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Induction of neutralizing antibodies in mice immunized with an amino-terminal polypeptide of *Streptococcus mutans* P1 protein produced bya recombinant *Bacillus subtilis* strain

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Abstract

The oral pathogen *Streptococcus mutans* expresses a surface protein, P1, which interacts with the salivary pellicle on the tooth surface or with fluid-phase saliva, resulting in bacterial adhesion or aggregation, respectively. P1 is a target of protective immunity. Its N-terminal region has been associated with adhesion and aggregation functions and contains epitopes recognized by efficacious antibodies. In this study, we used *Bacillus subtilis*, a gram-positive expression host, to produce a recombinant N-terminal polypeptide of P1 ($P1_{39–512}$) derived from the *S. mutans* strain UA159. Purified $P1_{39–512}$ reacted with an anti-full-length P1 antiserum as well as one raised against intact *S. mutans* cells, indicating preserved antigenicity. Immunization of mice with soluble and heat-denatured $P1_{39–512}$ induced antibodies that reacted specifically with native P1 on the surface of *S. mutans* cells. The anti-$P1_{39–512}$ antiserum was as effective at blocking saliva-mediated aggregation of *S. mutans* cells and better at blocking bacterial adhesion to saliva-coated plastic surfaces compared with the anti-full-length P1 antiserum. In addition, adsorption of the anti-P1 antiserum with $P1_{39–512}$ eliminated its ability to block the adhesion of *S. mutans* cells to abiotic surfaces. The present results indicate that $P1_{39–512}$, expressed and purified from a recombinant *B. subtilis* strain, maintains important immunological features of the native protein and represents an additional tool for the development of anticaries vaccines.

Keywords

Streptococcus mutans; P1 protein; recombinant proteins; *Bacillus subtilis*; antibody responses; saliva-binding region

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Introduction

Streptococcus mutans is a major etiologic agent contributing to the causation of dental caries, one of the most common human infectious diseases (Russell, 2000). Bacterial adherence to the tooth surface involves both sucrose-dependent and -independent mechanisms in which hydrogen bonds and hydrophobic interactions are formed between the bacterial cells and the salivary components present within dental pellicles (Gibbons, 1984; Gibbons *et al.*, 1986). *Streptococcus mutans* also interacts with other oral micro-organisms including primary colonizing commensal bacteria such as *Streptococcus gordonii* (Jenkinson & Lamont, 2005; Nobbs *et al.*, 2009). The complex multifactorial adherence process involves the M_r ~185 kDa, major surface antigen P1, originally identified as Antigen I/II (Russell & Lehner, 1978), and also referred to as PAc (Koga *et al.*, 1990).

P1 family proteins are expressed by almost all oral streptococci and orthologs have now also been identified in *Streptococcus pyogenes* and *Streptococcus agalactiae* (Zhang *et al.*, 2006). P1 is also required for saliva-mediated bacterial aggregation, possibly a nonimmune host defense mechanism, which is abolished in *S. mutans* isogenic mutants in which the *spaP* (*pac*) gene has been disrupted (Lee *et al.*, 1989; Koga *et al.*, 1990). The cariogenicity of *S. mutans* is diminished in the absence of P1 in a gnotobiotic rat model (Crowley *et al.*, 1999). P1 has been reported as a promising target for protective immunity in naturally sensitized humans and has been studied as a vaccine candidate in active immunization studies in primates and rodents (Lehner *et al.*, 1981; Michalek *et al.*, 2001b; Smith & Mattos-Graner, 2008; Shivakumar *et al.*, 2009). Passive immunization with certain, but not all, monoclonal antibodies against P1 has also been reported to confer protection against *S. mutans* colonization in macaques (Lehner *et al.*, 1985) and human subjects (Ma *et al.*, 1987).

The primary sequence of *S. mutans* P1 shows several distinct features (see Fig. 1a). The N-terminal portion encompasses the signal peptide (amino acid residues 1–38) and an alanine-rich region (A-region, amino acid residues 186–464). The A-region shows a seven-residue periodicity that adopts an extended alpha-helix structure (Larson *et al.*, 2010). The central region has a proline-rich segment (P-region, amino acid residues 840–963); between the A- and P-regions lies a segment commonly referred to as the variable or the V-region because this is where most of the sequence differences between *S. mutans* Antigen I/II family members are clustered (Brady *et al.*, 1991). The A- and P-regions of P1 interact, and these and segments flanking them contribute to the formation of complex discontinuous eptiopes (Rhodin *et al.*, 2004; Seifert *et al.*, 2004; van Dolleweerd *et al.*, 2004; McArthur *et al.*, 2007). At the C-terminal end, P1 displays cell wall and membrane-spanning regions, an LPXTG motif and a cytoplasmatic tail characteristic of cell surface-localized sortase substrates (Fischetti *et al.*, 1990; Ton-That *et al.*, 2004).

The A-region located within amino-terminal end of P1 has been reported to contribute to the interaction of *S. mutans* with salivary components, including salivary agglutinin (SAG), now known to represent the lung scavenger receptor cysteine-rich protein, gp340 (Prakobphol *et al.*, 2000). Because of its interactions with salivary constituents, the A-region has sometimes been referred as the saliva-binding region (SBR) (Nakai *et al.*, 1993; Hajishengallis *et al.*, 1995, 1998). This segment of P1 competitively inhibits both adherence of *S. mutans* to

immobilized SAG and aggregation in the presence of fluid-phase SAG (Crowley *et al.*, 1993); however, different determinants have been implicated in these two processes (Brady *et al.*, 1992). The A-region is also antigenic and encompasses both B-cell and T-cell epitopes (Takahashi *et al.*, 1991; Kelly *et al.*, 1995; Senpuku *et al.*, 1996). Antibodies against this segment of P1 have been reported to block the aggregation of *S. mutans* and to reduce the development of dental caries (Takahashi *et al.*, 1991; Munro *et al.*, 1993; Huang *et al.*, 2001; Michalek *et al.*, 2001a; Tsuha *et al.*, 2004).

Previous studies have reported the development of anti-caries vaccine approaches based on a recombinant P1 N-terminal region expressed in *Escherichia coli*. Usually, the recombinant *S. mutans* protein has been genetically fused to high-molecular-weight protein carriers and/or secreted into the periplasm of the host bacterial strains in order to reduce proteolytic degradation or simplify the purification process (Crowley *et al.*, 1993; Okahashi *et al.*, 1993; Hajishengallis *et al.*, 1995; Toida *et al.*, 1997; Matsumoto-Nakano *et al.*, 2008). Because hybrid proteins expressed in heterologous bacterial hosts can potentially lose important native-like immunological determinants, the generation of recombinant nonfused P1-derived peptides in an alternative expression system may result in an improved vaccine antigen and may contribute toward a better understanding of the functional and immunological features of the *S. mutans* protein.

Bacillus subtilis is a gram-positive spore-forming soil bacterium with a long history of industrial and technological uses, such as the production of proteases and fermented foods. Spores are also used as probiotics for different animal species and as growth promoters and biological control agents for several cultivated plants (Harwood, 1992; Paulitz & Bélanger, 2001; Ferreira *et al.*, 2005; Paccez *et al.*, 2006, 2007). The present knowledge of its physiology and genetics, as well as the availability of gene cloning and expression tools, make *B. subtilis* an important alternative to *E. coli* as a host for the expression of heterologous proteins with preserved biological activity devoid of lipopolysaccharide contamination that may affect immunological studies (Meima *et al.*, 2004; Westers *et al.*, 2004).

The aim of this study was to evaluate the immunological properties of a P1-derived polypeptide spanning the amino-terminus and encompassing the whole A-region and a short segment C-terminal, expressed and purified from a *B. subtilis* recombinant strain, and to determine its possible utility as a vaccine antigen.

Material and methods

Bacterial strains and growth conditions

All the bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* and *B. subtilis* strains were routinely cultivated in Luria–Bertani broth at 37 1C. Antibiotics were added to the growth media as necessary according to the strain and plasmids used. Competent *E. coli* cells were prepared using the $CaCl_2$-mediated transformation protocol (Sambrook & Russell, 2001), while natural *B. subtilis* competent cells were generated using the two-step transformation method (Cutting *et al.*, 1990). *Streptococcus mutans* strains were cultivated in brain–heart infusion broth for 16 h at 37 °C in 5% CO_2; PC3370 and

PC3370C strains were grown with tetracycline (10 μg mL^{-1}) and kanamycin (500 mg mL^{-1}), respectively.

Plasmid constructions

A *B. subtilis* strain expressing a polypeptide corresponding to the *S. mutans* to aa 39–512 ($P1_{39–512}$) of the P1 protein (see Fig. 1a) was obtained after cloning the coding sequence into the plasmid pHT08 under control of the P*grac* promoter (Nguyen *et al.*, 2007). The *spa*P region encoding the amino-terminus of P1 through the A-region and without a signal peptide was amplified by PCR as a 1.2-kb fragment using *S. mutans* UA159 chromosomal DNA as the template and primers FWsbr (5′-AAAggATCCATggATgAAACgACCACTAC) and RVsbr (5′-CgCgACgTCATTTggCTCAAgATCATA gAC) with restriction sites for BamHI and AatII (underlined sequences), respectively. The amplified fragment was cloned into the pGEM-T-Easy™ vector, resulting in the recombinant pGP1N vector. The pGP1N was digested with BamHI and AatII and the released fragment was subcloned into pHT08 to generate the recombinant plasmid named pLDV701. The correct nucleotide sequence of the cloned insert was confirmed by automated nucleotide sequencing.

Expression of the recombinant $P1_{39-512}$ by genetically modified *B. subtilis*

The plasmids pLDV701 and pHT08 were introduced into naturally competent *B. subtilis* WW02. One clone transformed with pLDV701 and another transformed with pHT08 was selected and the corresponding strains were named LDV701 and LDV700, respectively. Induction of protein expression and preparation of whole-cell protein extracts of genetically modified *B. subtilis* strains were performed as described previously (Paccez *et al.*, 2006; Nguyen *et al.*, 2007).

Purification of the recombinant $P1_{39-512}$ expressed by *B. subtilis* strain LDV701

Bacillus subtilis cells were suspended in buffer A (0.1 M Tris HCl, 0.5 M NaCl, pH 7.5) and lysozyme (800 μmg mL^{-1}) and incubated for 30 min to 37 1C. Sodium dodecyl sulfate (SDS) (0.01%) and phenylmethylsulfonyl fluoride (PMSF) (0.01 M) were added to the suspension and the cells were disrupted by sonication. The soluble supernatant was collected by centrifugation and subjected to affinity chromatography using a nickel–resin column in an AKTA FPLC device (Amersham Biosciences). Bound proteins were eluted with buffer B (0.1 M Tris HCl, 0.5 M NaCl, pH 7.5) containing final imidazole concentrations ranging from 0.05 to 1 M. The recombinant *S. mutans* P1 protein was purified from the previously described *E. coli* CG14 strain (Brady *et al.*, 1998). Expression of the recombinant protein was achieved following incubation of the *E. coli* CG14 strain in a medium containing 0.1 mM of IPTG for 4 h. The cell pellets were suspended in 0.1 M Tris HCl, 0.2 M NaCl and 4 M urea (pH 8.0) containing lysozyme (100 μg mL^{-1}) and incubated for 30 min to 37 °C. SDS (0.01%) and PMSF (0.01 M) were added to the suspension before sonic disruption of the cells. The soluble supernatant was applied in a nickel-affinity chromatography column and eluted under the same conditions as those described for the purification of the $P1_{39–512}$. The protein concentrations of the eluted fractions were determined using the BCA protein quantification assay (Pierce™), with bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots

SDS-PAGE was performed following standard procedures using a Mini Protean II vertical electrophoresis unit (Mini-protean, BioRad). Western blots were carried out following the incubation of nitrocellulose membranes with P1-specific (1 : 3000) or anti-$P1_{39–512}$-specific (1 : 2000) sera. The anti-P1 serum was raised in mice immunized with the recombinant P1 protein generated in *E. coli* while the anti-$P1_{39–512}$ serum was collected from mice immunized with the protein purified from *B. subtilis* LDV701 strain. Reactive bands were detected using a chemiluminescent kit (Super Signal™, Pierce), as described by the manufacturer.

Mouse immunizations and serum collection

BALB/c mice were supplied by the Isogenic Mouse Breeding Facility of the Department of Parasitology, Biomedical Sciences Institute (ICB), University of São Paulo (USP). All animal handling procedures were in accordance with the principles of the Brazilian code for the use of laboratory animals. Groups of five female mice, 6–8 weeks of age, were immunized subcutaneously with five doses of 10 μg of purified P1 or $P1_{39–512}$ proteins at 2-week intervals. The first dose was administered with complete Freund's adjuvant and the subsequent doses were administered with incomplete Freund's adjuvant. The recombinant $P1_{39–512}$ was administered as a soluble protein or after heat denaturation (boiling for 10 min) ($P1_{39–512}$d). Antisera against whole *S. mutans* PC3370 (anti-SmuΔP1) or PC3370C (anti-Smu) were raised by intravenous immunization of five female mice with five doses at 2-week intervals of 100 μL containing 7×10^9 CFU in phosphate-buffered saline (PBS) of whole cells harvested from cultures grown to an OD_{600}nm of 2. Serum samples were collected 2 weeks after the last inoculation, pooled and stored at −20 °C for subsequent analysis. Samples were tested for reactivity with full-length P1 and $P1_{39–512}$ antigens by an enzyme-linked immunosorbant assay (ELISA). Adsorption of anti-P1 serum samples with purified $P1_{39–512}$ or $P1_{39–512}$d was carried out in ELISA plates previously treated with 200 ng per well of purified recombinant protein as the solid phase-bound antigen. Aliquots of anti-P1 serum diluted 1 : 10^5 were applied to the plate wells and kept for 2 h at 37 °C. The serum samples were removed and checked for residual reactivity to the purified $P1_{39–512}$ in Western blots. The procedure was repeated until no reaction with the $P1_{39–512}$ was detected.

ELISA

The determination of specific antibody levels in serum samples was performed by ELISA and serial end-point titration in 96-well MaxiSorp (Nunc) plates coated with the recombinant truncated or full-length P1 proteins according to previously described procedures (Luiz *et al.*, 2008). Briefly, each purified protein tested (100 ng per well) diluted in PBS was added to the plates and kept for 16 h at 4 °C. Plates were washed twice with PBS–Tween 0.05% and then blocked with PBS–Tween – 5% milk for 2 h at 37 °C. One hundred microliters of twofold serial dilutions of the primary antibody beginning at 1/25 were added to the wells and incubated for 2 h at 37 °C. After a second washing step, peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma) (1/3000) was added and plates were incubated for 2 h at 37 °C. Following a final wash, the plates were

developed with *O*-phenylenediamine (0.4 mg mL^{-1}; Sigma) substrate solution containing 12% H_2O_2 and the reactions were stopped after addition of 2 M H_2SO_4. A_{492}nm was measured on a microplate reader (LabSystem). All samples were assayed in duplicate. An absorbance value of preimmune sera was used as a reference blank. Dilution curves were drawn for each sample and end-point titers were calculated as the reciprocal values of the last dilution with an optical density of 0.05.

Immunofluorescence analysis

Streptococcus mutans PC3370 and PC3370C strains were spread on glass slides, heat fixed and evaluated for reactivity with the anti-$P1_{39–512}$ antiserum, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Invitrogen™, Carlsbad, CA) as described by Homonylo-McGavin & Lee (1996). The anti-P1 antiserum and preimmune sera served as positive and negative controls. The stained cells were viewed using a Nikon TE300 inverted epifluorescence microscope (495 nm excitation, 525 nm emission).

Inhibition of saliva-mediated aggregation of *S. mutans*

Assays were carried out with clarified saliva, as described previously (Rundegren & Arnold, 1987). Aggregation and inhibition of aggregation assays in the presence of the test sera were performed according to a previously described method (Brady *et al.*, 1992). All tests were repeated independently at least three times.

Inhibition of *S.mutans* adhesion to immobilized saliva

Adhesion of *S. mutans* to immobilized saliva, and adherence inhibition mediated by different serum samples, were based on a method described previously (Jakubovics *et al.*, 2005). As a positive control reaction for inhibition of P1-mediated bacterial adhesion, we used 3 mM EDTA added to the bacterial cell suspension to interrupt the calcium-dependent interaction between P1 and gp340, as described previously (Crowley *et al.*, 1993). Nonimmune serum was used as a negative control and the *S. mutans* strain PC3370 devoid of P1 was also used as a negative control. The numbers of bound cells were calculated from a standard curve relating A_{600}nm to the cell number of *S. mutans*. All tests were independently repeated at least three times.

Statistical analyses

Results were analyzed in GRAPHPAD PRISM 5 and were expressed as mean ± SD. One-way ANOVA was used to compare the inhibition of adherence by anti-$P1_{39–512}$ sera. Statistically significant differences were considered with P values below 0.05.

Results

Expression of *S. mutans* $P1_{39-512}$ by genetically modified *B. subtilis* strains

The pLDV701 vector encodes the truncated recombinant *S. mutans* P1 polypeptide with an N-terminal histidine tag under control of the IPTG-inducible Pgrac promoter (Fig. 1b). The encoded protein has a predicted molecular mass of 55.7 kDa. A single band, with an apparent molecular mass of M_r~50 kDa, reactive with an anti-P1 antiserum was detected in

immunoblots of whole-cell extracts of the *B. subtilis* LDV701 strain following incubation with IPTG to induce protein expression. The same anti-P1-specific polyclonal serum detected only the M_r 185 kDa P1 protein in extracts of the *S. mutans* PC3370C-positive control strain. No cross-reacting bands were observed in extracts of *B. subtilis* transformed with pHT08 or the LDV701 strain cultivated in the absence of the inducer (Fig. 2a). The recombinant polypeptide accumulated in the cytoplasm of the *B. subtilis* LDV701 strain and approximately half of the protein was present in a soluble form (Fig. 2b and data not shown). Cellular extracts containing soluble proteins of *B. subtilis* strain LDV701 were loaded on a column of nickel-containing resin and the recombinant protein was eluted at 0.5 M imidazole concentration (Fig. 2c). A final protein yield of 15 mg L^{-1} of culture was achieved following incubation at 37 °C for 4 h in the presence of IPTG. No significant improvements in the recovery yields of the soluble protein were achieved following incubation of the *B. subtilis* at different growth temperatures, agitation levels or varying the inducer concentrations and induction periods in the presence of IPTG (data not shown).

Antibodies raised in mice immunized with $P1_{39\text{-}512}$ expressed in *B. subtilis* recognize the native and recombinant *S. mutans* P1 protein

Two groups of female BALB/c mice were immunized subcutaneously with five doses of the recombinant $P1_{39–512}$ protein: one group with the soluble protein purified from the *B. subtilis* LDV701 strain and another group immunized with the same protein boiled for 10 min ($P1_{39–512}$d). Antibodies raised in mice immunized with untreated or heat-denatured $P1_{39–512}$, as well as serum raised in mice immunized with purified P1 expressed in *E. coli*, were tested in immunoblots and showed no cross reaction with unspecific *B. subtilis* or *S. mutans* proteins (data not shown and Fig. 3). As expected, both sera reacted with $P1_{39–512}$ in whole-cell extracts of IPTG-treated *B. subtilis* LDV701 (Fig. 3). A major protein band of approximately 85 kDa was specifically detected in the whole-cell extract of *S. mutans* PC3370C by sera raised in mice against $P1_{39–512}$ or $P1_{39–512}$d. This probably represents a stable degradation product of the native streptococcal P1 protein (Fig. 3). That the 185 kDa P1 protein was not well recognized by the anti-$P1_{39–512}$ antisera suggests that epitopes contained within this truncated polypetide may be masked in the context of the full-length molecule, a property that has been observed previously when monoclonal antibody reactivity against full-length P1 and derivatives has been evaluated by Western blot (McArthur *et al.*, 2007).

The antiserum raised in mice immunized with P1, $P1_{39–512}$ or $P1_{39–512}$d was titrated in ELISA plates using purified full-length P1, $P1_{39–512}$ or $P1_{39–512}$d, as the solid phase-bound antigens. Not surprisingly, the highest measured titer was observed with the anti-P1 antiserum against the homologous full-length P1 protein (10^9) because additional epitopes would be contained within this molecule that are not present in the $P1_{39–512}$ derivative (Table 2). As shown in Table 2 and Fig. 4, ELISAs carried out with the anti-P1 antiserum against $P1_{39–512}$ demonstrated IgG titers of 10^4, while lower values (10^3) were obtained on plates coated with $P1_{39–512}$d. This suggests that at least some antigenic epitopes were affected by heat denaturation. When ELISA tests were carried out with antisera raised in mice immunized with $P1_{39–512}$ or $P1_{39–512}$d, the anti-P1 IgG titers were similar (7.0×10^3 and 8.4×10^3, respectively), while higher values were recorded for reactivity against

$P1_{39–512}$ in comparison with $P1_{39–512}$d (Table 2). Taken together, these results suggest that antibodies raised in mice immunized with $P1_{39–512}$ or $P1_{39–512}$d produced in *B. subtilis* recognize epitopes found in the *S. mutans* P1 protein and that the immunogenicity and antigenicity of $P1_{39–512}$ is diminished by heat denaturation. Additionally, the lower titer of anti-$P1_{39–512}$ and anti-$P1_{39–512}$d sera measured against full-length P1 compared with $P1_{39–512}$ again suggests that epitopes contained within the truncated construct may be masked in the intact protein.

To further evaluate whether epitopes present in the native *S. mutans* P1 protein are conserved in the recombinant $P1_{39–512}$, antisera raised in mice against the *S. mutans* PC3370 strain lacking the *spa*P gene encoding P1, or against PC3370C, the deletion mutant complemented with a plasmid encoding P1, were reacted with $P1_{39–512}$ and $P1_{39–512}$d polypetides in ELISA (Table 2). Antibodies generated against *S. mutans* PC3370C, but not those raised against PC3370, reacted with both of these antigens, further indicating that epitopes reflective of cell surface-localized streptococcal P1 are maintained in the recombinant $P1_{39–512}$ expressed in *B. subtilis*. As additional evidence of the specifi-city of the anti-$P1_{39–512}$ antiserum, *S. mutans* PC3370C and PC3370 were reacted with serum samples harvested from mice immunized with $P1_{39–512}$ or $P1_{39–512}$d. As shown in Fig. 5, PC3370C, but not PC3370, was labeled with both serum samples. These results indicate that the recombinant $P1_{39–512}$ polypeptide preserved surface-exposed epitopes, even after the heat treatment.

Streptococcus mutans saliva-mediated aggregation and adherence is inhibited by antisera raised in mice immunized with recombinant $P1_{39-512}$ protein

To evaluate the functional characteristics of antibodies raised in mice immunized with the recombinant $P1_{39–512}$ protein, we tested serum samples both for inhibition of saliva-mediated bacterial aggregation and for inhibition of *S. mutans* adherence to an abiotic surface. As demonstrated in Fig. 6a, anti-$P1_{39–512}$ and anti-$P1_{39–512}$d serum samples inhibited saliva-mediated aggregation of *S. mutans* cells at levels comparable to that from mice immunized with the full-length P1 protein (Table 2). The nonimmune mouse control serum did not show any effect on saliva-mediated *S. mutans* aggregation. The antiaggregation effect of the anti-P1 antiserum was partially, but not completely eliminated by adsorption with $P1_{39–512}$d, suggesting that antibodies against heat-sensitive epitopes contained within this poly-peptide may contribute to aggregation inhibition. In contrast, adsorption with $P1_{39–512}$ did not decrease the ability of the anti-P1 antiserum to inhibit aggregation and the inhibitory effect was even slightly improved (Fig. 6a). This suggests that the relative levels and balance of specific antibodies within the polyclonal anti-P1 reagent are a factor contributing to its biological function.

Experiments testing the inhibition of *S. mutans* adhesion to saliva-coated microplate wells showed that anti-P1, anti-$P1_{39–512}$d and anti-$P1_{39–512}$ antisera all significantly inhibited the binding of *S. mutans* PC3370C to the immobilized salivary constituents (Fig. 6b). The anti-$P1_{39–512}$ reagent was most effective in this regard. The interaction of P1 with gp340 is calcium-dependent and, as expected, *S. mutans* cells incubated in the presence of EDTA did not adhere to the saliva-coated microplate wells. A low level of background inhibition was

observed with the nonimmune mouse serum that was eliminated at the higher dilutions. No significant aggregation or adherence was observed with the *S. mutans* PC3370 P1-deficient negative control strain (data not shown). In contrast to the results observed in the aggregation inhibition assays, the anti-P1 serum adsorbed with $P1_{39–512}$ or $P1_{39–512}$d completely lost its ability to inhibit the adhesion of PC3370C to immobilized salivary constituents (Fig. 6b). It has long been recognized that bacterial aggregation and adherence represent distinct properties that can be distinguished by anti-P1 antibodies of differing specificities (Brady *et al.*, 1992). Importantly, the loss of adherence-inhibiting antibodies by adsorption with the purified *B. subtilis* $P1_{39–512}$ truncated polypeptide indicates that relevant functional epitopes are contained within this construction.

Discussion

The P1 adhesin of *S. mutans* represents an important target of protective immunity and represents a candidate antigen for anticaries vaccines (Shivakumar *et al.*, 2009). In this study, we expressed and purified a P1-derivatived polypep-tide using a heterologous prokaryotic protein expression system based on a genetically modified *B. subtilis* strains. Our results demonstrate that the truncated $P1_{39–512}$ poly- peptide produced by *Bacillus* is recognized by antibodies generated in mice immunized with either *S. mutans* whole cells or with the purified full-length P1 protein. Conversely, immunization of mice with the *B. subtilis*-derived recombinant $P1_{39–512}$ induced serum IgG antibodies that recognize the full-length molecule as well as the native protein expressed on *S. mutans* cells. Finally, the anti-$P1_{39–512}$ antibodies were shown to be functionally active as demonstrated by an ability to interfere both with aggregation and adhesion to saliva-coated surfaces of *S. mutans*.

Low cost and high yields are the two major reasons for the widespread acceptance of *E. coli* strains as bacterial hosts for the expression of heterologous proteins both by academic laboratories and by industry. Nonetheless, problems associated with reduced expression, generation of insoluble proteins and contamination with endotoxin, due to the presence of outer membrane fragments, may constrain the use of *E. coli* as a host for purification of heterologous proteins. *Bacillus subtilis* strains show a good capacity to express recombinant proteins with preserved biological activity and do not have lipopolysaccharides, representing a promising alternative for the expression and purification of recombinant proteins (Harwood, 1992; Terpe, 2006; Schumann, 2007). Indeed, some proteins derived from gram-positive bacteria, such as streptokinase, have a greater stability when produced in *B. subtilis* (Wong *et al.*, 1994). Similarly, the human interleukin-3 has been successfully expressed and purified from *B. subtilis* at recovery yields above 100 mg L^{-1} (Westers *et al.*, 2005). Our initial attempts to produce *S. mutans* $P1_{39–512}$ in *E. coli* failed due to low expression levels and the insolubility of the recombinant protein. In contrast, the same protein was expressed in *B. subtilis* at reasonable levels (15 mg L^{-1}) in a soluble state. Practical application of recombinant proteins generated in prokaryotic expression systems requires good expression levels and conservation of structural and functional features of the native protein. The recombinant $P1_{39–512}$ produced in *B. subtilis* was recognized by antisera generated against purified full-length recombinant P1 from *E. coli* as well as whole *S. mutans* cells. Several studies have shown that the P1 amino-terminal region containing the alanine-rich region is antigenic, as evidenced by reactivity of sera from patients naturally infected with *S. mutans*

(Takahashi *et al.*, 1991; Okahashi *et al.*, 1993; Senpuku *et al.*, 1996, 1998). Our data indicate that $P1_{39–512}$ preserves both antigenic and immunogenic determinants of the native *S. mutans* P1 protein. In the current work, with boiled and untreated polypeptides, we found that both heat-stable and heat-denaturable epitopes of P1, including those exposed at the cell surface, are contained within the recombinant $P1_{39–152}$. Interestingly, boiling the recombinant protein did not completely eliminate $P1_{39–512}$ antigenicity or immunogenicity, suggesting that both linear as well as heat-labile structural features play important roles in the immunological properties of P1.

The interaction of *S. mutans* with salivary components is a multifunctional process that determines whether the tooth surface will be colonized or not. The P1 protein interacts with salivary constituents adsorbed to the tooth surface leading to adherence (Lee *et al.*, 1988, 1989; Koga *et al.*, 1990). P1 also interacts with soluble fluid-phase components promoting aggregation and, presumably, elimination of *S. mutans* cells from the oral cavity (Ericson & Rundegren, 1983; Demuth *et al.*, 1988; Koga *et al.*, 1990). These dual roles of *S. mutans* P1 are contributed to by amino acid residues within $P1_{39–512}$ (Crowley *et al.*, 1993; Nakai *et al.*, 1993; Hajishengallis *et al.*, 1998; Matsumoto-Nakano *et al.*, 2008). Aggregation and adherence of *S. mutans* are inhibited by different anti-P1 monoclonal antibodies (Brady *et al.*, 1992), indicating that the composite specificity of the host immune response would represent a key feature in determining the neutralization activity of elicited antibodies to a specific vaccine immunogen. In our current study, antibodies generated in mice immunized with the recombinant $P1_{39–512}$ or $P1_{39–512}$d protein were effective in blocking both bacterial aggregation and the adherence of *S. mutans* to abiotic surfaces. These results indicate that relevant immunological determinants required both for adhesion and for aggregation of the native *S. mutans* protein are maintained in the recombinant protein expressed in *B. subtilis* cells even after the denaturation process.

Consistent with previous findings that P1 determinants mediating bacterial aggregation and adherence can be differentiated with certain monoclonal antibodies, experiments with the anti-P1 antiserum adsorbed with, and hence depleted of antibodies against $P1_{39–512}$ or $P1_{39–512}$d, demonstrated a difference in the relative effect on aggregation inhibition compared with adherence inhibition. The ability of the anti-P1 antiserum to inhibit aggregation was impaired more by adsorption with $P1_{39–512}$d compared with $P1_{39–512}$, suggesting that antibodies against linear epitopes may be more relevant for this biological activity. That immunization of mice with $P1_{39–512}$ induced the formation of antibodies better able to inhibit bacterial adherence to salivary components compared with antibodies elicited against $P1_{39–512}$d or full-length P1 is informative. First, this suggests that antibodies against heat-labile epitopes may contribute to adherence inhibition. Second, the higher degree of adherence inhibition induced by $P1_{39–512}$ compared with the intact molecule suggests an effect of truncation on immunodominance and that important determinants may be better exposed in the truncated poly-peptide. It has been shown previously that certain anti-P1 monoclonal antibodies can alter the adhesin's immunogeni-city by increasing epitope exposure. Furthermore, these results were replicated by immunization with truncated variants of the protein (Robinette *et al.*, 2009). The current results reiterate that less may in fact be more when it comes to induction of an optimal and efficacious response.

In conclusion, we have shown that a recombinant $P1_{39–512}$ polypeptide expressed and purified from *B. subtilis* cells preserves the relevant antigenic and immunogenic determinants of the native *S. mutans* adhesin. Because recombinant purified antigens represent an important approach for the development of vaccines against dental caries and systemic damage caused by *S. mutans* (Hajishengallis *et al.*, 1995; Toida *et al.*, 1997; Zhang *et al.*, 2002), the ability to utilize *B. subtilis* as a promising new expression system represents an additional tool for the generation of recombinant antigens with application in acellular-based vaccines.

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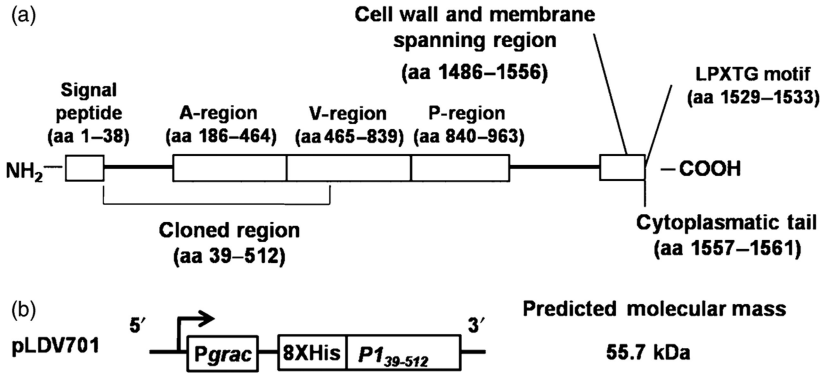

Fig. 1.

Schematic representation of the *Streptococcus mutans* P1 protein (a) and the recombinant gene encoding the truncated $P1_{39–512}$ polypeptide (b) cloned in *Bacillus subtilis* pLDV701 vector. The predicted molecular mass of the recombinant $P1_{39–512}$ protein is indicated on the right of (b).

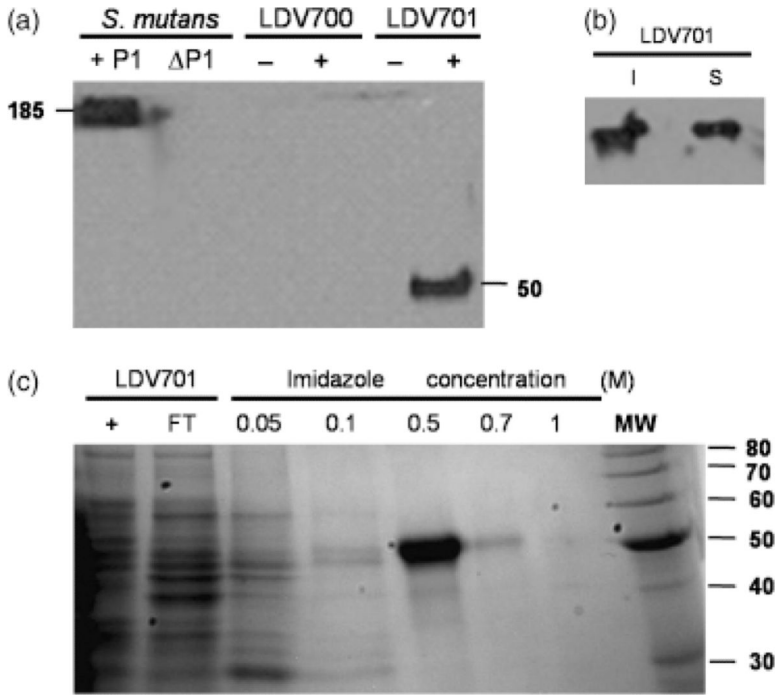

Fig. 2.

Detection and purification of the recombinant $P1_{39–512}$ protein expressed by *Bacillus subtilis* cells. (a) Immunodetection of P1 or $P1_{39–512}$ expressed by *Streptococcus mutans* PC3370C (1P1) or *B. subtilis* LDV701 (LDV701) strains, respectively. Negative controls include *S. mutans* PC3370 (ΔP1) and the *B. subtilis* LDV700 strain (LDV700). − and + denote cells induced or not with IPTG, respectively. Estimated molecular weights (kDa) of the reactive protein bands are indicated. (b) Immunodetection of recombinant $P1_{39–512}$ in the soluble (S) or insoluble (I) fractions of the *B. subtilis* LDV701 following induction with IPTG. (c) Purification of recombinant $P1_{39–512}$ expressed in *B. subtilis*. Imidazole concentrations used to elute the protein from the nickel-containing resin are indicated. Proteins were separated in 12.5% polyacrylamide gels and stained with Comassie Brilliant Blue. MW, molecular weight markers indicated in kilodaltons (kDa) at the right side of the figure.

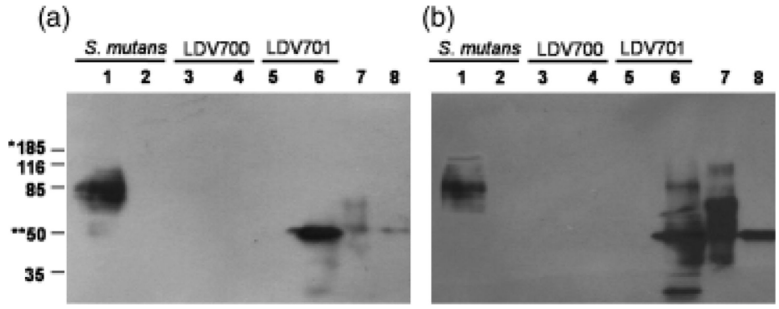

Fig. 3.

Antigen specificity of antibodies raised in mice immunized with $P1_{39–512}$. (a) Western blots developed with serum samples collected from mice immunized with $P1_{39–512}$ or (b) $P1_{39–512}$d. Samples: 1,*Streptococcus mutans* PC3370C strain; 2, *S. mutans* PC3370 strain; 3, *Bacillus subtilis* LDV700 strain following incubation without IPTG; 4, *B. subtilis* LDV700 strain following incubation with IPTG; 5, *B. subtilis* LDV701 strain following incubation without IPTG; 6, *B. subtilis* LDV701 strain following incubation with IPTG; 7, recombinant P1 protein; 8, $P1_{39–512}$ purified protein. Proteins were sorted in 12.5% polyacrylamide gels. The molecular weights of the reactive protein bands are indicated. (*) and (**) indicate the molecular weights of P1 and $P1_{39–512}$ proteins, respectively.

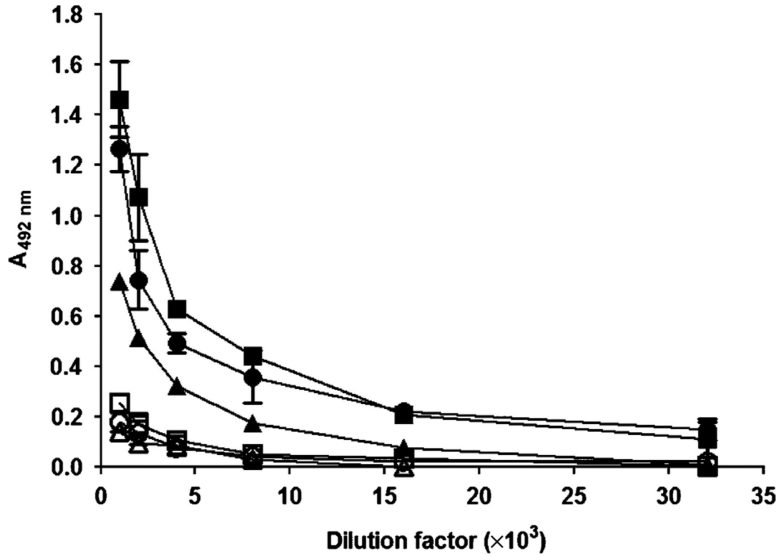

Fig. 4.

IgG titration in antisera raised in mice immunized with full-length P1, expressed in *Escherichia coli*, and with $P1_{39–512}$ or $P1_{39–512}$d, produced in recombinant *Bacillus subtilis*. Serum dilutions were reacted in ELISA plates coated with $P1_{39–512}$ (closed symbols) or heat-denatured $P1_{39–512}$d (open symbols). Symbols: (circles) anti-P1; (squares) anti-$P1_{39–512}$; and (triangles) anti-$P1_{39–512}$d.

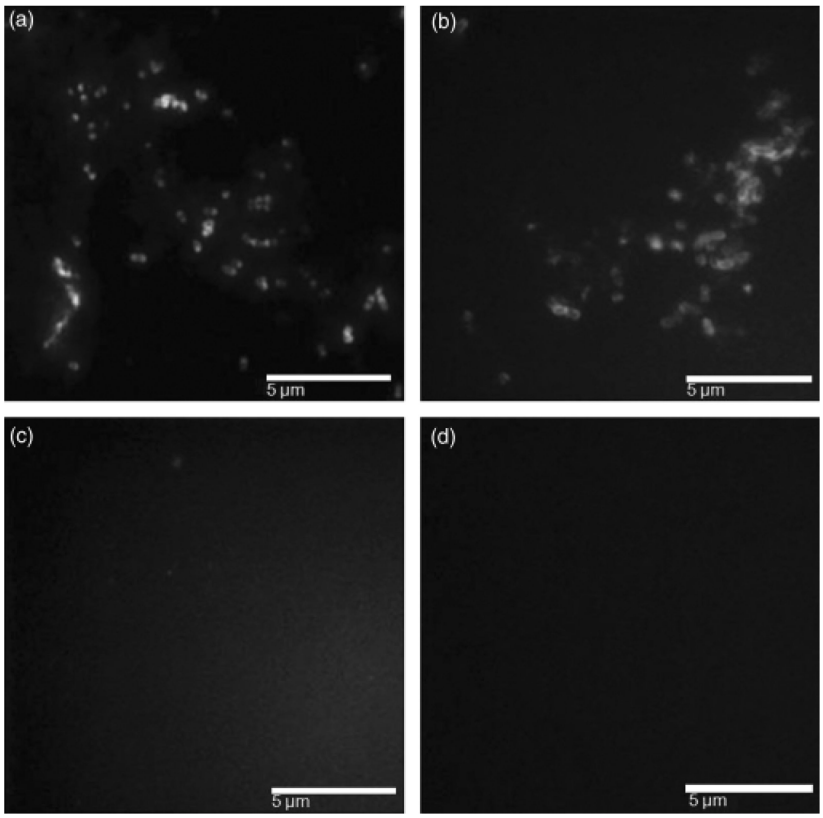

Fig. 5.

Immunofluorescence labeling of *Streptococcus mutans* cells using antisera from mice immunized with $P1_{39–512}$ or $P1_{39–512}$d. Samples: (a) *S. mutans* PC3370C strain reacted with the anti-$P1_{39–512}$ antiserum; (b) *S. mutans* PC3370C strain reacted with anti-$P1_{39–512}$ d antiserum; (c) *S. mutans* PC3370 (ΔP1) strain reacted with anti-$P1_{39–512}$ antiserum; (d) *S. mutans* PC3370 (ΔP1) strain reacted with anti-$P1_{39–512}$d serum. Labeled *S. mutans* cells were revealed with fluorescein isothiocyanate -conjugated goat anti-mouse IgG.

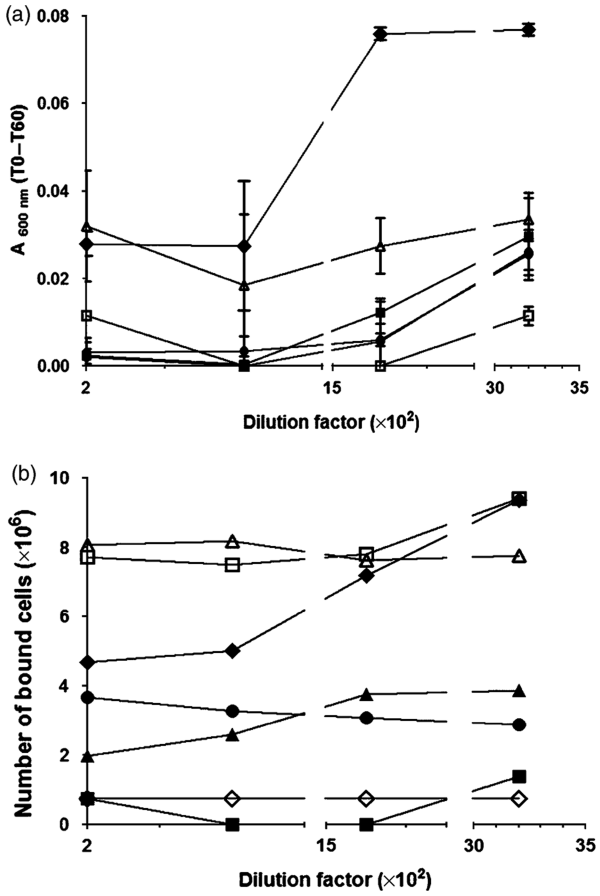

Fig. 6.

Functional characterization of antibodies raised in mice immunized with $P1_{39–512}$ produced in *Bacillus subtilis*. (a) Inhibition of saliva-mediated aggregation of *Streptococcus mutans* PC3370C cells. Samples containing bacterial cells and the tested serum were incubated for 5 min at 37 °C and the OD_{600nm} was measured (T0). After an additional 60-min incubation period at 37 1C, the OD (T60) was measured once more. The values are expressed as the difference between T0 and T60 for each tested serum dilution. Symbols: (◆) nonimmune serum; (●) anti-P1 serum; (■) anti-$P1_{39–512}$ serum; (▲) anti-$P1_{39–512}$d serum; (□) anti-P1 serum adsorbed with $P1_{39–512}$; and (△) anti-P1 serum adsorbed with $P1_{39–512}$d. (b) Inhibition of *S. mutans* adherence to immobilized saliva in microplate wells. *Streptococcus mutans* PC3370C cells were mixed with diluted tested serum samples, incubated for 30 min to 37 °C, and transferred to microplate wells coated with clarified saliva and incubated for an additional 2 h. Bound cells were revealed by staining with 0.5% crystal violet and determination of absorbance of the solubilized cell-bound stain at 600 nm. Symbols are the same as those shown in (a) and (◇) cells incubated in the presence of 3 mM EDTA. All tested sera were diluted in order to reach a final titer of 10^4. Data represent the average of three independent experiments. Values are expressed as mean ± SD.

Table 1

Bacterial strains and plasmids used in this work

	Main characteristics	References
Strains		
S. mutans		
UA 159	Km^r	Adjic *et al.* (2002)
PC3370	*S. mutans* NG8 *spaP*::*tet*^{r}	Crowley *et al.* (1999)
PC3370C	*S. mutans* NG8 *spaP*::*tet*^{r}*;* complemented with pMAD (pDL289+*spaP*)	Brady *et al.* (1998)
E. coli		
DH5α	*F*$^-$ *ϕ80dlacZΔM15Δ(lacZYA-argF) U169 deoR, recA1 endA1 hsdR17 (*r_k *-* m_k^+ *phoA supE44λ-thi-1 gyrA96 relA1*	Invitrogen™
CG14	M15(pREP4) carrying pCG14	Brady *et al.* (1998)
B. subtilis		
WW02	*leuA8 metB5 trpC2 hsrdRM1 amyE*::*neo*	Wehrl *et al.* (2000)
LDV700	WW02 carrying pHT08 [empty vector - IPTG-inducible promoter (Pgrac)]	This work
LDV701	WW02 carrying pLDV701 ($P1_{39\text{-}512}$ under the control of *Pgrac*)	This work
Plasmids		
pGEM-T-Easy™	Amp^r; operon lac;T-end	Promega™
pCG14	Derived from pQE-30 with a cloned *spa*P gene coding for 39 to 1561 amino acids of the P1 protein	Brady *et al.* (1992)
pGP1N	Amp^r; pGEM-T-Easy™ with cloned $P1_{39\text{-}152}$	This work
pHT08	Amp^r; Cm^r; IPTG-inducible *PgraC* promoter; His-tag	Nguyen *et al.* (2007)
pLDV701	Derived from pHT08 with cloned $P1_{39\text{-}152}$	This work

Table 2

Reactivity of antibodies raised in mice immunized with recombinant proteins or whole *Streptococcus mutans* cells with recombinant P1, $P1_{39\text{-}512}$ or $P1_{39\text{-}512}$d

	Plates coated with		
Antiserum	**P1**	**$P1_{39\text{-}512}$**	**$P1_{39\text{-}512}$d**
Anti-P1	1.65×10^9	1.3×10^4	6.3×10^3
Anti-P1 39-512	7.0×10^3	7.6×10^4	6.8×10^3
Anti-P1 39-512d	8.4×10^3	1.1×10^4	5.1×10^3
Anti-Smu*	ND	1.7×10^5	7.0×10^4
Anti-SmuΔP1†	ND	2.0×10^2	1.4×10^2

ND, not determined.

*Antisera raised in mice immunized with the whole cell of the *S. mutans* PC3370C strain.

†Antisera raised in mice immunized with the whole cell of the *S. mutans* PC3370 (P1-knockout) strain.