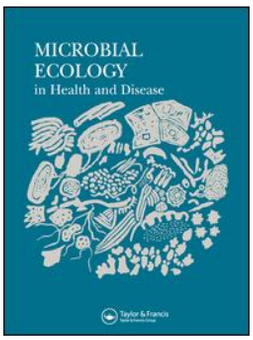

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Enhanced Growth of Complex Communities of Dental Plaque Bacteria in Mucin-Limited Continuous Culture

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The possibility that bacteria in plaque can grow at rates which are unaffected by the availability of dietary foods, because of their ability to metabolise salivary proteins, has been investigated by Keevil *et al.* using an *in vitro* continuous culture model. Plaque was collected aseptically from 13 school children, pooled and inoculated into a chemostat containing complex medium plus glucose. The principal genera enriched during growth at dilution rates of 0·05 and 0·2 h^{-1} (corresponding to mean generation times of 13·9 and 3·5 h, respectively, which might occur *in vivo*) were streptococci (including *Streptococcus mutans*) peptostreptococci, lactobacilli, fusiforms, veillonellae and *Bacteroides spp.* Addition of 0·1 per cent (w/v) mucin resulted in an increased growth yield, due to increases in the numbers of anaerobes, but the recovery of peptostreptococci and veillonellae decreased. Importantly, *Actinomyces spp.* increased and the appearance of spirochaetes was noted in all cultures fed with mucin. Withdrawal of glucose from mucin supplemented complex medium resulted in decreased growth yield, the loss of *Neisseria spp.* and decreased recovery of streptococci and lactobacilli. Replacement of the medium with artificial saliva containing 0·1 per cent (w/v) mucin enriched for *Bacteroides spp.*, but lower numbers of the other genera found in plaque were also maintained at both growth rates. This suggests that the chemostat can be used successfully to model the oral ecosystem, with salivary proteins providing the principal source of carbon and nitrogen for growth.

KEY WORDS—Mucin enrichment; plaque bacteria, mucin-limited continuous culture; enrichment of complex microbial communities.

INTRODUCTION

The antibacterial properties of saliva and its role in influencing the adherence of bacteria to teeth have been well established.[1–5] However, little interest has been shown in either the growth of the oral microflora or the possible role of saliva as a substrate. Nevertheless, the rapid colonisation of bacteria on tooth surfaces in the absence of diet[1,2,5] indicates that a continuous supply of nutrients, presumably the glycoproteins and proteins in saliva, can support the growth of the oral microflora. The few *in vitro* studies of oral bacteria grown on saliva[31] or on agar medium prepared from human saliva,[22] have provided supportive evidence towards this hypothesis. Cowman *et al.*[11,13] reported that saliva could not serve as a sole source of nutrients for oral streptococci but that they could utilise specific salivary proteins as a nitrogen source. For the complex plaque microflora, however, it has been shown that the carbohydrates from salivary glycoproteins can be rapidly metabolised.[3,12,32,33]

Mucin is the principal glycoprotein of saliva and may therefore be an important growth limiting substrate for the complex plaque microflora. To test this assumption, it was decided to grow complex communities of dental plaque bacteria under a variety of continuous culture conditions in the presence and absence of mucin, including one environment in which mucin constituted the principal carbon and nitrogen source. Oral bacteria are continuously bathed by replenished saliva, making conventional batch culture studies irrelevant from an *in vivo* modelling point of view. However, continuous culture provides a constant supply of fresh medium, strict control of the growth rate and environment,

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and reproducibility. These attributes have been used successfully to model the various environments of the oral cavity and maintain complex communities of oral bacteria.[19,24] In the present study, two dilution rates corresponding to mean generation times of 3·5 and 13·9 h were selected for investigation, since these relatively slow and fast growth rates have been measured at various sites in the oral cavity for bacteria growing in young or mature plaque.[2,5,14,34]

MATERIALS AND METHODS

Inocula

Plaque was kindly collected from 13 school children (aged 9 to 15 years) by Mr A. P. V. Pitter (Bath Dental Health Authority) and individual samples were immediately immersed in pre-reduced, serum-based transport medium[7] under an atmosphere of filter-sterilised, high purity nitrogen. The samples were chilled in ice and transported to the laboratory where they were pooled in an anaerobic cabinet, dispensed into 0·5 ml aliquots in 2 ml screw-cap ampoules and stored in liquid nitrogen to maintain viability.[36] The ampoules were removed from the liquid nitrogen, allowed to thaw slowly at room temperature and 0·45 ml of the plaque suspension inoculated directly into the chemostat containing 500 ml of medium. Continuous medium flow at the required dilution rate was initiated when the culture turbidity ($OD_{540_{nm}}$) increased to greater than 0·5 (see later). The remaining 50 µl of the plaque suspension was diluted in growth medium for identification and enumeration of the bacteria (see below). The composition of the inocula was similar to that described by Keevil *et al.*[19] and varied little on prolonged storage in the liquid nitrogen, even after 18 months.

Media

The medium used for continuous culture, and as a diluent for viable counts, was the slightly modified BM medium of Shah *et al.*[29] containing 0·5 per cent (w/v) glucose as the limiting nutrient[19] and supplemented with 5 mg ml^{-1} haemin and 1 mg ml^{-1} vitamin k_1 (Sigma) to promote growth of *Bacteroides spp.*[8] Alternatively, plaque bacteria were cultured in the defined saliva medium of Shellis[30] except that the principal glycoprotein, bovine submandibular mucin, was replaced with hog gastric mucin since this is not only cheaper but also is much more closely related to the human material than the bovine mucin.[17] The porcine material was obtained as a crude commercial preparation (Sigma) and was purified by dissolving 10 per cent (w/v) in 0·1 M NaCl, adjusting the pH to 7·0 with NaOH pellets and stirring overnight.[27] 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. The precipitate was pelleted by centrifugation, as before, and redissolved in 0·1 M NaCl before reprecipitation with 60 per cent (v/v) ethanol. This final, washed precipitate was lyophilised and stored at 5°C until required. Chemical analysis showed that the purified mucin was comprised of (w/w): 37 per cent hexosamine, 27 per cent total hexose, 10 per cent fucose, 20 per cent protein and 6 per cent neuraminic (sialic) acid (Beighton *et al.*, unpublished). This product has recently become commercially available in 50 g and 250 g quantities using our extraction procedure (product code 38105; BDH Ltd, Poole, UK) and gives essentially identical results to those described below. A 5 per cent (w/v) aqueous solution of mucin, pH 7·0, was autoclaved at 121°C for 15 min and added aseptically to the remainder of the defined medium which had been previously sterilised by passage through a 0·22 µm filter (Millipore). In several experiments sterilised mucin solutions were added to the BM complex growth medium, with or without glucose, to determine their contribution to the carbon supply of the culture and possible induction of mucin degrading enzymes.

Cultural conditions

The chemostat design was that described by Keevil *et al.*[19] The medium was introduced into the 500 ml of culture at a constant rate of 25 or 100 ml h^{-1} (dilution rates 0·05 or 0·2 h^{-1}, respectively) to give mean generation times of 13·9 h or 3·5 h, respectively. The pH was maintained automatically with 2 M NaOH and the temperature kept at 37°C with an external infra red lamp. The culture was grown anaerobically by surface gassing with filter-sterilised 5 per cent (v/v) carbon dioxide in white-spot (high purity) nitrogen (British Oxygen Company).

Bacterial identification

The microbial composition of inocula and in continuous culture was determined by isolation of serially diluted samples (in BM medium) on a variety of selective and non-selective media.[19] Briefly,

Table 1. Influence of dilution rate and medium composition on the yield of complex communities of oral bacteria growing in continuous culture at pH 7·0.

Medium	BMG*		BMGM*		BMM*		Saliva	
Dilution rate	0·05	0·2	0·05	0·2	0·05	0·2	0·05	0·2
Culture turbidity (OD_{540nm})	6·5	6·5	6·8	6·8	4·1	4·4	0·5	0·33
Yield (g dry wt. L^{-1})	2·5	2·18	2·75	2·68	1·75	2·18	0·32	0·23
Apparent Yglucose (g dry wt. mol^{-1})	90·3	78·7	99·3	96·8	—	—	—	—
Anaerobe conc.[a]	6700	5800	7270	6505	875	1760	330	150
Aerotolerant bacteria conc.[a]	3620	2807	1180	3895	765	212	6·15	3·7

*BM medium supplemented with glucose (BMG), glucose plus mucin (BMGM) or mucin (BMM). [a]Denotes concn. expressed as colony forming units $\times 10^{-6}$ ml^{-1}. The results are the mean of triplicate samples with errors of less than 3 per cent for culture turbidity, 6 per cent for yield and $Y_{glucose}$, and 15 per cent for bacterial concn.

these comprised 7 per cent (w/v) horse blood agar (Blood agar base No. 2, Oxoid) for total aerobic and anaerobic viable counts; horse blood agar supplemented with 2·5 μg ml^{-1} vancomycin (Sigma) for enumeration of Gram-negative bacteria and lactobacilli: the latter were resistant to this concentration of vancomycin and their identity was confirmed by growth on low pH Rogosa SL agar; TYC agar (London Analytical and Bacteriological Media) for streptococci and veillonella agar (Oxoid) for veillonellae. All plates, except those for aerobic bacteria, were incubated for 6 days at 37°C under an atmosphere of 10 per cent hydrogen: 10 per cent carbon dioxide: 80 per cent nitrogen (British Oxygen Company). Aerobic plates were incubated in a candle jar (effectively providing 5 per cent (v/v) carbon dioxide in air) for 3 days at 37°C. Each different colony type was counted and representative strains subcultured onto blood agar. These were tested for the ability to grow aerobically and for catalase activity. Copious extracellular polysaccharide (EPS)-producing streptococci were defined as those bacteria producing either hard, rubbery, undetachable colonies (*Streptococcus sanguis* and *Streptococcus mitior*), or large, soft, gelatinous colonies (*Streptococcus salivarius*), on TYC agar containing 5 per cent (w/v) sucrose. *S. mutans* gave small, irregular, crumbly, white colonies and was confirmed by the criteria of Hardie & Bowden.[16] Peptostreptococci were defined as obligately anaerobic, catalase-negative streptococci which grew on blood agar but not TYC agar. *Veillonella, Actinomyces, Bacteroides, Eubacterium, Capnocytophaga, Haemophilus* and *Neisseria spp.* were identified by the ability to grow aerobically or anaerobically, colonial morphology and appearance after Gram stain. Confirmation was obtained using the API Identification System. The presence of spirochaetes in samples was confirmed by dark field microscopy. Their viability was confirmed by growth on Spirochaete agar (Loesche,[23] 1976). The samples were inoculated onto 0·22 μm membrane filters (Oxoid) overlaid on the solid agar and the appearance of concentric rings of growth beneath the membrane noted.

RESULTS

The composition of the initial pooled plaque inocula was representative of that commonly stated in the literature[25,26] and has been described previously.[19] The complexities and interactions of the microbial consortia enriched at the relatively slow and fast growth rates which might be encountered in the oral cavity was reflected in the high culture turbidities and growth yields (Table 1). In particular, the molar growth yield for glucose was over 90 g dry wt. mol^{-1} in BMG medium at the slower growth rate, far in excess of the 35–50 g dry wt. mol^{-1} reported for pure cultures of oral bacteria growing in similar complex media.[21] The lower growth yield at the faster growth rate was most probably due to decreased numbers of aerotolerant anaerobes (bacteria such as streptococci which are able to grow aerobically but which do not exhibit classical respiration and whose growth is not necessarily stimulated by oxygen).

The principal genera isolated from the homogeneously mixed cultures growing in BM medium supplemented with glucose were streptococci

Table 2. Influence of complex and defined media containing glucose or mucin on the composition of complex communities of oral bacteria growing in continuous culture at a dilution rate of 0·05 h^{-1} and pH 7·0.

Medium	BMG	BMGM	BMM	Saliva
EPS streptococci	ND	ND	70	3·7
Low EPS streptococci	4100	1770	395	2·8
S. mutans	ND	10	ND	0·5
Peptostreptococci	2000	ND	25	2·5
Lactobacilli	96	110	0·3	0·002
Actinomyces spp.	ND	18	10	0·6
Veillonellae	580	10	10	2·5
Bacteroides spp.	300	5380	50	294
Fusiforms	220	7	45	26
Neisseria spp.	1·6	10	ND	ND
Capnocytophaga spp.	ND	ND	0·35	ND
Haemophilus spp.	ND	ND	ND	0·02
Treponema spp.	ND	D	D	D

The growth media were as described in Table 1. Results are expressed as colony forming units $\times 10^{-6}$ ml^{-1} and are the mean of samples from triplicate cultures with errors of less than 20 per cent. ND denotes not detected; D denotes detection of spirochaetes which could not be quantitated by the methods available.

Table 3. Influence of complex and defined media containing glucose or mucin on the composition of complex communities of oral bacteria growing in continuous culture at a dilution rate of 0·2 h^{-1} and pH 7·0.

Medium	BMG	BMGM	BMM	Saliva
EPS streptoccocci	215	3·7	17·5	0·4
Low EPS streptococci	2555	3590	205·5	5·6
S. mutans	1·6	0·65	0·3	0·002
Peptostreptococci	510	260	85	0·65
Lactobacilli	24	60	0·1	0·002
Actinomyces spp.	4	95	26·5	0·7
Veillonellae	17·5	11	50	3
Bacteroides spp.	2515	3365	1200	135·5
Fusiforms	7	3	95	4
Neisseria spp.	4	0·55	D	D
Capnocytophaga spp.	30	11	60	ND
Haemophilus spp.	200	D	0·06	0·005
Treponema spp.	ND	D	D	D

The growth media were as described in Table 1. Results are expressed as in Table 2 with similar errors between samples. ND denotes not detected; D denotes detection of either spirochaetes which could not be quantitated by the methods available or bacteria whose numbers were too low to quantitate ($< 10^2$) against the background growth of the predominant species on the agar media.

(including the suspected aetiological agent of caries, *S. mutans*), peptostreptococci, lactobacilli, fusiforms, veillonellae and *Bacteroides spp.* (Tables 2 and 3). The aerotolerant bacteria, constituting a third of the total population, were predominantly streptococci and lactobacilli whilst the fastidious anaerobes consisted mainly of *Bacteroides spp.* and peptostreptococci. The numbers of some of the bacteria recovered decreased at the faster growth rate, particularly peptostreptococci, lactobacilli, veillonellae and fusiforms. By contrast, *Bacteroides spp.* increased 8-fold. EPS-streptococci, *S. mutans, Actinomyces, Capnocytophaga, and Haemophilus spp.* were undetectable at the slow growth rate but were present in high numbers at the faster growth rate. There was no evidence of viable or non-viable spirochaetes in the communities by growth on selective medium or direct microscopy.

Addition of 0·1 per cent (w/v) mucin to the complex growth medium resulted in an apparent increase in numbers of fastidious anaerobes in the complex communities at both growth rates (Table 1). This correlated with an approximately 10 per cent increase in growth yield and the apparent $Y_{glucose}$ increased to almost 100 g dry wt. mol^{-1}. The observed population contributing to these increases was *Bacteroides spp.*, while apart from spirochaetes, the remaining anaerobes were presumably less competitive in this particular growth environment and their numbers decreased (Tables 2 and 3). The numbers of aerotolerant bacteria only increased at the faster growth rate, while the addition of mucin promoted the enrichment of *Actinomyces spp.* which were previously very low in the complex BM plus glucose medium. It should be noted that spirochaetes were now detectable in the communities by either dark field microscopy or growth on the relevant selective medium.

The contribution of mucin to the carbon supply of the cultures was further ascertained by removal of glucose from the mucin supplemented BM medium. This growth environment resulted in a lower culture turbidity and growth yield (Table 1). There was an overall decrease in aerotolerant and anaerobic bacteria, with a more significant decrease in numbers at the lower dilution rate of 0·05 h^{-1}, particularly *Bacteroides spp.* (Tables 2 and 3). These bacteria remained predominant at the faster growth rate but the slow growth rate favoured the growth of streptococci producing little polysaccharide on the relevant selective medium.

Fusiforms, *Capnocytophaga spp.* and streptococci producing copious polysaccharide on the relevant selective medium were also enriched in the absence of glucose. By contrast, *Neisseria spp.* were only barely detectable when the glucose was removed.

Replacement of the complex growth medium with a defined, artificial saliva medium, again containing 0·1 per cent (w/v) mucin, caused a significant decrease in culture turbidity, growth yield and the numbers of aerotolerant and anaerobic bacteria (Table 1). Representatives of all the genera were detected in the complex communities growing in this medium, at either growth rate, with the exception of *Neisseria* and *Capnocytophaga spp.* (Tables 2 and 3). The former were only barely detectable at the faster growth rate. In contrast to members of the other genera, the numbers of *Bacteroides spp.* became particularly high in proportion to the total numbers of anaerobes. Furthermore, although difficult to quantitate, the numbers of spirochaetes appeared greatly enhanced when detected by either growth on selective medium or by direct microscopy of culture samples. The total numbers of bacteria in the communities were markedly different at the two growth rates. At a dilution rate of 0·2 h^{-1} the population size was half of that at the 0·05 h^{-1} dilution rate. This was a reversal of the situation found in the previous growth environments where the faster growth rate tended to sustain a higher population size.

DISCUSSION

Plaque is a heterogeneous system which is composed of a liquid phase containing salivary components, bacteria and their products: principally polysaccharides which retain the acidic products arising from the fermentation of carbohydrates adjacent to the enamel and cause its demineralisation (caries). The composition of the microflora within the plaque is extremely complex and it has been found to vary, depending on its site in the oral cavity and its age.[25] Thus plaque can form at or beneath the gum (gingiva) margin and the altered communities are capable of causing inflammation (gingivitis) or tissue destruction and bone loss (periodontal diseases including juvenile periodontitis, rapidly-progressing periodontitis and refractory adult periodontitis). Previous studies have attempted to model the ecology of these diseases in the laboratory using a variety of batch or continuous culture techniques, and the latter have been laudable in attempting to investigate specific growth environments of physiological interest. However, these studies have usually utilised conventional complex media derived from animal sources with little regard for the actual composition of human saliva. As a consequence, members of the genera *Actinomyces* and *Treponema* are commonly found in the oral cavity but grew poorly or were absent in these laboratory studies,[8,9] even after several further inoculations to promote a full enrichment culture.[24]

We have attempted to reconcile these difficulties by growing the oral microflora in continuous culture using the defined saliva medium of Shellis.[30] This medium contains all of the principal ions found in saliva, together with the requisite low concentrations of amino acids, vitamins, albumin, alpha amylase etc. In particular, the medium contains bovine salivary mucin as the principal carbon and nitrogen source but this was replaced with purified hog gastric mucin. Mucous glycoproteins are characterised by a high carbohydrate: protein ratio.[10,27,28,35] Much of the structural analysis has been on gastrointestinal mucins from non-human sources because of the difficulty in obtaining sufficient quantities from human sources. There is, however, no compelling evidence that the structures of cognate gastrointestinal mucins are grossly different. As a control, the complex BM medium was also supplemented with mucin to ascertain its effects in the absence of the other salivary components. The present results have indicated that either of the growth media which contained the mucin was able to support the growth of the previously fastidious *Actinomyces* and *Treponema spp.* over prolonged periods. The growth of *Bacteroides spp.* was also enhanced in the presence of mucin, particularly at the slow growth rate. The artificial saliva medium was also able to successfully support the growth of many of the principal genera found in the oral cavity. Interestingly, the established complex community was reminiscent of sub-gingival plaque which exists in anaerobic pockets of low redox potential and contains few aerotolerant bacteria.[25] The latter included *Haemophilus spp.* which might have contained *Actinobacillus actinomycetemcomitans,* a species whose genus is currently in doubt. The strict anaerobes were dominated by *Bacteroides spp.* but also included spirochaetes, which appeared in great numbers although difficult to quantitate, and fusiforms. It is perhaps noteworthy that *Bacteroides* and *Treponema spp.*, fusiforms and *A. actinomycetemcomitans* have all been implicated as causative agents of the periodontal

diseases,[25] suggesting that the present growth environment has potential for use in a suitable model system of those disorders. Furthermore, *Actinomyces spp.* have been implicated in a particular form of caries, root surface caries,[25] and the present growth environment describes the first successful enrichment of members of this important genus in continuous culture. This finding supports the report by Bössman and Hoppe[6] that *Actinomyces spp.* are enriched on saliva-containing agar media.

It is likely that alterations in the growth environment other than in medium composition, such as pH, will also greatly affect the enrichment of particular species to model possible polymicrobial diseases. High numbers of streptococci, particularly *S. mutans,* and lactobacilli have been found in carious lesions and both grew successfully in the present study, albeit in comparatively low numbers. However, they, along with veillonellae, are the only members of a consortium enriched from plaque when the growth pH is similar to that of a carious lesion, namely pH 4·0.[9] Interestingly, a biofilm similar in composition to carious plaque is also formed when specific surfaces are immersed in the enriched culture.[20] Future work will investigate the influence of environment on the composition of the biofilms associated with the range of dental diseases described previously.

The ability of a specific microflora to grow continuously in the artificial saliva suggests that members of that consortium were able to metabolise the principal carbon and nitrogen source, mucin. This has been confirmed by various means. Firstly, the mucin fraction recovered from the spent culture medium contained only 30 per cent (w/w) of the original total hexose, fucose and neuraminic acid content and only 50 per cent (w/w) of the hexosamine and protein content (Beighton *et al.*, manuscript in preparation). Secondly, the consortium produced a range of cell-associated and extracellular exoglycosidases and peptidases capable of degrading the oligosaccharide side chains and protein backbone of the mucin, including high activities of fucosidases, xylosidases, glucuronidases, galactosidases, glucosidases, N-acetylhexosaminidases, sulphatase, and neuraminidase (Beighton *et al.*, manuscript in preparation). Thirdly, many of these enzymes were either induced or produced by newly predominant species when mucin was included in the growth medium, especially in the absence of glucose. The ability of the putative periodontal pathogens to produce proteolytic enzymes has been described as an important virulence factor for tissue destruction. However, little attention has been made to whether some of these activities are directed at degrading salivary and crevicular fluid components for growth. Further studies will determine whether all of the requisite enzymes are produced by individual strains, such as the enriched *Bacteroides spp.* described here and elsewhere,[4] or by the combined action of several strains.

In summary, the present study has described how mucin enriches for specific consortia during growth in complex or defined media. This will have profound consequences for developing laboratory media for the *in vitro* modelling of complex communities inhabiting other environments where mucin is prevalent. The structure of the glycoprotein used will also probably affect the composition of the microflora since in the gut there is a selection of bacteria that elaborate exoglycosidases capable of degrading the particular blood group substance secreted by the host.[18]

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