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Note

Evidence for mannosidase activities in *Streptococcus oralis* when grown on glycoproteins as carbohydrate source

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Abstract

Streptococcus oralis when cultured using ribonuclease B as the sole source of carbohydrate, selectively uses the sugars of the Man_5 glycoform as shown by HPAEC and MALDI–TOF mass spectrometric analyses. The organism is able to do this by producing novel α-(1→3), α-(1→6) and β-(1→4) mannosidase activities and these act in a concerted manner in what appears as a single-step process. The selective utilisation of Man_5 is explained by the absence of an α-(1→2) mannosidase which is required to initiate breakdown of the glycan chains present in the other glycoforms which are components of the glycoprotein. © 1998 Elsevier Science Ltd. All rights reserved.

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Streptococcus oralis is a major pathogen causing septicaemia in immunocompromised and neutropenic patients and those with malignancies. These infections are associated with complications including Adult Respiratory Distress Syndrome (ARDS), streptococcal shock and endocarditis which may result in high mortality rates [1–4]. In order to better understand how *S. oralis* proliferates in vivo, where carbohydrates which are essential for growth may be acquired from host glycoproteins, we have investigated and reported upon the manner by which the bacterium sustains itself in vitro using α_1-acid glycoprotein (AGP) as a model [5,6]. AGP is a heavily glycosylated (~ 40% carbohydrate by weight), acute phase glycoprotein possessing five complex-type, sialylated N-glycans, some containing sialyl Lewis x structures [7–9]. Our previous investigation found that *S. oralis* proliferated by degrading the glycan chains of AGP with α-sialidase, β-galactosidase, β-*N*-acetylglucosaminidase, α-fucosidase and α-and β-mannosidases. These enzymes released all monosaccharides except for protein bound GlcNAc which is *N*-linked to asparagines. Fluorogenic substrates failed however to show that fucosidase and mannosidase activities are produced by the organism. On occasions synthetic substrates are not able to demonstrate the presence of glycosidases, the recognition and action by these enzymes

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requiring more than a monosaccharide residue attached to a relatively simple aglycone, i.e., there can be a requirement for a certain oligosaccharide structure and perhaps a specific location within a polypeptide chain. From these earlier studies the production of mannosidases by *S. oralis* [specifically α-(1→3), α-(1→6) and β-(1→4)] was inferred from compelling circumstantial evidence. Mannosidase activities in *S. oralis* have, we believe, not been previously observed and have only rarely been reported to be present in other viridans streptococci [10]. In order to obtain further and more direct evidence for the production of mannosidases and to investigate their actions, in vitro growth of *S. oralis* was studied using bovine pancreatic ribonuclease B (RB) as the sole source of fermentable carbohydrate and these results are presented here. RB is a 15–16 kDa glycoprotein possessing a single *N*-glycosylation site and comprises a family of high-mannose type glycoforms (Fig. 1). The glycoforms Man_5 and Man_6 predominate, the oligosaccharide of the former terminating in α-(1→3) and α-(1→6) linked Man residues. Man_6 and the minor glycoforms (Man_7, Man_8 and Man_9) possess additional terminal α-(1→2) linked Man [11]. In order for *S. oralis* to utilise the monosaccharides of RB, bacterial exoglycosidase(s) capable of cleaving at least some of the terminal Man residues is therefore a requirement.

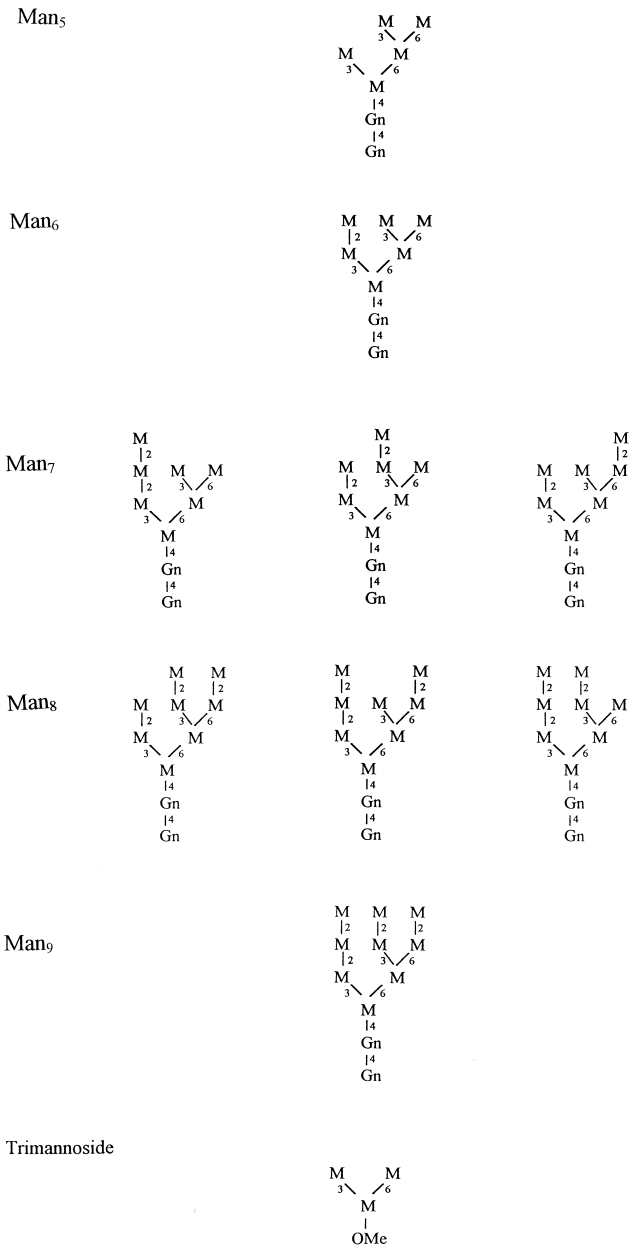

Fig. 1. The oligosaccharide structures present in the different glycoforms of RB [11] and of the core trimannoside. The monosaccharide abbreviations are: M = mannose, Gn = *N*-acetylglucosamine. All mannose residues except those connected to *N*-acetylglucosamine and *O*-methyl have the α configuration. The numbers indicate position of glycosidic linkage.

1. Results and discussion

It was found that *S. oralis* was able to grow under conditions where RB provided the sole source of fermentable sugar, although, as expected because of its lower carbohydrate content, growth was not as prolific on RB as on AGP. For comparison, under similar conditions, the 24 h post inoculum A_{620} had increased by 0.07 with RB and by 0.295 with AGP using the same concentration of each glycoprotein.

Oligosaccharides derived from RB were not detected in the culture medium indicating the absence of endoglycosidase activities. Following growth for 24 h on RB, glycans remaining on treated RB were removed by hydrazinolysis [12] and compared by HPAEC [13] with those obtained from control RB (Fig. 2). Man_6–Man_9 oligosaccharides were present after growth but the Man_5 oligosaccharide was virtually absent (< 7% of that originally present). Smaller saccharides such as chitobiose or those which would be expected from Man_1–Man_4 were not detected. MALDI spectra confirmed the absence of Man_5 derived and smaller glycans in these pools of oligosaccharides and the presence of those glycans derived from Man_6–Man_9 (Fig. 3). Identical results were obtained when the glycans were removed enzymatically with

PNGaseF (data not shown).[1] These results demonstrate that the organism was unable to utilise those glycoforms which possess a terminal α-(1→2) Man residue, i.e., the organism was only able to utilise carbohydrates present in the Man_5 glycoform. Further evidence of this selectivity was obtained from MALDI analysis directly on the RB-supplemented culture medium during growth. This showed over time (Fig. 4) reduced amounts and eventually the absence of the Man_5 glycoform with the formation of a new major species corresponding to ribonuclease A (the non-glycosylated form of RB) containing a single GlcNAc residue (mass 13,885) together with a new minor species containing two GlcNAc residues (mass 14,089). Man_6–Man_9 were present throughout growth and in addition species corresponding to Man_1–Man_4 (masses 14,250–14,737) were absent in the spectra. This suggests that the loss of Man from Man_5 occurs in an apparently single step and in addition demonstrates that none of the other glycoforms are affected by the action of α-(1→3) and α-(1→6) mannosidases. If they were then Man_3 and Man_4 would be produced from Man_6 and from one of the Man_7 isomers. The action of these mannosidases appears therefore to be inhibited by the proximity of any α-(1→2) linked Man. It is noteworthy that the trimannoside methyl 3,6-di-*O*-α-D-mannopyranosyl β-D-mannopyranoside (Fig. 1)

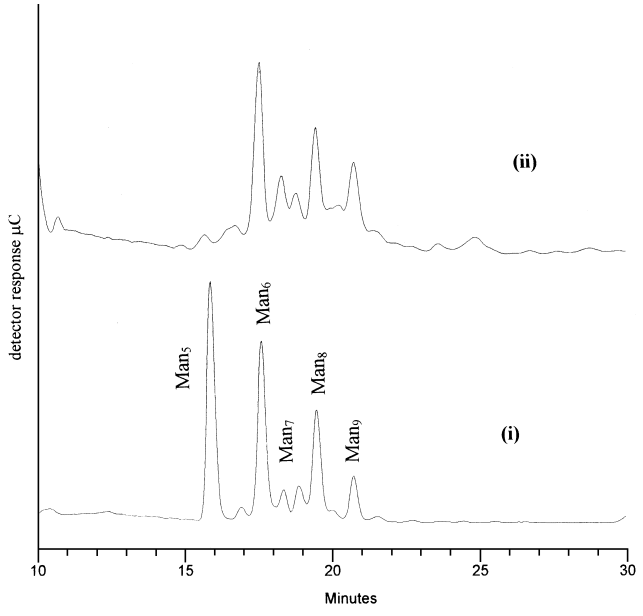

Fig. 2. HPAEC–PAD analyses of glycans during growth of *S. oralis*. The glycans were released by treatment with hydrazine, as described in Experimental from (i) control RB, (ii) 24 h *S. oralis* treated RB. The oligosaccharide species of Man_5–Man_9 are indicated.

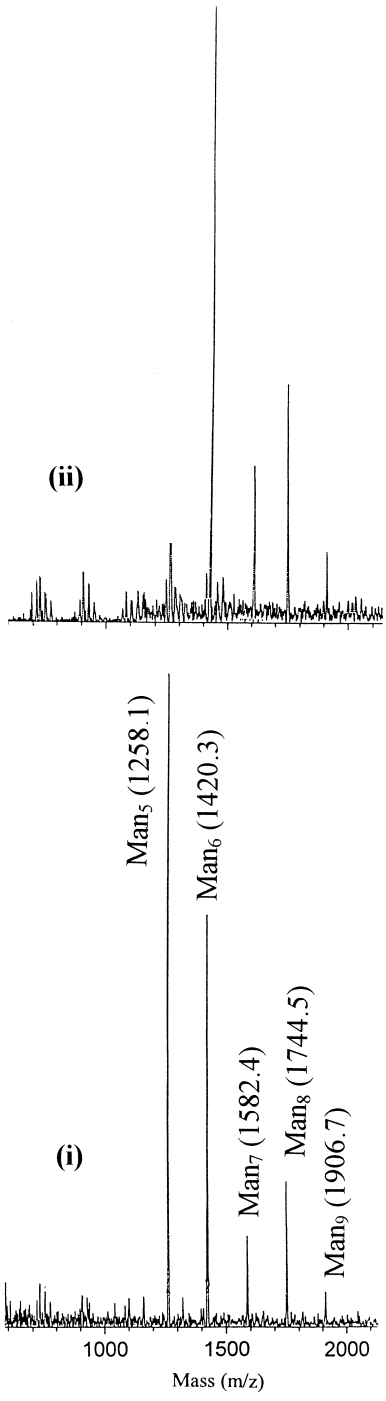

Fig. 3. MALDI spectra of glycan pools obtained from hydrazinolysis of (i) control RB and (ii) 24 h *S. oralis* treated RB. The matrix used was 2,5-dihydroxybenzoic acid. The glycan species are indicated with their calculated average mass in parenthesis. Observed values were within 0.5% of these values.

[1]The absence of smaller saccharides in the glycan pools prepared during the growth of *S. oralis* on AGP was also noted. The pentasaccharide core ($Man_3GlcNAc_2$) was (apart from the terminal GlcNAc) the smallest saccharide detected by either HPAEC or MALDI analysis [6].

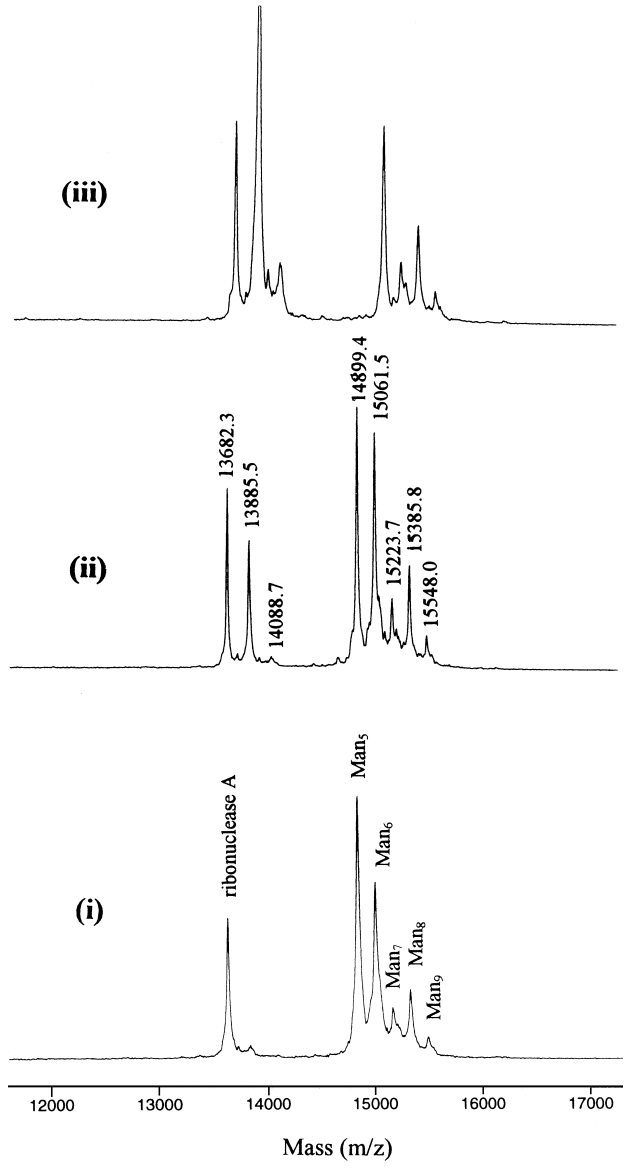

Fig. 4. MALDI spectra of RB of (i) control RB, (ii) 4 h *S. oralis* treated RB, (iii) 24 h *S. oralis* treated RB. The glycoforms are indicated in (i) and their average masses are shown in (ii). The matrix used was sinapinic acid. RB obtained from Sigma contains ribonuclease A [13]. The ion observed at *m*/*z* 14088.7 corresponds to the species containing two GlcNAc and no Man residues.

which is the methyl glycoside of the core trimannoside in N-linked glycans was unable to sustain growth of *S. oralis* and this trisaccharide glycoside was also unaffected by a culture of the organism which had grown with AGP as nutrient and in which mannosidases would be expected to be present. This lack of sustainable growth indicates that a structure larger than this trimannoside is necessary for mannosidase recognition. Such structural requirements are not unusual—molecular recognition by the endoglycosidase PNGase F when cleaving N-linked glycans, for example, requires not only an oligosaccharide chain lacking α-(1→3) core Fuc, but in addition the participating asparagine must be peptide bonded at both its amino and carboxyl termini.

The results presented here show that for *S. oralis*:

- endoglycosidase activity is absent since the culture media contained
  - (a) no free oligosaccharides
  - (b) intact Man_6–Man_9 glycoforms with their oligosaccharides remaining covalently attached to undegraded polypeptide
  - (c) a species corresponding to ribonuclease A carrying two GlcNAc residues which would result from the action of exo- but not endo-glycosidase activity
- the organism produces α-(1→3), α-(1→6), β-(1→4) but not α-(1→2) mannosidase activities
- the mannosidase activities act in a concerted manner with the individual residues of the glycans terminating in α-(1→3) and α-(1→6) Man only being liberated in what appears as a pseudo single-step process. This is borne out by the absence during growth, when Man_5 was still present, of species of glycoproteins (or derived glycans) with structures intermediate between Man_5 and those containing only GlcNAc.

The manner by which *S. oralis* degrades and utilises N-glycans of glycoproteins in vitro may be an important factor for the survival and proliferation of the organism in vivo. The action of mannosidases described here may also be operational in other bacteria and further studies will, it is hoped, provide more detailed mechanisms of their action. A clearer understanding of these mechanisms may then lead to the development of novel intervention strategies to combat these diseases and such strategies could be especially important because of the increasing incidence of antibiotic resistance of pathogenic bacteria [14].

2. Experimental

Materials.—PNGase F was obtained from New England BioLabs, Hitchin, UK. Methyl 3,6-di-*O*-α-D-mannopyranosyl β-D-mannopyranoside was obtained from Glycorex Fine Chemicals S-223 70

Lund, Sweden. 48% w/v NaOH and NaOAc were purchased from Fisons, Loughborough, UK. Polyacrylamide desalting column was from Pierce and Warriner Ltd., Chester, UK. Ultra-free (5000 NMWL) centrifugal filters were obtained from Millipore Products Ltd., Bedford, UK. All other materials and substrates including bovine pancreatic ribonuclease B were purchased from Sigma, Poole, UK. unless otherwise stated.

Methods.—Bacterial strain and culture. *S. oralis* strain AR3, isolated from subacute infective endocarditis, was routinely cultured on Fastidious Anaerobe Agar supplemented with 5% (v/v) defibrinated horse blood and incubated in an anaerobic atmosphere comprising 10% H_2, 80% N_2 and 10% CO_2 at 37 °C for 24 h. Single colonies were removed into 20 mL volumes of Brain Heart Infusion broth (BHI) and incubated until bacterial growth reached late log-phase. Minimal medium, in which streptococcal growth is dependent on the addition of a source of fermentable carbohydrate, was prepared at double strength, as previously described [15]. Minimal medium was supplemented with RB to a final concentration of 5 mg/mL in culture volumes of up to 2 mL and inoculated with 5% (v/v) of BHI starter culture of *S. oralis*. Cultures were incubated anaerobically for periods of up to 24 h and aliquots removed at intervals for determination of cell growth and glycan analyses. Cell growth was monitored by measuring the A_{620} of 200 μL aliquots of cultures in a 96-well plate-reading spectrophotometer (MCC340; ICN-Flow). Prior to further analyses, cells were pelleted from culture by centrifugation at 14,600 ***g*** for 5 min. The culture supernatant was then heated at 100 °C for 15 min to inactivate glycosidases. Aliquots of whole culture supernatant were retained for the direct analysis of free oligosaccharides and MALDI analysis and the remainder was stored at −20 °C prior to desalting by gel filtration, and oligosaccharide analyses.

Preparation of culture supernatants prior to oligosaccharide analyses. Glycoprotein-containing culture supernatants (500 μL aliquots) were applied to a 5 mL polyacrylamide, desalting column (6 kDa cut-off) equilibrated in and eluted with 0.2 M ammonium bicarbonate (pH 8.0). The void and included volumes were determined by calibrating with blue dextran and NaCl, respectively. The void volume fractions which contained all residual glycoprotein (confirmed by the BCA protein assay [16]), were pooled, lyophilised (three times) to remove ammonium bicarbonate, and stored at −20 °C prior to analyses.

Release of oligosaccharides by treatment with hydrazine. Glycoprotein dried under vacuum over phosphoric oxide was treated with anhydrous hydrazine (100 μL) at 95 °C for 4 h. The reaction mixture was processed as described [17] and the freed oligosaccharides analysed by HPAEC and MALDI.

Release of RB oligosaccharides by treatment with PNGase F. This was essentially carried out using the suppliers protocol. Briefly, the culture supernatant, following heat inactivation, was subjected to denaturing conditions (0.5% SDS, 1% β-mercaptoethanol, 100 °C, 10 min) and then treated with PNGase F (100 NEB units) in 0.05 M phosphate buffer (pH 7.5) at 37 °C for 4 h. The solution was then centrifuged using a 5000 NMWL centrifugal filter and the flow through fraction was further purified by passage through a C_{18} reversed phase cartridge (Waters) prior to analysis of the aqueous fraction by HPAEC and MALDI.

High pH anion exchange chromatographic analysis. HPAEC was performed using a Dionex DX500 system fitted with gradient pumps and a PAD detector (Dionex, UK). Data were collected and analysed using the Dionex Peaknet software. Eluents comprised 200 mM NaOH and 1.0 M NaOAc prepared in 18 MΩ water and these were degassed by sparging with helium for 30 min prior to chromatographic analyses. An appropriate volume of each sample was injected onto the column from an AS3500 autosampler (Thermoseparations, UK) equipped with a 100 μL sample loop. All separations were performed at a flow rate of 1 mL/min at ambient temperature. Oligosaccharides were analysed in 100 mM NaOH using a gradient of NaOAc (20–100 mM, 0–30 min).

MALDI analysis. This was performed with pulsed extraction on a Kratos Maldi 4 mass spectrometer, Kratos Analytical Ltd., Manchester, UK, and with delayed extraction on a Perseptive Voyager Elite (Perseptive Biosystems Ltd, Framingham, USA) operating in positive-ion and linear modes. Spectra were acquired following irradiation of samples with a nitrogen laser giving a 337 nm output with 3 ns pulse width and molecular ions accelerated at a potential of 20 kV. Pools of oligosaccharides released from approximately 20 nmol of glycoprotein were dissolved in 0.5 mL of 1% aqueous TFA and 0.5 μL of this was mixed with an equal volume of matrix solution on the target plate. The matrix solution for oligosaccharide

analysis was 2,5-dihydroxybenzoic-acid 10 mg/mL in 0.1% aqueous TFA/acetonitrile (2/1, v/v) and for glycoprotein analysis sinapinic acid 10 mg/mL in 0.1% aqueous TFA/acetonitrile (2/1, v/v). Bovine pancreas insulin (mass 5734.6) and bovine ribonuclease A (mass 13,682.3) were used as external standards for instrument calibration.

Acknowledgements

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