reaction (PCR) ligation mutagenesis approach (Ahn *et al.*, 2005; Lemos *et al.*, 2004). Primers 5′-arm1pstS (5′-gggTgCTCgAAATgTTgTTg-3′) and 3′-arm1pstS (5′-gggCTAgCggATCCAATAAAgTTA-3′) were used to amplify a 1000-bp region upstream of *pstS*. Primers 5′-arm2pstS (5′-gTTCTTTTAggATCC TgTgTggAg-3′) and 3′-arm2pstS (5′-AgAgCgAAgCC AAgATgg-3′) were used to amplify a 1-kb sequence downstream of *pstS*. The PCR products were digested with *Bam*HI and ligated to an NP:Km^r fragment digested with *Bam*HI. The ligation mixture was introduced into naturally competent *S. mutans* UA159 cells and bacteria were plated onto BHI agar containing kanamycin. Positive transformants were confirmed by PCR, sequencing and Western immunoblot. One recombinant clone carrying a precise deletion of the *pstS* gene (DR1 strain) was selected for further studies.

RNA isolation and reverse transcription-PCR

To confirm that the mutation of *pstS* did not affect the expression of downstream genes, RNA was extracted from cells grown to late-exponential phase (OD_{600} 0.8) in BHI broth as described elsewhere (Abranches *et al.*, 2006). A high-capacity cDNA reverse transcription kit containing random primers (First-Strand cDNA sysnthesis Kit, Invitrogen) was used to obtain cDNA from DNAse-treated RNA samples. Specific downstream amplification was performed with primers designed to amplify the *pstC1* gene (RTFwpstc1 – 5′-TggTAAACCATTACTTggggC 3′ and RTRvpstc1 – 5′-CAACCgTgAAAAACTgTCC ggC-3′). As control, the same reaction was carried out with the RNA samples before the cDNA transcription step.

Growth curves

The *S. mutans* UA159 and DR1 strains were grown for 16 h at 37°C with 5% CO_2 in FMC medium (Terleckyj *et al.*, 1975), with phosphate buffer replaced by 40 mM 3-(*n*-morpholino)propanesulfonic acid (MOPS; pH 7.0) and 10 mM KH_2PO_4 provided as phosphate source. The cells were collected by centrifugation, washed three times in citrate-buffered glucose (3% sodium citrate with 2% glucose) and resuspended in FMC medium at OD_{600} 0.3. Cells were inoculated into fresh FMC medium (1 : 25) containing different phosphate concentrations (1–30 mM KH_2PO_4). The cultures were incubated at 37°C and their cell densities were measured at OD_{600}. To analyse the impact of PstS inhibition by an anti-PstS antibody, strain UA159 was grown on BHI medium containing different serum dilutions (1 : 250; 1 : 500; 1 : 1000). As control, an antibody raised against a nude WB800 *S. mubtilis* strain and the DR1 mutant strain was used in 1 : 100 dilution.

Phosphate uptake

Streptococcus mutans strains were cultivated in MOPS-buffered (40 mM) FMC medium containing 10 mM Pi (KH_2PO_4) for 16 h at 37°C and 5% CO_2. Bacteria were centrifuged at 5000 ***g*** for 10 min, washed twice in Pi-free FMC and suspended in the same medium at OD_{600} 0.1. Cultures were then incubated for 6 h, at 37°C and 5% CO_2 to induce phosphate starvation. The Pi transport was assayed by incubating the cells for 5 min with 0.5 mM K_2HPO_4 and 10 μCi [^{32}P]Pi (IPEN, Sao Paulo, Brazil). One-hundred-microliter samples were withdrawn every 5 min, applied to nitrocellulose membrane disks (Millipore, Billerica, MA) on a manifold (Millipore) and immediately washed with citrate–glucose buffer (3% sodium citrate and 2% glucose) containing 50 mM

unlabelled KH_2PO_4. The filters were transferred to vials containing 5 ml scintillation cocktail (Perkin Elmer, Waltham, MA), and radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

Adhesion of *Streptococcus mutans* cells to a saliva-coated surface

Binding of *S. mutans* cells to a saliva-coated surface was determined in 96-well flat-bottom microtitre plates (Nalge Nunc International, Rochester, NY) as previously described, but with minor modifications (Nepomuceno *et al.*, 2007). Microtiter plates were coated with 50 μl clarified saliva for 1 h at room temperature (Barboza-Silva *et al.*, 2005). Cultures were prepared in BHI medium (Pi concentration 60 mM) at 37°C and 5% CO_2 at OD_{600} 0.5. Cultures were diluted 1 : 100 in biofilm medium (BM) broth (Loo *et al.*, 2000) supplemented with sucrose (10 mM) or glucose (20 mM) as carbon source, and aliquots (200 μl) were transferred to microtiter plate wells and incubated for 48 h at 37°C and 5% CO_2. Non-adherent cells were removed by gentle washing with saline and the bound bacteria were subsequently stained with 0.1% crystal violet dye for 15 min at room temperature. The plates were gently washed twice with saline, the cell-bound dye was solubilized by the addition of 200 μl acetic acid (33% volume/volume), and absorbance was measured at 600 nm in a microtiter plate reader.

Detection of extracellular polysaccharide production

Extracellular polysaccharide production by *S. mutans* strains was measured following a previously described procedure (Ogawa *et al.*, 2011). Suspensions of *S. mutans* strains UA159 and DR1 were cultivated in trypticase soy broth medium supplemented with 0.25% sucrose in six-well culture plates for 24 h. To observe polysaccharide in the biofilms formed by the *S. mutans* strains, the biofilms were rinsed twice with sterile distilled water and treated with 2 mg ml^{-1} dextran-Alexa Fluor 647 (10,000 molecular weight, anionic, and fixable; Invitrogen) in phosphate-buffered saline (pH 8.0) for 30 min at room temperature. Cells were washed three times with phosphate-buffered saline, spotted onto glass slides and cover slips were applied. Labeled extracellular polysaccharide was observed using a confocal microscope (Laser Scanning Microscopy, LSM 510 META, Zeiss).

pH measurement

Cultures of *S. mutans* UA159 and DR1 were grown in BHI medium and the pH of the medium was measured before growth and after the cultures reached stationary phase. Measurements were made in three independent experiments.

Statistical analyses

All quantitative data are expressed as means ± SD of at least three independent experiments. Student's *t*-test was employed to compare mean values, and *P* values <0.05 indicate statistically significant differences.

RESULTS

Structure of the *pst* operon

Five structural genes (*pstS*, *pstC1*, *pstC*, *pstB* and *smu.1134*) encoding the components of the Pst system were found in the genome of the *S. mutans* UA159. In addition, a *phoU* orthologous sequence was found in the last cistron of the operon (Fig. 1). The first gene, *pstS*, encodes a 30.8-kDa phosphate-binding protein. The PstS protein has a predicted signal peptide of 31 amino acid (aa) residues and an N-terminal cysteine residue through which the protein binds to membrane lipids following processing of the signal peptide. The next two genes, *pstC1* and *pstC*, overlap by 11 bp and encode 31.7- and 33-kDa polypeptides, respectively, that constitute the membrane permease, inferred by the presence of hydrophobic membrane-spanning domains in both proteins. The ATP-binding components of the *S. mutans* Pst system are encoded by two genes, *pstB* and *smu.1134*, which encode proteins with molecular masses 30.6 and 28 kDa, respectively. Both proteins carry typical Walker A and B motifs common to nucleotide-binding proteins and a consensus signature sequence, LSGGQ, usually found among ABC superfamily members. Overall, the *pst* operon of *S. mutans* is very similar to those found in other *Streptococcus* species. Amino acid sequence analysis of *Streptococcus* Pst orthologs suggests that the species most closely

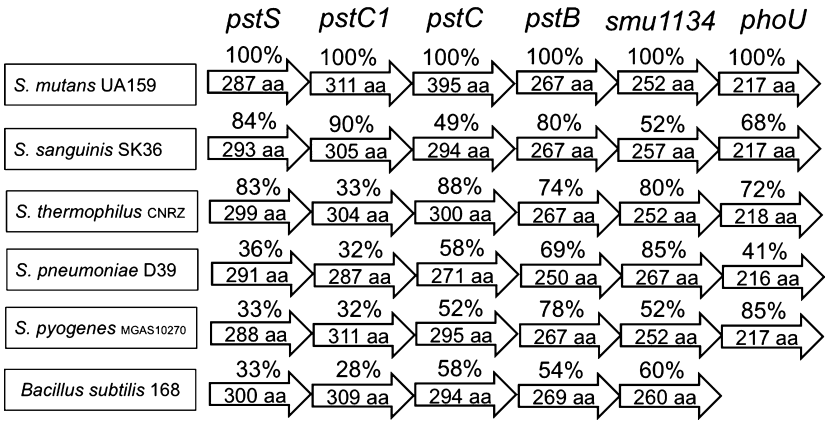

Figure 1 Schematic representation of the *pst* operon organization found among different *Streptococcus* species. The species are indicated in the boxes at the left side of the figure. The *pst* genes are indicated by open arrows and the ortholog identity values are percentage values shared by the different orthologs with regard to the respective *S. mutans* ortholog. The numbers of amino acid (aa) residues for each ortholog protein are indicated inside the open arrows. The *pst* operon of *Bacillus subtilis* is included as reference for a distinct gram-positive bacterial species.

related to *S. mutans* are *Streptococcus sanguinis* and *Streptococcus thermophilus*. The PstS protein of *S. mutans* shares 84% and 83% identity values with the *S. sanguinis* and *S. thermophilus* orthologs, respectively (Fig. 1).

Phenotypic effects of *pstS* gene inactivation

To evaluate the role of the Pst system and, particularly, of the PstS protein in the physiology and pathogenesis of *S. mutans* UA159, we generated a non-polar mutation in the *pstS* gene by replacing the *pstS* gene with a kanamycin-resistance cassette (Fig. 2). One recombinant clone, named DR1, was selected, and deletion of the *pstS* gene was confirmed by DNA sequencing and Western blot with specific anti-PstS serum. Amplification of the *pstC1* gene, located downstream of the *pstS* gene, using cDNA of both US159 and DR1 strains demonstrated that deletion of the *pstS* gene in the DR1 strain was non-polar and, therefore, did not interfere with expression of downstream genes (Fig. 3).

Streptococcus mutans DR1 was unable to grow in FMC medium supplemented with 1 mM Pi and displayed a reduced growth rate when cultured in medium containing a 30-fold higher Pi concentration (30 mM) (Fig. 4). At 43 mM Pi, there was no significant difference in the growth rates between the wild-type and the *pstS* mutant. These results indicated that the impaired growth of the *pstS* mutant at low

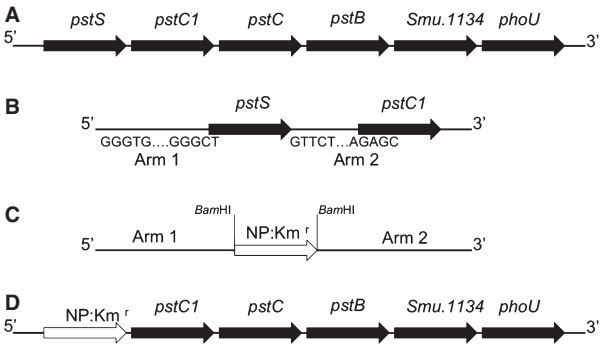

Figure 2 Schematic representation of the *Streptococcus mutans pst* operon and the strategy used to generate a *pstS*-deleted mutant. (A) Genetic organization of the *pst* operon of *S. mutans* UA159. (B) Location of the Arm1 and Arm2 flanking sequences generated to delete the *pstS* gene. (C) DNA fragment generated after linking Arm1 and Arm2 to the kanamycin-resistance cassette. (D) *pstS* deletion after a double recombination event.

phosphate concentration was the result of a defective Pst system. Addition of an anti-PstS serum, generated in mice immunized with a recombinant protein generated in the *B. subtilis* WB800 strain, to the growth medium inhibited growth of *S. mutans* UA159 but not of DR1 strain (Fig. 4C). As a control, a serum raised in mice immunized with the *B. subtilis* WB800 strain (final dilution 1 : 100) did not interfere with the growth of either UA159 or DR1 strains. These results suggest that a functional PstS protein is necessary for optimal growth of the *S. mutans* UA159. Finally, we evaluated the impact of *pstS* deletion on the

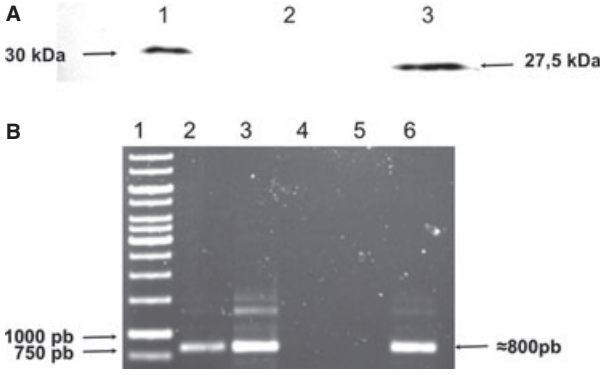

Figure 3 (A) Detection of the PstS protein in whole cell extracts on *Streptococcus mutans* UA159 and DR1 with an anti-PstS mouse serum. (i) Whole cell extracts of *S. mutans* UA159; (ii) whole cell extracts of *S. mutans* DR1; (iii) purified recombinant *S. mutans* PstS protein. (B) Reverse transcription-polymerase chain reaction of the *pstC1* gene of *S. mutans* UA159 and DR1 strains. (i) Molecular weight (Fermentas™); (ii) cDNA of *S. mutans* UA159; (iii) cDNA of *S. mutans* DR1; (iv) negative control using RNA from *S. mutans* DR1; (v) negative control using RNA from *S. mutans* UA159; (vi) positive control using chromosomal DNA from *S. mutans* UA159.

capability of *S. mutans* to transport Pi into the cell. As shown in Fig. 5, phosphate uptake in the DR1 mutant was reduced by approximately 45%, when compared with the parental UA159 strain.

Effects of *pstS* mutation on potential virulence traits

To evaluate the role of the *pstS* deletion on adhesion of *S. mutans* cells to saliva-coated surfaces, the wild-type and *pstS* mutant were cultivated in media containing excess Pi and glucose or sucrose for 48 h and allowed to bind to plastic surfaces coated with saliva. As shown in Fig. 6, binding of the DR1 strain, grown in the presence of sucrose (to promote biofilm formation) to the saliva-coated surface was reduced by approximately two-fold when compared with the parental strain. Although adhesion of cells cultivated in glucose was lower than that observed in cells cultivated in sucrose-containing medium no difference in the adhesion patterns was observed with the two tested strains. Indeed, secretion of extracellular polysaccharide by the *pstS*-deleted *S. mutans* strain was reduced with regard to the parental strain, as demonstrated after labeling with dextran-Alexa Fluor (Fig. 7). In contrast, no difference in acidogenicity was detected between the DR1 mutant and US159 strains, as evaluated by the pH of the medium after the growth of the strains (Table 2). Finally, we evaluated the aciduricity of the two strains and

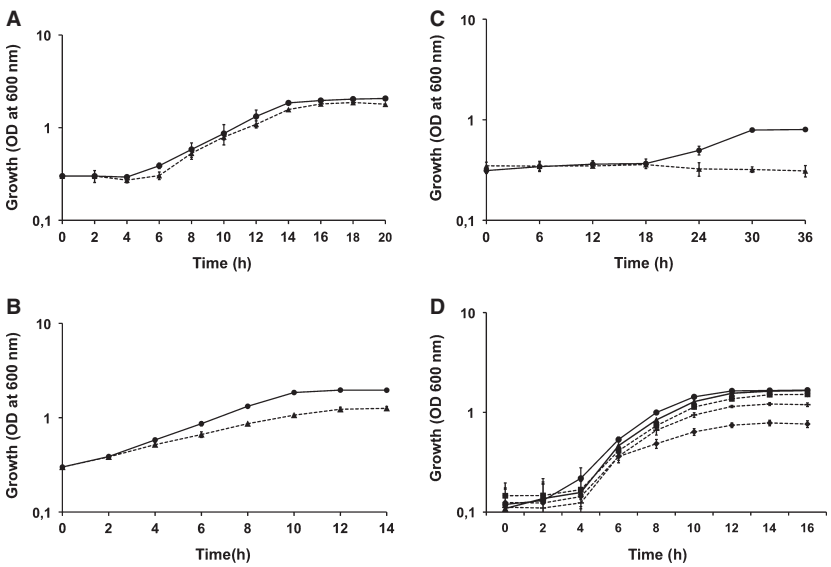

Figure 4 Growth curves at different phosphate concentrations, and phosphate uptake by *Streptococcus mutans* strains UA159 and DR1. (A) Growth curves of *S. mutans* UA159 and DR1 in FMC medium supplemented with 43 mM Pi. (B) Growth curves of *S. mutans* UA159 and DR1 in FMC medium supplemented with 30 mM Pi. (C) Growth curves of *S. mutans* UA159 and DR1 in FMC medium supplemented with 1 mM Pi. Strains of *S. mutans*: (●) UA159, (▲) DR1. Each curve was independently repeated three times. Values are expressed as means (±SD). (D) Growth in the presence of anti-PstS mouse serum. Samples: (●) UA159 with anti-*Bacillus subtilis* whole cell serum (1 : 100), (▲) DR1 with anti-PstS serum (1 : 250), (■) UA159 with anti-PstS serum (1 : 1000), (–) UA159 with anti-PstS serum (1 : 500), (◆) UA159 with anti-PstS serum (1 : 500). Each curve was independently repeated three times. Values are expressed as means (±SD).

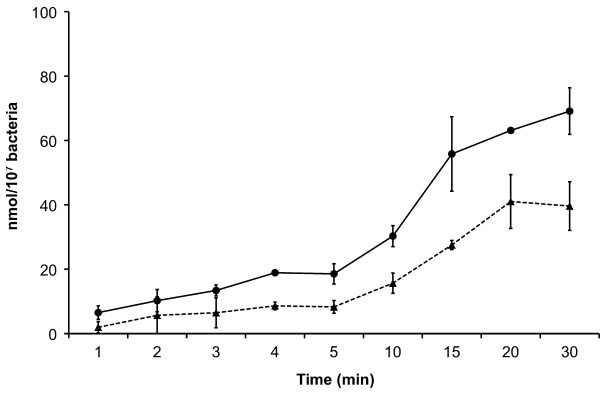

Figure 5 Phosphate uptake by *Streptococcus mutans* UA159 and DR1. Symbols: *S. mutans* strain UA159 (●) or DR1 (▲). Values are expressed as nanomoles of intracellular phosphate per 10^7 cells. Each point represents the average (±SD) of three independent experiments.

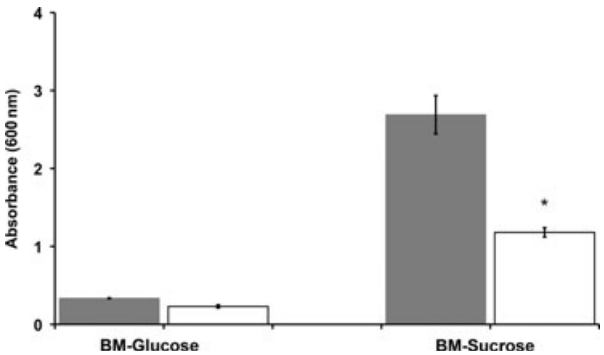

Figure 6 Effect of *pstS* deletion on adhesion to a saliva-coated surface. *Streptococcus mutans* UA159 (gray bar) and DR1 (open bar) strains were incubated in polystyrene microtiter wells for 48 h in BM medium containing glucose or sucrose. *Statistically significant differences ($P > 0.05$).

no significant difference was observed (data not shown).

DISCUSSION

In this paper, we investigated for the first time the Pst system of *S. mutans*. Our data showed that deletion of the *pstS* gene, encoding the phosphate-binding protein, affected growth of *S. mutans* in minimal medium with low or intermediate phosphate concentrations and reduced by approximately half the uptake of extracellular Pi. Of particular relevance was the observation that the PstS-deleted strain showed a significant reduction in binding to saliva-coated surfaces when cultivated in the presence of sucrose. This was concomitant with reduction in production of extracellular polysaccharides, which are necessary for adhesion to the tooth surface and biofilm formation, and essential steps in the formation of tooth cavities. Altogether, these results demonstrate that the Pst uptake system plays an important role in the physiology and pathogenesis of *S. mutans*.

The *pst* operon of *S. mutans* is similar to those found in other bacterial species, such as *B. subtilis* and other *Streptococcus* species. Interestingly, the phosphate-binding proteins (PstS) of *S. mutans*, *S. sanguinis* and *S. thermophilus* are highly conserved (aa residue identity >80%) but more distantly related (<40% identity) to the orthologs found in *Streptococcus pyogenes* and *S. pneumoniae*. The nucleotide-binding components of ABC transporters are usually more conserved than the membrane permeases, whereas the substrate-binding component usually show more divergent aa sequences but preserved structural features (Igarashi *et al.*, 2001). Because phosphate concentrations in the oral cavity environment are not expected to reach limiting values, the conservation of the Pst components in *S. mutans* and other *Streptococcus* species suggests that this uptake system may have additional physiological or regulatory roles in this bacterial species.

The fact that Pi uptake was reduced but not ablated in the *pstS* mutant indicated that the Pst system is not the only Pi-transport system in *S. mutans*. The presence of a functional high-affinity phosphate uptake system in an oral bacterial pathogen would be dispensable because this nutrient is readily available through acid solubilization of hydroxyapatite (García-Godoy & Hicks, 2008; Luoma, 1968). However, such an uptake system would play a relevant role in bacterial cells entering the bloodstream and promoting extra-oral infections in organs where Pi concentration would be limiting (Horaud & Delbos, 1984). Indeed, deletion of *pstS* caused a significant reduction in *S. pneumoniae* virulence as demonstrated in experimental murine models following administration of bacterial cells into the peritoneal cavity (Orihuela *et al.*, 2001). Collectively, these observations indicate that the Pst system in different streptococcal species affects both the physiology and virulence of different strains reflecting either a reduction in the intracellular phosphate pool or a differential regulation of genes, such as those under the control of the Pho regulon (Lamarche *et al.*, 2008).

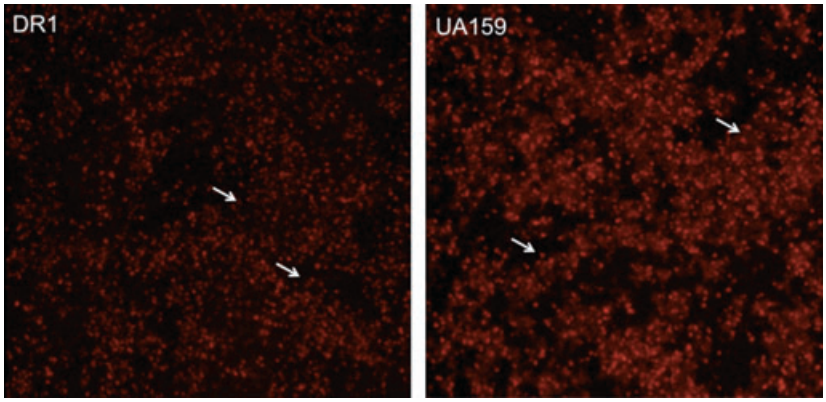

Figure 7 Production of extracellular polysaccharide by *Streptococcus mutans* UA159 or DR1. Dextran-Alexa Fluor-labeled extracellular polysaccharide and cells were monitored by confocal microscopy. The left panel represents strain UA159 and the right panel represents DR1. The arrows indicate bright cells surrounded by secreted polysaccharide. Final magnification ×600. The experiment was independently repeated three times with similar results observed.

Table 2 Acid production (acidogenicity) by *Streptococcus mutans* UA159 and DR1

Streptococcus mutans strains	Initial pH[1]	Final pH[1]
UA159	7.1 ± 0.3	5.4 ± 0.3
DR1	7.1 ± 0.3	5.0 ± 0.4

[1]Values represent the means of three independent experiments.

The *pstS*-deleted *S. mutans* strain showed a reduced growth rate when cultivated at intermediate or low phosphate concentrations. A similar growth defect was reported in a Pst-deficient *S. pneumoniae* mutant strain even at rather high phosphate concentrations (Novak *et al.*, 1999). In accordance with our results with *S. mutans*, growth impairment was also observed with *Proteus mirabilis* and *Mycobacterium smegmatis* strains exposed to antibodies targeting different Pst system components (Gebhard *et al.*, 2006; Jacobsen *et al.*, 2008). Reduction of the intracellular phosphate concentration may affect the expression of multiple genes and physiological aspects other than the phosphate metabolism may be disturbed by a defective Pst system. On the other side, mutations at the *pstS* gene, even at phosphate concentrations that would not cause any nutritional stress, are known to exert pleiotropic effects and indirectly affect expression of diverse traits, under *in vitro* and *in vivo* conditions, in different bacterial species (Lamarche *et al.*, 2008).

Adherence to abiotic surfaces and subsequent biofilm formation are two main steps in the etiology of caries formation by *S. mutans* strains. The initial reversible and sucrose-independent stage promotes binding of bacteria to the tooth surface using electrostatic charges and hydrophobic interactions of surface components, followed by the action of specific adhesins that recognize saliva components bound to the tooth surface (Burne, 1998). The second and irreversible sucrose-dependent stage involves production of insoluble polysaccharides that contribute both to adhesion to the tooth surface and the formation of the complex biofilm structures (Hamada *et al.*, 1984; Bowen *et al.*, 1991; Crowley *et al.*, 1999). Here we showed that deletion of the *pstS* gene affects binding of *S. mutans* to saliva-coated surfaces in cells cultivated in the presence of sucrose. The concomitant reduction in adhesion to abiotic surfaces and secretion of extracellular polysaccharides indicated that the virulence of *S. mutans* UA159 is negatively regulated by the Pst system. Indeed the presence of a functional Pst system, under control of the Pho regulon, is required for proper adhesion of enteropathogenic *E. coli* (EPEC) strains to epithelial cells and for *Citrobacter rodentium* to colonize the intestinal epithelia and cause diarrhea in rabbits (Cheng *et al.*, 2009). Similar results were also reported by Ferreira & Spira (2008) in which EPEC mutants defective in *pst* components were defective in adhesion to epithelial cells. Additional evidence linking biofilm production and the Pst system has also been reported for *Pseudomonas aureofaciens* and *Proteus mirabilis* strains (Monds *et al.*, 2001; O'May *et al.*, 2009). Altogether, these results suggest that the Pst system influences both

adhesion and biofilm formation in different bacterial species. In the case of *S. mutans*, further studies are required to demonstrate the role of this nutrient uptake system in virulence under *in vivo* conditions both at the dental surface and following systemic dissemination.

ACKNOWLEDGEMENTS

We appreciate the collaboration of Alexsander S. Souza and Cecília M. Abe for kindly support the microscope experiment, Loren Cristina da Silva and Eduardo Gimenes for technical support at work, and FAPESP and CNPq for financing this project.

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