PATHOGENIC MECHANISMS

Production of specific glycosidase activities by *Streptococcus intermedius* strain UNS35 grown in the presence of mucin

K. A. HOMER, R. A. WHILEY* and D. BEIGHTON

Oral Microbiology, Royal College of Surgeons Department of Dental Sciences, Faculty of Clinical Dentistry, King's College School of Medicine and Dentistry, Caldecot Road, Denmark Hill, London SE5 9RW and * Department of Oral Microbiology, London Hospital Medical College, Turner Street, Whitechapel, London E1 2AD

Summary. An isolate of *Streptococcus intermedius* from a brain abscess showed neuraminidase (sialidase), β -D-galactosidase, N-acetyl- β -D-glucosaminidase and N-acetyl- β -D-galactosaminidase activities. The optimal pH values of these enzymes were 5.5–6.0, 5.5–6.0, 5.0–5.5 and 5.0–5.5, respectively. The k_m of the enzymes varied according to whether the type of substrate was chromogenic or fluorogenic; sialidase was most active at the lowest substrate concentrations, with a k_m of 0.01 mM. In semi-defined medium, with porcine gastric mucin—a model glycoprotein—as the sole source of fermentable carbohydrate, levels of the glycosidases were significantly increased. Addition of glucose to the mucin-containing medium, or growth of cells in media supplemented with glucose alone, repressed glycosidic activities and the majority of these were cell-associated. *S. intermedius* cells from cultures grown with mucin were able, simultaneously, to transport *via* sugar:phosphoenolpyruvate phosphotransferase (PTS) systems, monosaccharides which are constituents of carbohydrate side chains of glycoproteins. These cells also possessed significant levels of neuraminase-pyruvate lyase, involved in the intracellular catabolism of neuraminic acid; this was absent from cells grown with glucose. These mechanisms, collectively, may facilitate the persistence and growth of *S. intermedius* *in vivo*.

Introduction

The “*Streptococcus milleri* group” (SMG) are members of the normal oral, urogenital and gastrointestinal flora and are isolated frequently from a wide range of clinical conditions.^{1–4} Recent taxonomic studies have indicated that the SMG is composed of three distinct species: *S. anginosus*, *S. constellatus* and *S. intermedius*.⁵ Of these, *S. intermedius* is particularly associated with deep-seated, purulent abscesses of the brain and liver.^{6,7} *S. intermedius* strains produce glycosaminoglycan-depolymerising activities, including hyaluronidase and chondroitin sulphate depolymerase,^{8–10} and glycosidic enzyme activities including β -D-galactosidase, N-acetyl- β -D-glucosaminidase, N-acetyl- β -D-galactosaminidase and neuraminidase (sialidase).^{7,11} Collectively, these activities may enable *S. intermedius* to degrade human tissue constituents and facilitate the growth of *S. intermedius* *in vivo*, and contribute to its pathogenicity.

In this investigation, the specific glycosidase ac-

tivities of *S. intermedius* strain UNS35, isolated from a brain abscess, were partially characterised and the influence of a model glycoprotein, porcine gastric mucin,^{12–14} on their production was studied. The ability of *S. intermedius* to transport glycoprotein-derived carbohydrates *via* sugar:phosphoenolpyruvate phosphotransferase (PTS) systems was also investigated to determine the potential of *S. intermedius* to transport glycoprotein-derived monosaccharides.

Materials and methods

Growth and maintenance of *S. intermedius*

Stock cultures of *S. intermedius* strain UNS35 were stored in cryostat tubes (Gibco, Paisley) containing Brain Heart Infusion Broth (BHI; Unipath Ltd, Basingstoke, Hampshire) and glycerol 50% v/v at –70°C. Bacteria were subcultured routinely on to Fastidious Anaerobe Agar (FAA; Lab M, Salford, Lancs) containing horse blood 5% v/v, and incubated at 37°C for 2 days in an anaerobic cabinet (Don

Whitley, Shipley, West Yorks) with an atmosphere of N₂ 80 %, CO₂ 10 %, H₂ 10 %.

Preparation of cell suspensions and crude cell-free extracts

Cell suspensions (OD₆₂₀ = 2.0) were prepared from 2-day-old FAA cultures by suspending cells in ice-cold 50 mM Tris-HCl buffer, pH 7.5 (Tris buffer). Cell-free extracts were prepared by dispensing 2-ml volumes into 5-ml glass screw-capped bottles and, with 0.5 g of glass Ballotini (Grade 12, BDH, Poole, Dorset), shaking them in a Mickel disintegrator (The Mickel Laboratory Engineering Company Ltd, Gomshall, Surrey) for 10 min at 4°C. Cell debris was removed by centrifugation (MSE Microfuge; 13000 rpm, 5 min, 4°C) and supernates were used in glycosidase assays.

Protein estimation

Protein concentrations of cell suspensions and extracts were determined with the Coomassie Blue dye-binding assay by means of a commercial kit (Kit No. 610-A; Sigma); all volumes were reduced proportionately. Samples (1.25 ml) of dye reagent were dispensed into disposable cuvettes (Elkay Laboratory Products, UK, Ltd, Basingstoke, Hampshire) and 25 µl of sample were added. After mixing thoroughly, the absorbance of the dye-sample mixture was determined at 595 nm. Protein concentrations were calculated by comparison with a curve constructed with bovine serum albumin (Sigma) prepared in Tris buffer.

Preparation of glycosidase substrates

Glycosidic activities were measured with chromogenic (*p*-nitrophenol-linked; pNP) and fluorogenic (4-methylumbelliferyl-linked; 4MU) substrates. The chromogenic substrates pNP-N-acetyl-β-D-galactosaminide, pNP-N-acetyl-β-D-glucosaminide, pNP-β-mannoside, pNP-α-mannoside and pNP-β-D-galactoside were prepared at 10 mM. The fluorogenic substrates 4MU-N-acetyl-β-D-galactosaminide, 4MU-N-acetyl-β-D-glucosaminide, 4MU-β-mannoside, 4MU-α-mannoside, 4MU-β-D-galactoside and 2'-(4MU)-α-D-N-acetylneuraminic acid were prepared at 1 or 10 mM. All substrates were obtained from Sigma and were freshly prepared. Each substrate was dissolved in dimethylsulphoxide (BDH) and then distilled water to give a final dimethyl sulphoxide concentration of 20 % v/v.

Determination of optimal pH values

The range of buffers used at 0.2 M comprised: citric acid-trisodium citrate, pH 3.0–6.0; NaH₂PO₄-Na₂HPO₄, pH 6.0–8.0; Tris-HCl, pH 7.5–8.9. Assays utilising chromogenic substrates contained: 35 µl of 0.2 M buffer; 10 µl of 10 mM pNP-linked substrate; 10 µl of cell suspension or crude cell-free extract; and distilled water to a final volume of 100 µl. Assays utilising fluorogenic substrates consisted of: 35 µl of

0.2 M buffer; 25 µl of 1 mM 4MU-linked substrate; 10 µl of cell suspension or crude cell-free extract; and distilled water to 100 µl. All assays were set up in duplicate in transparent 96-well microtitration trays (ICN-Flow, High Wycombe, Buckinghamshire) and were initiated by the addition of cell suspension or cell-free extract. Control assays contained all assay components except bacterial cell preparations and the total volume of these assays was maintained at 100 µl by the addition of an appropriate amount of Tris buffer. Assays were incubated at 37°C so that the rate of increase in product formation was linear with respect to time and were terminated by the addition of 100 µl of 0.5 M NaHCO₃-Na₂CO₃, pH 10.2.

The release of pNP was determined at 405 nm in a 96-well plate-reading spectrophotometer (ICN-Flow), and by comparison with the absorbance of standard concentrations of pNP treated with the NaHCO₃-Na₂CO₃ buffer. The release of 4MU was determined in a fluorescence spectrophotometer fitted with a plate-reading attachment (LS-3B, Perkin-Elmer, Beaconsfield, Herts); excitation and emission wavelengths were 380 nm and 460 nm, respectively. Concentrations of liberated 4MU were calculated by comparison of fluorescence values with those obtained from standard concentrations of 4MU treated with the NaHCO₃-Na₂CO₃ buffer.

Determination of Michaelis constants

The Michaelis constants (*k_m*) for N-acetyl-β-D-galactosaminidase, N-acetyl-β-D-glucosaminidase and β-D-galactosidase activities were determined with citrate buffer, pH 5.5. The *k_m* for neuraminidase was determined in citrate buffer, pH 6.0. Assays contained: 35 µl of 0.2 M citrate buffer; 10 µl of crude cell-free extract; variable volumes of 10 mM substrate; and water to a final volume of 100 µl. Substrates were included up to a final concentration of 5.5 mM and all assays were set up in duplicate. Control assays were included as before, and all assays were incubated at 37°C and terminated by the addition of 100 µl of 0.5 M NaHCO₃-Na₂CO₃ buffer while the rate of reaction was linear.

Units of enzyme activity

Glycosidic enzyme activities were expressed as units/mg of protein, where 1 unit was the amount of enzyme required to catalyse the production of 1 nmol of pNP or 4MU/min.

Growth of S. intermedius in semi-defined media and induction of glycosidic activities

Bacteria were grown in a semi-defined medium as described previously,^{15,16} to which either porcine gastric mucin (Sigma; a model glycoprotein) or glucose was added. Mucin was dissolved at 1 % w/v in 10 mM potassium phosphate buffer, pH 7.5, and sterilised by autoclaving at 121°C for 15 min. Glucose, in the same buffer, was sterilised by filtration (0.2-µm

Figure 1 pH Optimum of *S. intermedius* N-acetyl- β -D-glucosaminidase in crude cell-free extracts from cultures grown on FAA. Activity is given as a percentage of the maximum for each N-acetyl- β -D-glucosaminidase substrate (chromogenic or fluorogenic). Buffers: ●, citrate; ■, sodium phosphate; ▲, Tris-HCl. Substrates: —, chromogenic (pNP-linked); ----, fluorogenic (4MU-linked).

pore size filters; Sigma). *S. intermedius* grown on FAA was inoculated into 20 ml of pre-reduced BHI and incubated anaerobically at 37°C until the late exponential phase; 200- μ l samples were used to inoculate 5-ml volumes of the semi-defined media containing mucin, glucose (at final concentrations of 0.5% w/v and 10 or 50 mM, respectively) or mucin and glucose. Cultures were set up in triplicate and incubated anaerobically at 37°C for 24 h. Cells were harvested by centrifugation, washed in 50 mM sodium phosphate buffer (pH 7.5) and both culture supernates and cell pellets were stored at -20°C. Cells and culture supernates were thawed at ambient temperature and stored on ice. Cell pellets were resuspended in ice-cold sodium phosphate buffer and glycosidic activities were determined with fluorogenic substrates. Assays were performed as described above with substrates added at 0.25 mM, except for 2'-(4MU)- α -D-N-acetylneuraminic acid which was added at 25 μ M, and the specific activities were calculated.

Determination of phosphoenolpyruvate:sugar phosphotransferase (PTS) activities

S. intermedius was grown anaerobically for 18 h in

pre-reduced semi-defined medium supplemented with mucin 0.5% or 10 mM glucose. Cells were harvested by centrifugation (4000 rpm, 10 min), washed by centrifugation in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) and resuspended to give an $OD_{620} = 2.0$. Cells were decriptified by adding toluene:acetone (1:4) 1% v/v and mixing at maximum speed for 90 s on a vortex mixer. PTS activities were determined by the method of Slee and Tanzer.¹⁷ Assays contained: 25 mM potassium phosphate buffer, pH 7.0; 1 mM phosphoenolpyruvate; 2 units of lactate dehydrogenase; 0.1 mM NADH; 1 mM $MgCl_2$; 10 mM sodium fluoride; an appropriate volume of decriptified cell suspension; and 10 mM monosaccharide; in a total volume of 1 ml. All monosaccharides were prepared in distilled water; they were glucose, galactose, mannose, fucose, N-acetylglucosamine, N-acetylgalactosamine and, as a negative control, mannitol.

Assay mixtures (without carbohydrate) were prepared in disposable UV-transparent cuvettes (Elkay) and pre-incubated at 37°C for 10 min. Reactions were initiated by the addition of monosaccharides and oxidation of NADH was monitored at 340 nm. Each assay was performed in duplicate; distilled water

replaced the carbohydrate solution in control assays. The number of moles of NADH oxidised in each reaction was calculated by reference to a standard curve and specific activities of PTS systems, as nmol of NADH oxidised/min/mg of protein, were calculated.

Assay of N-acetylneuraminase-pyruvate lyase activity

S. intermedius was grown in the semi-defined medium supplemented with mucin 0.5% w/v or 10 mM glucose, washed by centrifugation and stored at -20°C . Assays contained: 25 mM potassium phosphate buffer, pH 7.0, 2 units of lactate dehydrogenase; 0.1 mM NADH; an appropriate volume of cell-free extract prepared from the frozen cell pellets; and 10 mM N-acetylneuraminic acid (Sigma); in a total volume of 1 ml. Assays were pre-incubated at 37°C for 10 min. Reactions were initiated by the addition of N-acetylneuraminase and the oxidation of NADH was monitored at 340 nm. Control assays contained distilled water in place of N-acetylneuraminic acid and specific activities were expressed as μmol of NADH oxidised/min/mg of protein.

Results

Optimal pH values of glycosidases

A typical profile of the relationship between pH and N-acetyl- β -D-glucosaminidase activity of cell-free extract is shown in the figure. The pH profiles for the other glycosidases produced by *S. intermedius* strain UNS35 indicated that the optimal pH value was between 5.0 and 5.5 for both N-acetylglucosaminidase and N-acetylgalactosaminidase and between 5.5 and 6.0 for β -D-galactosidase and neuraminidase (table I). Optimal pH values were identical with both chromogenic and fluorogenic substrates and the pH profiles for whole cells were indistinguishable from those of crude cell-free extracts (data not shown). Each glycoside functioned over a wide pH range. N-acetylglucosaminidase and N-acetylgalactosaminidase exhibited $> 50\%$ activity over the pH range 4.5–6.5, β -D-galactosidase in the range pH 5.0–7.2 and neuraminidase in the range pH 4.8–7.1 (table I). However, the absolute activity of each glycosidase was dependent upon the leaving group of the synthetic substrate (table II). The specific activity of the β -D-galactosidase enzyme was the highest and that of the neuraminidase the least, irrespective of whether the substrate used was chromogenic or fluorogenic. Activities of β -D-mannosidase and α -D-mannosidase were very low; < 0.1 units/mg of protein were detected with both chromogenic and fluorogenic substrates.

k_m Determinations

The k_m values varied markedly among glycosidases. Neuraminidase had the greatest affinity for the substrate, as demonstrated by the low value of k_m , whereas

β -D-galactosidase had the least, irrespective of the leaving group (table III); k_m values were consistently lower with fluorogenic substrates.

Induction of glycosidases

Cultures of *S. intermedius* grown with mucin exhibited high levels of β -D-galactosidase, N-acetyl- β -D-glucosaminidase, N-acetyl- β -D-galactosaminidase and neuraminidase (sialidase) activities, but no β -mannosidase or α -mannosidase activities were detected (table IV). Addition of 10 mM glucose to the mucin-containing medium decreased the levels of supernate and cell-associated glycosidases and the repression of glycosidase activities was most pronounced in cultures containing 50 mM glucose. Cells from cultures grown with glucose had specific activities 20% of those found in cells grown with mucin and

Table I. Optimal pH values of *S. intermedius* glycosidases

Enzyme	pH value	
	Optimal	Range*
β -D-galactosidase	5.5–6.0	5.0–7.2
N-acetyl- β -D-glucosaminidase	5.0–5.5	4.5–6.5
N-acetyl- β -D-galactosaminidase	5.0–5.5	4.5–6.5
Sialidase	5.5–6.0	4.8–7.1

* Range within which enzyme expressed $> 50\%$ of maximal activity.

Table II. Specific activities* of *S. intermedius* UNS35 glycosidases

Enzyme	Activity with pNp-linked substrate	Activity with 4MU-linked substrate
β -D-galactosidase	935	627
N-acetylglucosaminidase	569	414
N-acetylgalactosaminidase	139	136
Neuraminidase	...	32
α -mannosidase	< 0.1	< 0.1
β -mannosidase	< 0.1	< 0.1

* Crude cell-free extracts were prepared from cultures of *S. intermedius* UNS35 grown on FAA. Chromogenic substrates and fluorogenic substrates were included in the assays at final concentrations of 1 mM and 0.5 mM, respectively. Assays were performed at optimum pH and activities are expressed in units/mg of protein.

Table III. k_m of glycosidases produced by *S. intermedius*

Enzyme	k_m (mM) with pNp-linked substrate	k_m (mM) with 4MU-linked substrate
β -D-galactosidase	1.56	0.17
N-acetylglucosaminidase	0.17	0.04
N-acetylgalactosaminidase	0.38	0.09
Neuraminidase	—	0.01

Chromogenic and fluorogenic substrates were included in the assays at concentrations up to 5.5 mM. Data are shown for each enzyme at its optimum pH (figure).

Table IV. Effect of glucose and mucin on the production of glycosidases by *S. intermedius*

Glycosidase	Activity (units/mg of protein) after growth with				
	Mucin	Mucin + 10 mM glucose	Mucin + 50 mM glucose	10 mM glucose	50 mM glucose
Sialidase					
cell-associated	42	8	1	14	3
supernate	17	4	< 1	< 1	1
β -D-galactosidase					
cell-associated	297	181	12	53	34
supernate	186	43	< 1	1	6
β -N-acetylglucosaminidase					
cell-associated	377	245	37	74	39
supernate	228	47	3	22	19
β -N-acetylgalactosaminidase					
cell-associated	214	106	11	42	41
supernate	64	12	1	4	6

Table V. PTS activities of cells of *S. intermedius* grown with glucose or mucin

Monosaccharide in assay	Activity (nmol of NADH oxidised/min/mg of protein) after growth with	
	glucose	mucin
Glucose	28.4	43.5
N-acetylglucosamine	25.3	40.3
N-acetylgalactosamine	< 1	< 1
Fucose	< 1	< 1
Galactose	< 1	3.7
Mannose	33.6	49.1
Mannitol	< 1	< 1

glycosidic activities were significantly less in culture supernates from these cultures.

Sugar PTS activities

Cells of *S. intermedius* grown with glucose transported glucose, N-acetylglucosamine and mannose at comparable rates (table V) but N-acetylgalactosamine, fucose and mannitol were not transported. Cells grown with mucin retained the ability to transport glucose, N-acetylglucosamine and mannose at increased rates and additionally exhibited a low level of PTS activity for galactose.

Neuraminate-pyruvate lyase activity

The ability of *S. intermedius* to utilise N-acetylneuraminate was investigated indirectly by determining the level of intracellular neuraminate-pyruvate lyase. No significant activity (< 1 nmol/min/mg) was detected in cell-free extracts of cells grown with glucose but considerable activity (93 nmol of NADH oxidised/min/mg of protein) was detectable in cells grown with mucin.

Discussion

S. intermedius strain UNS35 produced β -D-galactosidase, N-acetyl- β -D-glucosaminidase, N-acetyl- β -D-galactosaminidase and neuraminidase (sialidase) but not α - or β -mannosidase. Studies with oral streptococci have indicated that the ability to elaborate these activities, determined with synthetic substrates, was a measure of the ability of these bacteria to degrade oligosaccharide side chains of native glycoproteins.^{12, 13, 18-20} Therefore, such activities should provide *S. intermedius* with sufficient metabolic capability to degrade the greater proportion of the external portions of oligosaccharide side-chains of glycoproteins. Many of the host-derived glycoproteins likely to be encountered by *S. intermedius* *in vivo* are of the "mucin-type", in which the oligosaccharide chains are conjugated *via* O-glycosidic linkage to a serine or threonine residue of the protein backbone.²¹ Among the principle monosaccharides of these glycans are sialic acid, galactose, N-acetylglucosamine and N-acetylgalactosamine. The range of exoglycosidic enzymes investigated here was chosen to reflect the composition of these molecules.

The optimal pH values of these enzymes were below neutrality (pH 5.0-6.0), but each was active over a wide pH range. This may be advantageous *in vivo*, ensuring the ability to degrade host tissue components in a range of environmental conditions, ranging from deep-seated purulent abscesses to the healthy tooth surface. The k_m of these enzymes varied according to the type of substrate used. Of interest is the low k_m , and therefore high affinity, of the sialidase for its substrate. The k_m for the *S. intermedius* sialidase is lower than that reported for the enzyme from *Vibrio cholerae*, *Clostridium perfringens* or *Arthrobacter urea-fasciens*.²¹ This enzyme has been implicated as a bacterial virulence determinant for a number of species including *V. cholerae* and *Bacteroides fragilis*.^{22, 23} The high affinity of the sialidase for the substrate is

important because of the role of sialic acids in a range of essential cell functions and because sialic acids always occupy terminal positions on oligosaccharide sidechains. Their removal is a primary event in glycoprotein deglycosylation.²⁴

Porcine gastric mucin has been used previously as a model glycoprotein because of its marked structural similarity to certain human glycoproteins. When grown in minimal medium supplemented with mucin as a sole source of fermentable carbohydrate, significantly raised levels of β -D-galactosidase, N-acetyl- β -D-glucosaminidase, N-acetyl- β -D-galactosaminidase and neuraminidase were expressed by *S. intermedius*. Glucose markedly repressed these glycosidic activities. Therefore, it is likely that, *in vivo*, where levels of free fermentable carbohydrate are low, these glycosidic enzymes may be fully derepressed. The expression of these activities within tissues would facilitate bacterial survival in the way suggested for *S. oralis* associated with septicaemia in neutropenic cancer patients.²⁵

The ability of bacteria to attach to host tissue²⁶ and to produce enzyme activities with the potential to liberate monosaccharides from glycoproteins is unlikely, alone, to be sufficient to ensure abscess formation. The bacteria must also be able to utilise liberated host-tissue components. Therefore, the ability of *S. intermedius*, grown with glucose and mucin, to transport *via* PTS systems the principal carbohydrates of oligosaccharide sidechains was also studied. Cultures grown with glucose exhibited PTS activities for glucose, N-acetylglucosamine and mannose, as reported

previously for *S. mutans* and *S. sobrinus*.¹⁶ N-acetylglucosamine, mannose and glucose are probably taken up by a common PTS system.²⁷ Cells grown with mucin transported these sugars at elevated rates and, in addition, exhibited low PTS activity with galactose, as has been reported with lactobacilli.^{27, 28} The possibility that galactose may also be transported *via* transport mechanisms other than the PTS was not investigated in this study.

Growth of *S. intermedius* in the presence of mucin induced neuraminate-pyruvate lyase activity. Direct transport of sialic acid into *S. intermedius* cells was not measured, but sialic acid is transported *via* specific permeases which are independent of phosphoenolpyruvate in other genera.²⁹ However, neuraminate-pyruvate lyase initiates the catabolism of neuraminate to pyruvate and N-acetylmannosamine. The pattern of induction of this enzyme in *S. intermedius* has also been reported for *Escherichia coli*, in which neuraminate-pyruvate lyase activity is induced only during growth on a sialic acid-containing substrate.^{30, 31}

These data indicate that *S. intermedius* grown with mucin exhibits increased levels of glycosidase activities and also the ability to transport simultaneously, and potentially utilise, the principal carbohydrate moieties of glycoprotein oligosaccharide side chains. These abilities might confer an advantage *in vivo* and contribute to the ability of *S. intermedius* to grow at sites anatomically removed from its normal habitat, the oral cavity.

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