

BACTERIAL METABOLISM

Distribution of endo- β -N-acetylglucosaminidase amongst enterococci

G. ROBERTS, K. A. HOMER, E. TARELLI, J. PHILPOTT-HOWARD, L. A. DEVRIESE* and D. BEIGHTON

Oral Microbiology, Guy's, King's and St Thomas' Dental Institute, King's College London, London, SE5 9RW, UK and *Faculty of Veterinary Medicine, University of Gent, Gent, Belgium

Enterococci are becoming increasingly important nosocomial pathogens, a fact mainly attributed to their antimicrobial resistance profiles. However, the enzymic activities required for these organisms to proliferate *in vivo* have received little attention. *Enterococcus faecalis* has been shown previously to produce an endo- β -N-acetylglucosaminidase activity which cleaves high mannose-type glycans in glycoproteins between the N-acetylglucosamine residues of the pentasaccharide core. This study investigated the distribution of this endoglycosidase activity amongst the other enterococcal species. Ribonuclease B, a high mannose-type glycoprotein, was used as a substrate and endoglycosidase activity was demonstrated by a combination of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry and high pH anion-exchange chromatography. Endo- β -N-acetylglucosaminidase activity was present in 10 of the 18 enterococcal species isolated from both human and animal sources, including all *E. faecalis* strains. The most notable exception was the lack of this activity in all *E. faecium* isolates tested. All enterococcal species possessing endoglycosidase activity utilised the liberated glycans to support bacterial growth.

Introduction

Enterococci are ubiquitous in nature, and are isolated mainly from the gastrointestinal tract of man and other animals including birds and insects, with some species also isolated from soil, plants and water [1, 2]. Formerly classified as group D streptococci along with *Streptococcus bovis*, enterococci were transferred to a separate genus in the mid-1980s based on 16S rRNA sequence analysis [3]. There are currently 18 recognised enterococcal species: *E. asini*, *E. avium*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. dispar*, *E. durans*, *E. faecalis*, *E. faecium*, *E. flavescens*, *E. gallinarium*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, *E. raffinosus*, *E. saccharolyticus* and *E. sulfureus* [4, 5], which can be divided into five phenotypic groups based on acid formation from mannitol and sorbose and the hydrolysis of arginine [5].

Over the last two decades the enterococci have increased in importance as nosocomial pathogens,

which has mainly been attributed to their resistance to a wide range of clinically relevant antibiotics. *E. faecalis* and *E. faecium* account for *c.* 95% of all human enterococcal infections [6], although all species of enterococci – with the exception of *E. mundtii* – have been isolated from human blood cultures [7]. Enterococci are capable of causing a wide range of diseases including urinary tract and soft tissue infections, post-operative endophthalmitis, bacteraemia and endocarditis [8–10].

Previous work has revealed the presence of an extracellular endo- β -N-acetylglucosaminidase activity in *E. faecalis* which cleaves high mannose-type glycans between the two N-acetylglucosamine residues of the pentasaccharide core (Fig. 1). The liberated glycans were subsequently degraded and the released monosaccharides (mannose and N-acetylglucosamine) were utilised for bacterial growth [11]. The present study investigated the distribution of this endo- β -N-acetylglucosaminidase activity among the other enterococcal species with ribonuclease B (RNAase B) as a substrate. RNAase B (14.9–15.5 kDa) possesses a single N-glycosylation site occupied by one of a family of five high mannose-type glycoforms (Man₅–Man₉) [12]. The study examined the ability of enterococcal species to

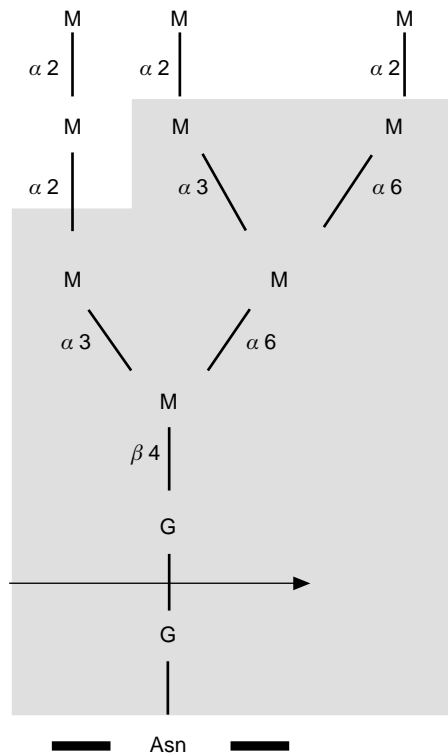


Fig. 1. Cleavage site of endo- β -N-acetylglucosaminidase on high mannose-type glycans. Highlighted structure represents the Man_5 glycoform (M, mannose; G, N-acetylglucosamine; Asn, asparagine). Mannose residues are linked via $\alpha(1 \rightarrow 3)$, $\alpha(1 \rightarrow 6)$ and $\beta(1 \rightarrow 4)$ linkages (labelled $\alpha 3$, $\alpha 6$, $\beta 4$ respectively). Man_6 – Man_9 are formed by the addition of $\alpha(1 \rightarrow 2)$ -linked mannose residues ($\alpha 2$). Arrow indicates the cleavage site of the *E. faecalis* endo- β -N-acetylglucosaminidase.

cleave high mannose-type glycans and utilise the released carbohydrates for growth.

Materials and methods

Bacterial strains and routine culture

A total of 91 enterococcal strains, isolated from a wide variety of sources, and representing all 18 enterococcal species, was used in this study (Table 1). Stock cultures were stored at -70°C in cryovials (Protect; Technical Service Consultants, Heywood, Lancashire) and were subcultured routinely on to Columbia base agar (Oxoid) supplemented with defibrinated sterile horse blood (TCS Microbiology, Buckinghamshire) 5% v/v and incubated aerobically at 37°C for 1 day.

Growth of isolates on minimal medium supplemented with RNAase B

Minimal medium was prepared essentially as described by Lacks and Hotchkiss [13] and Tomasz and Hotchkiss [14], but with the omission of glucose and bovine serum albumin, and sterilised by filtration ($0.2 \mu\text{m}$ pore size Acrodisc filter; Pall Gelman Sciences, Northampton). Stock solutions of RNAase

B (bovine pancreatic; Sigma) 10 mg/ml and 20 mM mannose were prepared in de-ionised water and filter-sterilised. Growth media were prepared by mixing equal volumes of minimal medium with the stock solutions of RNAase B or mannose in sterile 28-ml screw-capped containers (Sterilin). Carbohydrate-free medium control cultures were also prepared by mixing equal volumes of minimal medium with filter-sterilised de-ionised water.

Bacterial cell suspensions were prepared by suspending four or five colonies from blood agar plates in 1 ml of filter-sterilised 50 mM sodium phosphate buffer (pH 7.5); the A_{620} of 200- μl volumes was standardised to 0.3 (Titertek Multiscan MCC340; ICN Flow, ICN Biomedicals, Thame, Oxfordshire).

The ability of each strain to grow on RNAase B and mannose was determined by inoculating 200- μl volumes of RNAase B- or mannose-supplemented medium in sterile microtitration plate wells (Sterilin) with a 5% v/v suspension of each isolate. Growth of the isolates was monitored over 24 h at 37°C in a shaking, plate-reading spectrophotometer (iEMS; Lab-systems, Life Sciences International, Hampshire), with the A_{620} used as a measure of growth. Control cultures comprised inoculated carbohydrate-free media and uninoculated RNAase B-supplemented medium. Growth was defined as an increase in A_{620} of >0.03 over the carbohydrate-free controls.

At the end of the incubation period the RNAase B-grown cultures were centrifuged (11 600 g, 3 min) and the supernates were decanted and stored at -20°C before analysis by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry.

MALDI-TOF analysis of residual RNAase B

Residual RNAase B glycoforms were detected with a MALDI-TOF mass spectrometer with delayed extraction (Voyager Elite Workstation; PerSeptive Biosystems, Framingham, MA, USA) as previously described by Roberts *et al.* [11]. Briefly, culture supernates were diluted 1 in 5 with trifluoroacetic acid (TFA; Sigma) 0.1%; then 0.5 μl of each sample was applied to the sample plate and overlaid with 0.5 μl of a solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid; Sigma-Aldrich, Gillingham, Dorset) 10 mg/ml in acetonitrile 33% v/v in TFA 0.1% and air-dried. Spectra were acquired under linear mode with delayed extraction following irradiation with a nitrogen laser giving a 337-nm output with a 3-ns pulse width and molecular ions accelerated at a potential of 25 kV. Ribonuclease A (RNAase A), the non-glycosylated form of the protein and a component of the RNAase B preparation, was used as an internal mass calibrant (13 682 Da) and as a standard for calculating relative peak intensities.

Table 1. Enterococcal species used in this study

Species group*	Species	n	Strain	Origin
Group I	<i>E. avium</i>	1	DSM 20679 ^T	Human
	<i>E. malodoratus</i>	1	DSM 20681 ^T	Gouda cheese
	<i>E. raffinosus</i>	1	NCTC 12192 ^T	Human
	<i>E. pseudoavium</i>	1	DSM 5632 ^T	Cattle
	<i>E. saccharolyticus</i>	1	DSM 20726 ^T	Straw bedding
Group II	<i>E. faecalis</i>	29	BC001-BC009, NF011-NF020	Human
			VT31a, VT25, VT26a, VT29, VT30a, VT32	Pig
			PT12b, PT25	Poultry
			ATCC 29212 ^T	Human
			V582 ^S	Human
	<i>E. faecium</i>	20	F9, S386	Cattle
			VT11, VT15, STR792, V3, V41, V51, V67, V72	Pig
			PT13, PT17, PT26, PT35, KH4, K3, K14, K30, K110	Poultry
			NCTC 7171 ^T	Human
			ATCC 25788 ^T	Plant
			CCM 4239 ^T	Human
			DSM 4838 ^T	Soil
			DSM 20628 ^T	Poultry
Group III	<i>E. durans</i>	15	V18, V23, V24, V27, V55, V69, V78	Pig
			K28, K36, KS36, KH2, KH36, KH37, PT12b	Poultry
			NCTC 8307 ^T	Dried milk
	<i>E. hirae</i>	8	DSM 20160 ^T	Unknown
			DSM 3320	Unknown
			VT1, VT4, VT6, VT12, VT13, VT21	Pig
			NCFB 2821 ^T	Human
Group IV	<i>E. dispar</i>	1	NCFB 2821 ^T	Human
	<i>E. sulfureus</i>	1	DSM 6905 ^T	Plant
	<i>E. cecorum</i>	6	RS33, RS259, S566	Cattle
S510, S512, S593			Pig	
Group V	<i>E. columbae</i>	1	DSM 7374 ^T	Pigeon
Miscellaneous	<i>E. asini</i>	1	DSM 11492 ^T	Donkey

^TType strains. ATCC, American Type Culture Collection; CCM, Czech Collection of Microbes; DSM, German Collection of Microorganisms; NCTC, National Collection of Type Cultures; NCFB, National Collection of Food Bacteria. ^S*E. faecalis* sequenced strain (<http://www.tigr.org/tdb/mdb/mdb.html>).

* Grouped according to [5].

Detection of free RNAase B-derived glycans

To elucidate whether RNAase B deglycosylation by enterococcal species occurred as a result of the production of endo- β -N-acetylglucosaminidase activity, culture supernates were screened for the presence of free RNAase B-derived glycans over a period of 24 h.

Cell suspensions of one isolate from each species capable of deglycosylating RNAase B were prepared as described above, 5% v/v was inoculated into RNAase B-supplemented minimal medium and these were incubated aerobically at 37°C. Samples of culture were removed at intervals over a period of 24 h and growth was monitored by measurement of the A₆₂₀. Control cultures were included as described above.

Heat-inactivated culture supernates (100°C for 10 min) were analysed by high pH anion-exchange chromatography (HPAEC) for the presence of RNAase B-derived glycans following a 1 in 4 dilution with 18 MΩ water. RNAase B-derived Man₅₋₉-GlcNAc glycans were prepared from RNAase B with a commercial endo- β -N-acetylglucosaminidase H preparation (New England

Biolabs, Hitchin, Hertfordshire) and used as external standards. HPAEC was performed as described previously by Byers *et al.* [15] with 100- μ l volumes of the diluted samples injected on to a 250 \times 4-mm Carbopac PA1 column. Separations were performed with a flow rate of 1 ml/min at ambient temperature and individual glycans were resolved in 100 mM sodium hydroxide with a gradient of sodium acetate (10–60 mM over 0–30 min).

Assay for glycosidase activities with fluorogenic substrates

The α - and β -mannosidase activities which are required to degrade high mannose-type glycans were determined for enterococcal cell suspensions with 4-methylumbelliferyl (4-MUB)-linked fluorogenic substrates (4-MUB- α -D-mannopyranoside and 4-MUB- β -mannopyranoside; Sigma). The substrates were included in the assays at a final concentration of 100 μ M as described previously [16]. The release of 4-methylumbelliferone was monitored by recording fluorescence values at 37°C in a shaking, plate-reading fluorimeter (Fluorescan-Ascent

FL, Labsystems) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Sodium phosphate buffer replaced the bacterial cell suspensions in the control assays. The presence of each activity was recorded as positive when the increase in fluorescence above the controls over 24 h was >25 fluorescence units (equivalent to 0.05 nmol of 4-MUB released).

Results

Growth of enterococci on RNAase B

Ten of the 18 enterococcal species utilised RNAase B to support bacterial growth. These included all *E. cecorum*, *E. durans*, *E. hirae* and *E. faecalis* isolates, together with the *E. casseliflavus*, *E. dispar*, *E. flavescens*, *E. gallinarium*, *E. mundtii* and *E. sulfureus* type strains (Table 2). None of the *E. faecium* isolates exhibited growth on RNAase B. Mannose supported the growth of all 18 enterococcal species (data not shown).

Deglycosylation of RNAase B by enterococci

MALDI-TOF analysis of intact RNAase B produced a characteristic spectrum corresponding to the five glycoforms (Man₅–Man₉) with an additional peak at 13 682 m/z corresponding to the molecular mass of RNAase A, the non-glycosylated form of the protein (Fig. 2a). Analysis of the culture supernates after incubation for 24 h revealed the complete deglycosylation of RNAase B by six of the 18 species, with no Man₅–Man₉ glycoforms detectable and the appearance of a new species corresponding to the molecular mass of RNAase B with a single N-acetylglucosamine residue attached (13 885 m/z). A second, minor species was also observed corresponding to the protein with two N-acetylglucosamine residues (14 088 m/z) (Fig. 2b). This pattern of RNAase B deglycosylation was observed with all *E. faecalis* isolates and with six of

seven *E. hirae* isolates, nine of 15 *E. durans* isolates, one of six *E. cecorum* isolates and the *E. casseliflavus*, *E. flavescens*, *E. gallinarium* and *E. mundtii* strains (Table 2). A further six *E. durans*, five *E. cecorum* and one *E. hirae* isolates, and the *E. dispar* and *E. sulfureus* type-strains showed partial deglycosylation of the RNAase B characterised by a reduction in the relative intensities of the Man₅–Man₉ glycoforms and the appearance of the 13 885 m/z species (Fig. 2c). The other eight enterococcal species showed no ability to degrade RNAase B with spectra identical to that of intact RNAase B.

Endo- β -N-acetylglucosaminidase activity in enterococci

The culture supernates of all species exhibiting an ability to deglycosylate RNAase B contained intact free RNAase B-derived glycans during exponential growth. These glycans eluted with retention times identical to those of glycans released by the action of a commercially available endo- β -N-acetylglucosaminidase H on RNAase B (Fig. 3) and corresponded to Man₅₋₉-GlcNAc. These liberated glycans were absent from the 24-h culture supernates in all the enterococcal species, indicating the utilisation of the glycans to support growth.

Glycosidase activities

No α -mannosidase activity was detected among the 91 enterococcal isolates with the fluorometric substrate. β -Mannosidase activity was detected in 14 enterococcal species (Table 2). All 10 enterococcal species which degraded RNAase B glycoforms possessed β -mannosidase activity, as did *E. asini*, *E. faecium*, *E. pseudo-avium* and *E. malodoratus*, which did not deglycosylate RNAase B.

Table 2. Growth and enzymic activities of enterococcal species

Species group*	Species	Growth on RNAase B†	Endo- β -N-acetylglucosaminidase†	α -Mannosidase†	β -Mannosidase†
Group I	<i>E. avium</i>	–	–	–	–
	<i>E. malodoratus</i>	–	–	–	+
	<i>E. raffinosus</i>	–	–	–	–
	<i>E. pseudoavium</i>	–	–	–	+
	<i>E. saccharolyticus</i>	–	–	–	–
Group II	<i>E. faecalis</i>	+	+	–	+
	<i>E. faecium</i>	–	–	–	+
	<i>E. casseliflavus</i>	+	+	–	+
	<i>E. flavescens</i>	+	+	–	+
	<i>E. mundtii</i>	+	+	–	+
	<i>E. gallinarium</i>	+	+	–	+
Group III	<i>E. durans</i>	+	+	–	+
	<i>E. hirae</i>	+	+	–	+
	<i>E. dispar</i>	+	+	–	+
Group IV	<i>E. sulfureus</i>	+	+	–	+
	<i>E. cecorum</i>	+	+	–	+
Group V	<i>E. columbae</i>	–	–	–	–
Miscellaneous	<i>E. asini</i>	–	–	–	+

*Grouped according to [5].

†Determined as described in *Materials and methods*.

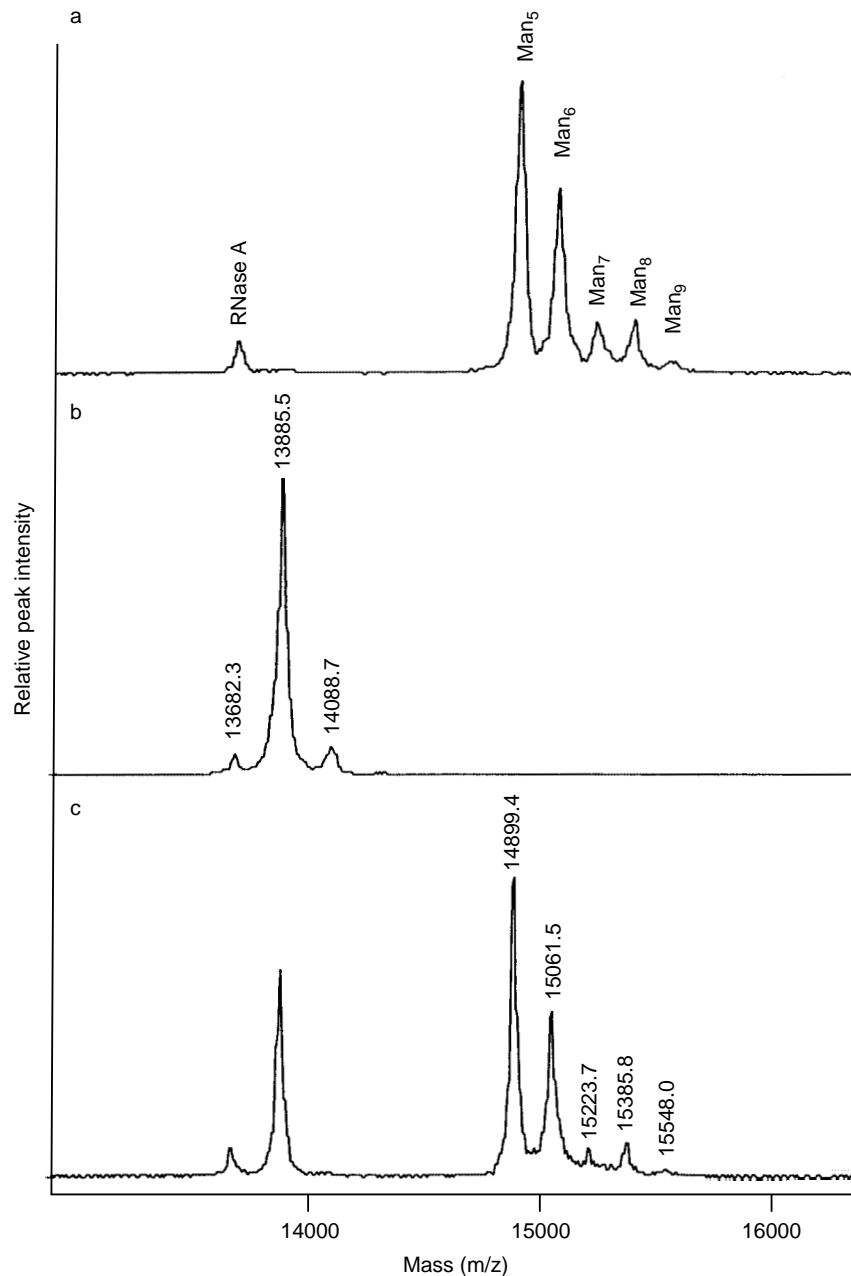


Fig. 2. MALDI-TOF spectra of (a) intact RNAase B, (b) RNAase B in culture supernatates after incubation with *E. faecalis* and (c) RNAase B in culture supernatates after incubation with *E. dispar*.

Discussion

This study investigated the distribution of endo- β -N-acetylglucosaminidase activity among the enterococci. By means of a combination of growth studies, MALDI-TOF mass spectrometry and HPAEC analysis the study identified 10 enterococcal species that produce endo- β -N-acetylglucosaminidase, i.e., *E. casseliflavus*, *E. cecorum*, *E. dispar*, *E. durans*, *E. faecalis*, *E. flavesens*, *E. gallinarium*, *E. hirae*, *E. mundtii* and *E. sulfureus*. This activity cleaved the high mannose-type glycoforms present on RNAase B between the two N-acetylglucosamine residues of the pentasaccharide core, the cleavage site also observed for the *Streptomyces plicatus* endo- β -N-acetylglucosaminidase H, releasing

free RNAase B-derived glycans (Man₅₋₉-GlcNAc). Previous studies on *E. faecalis* have shown the action of at least one mannosidase on free RNAase B-derived glycans resulting in the production of free mannose, which supported bacterial growth [11]. All endo- β -N-acetylglucosaminidase-producing enterococci were able to utilise the RNAase B glycans to support growth. No endo- β -N-acetylglucosaminidase activity was detected in the remaining eight enterococcal species and these species were unable to deglycosylate RNAase B or utilise the attached glycans to support bacterial growth. These results suggest that a mechanism of glycan degradation occurs in the endo- β -N-acetylglucosamine-producing enterococcal species similar to that observed previously in *E. faecalis* [11].

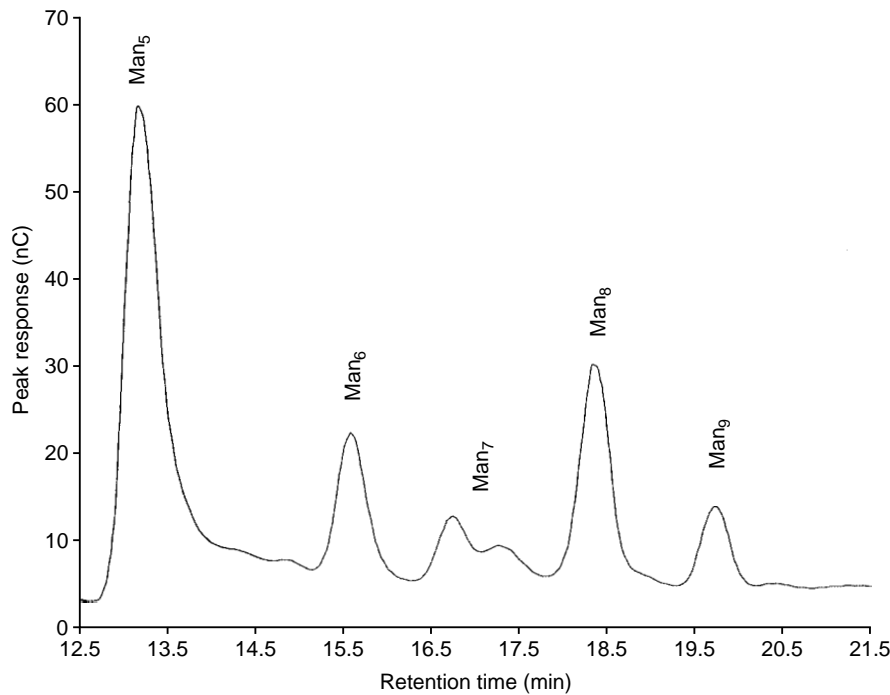


Fig. 3. HPAEC analysis of culture supernates during growth of *E. faecalis* on RNAase B. Peaks correspond to free Man₅–Man₉ glycoans with a single N-acetylglucosamine residue.

A full complement of $\alpha(1 \rightarrow 2)$, $\alpha(1 \rightarrow 3)$, $\alpha(1 \rightarrow 6)$ and $\beta(1 \rightarrow 4)$ mannosidase activities is required to fully degrade glycans liberated by the action of endo- β -N-acetylglucosaminidase on RNAase B. No α -mannosidase activity was detected in the enterococcal isolates with the appropriate fluorometric substrate; however, all enterococcal isolates producing endo- β -N-acetylglucosaminidase exhibited β -mannosidase activity. Four of the non-endoglycosidase-producing enterococcal species also produced β -mannosidase activity. Several reports have shown that the ability of bacteria to hydrolyse synthetic substrates, and in particular α -mannosidase substrates, do not necessarily represent the ability of bacteria to degrade native glycans [15, 17–19].

The distribution of the endo- β -N-acetylglucosaminidase activity amongst the enterococci is on the whole consistent with the phenotypic grouping system [5] with no activity in groups I and V and activity in all members of groups III and IV. One exception is the absence of endo- β -N-acetylglucosaminidase in *E. faecium*, a member of group II, whereas all other members of this group were positive. The function of this endo- β -N-acetylglucosaminidase activity in enterococci is unknown but may play a role in the ability of these organisms to proliferate *in vivo*. High mannose-type glycans are constituents of a range of host glycoproteins that are likely to be encountered by these bacteria *in vivo*, including laminin, which is ubiquitous in the gastrointestinal tract and a component of the basement membranes underlying the vascular epithelium [20, 21]. Furthermore, these oligosaccharides are found on platelet-derived glycoproteins,

including thrombospondin and the major platelet integrin, GP IIb/IIIa [22, 23] and may be of relevance to the growth of enterococci in cases of infective endocarditis. If expressed *in vivo*, the enterococcal endo- β -N-acetylglucosaminidase, in combination with mannosidase activities which cleave mannose residues from liberated glycans, could play a role in bacterial survival and growth in these environments, thus contributing to pathogenicity.

MALDI-TOF mass spectrometry is particularly applicable to the investigation of biological materials due to the rapid analysis times and a relatively high tolerance to salts and other impurities. This methodology was used in the present study to investigate endo- β -N-acetylglucosaminidase activity in enterococci. For laboratories not equipped with a MALDI-TOF mass spectrometer, this enzymic activity can also be monitored by conventional SDS-PAGE analysis [11] and may be used in the clinical microbiology setting to distinguish between *E. faecalis* and *E. faecium* isolates.

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