A novel glucan-binding protein with lipase activity from the oral pathogen \textit{Streptococcus mutans}

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\textit{Streptococcus mutans} produces extracellular glucosyltransferases (GTFs) that synthesize glucans from sucrose. These glucans are important in determining the permeability properties and adhesiveness of dental plaque. GTFs and the GbpA glucan-binding protein are characterized by a binding domain containing a series of 33-amino-acid repeats, called ‘A’ repeats. The \textit{S. mutans} genome sequence was searched for ORFs containing ‘A’ repeats, and one novel gene, \textit{gbpD}, which appears to be unique to the mutans group of streptococci, was identified. The GbpD sequence revealed the presence of three ‘A’ repeats, in the middle of the protein, and a novel glucan-binding assay showed that GbpD binds to dextran with a \(K_D\) of 2–3 nM. Construction of truncated derivatives of GbpD confirmed that the ‘A’ repeat region was essential for binding. Furthermore, a \textit{gbpD} knockout mutant was modified in the extent of aggregation induced by polymers derived from sucrose. The N-terminus of GbpD has a signal sequence, followed by a region with no homologues in the public databases, while the C-terminus has homology to the \(\alpha/\beta\) hydrolase family (including lipases and carboxylesterases). GbpD contains the two regions typical of these enzymes: a GxSxG active site ‘lipase box’ and an ‘oxyanion hole’. GbpD released free fatty acids (FFAs) from a range of triglycerides in the presence of calcium, indicating a lipase activity. The glucan binding/lipase bifunctionality suggested the natural substrate for the enzyme may be a surface macromolecule consisting of carbohydrate linked to lipid. The \textit{gbpD} mutant was less hydrophobic than wild-type and pure recombinant GbpD reduced the hydrophobicity of \textit{S. mutans} and another plaque bacterium, \textit{Streptococcus sanguinis}. GbpD bound to and released FFA from lipoteichoic acid (LTA) of \textit{S. sanguinis}, but had no effect on LTA from \textit{S. mutans}. These results raise the intriguing possibility that GbpD may be involved in direct interspecies competition within the plaque biofilm.

INTRODUCTION

The ability of \textit{Streptococcus mutans} to bind to high-molecular-mass glucans synthesized from sucrose is recognized as an important determinant in the formation of dental plaque and tooth decay (Hamada & Slade, 1980; Loesche, 1986). These polymers are of importance in adhesive interactions in plaque, mediating the attachment of bacteria to teeth, and providing a stable matrix for the maintenance of the plaque biofilm (Colby et al., 1999) as well as acting as energy stores enabling the survival of bacteria in plaque (Colby et al., 1995b). Extracellular glucans also affect the permeability of materials through the plaque biofilm thereby affecting solute concentration and pH near the tooth (van Houte et al., 1989). Thus, the proteins that enable bacteria to bind glucans have attracted interest as potential targets for inhibition of the caries process.

The extracellular glucosyltransferase (GTF) enzymes that act upon sucrose to produce glucan have a modular structure with distinct catalytic and glucan-binding domains (GBDs) (Colby & Russell, 1997; Monchois et al., 1999). The C-terminal one-third of GTF has been identified as a GBD and has been shown to be responsible for the ability of GTF to bind dextran, by experiments in which GTF was truncated by protein engineering (Abo et al., 1991; Ferretti et al., 1987) and by proteolysis of GTF and capture of dextran-binding fragments (Mooser & Wong, 1988). The GBD consists of a number of closely related amino acid repeat units that were first demonstrated in GTF-I from a strain originally identified as \textit{Streptococcus sobrinus}, later reclassified as \textit{Streptococcus downei} (Ferretti et al., 1987; Whiley et al., 1988). GTF-I contains six ‘A’ repeats of 33 amino acids and two ‘B’ repeats each of 48 amino acids. ‘A’ repeats have since been identified in all known streptococcal GTF and in the related dextransucrases of...
Protein, GbpD. The discovery and analysis of a novel glucan-binding protein aims to enable a systematic search for novel Gbps using known techniques and the availability of the genome sequence. Recent advances in genomic and bioinformatics technologies and the availability of the genome sequence of *Streptococcus pneumoniae* have suggested that all longer repeats are derived from a basic 'YG' repeat unit (Giffard & Jacques, 1994). The 'A' repeats show similarity to repeats found in binding proteins in other organisms, notably the choline-binding autolysins and surface proteins of *Streptococcus pneumoniae* and the toxins A and B of *Clostridium difficile*, which are also characterized by conserved aromatic amino acids and glycines (Wren, 1991; Wren et al., 1991; Yother & Briles, 1992; Garcia et al., 1998; Janeček et al., 2000).

In addition to the GTF, oral streptococci possess several glucan-binding proteins (Gbps) of unknown enzymic activity (Banas & Vickerman, 2003; Matsumura et al., 2003). Three Gbps have been identified in *S. mutans*: GbpA (Banas et al., 1990; Russell, 1979a), GbpB (Smith et al., 1994) and GbpC (Sato et al., 1997). GbpA contains five repeats, similar to those of GTF-I, and deletion of the gbpA gene alters sucrose-dependent biofilm formation in vitro and results in increased caries in a rat model (Banas & Gilmore, 1994). The GbpB gene corresponds to 57 individual sucrase sequences and GbpA (corresponding to 57 individual repeats) were searched by the motif-searching programs MEME and MAST (Bailey & Elkan, 1994; Bailey & Gribskov, 1998, http://meme.sdsc.edu/meme/website/) to identify the consensus 'A' repeat sequence: WYYFDNNGYAVTGLQTINGQHLYFDANGVQVKG. The TBLASTN program (Altschul et al., 1990) was used to search the *S. mutans* UA159 genomic sequence data at http://www.genome.ou.edu/smutans.html to identify ORFs containing matches to the consensus 'A' repeat. ORFs were characterized with the aid of the Virulogenome website (http://www.vge.ac.uk) and analysed with the commercial packages DNASTar and OMIGA, as well as a range of web-based sequence analysis programs.

**Identification of the gbpD gene.** All available GTF and dextran-sucrase sequences and GbpA (corresponding to 57 individual repeats) were searched by the motif-searching programs MEME and MAST (Bailey & Elkan, 1994; Bailey & Gribskov, 1998, http://meme.sdsc.edu/meme/website/) to identify the consensus 'A' repeat sequence: WYYFDNNGYAVTGLQTINGQHLYFDANGVQVKG. The TBLASTN program (Altschul et al., 1990) was used to search the *S. mutans* UA159 genomic sequence data at http://www.genome.ou.edu/smutans.html to identify ORFs containing matches to the consensus 'A' repeat. ORFs were characterized with the aid of the Virulogenome website (http://www.vge.ac.uk) and analysed with the commercial packages DNASTar and OMIGA, as well as a range of web-based sequence analysis programs.

**Cloning, expression and purification of his-tagged GbpD protein.** A recombinant plasmid expressing GbpD, but excluding the signal sequence, was constructed by PCR cloning of the relevant DNA fragment into the vector pQE30 (Qiagen) such that the coding sequence of the GbpD fragment was in-frame with an N-terminal His$_6$ tag. The primers used were as follows: forward primer DS23 (GGCGGATCTGACGACAGCATGAATCT) incorporated a BglII restriction site (underlined) that gives sticky ends compatible with the BamHI site on the vector; the reverse primer DS24 (GGCGAAGCTTATATTACCTTCTGGCTGACC) incorporated a HindIII restriction site (underlined) compatible with that on the vector. The His$_6$-tagged GbpD was called GbpD2.

**METHODS**

**Bacterial strains and growth conditions.** *S. mutans* strain UA159 was used for the identification and cloning of *gpbD*. Other *S. mutans* strains and oral streptococcal species used are listed in the relevant Results section. *Escherichia coli* XL1 Blue was used in all cloning and protein expression procedures. *S. mutans* strains were grown in Todd–Hewitt Broth (Oxoid) supplemented with 5% yeast extract (THYE) and 5% sucrose, when required, or in a modified chemically-defined minimal medium containing Casamino acids (CASM; Russell, 1979b). The cultures were incubated at 37°C in candle jars. Utilization of β-glucosides was performed as described by Colby et al. (1995a) and adhesion to nicothine wares immersed in THYE containing 5% sucrose was examined as described by Colby et al. (1999). *E. coli* strains were grown in Luria–Bertani broth (LB) containing ampicillin (100 μg ml$^{-1}$) where required. For solid media, bacteriological agar (Agar no 1, Oxoid) was added at a final concentration of 1-5%.

**Molecular biology techniques.** Standard DNA manipulations were carried out using protocols described in Sambrook & Russell (2001). Restriction enzymes were obtained from New England Biolabs and used according to the manufacturer’s instructions. Large-scale plasmid extractions for DNA sequencing were carried out using the Plasmid Midi-Kit (Qiagen). DNA sequencing was carried out at the Molecular Biology Unit, University of Newcastle, using Thermosequenase and dye terminator chemistry on an ABI377 sequencer (Amersham). DNA sequence was assembled and analysed using OMIGA v2.0 software (Oxford Molecular). PCR reactions were carried out using the high-fidelity, pre-mixed Extensor Long PCR Master Mix (ABgene) without oil overlays, using cycling conditions recommended by the manufacturer, on a GeneAmp9700 thermal cycler (Applied Biosystems). All PCR primers were custom synthesized by Genset. PCR products for cloning were separated by electrophoresis and purified from the agarose gel using the Qiagen II kit (Qiagen). Electrophoresis of proteins and Western blotting were carried out using the standard protocols described in Sambrook & Russell (2001).

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For affinity purification, an AKTA Prime Chromatography instrument (Amersham Pharmacia) with the pre-programmed HisTag purification protocol was used for subsequent purification steps: the lysate was loaded onto a nickel-charged Hitrap chelating column (Amersham Pharmacia) equilibrated with 1× binding buffer +20 mM imidazole (BBI). After washing with 10 column volumes of BBI to remove unbound material, the his-tagged GbpD derivatives were eluted with an imidazole gradient of 20 mM–500 mM and collected in 1 ml fractions. Purity of the fractions was monitored by SDS-PAGE.

Construction of an insertion mutant of gbpD. An internal fragment from the 5′ end of the gbpD coding sequence was PCR amplified from S. mutans UA159 chromosomal DNA using primers designed using the genome sequence. The primers DS21 (TCCGCCGGGTGGTTTATGGTATTTGCG) and DS22 (TCCCCTCCGGATTGGGTTCTTCTGCTTCA) incorporated an Xmal site (underlined) at either end and gave a fragment of ~400 bp corresponding to amino acids 16–145 of the GbpD sequence. This was cloned into the Xmal site of the suicide vector pVA8912 (Malke et al., 1994; Simpson & Russell, 1998) using primer-derived Xmal sites to give plasmid pBA1. S. mutans UA159 cells were transformed with pBA1 using the biofilm transformation method of Li et al. (2001). Cells with the plasmid integrated into the chromosome were selected on erythromycin. The correct location of the insertion was confirmed using PCR with primers within the insert and flanking regions.

Binding assays. To measure binding of dextran by GbpD, the microtitre plate assay of Lis et al. (1995) was modified, utilizing the Ni-affinity of the histidine tag to immobilize the protein (Shah & Russell, 2002). Cleared lysates of induced E. coli cells carrying the overexpression plasmids or vector controls were prepared as described above. Preliminary experiments revealed that >5 µl of cleared lysate was sufficient to saturate the binding capacity of the Ni-NTA-coated wells (data not shown); therefore as a standard, 50 µl of cleared lysate was added to Ni-NTA-coated 96-well HisSorb plates (Qiagen) in PBS containing 0.05 % (v/v) Tween 20 (PBST) to a final volume of 200 µl and incubated overnight at 4°C. The protein solutions were removed and the wells washed four times for 1 min with PBST. Two hundred microlitres of a 100 µg ml−1 solution of biotin-dextran (Fluka) in PBS +0.2 % BSA was added and incubated for 10 min. After washing as before, wells were incubated with 200 µl of a 1:20000 dilution of Extravidin–alkaline phosphatase conjugate (Sigma) in PBS +0-2 % BSA for 30 min and washed again. One hundred microlitres of phosphate substrate solution (1 mg ml−1 p-nitrophenyl phosphate Sigma 104 substrate, 28 mM NaHCO3, 22 mM NaCO3, 5 mM MgCl2) was added and colour change monitored by readings at 405 nm in a Titertek Multiskan MCC 340 Plate Reader. For competition studies, competitors were added and the plates incubated at room temperature for 10 min prior to the addition of biotin-dextran.

Binding of His-tagged GbpD by cells was assayed by a whole-cell ELISA as described by Russell & Beighton (1982). Streptococci from overnight cultures were immobilized in microtitre tray and treated successively with pure recombinant GbpD2, mouse antibody to RGS-His (Qiagen) and finally rabbit anti-mouse alkaline phosphatase conjugate assayed by p-nitrophenylphosphate as above.

Hydropathicity assay. Hydrophobicity of S. mutans UA159 and the gbpD mutant was determined by the hexadecane partition assay as described by Hogg & Manning (1987).

Electroblotting and detection with biotin-dextran. Samples were run on SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were kept overnight in 20 mM PBS, pH 7-3, containing 3 % (w/v) BSA (initial heat-shock fraction, Sigma) to block non-specific binding, and washed three times for 15 min in PBST. Biotin-dextran (100 µg ml−1; Fluka) in PBS +0-2 % BSA were then added. After incubation at room temperature with shaking, the membranes were washed in PBST for 45 min with three buffer changes and then incubated for 1 h at room temperature with a 1:20000 dilution of Extravidin–alkaline phosphatase conjugate (Sigma) in PBS +0-2 % BSA. Proteins that bound to dextran were revealed by a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium colour reaction using reagents supplied by Zymed according to the manufacturer’s instructions. Conventional Western blotting was performed with rabbit antiserum raised against purified recombinant GbpD2.

Enzyme activity assays. Activities on carbohydrates were tested on synthetic substrates linked to 4-methylumbelliferone (4MU). Fluorogenic assays were set up in 96-well microtitre plates as follows. Fifty microlitres of a 100 µg ml−1 solution of 4MU-β-D-glucoside or 4MU-β-D-celllobioside (Sigma) were mixed with 130 µl of 50 mM TES (Sigma), pH 7-5, and either purified GbpD2 (5 µg ml−1) or cell-free extract from induced E. coli cells carrying the GbpD overexpression plasmid. The plates were incubated at 37°C for up to 24 h and fluorescence was visualized under UV light. In each case, cell free extract from E. coli carrying the pQE30 vector was used as a negative control.

Lipase and esterase activities were tested as above but in the presence of 1 mM CaCl2, on 6,8-difluoro-4MU-octanoate and ELF97 esterase substrate (Molecular Probes), respectively. Lipase activity on triglycerides was tested as follows. The triglycerides were stored as 10 % (v/v) emulsions in 10 % (w/v) gum Arabic and used at a final concentration of 0.5 %. Assays were carried out in buffers made with 50 mM MES, pH 6.5, or Tris/HisCl pH 7-0 and 7-5, containing 0.2 % Triton X-100. CaCl2 was added at 1 mM where necessary. E. coli LPS (Sigma; 25 µg ml−1 final concentration in the reaction), S. mutans LTA (12.5 µg ml−1), S. sanguis LTA (12.5 µg ml−1), LTA from S. mutans and S. sanguis was prepared as described by Sutcliffe & Hogg (1993) and was kindly provided by Dr S. D. Hogg, University of Newcastle. Cleared lysate from induced cultures expressing GbpD2 was added to the substrate and the reactions were incubated at 37°C for 2–4 h. Cleared lysate from cells carrying vector pQE30 were used as the negative control. Lipase activity was monitored by measuring the release of free fatty acids (FFAs) using the Novák assay (Novák, 1965). For spectrophotometer readings, 200 µl samples of the final colour reaction were transferred to wells in a 96-well microtitre plate and readings taken at 500 nm in a Titertek Multiskan MCC340 plate reader.

RESULTS

Discovery of gbpD

The MEME motif discovery tool (Bailey & Elkan, 1994) provides an automated system for identification of repeated motifs within a protein, whilst the related MAST tool (Bailey & Gribskov, 1998) searches for occurrence of a motif in a collection of sequences. When the MAST program was used to seek for a recurrent motif in 16 GTF and dextranucrase sequences available in GenBank, it identified a 33-aminoacid repeat closely matching the ‘A’ repeat in all the sequences, confirming that it is the distinctive building unit found in these proteins. The MEME consensus sequence differs only slightly from that originally described for the repeats in Gtf-I (Ferretti et al., 1987). The most striking feature of the ‘A’ repeat (Fig. 1) is the strong

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conservation of the aromatic amino acids and glycine (Banas et al., 1990).

The 33-amino-acid ‘A’ repeat consensus sequence was used to probe the S. mutans genome (Ađiĉ et al., 2002), translated in all reading frames, to search for ORFs that contained matching motifs. As expected, the search revealed the ORFs for those proteins known to contain ‘A’ repeats: Gtf-B, Gtf-C, Gtf-D and GbpA. In addition, a novel ORF containing ‘A’ repeats was discovered at positions 719 789–721 969 on the completed genome sequence. The ORF was named gbpD to follow previous nomenclature (Banas et al., 1990; Sato et al., 1997; Hazlett et al., 1998). Upstream of gbpD, and transcribed in the opposite direction, is an ORF encoding a protein with homology to the natural resistance-associated macrophage protein (Nramp) family characterized as divalent cation-transport proteins in many prokaryotic and eukaryotic organisms (Jakubovics & Jenkinson, 2001). The gbpD gene is preceded by a putative ribosome-binding site (AGAAAG) 10 bp upstream from the start codon and terminated by three stop codons (TAATAAA) followed by a hairpin loop structure necessary for lipase activity and may form part of the ‘catalytic triad’ with the serine of the lipase box (Arpigny & Jaeger, 1999). Analysis with the Interpro motif-seeking program (Apweiler et al., 2001) or a recently described structure modelling server (Dogouet et al., 2002) also showed that the best matches were to esterases and lipases of the α/β-hydrolase family of enzymes. Members of this family show a wide range of substrate specificities (Nardini & Dijkstra, 1999). Alignment of the lipase box and flanking regions of GbpD from S. mutans (Smu GbpD, AAN58492) with BglB from S. mutans (Smu BglB, AAF89976), PHBd from Alcaligenes faecalis (Afe, A32235) and Paucimonas lemioignei (Ple, P52090), Homo sapiens bile-salts activated lipase (Hsa BAL, P19835) and GehC lipase from Staphylococcus epidermidis (Sep GehC, Q02510). Identical residues of the lipase box are indicated by asterisks. (d) Alignment of the oxyanion hole region of the same proteins as in (c); the oxyanion hole is underlined. In each case residues identical in >50% of the sequences described are shown in bold.

**Sequence analysis of the GbpD peptide**

The gbpD ORF encodes a putative protein of 726 amino acids with a potential 36-amino-acid signal sequence at the N-terminus. The mature peptide is predicted to have a molecular mass of 76 kDa. Fig. 1 shows a schematic illustration of the main features of the peptide and alignments of the relevant motifs. There are three ‘A’ repeats of which the third is the least homologous to the consensus. The region N-terminal to the repeats does not have any homology to other proteins in the public databases. A BLASTP search showed that the C-terminal region has highest homology (~40% identity over a 350-amino-acid region) to BglB, encoded by an ORF of unknown function in a region of the S. mutans genome that is involved in utilization of β-glucosides (Cote et al., 2000) and to polyhydroxybutyrate depolymerases (PHBd). There is also homology to a variety of lipases and esterases. This homology is highest around the ‘lipase box’ and ‘oxyanion hole’ motifs typical of lipases and esterases (Bell et al., 2002). In addition, there are a number of characteristic aspartate and histidine residues after the lipase box that are thought to be involved in co-ordinating the calcium ion necessary for lipase activity and may form part of the ‘catalytic triad’ with the serine of the lipase box (Arpigny & Jaeger, 1999).
Dextran binding by GbpD

*S. mutans* strain UA159 was used as the parental strain for insertional inactivation of *gbpD* as described in Methods. The site of insertion means that residual expression of *gbpD* would yield only a truncated protein of ~100 amino acids, lacking the ‘A’ repeats and downstream sequences. Both strains were grown overnight and the culture medium was concentrated 50-fold by ultrafiltration after removal of cells by centrifugation. SDS-PAGE and electroblotting of concentrated culture supernatant of *S. mutans* UA159 with biotin-labelled dextran detected GTF, GbpA and a dextran-binding band of approximately 75 kDa, which correlates well with the calculated molecular mass of 76 kDa for the mature GbpD peptide. This 75 kDa band was absent from the *gbpD* mutant (Fig. 2). Specific antiserum raised against GbpD2 detected GbpD in Western blots and also reacted with GbpA and GTF (data not shown). These results confirm that GbpD is expressed, exported and binds to dextran. When sucrose was present in the growth medium, GbpD became cell-associated, as observed previously for other Gbps (Douglas & Russell, 1982).

The *gbpD* mutant was indistinguishable from the wild-type with respect to growth rate and colony morphology on solid media in the presence of glucose or sucrose. However, in broth cultures grown in the presence of sucrose, the mutant showed much looser packing of the aggregates than wild-type (Fig. 3). *gbpD* inactivation also had a marked effect on adhesion of cells to nichrome wires. *S. mutans* UA159 formed a robust, stable biofilm on nichrome wires in the presence of sucrose whereas the biofilm formed by the *gbpD* mutant was very fragile. Even the slight perturbation of removing the tube rack from the incubator resulted in detachment of the biofilm from the wire, indicating that GbpD contributes to the phenomenon of sucrose-dependent adherence.

Dextran binding by histidine-tagged GbpD derivatives

A series of histidine-tagged truncated derivatives of GbpD was constructed. Inducible expression and solubility of the different proteins in *E. coli* was confirmed by SDS-PAGE. Dextran-binding activity was measured by immobilizing the induced proteins on nickel-coated wells in microtitre plates and measuring binding of biotinylated dextran to the immobilized protein by detection with Extravidin–alkaline phosphatase conjugate. The constructs and their dextran-binding activities are shown in Fig. 4. GbpD2, which is the full-length GbpD without the signal sequence, has the highest dextran-binding activity. Removal of the sequence N- or C-terminal to the repeats resulted in around 65% reduction in binding activity (GbpD3 and GbpD4) while removal of both the N- and C-terminal regions (GbpD5) resulted in abolition of binding activity. These results indicate that the three repeats alone are not capable of binding to dextran and that the regions flanking the ‘A’ repeats contribute to the dextran-binding activity of GbpD perhaps by ensuring correct folding. Assays of GbpD2 binding to varying concentrations of biotin-dextran revealed a $K_D$ in the 0·1–0·2 μg ml$^{-1}$ (2–3 nM) range.

Fig. 2. Identification of dextran-binding proteins in culture supernatants by Western blotting with biotin-dextran. The ~75 kDa band corresponding to GbpD is indicated by the down arrow in the *S. mutans* UA159 lane; this band is missing in the *gbpD* mutant strain. The other dextran-binding proteins identified with this technique are the GTFs and GbpA, which migrate at positions expected for proteins of ~150 kDa and ~74 kDa (Russell, 1979a), respectively.

Fig. 3. Appearance of cultures of *S. mutans* UA159 (left) and its isogenic *gbpD* mutant (right) after overnight growth in Todd–Hewitt yeast extract broth supplemented with 5% sucrose.
Enzymic activity of GbpD

The high homology of the C-terminus to BglB and PHBd suggested that GbpD might be able to hydrolyse β-glucosides and/or PHB. The gbpD mutant strain was identical to the wild-type for metabolism of the β-glucosides amygdalin, arbutin, cellobiose, aesculin and gentibiose. Purified recombinant GbpD2 was tested under different conditions on a variety of β-linked substrates. No β-glucosidase activity was demonstrated on the substrates: β-D-glucoside, cellobiose, laminarin azure, AZCL pachyman, lichenan or the fluorogenic substrate 4-methylumbelliferyl-β-D-cellobiose, under a range of conditions (pH 4–8) in various buffers (Tris, phosphate, MES and TES), and in the presence or absence of various cations (Mg²⁺, Ca²⁺, Mn²⁺). Similarly experiments for detecting PHBd activity by monitoring absorbance change of a suspension of PHB yielded no evidence for a PHBd activity under a similar variety of conditions.

GbpD has homology to esterases and lipases and possesses motifs typical of this family of enzymes. Initial attempts to detect lipase/esterase activity of GbpD carried out using fluorogenic substrates (ELF 97 and 6,8-difluoro-4-methylumbelliferylcaproate; Molecular Probes) or chromogenic substrates (p-nitrophenylcaproate; Sigma) did not reveal any such activity. The lipase activity of GbpD was also tested by monitoring the release of FFAs from triglyceride emulsions in gum Arabic (Fig. 5). GbpD was able to release FFA from tricaprylin, triolein and to a lesser degree from tricaprin, indicating that GbpD does indeed have a lipase activity. This activity was dependent on the presence of Ca²⁺ ions. There was no detectable release of FFA in the absence of calcium and activity was abolished by the addition of EDTA.

A ‘natural’ substrate for GbpD?

What might be natural substrates available for a glucan-binding, lipolytic enzyme in dental plaque? S. sanguinis lipoteichoic acid (LTA) is a candidate since it contains α-1,6-linked isomalto-oligosaccharides and lipid. In a whole-cell ELISA, GbpD2 bound to S. sanguinis but not to any of the other species of oral streptococci tested (S. mutans UA159, S. sobrinus, S. mitis, S. oralis, S. gordonii, S. salivarius and S. downei; not shown). In order to explore further binding of GbpD to LTA, competitive binding assays were carried out. LTA at 40 μg ml⁻¹ was added to GbpD2 bound to a series of Ni-NTA-labelled 96-well plates. After incubation for 10 min, biotin-dextran was added at different concentrations and binding was assayed as described in Methods. The fact that an equimolar concentration of unlabelled dextran (10 μg ml⁻¹) gave a 50% reduction in signal shows that biotinylation does not interfere with binding. The results (Fig. 6) showed that LTA of S. sanguinis competed with biotin-dextran for binding to immobilized GbpD2, albeit not as effectively as non-labelled dextran. LTA from S. mutans was not an effective competitor for binding to GbpD2 (not shown). GbpD2 could release FFA from LTA of S. sanguinis but not

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**Fig. 4.** Dextran-binding ability of histidine-tagged derivatives of GbpD from lysates of recombinant E. coli (higher phosphatase activity equates to higher binding activity). (a) Schematic illustration of the different GbpD constructs; (b) alkaline phosphatase activity measured as an indicator of glucan-binding capacity. pQE30 (negative control) represents binding by lysate of E. coli carrying the vector plasmid. Data shown were obtained at 30 min after addition of chromogenic substrate and are means of three independent assays each with triplicates; error bars indicate SD.

**Fig. 5.** Release of FFAs from different substrates by GbpD2 in lysates of recombinant E. coli at pH 6.5 (white bars), pH 7.0 (hatched bars) and pH 7.5 (black bars). Data shown are means of triplicate measurements; error bars indicate SD.
A novel glucan-binding protein, GbpD, was identified in *S. mutans* by searching for the 33-amino-acid ‘A’ repeat originally identified as the major building blocks of the GBDs that make up the C-terminal third of streptococcal GTF and are also found in the non-enzymic glucan-binding protein GbpA of *S. mutans* (Banas *et al.*, 1990). The deduced peptide sequence revealed three of the 33-amino-acid ‘A’ repeats located internally, in the first one-third of the protein. Recent reports have identified stretches of sequence matching two or three of the ‘A’ repeats near the N-terminus of GTF and also dextran sucrases and alternansucrase of *L. mesenteroides* (Janecˇek *et al.*, 2000; Shah & Russell, 2002). The three internal repeats found in GbpD thus resemble this arrangement, though there is no similarity between the proposed catalytic domain of GbpD and that of the glucansucrases. Results presented here show that presence of the ‘A’ repeats of GbpD is essential for binding of dextran in Western blots and microtitre plate assay. The appearance of cultures of a mutant in which *gbpD* has been insertionally inactivated shows that GbpD contributes to the cohesiveness of aggregates and adhesion to a hard surface, which are known to be mediated by glucans, particularly dextran. This appearance resembles that seen in a *gbpA* knockout mutant (Banas & Gilmore, 1991). In *S. mutans*, there now are three GTFs and three Gbps known to contribute to the phenomenon of sucrose-dependent aggregation and adhesion (Banas & Vickerman, 2003). The discovery of a binding domain linked to a lipase was unexpected but another recently described example is the staphylococcal GehD lipase that has a collagen-binding activity (Bowden *et al.*, 2002). The role of this lipase and the significance of its ability to bind collagen are not clearly understood.

Clues to a function of GbpD additional to glucan binding came from homology searches with the proposed catalytic domain. The strongest homology was with the BglB protein of *S. mutans*. BglB is encoded by an ORF in the *bgl* regulon of *S. mutans* that is involved in transport and hydrolysis of β-glucosides (Cote *et al.*, 2000). We could not demonstrate any involvement of GbpD in β-glucoside utilization by *S. mutans* using fluorogenic substrates or β-glucan polymers. It should be noted, however, that an inability to demonstrate a particular enzymic activity with synthetic chromogenic or fluorogenic substrates does not necessarily mean that the activity is not present; for example, streptococcal mannosidases that degrade the Man₅ glycan of RNase B failed to degrade 4-methylumbelliferyl- or p-nitrophenol-linked mannoside substrates (Homer *et al.*, 2001). The function of BglB is not known but it lacks a signal sequence so would be predicted to be in the cytoplasm. It is interesting that *S. mutans* produces two homologous proteins, one of which is predicted to be extracellular (GbpD) and one that is predicted to be cytoplasmic (BglB) and may have the same substrate specificity.

Since sequence homology with various lipases and esterases was detected, GbpD was subjected to tests for lipase and esterase activities on synthetic fluorogenic and chromogenic substrates. No lipase or esterase activities could be demonstrated using these substrates. However, GbpD was

![Graph showing competition with biotinylated dextran for binding to GbpD2](Image)

**Fig. 6.** Competition with biotinylated dextran for binding to GbpD2. The concentration of biotinylated dextran was increased whilst the concentration of competitor was kept constant. The competitors diluted in PBS + 0.2% BSA were: None (△, no competitor control), Dextran T70 (▽, unlabelled dextran at 10 μg mL⁻¹) and *S. sanguinis* LTA (■, *S. sanguinis* LTA at 40 μg mL⁻¹). The data shown are the means of triplicates; error bars, indicating SD, are too small to be visible.

from *S. mutans* LTA, *E. coli* LPS or saliva (Fig. 5). The activity on *S. sanguinis* LTA was dependent on the presence of calcium.

**Effect of GbpD on hydrophobicity**

Since GbpD is an extracellular lipolytic enzyme, it seemed possible that it might modify the lipid composition of the *S. mutans* surface and hence influence its hydrophobicity. The percentage hydrophobicity, calculated from the proportion of cells associated with the hexadecane phase, was 66% for wild-type *S. mutans* UA159 while the *gbpD* mutant was altered significantly, with hydrophobicity reduced to 52%.

**Distribution of GbpD**

The *gbpD* gene was first identified in the genome sequence of *S. mutans* strain UA159. To investigate the distribution of *gbpD*, PCR reactions using primers DS23 and DS24 (see Methods) were carried out on genomic DNA isolated from a variety of other *S. mutans* strains. All of 10 strains tested yielded an amplified band corresponding to *gbpD* of UA159 and expression of GbpD was confirmed by Western blotting with specific antibody. A homologue of the *gbpD* gene was also found in the partially sequenced *S. sobrinus* genome (http://www.tigr.org/) but was not found in the partially sequenced genomes of *S. gordonii*, *S. mitis* or in any other publicly available complete or partial genome sequences.

**DISCUSSION**

A novel glucan-binding protein, GbpD, was identified in *S. mutans* by searching for the 33-amino-acid ‘A’ repeat originally identified as the major building blocks of the

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**File Reference:** S. mutans glucan-binding lipase

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able to release FFAs from triglycerides in the presence of calcium, indicating that GbpD does indeed have a lipase activity. What is the natural substrate for GbpD in its environmental niche? As an extracellular enzyme, GbpD might encounter secreted products of S. mutans itself and the products of other plaque bacteria. Since GbpD is a glucan-binding, lipolytic enzyme, we therefore tested its effect on S. mutans LTA and also LTA of S. sanguinis, which contains diglucosyl-diacylglycerol as the glycolipid substituted with \( \alpha-1,6 \)-linked isomalto-oligosaccharide (Kochanowski et al., 1993). It has previously been reported that GTF of S. sanguinis interacts with its LTA, presumably through the GBD (Chiu & Baker, 1994). GbpD2 bound selectively to whole cells of S. sanguinis but not to any other oral streptococci. Furthermore, S. sanguinis LTA was able to compete with biotin-dextran for binding to GbpD and served as a substrate for the lipase activity, with release of FFA. In contrast, S. mutans LTA neither bound nor served as substrate. The specificity of the lipase activity may be due to the fact that GbpD binds to S. sanguinis LTA via the ‘A’ repeat motif, thereby facilitating access to the ester bond linking the fatty acid moiety. We cannot exclude the possibility that GbpD might attack S. mutans LTA, particularly during growth on sucrose. Under these conditions, GbpD would remain entrapped close to the cell surface within the glucan matrix, which contains both dextran and LTA (Rolla et al., 1980). If GbpD lipase is active at the cell surface, it might intuitively be expected that reduced lipolysis in the gbpD knockout mutant would result in an increase in hydrophobicity. In fact, the reverse is observed and the mutant is less hydrophobic than the wild-type. It is not yet apparent whether this is due to loss of GbpD protein itself or to a consequential change in the amount or location of other surface components such as lipoproteins. There is close interaction between the various surface macromolecules and alteration in one can have substantial effects on others, with consequences for overall surface properties (Harrington & Russell, 1993).

Enzymes that can modify or degrade surface LTA have important roles in modulating the surface properties of cells. One such enzyme is Pce, the teichoic acid phosphorylcholine esterase of S. pneumoniae and S. oralis, which is able to remove phosphorylcholine from cell wall teichoic and LTAs (De las Rivas et al., 2001; Ronda et al., 1991; Vollmer & Tomasz, 2001). S. mutans does not appear to possess a homologue of Pce and there is no sequence homology between the enzymic domains of GbpD and Pce. Although the first 12 amino acids of the ‘A’ repeat show high homology to repeats found in Pce and other choline binding proteins of S. pneumoniae (Garcia et al., 1998; Janeček et al., 2000), this sequence similarity is not reflected in binding specificity and choline was unable to compete with biotin-dextran for binding to GbpD (data not shown). The similarity between Pce and GbpD is thus limited to the fact that both consist of a binding domain and a catalytic domain. All experimental evidence available suggests that the ‘A’ repeats of GbpD confer specificity of binding only to dextran or LTA containing \( \alpha-1,6 \)-linked isomalto-oligosaccharide units.

The ability of S. mutans GbpD to release FFAs from another plaque species, S. sanguinis, raises the intriguing possibility that it may modulate the surface of at least one organism within its ecosystem with which S. mutans competes for resources. Such inter-bacterial competition was recently shown to occur between S. pneumoniae and Neisseria meningitidis and Haemophilus influenzae, all pathogens of the human respiratory tract. S. pneumoniae produces a neuraminidase that desialylates the LPS of the other two species, thereby altering their surface properties and rendering them more susceptible to host defences (Shakhnovitch et al., 2002). In normal plaque, S. mutans is rare but in plaque associated with dental caries it is the dominant species. In contrast, S. sanguinis is found in high numbers in ‘healthy’ plaque but is rare in carious plaque (Becker et al., 2002). Thus there is a reciprocal relationship between these species. The high proportion of S. mutans in caries-associated plaque has been attributed to its competitive advantage over other plaque species during periods when plaque is at a low pH. Although this may well be a major factor, this study suggests that S. mutans may have another weapon that directly affects competitors and gives it advantage. Furthermore, the pH optimum for the GbpD lipase activity suggests that it will be most effective during periods between sugar exposure, when plaque pH is raised by the buffering power of saliva.

The discovery of GbpD adds to the number of proteins containing a GBD. We have previously reported the antigenic cross-reaction between GTF and GbpA (Russell, 1990) and there is reciprocal cross-reaction between GTF, GbpA and GbpD. One consequence is that any caries vaccine based upon one of these proteins, or an isolated GBD, is likely to have multiple effects upon the growth and properties of S. mutans and proteins of other plaque streptococci with proteins containing ‘A’ repeats. Identification of the various proteins capable of binding glucans will contribute to our understanding of the complex interactions occurring in plaque.

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