Metabolism of glycoprotein-derived sialic acid and *N*-acetylglucosamine by *Streptococcus oralis*

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Nine strains of *Streptococcus oralis*, isolated from blood cultures of patients with infective endocarditis or from the oral cavity as part of the normal flora, were examined for their ability to elaborate sialidase (neuraminidase) and *N*-acetylglucosaminidase, enzymes which are involved in the degradation of glycoproteins. Both glycosidases were induced when bacteria were grown in a minimal medium supplemented with porcine gastric mucin, a model glycoprotein, and repressed when growth occurred in the presence of glucose. Cell-free extracts of mucin-grown cultures expressed elevated levels of *N*-acetylneuraminate pyruvate-lyase (the first intracellular enzyme in the pathway of *N*-acetylneuraminate catabolism), *N*-acetylglucosamine (GlcNAc)-6-phosphate deacetylase and glucosamine-6-phosphate deaminase (enzymes involved in the intracellular catabolism of GlcNAc 6-phosphate); activity of each of these intracellular enzymes was markedly repressed when bacteria were grown in the presence of glucose. Three strains of *S. oralis* were also grown in media supplemented with α_1 -acid glycoprotein, a major component of human plasma. Cells from these cultures expressed high levels of sialidase, *N*-acetylglucosaminidase, and the intracellular enzymes involved in the catabolism of *N*-acetyl-sugars released by the action of these glycosidases. High-resolution ¹H-NMR spectroscopy of spent culture supernatants revealed that sialic acid and GlcNAc residues of the molecularly mobile oligosaccharide side-chains of α_1 -acid glycoprotein had been hydrolysed and the released sugars internalized by the bacteria. These data indicate that *S. oralis* has the ability to hydrolyse constituents of oligosaccharide side-chains of host-derived glycoproteins and to utilize simultaneously these released carbohydrates. The biochemical characteristics induced by the growth of *S. oralis* on glycoproteins may play a role in the survival and persistence of these bacteria at the infection site *in vivo*.

Keywords: endocarditis, glycoprotein metabolism, glycosidases, ¹H-NMR, *Streptococcus oralis*

INTRODUCTION

Infective endocarditis is a potentially fatal disease of the heart valves and endocardium. Among reported cases where an organism has been isolated from blood culture, 50–60% are attributable to streptococci, and members of the viridans group account for approximately half of these infections (Manford *et al.*, 1992; Bouvet *et al.*, 1994). Until

recently, *Streptococcus oralis* and *Streptococcus sanguis*, derived from the oral cavity, were considered to be the major aetiological agents of this disease. However, in the light of the changes in the taxonomy of the viridans streptococci and improvements in the reliability of their identification (Beighton *et al.*, 1991; Killian *et al.*, 1989), it appears that the majority of these infections are associated with the presence of *S. oralis* (Bouvet *et al.*, 1994).

S. oralis is a member of the normal oral flora, and may enter the bloodstream as a result of dental procedures, most commonly tooth extraction (Knox & Hunter, 1991). Damage to the surface of the heart prior to the entry of

Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; NPL, *N*-acetylneuraminate pyruvate-lyase; PGM, porcine gastric mucin.

these organisms into the bloodstream, often as a result of a congenital defect or a previous episode of rheumatic fever, or the presence of prosthetic valves have been considered to be major, but not essential, factors in the adhesion of bacteria to cardiac tissue (Douglas, 1993). Although antibiotic prophylaxis prior to dental treatment has been shown to be effective in preventing infections in those most at risk, identifying such subjects remains a matter of some concern and mortality levels remain high (Oakley, 1979).

The precise mechanisms by which streptococci initiate endocarditis and contribute to its progression remain unclear, but have been the subject of numerous studies. Following entry into the bloodstream, bacteria must adhere to sterile platelet-fibrin vegetations on the surface of cardiac tissue and replicate, resulting in the deposition of additional platelets, consequently increasing the size of the vegetation. It has been suggested that the production of extracellular polysaccharides by streptococci plays a role in virulence in endocardial disease (Munro & Macrina, 1993). Additionally, streptococci isolated from blood culture of patients with infective endocarditis show a marked ability to bring about platelet aggregation *in vitro*, with a presumed role in attachment and increase in vegetation size at the site of infection (Ford *et al.*, 1993). These are processes involved in the streptococcal adherence to cardiac tissues and do not account for the growth of bacteria at the site of attachment. The source of nutrient which supports bacterial growth within this environment remains to be established. Serum glycoproteins should, however, provide a source of both carbon and nitrogen for bacteria with sufficient metabolic capacity to degrade these macromolecules. Glycoprotein degradation may be achieved by the concerted action of a range of specific exoglycosidase and non-specific protease activities, which release monosaccharides from the glycan side-chain and peptides from the protein backbone. These constituents may be transported and catabolized as a source of nutrient.

In the study presented, we examined the ability of nine strains of *S. oralis* (four strains isolated from the oral cavity and five from blood culture of patients with endocarditis) to elaborate sialidase and *N*-acetylglucosaminidase when grown in the presence of a model glycoprotein, porcine gastric mucin (PGM; Beighton *et al.*, 1988; Bradshaw *et al.*, 1989; van der Hoeven *et al.*, 1990). The further catabolism of the released glycoprotein-derived monosaccharides has been investigated by measuring levels of intracellular enzymes which are associated with their metabolism. Additionally, we provide data that indicate that *S. oralis* strains elaborate a similar array of enzymes when grown in the presence of an authentic host-derived glycoprotein, human α_1 -acid glycoprotein. The ability of these same strains to cleave *N*-acetylneuraminic acid (NeuAc) and *N*-acetylglucosamine (GlcNAc) from the oligosaccharide side-chains of α_1 -acid glycoprotein and internalize the released sugars has been demonstrated using high-resolution (600 MHz) proton (^1H)-NMR spectroscopy. The multicomponent analytical ability of this technique has previously been applied to a

number of biofluids, including serum and synovial fluid (Grootveld *et al.*, 1991, 1993; Naughton *et al.*, 1993; Nicholson *et al.*, 1983, 1984) and has been used here to investigate glycoprotein metabolism by *S. oralis*.

METHODS

Growth and maintenance of bacteria. Stock cultures of nine strains of *S. oralis* isolated from the oral cavity (strains S16/5, S14/8, N6 3 and N2 26) or from blood culture of patients with infective endocarditis (strains AR37, AR19, AR7, AR5 and AR3) were stored at -70°C in cryovials (Protect, Lab M). Bacteria were identified using a range of fluorogenic glycosidase assays and conventional carbohydrate fermentation tests, as previously described by Beighton *et al.* (1991). Bacterial strains were routinely subcultured onto Fastidious Anaerobe Agar (Amersham) supplemented with 5% (v/v) horse blood (FAA) and incubated in an anaerobic cabinet (Don Whitley) at 37°C for 2 or 3 d. Individual colonies of each strain were used to inoculate 20 ml volumes of pre-reduced Brain Heart Infusion broth (BHI; Oxoid) and cultures were incubated anaerobically until late-exponential phase.

Growth of bacteria in minimal medium. Minimal medium was prepared as previously described by Homer *et al.* (1993), but with all components at double concentration, and sterilized by autoclaving (121°C , 15 min). Filter-sterilized solutions (0.2 μm pore-size filters; Millipore) of MgSO_4 , cysteine.HCl and Na_2CO_3 were added to final concentrations of 0.4, 0.5 and 4.4 g l^{-1} , respectively, followed by the addition of a filter-sterilized vitamin solution (2 ml l^{-1}) (Homer *et al.*, 1993). Substrates for use in growth studies were prepared in 10 mM potassium phosphate buffer (pH 7.0) and comprised: 20 mM glucose, 20 mM GlcNAc, 20 mM NeuAc (derived from *Escherichia coli*; Sigma), 1% (w/v) PGM (Sigma) and 1% (w/v) α_1 -acid glycoprotein (from human serum; Sigma). All substrates were sterilized by filtration, with the exception of mucin, which was sterilized by autoclaving, this treatment having no effect on the availability of the carbohydrate side-chains to bacteria (Beighton *et al.*, 1988). Each substrate was mixed in equal parts with minimal medium in sterile containers (Sterilin) in 20 ml or 5 ml final volumes, with the exception of medium containing α_1 -acid glycoprotein (2 ml final volume). Aliquots (5%, v/v) of late-exponential phase BHI cultures of each strain were used to inoculate minimal media and the cultures were incubated anaerobically at 37°C for 24 or 48 h. Growth of bacteria was monitored by removing 200 μl aliquots of each culture into a 96-well microtitre plate and measuring the increase in OD_{620} using a plate-reading spectrophotometer (Titertek, Multiscan MCC340; ICN-Flow).

Preparation of bacterial cultures for enzymic and spectroscopic analyses. Aliquots (1 ml) of each culture were removed into microcentrifuge tubes with a 1.5 ml nominal volume and centrifuged (MSE Microcentrifuge, 13000 r.p.m., 10 min) to pellet cells. Supernatants were removed and stored at -20°C for subsequent analysis of glycosidase activity, residual carbohydrate and lactate concentration. Cells were resuspended in 1 ml 50 mM sodium phosphate buffer (pH 7.5), washed by centrifugation and pellets were stored at -20°C . Immediately prior to use, cells were resuspended in 1 ml of the same buffer and whole-cell suspensions were used for assay of glycosidase activity. Cell-free extracts of these same suspensions were prepared by adding ballotini (0.25 g, size 12; Jencons) to each 1 ml of cell suspension in a glass bijoux bottle and treating in a Mickle disintegrator (Mickle Engineering) for 10 min at 4°C . Cell debris was removed by centrifugation (13000 r.p.m., 5 min)

and supernatants were used as a source of intracellular enzymes of NeuAc and GlcNAc metabolism. All assays for enzyme activities were performed in duplicate and in all cases duplicate values did not differ by more than 10%. Mean values for enzyme activities are reported.

Assay of glycosidase activities. *N*-Acetylglucosaminidase and sialidase (*N*-acetylneuraminidase) activities were determined using fluorogenic 4-methylumbelliferyl (4-MU)-linked substrates (Sigma). 4-MU-GlcNAc and 2'-(4-MU)- α -D-NeuAc were dissolved in a minimal volume of dimethylsulphoxide and prepared to a final concentration of 0.1 mM in distilled water. Assays contained: 70 μ l 0.2 M sodium phosphate buffer (pH 7.5), 100 μ l substrate and 30 μ l culture supernatant or cell suspension. Control assays contained 30 μ l 50 mM sodium phosphate buffer (pH 7.5) in place of cells or supernatants. Assays were set up in 96-well microtitre trays, incubated at 37 °C and fluorescence values were recorded in a fluorimeter (Perkin Elmer LS-3B) fitted with a plate-reading attachment at excitation 380 nm and emission 460 nm. 4MU released by the action of bacterial glycosidases was quantified by comparison of fluorescence values with those obtained for standard concentrations of authentic methylumbelliferone.

Assay of *N*-acetylneuraminate pyruvate-lyase (NPL). NPL (EC 4.1.3.3; NeuAc $\rightarrow$ *N*-acetylmannosamine + pyruvate) activity was determined using a modification of the method described by Comb & Roseman (1962). Pyruvate formed by the action of NPL is converted to lactate by the action of exogenous lactate dehydrogenase with the concomitant conversion of NADH to NAD. Assays were set up in microcentrifuge tubes and contained 25 μ l 0.2 M sodium phosphate buffer (pH 7.5), 10 μ l 0.1 M NeuAc and an appropriate volume of cell-free extract in a total volume of 100 μ l. Control assays contained 10 μ l distilled water in place of NeuAc. Assays were incubated at 37 °C for 1 h and the reaction was terminated by heating at 100 °C for 3 min. Following cooling to ambient temperature, 50 μ l 0.2 M sodium phosphate buffer (pH 7.5), 50 μ l distilled water and 50 μ l 1 mM NADH were added to each assay. Duplicate aliquots (200 μ l) of each sample were dispensed into a 96-well microtitre tray and the A_{340} was recorded (Titertek Multiscan MCC340). Lactate dehydrogenase (Sigma; 0.2 units) was added to each well and trays were incubated at 37 °C. The A_{340} was measured periodically until a minimum value was recorded. The number of mol NADH converted to NAD was determined by comparison with a standard curve of the A_{340} values of different concentrations of NADH.

Assay of GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase. The two reactions GlcNAc 6-phosphate + H₂O $\rightarrow$ glucosamine 6-phosphate + acetate and glucosamine 6-phosphate + H₂O $\rightarrow$ fructose 6-phosphate + NH₃ were catalysed by GlcNAc-6-phosphate deacetylase (EC 3.5.1.25) and glucosamine-6-phosphate deaminase (EC 5.3.1.10), respectively. GlcNAc is transported via the phosphoenolpyruvate:sugar phosphotransferase system, being phosphorylated to form GlcNAc 6-phosphate, which is then catabolized. Assays for enzymes of the intracellular catabolism of GlcNAc 6-phosphate were carried out essentially as previously described by Homer *et al.* (1993). The A_{340} due to the formation of NADPH was monitored while the rate of the reaction was still linear and the number of mol NADPH formed was calculated by comparison with a standard curve.

Protein estimations. The protein concentration of whole-cell suspensions or cell-free extracts was determined using the Coomassie Blue dye-binding assay (Sigma kit no. 610-A). The estimation was carried out according to the manufacturer's instructions, except that assays were reduced proportionately to use 25 μ l sample and 1.25 ml dye reagent. The A_{580} was recorded

in a recording spectrophotometer (Shimadzu UV-160 A) and protein concentrations were calculated by comparison with a standard curve of BSA (Sigma).

Assay of residual carbohydrate and lactate. Residual GlcNAc concentrations in culture supernatants were estimated according to the method of Homer *et al.* (1993), using 4-(*N,N*-dimethylamino)-benzaldehyde reagent. GlcNAc concentrations in culture supernatants were calculated by comparison with a series of GlcNAc standards up to 1.0 mM treated in the same manner.

NeuAc concentrations were estimated using a modification of the method described by Aminoff (1961). Samples of culture supernatants (250 μ l) were added to 125 μ l 25 mM periodic acid in 62.5 mM sulphuric acid and incubated at 37 °C for 30 min. Aliquots (100 μ l) of 2% (w/v) sodium arsenite in 0.5 M hydrochloric acid were added and, following the disappearance of the yellow colour of the liberated iodine, 1.0 ml 0.1 M 2-thiobarbituric acid was mixed into the solution. Samples were heated at 100 °C for 7.5 min and, after allowing assays to cool to room temperature, the assays were extracted using 2.5 ml *n*-butanol. The assays were centrifuged and the A_{540} of 200 μ l aliquots of the organic phase of each sample was measured in a plate-reading spectrophotometer and concentrations of residual NeuAc were calculated by comparison with standards to a concentration of 1 mM.

Residual glucose concentrations in culture supernatants were determined using Sigma kit no. 510 according to the manufacturer's instructions except that reactions were scaled-down to give a final total volume of 200 μ l. The A_{450} was measured and concentrations were determined from a series of glucose standards prepared up to 1.0 mM.

The production of lactate was measured using Sigma kit no. 826-UV, in which lactate is converted to pyruvate in the presence of exogenous lactate dehydrogenase with the concomitant conversion of NAD to NADH. Samples of culture supernatants (20 μ l) were placed in a 96-well microtitre tray and 180 μ l reagent (comprising lactate dehydrogenase, NAD and buffer) was added. Assays were incubated at 37 °C for 30 min and the A_{340} was measured. Lactate standards (up to 10 mM) were treated in the same manner and the lactate concentrations in culture supernatants were calculated accordingly.

NMR spectroscopy. Supernatants from cultures of *S. oralis* grown in minimal medium supplemented with α_1 -acid glycoprotein as the major source of carbohydrate for growth and controls (uninoculated media) were thawed at ambient temperature. Aliquots (0.5 ml) were placed in 5 mm diameter NMR tubes and 0.06 ml ²H₂O was added to each to provide a field frequency lock. ¹H-NMR measurements on control samples were conducted on a Bruker AMX-400 spectrometer [University of London Inter-collegiate Research Services (ULIRS), King's College (Strand Campus), University of London] operating in quadrature detection mode at an operating frequency of 400.14 MHz for ¹H. The broad resonances arising from the protein component of the α_1 -acid glycoprotein were suppressed by the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [90°(-*t*-180°-*t*)₁₂₈, *t* = 1 ms]. ¹H-NMR measurements on spent culture supernatants were carried out using a 600 MHz facility in order to obtain increased resolution and sensitivity. Measurements were conducted on a Bruker AMX-600 spectrometer (ULIRS, Queen Mary and Westfield College Facility, University of London) operating in quadrature detection mode at an operating frequency of 600.13 MHz for ¹H. All spectra were obtained at a probe temperature of 298 K. The broad resonances arising from the protein component of the α_1 -acid glycoprotein were suppressed by the Hahn spin-echo sequence [D(90° *x*-*t*-180° *y*-*t*-collect)], which was repeated 128–256 times

with $t = 68$ ms. The intense water signal was suppressed by presaturation with gated decoupling during the delay between pulses.

Chemical shifts of resonances in spectra of culture supernatants were referenced to external sodium 3-trimethylsilyl[2,2,3,3- $^2\text{H}_4$]propionate ($\delta = 0.00$ p.p.m.). The methyl group resonances of alanine ($\delta = 1.487$ p.p.m.), lactate ($\delta = 1.330$ p.p.m.) and valine ($\delta = 1.050$ p.p.m.) served as secondary internal references.

RESULTS

Production of sialidase and *N*-acetylglucosaminidase by *S. oralis* strains

All *S. oralis* strains elaborated sialidase and *N*-acetylglucosaminidase when cultured on FAA (Table 1). Under the assay conditions, the specific activity of the sialidase

Table 1. Glycosidase activities of FAA-derived *S. oralis* cells

Activities are shown as nmol substrate hydrolysed min^{-1} (mg protein) $^{-1}$.

Strain	Sialidase activity	<i>N</i> -Acetylglucosaminidase activity
S16/5	16.8	10.6
S14/8	12.3	7.6
N6 3	5.6	4.1
N2 26	9.6	3.2
AR37	7.1	3.5
AR19	13.7	5.7
AR7	3.0	2.5
AR5	3.1	2.5
AR3	1.6	1.2

was always greater than that of the *N*-acetylglucosaminidase for individual strains, ranging from 1.6–16.8 and 1.2–10.6 nmol min^{-1} (mg protein) $^{-1}$, respectively, and there were no overall differences between strains isolated from the oral cavity or from patients with endocarditis.

The effect of growth in minimal medium supplemented with either glucose or PGM on *S. oralis* glycosidase activity is demonstrated in Table 2. Growth in the presence of glucose resulted in decreased levels of both cell- and supernatant-associated sialidase and *N*-acetylglucosaminidase activity for all strains when compared with the corresponding PGM-derived cultures. This repression of activity in glucose-grown cultures was most notable for *N*-acetylglucosaminidase. Cell- and supernatant-associated activity of this enzyme was undetectable in five and seven of the nine *S. oralis* strains, respectively, when grown in medium containing 10 mM glucose. Irrespective of culture conditions, cell-associated sialidase and *N*-acetylglucosaminidase activities were always higher than the corresponding supernatant levels, with the exception of the *N*-acetylglucosaminidase levels of PGM-grown cultures of strain AR19. In almost every case, the sialidase activity was higher than that of the *N*-acetylglucosaminidase activity for a given strain under each culture condition (Table 2), a similar pattern to that exhibited by FAA-derived cells (Table 1).

Intracellular enzymes of sialic acid and GlcNAc metabolism

All *S. oralis* strains grew in the minimal medium supplemented with glucose, reducing the substrate concentration to undetectable levels after 24 h growth. The major end-product of glucose fermentation was lactate, with all nine strains producing this metabolite to a concentration of > 10 mM (data not shown). Incomplete utilization of NeuAc was exhibited for some strains of *S.*

Table 2. Cell-associated and supernatant glycosidase activities of *S. oralis* strains grown in minimal medium supplemented with glucose or PGM

Activities are shown as nmol substrate hydrolysed min^{-1} (mg protein) $^{-1}$.

Strain	Sialidase activity				<i>N</i> -Acetylglucosaminidase activity			
	Glucose-grown		PGM-grown		Glucose-grown		PGM-grown	
	Cell-associated	Supernatant	Cell-associated	Supernatant	Cell-associated	Supernatant	Cell-associated	Supernatant
S16/5	52	26	115	79	21	19	45	35
S14/8	13	<1	34	30	<1	<1	28	27
N6 3	11	10	36	29	8	<1	93	31
N2 26	<1	<1	44	34	<1	<1	33	29
AR37	11	<1	50	39	<1	<1	36	29
AR19	8	7	37	32	<1	<1	22	42
AR7	5	<1	25	18	<1	<1	19	18
AR5	8	5	72	43	6	<1	27	21
AR3	46	14	57	37	15	10	27	25

Table 3. NPL activities of *S. oralis* strains grown in minimal medium supplemented with glucose, PGM or NeuAc

Activities are shown as nmol pyruvate formed min⁻¹ (mg protein)⁻¹.

Strain	Glucose-grown	PGM-grown	NeuAc-grown
S16/5	37	85	113
S14/8	47	210	288
N6 3	11	91	503
N2 26	25	245	562
AR37	17	226	323
AR19	7	85	78
AR7	12	227	71
AR5	27	251	335
AR3	46	165	399

oralis, but levels of this nutrient were reduced by at least 70 % following growth (data not shown). In contrast to growth in the presence of glucose, no significant increases in the concentration of lactate were measured when *S. oralis* strains were grown in media supplemented with PGM or NeuAc.

The specific activity of the intracellular NPL for the *S. oralis* strains grown in the presence of glucose, PGM or NeuAc is shown in Table 3. Glucose-grown cultures of all strains exhibited the ability to hydrolyse NeuAc into *N*-acetylmannosamine and pyruvate, but levels of activity were always considerably less than those of cells grown in the presence of PGM or NeuAc. Growth in the presence of PGM resulted in increased specific activities for NPL,

with 2.3–18.6-fold increases in levels of activity when compared with glucose-grown cultures. When the minimal medium was supplemented with NeuAc (the natural substrate for the induction of NPL activity) and used for the growth of the *S. oralis* strains, levels of enzyme activity were, in most cases, higher than those observed for the PGM-grown cultures.

When grown in culture medium supplemented with GlcNAc, all strains of *S. oralis* produced lactate as the major end-point of catabolism of this sugar (all strains producing >10 mM lactate from 10 mM GlcNAc). GlcNAc was completely metabolized within 24 h, with no residual sugar detectable in culture supernatants (data not shown). Intracellular enzymes of GlcNAc metabolism were expressed at low levels of activity in glucose-grown cultures of *S. oralis* (Table 4). The levels of GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase were elevated in both PGM- and GlcNAc-grown cultures when compared with the levels of activity in cells grown in the presence of glucose. When GlcNAc, the inducing sugar for these enzymes, was supplied as the major source of carbohydrate for growth, activities were generally higher than those observed for PGM-grown cultures. Irrespective of culture conditions, the GlcNAc-6-phosphate deacetylase specific activity was usually lower than that of the glucosamine-6-phosphate deaminase.

Effect of growth in the presence of α_1 -acid glycoprotein on levels of glycoprotein-metabolizing enzymes

S. oralis strains S16/5, N2 26 and AR3 were grown in minimal medium with α_1 -acid glycoprotein as the major source of carbohydrate. Under these conditions, sialidase and *N*-acetylglucosaminidase activities were elevated to levels higher than those observed when growth occurred

Table 4. GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase activities of *S. oralis* strains grown in minimal medium supplemented with glucose, PGM or GlcNAc

Activities are shown as nmol min⁻¹ (mg protein)⁻¹.

Strain	Glucose-grown		PGM-grown		GlcNAc-grown	
	GlcNAc-6-phosphate deacetylase	Glucosamine-6-phosphate deaminase	GlcNAc-6-phosphate deacetylase	Glucosamine-6-phosphate deaminase	GlcNAc-6-phosphate deacetylase	Glucosamine-6-phosphate deaminase
S16/5	17	65	100	77	49	147
S14/8	<1	26	10	40	83	238
N6 3	<1	8	22	33	115	268
N2 26	2	15	12	34	89	42
AR37	2	17	11	39	118	152
AR19	<1	<1	90	86	47	90
AR7	1	5	14	47	134	319
AR5	<1	4	62	41	78	494
AR3	12	72	139	261	202	235

Table 5. Effect of growth in the presence of α_1 -acid glycoprotein on the production of enzymes metabolizing *N*-acetyl-sugarsActivities are shown as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Strain	Sialidase		N-Acetylglucosaminidase		NPL	GlcNAc-6-phosphate deacetylase	Glucosamine-6-phosphate deaminase
	Cell-associated	Supernatant	Cell-associated	Supernatant			
S16/5	106	120	78	54	86	67	110
N2 26	134	93	111	75	114	36	141
AR3	73	55	45	40	256	41	122

Fig. 1. Typical 400 MHz ^1H CPMG spectrum of minimal medium supplemented with α_1 -acid glycoprotein. Val, valine- CH_3 ; Lac- CH_3 and Lac-CH, lactate- CH_3 and lactate-CH, respectively; Ala, alanine- CH_3 ; A, acetate- CH_3 ; Tyr, tyrosine aromatic ring protons; Phe, phenylalanine aromatic ring protons; Form, formate-H. The arrow denotes signals in the 2.00–2.10 p.p.m. region of the spectrum attributable to *N*-acetyl- CH_3 group protons of α_1 -acid glycoprotein. The inset shows the 1.00–3.00 p.p.m. region of the spectrum of α_1 -acid glycoprotein in phosphate buffer (pH 7.0). Resonances at 2.04 and 2.08 p.p.m. (NAc) arise from $-\text{NHCOCH}_3$ group protons of α_1 -acid glycoprotein.

in the presence of glucose (Table 5). In the majority of instances the greater proportion of the glycosidase activity was cell-associated. In parallel with the increase in glycosidic activity, growth in the presence of α_1 -acid

glycoprotein also had a significant effect on the production of intracellular enzymes of NeuAc and GlcNAc 6-phosphate metabolism (Table 5). In this case, the specific activities of NPL, GlcNAc-6-phosphate deacetylase and

Fig. 2. Typical 600 MHz ^1H Hahn spin-echo spectrum of α_1 -acid glycoprotein-supplemented minimal medium following growth of *S. oralis* strain S16/5. Ile, isoleucine- CH_3 ; Val, valine- CH_3 ; Eth, ethanol- CH_3 ; Lac- CH_3 and Lac-CH, lactate- CH_3 and lactate-CH, respectively; Ala, alanine- CH_3 ; A, acetate- CH_3 ; Form, formate-H. The arrow denotes the region of the spectrum in which *N*-acetyl- CH_3 group protons would arise. The inset shows the expanded 1.8–2.4 p.p.m. region of this spectrum. The area denoted by the arrow shows that no resonances attributable to COCH_3 group protons are detectable.

glucosamine-6-phosphate deaminase were greater than those observed when these strains were grown in the presence of glucose (Tables 3, 4 and 5).

^1H -NMR investigation of α_1 -acid glycoprotein metabolism by *S. oralis*

Fig. 1 shows the 0.00–9.00 p.p.m. regions of the 400 MHz CPMG ^1H -NMR spectrum of minimal medium containing untreated human α_1 -acid glycoprotein. The broad resonances arising from the protein moiety of the glycoprotein are suppressed and only resonances attributable to the presence of low-molecular-mass metabolites and the molecularly mobile protons of NeuAc and GlcNAc of the branching carbohydrate side-chains are detectable. The multicomponent analytical capacity of this technique is demonstrated in Fig. 1 (untreated control), in which a variety of low-molecular-mass components of the minimal medium were identifiable. In the high field (aliphatic) region of the spectrum, resonances arising from the methyl group protons of valine, lactate, alanine and acetate were readily detectable at 1.050, 1.330, 1.487 and 1.920 p.p.m., respectively. The low field (aromatic) region of the spectrum exhibited

signals in the 6.7–8.5 p.p.m. chemical-shift range attributable to tyrosine, phenylalanine and low levels of formate, the latter arising as a minor contaminant of culture media components.

Intense broad resonances at 2.00–2.10 p.p.m. (Fig. 1 and inset) arise from the *N*-acetyl- CH_3 group protons of the *N*-acetylated sugars (NeuAc and GlcNAc) which are components of the α_1 -acid glycoprotein molecularly mobile carbohydrate side-chains. The inset shows the expanded 1.00–3.00 p.p.m. region of the spectrum given in Fig. 1, the chemical-shift region in which the *N*-acetyl-sugars of the α_1 -acid glycoprotein give rise to NMR-detectable signals. Comparison of Figs 1 and 2 (control and *S. oralis* S16/5-grown supernatants, respectively) indicated that the relative peak intensities of the ethanol and formate resonances [$\delta = 1.21$ (*t*) and 8.46 p.p.m. (*s*), respectively] were substantially greater in the spent culture supernatant than in the control medium, demonstrating that these metabolites were produced during growth of the *S. oralis* strains in the medium containing α_1 -acid glycoprotein. Comparable results were obtained for *S. oralis* strains N2 26 and AR3 (data not shown). Fig. 2 demonstrates that the relative peak intensities rep-

representative of the *N*-acetyl-sugars of α_1 -acid glycoprotein (2.04 and 2.08 p.p.m.) were reduced to virtually undetectable levels in the spent culture supernatant of *S. oralis* strain S16/5. These data indicate that *S. oralis* strains cleaved the *N*-acetyl-sugars from the oligosaccharide side-chains of α_1 -acid glycoprotein. Additionally, these spectra provided no evidence of free NeuAc or GlcNAc in the culture supernatants, indicating that both of these *N*-acetyl-sugars were transported and metabolized by the bacteria.

DISCUSSION

The production of glycosidases by members of the viridans streptococci has been used as a taxonomic tool for several years, resulting in the more reliable identification of individual species (Beighton *et al.*, 1991; Killian *et al.*, 1989). Consequently, *S. oralis* is becoming recognized as an important pathogen associated with a number of infections of clinical importance, including endocarditis (Bouvet *et al.*, 1994) and septicaemia and acute lung infections in neutropaenic cancer patients (Beighton *et al.*, 1994). This species is characterized, at least in part, by its ability to elaborate sialidase and *N*-acetylglucosaminidase (Beighton *et al.*, 1991; Beighton & Whiley, 1990) and it has been suggested that these enzymes play a role in the virulence of *S. oralis* in cases of septicaemia occurring in neutropaenic cancer patients by virtue of their ability to degrade host-derived glycoproteins (Beighton *et al.*, 1994). In this study, we have investigated the production of these glycosidases and of intracellular enzymes involved in the catabolism of the released *N*-acetyl-sugars when *S. oralis* is grown in the presence of glycoproteins.

Here we have used PGM, an O-linked glycoprotein, as the major source of carbohydrates for the growth of *S. oralis* in a semi-defined medium. PGM was used as it has been used widely in other investigations with mixed bacterial cultures as a model glycoprotein to determine the ability of oral bacteria to degrade glycoproteins and to release nutrients for growth (Beighton *et al.*, 1988; Bradshaw *et al.*, 1989, 1994; van der Hoeven *et al.*, 1990). The structural complexity of all glycoproteins, including PGM, arises from the arrangement and relative content of a limited number of monosaccharides. The oligosaccharide side-chains of PGM, which consist primarily of sialic acid, GlcNAc, *N*-acetylgalactosamine, fucose and galactose, are primarily of the O-linked, or mucin-type, and are linked to the peptide backbone via the hydroxyl group of a serine or threonine residue (Montreuil *et al.*, 1994). Growth of all nine *S. oralis* strains in minimal medium with PGM as the major source of carbohydrate resulted in higher levels of sialidase and *N*-acetylglucosaminidase than when these bacteria were grown in the same medium supplemented with glucose. Observations regarding the production of glycosidases in the dental plaque of macaque monkeys have also demonstrated the increased production of these enzyme activities in the plaque of fasted animals, when bacterial growth is supported by salivary mucins, in comparison to the levels

in the plaque of monkeys receiving a conventional diet (Beighton & Smith, 1986; Smith & Beighton, 1986; Beighton *et al.*, 1986). Continuous culture studies using mixed dental plaque samples have also demonstrated increased levels of these enzyme activities when PGM was provided as the major source of carbohydrate, with a strong correlation between the level of glycosidase activity and the removal of the respective monosaccharides from PGM (Beighton *et al.*, 1988).

Growth of *S. oralis* in PGM-containing media also resulted in changes in the intracellular levels of specific enzyme activities involved in the catabolism of sialic acid and GlcNAc. Specifically, cells grown on media supplemented with PGM exhibited increased levels of NPL, the first enzyme in the intracellular catabolism of neuraminate, which catalyses the formation of *N*-acetylmannosamine and pyruvate from neuraminate. These cells also possessed elevated levels of both GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase, which are involved in the catabolism of GlcNAc 6-phosphate. The cultures grown using PGM therefore exhibited, simultaneously, the ability to catabolize both NeuAc and GlcNAc derived from PGM.

It is expected that *S. oralis* strains, when associated with systemic diseases such as endocarditis or septicaemia, will be exposed to human serum glycoproteins, including α_1 -acid glycoprotein. We therefore studied the effects of the inclusion of this glycoprotein on the production of enzyme activities by *S. oralis*. Human α_1 -acid glycoprotein contains primarily *N*-linked glycans which are similar in structure to those of PGM, except that the end adjacent to the peptide backbone is composed of mannose units and the side-chain is linked to the peptide via the amide group of an asparagine residue (Montreuil *et al.*, 1994). Three *S. oralis* strains (S16/5, N2 26 and AR3) were grown in media supplemented with α_1 -acid glycoprotein and, as with PGM, the cell- and supernatant-associated levels of sialidase and *N*-acetylglucosaminidase were increased when compared with those found for these strains grown in the presence of glucose. $^1\text{H-NMR}$ spectroscopy demonstrated both cleavage and utilization of the *N*-acetyl-sugars from the native glycoprotein. No free neuraminate or GlcNAc was detected by $^1\text{H-NMR}$ spectroscopy in the spent culture supernatants of bacteria grown in the presence of α_1 -acid glycoprotein, demonstrating that they were able to transport the released monosaccharides. In the α_1 -acid-glycoprotein-grown cells, elevated levels of the intracellular enzymes NPL, GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase, required for the intracellular catabolism of the *N*-acetyl-sugars, were demonstrable. Taken together, these data indicate that *S. oralis* strains, grown in the presence of α_1 -acid glycoprotein, possessed the metabolic capacity to degrade the glycan chains of the α_1 -acid glycoprotein and to utilize the *N*-acetyl-sugars as a source of carbon and subsequently energy for growth.

When *S. oralis* strains were grown in media supplemented with either glucose or GlcNAc, significant levels of lactate were formed as we have previously reported for

Streptococcus mutans (Homer *et al.*, 1993). However, when they were grown in media supplemented with either PGM, NeuAc or α_1 -acid glycoprotein, no significant lactate production was demonstrable, which indicates that growth of the *S. oralis* strains on the glycoproteins and NeuAc was essentially carbon (C)-limited. Although no measurements were made of any metabolic end-product except lactate in the PGM and NeuAc cultures, the ^1H -NMR spectra of the culture supernatants from the media containing α_1 -acid glycoprotein demonstrated the formation of formate and ethanol. It was not possible to determine the production of acetate due to the composition of the basal medium. The formation of these mixed end-products and no detectable lactate supports the premise that in the glycoprotein-supplemented cultures the bacteria were C-limited (Yamada & Carlsson, 1975, 1976). The C limitation most likely arose either due to the rate at which carbohydrates were released from the oligosaccharide side-chains by the action of the bacterial glycosidases or as a consequence of the low extracellular carbohydrate concentrations. If the NeuAc-grown cells were also C-limited, as would seem likely, the C-limitation may arise, not due to the low concentrations of NeuAc in the media but as a consequence of the rate of transport of NeuAc into these cells.

It has been suggested that the production of sialidase functions as a virulence determinant for a number of important human pathogens, including *Bacteroides fragilis* and *Vibrio cholerae* (Russo *et al.*, 1990; Vimr *et al.*, 1988). Its role in pathogenicity has been considered to be by virtue of its ability to destroy host tissue and disrupt a wide range of cellular functions which are mediated by glycoprotein-associated sialic acid (Schauer, 1982, 1985). We provide evidence here for an additional role for this enzyme in the disease process, namely in the nutrition and growth of bacteria. Liberation and utilization of N-acetyl-sugars (including NeuAc and GlcNAc) from host-derived glycoproteins is likely to play a role in the persistence and survival of *S. oralis* and other infecting micro-organisms *in vivo*.

We have demonstrated that when *S. oralis* strains are grown in the presence of glycoproteins (PGM or α_1 -acid glycoprotein), sialidase and N-acetylglucosaminidase are induced and simultaneously the intracellular enzymes involved in the catabolism of NeuAc and GlcNAc 6-phosphate are also induced. The regulation of production of these enzymes by *S. oralis*, grown in the presence of glycoproteins, merits further investigation.

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