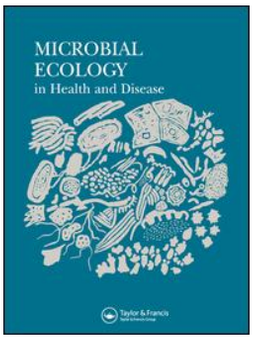

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Increased Degradative Enzyme Production by Dental Plaque Bacteria in Mucin-limited Continuous Culture

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Complex communities of dental plaque bacteria were grown under a variety of continuous culture conditions including one in which hog gastric mucin constituted the principal carbon and nitrogen sources. Washed cells and culture supernatants were assayed for fifteen exoglycosidase activities and for the ability to hydrolyse eleven synthetic peptidase substrates. Under all conditions, cultures elaborated a wide range of exoglycosidases and several peptidase activities that were principally cell-associated. Highest exoglycosidase activities were obtained with cultures growing under conditions in which hog gastric mucin constituted the principal carbon and nitrogen sources. Under these conditions, the principal species isolated were *Bacteroides spp.* and there was extensive utilisation of protein and carbohydrate moieties of the hog gastric mucin. These data support the hypothesis that supra-gingival plaque bacteria are able *in vivo* to obtain their nutritional requirements from salivary glycoproteins.

KEY WORDS — Dental plaque; Chemostat; Glycosidases; Proteases; Mucin.

INTRODUCTION

Dental plaque is a complex microbial community whose metabolic activities may lead to the initiation of dental caries and periodontal diseases. Studies of the normal flora in macaque monkeys[4,6] and of *Streptococcus mutans* and *Actinomyces viscosus* associated with gnotobiotic rats[3] have shown bacterial growth rates are generally not influenced by the composition or availability of the host's diet. In these studies, and in another of developing dental plaque in humans,[34] the doubling times of most of the bacterial species studied were of the order of 2–4 hours. The major source of nutrients for supra-gingival plaque bacteria in the absence of the host's diet, is most likely to be the constituents of saliva.[19]

We have previously shown that specific activities of exoglycosidases, having the potential to degrade oligosaccharide side chains of host glycoproteins, are increased in the plaque of fasted macaque monkeys when compared to levels found in the plaque of fed animals.[5,32] The specific activities of a wide range of arylamidase and proteolytic activities are also increased in the plaque of fasted monkeys.[33] Based on these studies we have proposed that the collective action of the exoglycosidases and the proteases sustain the growth and maintenance of the oral flora in the absence of the host's diet.

To substantiate this hypothesis dental plaque bacteria have been grown under a variety of continuous culture conditions including one in which a model glycoprotein, hog gastric mucin, was supplied as the principal carbon and nitrogen source.

MATERIALS AND METHODS

Media

The basal medium used for most of the continuous culture studies, and as a diluent for viable counts, was the slightly modified BM medium of Shah *et al.*[28] supplemented with 5 mg l^{-1} haemin (Sigma) and 1 mg l^{-1} vitamin K_1 to promote the growth of *Bacteroides spp.*[18] Plaque bacteria were also cultured in the defined synthetic medium of Shellis[30] except that the principal glycoprotein, bovine submandibular mucin, was replaced with hog gastric mucin. This was obtained as a crude, commercial preparation (Sigma, type II) by a modification of the method of Miller and Hoskins.[22] The mucin was purified by dissolving 10 per cent (w/v) in 0·1 M

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NaCl, adjusting the pH to 7·0 with NaOH pellets and stirring overnight. The impurities were pelleted by centrifugation at 10 000 g for 10 min and the dissolved mucin precipitated by adding ethanol to 60 per cent (v/v) final concentration, this was redissolved and precipitated as before. The final precipitate was lyophilised and stored until required. An aqueous 5 per cent (w/v) stock solution of mucin, pH 7·0, was autoclaved at 121°C for 15 min. Autoclaving mucin at pH 7·0 does not effect the biological availability of its constituents (Hoskins, personal communication). Sterile mucin was added at a final concentration of 0·1 per cent to the remainder of the defined medium, which had been sterilised by passage through a 0·22 μm filter (Millipore). The basal medium was supplemented with glucose (0·5 per cent w/v) as the growth limiting nutrient and with sterilised mucin, with or without glucose, to determine their effects on the levels of exoglycosidic and proteolytic enzymes in culture.

Steady state for each culture condition was achieved twice during independent chemostat runs and for each run the enzyme activities in the cell pellets and culture supernatants were determined in duplicate. The variability for the enzyme estimations between chemostat runs was approximately 15 per cent while variation within runs was only 5 per cent. Therefore differences in enzyme activities of 30 per cent may be regarded as significantly different.

Inoculation of chemostats

Dental plaque was collected from thirteen schoolchildren (aged 9–15 years), and transported in pre-reduced, serum-based medium.[8] Samples were pooled in an anaerobic cabinet, dispensed into aliquots and stored in liquid nitrogen to maintain viability[37] until required to inoculate the chemostat. The chemostats were inoculated with the plaque suspension and allowed to grow as a batch culture until the culture turbidity (OD 450 nm) increased to greater than 0·5. The composition of the inoculum was similar to that described by Keevil *et al.*[18] and varied little on prolonged storage, even after 18 months. Additional information is given in Glenister *et al.*[14]

Chemostat design

The apparatus used was that described by Keevil *et al.*[18] The medium was introduced into the vessel containing 500 ml of culture at a constant rate of 25 or 100 ml h^{-1} to give dilution rates of 0·05 h^{-1} and 0·2 h^{-1} respectively (equivalent to a mean generation time of 13·9 h and 3·5 h respectively). The pH was maintained at 7·0 ± 0·1 automatically by the addition of 2 M NaOH and the temperature kept at 37 ± 0·5°C with an external infra red lamp. Cultures were grown anaerobically by surface gassing with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide.

Cultural studies

Full details on the isolation, identification and enumeration of bacteria in the inoculum and in samples taken from the chemostats are presented elsewhere.[14]

Collection of cells

Cells were collected during steady state conditions from the chemostat sample port into sterile containers. Cells were harvested by centrifugation at 10 000g for 10 min, resuspended in their original volume of 0·85 per cent (w/v) NaCl and finally pelleted by centrifugation as before. Washed cells from all cultures, except those growing on the defined synthetic medium, were resuspended in 5 times their original volume in 0·85 per cent (w/v) NaCl. Cells growing in the defined synthetic medium were resuspended to the original volume in saline. All culture supernatants were clarified by microfiltration through a chemically inert filter (Gelman Sciences, Nylaflow, 0·22 μm diameter).

Protein assay

Aliquots of the bacterial suspensions were extracted with 0·5 M NaOH as described previously[5] and after neutralisation by the addition of 0·5 M HCl assayed for protein by the Coomassie Brilliant blue binding assay (Pierce Chemical Company) using bovine serum albumin (Sigma) as standard.

Enzyme assays

All exoglycosidases, with the exception of neuraminidase, were assayed using the appropriate para-nitrophenylglycoside substrate (Sigma) in the assay system described previously.[5] One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmole of 4-nitrophenol h^{-1} at 37°C. Neuraminidase was assayed using N-acetylneuramin lactose (Sigma) as described previously.[5]

Table 1. Carbohydrate and protein moieties of hog gastric mucin (HGM) remaining after growth of dental plaque bacteria in the defined medium, $D = 0{\cdot}2\ h^{-1}$

	Carbohydrate and protein moiety of HGM ($\mu g\ ml^{-1}$)				
	Total hexose	Fucose	Hexosamine	Neuraminic acid	Protein
Uninoculated medium	385	145	531	85	280
Spent medium	130	50	237	30	150
Per cent loss	66	65	55	65	46

One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmole of neuraminic acid h^{-1} at 37°C.

Arylamidase activities were assayed using the appropriate N-L-aminoacyl-2-napthylamine substrate (Sigma) essentially as described by Oya *et al.*[25] The assay system contained: Tris-maleate buffer, pH 7·0, 10 μmole; substrate, 0·1 μmole; culture fraction, 50 μl and water to 0·25 ml. Incubations were performed at 37°C for up to 8 h depending on the rate of reaction. Reactions were terminated by the addition of 600 μl of 10 per cent (v/v) Tween 20 in 1 M sodium acetate buffer, pH 4·2, containing 120 μg of the stabilised diazonium salt, Fast Garnet GBC. After at least 10 min, the absorbance at 530 nm was measured against a suitable reagent blank. One unit of activity was defined as the amount of enzyme causing an increase of one absorbance unit at 530 nm h^{-1} in a cell path of 1 cm.

Glycyl prolyl dipeptidase activity was measured essentially as described by Fukasawa & Harada.[13] The incubation mixture contained: Tris-maleate buffer, pH 7·0, 10 μmole; glycyl-L-proline 4-nitroanilide 0·15 μmole; cell-fraction, 50 μl and water to 0·25 ml. After incubation for 3 h at 37°C, 600 μl of 50 mM Tris-maleate buffer was added to the reaction mixture and the absorbance of the 4-nitroaniline liberated was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmole of 4-nitroaniline h^{-1} at 37°C.

The specific activities of the cell-associated exoglycosidases and peptidases are expressed as units of enzyme activity (mg of bacterial protein)$^{-1}$. The specific activities of extracellular exoglycosidases are expressed as units of enzyme activity (mg of bacterial protein)$^{-1}$.

Chemical analysis

Mucin was precipitated from unused and spent media by the addition of ice-cold ethanol to a final concentration of 60 per cent (v/v). After incubation on ice for at least 30 min, the precipitated mucin was collected by centrifugation (11 000 g) for 10 min at 4°C, washed once with ice-cold 60 per cent (v/v) ethanol, pelleted again by centrifugation, and redissolved in the original volume of 0·85 per cent (w/v) NaCl prior to chemical analyses. Mucin was assayed for total hexoses by the anthrone method[23] using D-galactose as standard; for L-fucose by the method of Dische and Shettles,[11] using L-fucose as standard; for hexosamines, following its hydrolysis for 16 h at 100°C in 2 N HCl, by the method of Boas[7] using D-glucosamine HCl as standard and, for neuraminic acid by the method of Warren[36] after hydrolysis with H_2SO_4 for 60 min at 80°C. 'True-hexoses' in the mucin preparation were calculated by subtracting the contribution of L-fucose to the anthrone reaction.[22] Mucin protein was determined using a modified Lowry method.[22]

RESULTS

Parameters of growth in the chemostat

Glucose could not be detected in the supernatants of the cultures growing on the complex BM plus glucose medium, in the presence or absence of mucin, at either growth rate. Chemical analyses on the spent medium from the culture growing at $D = 0{\cdot}2\ h^{-1}$ on defined synthetic medium revealed that there was a considerable, but not complete, loss of both mucin sugars (total hexoses, fucose, hexosamines, and neuraminic acid) and protein (Table 1).

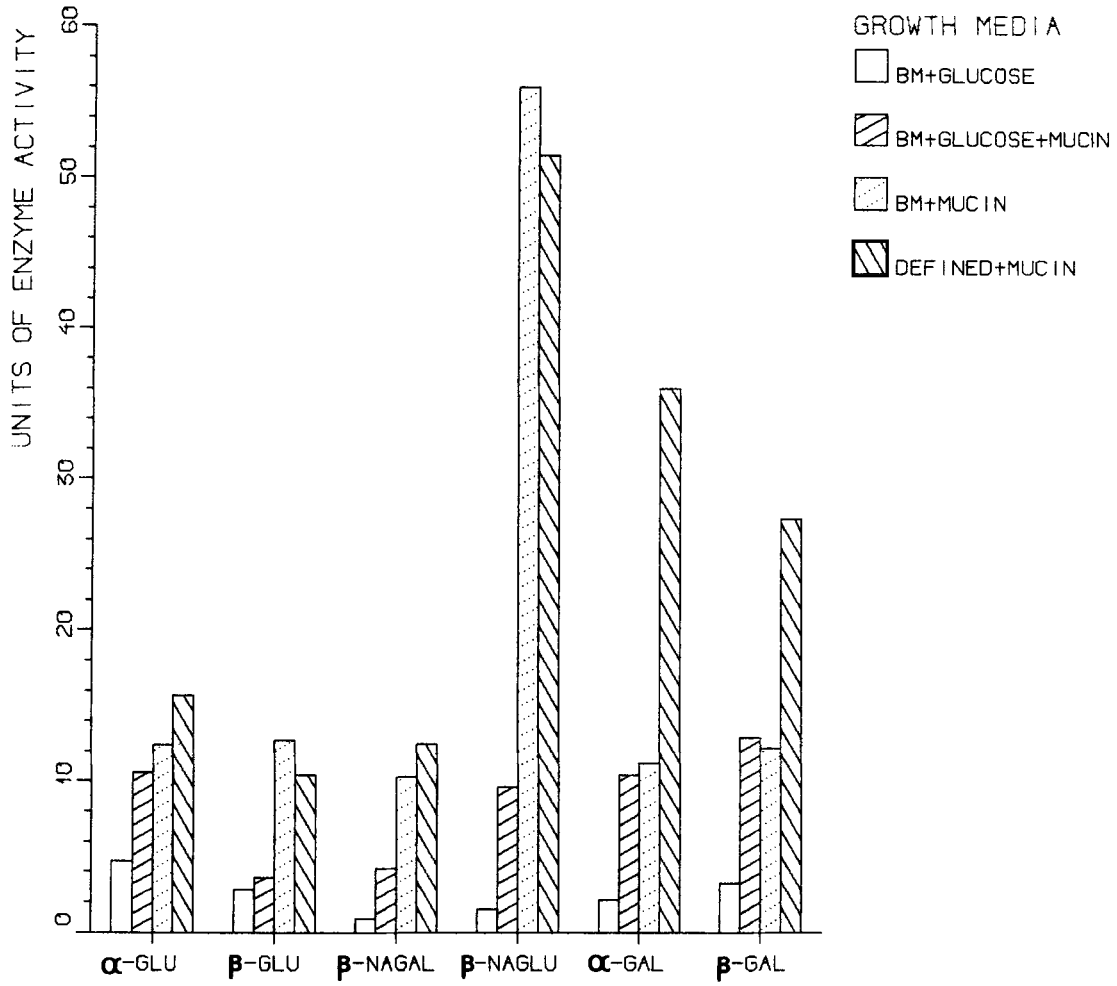

Figure 1a

Exoglycosidase activities of washed cells and culture filtrates

The specific activities of ten cell-associated enzymes in the cultures growing at $D = 0{\cdot}2\ h^{-1}$ are presented in Fig. 1a, b. The activities of these enzymes in the basal medium were in every case low. Supplementation of the basal media with hog gastric mucin increased the levels of most enzyme activities, the exceptions being β-D-glucosidase and β-D-xylosidase. Omission of glucose increased the levels of α-fucosidase, β-N-acetyl-D-galactosaminidase and in particular β-N-acetyl-D-glucosaminidase and β-D-xylosidase. High levels of enzyme activities were also attained in the culture growing on the defined synthetic medium. Similar results were also obtained with the cultures growing at the slower dilution rate of $0{\cdot}05\ h^{-1}$ (data not shown).

The same ten activities were demonstrable in the culture filtrates of cells grown at both dilution rates and, as with cell-associated enzymes, levels were also sensitive to the presence of mucin in the growth medium (Table 2).

Levels of β-L-fucosidase, α-N-acetyl-D-galactosaminidase, α-N-acetyl-D-glucosaminidase and α-D-mannosidase were reproducibly low and unvarying in all cultures, irrespective of the media composition or of the growth rate (data not shown).

Proteolytic and arylamidase activities of washed cells and culture filtrates.

Eleven of the peptide substrates were hydrolysed by cell pellets of all cultures, with N-leucyl-, N-prolyl-2-naphthylamines and glycyl-proline

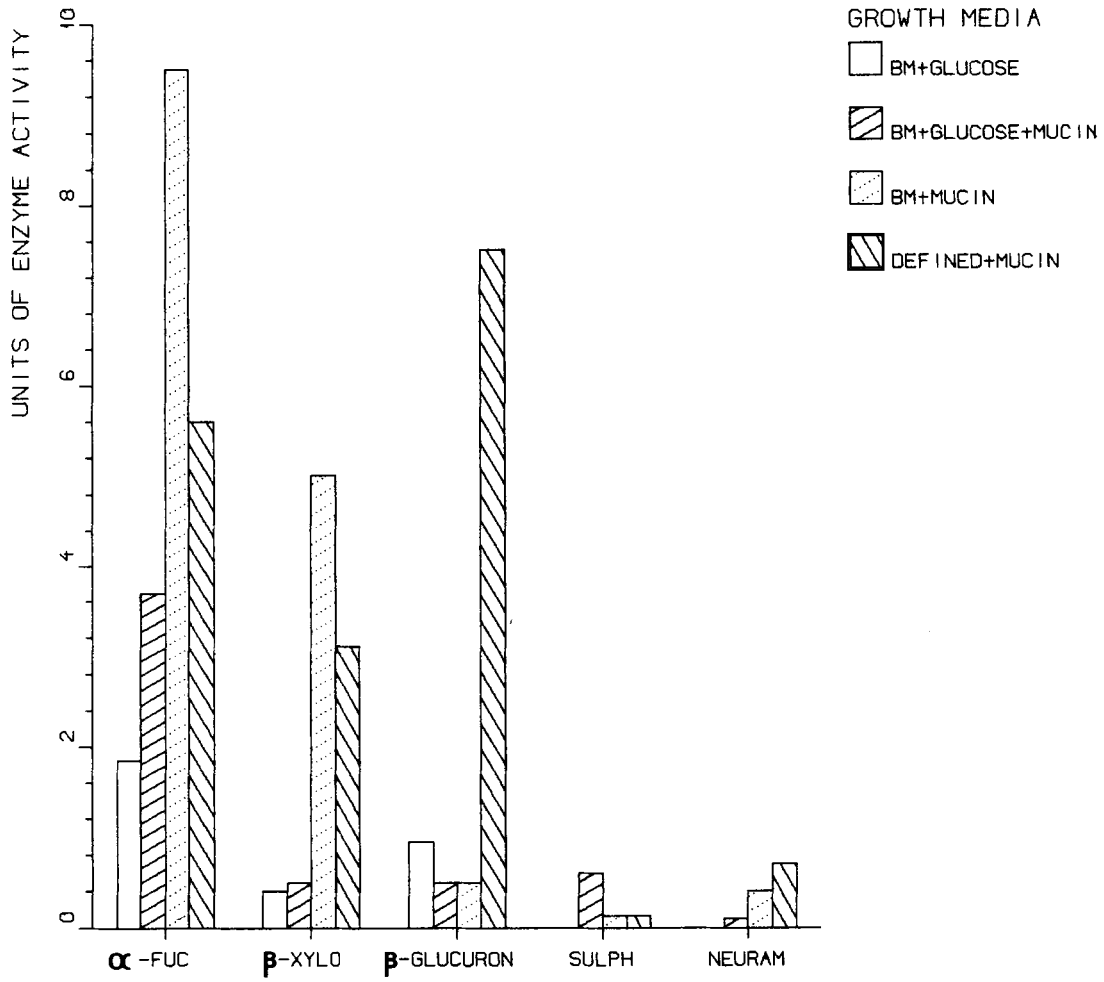

Figure 1b

Figure 1a and b. Specific activities of cell-associated exoglycosidase from cultures of dental plaque bacteria grown under different cultural conditions ($D = 0{\cdot}2\ h^{-1}$). Variation between duplicate assays from the same chemostat culture was approximately 5 per cent and variation between different chemostat cultures approximately 15 per cent

Abbreviations: α-GLU = α-glucosidase; β-GLU = β-glucosidase; β-NAGAL = β-N-acetylgalactosaminidase; β-NAGLU = β-N-acetylglucosaminidase; α-GAL = α-galactosidase; β-GAL = β-galactosidase; α-FUC = α-fucosidase; β-XYLO = β-xylosidase; β-GLUCURON = β-glucuronidase; SULPH = sulphatase; NEURAM = neuraminidase

4-nitroanilide being hydrolysed most rapidly. In contrast to the findings with cell-associated exoglycosidases, supplementation of the glucose-containing basal medium with hog gastric mucin had only minor effects on the rates of hydrolysis of the peptidase substrates by the bacterial preparations. However, the omission of glucose from this medium increased the rates of hydrolysis of valyl-, prolyl-, seryl-, threonyl-, glutamyl- and tryosyl- 2-naphthylamines by cell-pellets of cultures growing at both dilution rates. The specific activities for the cultures grown at $D = 0{\cdot}2\ h^{-1}$ are shown in Fig. 2a, b; the specific activities for the cultures grown at $D = 0{\cdot}05\ h^{-1}$, were essentially the same (data not shown). The rates of hydrolysis of the peptide substrates by pelleted cells of cultures grown in the defined synthetic medium were comparable with and in most instances higher than rates obtained with cells from the glucose supplemented basal medium; however, the level of glycylprolyl dipeptidase was lowest in the cells from the defined synthetic medium.

Table 2. Specific activities of exoglycosidases in culture supernatants of dental plaque bacteria grown at $D = 0{\cdot}2\ h^{-1}$. Variation between duplicate assays from the same chemostat culture was approximately 5 per cent and variation between different chemostat cultures approximately 15 per cent

Enzyme activity*	Media			
	BM + glucose	BM + HGM + glucose	BM + HGM (no glucose)	Defined (no glucose)
α-fucosidase	0·7	4·5	5·2	ND
α-galactosidase	0·9	14·6	13·5	15·5
β-galactosidase	2·0	18·7	37·2	30·6
α-glucosidase	3·3	10·6	8·7	16·1
β-glucosidase	0·4	1·0	1·7	1·8
β-N-acetylglucosaminidase	0·5	37·5	16·2	18·9
β-N-acetylgalactosaminidase	0·5	5·1	6·7	8·4
β-xylosidase	0·0	0·4	2·3	2·5
β-glucuronidase	0·0	0·3	0·4	1·1
sulphatase	0·0	0·7	1·1	0·3
neuraminidase	0·0	0·04	0·07	0·13

*Units of enzyme activity are as given in the Materials and Methods.
ND, Not determined.

The rates of hydrolysis of the peptidase substrates by all supernatant fractions were too low for their reliable determination.

DISCUSSION

The overall similarity of bacterial growth rates in supragingival dental plaque of both fed and fasted animals[3-6] suggests that the bacterial populations gain a major proportion of their nutritional requirements from their immediate environment and primarily the glycoprotein and protein fractions of saliva.[5,10,17,20,21] To degrade such complex molecules the bacteria individually, or the dental plaque collectively, must produce a wide range of glycosidases and proteases.

The activity of proteolytic enzymes purified from potentially pathogenic bacteria, indigenous to the oral cavity, has been determined. Trypsin-like enzymes have been purified from *Bacteroides gingivalis*[38] and *Treponema denticola*.[24] The enzyme from *B. gingivalis* hydrolysed albumin and ovalbumin whereas the enzyme from *T. denticola* exhibited no activity towards a number of proteins. Glycylprolyl dipeptidase purified from *B. gingivalis* was found to hydrolyse collagen.[1]

Although there have been numerous reports on the production of arylamidases by oral bacteria[12,25,27,31] none of these enzymes has been characterised in detail. However, the substrate specificities of the leucine arylamidases from two non-oral bacteria, *Pseudomonas aeruginosa*[26] and *Treponema phagedenis*,[35] would suggest that cognate arylamidases from oral bacterial isolates have the capability to hydrolyse short peptides. Furthermore, the purified arylamidases from *P. aeruginosa* and *T. phagedenis* hydrolysed several N-amino acid-2-naphthylamines; it is therefore unlikely that the cell-associated arylamidase activities represent the activities of 'individual' arylamidases.

Oral bacteria elaborate a wide range of exoglycosidases and we have previously shown that it is possible to quantitate reproducibly the levels of exoglycosidases in the supragingival plaque of macaque monkeys.[6,32] The specificities of the glycosidases suggest that the oligosaccharide side chains of salivary glycoproteins constitute a potential nutrient source for plaque bacteria *in vivo*. Salivary glycoproteins include the high molecular weight mucins which usually contain greater than 50 per cent carbohydrate.[2,15,29] In the present study hog gastric mucin prepared from a commercial source was employed in the cultural studies. This mucin is similar in structure to several mammalian salivary glycoproteins[15] and unlike salivary mucins can be

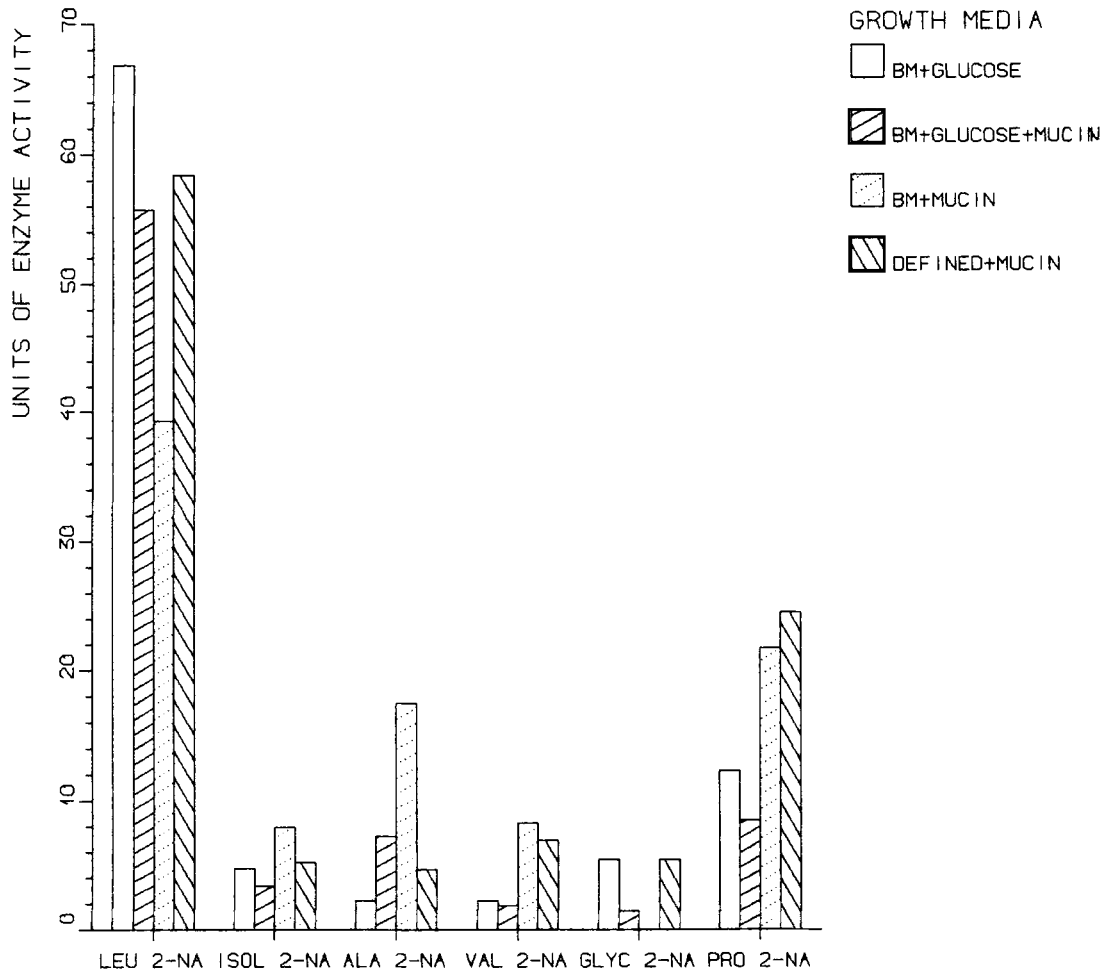

Figure 2a

isolated in the yields and purity necessary for the type of study described here.

Details of the bacterial data derived from these chemostat studies have been reported previously[14] and will only be dealt with briefly here. They do however demonstrate that the changes in the composition of the medium produced marked shifts in the composition of the bacterial populations. The principal genera isolated from the basal medium supplemented with the glucose were streptococci, peptostreptococci, lactobacilli, fusobacteria, veillonellae and *Bacteroides spp.*[9] Addition of the mucin to this community resulted in decreased levels of peptostreptococci and veillonellae but increased levels of *Actinomyces spp.*[19] The omission of glucose from the mucin supplemented medium resulted in undetectable levels of *Neisseria spp* and lower levels of streptococci and lactobacilli. Cultures growing on the defined synthetic medium were enriched for *Bacteroides spp.* and lower numbers of the other genera were also isolated.

The changes in the levels of exoglycosidases was no doubt due, at least in part, to alterations in the bacterial population occurring with each change in the medium composition. However it is possible that the introduction of the glycoprotein induced the expression of enzymes not synthesized under conditions of glucose limitation.

The range of glycosidic activities produced by the bacterial consortia growing in the chemostats under each of the conditions in which mucin was available was similar to that produced by macaque

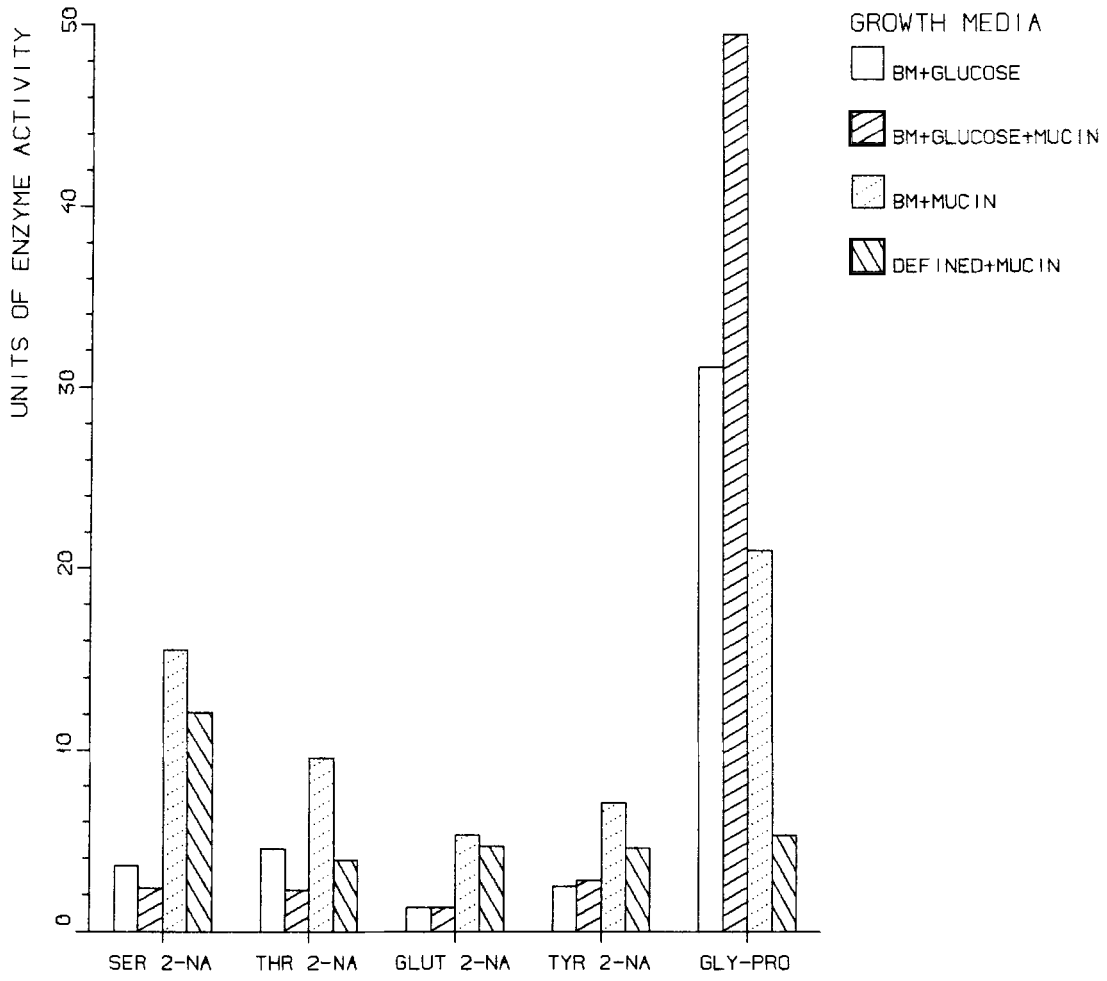

Figure 2b

Figure 2a and b. Cell-associated rates of hydrolysis of protease and arylamidase substrates by cultures of dental plaque bacteria grown under different cultural conditions ($D = 0{\cdot}2\ h^{-1}$). Variation between duplicate assays from the same chemostat culture was approximately 5 per cent and variation between different chemostat cultures approximately 15 per cent

Abbreviations; SER 2-NA = seryl-2-naphthylamine; THR 2-NA = threonyl-2-naphthylamine; GLUT 2-NA = glutamyl-2-naphthylamine; TYR 2-NA = tyrosyl-2-naphthylamine; GLY-PRO = glycyl-L-proline-4-nitroanilide; LEU 2-NA = leucyl-2-naphthylamine; ISOL 2-NA = isoleucyl-2-naphthylamine; ALA 2-NA = alanyl-2-naphthylamine; VAL 2-NA = valyl-2-naphthylamine; GLYC 2-NA = glycyl-2-naphthylamine; PRO 2-NA = prolyl-2-naphthylamine

supra-gingival plaque *in vivo*. The reason for this similarity is no doubt due to the restricted number of carbohydrates found in the oligosaccharide side-chains of all mammalian glycoproteins. Hog gastric mucin has a N-acetylglucosamine: N-acetylgalacto-samine: galacose: fucose: N-acetylneuraminic acid ratio of 2·8 : 1 : 2·9 : 1·9 : 0·2.[2] It is therefore of interest that the specific activities of the enzymes potentially responsible for the liberation of these moieties from oligosaccharide sidechains are increased in those cultures supplemented with hog gastric mucin.

Although the specific activity of neuraminidase increased in the cultures supplemented with mucin these increases do not appear sufficient to account for the 65 per cent utilisation of neuraminic acid from the defined medium. The reason for this might be that the bacterial neuraminidases have a lower affinity for the synthetic substrate than for the natural substrate incorporated into the culture

medium. All proteolytic and arylamidase activities reported to be present in macaque dental plaque were also produced in the mucin-supplemented chemostats.

Selection for mucin-degrading bacteria would be greatest in the chemically defined medium having the mucin as the principal carbon and nitrogen source. Bacterial growth in this medium was accompanied by extensive utilisation of the sugar moieties of the oligosaccharide side chains and of the amino acids of the protein backbone of the mucin. It remains to be established whether degradation of the mucin was accomplished by strains producing all the requisite exoglycosidases and proteases or by the combined action of several strains that individually produce only a few degradative enzymes. We are attempting to clarify this point by characterising the glycosidases and proteases produced by predominant organisms in the mucin-enriched cultures.

These data clearly show that dental plaque bacteria are able to grow in an environment in which a glycoprotein is the major source of both N and C, supporting the hypothesis that dental plaque bacteria are able, *in vivo,* to obtain their nutritional requirements from host macromolecules, including salivary glycoproteins. However the structure of the glycoprotein used will influence the composition of the climax microbial community. Thus, in the gut there is a selection for bacteria that elaborate exoglycosidases capable of degrading the particular blood group substance secreted by the host.[16]

The role played by each of the members of the dental plaque community *in vivo* in the degradation of salivary macromolecules is clearly complex. There are intricate food webs to be determined and this information will provide greater insights into the pathogenicity and ecology of dental plaque bacteria.

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