Environmental regulation of glycosidase and peptidase production by *Streptococcus gordonii* FSS2

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The synthesis of cell-associated and secreted proteins by *Streptococcus gordonii* FSS2, an infective endocarditis (IE) isolate, was influenced by both environmental pH and carbon source. Controlling the pH at 7.5 in stirred batch cultures showed that cell-associated and secreted protein concentrations were increased during late exponential and stationary phase by 68% and 125%, respectively, compared with similar cultures without pH control. The expression of five glycosidase and eight peptidase activities were examined using fluorogen-labelled synthetic substrates. Enzyme activities were significantly down-regulated during exponential growth, increasing during stationary phase ($P < 0.01$) whether the culture pH was controlled at pH 7.5 or allowed to fall naturally to pH 4.4. Culture-supernatant activities were significantly increased ($P < 0.05$) when the pH was maintained at 6.0 or 7.5, indicating modulation of enzyme activity by pH. Growth under nitrogen-limitation/glucose-excess conditions resulted in a significant repression of cell-associated glycosidase activities ($P < 0.01$), whilst in the supernatant, activities were generally reduced. The expression of peptidase activities in the culture supernatant did not significantly change. The results suggest a possible role for catabolite repression by glucose in regulating enzyme expression. When *S. gordonii* FSS2 was cultured with 50% (v/v) added heat-inactivated foetal bovine serum, several cell-associated enzyme activities increased initially but were then reduced as the culture time was extended to 116 h. Culture-supernatant enzyme activities (*N*-acetyl- β -D-glucosaminidase, *N*-acetyl- β -D-galactosaminidase, thrombin, Hageman factor, collagenase and chymotrypsin), however, were significantly increased ($P < 0.01$) over the same time period. The findings indicated that most of the important glycosidases synthesized by *S. gordonii* FSS2 were down-regulated by acid growth conditions and may also be subject to catabolite repression by glucose but conversely may be up-regulated by growth in serum. These results may have implications for streptococcal growth in an IE vegetation and in the mouth between meals or during sleep.

Keywords: Infective endocarditis, *Streptococcus gordonii*, pH, enzymes

INTRODUCTION

Streptococcus sanguis and *Streptococcus gordonii*, members of the *Streptococcus mitis* group (Whiley & Beighton, 1998), are primary colonizers of the oral

cavity where they form an important part of the microbial community that comprises healthy dental plaque. *S. sanguis* and *S. gordonii*, with other streptococci in the *mitis* group of organisms, are also major causative organisms of the disease infective endocarditis

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Abbreviations: 7-AMC, 7-amido-4-methylcoumarin; Ca, activated protein C; CDM, chemically defined medium; FBS, foetal bovine serum; IE, infective endocarditis; 4-MU, 4-methyl-umbelliferyl; PAS, periodic acid/Schiff; Xa, activated protein X.

(IE), where it is thought that poor dental hygiene can result in the streptococci entering the bloodstream from dental plaque. Once in the bloodstream, the streptococci have been shown to cause haemodynamic changes in the rabbit model of experimental endocarditis where intermittent electrocardiographic abnormalities and pre-ventricular contractions are observed in the initial 3–7 min after infusion of the bacteria (Meyer *et al.*, 1998). Blood catecholamine concentration, thrombocytopenia, the accumulation of platelets in the lungs and ventricular axis deviation also showed dose dependency (Meyer *et al.*, 1998; Herzberg & Meyer, 1996). Streptococcal species are also able to adhere to (Ke Gong *et al.*, 1998) and aggregate platelets (Herzberg, 1996; Herzberg *et al.*, 1992; Ford & Douglas 1997; Manning *et al.* 1994a, b), as well as adhere to damaged areas of the heart valve, resulting in the formation of a platelet fibrin vegetation (Lowrance *et al.*, 1988; Manning *et al.*, 1994b).

Several studies have examined the enzymes produced by streptococci that enable them to metabolize complex molecules, mainly concentrating on the model glycoprotein, pig gastric mucin. Homer *et al.* (1996) examined the ability of *Streptococcus oralis* to synthesize sialidase and *N*-acetylglucosaminidase, enzymes important in the metabolism of glycoproteins, and found that both were induced when grown in the presence of pig gastric mucin (Rafay *et al.*, 1996). It has also been observed that growth in pig gastric mucin leads to higher levels of bacterial glycosidase activity, whilst some protease activities are repressed. Conversely, the presence of glucose represses glycosidase activity (Rafay *et al.*, 1996; Beighton *et al.*, 1995). Mayo *et al.* (1995) showed that *S. gordonii* FSS2 grown in a complex medium produced a variety of glycosidase and peptidase enzymes which were modulated by both growth rate and environmental pH. Enzyme activities were generally higher at pH 6.5 than at 5.5 or 7.5. The exception was thrombin-like activity which was found to be fivefold higher at more neutral pH than under more acidic conditions. Another activity, Hageman factor, was apparently subject to catabolite repression by glucose, as activity was threefold higher in cultures grown on galactose compared with those grown on glucose. Enzymes important for catabolism in *S. gordonii* FSS2 therefore seem to be regulated by environmental conditions.

The shift from a low oral pH to blood pH has been shown in *S. gordonii* to induce or up-regulate the expression of several genes including one, *msrA*, which is involved in protection against oxidative stress and enhanced bacterial growth (Vriesema *et al.*, 2000). In this work we investigate the effects of environmental pH and carbon source on the expression of cell-associated and secreted enzymes of *S. gordonii* grown in a fully defined medium.

METHODS

Bacterial strain. The streptococcal strain used in this work was originally isolated from an IE patient at Freeman Hospital, Newcastle-upon-Tyne, UK (Manning *et al.*, 1994b; Mayo *et al.*, 1995) and was at the time designated as *S. sanguis* FSS2.

Recent taxonomic studies on streptococci (Whiley & Beighton 1998) have indicated that the majority of *S. sanguis* isolates are unable to produce α -L-fucosidase, *N*-acetyl- β -D-galactosaminidase, *N*-acetyl- β -D-glucosaminidase and alkaline phosphatase, whilst *S. gordonii* strains are generally able to produce these enzyme activities. The presence of these enzyme activities as well as taxonomic testing using the API ID32 strep test kit (bioMérieux Vitek) indicated that the streptococcal strain should be designated *S. gordonii*.

Culture media. *S. gordonii* FSS2 was stored as brain-heart infusion broth (Oxoid)/20% (v/v) glycerol stocks at -70°C and was routinely grown on Columbia blood agar plates (Oxoid) with 5% (v/v) horse blood. Batch cultures were grown on a filter-sterilized (0.2 μm pore; Sartorius) chemically defined medium (CDM) modified from that described by Terleckyj *et al.* (1975) and Wittenberger *et al.* (1978), containing (mg l $^{-1}$ unless stated otherwise): trisodium citrate, 250; NH_4SO_4 , 600; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 11; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 700; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 18; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.15; NaCl, 2000; NaHCO_3 , 500; adenine, 20; guanine, 20; uracil, 20; thiamin, 8; pyridoxamine, 2; calcium pantothenate, 8; riboflavin, 8; nicotinamide, 4; *p*-aminobenzoic acid, 0.4; biotin, 0.2; folic acid, 0.2; inositol, 2; thiamin, 2; L-glutamic acid, L-glycine and L-cysteine, 3 mM; L-arginine, L-lysine, L-isoleucine, L-leucine, L-methionine, L-tryptophan, L-tyrosine, L-histidine, L-proline, DL-alanine, L-aspartic acid, L-phenylalanine, L-asparagine, L-serine, L-threonine, L-valine and L-glutamine all 1 mM; 30 mM potassium phosphate buffer pH 7.5; 0.5% (w/v) glucose (27.8 mM). The medium was brought to pH 7.5 by the addition of 5 M KOH. All chemicals were of analytical grade and were purchased from either Sigma or Merck. In one series of experiments, 50% (v/v) foetal bovine serum (FBS; Commonwealth Serum Laboratories), heated at 80°C for 30 min to inactivate serum-enzyme activities, was added to CDM devoid of glucose.

Growth conditions. *S. gordonii* FSS2 was grown at 37°C as a 100 ml stirred, pH-controlled batch culture gassed with 5% (v/v) CO_2 /95% (v/v) N_2 at a rate of 50 ml min $^{-1}$. The pH was controlled by automatic addition of a mixture of 2 M NaOH and 2 M KOH (to avoid changes in the sodium:potassium ratio of the medium during growth; Pitty & Jacques, 1989). Batch cultures were inoculated at 15% (v/v) from a 20 ml static batch culture grown for 18 h in CDM. In some experiments, CDM was modified to provide carbon-excess/nitrogen-limited growth conditions by removing the ammonium sulphate and adding one-third the concentration of amino acids (1 mM glutamic acid, glycine and cysteine; 0.33 mM all other amino acids). This resulted in residual glucose [0.93 mg ml $^{-1}$ (5.2 mM)] being detected in the medium at stationary phase.

Bacteria were harvested by centrifugation (27000 g, 4°C , 5 min), washed three times in 50 mM potassium phosphate buffer pH 7.5 containing 0.02% (w/v) sodium azide and resuspended to an OD_{600} 1.00 ± 0.05 . All determinations of cell-associated protein concentration were made at OD_{600} . Supernatants were filtered through 0.2 μm pore membranes (Sartorius) prior to assaying for enzyme activity. In some experiments, bacteria were resuspended in 50 mM citrate/phosphate buffer at pH 4.0, pH 5.0 or pH 6.0 and incubated at 37°C for 2 h before being centrifuged (27000 g, 4°C , 5 min) and resuspended in 50 mM potassium phosphate buffer pH 7.5 prior to assaying for enzyme activities.

The ability of *S. gordonii* FSS2 to utilize FBS as a source of nutrients was determined in pH-controlled stirred cultures by the addition of an equal volume of heat-inactivated FBS to the

defined medium devoid of glucose. After treatment, the only residual enzyme activities in FBS were thrombin [5.7 (4.1 – 7.4) nmol h^{-1} (100 ml culture) $^{-1}$] and Hageman factor [9.8 (8.1 – 11.4) nmol h^{-1} (100 ml culture) $^{-1}$] representing 0.2% and 0.02% , respectively, of the initial activities prior to heating at 80°C . Bacterial growth was monitored spectrophotometrically at OD_{600} and by the number of viable cells in 1 ml culture samples after being sonicated (10°C , 5 min, 130 W) in a cup horn (Branson model 450 sonifier, Branson Ultrasonics) to break up aggregates of bacteria before being diluted and plated on Columbia 5% (v/v) horse-blood agar.

A partial separation of the 'fibrous' precipitate from streptococcal cells grown on FBS was achieved by a slow-speed centrifugation (500 g, 22°C , 5 min). Examination by light microscopy of the pellet after centrifugation indicated that few bacterial cells were present.

Enzyme assays. Four glycosidase and eight peptidase activities were measured using a range of synthetic substrates attached to the fluorogens, 4-methylumbelliferone or 7-amido-4-methylcoumarin (7-AMC), that were purchased from Sigma. The substrates (with the activity they detected shown in parentheses) were: 4-methylumbelliferyl (4-MU) β -D-glucoside (β -D-glucosidase); 4-MU- β -D-galactoside (β -D-galactosidase); 4-MU-N-acetyl- β -D-glucosaminide (N-acetyl- β -D-glucosaminidase); 4-MU-N-acetyl- β -D-galactosaminide (N-acetyl- β -D-galactosaminidase); N-tert-butoxycarbonyl-Val-Pro-Arg-7-AMC (thrombin); N-tert-butoxycarbonyl-Leu-Ser-Thr-Arg-7-AMC [activated protein C (Ca)]; N-tert-carbobenzoyloxy-Phe-Arg-7-AMC (kallikrein); N-tert-carbobenzoyloxy-Lys-7-AMC (Hageman factor); N-tert-butoxycarbonyl-Ile-Glu-Gly-Arg-7-AMC [activated protein X (Xa)]; N-tert-butoxycarbonyl-Glu-Lys-Lys-7-AMC (plasmin); N-succinyl-Leu-Leu-Val-Tyr-7-AMC (chymotrypsin); and N-succinyl-Gly-Pro-Leu-Gly-Pro-7-AMC (collagenase) (Mayo *et al.*, 1995; Oakey *et al.*, 1995). All substrates were dissolved in a minimal amount of dimethylsulphoxide and then diluted to $100\text{ }\mu\text{g ml}^{-1}$ in 50 mM potassium phosphate buffer pH 7.5 containing 0.02% (w/v) sodium azide. Enzyme assays were performed in duplicate at room temperature (18 – 20°C) using a Perkin-Elmer Luminescence spectrometer LS50-B with a microtitre plate assembly using white 96-well microtitre plates (Perkin-Elmer). The assay volume of $200\text{ }\mu\text{l}$ consisted of $50\text{ }\mu\text{l}$ substrate, $50\text{ }\mu\text{l}$ bacterial cells or $10\text{ }\mu\text{l}$ culture supernatant diluted to $150\text{ }\mu\text{l}$ with 50 mM potassium phosphate buffer pH 7.5 . The controls contained $50\text{ }\mu\text{l}$ substrate and $150\text{ }\mu\text{l}$ 50 mM potassium phosphate buffer pH 7.5 . Fluorescence readings were taken at 0 , 10 , 20 , 30 , 40 , 50 , 60 and 120 min and at 24 h. The maximum rate of release of fluorogen was determined and calculated from a standard curve of authentic 4-MU or 7-AMC and expressed as specific activity for cell-associated enzymes [nmol h^{-1} ($\text{mg protein})^{-1}$] or total activity for culture-supernatant activities [nmol h^{-1} (100 ml culture) $^{-1}$].

Enzyme synthesis. To confirm that *de novo* enzyme synthesis was occurring, cells from a 20 ml CDM static batch culture containing 30 mM potassium phosphate buffer pH 7.5 and grown for 18 h were harvested as above and resuspended in 5 ml phosphate buffered saline. A portion of the resuspended cells was reserved to determine enzyme activities. Of the remaining cells, 1 ml was inoculated into duplicate pre-warmed CDM cultures containing 100 mM potassium phosphate buffer pH 7.5 , or duplicate CDM cultures supplemented with 100 mM potassium phosphate buffer pH 7.5 and 50% (v/v) heat-inactivated FBS. Increasing the concentration of phosphate buffer raised the terminal pH of the culture. Chloramphenicol was added to a final concentration of $100\text{ }\mu\text{g ml}^{-1}$ to one of each of the duplicate cultures to inhibit

protein synthesis. The cultures were incubated for 6 h in an anaerobic jar as described above before being harvested by centrifugation (27000 g, 4°C , 5 min) and washed three times in 50 mM potassium phosphate buffer pH 7.5 containing 0.02% (w/v) sodium azide. The cells were finally resuspended to an OD_{600} 1.00 ± 0.05 for determination of enzyme activities. To confirm that chloramphenicol was inhibiting protein synthesis, static batch cultures of *S. gordonii* were grown in CDM supplemented with 185 kBq [^3H]leucine (Amersham Life Sciences). After harvesting and washing, the level of incorporation of [^3H]leucine into the cells was determined using a Packard 1500 Tricarb Liquid scintillation analyser (Canberra Packard).

Analytical procedures. The concentration of bacterial cell-associated proteins (at OD_{600} 1.00 ± 0.05) and the protein concentration in the culture supernatant were determined by the Pierce Coomassie Protein Plus Assay Reagent (Pierce) using bovine serum albumin as the standard. The concentration of residual glucose in CDM and in serum was determined using a glucose oxidase test kit (Sigma). Statistical analysis, Friedman non-parametric repeated-measures test, two-tailed, was carried out using the statistical program Instat v2.02 (Graphpad Inc.). SDS-PAGE analysis of FBS culture components was carried out on duplicate 10% gels (Laemmli, 1970). One gel was stained for protein with Gelcode Blue Coomassie blue (Pierce) and the other stained for carbohydrate [periodic acid/Schiff (PAS) stain] using the method described by Pitty *et al.* (1989). Protein molecular masses were estimated using Bio-Rad broad range molecular mass markers.

RESULTS

Effect of pH control on the expression of enzyme activities

To examine the effect of pH on growth and protein expression by *S. gordonii* FSS2, 100 ml stirred batch cultures were grown with and without pH control. Cultures grown without pH control (Fig. 1a) had a rapid reduction in pH after a lag period of approximately 1 h. By the onset of stationary phase the culture pH had fallen to pH 4.7 and by late stationary phase (7 h) had fallen further to pH 4.4 . Over the whole period of growth, the amount of both cell-associated and culture-supernatant protein varied very little. The cell-associated protein concentration at the first sample point (1.5 h) was measured as $86.3 \pm 4.0\text{ }\mu\text{g ml}^{-1}$ and as $79.4 \pm 0.5\text{ }\mu\text{g ml}^{-1}$ by late stationary phase. Over the same period, the protein concentration of the culture supernatant increased by only 28.8% . In contrast, when the pH of the culture was controlled at pH 7.5 (Fig. 1b), the cell-associated protein concentration increased marginally during the exponential phase of growth but increased by 68.2% once the culture had entered stationary phase. Similarly, the concentration of protein observed in the culture supernatant increased gradually during exponential growth but rose rapidly in stationary phase to give an overall increase of 125% over that observed during the lag phase of growth. When the pH was set at an intermediate level (pH 6.5), the cell-associated protein concentration was unchanged between exponential phase ($63.7 \pm 0.7\text{ }\mu\text{g ml}^{-1}$) and stationary phase ($63.7 \pm 2.4\text{ }\mu\text{g ml}^{-1}$). Culture-supernatant protein concentration, in contrast, increased

Fig. 1. Cell-associated and secreted protein production by *S. gordonii* FSS2 grown (a) without pH control and (b) at pH 7.5. ●, pH; □, OD₆₀₀; ■, supernatant protein concentration (μg ml⁻¹); ▼, cell-associated protein concentration [μg (ml cells OD₆₀₀ 1.00)⁻¹]. Values represent the mean and bars show standard deviation ($n = 2$).

Table 1. Cell-associated enzyme specific activities [nmol h⁻¹ (mg protein)⁻¹] of *S. gordonii* FSS2 at exponential and stationary phases

ND, Not detected [< 2 nmol h⁻¹ (mg protein)⁻¹]. Values are the mean of two assays; replicates all showed less than twofold difference.

Enzyme	No pH control, exponential phase	No pH control, stationary phase	pH 7.5, exponential phase	pH 7.5, stationary phase	pH 6.5, stationary phase	Nitrogen-limited/glucose-excess, stationary phase
β-D-Galactosidase	ND	27.7	7.5	66.8	11.9	ND
N-Acetyl-β-D-glucosaminidase	7.3	51.3	20.7	218.1	123.3	7.8
N-Acetyl-β-D-galactosaminidase	ND	9.7	3.3	42.6	20.6	ND
β-D-Glucosidase	ND	46.4	11.9	352.2	150.7	ND
Thrombin	ND	20.2	7.9	97.9	35.4	14.2
Plasmin	ND	2.5	ND	ND	3.9	5.2
Kallikrein	ND	20.3	5.5	ND	12.0	ND
Hageman	1701	2353.7	934.3	962.3	1863.0	279.1
Collagenase	33.5	23.4	24.9	27.5	36.9	37.7
Ca	7.7	38.5	12.9	29.6	46.0	37.5
Xa	12.5	9.0	6.7	6.9	11.9	4.2
Chymotrypsin	4.5	32.0	15.0	15.7	64.1	34.9

Table 2. Enzyme total activities in culture supernatant [nmol h⁻¹ (100 ml culture)⁻¹] of *S. gordonii* FSS2 at exponential and stationary phase

ND, Not detected [< 2 nmol h⁻¹ (100 ml culture)⁻¹]. Values are the mean of two assays; replicates all showed less than twofold difference except where indicated.

Enzyme	No pH control, exponential phase	No pH control, stationary phase	pH 7.5, exponential phase	pH 7.5, stationary phase	pH 6.5, stationary phase	Nitrogen-limited/glucose-excess, stationary phase
β -D-Galactosidase	ND	ND	ND	14.0	ND	ND
N-Acetyl- β -D-glucosaminidase	ND	ND	ND	190.3	ND	ND
N-Acetyl- β -D-galactosaminidase	ND	ND	ND	26.4	ND	ND
β -D-Glucosidase	ND	ND	ND	ND	ND	ND
Thrombin	ND	ND	14.8	26.5	2.2	7.5
Plasmin	ND	ND	ND	ND	ND	ND
Kallikrein	ND	ND	ND	5.1	ND	ND
Hageman	ND	120.4 (75.5–165)	95.6	662.1	112.7	1410.8
Collagenase	ND	14.5 (0.0–29.0)	58.8	70.0	21.7	81.3
Ca	ND	ND	23.5	43.5	2.4	38.3
Xa	ND	ND	7.1	2.3	ND	ND
Chymotrypsin	ND	ND	30.4	17.7	19.2	31.3

from 7.6 ± 0.1 $\mu\text{g ml}^{-1}$ in exponential phase to 12.7 ± 0.1 $\mu\text{g ml}^{-1}$ at stationary phase (a 67% increase) (data not shown).

Effect of pH and carbon source on glycosidase and peptidase activities

Cell-associated and culture-supernatant enzyme activities were determined at both mid-exponential and stationary phases. The cell-associated specific activities were significantly increased in stationary phase over those measured in mid-exponential phase in both non-pH-controlled ($P = 0.007$) and pH 7.5 cultures ($P = 0.012$) (Table 1). Cell-associated enzyme activities at stationary phase were not significantly different ($P > 0.05$) between any of the growth conditions. Glucose-excess/nitrogen-limitation conditions at stationary phase (Table 1) resulted in a significant reduction in cell-associated glycosidase activities when compared with cells grown at pH 7.5 ($P < 0.01$) while there was no significant change in the peptidase activities (Table 1).

To compare the enzyme activities in the culture supernatant for all the culture conditions, the total enzyme activities for the entire 100 ml culture [nmol h⁻¹ (100 ml culture volume)⁻¹] were calculated. In the absence of pH control, no activities were found at mid-exponential phase and only a very reduced level of both Hageman factor and collagenase activity was detected in stationary phase (Table 2). In cultures controlled at pH 7.5, none of the glycosidase activities was detected in mid-exponential phase whilst all the peptidase activities with

the exception of plasmin and kallikrein were detected (Table 2). In stationary phase at pH 7.5, however, all activities with the exceptions of plasmin and β -D-glucosidase were detected and were significantly increased in comparison with mid-exponential-phase activities ($P = 0.03$) and with the stationary phase no pH control activities ($P < 0.01$) (Table 2).

To confirm that *de novo* protein synthesis was responsible for the increase in protein expression, duplicate cultures were incubated in the presence or absence of 100 μg chloramphenicol ml⁻¹. When compared with the activities present in the inoculum, the accumulation of enzyme activities was significantly reduced by the addition of chloramphenicol in the presence ($P < 0.001$) or absence ($P < 0.05$) of 50% (v/v) FBS. Inhibition of protein synthesis by chloramphenicol was confirmed by the reduction of the incorporation of [³H]leucine into the cell. In the presence of chloramphenicol, incorporation of radioactivity into *S. gordonii* cells was reduced by 99.7% compared with control cells grown in the absence of chloramphenicol (data not shown).

Effect of glucose-excess/nitrogen-limited conditions on enzyme activities

At stationary phase, in cultures grown under glucose excess/nitrogen limitation at pH 7.5, the cell-associated protein concentration (50.1 ± 0.3 $\mu\text{g ml}^{-1}$) was approximately 50% of the value for the other culture conditions. Culture-supernatant protein concentration (19.9 ± 0.1 $\mu\text{g ml}^{-1}$) was similar to the pH 7.5 culture grown on

Fig. 2. *Streptococcus gordonii* FSS2 growth curve on 50% (v/v) FBS pH 7.5. □, OD₆₀₀; ■, viable count (c.f.u. ml⁻¹).

CDM but nearly double the concentration of cultures grown with no pH control or at pH 6.5 on CDM. Cell-associated and supernatant glycosidase activities were undetectable, whilst peptidase activities were not significantly different from the other culture conditions (Tables 1 and 2).

Effect of incubating cells in various pH buffers

Washed cell suspensions of *S. gordonii* were incubated at 37 °C in 50 mM citrate/phosphate buffer at pH 4.0, 5.0 or 6.0 for 2 h. Incubation at pH 4.0 resulted in the total loss of measurable activity for all enzymes except *N*-acetyl- β -D-glucosaminidase, β -D-glucosidase and Hageman factor. These enzymes retained 50%, 30% and 1% of their pre-incubation activities, respectively. Incubation at pH 5.0 or above had little effect on any of the enzyme activities (data not shown).

Effect of growth on 50% (v/v) FBS on the expression of enzyme activities

Cultures were grown in CDM medium without glucose and supplemented with 50% (v/v) FBS; 50% (v/v) FBS contained 0.52 mg glucose ml⁻¹. The inoculum for the cultures was centrifuged to pellet the cells and resuspended in a small volume of 50 mM potassium phosphate buffer pH 7.5 prior to inoculation into the batch culture vessel. Culturing in FBS was repeated three times and a representative growth curve is shown in Fig. 2. Two hours after inoculation, the culture OD₆₀₀ had reached 0.57 and no glucose could be detected in the culture supernatant. The culture optical density then fell to an OD₆₀₀ of 0.25 after 24 h. The viable count, which had increased from 3.0 × 10⁸ c.f.u. ml⁻¹ at inoculation to 1.4 × 10⁹ c.f.u. ml⁻¹ at 2.5 h, also declined until at 24 h it was 3.0 × 10⁸ c.f.u. ml⁻¹. However, over the next 92 h the culture OD₆₀₀ increased to 0.95 while the viable count declined slightly to 7.2 × 10⁷ c.f.u. ml⁻¹ (Fig. 2). Centrifugation of culture samples and examination by Gram staining and light microscopy revealed that a

fibrous precipitate was being formed in the culture. The precipitate had little solubility in 50 mM potassium phosphate buffer pH 7.5 or deionized water.

The precipitate was separated from the majority of *S. gordonii* cells (as observed by light microscopy) by low-speed centrifugation (500 g, 22 °C, 5 min) and was washed six times with 50 mM Tris/HCl buffer pH 7.5 to remove serum components. The composition of the washed precipitate was examined by SDS-PAGE following dissolution by boiling in SDS-PAGE sample buffer with 5% (v/v) 2-mercaptoethanol for 10 min. Following centrifugation, the sample was separated on duplicate 10% SDS-PAGE gels and either stained with Coomassie blue for protein or with PAS stain to detect carbohydrate (data not shown). Analysis of the proteins present in the SDS-PAGE gel of the precipitate indicated that it consisted of serum components. PAS staining indicated that carbohydrate moieties of the serum glycoproteins were still detectable with no obvious differences between serum and the precipitate. To determine if residual fibrinogen was also contributing to the precipitate by forming fibrin, FBS was incubated with 2 U thrombin at 37 °C for 48 h. No coagulation or precipitate was formed, indicating that there was no residual fibrinogen in the FBS (data not shown).

Effect of FBS on enzyme activities in late-stationary phase

Cell-associated specific enzyme activities in FBS cultures (Table 3) were initially increased (at 2.5 h and 5.5 h) from those detected at inoculation. However, over the extended time period of the culture, enzyme activities were generally reduced, though not significantly (Table 3). Cell-associated enzyme activities were not significantly different from those activities found in cultures grown at pH 7.5 on CDM when compared with any time point in the FBS culture.

Analysis of the enzyme total activities indicated that β -D-glucosidase, plasmin and Xa activities were not detected

Table 3. Cell-associated enzyme specific activities [$\text{nmol h}^{-1} (\text{mg protein})^{-1}$] of *S. gordonii* FSS2 grown on 50 % FBS, pH 7.5

ND, Not detected [$< 2 \text{ nmol h}^{-1} (\text{mg protein})^{-1}$]. Values are the mean of two assays; replicates all showed less than twofold difference.

Enzyme	0 h	2.5 h	5.5 h	24 h	72 h	96 h	116 h
β -D-Galactosidase	ND	48.5	56.2	20.9	15.5	6.7	3.5
N-Acetyl- β -D-glucosaminidase	6.2	704.5	677.2	235.8	140.5	78.1	42.3
N-Acetyl- β -D-galactosaminidase	ND	149.9	132.0	49.5	21.0	12.6	8.3
β -D-Glucosidase	ND	32.1	89.4	35.2	14.7	13.2	7.7
Thrombin	ND	23.9	68.7	183.0	165.7	130	73.1
Plasmin	2.9	8.4	13.2	4.1	ND	ND	ND
Kallikrein	16.9	ND	2.6	ND	ND	ND	ND
Hageman	1403	1130.0	1156.3	1055.4	1818.4	1214.5	779.2
Collagenase	5.0	6.9	3.1	20.3	29.2	24.4	10.5
Ca	ND	13.1	9.4	8.2	18.3	ND	9.9
Xa	2.2	ND	ND	ND	ND	ND	ND
Chymotrypsin	2.2	11.9	5.8	13.8	18.3	14.9	16.6

Table 4. Culture-supernatant enzyme total activities [$\text{nmol h}^{-1} (100 \text{ ml culture})^{-1}$] for *S. gordonii* FSS2 grown on 50 % (v/v) FBS, pH 7.5

ND, Not detected [$< 2 \text{ nmol h}^{-1} (100 \text{ ml culture})^{-1}$]. Values are the mean of two assays; all replicates showed less than twofold difference except where indicated.

Enzyme	0 h [Uninoculated 50 % (v/v) FBS]	2.5 h	5.5 h	24 h	72 h	96 h	116 h
β -D-Galactosidase	ND	ND	ND	120.6	181.3	209.1	168.5
N-Acetyl- β -D-glucosaminidase	ND	443.3	833.8	2752.2	3313.8	3733.7	4064.1
N-Acetyl- β -D-galactosaminidase	ND	58.6	116.4	574.9	645.0	756.3	732.8
β -D-Glucosidase	ND	ND	ND	ND	ND	ND	ND
Thrombin	5.7	32.0	58.1	82.3	123.6	171.6	177.2
Plasmin	ND	ND	ND	ND	ND	ND	ND
Kallikrein	ND	ND	ND	ND	ND	4.1 (2.2–5.9)	ND
Hageman	9.8	995.1	2579.6	7636.0	8445.1	10269.0	12531
Collagenase	ND	6.8 (0.0–13.6)	39.8	137.8	170.2	222.3	211.0
Ca	ND	ND	15.3	14.1 (7.8–20.4)	ND	ND	ND
Xa	ND	ND	ND	ND	ND	ND	ND
Chymotrypsin	ND	ND	ND	35.6	51.9	107.3	115.6

at any time in the culture supernatant (Table 4). Of those activities detected, there was a significant increase in both glycosidase and peptidase activities between 0 h and 96 h ($P < 0.001$); 0 h and 116 h ($P < 0.01$); 2.5 h and 96 h ($P < 0.01$); 2.5 h and 116 h ($P < 0.05$) (Table 4). When compared with the pH 7.5 cultures grown on CDM, FBS culture-supernatant activities were significantly increased at all time points after 24 h ($P < 0.05$).

DISCUSSION

S. gordonii is found as a normal commensal organism in the oral cavity and as an opportunistic pathogen in thrombotic vegetations on the heart valve. To be able to

survive and grow in these two very different conditions it must be able to adapt its physiology to the respective environments. The oral cavity, for instance, is subject to periodic high loadings of dietary carbohydrate with subsequent low pH as the carbohydrate is metabolized to its acidic by-products. In contrast, between meal times and at night during sleep, easily metabolizable carbohydrate is virtually absent and a more neutral pH occurs due to buffering by saliva. Under these conditions the bacterium must scavenge nutrients from salivary components. This study has shown that under these latter conditions glycosidase and peptidase enzymes are up-regulated. Growth of *S. gordonii* FSS2 at low pH showed that an acidic environment decreased the

production of both cell-associated protein and culture-supernatant protein. This observation was paralleled by the reduction of glycosidase and peptidase activities at low pH.

Previous chemostat studies showed that glucose, although present at a low level ($9\text{--}15\ \mu\text{g ml}^{-1}$), was still detectable in all steady-state conditions (Mayo *et al.*, 1995). In the current batch-culture studies, no glucose was detectable by stationary phase. When grown under glucose-excess/nitrogen-limited conditions, it was noted that glycosidase expression was significantly repressed whereas peptidase expression was virtually unchanged. This suggested that the presence of glucose was repressing the expression of the glycosidase enzymes. Catabolite repression of glycosidases by glucose in *S. oralis* has been previously reported (Rafay *et al.*, 1996).

In contrast to the oral environment, in an IE vegetation large fluctuations in carbohydrate concentration and therefore in acidity are unlikely to occur. The pH of the local environment would therefore be expected to be more stable with a near-neutral pH similar to that in the oral cavity in the absence of dietary carbohydrate. Even though it is not known what nutrients are available to streptococci in a platelet fibrin vegetation, serum glucose is present in the blood at $1\ \text{mg ml}^{-1}$. The relative availability of blood glucose to bacteria in a vegetation is not known; however, transmission electron microscopic images of sections through staphylococcal IE vegetations show densely packed bacterial cells embedded in an amorphous fibrous matrix, probably comprising fibrin, platelets and other host and bacterial products, with no bacteria visible on the surface (Marrie *et al.*, 1987; Ferguson *et al.* 1986). When the surface of a damaged vegetation is examined (Marrie *et al.*, 1987), the exposed subsurface shows large numbers of bacteria covered by a 'flocculent material' which has been described as being similar to the exopolysaccharide material found by Mills *et al.* (1984). Durack & Beeson (1972) have also reported that bacteria near the surface of the vegetation are metabolically active whilst those buried deeper are metabolically inactive. These findings would imply that those metabolically active bacteria in the surface layers of the vegetation are exposed to glucose from blood. However, the nutritional status of the bacteria in the bulk of the vegetation is unknown but they may be existing in near-starvation conditions. The increased expression of glycosidases at pH 7.5 and when also cultured in the presence of serum indicate an adaptive response to the available nutrients in the environment. In our studies, serum clearly up-regulates the synthesis and secretion of the glycosidases by *S. gordonii* that would be required for acquisition of oligosaccharides from host sources.

This study also showed that *S. gordonii* is able to regulate specific peptidase activities involved in clot formation [i.e. thrombin, Hageman factor (lysine peptidase) and Xa] as well as clot dissolution (i.e. plasmin, kallikrein and Ca). Hageman factor was the most

abundant activity found under any condition. It was the least affected by pH and was continuously accumulated in the culture supernatant when *S. gordonii* was grown on FBS. The ability of *S. gordonii* FSS2 to synthesize enzymes similar to those that promote the formation of clots would amplify the effect of clot formation already stimulated by the platelet-aggregating ability of this IE strain when it first enters the bloodstream (Manning *et al.*, 1994a, b). Conversely, activities that promote clot dissolution would clearly assist in the penetration and release of potential nutrients into the clot in close proximity to the bacteria. It is perhaps pertinent to speculate that clot dissolution on a larger scale may also assist in the dissemination of an IE infection to other sites in the body. IE vegetations are known to be friable and to fragment, spreading the infection to other sites where they cause abscesses in organs such as the lungs, liver and kidneys (Ferguson *et al.*, 1986).

In conclusion, the glycosidases and peptidases of *S. gordonii* have been shown to be up-regulated in neutral pH environments and in the presence of serum. The increased expression of these enzymes would greatly assist *S. gordonii* in acquiring nutrients, particularly in the environment of the IE vegetation, but also in 'healthy' dental plaque or between meal times when it relies on intrinsic sources of nutrients for survival.

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