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Identification and characterization of amylase-binding protein C from *Streptococcus mitis* NS51

J. Vorrasi, B. Chaudhuri, E.M. Haase and F.A. Scannapieco

Department of Oral Biology, 109 Foster Hall, School of Dental Medicine, State University of New York at Buffalo, Buffalo, NY, USA

Correspondence: Frank A. Scannapieco, Department of Oral Biology, 109 Foster Hall, School of Dental Medicine, State University of New York at Buffalo, Buffalo, NY 14214, USA Tel.: + 1 716 829 3056; fax: + 1 716 829 3942; E-mail: fas1@buffalo.edu

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SUMMARY

A substantial proportion of the streptococcal species found in dental plaque biofilms are able to interact with the abundant salivary enzyme α-amylase. These streptococci produce proteins that specifically bind amylase. An important plaque species, *Streptococcus mitis*, secretes a 36-kDa amylase-binding protein into the extracellular milieu. Proteins precipitated from *S. mitis* NS51 cell culture supernatant by the addition of purified salivary amylase were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a membrane, and a prominent 36-kDa band was cut from the membrane and sequenced to yield the N-terminal amino acid sequence DSQAQYSNGV. Searching the *S. mitis* genome sequence database revealed a single open reading frame containing this sequence, and the gene was amplified by the *S. mitis* genomic DNA polymerase chain reaction. The coding region of this open reading frame, designated amylase-binding protein C (AbpC), was cloned into an *Escherichia coli* expression vector and the recombinant AbpC (rAbpC) was purified from the soluble fraction of the *E. coli* cell lysate. Purified AbpC was found to interact with immobilized amylase, confirming AbpC as a new streptococcal amylase-binding protein.

INTRODUCTION

Interactions between salivary components and oral bacteria are thought to play an important role in the ecology of oral biofilms (Douglas, 1994; Scannapieco, 1994; Rudney, 2000). Amylase, the most abundant enzyme in human saliva, specifically binds to several species of oral streptococci (Douglas, 1983; Scannapieco *et al.*, 1989; Douglas *et al.*, 1990; Kilian & Nyvad, 1990). One or more bacterial receptors mediate the binding of amylase to the streptococcal surface (Douglas, 1990; Scannapieco *et al.*, 1992). Much of our current knowledge about the mechanism of interaction of amylase with oral bacteria derives from the study of two amylase-binding proteins (AbpA and AbpB) produced by *Streptococcus gordonii* (Rogers *et al.*, 2001; Li *et al.*, 2002; Chaudhuri *et al.*, 2007). Both of these proteins appear to be expressed transiently on the cell surface before being released into the extracellular milieu in soluble form. AbpA is a 20-kDa protein that is unique to *S. gordonii*, and is essential for amylase binding to the cell surface (Rogers *et al.*, 2001). AbpB, an 82-kDa protein, shares sequence homology with other bacterial dipeptidases and appears to play a crucial role in *S. gordonii* oral colonization (Tanzer *et al.*, 2003; Chaudhuri *et al.*, 2008). To date, however, little is known about the amylase-binding proteins of other species of oral streptococci.

Streptococcus mitis NS51 releases a 36-kDa amylase-binding protein into the culture medium during growth (Gwynn & Douglas, 1994). The goal of this study was to identify the gene encoding this protein, to express and purify the polypeptide, and to verify its function *in vitro*. This information advances knowledge of the structure and functions of streptococcal amylase-binding proteins.

METHODS

Bacterial strains and growth conditions

Streptococcus mitis NS51 was cultivated from frozen stocks on tryptic soy blood agar and incubated at 37°C for 16 h in a candle jar. For routine experiments the bacteria were cultured in tryptic soy broth (Difco, Detroit, MI) containing 0.5% yeast extract (TSBY). *Escherichia coli* was grown in Luria–Bertani broth with constant shaking at 37°C and maintained on Luria–Bertani agar.

Precipitation of amylase-binding protein from *S. mitis* culture supernatants

Cell-free supernatant was collected from overnight cultures by centrifugation at 5000 ***g*** for 10 min at room temperature, followed by filtration through a 0.4-μm filter (Corning Inc, Corning, NY). Precipitation of amylase-binding proteins was induced by the addition of 50 μg/ml of purified human salivary amylase (A1031 Type XIII-A; Sigma Aldrich, St Louis, MO) to the culture supernatant (Li *et al.*, 2002; Chaudhuri *et al.*, 2007). After incubation at room temperature for 2 h, the resulting precipitate was recovered by centrifugation at 5000 ***g*** for 10 min, suspended in sample buffer [0.06 M Tris–HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.05% 2-mercaptoethanol, and 0.00125% bromophenol blue], boiled for 3 min, and resolved by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Amylase ligand-binding assay

The amylase ligand-binding assay was performed as previously described (Gwynn & Douglas, 1994; Rogers *et al.*, 2001). Briefly, proteins separated by SDS–PAGE were electrotransferred onto Immobilon-P membrane (Millipore, Bedford, MA). After washing and blocking with 5% non-fat milk in 10 mM Tris-buffered saline containing 0.05% Tween 20 (TBST), the membrane was incubated with 1% purified amylase (Sigma) in TBST for 1 h. Following washing in TBST, the membrane was incubated with polyclonal anti-human α-amylase antibody (used at a dilution of 1/500) (Sigma) in TBST, washed in TBST, and then incubated with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega, Madison, WI). Finally, the blot was developed using the ProtoBlot Western Blot AP system (Promega).

Protein sequencing

Resolved proteins were electrotransferred from SDS–PAGE gel onto Immobilon-P membrane (Millipore). Proteins were stained with Coomassie brilliant blue for 1 min, destained in a 40% methanol : 10% acetic acid solution in water, and washed in water. Portions of the membrane containing the target protein band were excised and subjected to N-terminal sequencing [Proseq Inc., Boxford MA].

Genomic DNA isolation from *S. mitis* NS51

Chromosomal DNA from *S. mitis* was prepared according to a modification of a previously described method (Rogers *et al.*, 1998). Briefly, *S. mitis* cells were grown overnight in 5 ml TSBY containing 0.5% glycine. Following centrifugation, the cell pellet was suspended in 0.5 ml GET [50 mM glucose, 10 mM ethylenediamine tetraacetic acid (EDTA), 25 mM Tris–HCl, pH 7.5] buffer containing lysozyme (1 mg/ml), 50 U mutanolysin (Sigma), and was incubated at 37°C for 1 h. After lysis with 0.1 ml 20% SDS and 50 μl of 2% Sarkosyl (Sigma), the lysate was mixed with 0.15 ml 5 M sodium percholate. Following extraction of the lysate with phenol : chloroform (1 : 1), genomic DNA was precipitated from the top aqueous layer by adding an equal volume of isopropanol. The DNA precipitate was washed with 70% ethanol, dissolved in Tris–EDTA buffer and stored at −20°C.

Amplification of the *S. mitis* gene encoding amylase-binding protein C *(abpC)*

The target region was amplified by the polymerase chain reaction (PCR) from *S. mitis* NS51 genomic DNA using OBC38, forward primer (5′-GAA AAC

CCT TGA CAA ATC TTG C-3′) and OBC39, reverse primer (5′-AAA CTG CTG CAC TTG CCA TT-3′). A 1208-base-pair (bp) PCR product was purified from a 1% agarose gel and sequenced using an automatic DNA sequencer (ABI Prism, Foster City, CA). The expression vector, pET32Xa/LIC (Novagen, Madison, WI) was used to clone and express *abpC*. The DNA fragment comprising the open reading frame was amplified by PCR from the *S. mitis* genomic DNA template using OBC40, forward primer (5′-GGT ATT GAG GGT CGC GAT TCA CAA GCA CAA TAC AGT AAT GGT -3′) and OBC41, reverse primer (5′-AGA GGA GAG TTA GAG CCA AAC TGC TGC ACT TGC CAT T-3′). A 1074-bp DNA fragment was gel purified, treated with T4 DNA polymerase and annealed with an isopropyl-d-thiogalactopyranoside (IPTG) inducible linear pET32-Xa/LIC expression vector (Novagen). Recombinant plasmid (p226-1) was introduced by chemical transformation into NovaBlue Singles™ Competent Cells (Novagen), and selected on Luria–Bertani agar supplemented with ampicillin (100 μg/ml). Plasmids containing inserts of correct size and orientation were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega), and the cloned region was verified by sequencing.

Expression of *abpC* in *E. coli*

The *abpC* plasmid p226-1 was transformed into BL21 (DE3) pLysS competent cells (Novagen) with selection for ampicillin (50 μg/ml) resistance. A positive transformant was then induced with IPTG for 2 h at 37°C with constant shaking. Induced cells were harvested by centrifugation, and suspended in 10 ml of EasyLyse™ Bacterial Protein extraction solution (5 mM Tris–HCl pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 0.1 mM $MgCl_2$ and 20 ml EasyLyse™ enzyme mix) (Epicentre, Madison, WI). The soluble supernatant was collected following centrifugation (10,000 ***g***) at room temperature, and total protein concentrations were estimated using BCA (bicinchoninic acid) reagent (Bio-Rad, Hercules, CA).

Purification of recombinant AbpC

Recombinant AbpC (rAbpC) was purified by using Ni^{2+}-charged His·bind cellulose cartridges (Novagen). The soluble supernatant containing rAbpC was loaded onto a cartridge equilibrated with binding buffer (0.5 M NaCl, 20 mM Tris–HCl, pH 7.9; 5 mM imidazole). The cartridge was washed twice with binding buffer and once with wash buffer (0.5 M NaCl, 20 mM Tris–HCl, 60 mM imidazole, pH 7.9). Finally, the rAbpC was recovered with elution buffer (0.5 M NaCl, 20 mM Tris–HCl, 1 M imidazole, pH 7.9). Eluted rAbpC was dialysed overnight against 50 mM Tris–HCl (pH 8) at 4°C and stored in aliquots at −20°C.

RESULTS

Previous studies have reported that amylase-binding proteins may be precipitated from *S. gordonii* culture supernatant by the addition of purified salivary amylase (Li *et al.*, 2002; Chaudhuri *et al.*, 2007). We used this approach to precipitate amylase-binding proteins from *S. mitis* culture supernatant. Purified amylase was added to *S. mitis* cell-free culture supernatant at a concentration previously found to be optimal for precipitation of such proteins from *S. gordonii* culture supernatants (Chaudhuri *et al.*, 2007). SDS–PAGE analysis of the precipitate obtained from the culture supernatant showed the presence of amylase along with a protein with molecular mass about 36 kDa (Fig. 1A, lane 3). This protein was designated amylase-binding protein C (AbpC). A ligand-binding Western-blot assay using salivary amylase as a probe confirmed that AbpC from both the supernatant and precipitate retained amylase-binding activity (Fig. 1B, lanes 2 and 3). N-terminal amino acid (aa) sequencing of this protein identified 10 aa residues as: DSQAQYSNGV.

The initial BLAST search using the 10 N-terminal aa sequence against the *S. mitis* NCTC 12261 genome sequence database available at The Institute of Genome Research (TIGR) web site (http://www.tigr.org) revealed an open reading frame designated SMT1193 (co-ordinates: 1188145–1187990) encoding a polypeptide with 100% part sequence homology. However, while the estimated size of the gene encoding AbpC (36 kDa) was about 981 bp, the size of SMT1193 was 155 bp. The DNA fragment found to encode the complete coding sequence for *abpC* was amplified by PCR from *S. mitis* genomic DNA using OBC38 (forward primer) and OBC39 (reverse primer). The OBC38 and OBC39 primers were designed using the sequences 100 bp upstream and

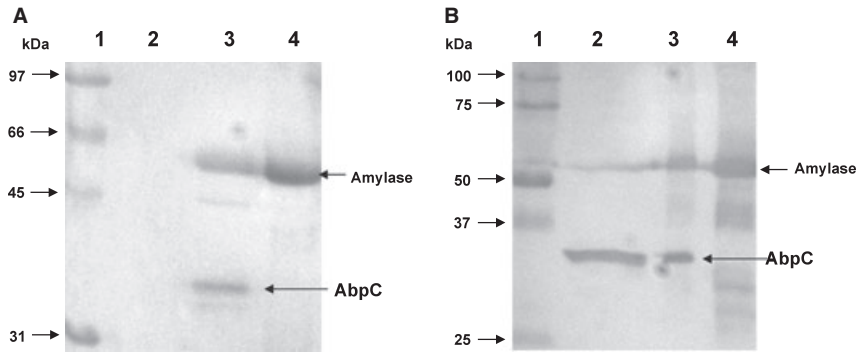

Figure 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of amylase precipitate. (A) Coomassie-blue-stained 12% SDS–PAGE gel. Lane 1, molecular mass standards in kDa; lane 2, 18 h cell-free culture supernatant of *Streptococcus mitis* NS51; lane 3, amylase precipitate from culture supernatant of NS51; and lane 4, purified salivary amylase (2 μg). (B) Amylase ligand binding assay. Lane 1, molecular mass standards in kDa; lane 2, cell-free culture supernatant; lane 3, precipitate from 1 ml supernatant; and lane 4, 0.2 μg purified amylase.

1100 bp downstream, respectively, of the start codon of SMT1193. Nucleotide sequencing of the DNA fragment (1208 bp) obtained from PCR revealed a putative 879-bp open reading frame that may encode AbpC (GenBank Accession number: EF989012.1). The overall G + C content of *abpC* was 40 mol %, which is within the range noted for streptococcal genes (34–46 mol %).

To obtain a DNA fragment more closely comprising only the putative open reading frame for the 36-kDa AbpC, primers OBC40 (forward primer) and OBC41(reverse primer) were used in PCR to obtain a 1074-bp DNA fragment, the identity of which was confirmed by nucleotide sequencing. The DNA fragment was cloned in-frame into an IPTG-inducible pET32Xa/LIC expression vector (not shown). SDS–PAGE analysis detected the presence of rAbpC in the soluble fraction of cell lysate (Fig. 2A). The rAbpC was purified from the soluble fraction and SDS–PAGE of the purified material stained with Coomassie blue showed a

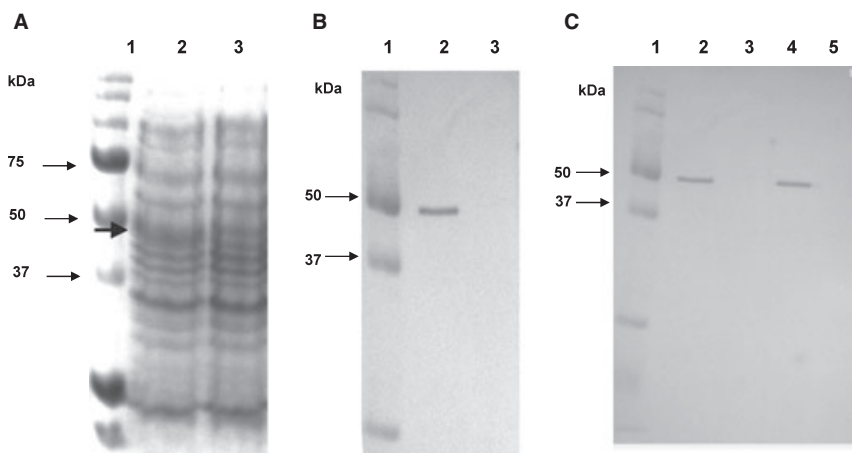

Figure 2 Expression and purification of recombinant amylase-binding protein C (rAbpC). (A) Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel of total cell lysate showing expression of rAbpC (arrow) in *Escherichia coli*. Lane 1, molecular mass standards in kDa; lanes 2 and 3, isopropyl-d-thiogalactopyranoside (IPTG)-induced or non-induced *E. coli* cell lysates, respectively. (B) Coomassie-stained SDS–PAGE gel of purified rAbpC. Lane 1, molecular mass standards in kDa; lane 2, purified rAbp (2 mg) from IPTG-induced *E. coli*; and lane 3, sample from non-induced culture as control. (C) Amylase-ligand binding assay of rAbpC. Lane 1, molecular mass standards in kDa; lanes 2 and 3, purified AbpC from induced or non-induced cells, respectively; lanes 4 and 5, purified AbpC from induced or non-induced cells, respectively.

single band of approximately 46 kDa (Fig. 2B), consistent with the calculated mass of AbpC (36 kDa) plus the 10 kDa N-terminal His tag from pET32Xa/LIC. A ligand binding Western blot assay of purified rAbpC using salivary amylase as a probe confirmed that rAbpC retained amylase-binding activity (Fig. 2C, lanes 2 and 4). The complete nucleotide and inferred aa sequences of *abpC* and AbpC from *S. mitis* NS51 can be found in GenBank, Accession number EF989012.1.

Further sequence analysis of *abpC* was performed on the annotated Oral Pathogen Database (ORALGEN) (http://www.oralgen.lanl.gov) using the draft genome of *S. mitis* NCTC 12261 obtained from TIGR. Not only did the nucleotide sequence near the N terminus of the translated protein AbpC have nearly 100% homology with gene SMT1193 (coordinates: 1118145–1187790), a large portion of the C-terminal region had nearly 97% homology with the immediate downstream gene SMT1192 (1187870–1187241). In the database sequence there is an intergenic region IGR996 (1187871–1187989) between SMT1193 and SMT1192. Only 46% of nucleotides in the corresponding region from *abpC* were identical to IGR996. Hence, the combined total (904 bp) of SM1193 (156 bp), IGR996 (118 bp), and SM1192 (630 bp) closely approximates to *abpC* (879 bp) (Fig. 3).

Analysis of the deduced aa sequence revealed that the first 31 aa residues of AbpC are consistent with a hydrophobic signal peptide characteristic of secreted proteins (Fig. 4). This signal peptide was followed by 10 aa residues, representing the N-terminal region of secreted AbpC, which shared 100% identity with the N-terminal sequence obtained for native AbpC purified from *S. mitis* culture supernatant. While analysis of the deduced aa sequence did not show the presence of any lipoprotein [L(S/A)(A/G)C(S/G)] consensus sequence [L(S/A)(A/G)C(S/G)] (Sutcliffe & Russell, 1995; Navarre & Schneewind, 1999), a membrane-spanning domain of 15 aa residues (position 13–27) was noted within the N-terminal region of AbpC (http://us.expasy.org; 'DAS'-Transmembrane Prediction Server). Further, aa sequence analysis using Prosite software from the Swiss Institute of Bioinformatics revealed that the aa sequence starting at position 210 through 285 contains four cell-wall-binding repeats (CW), previously found in two bacterial gram-positive protein families: choline-binding proteins and glucosyltransferases (Shah *et al.*, 2004).

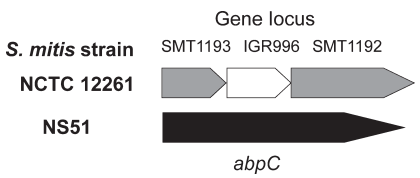

Figure 3 Representation of *abpC* gene in *Streptococcus mitis* NS51 compared with *S. mitis* NCTC12261.

DISCUSSION

In this study we identified a gene that encodes a new amylase-binding protein in *S. mitis*, which we have designated AbpC. The protein is composed of 292 aa residues, with a hydrophobic signal peptide comprising the first 31 N-terminal aa residues. Native AbpC secreted into the culture medium had a molecular mass of 36 kDa (Gwynn & Douglas, 1994). However, molecular weight calculation of the deduced aa sequence of AbpC suggests a value of 32 305. The observed molecular mass of AbpC (36 kDa) could be the result of post-translational modification or simply of aberrant migration in electrophoresis.

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5′ ATG AAA AAT GAA GGA GAT AAA ACG ATG AAA AAA GTT TTA TTG ACA AGC
   M   K   N   E   G   D   K   T   M   K   K   V   L   L   T   S

          54                  63                  72                  81
   GCA GTC GCT CTT GCA GCA TTT GGT GCT GTA CAA GCA GTT TCA GCT GAT
   A   V   A   L   A   A   F   G   A   V   Q   A   V   S   A   D

      99                  108                 117                 126                 135
   TCA CAA GCA CAA TAC AGT AAT GGT GTA GTA CCA GAG GCT AAC CAA TAT 3′
   S   Q   A   Q   Y   S   N   G   V   V   P   E   A   N   Q   Y
```

Figure 4 Partial nucleic acid and deduced amino acid (aa) sequence of the *abp*C gene from *Streptococcus mitis*. The aa residues in bold letters indicate the deduced signal peptide; underlined aa sequence denotes homology with N-terminal aa residues of the purified 36-kDa amylase-binding protein C (AbpC).

Repeated attempts to construct a *S. mitis* mutant deficient in AbpC have been unsuccessful because we have been unable to naturally transform or electrotransform *S. mitis* NS51. For the expression of AbpC, a pET vector system was chosen based on its incorporation of an IPTG-inducible promoter. rAbpC had a molecular mass of 46 kDa, in accordance with the theoretical mass prediction of AbpC, plus the N-terminal tag from the pET32Xa/LIC vector. Sequence comparison analysis using Prosite software (http://us.expasy.org) revealed the presence of CW repeats within the AbpC sequence. The CW repeat is a approximately 20 aa residue module found in choline-binding proteins and glucosyltransferases of gram-positive bacteria (Shah *et al.*, 2004). However, the relevance of such CW repeats in AbpC in the context of *S. mitis* colonization of the oral environment awaits further study.

Several species of oral streptococci are known to bind amylase (Douglas, 1983; Scannapieco *et al.*, 1989). Together, the streptococci producing amylase-binding proteins comprise a sizable proportion of the dental plaque biofilm microflora (Scannapieco *et al.*, 1994). It is curious that the amylase-binding proteins seem widely divergent in size and number between species (Gwynn & Douglas, 1994). What little sequence data that are available suggest that these proteins are very dissimilar between species. For example, AbpA, a 20-kDa amylase-binding protein from *S. gordonii*, appears to have no homology with any previously reported proteins. Likewise, no significant similarity was found between *S. mitis* AbpC and any other bacterial protein accessible through BLAST searching, and no obvious sequence relationships were noted among AbpA, AbpB, and AbpC. In contrast, AbpB appears to be a dipeptidase with orthologous proteins distributed among many bacterial species (Chaudhuri *et al.*, 2008). To understand better the structural basis for amylase binding and the evolutionary relationships between these proteins, further research into the structures and functions of these and additional amylase-binding proteins is required.

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