Identification and analysis of a gene (*abpA*) encoding a major amylase-binding protein in *Streptococcus gordonii*

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Oral streptococci such as *Streptococcus gordonii* bind the abundant salivary enzyme α -amylase. This interaction may be important in dental plaque formation and metabolism, thus contributing to the initiation and progression of dental caries and periodontal disease, the two most common plaque-mediated diseases. The conjugative transposon Tn916 was used to insertionally inactivate gene(s) essential to the expression of amylase-binding components of *S. gordonii* Challis, and a mutant deficient in amylase-binding (Challis Tn1) was identified. While wild-type strains of *S. gordonii* released both 20 kDa and 82 kDa amylase-binding proteins into culture supernatants, Challis Tn1 expressed the 82 kDa but not the 20 kDa protein. The 20 kDa amylase-binding protein was isolated from culture supernatants of *S. gordonii* Challis by hydroxyapatite chromatography. A partially purified, functionally active 20 kDa protein was sequenced from blots, and the N-terminal sequence obtained was found to be DEP(A)TDAAT(R)NND. A novel strategy, based on the single-specific-primer polymerase chain reaction technique, enabled the gene inactivated by Tn916 to be cloned. Analysis of the resultant nucleotide sequence revealed an open reading frame of 585 bp, designated amylase-binding protein A (*abpA*), encoding a protein of 20 kDa (AbpA), immediately downstream from the insertion site of Tn916. This protein possessed a potential signal peptide followed by a region having identity with the N-terminal sequence of the 20 kDa amylase-binding protein. These results demonstrate the role of the 20 kDa protein in the binding of amylase to *S. gordonii*. Knowledge of the nature of amylase-binding proteins may provide a better understanding of the role of these proteins in the colonization of *S. gordonii* in the oral cavity.

Keywords: biofilms, dental plaque, salivary proteins, microbial adhesion/adherence, amylase-binding protein

INTRODUCTION

Several mammalian enzymes are known to specifically bind to bacteria. For example, plasmin (Lottenberg *et al.*, 1992), lysozyme (Tellefson & Germaine, 1986; Cimasoni *et al.*, 1987; Roger *et al.*, 1994) and lactoperoxidase (Roger *et al.*, 1994) all bind to streptococci to

influence their adhesion to and colonization of host surfaces. α -Amylase, the most abundant enzyme in human saliva (Aguirre *et al.*, 1987), also binds with high affinity to a number of the streptococci that are early colonizers of the tooth, including *Streptococcus gordonii*, *S. mitis*, *S. parasanguis*, *S. crista* and *S. salivarius* (Douglas, 1983; Scannapieco *et al.*, 1989, 1994; Douglas *et al.*, 1990; Kilian & Nyvad, 1990; Handley *et al.*, 1991; Gwynn & Douglas, 1994). Amylase-binding bacteria constitute a substantial proportion of the total cultivable flora from human teeth (Tseng *et al.*, 1992; Scannapieco *et al.*, 1994), and amylase-binding bacteria appear to

Abbreviations: BHI, brain heart infusion broth; HPS, human parotid saliva; TBST, Tris-buffered saline with Tween 20.

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colonize only the mouths of animals having salivary amylase activity (Scannapieco *et al.*, 1994). Amylase may also serve as an adherence receptor for amylase-binding bacteria (Scannapieco *et al.*, 1995). Thus, amylase, together with other salivary components, may play an important role in the formation of dental plaque (Douglas, 1994; Scannapieco, 1994).

Amylase binds to *S. gordonii* through high-affinity receptors that appear to cluster around cell-division sites on the surface of actively dividing cells (Scannapieco *et al.*, 1992). Bacterial cells in the exponential phase of growth are capable of binding more amylase than those in stationary phase (Scannapieco *et al.*, 1992). The interaction of amylase with the surface of *S. gordonii* has been shown to be sensitive to treatment with proteases and periodate, suggesting that the amylase receptor is a protein or a glycoprotein (Douglas, 1990; Scannapieco *et al.*, 1992). Further work suggested that amylase-binding components of 20 and 82 kDa are associated with the cell surface and released into the culture supernatants of *S. gordonii* strains (Douglas, 1990; Gwynn & Douglas, 1994). Furthermore, an alkali-soluble 20 kDa component was extracted from *S. gordonii* G9B and interacted with immobilized amylase (Scannapieco *et al.*, 1992). Taken together, these findings suggest that amylase binding to *S. gordonii* may be mediated by a multi-component, cell-surface-associated protein receptor that is subsequently released into the culture supernatant as the cell matures.

Transposon-mediated insertional gene inactivation has proven to be a powerful tool for the inactivation and cloning of bacterial genes (Clewell, 1981; Gawron-Burke & Clewell, 1984; Harris *et al.*, 1992). Transposon mutagenesis is particularly useful in identifying genes and gene products important to the expression of specific phenotypes for which screening assays are available. *S. gordonii* Challis, an amylase-binding strain, is particularly amenable to such an approach since it exhibits natural competence (LeBlanc & Hassell, 1976) and therefore provides a convenient host for gene transfer.

The present study was performed to attempt to definitively identify and sequence amylase-binding component(s) of *S. gordonii* and to further explore the relationship between the 82 and 20 kDa amylase-binding components. The 20 kDa amylase-binding protein was isolated and its N-terminal sequence determined. Further, a mutant of *S. gordonii* Challis deficient in amylase-binding was constructed following insertional gene inactivation using the conjugative transposon Tn916. The disrupted gene was then cloned employing a strategy based on the single-specific-primer polymerase chain reaction technique (Shyamala & Ames, 1989; Novak *et al.*, 1997). Analysis of the disrupted nucleotide sequences revealed an open reading frame consistent with a gene encoding the putative 20 kDa amylase-binding protein.

METHODS

Bacterial strains, plasmids and culture conditions. *S. gordonii* strains Challis (NCTC 7868) and G9B, *S. gordonii* Challis Tn1 (this study), *S. sanguis* 10556, *Escherichia coli* strains DH1 (host for pAM620), JM109 and INV α (Invitrogen), and plasmids pAM620 (Yamamoto *et al.*, 1987), pCR2.1 (Invitrogen), pVA856 (Macrina *et al.*, 1983) and pCR2KB-7 (this study) were studied. The streptococcal strains were inoculated from frozen stocks on tryptic soy blood agar plates supplemented with 0.5% (w/v) yeast extract (TSBY agar; Difco) or TSBY agar supplemented with tetracycline (10 $\mu\text{g ml}^{-1}$) (TSBY-T; Sigma). Streptococcal strains were routinely cultured in either defined medium (Terleckyj *et al.*, 1975), in TSBY broth or in brain heart infusion broth (BHI; Difco). The mutant strain was cultured in media supplemented with or without 10 $\mu\text{g tetracycline ml}^{-1}$. Cultures were typically incubated for 16–18 h at 37 °C in a candle jar. *E. coli* strains were grown under aerobic conditions with shaking for 12–16 h at 37 °C in Luria–Bertani broth (LB) and maintained on LB agar. Strains containing recombinant clones were plated on LB agar containing 40 $\mu\text{g X-Gal ml}^{-1}$ (Sigma), 22 $\mu\text{g IPTG ml}^{-1}$ (Sigma), and 100 $\mu\text{g ampicillin ml}^{-1}$ (Sigma).

Collection of saliva and purification of amylase. Human parotid saliva (HPS) was collected into chilled tubes from healthy donors using a modified Carlsen–Crittenden apparatus (Curby, 1953) following stimulation with 2% citric acid. The HPS was dialysed against distilled water and lyophilized. Salivary amylase was purified from lyophilized HPS as previously described (Scannapieco *et al.*, 1989). For some experiments, whole saliva was collected by expectoration into a chilled tube, clarified by centrifugation, and used as a source of amylase.

Bacterial growth studies. *S. gordonii* Challis was grown in continuous culture at a range of dilution rates in a LH 210 series chemostat (LH Engineering) having a pot volume of 1 l at 37 °C at pH 7.0 in defined medium supplemented with arginine (100 $\mu\text{g ml}^{-1}$), so that the cells were carbon limited. The OD₆₀₀ and purity of the cultures were checked every 24 h, and ten generation times at a given dilution rate were allowed to elapse to reach steady state. Cells were grown at dilution rates of between 0.264 and 0.048 $\text{ml}^{-1} \text{h}^{-1}$, which corresponded to mean generation times of between 2.6 and 14.5 h. Samples from each steady-state culture were collected, the cells were washed in 50 mM phosphate buffer, pH 7.0, and resuspended in the same buffer to OD₆₀₀ 1.0 (approx. 4.2×10^8 cells ml^{-1}). Aliquots of 1 ml cell suspension were then centrifuged and the cell pellet resuspended in 1 ml whole clarified saliva for 1 h at 37 °C. After incubation, the suspensions were centrifuged and the supernatants retained. Both the cells and supernatants were assayed for amylase activity using a commercial coupled assay kit (Sigma) as previously described (Douglas *et al.*, 1992). For comparison, batch grown cells in BHI were also assayed for their ability to bind amylase. The saliva used as the source of amylase was from the same batch and was maintained as aliquots at –20 °C in order to standardize the amount of enzyme present in each assay between chemostat samples.

For construction of growth curves, streptococcal strains were first cultured in 10 ml BHI at 37 °C in a candle jar overnight. The cultures were diluted 1:100 in the appropriate broth and incubated in a candle jar at 37 °C until growth reached mid-exponential phase (OD₆₀₀ 0.6). Each of the cultures were again diluted 1:100 into multiple 10 ml aliquots of the same medium and incubated at 37 °C in a candle jar without shaking. Growth was assessed hourly for 12 h and at 18 h by

measurement of the OD₆₀₀ of each broth culture. The bacterial cells were then removed by centrifugation at 5000 g for 10 min and the supernatants stored at -20 °C. Proteins in the culture supernatants were precipitated by the addition of an equal volume of saturated ammonium sulfate, pH 7.2, and left overnight at 4 °C. Precipitated material was collected by centrifugation at 5000 g for 30 min, reconstituted in 10 mM PBS, pH 7.0, exhaustively dialysed against distilled water and lyophilized.

SDS-PAGE and Western blotting. To optimally separate lower-molecular-mass proteins (< 15 kDa), SDS-PAGE was performed as described by Giulian *et al.* (1983). Alternatively, proteins of molecular mass > 15 kDa were separated by SDS-PAGE (Laemmli, 1970). Apparent molecular masses were determined by comparison of the relative migration of bands of interest with known standard proteins. Gels were stained with 0.025% Coomassie brilliant blue R-250 (Bio-Rad) or with silver, using dichromate (Merril *et al.*, 1982).

Western blotting (Burnette, 1981) was performed by electro-transfer of gels to Immobilon-P membranes (Millipore) for 45 min at 100 mA using a TE-70 SemiPhor semi-dry transfer unit (Hoefer). Membranes were blocked with 3% (w/v) nonfat dry milk in Tris-buffered saline, pH 8.0, 0.05% (v/v) Tween-20 (TBST), washed with TBST and incubated with primary rabbit antibody diluted in 1% (w/v) nonfat dry milk in TBST. Primary antibodies utilized included rabbit polyclonal antisera to whole cells of *S. gordonii* (anti-G9B), to the purified 20 kDa amylase-binding protein (anti-20 kDa) (Scannapieco *et al.*, 1992) and to purified amylase (Scannapieco *et al.*, 1989). The membranes were subsequently incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) diluted in 1% (w/v) nonfat dry milk in TBST and developed with the ProtoBlot Western Blot AP system (Promega).

Amylase-binding assays. Soluble ¹²⁵I-labelled amylase binding to whole bacterial cells was investigated as previously described (Scannapieco *et al.*, 1989). The binding of amylase by streptococcal components was assessed using a solid-phase amylase ligand-binding assay as previously described (Douglas, 1990; Gwynn & Douglas, 1994). Briefly, streptococcal extracts or purified molecules in PBS were dotted on nitrocellulose (MSI). Alternatively, components were separated by SDS-PAGE and electrotransferred to Immobilon-P membranes or nitrocellulose. Blots were incubated with lyophilized HPS (1 mg ml⁻¹ in 3%, w/v, nonfat dry milk in TBST) for 1 h, washed and incubated with antibody to purified human salivary amylase in 1% (w/v) nonfat dry milk in TBST for 30 min. The blots were then washed, incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) or to horseradish-peroxidase-conjugated swine anti-rabbit IgG and developed with appropriate reagents.

Transformation of *S. gordonii* Challis and screening for amylase-binding deficient mutants. Plasmids were isolated as described by Birnboim & Doly (1979). Prior to transformation, competence was induced in *S. gordonii* Challis as described by Behnke (1981). Transformants were selected by plating cells onto TSBY-T agar and incubating at room temperature for 4 d in an anaerobic chamber (85% N₂, 10% H₂, 5% CO₂).

Transformants deficient in amylase binding were screened for by using a replica-plating method (Tseng *et al.*, 1992). Colonies (200–300 per plate) were transferred onto nitrocellulose membranes overlaid onto TSBY-T agar using a Replipad (Schleicher & Schuell). The membranes were in-

cubated at 37 °C under anaerobic conditions for 48 h, blocked in PBS with 0.1% (w/v) lipid-free BSA for 30 min and incubated in 20 ml PBS containing 10⁶ c.p.m. ¹²⁵I-labelled amylase (~1 µg) for 30 min with gentle agitation. The membranes were then washed with PBS until the c.p.m. eluted approached background levels, air dried and subjected to autoradiography. The inability of selected clones to bind amylase was confirmed by the solution-phase assay described above. Mutant strains unable to bind amylase were compared to the wild-type strain by Gram staining, evaluation of colony morphology on blood and mitis salivarius agars, comparison of biochemical metabolic profiles using the API Rapid Strep kit (bioMérieux Vitek) and by demonstrating that the mutant retained natural competence after transformation with a plasmid (pVA856) bearing an alternate antibiotic marker (erythromycin).

Determination of insertion number and genetic linkage studies. Streptococcal genomic DNA was isolated from the mutant (Challis Tn1) and wild-type strains as described by Coykendall & Gustafson (1985) with the following modification. The bacteria were first incubated for 1 h at 37 °C with 150 µg each of lysozyme and mutanolysin ml⁻¹ (Sigma). The number of Tn916 insertions present in the transformants was determined by treatment of genomic DNA isolated from the mutant strain with *Hind*III (Gibco-BRL). The restricted DNA was electrophoresed in 1.0% (w/v) agarose gels and Southern blots were prepared by capillary transfer of DNA from gels to Hybond-N membranes (Amersham). Nucleic acid was cross-linked to the membranes by UV light fixation. Hybridization was performed according to the Photogene nucleic acid detection system version 2.0 (Gibco-BRL) protocol and the blots were probed with *Eco*RI-digested pAM620 biotinylated using the BioPrime system (Gibco-BRL). Southern blots were visualized using the Photogene nucleic acid detection system version 2.0 (Gibco-BRL).

Isolation and sequencing of the 20 kDa amylase-binding component from *S. gordonii* supernatants. Supernatant from BHI cultures of *S. gordonii* was dialysed against 10 mM potassium phosphate buffer, pH 7.0, and then loaded onto an HTP hydroxyapatite cartridge (5 ml; Bio-Rad) at 4 °C. Bound proteins were eluted over a gradient of 10 to 400 mM potassium phosphate, pH 7.0, at a flow rate of 0.5 ml min⁻¹, collecting 2 ml fractions. Samples of these fractions were monitored by measuring A₂₈₀ and analysed by dotting (2.5 µl) onto nitrocellulose. The membrane was then blocked with 5% (w/v) haemoglobin, incubated in salivary amylase followed by horseradish-peroxidase-conjugated swine anti-rabbit IgG and visualized using appropriate developing reagents. Positive fractions were pooled and dialysed against 10 mM phosphate buffer, pH 7.0, and lyophilized. Proteins were analysed by SDS-PAGE and the amylase ligand-binding assay.

Samples of the purified amylase-binding protein were run on 15% SDS-PAGE gels. Gels were equilibrated with several changes of 10 mM CAPS, pH 11.0, containing 10% (v/v) methanol pH 11.0, then blotted onto a ProBlott (Applied Biosystems) membrane that had been pre-soaked in methanol. Blotting was carried out at 50 V for 30 min in equilibrating buffer. Protein bands were visualized by staining with 0.1% (w/v) Coomassie brilliant blue R-250. The protein band was then cut out of the blot and sequenced in an Applied Biosystems model 476 A sequencer by the Edman degradation reaction. The phenylthiohydantoin amino acid derivatives liberated by the degradation reaction were detected and analysed by measuring A₂₅₄.

Trypsin digestion of cell pellets and supernatants. Trypsin

(DPCC treated, type XI; Sigma), was coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) and titrated for activity against bovine serum albumin (fraction V; Sigma) to determine the minimum concentration of enzyme necessary for digestion. Bacterial cells were centrifuged, and supernatants were removed and stored at -20°C . Cell pellets were suspended in PBS, adjusted to OD_{600} 10, and $10\ \mu\text{l}$ trypsin-coupled beads was added to $1\ \text{ml}$ of the standardized cell suspension and mixed end-over-end at 37°C for 30 min. Supernatants were dialysed for 3 d against distilled water and lyophilized. Culture supernatants ($100\ \mu\text{l}$), first adjusted to pH 8.0 with $0.1\ \text{M}$ Tris, pH 9.5, were mixed with $2\ \mu\text{l}$ trypsin-coupled beads and incubated for 5, 10, 30 and 60 min at 37°C . Hydrolysis was stopped by centrifuging the suspension for 2 min at $12000\ g$ and boiling the supernatants for 2 min. Controls for each experiment included the cell pellet or supernatant without trypsin incubated at 37°C for 0 h and 1 h and trypsin in $0.1\ \text{M}$ Tris/HCl, pH 8.0, incubated alone at 37°C for 0 h and 1 h. Samples were dissolved or diluted in the appropriate sample buffer, separated by SDS-PAGE, electroblotted and examined by the amylase ligand-binding assay or immunostaining with the anti-20 kDa antibody.

PCR cloning of the disrupted amylase-binding-protein gene. *Hind*III/*Kpn*I-restricted Challis Tn1 genomic DNA was shotgun-cloned into pBluescript II SK+ (Stratagene) and ligated with T4 DNA polymerase (Gibco-BRL) at 14°C overnight. PCR primers based on the published sequences of both the vector (5'-GGTGCCATAGGTGTGAATCA-3'; SK primer) and Tn916 (5'-AGAGAAGCAACAGGAGCGTC-3'; Tn916L) were used to amplify the ligation reaction for the rapid identification of the disrupted nucleic acid sequence. After initial denaturation for 3 min at 94°C , 30 cycles were performed as follows: 1 min at 94°C , 2 min at 55°C and 3 min at 72°C . This was followed by 10 min at 72°C for completion of the extension reaction. Partial sequencing of the PCR product was performed by the Nucleic Acid Sequencing Facility at the State University of New York at Buffalo. To eliminate vector sequences, a new primer was synthesized and designated PCRf (5'-ATCCGTTTGGTCCCATAGGT-3') which was used to generate a PCR product from Challis Tn1 DNA using the identical thermocycler program described above. The resulting PCR product contained a portion of Tn916 and streptococcal DNA. This product was subcloned into pCR2.1 (Invitrogen) and the newly constructed plasmid designated pCR2KB-7.

Analysis of the cloned streptococcal DNA fragment. To confirm the insertion of the PCR-amplified fragment into pCR2KB-7, PCR was performed using pCR2KB-7 as the template DNA and PCRf and Tn916L as the reaction primers. Challis Tn1 genomic DNA served as the positive control and pCR2.1 as the negative control. The reaction conditions were identical to those described above. The PCR product from Challis Tn1 was subsequently biotinylated and used to probe Southern blots containing Challis and Challis Tn1 genomic DNA, the PCR amplification product that was subcloned into pCR2.1 and the vector (pCR2.1) alone. Southern blotting conditions were as described above.

Nucleotide sequencing and analysis. Single-stranded DNA was prepared using the M13K07 helper phage (Promega) according to the method of Yanisch-Perron *et al.* (1985). Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 DNA polymerase, [^{32}S]dATP αS and Sequenase kit reagents (United States Biochemical). Sequence data, which were confirmed by the Nucleic Acid Sequencing Facility at the

State University of New York at Buffalo, were analysed with HIBIO DNASIS (Hitachi Software Engineering) and Statistical Analysis of Protein Sequences (SAPS) (Brendel *et al.*, 1992) software. Nucleotide and deduced amino acid sequences were compared with previously published genes using BLAST (Altschul *et al.*, 1990) and PROSITE (Bairoch *et al.*, 1996) software.

RESULTS

Generation of a mutant of *S. gordonii* Challis deficient in amylase-binding

The naturally competent *S. gordonii* strain Challis (LeBlanc & Hassell, 1976) was mutagenized with the conjugative transposon Tn916. A transformation rate of approximately 10^{-4} was observed and 6000 colonies were initially screened for the non-amylase-binding phenotype using a replica-plating method (Tseng *et al.*, 1992). The initial screening identified 30 colonies that appeared to be amylase-binding deficient (AB^-), which were subcultured and rescreened using the liquid-phase [^{125}I]-labelled amylase binding assay. This screening yielded a single AB^- mutant strain (Challis Tn1). The binding of amylase to Challis Tn1 was found to be significantly less than that of the wild-type and nearly identical to that observed with *S. sanguis* 10556, a strain known not to bind amylase (Fig. 1). The colony morphology of Challis Tn1 and the wild-type was indistinguishable on both blood and mitis salivarius agar. In addition, no metabolic differences were noted when the mutant and wild-type strains were compared using the API Rapid Strep system, and the competence of Challis Tn1 was not affected as demonstrated by successful transformation with pVA856.

The stability of Challis Tn1 in the absence of tetracycline was assessed. The proportion of tetracycline-resistant colonies observed in cultures in the absence of tetracycline diminished with each subsequent passage so that after five passages only 50% of the total c.f.u. were tetracycline resistant.

Fig. 1. Binding of [^{125}I]-labelled amylase to *S. gordonii* Challis (▲), Challis Tn1 (△) and *S. sanguis* 10556 (○). Data points represent the mean value of duplicate samples.

Table 1. Relationship between growth rate and amylase-binding ability in *S. gordonii*

The values are the means of duplicate determinations, which were within 10% of each other.

Generation time (h)	Amount of amylase bound (U ml ⁻¹)	Percentage of amylase activity removed from saliva
2.6	38.6	32.1
4.6	22.2	28.0
6.4	20.6	25.2
14.5	18.2	20.0

Determination of Tn916 insertion number and genetic linkage studies

Southern blots of *Hind*III digests of wild-type and Challis Tn1 genomic DNA were probed with the biotinylated pAM620, which contains Tn916. Since Tn916 has a single *Hind*III restriction site, digestion with this enzyme would yield two fragments per insert following digestion with *Hind*III. Only a single insertion of Tn916 was noted in Challis Tn1 as demonstrated by the presence of only two hybridization bands following digestion with *Hind*III. The 15 kb and 8.2 kb bands associated with the Challis Tn1 genomic DNA were absent in the wild-type genomic DNA, and the pAM620 probe did not hybridize with *S. gordonii* wild-type DNA, suggesting there were no significant homologies between the plasmid vector, transposon and wild-type chromosomal DNA.

Linkage of the Tn916 insertion with the loss of amylase binding was evaluated by transformation of wild-type *S. gordonii* Challis with Challis Tn1 genomic DNA. Following selection on tetracycline-containing plates, 25 transformants were randomly selected and evaluated for amylase binding. None of these secondary transformants bound amylase at levels greater than that seen for *S. sanguis* 10556, the negative control. In addition, the hybridization patterns observed for the original mutant and three randomly selected transformants were identical. Together, these data demonstrate the genetic linkage between the AB⁻ phenotype and the insertion of Tn916 in Challis Tn1.

Kinetics of the release of amylase-binding protein into culture supernatants

To determine the effect of growth rate on amylase-binding ability, *S. gordonii* Challis was grown in continuous culture at four different growth rates (Table 1). As the mean generation time decreased (i.e. the faster the growth rate), the more amylase the bacteria were able to bind. At the fastest growth rate studied (mean generation time 2.6 h; dilution rate 0.264 ml⁻¹ h⁻¹ l⁻¹), the amount of amylase bound per 4.2 × 10⁸ cells was more than double the level bound by cells grown at the slowest growth rate (14.5 h; 0.048 ml⁻¹ h⁻¹ l⁻¹). The

mean amount of amylase bound by cells grown in BHI batch culture was 57 U ml⁻¹, which was 47% more than the amount bound by cells grown at the fastest growth rate studied in the chemostat.

When the growth of wild-type *S. gordonii* Challis was compared to that of the AB⁻ mutant Challis Tn1 in the absence of tetracycline, the growth curves were nearly identical at all time points, demonstrating that normal growth was not significantly disrupted by the insertion of Tn916 into the chromosome. When tetracycline was included in the growth medium of Challis Tn1, the lag phase of the mutant was prolonged by approximately 1 h, although the duration of the exponential phase remained unchanged.

The appearance of amylase-binding proteins in the culture supernatant at various times during the growth cycle was also determined by the amylase ligand-binding assay. When standardized supernatant concentrates were analysed in the amylase ligand-binding assay (Fig. 2a) both the 20 kDa and 82 kDa components were found to be present in stationary-phase culture supernatants of wild-type *S. gordonii* G9B and Challis. The 20 kDa component was also detected at 1 h and continued to increase in concentration during the next 12 h, reaching a plateau during the stationary phase. In control experiments, the 20 kDa and 82 kDa components were not detected when amylase was omitted from the blocking step of the amylase ligand-binding assay (Fig. 2b). Similar blots probed with the anti-20 kDa antibody clearly demonstrated that the 20 kDa component was produced by the wild-type strain, but was absent from the mutant strain (Fig. 2c).

The 20 kDa component was not observed to be present on the cell surface or in the culture supernatant of the mutant strain Challis Tn1 at any time during the growth cycle. However, the mutant strain cultured with or without tetracycline always expressed the 82 kDa component, which, like the wild-type strains, was not detected until the late-exponential phase of growth. When surface extracts and culture supernatants of Challis Tn1 were evaluated by silver-stained SDS-PAGE and immunoblotting using anti-G9B antibody, only the 20 kDa component appeared to be absent from the mutant (Fig. 3) and no additional stained components were detected.

To further characterize the relationship between the 20 kDa and 82 kDa components, supernatant samples were prepared by incubation at 37 °C, at 100 °C for 90 s with or without β-mercaptoethanol and at 100 °C for 30 min in SDS-PAGE sample buffer. When gels were run under denaturing conditions the apparent molecular masses of both the 20 kDa and the 82 kDa components were not altered by any of the sample conditions employed, as determined by the amylase ligand-binding assay.

Isolation of the amylase-binding components

Hydroxyapatite chromatography was performed to isolate the 20 kDa component from BHI-cultured cells

Fig. 2. Expression of amylase-binding proteins in stationary-phase supernatants applied to 20% Giulian gels in the amylase ligand-binding assay and Western blot. (a) Amylase ligand-binding assay (blot probed with amylase followed by anti-amylase antibody); (b) ligand-binding assay without amylase, probed with anti-amylase antibody; (c) blot probed with anti-20 kDa antibody. Lane 1, *S. gordonii* Challis wild-type; lane 2, Challis Tn1; lane 3, *S. gordonii* G9B; lane 4, purified amylase (1.5 µg). Molecular mass markers are indicated in kDa.

Fig. 3. Immunoblot (12.5% Laemmli gel) of stationary-phase cell pellets and supernatants from *S. gordonii* Challis wild-type strain and the AB⁻ mutant Challis Tn1 probed with antibody to *S. gordonii* G9B whole cells. Lane 1, *S. gordonii* Challis wild-type cell pellet; lane 2, wild-type supernatant; lane 3, Challis Tn1 cell pellet; lane 4, Challis Tn1 supernatant. Molecular mass markers are indicated in kDa.

of *S. gordonii* Challis. Fractions containing amylase-binding activity, as determined by the amylase ligand-binding assay, were eluted from the hydroxyapatite matrix with 300–400 mM potassium phosphate buffer and had as their major constituent the 20 kDa component. The N-terminal sequence of the 20 kDa amylase-binding protein was determined on directly blotted material and no other contaminating proteins in that region of the blot were noted. The amino acid sequence obtained was found to be DEPTDAATNND. The P at position 3 was equivocal and might be identified as A, and the T at position 8 was equivocal and might be identified as R.

Trypsin sensitivity of the 20 kDa amylase-binding component

Cell pellets from *S. gordonii* G9B were digested with trypsin to provide further evidence of the protein nature of this component. A time-course study demonstrated

that the 20 kDa protein disappeared by 30 min with the concomitant appearance of 10 kDa and 7.7 kDa fragments, which were reactive with the anti-20 kDa antibody (Fig. 4c). Similarly, the 20 kDa protein obtained from culture supernatant was initially digested into 12 kDa and 11 kDa fragments, with increasing amounts of a 7.6 kDa and other lower-molecular-mass fragments appearing as digestion progressed (Fig. 4b). Furthermore, when the trypsin-treated culture supernatants were tested in the amylase ligand-binding assay, the 20 kDa immunoreactive fragment was not apparent after digestion (Fig. 4a). However, no functional fragments smaller than 18 kDa were reliably detected by this assay. The same results were obtained when *S. gordonii* G9B was evaluated (data not shown).

Cloning and sequencing of the gene encoding the amylase-binding protein

Since previous attempts to clone the gene or genes encoding amylase-binding components proved problematical, a novel strategy was devised for cloning the disrupted gene from Challis Tn1 (Fig. 5). After ligation of the *Hind*III/*Kpn*I-restricted Challis Tn1 genomic DNA into pBluescript II SK⁺, the vector-specific primer (SK) and a primer for the left end of Tn916 (Tn916L) were used to amplify the ligation reaction. A 2 kb product was detected by electrophoresis and sequences from pBluescript II SK⁺, *S. gordonii* and Tn916 were identified by partial DNA sequencing. PCR amplification of the vector alone failed to produce a 2 kb product.

To remove plasmid DNA from the PCR product, PCR of genomic Challis Tn1 DNA was performed using the original Tn916L primer as well as PCR2, a primer based on sequence data from the initial PCR product described above, which was nested downstream from the insertion site in pBluescript II SK⁺. A 2 kb product generated by PCR was biotinylated and used as a probe for Southern blotting. In addition to binding as expected with Challis Tn1 DNA, the probe also hybridized with wild-type Challis genomic DNA and with itself. Importantly, it failed to hybridize with pBluescript II SK⁺ or PCR2.1,

Fig. 4. Time course of trypsin digestion of the 20 kDa protein from *S. gordonii* Challis. (a) Supernatant probed by amylase ligand-binding assay; (b) supernatant probed with anti-20 kDa antibody; (c) cell pellet probed with anti-20 kDa antibody. For (a) and (b): lane 1, 0 h, no trypsin; lane 2, 0 h, trypsin; lane 3, 5 min, trypsin; lane 4, 10 min, trypsin; lane 5, 30 min, trypsin; lane 6, 1 h, trypsin. For (c): lane 1, 0 h, no trypsin; lane 2, 30 min, trypsin; lane 3, 30 min, no trypsin. Molecular mass markers are indicated in kDa.

Fig. 5. Strategy for cloning the amylase-binding protein (*abpA*) gene of *S. gordonii* Challis.

the vector used for the subsequent subcloning of the PCR fragment, demonstrating that the plasmid vector sequences had been eliminated.

Subcloning of the amplified streptococcal DNA

The T/A cloning vector pCR2.1 was used for cloning the PCR product. The 2 kb PCR product (which represents the streptococcal sequence downstream from the Tn916 insertion site) was ligated into pCR2.1 and

the new plasmid construct was designated pCR2KB-7. When this construct was incubated with *Kpn*I, a fragment of 5.9 kb was obtained, thus confirming the presence of a 2 kb insert within the construct. When this construct was subjected to PCR using the PCRF and Tn916L primers, a fragment of 2 kb was obtained, while a similar reaction using the pCR2.1 vector alone yielded no product. When Challis Tn1 genomic DNA was PCR amplified using the same primers, a product of 2 kb was also obtained. These data suggest that the streptococcal sequence downstream from the Tn916 insertion site was successfully cloned.

Nucleotide sequencing and sequence analysis

A total of 1013 bases of the cloned streptococcal 2 kb PCR product were sequenced in both directions. A sequence corresponding to the 3' end of Tn916 was found at the 5' end of the cloned fragment. A potential open reading frame of 585 bp encoding a protein of 195 amino acid residues was noted 15 bp downstream from the junction of Tn916. This open reading frame encodes a protein with a predicted molecular mass of 20 505 Da. This closely approximates the molecular mass of the 20 kDa amylase-binding protein previously identified from *S. gordonii* Challis (Douglas, 1990; Scannapieco *et al.*, 1992; Gwynn & Douglas, 1994). This open reading frame was designated *abpA* (amylase-binding protein A). A potential Shine-Dalgarno sequence, AAGGAGA, was noted 7 bp upstream from the putative ATG start codon of *abpA*. This gene was terminated by a TAA codon, with two stop codons noted in a stretch of 12 nucleotides and followed by seven thymidines. This

		9		18		27		36		45		54							
5'	ATG	AAA	AAA	GTT	TTA	TTG	TCA	AGC	GTG	GTA	GCA	CTT	ACA	TTA	TTT	GCA	GCT	GCA	
1	M	K	K	V	L	L	S	S	V	V	A	L	T	L	F	A	A	A	18
			63			72			81			90			99				
	GCA	CCA	GTT	TTC	TCT	GCT	GAT	GAA	GCT	ACT	GAT	GCA	GCT	CGT	AAC	AAC			3'
	A	P	V	F	S	A	D	E	A	T	D	A	A	R	N	N			35

Fig. 6. Partial nucleic acid and deduced amino acid sequence of the *abpA* gene. Amino acids in bold letters indicate the deduced signal peptide; underlined italicized amino acid sequence denotes homology with N-terminal amino acids of the purified 20 kDa protein.

motif is typical of previously reported *E. coli* and streptococcal transcription terminators (Suvorov *et al.*, 1997). The overall G + C content of *abpA* was 38 mol %, which is within the range noted for streptococcal genes (34–46 mol %). Based on this sequence, it is clear that Tn916 inserted immediately upstream of the *abpA* gene to inhibit the expression of the AbpA protein at the level of transcription.

Analysis of the deduced amino acid sequence revealed that the first 24 amino acids of the putative protein are consistent with a hydrophobic signal peptide characteristic of secreted proteins (Fig. 6). This signal peptide was followed by a region of 11 amino acids, representing the N-terminal region of the secreted protein, which shared identity (at least 9 and probably all 11 residues) with the N-terminal sequence data obtained for the 20 kDa amylase-binding protein purified from *S. gordonii* Challis as described above.

No potential membrane-spanning or lipoprotein [L(S/A)(A/G)C(S/G) (Sutcliffe & Russell, 1995)] consensus sequences were noted. However, a potential cell-wall anchor hexapeptide motif compatible with those of other Gram-positive surface proteins may be present within the C-terminal region of AbpA [LPKTS; residues 188–193 (Schneewind *et al.*, 1995)]. Further analysis of the deduced amino acid sequence by BLAST searching revealed no significant homology with any known proteins produced by other bacteria.

As described above, partial digestion of AbpA with trypsin yielded fragments of 12, 11, 10 and 7.7 kDa. Inspection of the predicted sequence of AbpA revealed potential tryptic fragments as follows. Digestion at residue 92 (lysine) would yield major fragments of 7.7 and 11 kDa. Digestion at residue 107 (arginine) would yield a fragment of 10 and 7.7 kDa, while digestion at residue 75 (arginine) would yield a 12 kDa fragment.

DISCUSSION

The results of the present study confirm the presence of 20 kDa and 82 kDa amylase-binding components on the surface of *S. gordonii* (Douglas, 1990; Scannapieco *et al.*, 1992; Gwynn, 1995). These results also extend previous studies by establishing that the 20 kDa component is essential for the binding of amylase to the cell surface. While both *S. gordonii* Challis and G9B produced amylase-binding components of similar apparent molecular mass (20 and 82 kDa), the AB⁻ mutant Challis Tn1 produced the 82 kDa but not the 20 kDa protein.

A number of observations suggest that the 20 and 82 kDa components are structurally distinct. Present and previous studies revealed that while the 20 kDa protein was released into the medium from the lag phase through the stationary phase of growth, the 82 kDa protein was released only during the late-exponential and stationary phases (Gwynn & Douglas, 1994). Growth of *S. gordonii* Challis in the presence of a variety of protease inhibitors such as EDTA, PMSF, SDS and trypsin inhibitor failed to prevent the appearance of the low-molecular-mass component (Gwynn & Douglas, 1994). When applied to denaturing SDS-PAGE, neither the 20 kDa nor the 82 kDa component was heat modifiable and both proteins appeared to lack intermolecular disulfide linkages, suggesting that each component was monomeric. Subsequent nucleic acid sequence analysis of the cloned gene revealed no cysteine residues within this protein, thus supporting these observations. Furthermore, while the ability of the 82 kDa protein to bind amylase in the amylase ligand-binding assay was abolished by periodate treatment, binding of the 20 kDa protein was unaffected, suggesting the involvement of carbohydrate in the binding of amylase to the 82 kDa component but not to the 20 kDa component (Gwynn & Douglas, 1994). Amylase binding to the 82 kDa protein was prevented by the presence of the amylase substrate analogue acarbose, whereas the binding of amylase to the 20 kDa protein was unaffected, suggesting different sites of attachment on the amylase molecule (Gwynn & Douglas, 1994). Antibody raised to the 20 kDa protein did not appreciably react with the 82 kDa component. Finally and most notably, the 20 kDa protein, while present in the wild-type strain, was found to be completely absent from an amylase-binding-deficient mutant of *S. gordonii* Challis. Indeed, the only detectable difference in protein profiles between stationary phase wild-type and mutant strains was the absence of the 20 kDa protein from Challis Tn1. No significant difference was noted in the generation time between the wild-type and mutant strains once exponential phase was reached, indicating that lack of the 20 kDa protein did not affect growth.

It has been suggested that the 20 kDa component is a protein, mostly supported by the observation that amylase-binding to these bacteria is inhibited by treatment of the cells with proteases. The present trypsin digestion studies revealed that the 20 kDa component is indeed degraded into components of 7.6–12 kDa, providing further evidence for the protein nature of this component. The distribution of the amylase-binding components on the cell surface of *S. gordonii* is confined

to the cell division septa on dividing cells or to polar zones on single cells (Scannapieco *et al.*, 1992). As these regions of the cell are the proposed sites for formation of new cell wall material (Higgins & Shockman, 1970), it has been suggested that the 20 kDa protein is found only on nascent cell wall and is then released into the culture medium. Implicit in such a concept is that cells not in the process of dividing will probably be unable to bind amylase or will have a reduced binding capacity. This is supported by results of the present study which demonstrate that cells growing and dividing more rapidly in a chemostat bound more amylase than cells growing slowly in the same medium. It is presently not known if slow-growing cells express lower levels of AbpA on the cell surface, or if this protein is masked on these cells. The finding of a potential cell-wall-anchoring motif at the C-terminus of AbpA (Schneewind *et al.*, 1995) is consistent with experimental evidence that amylase-binding to intact cells and cell walls can be abolished by trypsin. The presence of the molecule in culture supernatants may be due to physiological turnover and release of peptidoglycan fragments with linked proteins into the culture medium. However, the AbpA sequence differs from other anchors in that it lacks the 23 C-terminal hydrophobic amino acids thought to be typical of cell-wall proteins.

The role of the 82 kDa protein in amylase binding is presently unclear. This component may function as part of a heterotypic amylase receptor; the 20 kDa protein may influence the conformation of the 82 kDa protein. On the intact cell, the 82 kDa protein may not present a functional conformation in the absence of the 20 kDa protein. Thus, in this model, the 20 kDa protein would be essential for amylase binding to intact cells. Determination of the function of the 82 kDa protein awaits the construction of a mutant deficient in the 82 kDa protein. Of course, the possibility also exists that the 82 kDa protein fortuitously binds amylase in the *in vitro* ligand-binding studies but does not actually contribute to amylase binding to intact cells.

As the available evidence suggested that amylase binding to *S. gordonii* is probably mediated by a surface protein(s), initial attempts were made to clone the gene(s) for the amylase-binding protein(s) from this organism, but these were unsuccessful. For example, genomic libraries of *S. gordonii* Challis DNA prepared in the vectors λ gt11 and λ GEM-11 were screened with antibody prepared against the amylase-binding 20 kDa component isolated from SDS-PAGE gels (Scannapieco *et al.*, 1992), as well as with radioiodinated amylase. Both libraries contained abundant recombinant clones expressing antigens cross-reactive with antibody prepared against whole cells of *S. gordonii* G9B. However, of over 100 000 recombinants screened, no positive clones expressing antigen or functional receptor activity were obtained.

Alternative approaches were therefore sought to identify genes involved in amylase binding to these streptococci. Insertional gene inactivation using the conjugative transposon Tn916 resulted in the isolation of a single

mutant exhibiting a non-amylase-binding phenotype. Only a single insertion of Tn916 was observed in transformants of *S. gordonii* Challis as opposed to the multiple inserts previously reported (Gawron-Burke & Clewell, 1984). This is significant in that multiple transposon insertions would make analysis of the transformant much more difficult. Genetic linkage between the insertional event and the loss of amylase-binding activity established that the observed phenotype was probably not the result of a spontaneous mutation but was due to the insertion of Tn916 into the bacterial chromosome just upstream from the *abpA* gene. Since the inverted repeat regions of Tn916 direct its insertion into homologous regions of the bacterial chromosome that are A/T rich, Tn916 appears to have inserted into such regions upstream from *abpA*. The relatively large size of Tn916 and the lack of known promoters at its 3' end that could mediate transcription of *abpA* by readthrough would thus prevent transcription of *abpA* as either a monocistronic or polycistronic message. The inability to detect AbpA by immunoblot or ligand-binding assays lends support to such a hypothesis.

The use of the single-specific-primer PCR amplification technique (Shyamala & Ames, 1989; Novak *et al.*, 1997) proved to be a simple and rapid method for obtaining DNA flanking the Tn916 insertion site. Due to the relatively large size of Tn916, analysis of the surrounding DNA often requires a number of manipulations that greatly increase the complexity of the cloning process (Caparon & Scott, 1987; Caufield *et al.*, 1990). PCR screening of the ligation reaction was very efficient, obviating the need for the more labour-intensive construction and screening of genomic or cDNA libraries. Novak *et al.* (1997) used this technique to amplify fragments of up to 3.2 kb and predicted amplicons of up to 35 kb may be amplified using long PCR methodologies. The application of this technique, as demonstrated by this study, should prove very useful for cloning nucleotide sequences flanking known regions.

Concluding remarks

In summary, a mutant unable to bind amylase was selected following transposon mutagenesis. This mutant was unable to produce a 20 kDa protein found previously to bind amylase. The gene encoding this protein was subsequently cloned and sequenced; sequence analysis indicated that it encoded a unique secreted, cell-associated protein. These results demonstrate the importance of the 20 kDa protein in the binding of amylase to *S. gordonii*. Future studies will focus on the functional characterization of mutants deficient in amylase-binding in models of oral adhesion and colonization.

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