Effect of oxygen, inoculum composition and flow rate on development of mixed-culture oral biofilms

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The effect of aeration on the development of a defined biofilm consortium of oral bacteria was investigated in a two-stage chemostat system. An inoculum comprising 10 species, including both facultatively anaerobic and obligately anaerobic bacteria, and species associated with oral health and disease, was inoculated into an anaerobic first-stage chemostat vessel. The effluent from this chemostat was linked to an aerated [200 ml CO₂/air (5:95, v/v) min⁻¹] second-stage vessel, in which removable hydroxyapatite discs were inserted to allow biofilm formation. Comparisons were made of planktonic and biofilm communities in the aerated second-stage vessel by means of viable counts. Both planktonic and early biofilm communities were dominated by *Neisseria subflava*, comprising > 40% of total c.f.u. in the fluid phase, and > 80% of c.f.u. in 2 h biofilms. Obligate anaerobes persisted in this mixed culture, and succession in biofilms led them to predominate only after 7 d. Despite the continuous addition of air, the dissolved oxygen tension (dO₂) within the culture remained low (< 5% of air saturation), and the redox potential (E_h) was –275 mV. In order to assess the significance of the presence of *N. subflava* in community development, a subsequent experiment omitted this aerobe from the inoculum, to produce a nine-species culture. The planktonic phase was predominated by three streptococcal species, *Prevotella nigrescens* and *Fusobacterium nucleatum*. Biofilms again underwent successional changes, with anaerobes increasing in proportion with time. In contrast to the culture including *N. subflava*, dO₂ was 50–60% of air saturation, and the E_h was +50 mV. In the final experiment, the rate of addition of first-stage culture was reduced to 1/10 of that in the previous experiment, in order to determine whether anaerobes were growing, rather than merely persisting in the aerated culture. The data for the planktonic phase indicated that the anaerobes were growing in aerated (dO₂ 40–50%, E_h +100 mV) conditions. Once again, anaerobes increased in proportion in older biofilms. The study indicates that mixed cultures can protect obligate anaerobes from the toxic effects of oxygen, both in the biofilm and planktonic modes of growth.

Keywords: biofilm, oxygen, dental plaque microbiology, mixed cultures, chemostat

INTRODUCTION

Dental plaque is the aetiological agent of two of the most prevalent diseases affecting industrialized societies,

namely dental caries and periodontal disease. These diseases appear to result from a perturbation of the balance which normally exists between the host and the resident oral microflora. The factors responsible for this perturbation may be external (e.g. diet, antibiotic treatment) or host-related (e.g. immune deficiency, low saliva flow rate). In order to study some of these factors, we have developed model systems incorporating mixed cultures of oral bacteria grown in chemostats, generated

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Abbreviation: HA, hydroxyapatite.

from inocula of either undefined (Marsh *et al.*, 1983) or defined (McKee *et al.*, 1985; Bradshaw *et al.*, 1989b) composition. The chemostat allows close control of environmental factors and enables single parameters to be varied independently. Thus cause-and-effect relationships may be established between changes in key environmental factors and the composition and metabolism of this model microbial community. We have used this system to study the effects of pH and carbohydrate pulses (McDermid *et al.*, 1986; Bradshaw *et al.*, 1989a), fluoride (Bradshaw *et al.*, 1990), antimicrobial agents (McDermid *et al.*, 1987; Bradshaw *et al.*, 1993) and sugar alcohols (Bradshaw & Marsh, 1994). However, these studies were carried out in a homogeneous chemostat system, while dental plaque is a biofilm. Bacteria in biofilms can have markedly different properties to their planktonic counterparts (Costerton *et al.*, 1987). More recently, therefore, we have modified the chemostat system to allow the development of mixed culture biofilms on hydroxyapatite (HA) discs, which may be removed and replaced aseptically (Bradshaw *et al.*, 1996).

The biofilms that developed in the chemostat system were reproducible both within a chemostat run and between independent chemostat runs. The species composition of biofilms reflected the liquid phase of the culture, and was dominated by anaerobes, even after a few hours (Bradshaw *et al.*, 1996). Such rapid colonization of HA surfaces by anaerobes does not reflect the development of dental plaque observed *in vivo* (Ritz, 1967; Hardie & Bowden, 1976), and did not allow the study of succession events during early biofilm formation. In order to attempt to prolong this succession period, a novel two-stage model system was developed, in which the first-stage was a conventional anaerobic chemostat [gas phase: CO₂/N₂ (5:95, v/v)] which acted as an inoculum for an aerated second-stage [gas phase: CO₂/air (5:95, v/v)], in which the HA surfaces were inserted to allow biofilm development. This system was used to study the effect of oxygen and culture composition on the development of planktonic and biofilm communities. The study also investigated whether the 'obligately anaerobic' species could persist and/or grow in aerated conditions when part of a microbial community.

METHODS

Two-stage chemostat. A two-stage chemostat system was constructed using twin Anglicon (Brighton Systems) controllers, with a conventional culture vessel (500 ml working volume) connected via the overflow weir of the first-stage to a second-stage 'biofilm' vessel. The second-stage vessel (750 ml working volume) had a modified lid with multiple ports to allow the insertion and removal of indented HA discs (7 mm diameter; Calcitek), which were suspended from rubber bungs with titanium wire, as described previously by Bradshaw *et al.* (1996).

Inoculum and growth conditions. Ten bacterial species were inoculated from a pooled inoculum, stored in the gas phase above liquid nitrogen, as described previously by Bradshaw *et al.* (1989b), into the first-stage chemostat. The bacteria were *Streptococcus mutans* R9, *Streptococcus oralis* EF186, *Streptococcus*

sanguis 209, *Lactobacillus casei* AC413, *Actinomyces naeslundii* WVU627, *Neisseria subflava* A1078, *Veillonella dispar* ATCC 17745, *Porphyromonas gingivalis* W50, *Prevotella nigrescens* T588 and *Fusobacterium nucleatum* ATCC 10953. The inoculum included facultative anaerobes (*N. subflava*, *S. mutans*, *S. oralis*, *S. sanguis*, *A. naeslundii*, *L. casei*) and bacteria which are conventionally regarded as obligate anaerobes (*V. dispar*, *Por. gingivalis*, *Pre. nigrescens*, *F. nucleatum*). The chemostat growth medium was diluted BM medium (McKee *et al.*, 1985) supplemented with hog gastric mucin (BMHGM; Bradshaw *et al.*, 1989a), supplied to the culture at a rate of 50 ml h⁻¹ (corresponding to a dilution rate, *D*, of 0.1 h⁻¹, and a mean generation time of 6.9 h). The chemostat was maintained automatically at 37 ± 0.1 °C and pH was controlled at 7.0 ± 0.1 by the automatic addition of 2 M NaOH, with a gas phase of 5% CO₂ in N₂ (Bradshaw *et al.*, 1989a). Once this culture had reached steady-state (after 4–5 d at *D* = 0.1 h⁻¹), the overflow weir of the first-stage was connected to the second-stage 'biofilm' chemostat, into which HA discs could be inserted for varying time periods. The gas phase in the second chemostat was 5% CO₂ in air, sparged at a rate of 200 ml min⁻¹. BMHGM medium was supplied to the second-stage vessel at 75 ml h⁻¹ to give a total *D* in the second-stage chemostat of 0.17 h⁻¹, and the pH was again controlled at 7.0 ± 0.1. Dissolved oxygen tension (dO₂) was monitored with a polarographic electrode (Uniprobe). Following a period to allow the second-stage vessel to reach steady-state (at least 10 × mean generation time, around 3–4 d), HA discs were inserted, and planktonic sampling was commenced.

In a subsequent experiment, the primary oxygen-utilizing species in the culture, *N. subflava*, was omitted from the inoculum, so that a nine-membered culture developed. Finally, the system was reconfigured to reduce the rate of addition of first-stage culture to the second, aerated phase, to 5 ml h⁻¹ (i.e. 1/10 of the overflow from the first-stage chemostat), thus giving a total *D* of 0.11 h⁻¹. Once again, a steady state (at least 3–4 d) was established prior to commencing experiments. The purpose of this experiment was to determine whether the anaerobic species were actually growing in the aerated phase.

Community composition. The bacterial composition of biofilm and planktonic communities was determined by decimal dilution of samples, followed by viable counts on a range of selective and non-selective agar media, as described previously by Bradshaw *et al.* (1993).

Statistical methods. Viable counts were calculated as c.f.u. ml⁻¹. For the purposes of expressing data, a logarithmic transformation of data was performed, and means of log₁₀(c.f.u. ml⁻¹) ± standard deviation (SD) were calculated. Direct statistical comparisons between experiments were not possible because each experiment represented a single run of the chemostat. However, previous studies (Bradshaw *et al.*, 1993, 1996) have indicated the reproducibility of this system, and therefore qualitative comparisons were made on this basis. Comparisons of the longitudinal trends in biofilm development within each run were carried out by the nonparametric Jonckheere–Terpstra test (Hollander & Wolfe, 1973), using StatXact software (Cytel).

RESULTS

Community development in a ten-membered, aerated bacterial culture

The numbers of each species in planktonic cultures and biofilms in the aerated, second-stage chemostat vessel are shown in Table 1 as log₁₀(c.f.u. ml⁻¹) or log₁₀(c.f.u. cm⁻²) ± SD, respectively. The planktonic culture was

Table 1. Composition of the planktonic and biofilm communities in an aerated second-stage chemostat

Results are shown as $\log_{10}(\text{c.f.u. ml}^{-1})$ (culture) or $\log_{10}(\text{c.f.u. cm}^{-2})$ (biofilms) $\pm$ SD. The percentage of total c.f.u. values are shown in parentheses; n = number of determinations within a chemostat run.

Species	Bacterial numbers				
	Culture steady state ($n = 6$)	1 h biofilm ($n = 3$)	1 d biofilm ($n = 3$)	4 d biofilm ($n = 3$)	7 d biofilm ($n = 3$)
<i>S. mutans</i>	5.05 $\pm$ 0.92 (0.08 %)	4.94 $\pm$ 0.27 (0.5 %)	4.08 $\pm$ 0.59 (0.2 %)	4.28 $\pm$ 0.13 (0.1 %)	4.76 $\pm$ 0.41 (0.2 %)
<i>S. oralis</i>	6.56 $\pm$ 0.26 (2.7 %)	5.39 $\pm$ 0.13 (1.3 %)	5.62 $\pm$ 0.27 (7.0 %)	5.42 $\pm$ 0.10 (1.4 %)	5.99 $\pm$ 0.32 (2.6 %)
<i>S. sanguis</i>	6.33 $\pm$ 0.67 (1.6 %)	5.32 $\pm$ 0.09 (1.1 %)	5.10 $\pm$ 0.05 (2.1 %)	5.24 $\pm$ 0.15 (0.9 %)	5.69 $\pm$ 0.08 (1.3 %)
<i>L. casei</i>	4.53 $\pm$ 0.93 (0.03 %)	4.31 $\pm$ 0.30 (0.1 %)	3.77 $\pm$ 0.98 (0.1 %)	3.15 $\pm$ 0.09 (0.007 %)	4.66 $\pm$ 0.35 (0.1 %)
<i>A. naeslundii</i>	4.20 $\pm$ 0.41 (0.01 %)	3.21 $\pm$ 0.46 (0.009 %)	3.99 $\pm$ 0.48 (0.2 %)	3.83 $\pm$ 0.53 (0.04 %)	3.96 $\pm$ 1.07 (0.02 %)
<i>Por. gingivalis</i>	7.17 $\pm$ 0.16 (11.1 %)	5.63 $\pm$ 0.29 (2.9 %)	5.90 $\pm$ 0.19 (13.4 %)	6.45 $\pm$ 0.34 (14.9 %)	6.99 $\pm$ 0.13 (25.6 %)
<i>Pre. nigrescens</i>	7.10 $\pm$ 0.26 (9.4 %)	5.77 $\pm$ 0.28 (3.2 %)	5.96 $\pm$ 0.12 (15.4 %)	6.04 $\pm$ 0.47 (5.8 %)	7.02 $\pm$ 0.21 (27.4 %)
<i>F. nucleatum</i>	7.44 $\pm$ 0.11 (20.6 %)	6.10 $\pm$ 0.23 (6.8 %)	5.93 $\pm$ 0.21 (14.3 %)	6.59 $\pm$ 0.32 (20.6 %)	6.81 $\pm$ 0.31 (16.9 %)
<i>V. dispar</i>	7.04 $\pm$ 0.20 (8.2 %)	5.45 $\pm$ 0.38 (1.5 %)	5.61 $\pm$ 0.20 (6.9 %)	5.82 $\pm$ 0.10 (3.5 %)	6.48 $\pm$ 0.29 (7.9 %)
<i>N. subflava</i>	7.79 $\pm$ 0.90 (46.2 %)	7.19 $\pm$ 0.23 (83.2 %)	6.38 $\pm$ 0.20 (40.4 %)	7.00 $\pm$ 0.22 (52.8 %)	6.84 $\pm$ 0.35 (18.1 %)
Total c.f.u.	8.125	7.270	6.773	7.277	7.616

Fig. 1. Proportion of anaerobic and facultative species in planktonic culture and biofilms in a 10-species, aerated mixed culture. ■, Facultative anaerobes; ▨, obligate anaerobes; 100 % values correspond to total c.f.u. shown in Table 1.

dominated by *N. subflava*, which comprised more than 45 % of the total c.f.u. at steady-state. Anaerobic species, especially *Por. gingivalis* and *F. nucleatum* remained in relatively high numbers in the culture, despite the constant sparging with 200 ml 5 % CO_2 in air min^{-1} , while the numbers of streptococci, *L. casei* and *A. naeslundii* were low. The early biofilms (1 h) consisted mainly of *N. subflava* (> 80 % of total c.f.u.), although later biofilms, particularly after 7 d, were predominated by anaerobes (> 70 % of c.f.u.), while the proportion of *N. subflava* was reduced. The proportions of anaerobes increased significantly with time in biofilms (Jonckheere–Terpstra

test, $P < 0.03$). The levels of streptococci in all biofilms remained low. Fig. 1 summarizes the overall shifts in proportions of strict anaerobes (*Por. gingivalis*, *F. nucleatum*, *Pre. nigrescens*, *V. dispar*) observed during biofilm development, compared with the steady-state planktonic phase.

Despite the relatively rapid rate of addition of oxygen to the chemostat culture, the dO_2 at steady state remained low (< 5 % of air saturation). The steady-state E_h of the culture was –250 to –300 mV.

Community development in the absence of *N. subflava*

As *N. subflava* had predominated within the early biofilms, presumably in response to the aeration of the environment, the effect of deleting this more aerotolerant species from the inoculum on community development was investigated. The composition of planktonic cultures and biofilms in the aerated chemostat without *N. subflava* is shown in Table 2. The planktonic culture was predominated by *S. oralis*, *F. nucleatum*, *Pre. nigrescens*, *S. mutans* and *V. dispar*. Although anaerobic bacteria remained in relatively high numbers in planktonic culture (Table 2), the count of *Por. gingivalis* was lower than that found in the culture which included *N. subflava* (Table 1).

Numbers of bacteria in young biofilms (2 h and 4 h) without *N. subflava* were lower compared with 1 h biofilms developed in the culture with *N. subflava* (compare Tables 2 and 1), but had increased proportions of streptococci. These differences were not statistically tested. The proportions of anaerobes were again increased in later biofilms (1 d and 4 d), though this increase only bordered on significance (Jonckheere–Terpstra test, $P =$

Table 2. Composition of the planktonic and biofilm communities in an aerated second-stage chemostat in which *N. subflava* had been omitted from the culture

Results are shown as $\log_{10}(\text{c.f.u. ml}^{-1})$ (culture) or $\log_{10}(\text{c.f.u. cm}^{-2})$ (biofilms) $\pm$ SD. The percentage of total c.f.u. values are shown in parentheses; *n* = number of determinations within a chemostat run.

Species	Bacterial numbers				
	Culture steady state (<i>n</i> = 5)	2 h biofilm (<i>n</i> = 3)	4 h biofilm (<i>n</i> = 1)	1 d biofilm (<i>n</i> = 1)	4 d biofilm (<i>n</i> = 2)
<i>S. mutans</i>	7.30 $\pm$ 0.10 (15.6 %)	5.64 $\pm$ 0.04 (15.2 %)	5.36 (7.1 %)	5.85 (13.8 %)	6.11 $\pm$ 0.30 (4.8 %)
<i>S. oralis</i>	7.51 $\pm$ 0.08 (25.3 %)	5.74 $\pm$ 0.06 (19.2 %)	5.77 (18.3 %)	5.94 (17.0 %)	6.30 $\pm$ 0.22 (7.4 %)
<i>S. sanguis</i>	6.27 $\pm$ 0.78 (1.4 %)	4.99 $\pm$ 0.21 (3.4 %)	4.45 (0.9 %)	5.15 (2.8 %)	4.99 $\pm$ 0.43 (0.4 %)
<i>L. casei</i>	5.31 $\pm$ 0.47 (0.2 %)	4.31 $\pm$ 0.65 (0.7 %)	4.75 (1.7 %)	4.75 (1.1 %)	5.37 $\pm$ 0.32 (0.9 %)
<i>A. naeslundii</i>	5.64 $\pm$ 1.26 (0.3 %)	4.43 $\pm$ 0.58 (0.9 %)	4.60 (1.2 %)	4.71 (1.0 %)	3.81 $\pm$ 1.16 (0.02 %)
<i>Por. gingivalis</i>	6.72 $\pm$ 0.71 (4.1 %)	4.48 $\pm$ 0.43 (1.1 %)	3.93 (0.3 %)	4.75 (1.1 %)	5.84 $\pm$ 0.12 (2.6 %)
<i>Pre. nigrescens</i>	7.33 $\pm$ 0.43 (16.7 %)	5.94 $\pm$ 0.15 (30.4 %)	5.88 (23.5 %)	6.14 (26.9 %)	7.06 $\pm$ 0.08 (42.6 %)
<i>F. nucleatum</i>	7.45 $\pm$ 0.09 (22.0 %)	5.70 $\pm$ 0.12 (17.5 %)	5.94 (27.0 %)	6.10 (24.6 %)	6.86 $\pm$ 0.11 (26.9 %)
<i>V. dispar</i>	7.26 $\pm$ 0.09 (14.2 %)	5.52 $\pm$ 0.13 (11.6 %)	5.81 (20.0 %)	5.78 (11.8 %)	6.59 $\pm$ 0.09 (14.4 %)
Total	8.1066	6.467	6.502	6.709	7.431

Fig. 2. Proportion of anaerobic and facultative species in planktonic culture and biofilms in a nine-species, aerated mixed culture, in which *N. subflava* was omitted. ■, Facultative anaerobes; ▨, obligate anaerobes; 100 % values correspond to total c.f.u. shown in Table 2.

0.052). Fig. 2 summarizes the data in terms of proportions of strict anaerobes in developing biofilm populations and those persisting in the steady-state planktonic culture. The total count of the biofilm communities was lower in the absence of *N. subflava* during the first 4 d of development (Table 2 versus Table 1), though the difference observed was within the range found for similar biofilms in a previous study (Bradshaw *et al.*, 1996).

In this culture, in contrast to that incorporating *N. subflava*, the dO_2 was around 50–60 % of air saturation, and the E_h was around +50 mV.

Community development in the absence of *N. subflava*, with reduced flow rate

The data above established unequivocally that 'obligately anaerobic' species persisted in a microbial community grown in an aerated environment. The rate of addition of first-stage culture containing these anaerobes (50 ml h^{-1}) in the experiment described above, however, made it impossible to establish categorically whether the anaerobes were actually growing in the aerated stage or were present simply as a consequence of the volume of first-stage culture added. In order to clarify this, the system was reconfigured so that the rate of addition of first-stage culture to the second-stage was reduced to 1/10 of that of the previous experiment. The composition of planktonic cultures and biofilms from this experiment is shown in Table 3. The planktonic culture was again predominated by the three streptococcal species, *F. nucleatum*, *Pre. nigrescens* and *A. naeslundii*. The total planktonic population was reduced by approximately threefold compared with the study in which 50 ml h^{-1} conventional culture h^{-1} was added. This reduction appeared to be quantitative, with the proportions of different bacteria, including the anaerobic species, remaining relatively unaffected. The numbers of bacteria in young biofilms were reduced compared with the 50 ml h^{-1} addition culture ($10^{5.8} \text{ cm}^{-2}$ versus $10^{6.5} \text{ cm}^{-2}$ for 2 h biofilms) (Tables 2 and 3) and this difference was greater than that seen in biofilms of this age previously (Bradshaw *et al.*, 1996). These differences were less notable in older biofilms, and after 7 d the numbers were comparable with those seen in the culture including *N. subflava* (Tables 1 and 3). Bacterial succession occurred, with the proportions of anaerobes increasing significantly in older biofilms (Jonckheere–Terpstra test, $P < 0.0003$), notably after 4 and 7 d growth. Fig. 3 summarizes these shifts, in comparison with the steady-state planktonic

Table 3. Composition of the planktonic and biofilm communities in an aerated second-stage chemostat (lacking *N. subflava*), in which the rate of culture addition was reduced tenfold

Results are shown as $\log_{10}(\text{c.f.u. ml}^{-1})$ (culture) or $\log_{10}(\text{c.f.u. cm}^{-2})$ (biofilms) $\pm$ SD. The percentage of total c.f.u. values are shown in parentheses; *n* = number of determinations within a chemostat run.

Species	Bacterial numbers					
	Culture steady state (<i>n</i> = 6)	2 h biofilm (<i>n</i> = 3)	4 h biofilm (<i>n</i> = 2)	1 d biofilm (<i>n</i> = 3)	4 d biofilm (<i>n</i> = 3)	7 d biofilm (<i>n</i> = 3)
<i>S. mutans</i>	6.90 $\pm$ 0.28 (13.8 %)	5.04 $\pm$ 0.38 (18.8 %)	5.25 $\pm$ 0.28 (9.2 %)	5.55 $\pm$ 0.04 (11.6 %)	5.20 $\pm$ 0.12 (6.7 %)	6.46 $\pm$ 0.31 (12.5 %)
<i>S. oralis</i>	6.99 $\pm$ 0.16 (17.1 %)	5.03 $\pm$ 0.40 (18.3 %)	5.44 $\pm$ 0.06 (14.3 %)	5.84 $\pm$ 0.15 (22.7 %)	5.56 $\pm$ 0.09 (15.3 %)	6.36 $\pm$ 0.31 (9.9 %)
<i>S. sanguis</i>	6.89 $\pm$ 0.21 (13.5 %)	5.08 $\pm$ 0.30 (20.6 %)	5.74 $\pm$ 0.09 (28.6 %)	5.62 $\pm$ 0.18 (13.7 %)	5.50 $\pm$ 0.23 (13.3 %)	5.93 $\pm$ 0.13 (3.7 %)
<i>L. casei</i>	6.06 $\pm$ 0.31 (2.0 %)	4.10 $\pm$ 0.29 (2.2 %)	5.29 $\pm$ 0.02 (10.1 %)	5.63 $\pm$ 0.18 (14.0 %)	5.38 $\pm$ 0.24 (10.1 %)	5.36 $\pm$ 0.40 (1.0 %)
<i>A. naeslundii</i>	6.76 $\pm$ 0.35 (10.0 %)	5.02 $\pm$ 0.37 (17.9 %)	5.68 $\pm$ 0.04 (24.9 %)	5.57 $\pm$ 0.36 (12.2 %)	5.19 $\pm$ 0.33 (6.5 %)	5.40 $\pm$ 0.27 (1.1 %)
<i>Por. gingivalis</i>	6.69 $\pm$ 0.42 (8.5 %)	3.79 $\pm$ 0.74 (1.1 %)	3.93 $\pm$ 0.25 (0.4 %)	4.07 $\pm$ 0.54 (0.4 %)	4.28 $\pm$ 0.76 (0.8 %)	6.30 $\pm$ 0.53 (8.6 %)
<i>Pre. nigrescens</i>	6.87 $\pm$ 0.23 (12.9 %)	4.10 $\pm$ 0.60 (2.2 %)	4.26 $\pm$ 0.05 (0.9 %)	4.77 $\pm$ 0.56 (1.9 %)	5.49 $\pm$ 0.23 (13.0 %)	6.88 $\pm$ 0.36 (32.9 %)
<i>F. nucleatum</i>	6.96 $\pm$ 0.96 (15.9 %)	4.91 $\pm$ 0.10 (13.9 %)	5.14 $\pm$ 0.02 (7.2 %)	5.66 $\pm$ 0.03 (15.0 %)	5.75 $\pm$ 0.20 (23.7 %)	6.75 $\pm$ 0.34 (24.4 %)
<i>V. dispar</i>	6.55 $\pm$ 0.20 (6.2 %)	4.48 $\pm$ 0.30 (5.2 %)	4.92 $\pm$ 0.07 (4.3 %)	5.41 $\pm$ 0.20 (8.4 %)	5.40 $\pm$ 0.12 (10.6 %)	6.14 $\pm$ 0.13 (6.0 %)
Total	7.760	5.766	6.29	6.484	6.376	7.363

Fig. 3. Proportion of anaerobic and facultative species in planktonic culture and biofilms in a nine-species, aerated mixed culture, in which *N. subflava* had been omitted. Culture addition from the first-stage was at a reduced (1/10) flow rate. ■, Facultative anaerobes; ▨, obligate anaerobes; 100 % values correspond to total c.f.u. shown in Table 3.

culture. The oxygen tension in steady-state culture was between 25 and 50 %, and the measured E_h was between +90 mV and +140 mV.

DISCUSSION

Obligately anaerobic species were able to persist in high numbers in a ten-membered mixed culture, both in the biofilm and in the planktonic phase, even when the culture was sparged with 5 % CO_2 in air. Under these conditions, *N. subflava* was the major organism during the early stages of biofilm formation, and comprised > 45 %

of c.f.u. in the steady-state planktonic phase. The proportions of *N. subflava* found in young biofilms in this study were far higher compared with biofilms developed anaerobically, which were described in a previous study (Bradshaw *et al.*, 1996). The anaerobic populations in biofilms in the present study increased significantly in proportion over time, but predominated only after 4–7 d. This is similar to the successional changes observed during the development of plaque in humans (Ritz, 1967; Hardie & Bowden, 1976). The environmental conditions within the mixed culture remained relatively anaerobic, with the dO_2 of the planktonic phase only just measurable, and with a highly negative E_h , despite continuous sparging with 5 % CO_2 in air. This E_h was as low as that which was measured in conventional anaerobic mixed cultures of oral bacteria (McKee *et al.*, 1985). Since the numbers of *N. subflava* were so high, it was assumed that the growth and metabolism of this organism must be accounting for a substantial proportion of the oxygen consumption in the culture. The advantage of using a defined inoculum approach (Bradshaw *et al.*, 1989b) was exploited to determine the significance of the role of *N. subflava* in providing an environment conducive to the growth of the strict anaerobes, by constructing a new, nine-species inoculum from which *N. subflava* was omitted. In the absence of this aerobe, obligate anaerobes were still able to persist in planktonic and biofilm communities, but were found in slightly lower numbers in the younger biofilms. The loss of *N. subflava* was compensated for by an increase in the total numbers of streptococci in both planktonic and biofilm phases, though this increase was not statistically tested. Streptococci were found in lower proportions in early biofilms than in *de novo* dental plaque, presumably reflecting their proportions in the planktonic phase in this model system. Anaerobic species increased significantly in proportion, so that after 4 d they were predominant. The assumption that *N. subflava* had been

responsible for the majority of the oxygen consumption was supported by the observation that in the culture without *N. subflava*, oxygen was now readily detectable, and the E_h was positive. The persistence of bacteria conventionally regarded as obligate anaerobes in high numbers in such an aerated environment in both the planktonic and biofilm phases of growth was unexpected.

The experimental design had a conventional anaerobic chemostat feeding into an aerated second-stage that contained surfaces available for colonization. The rate of addition from the first-stage chemostat in the experiments discussed above made it difficult to determine to what extent the anaerobes were actually reproducing in the second-stage. When the rate of addition was reduced tenfold, the total numbers of anaerobes in the planktonic phase fell by less than threefold, confirming that they were not merely persisting in the culture, but were actually reproducing. These anaerobes were able to colonize the HA surfaces and eventually grow to predominate within the biofilm community after 7 d. This indicated that in this mixed culture, anaerobes could reproduce in aerated conditions, presumably as a result of the presence of facultative species, as part of a microbial community. This is a comparable situation to the persistence of sulphate-reducing bacteria in anaerobic micro-niches in biofilms associated with corrosion that develop on mild steel immersed in an aerated aqueous phase (Hamilton, 1987; Dexter, 1993; Lee *et al.*, 1995). This type of interaction may also be one of the explanations for the almost ubiquitous occurrence of facultative and obligately anaerobic species in microbial consortia isolated from abscesses (Fabricus *et al.*, 1982; Brook, 1985). Collectively, these findings address the issue that has been raised recently as to whether biofilms are true microbial communities or whether they represent more-or-less 'random accretions of organisms deposited in a haphazard manner on a solid surface, united only by their ability to adhere and proliferate in such an environment' (Wimpenny, 1994). Data from a range of habitats in the mouth imply that these oral biofilms are not arranged randomly; rather, they represent an organized and interactive consortium of micro-organisms. Such an arrangement enables individual species to combine metabolically to deal most efficiently with the prevailing physico-chemical conditions. This could be the utilization of oxygen (or the ability to persist in high redox environments), or the ability to act in a concerted manner to catabolize endogenous substrates (e.g. host glycoproteins) as nutrient sources (Bradshaw *et al.*, 1994).

It is well established that oxygen gradients can exist within biofilms, with anoxic conditions being established close to the biofilm surface (Hamilton, 1987; Dexter, 1993). Depths of 8–12 µm were sufficient for oxygen to become limiting in artificial biofilms of nitrifying bacteria (Cox *et al.*, 1980) or oral bacteria (Coulter & Russell, 1976). The biofilms in this system have a heterogeneous structure, with depths ranging from 5 to 22 µm (Bradshaw *et al.*, 1996). It may be that the older biofilms are capable of being organized spatially to protect the strict anaerobes within the community, thus resulting in their increasing

predominance with time in all three experiments described in this study. Although no structural or species distribution studies were carried out on the present biofilms, *N. subflava* appeared to be predominant in the upper levels and *F. nucleatum* in the lower layers of biofilms generated from a similar inoculum in a constant-depth (300 µm) film fermenter (Kinniment *et al.*, 1996). Similarly, *Neisseria* spp. have been observed in the upper layers of mature human plaque whereas *Veillonella* spp. were located in the deeper layers of the biofilm (Ritz, 1969). The observation that bacteria which, by normal criteria, are defined as obligate anaerobes can grow in aerated conditions in the planktonic phase is somewhat more difficult to explain. Kolenbrander (1988) demonstrated a number of highly specific coaggregations between oral bacteria, and has suggested that these could provide a mechanism by which successional development might occur during dental plaque development. This mechanism could also operate in the planktonic phase, and clumps of bacteria thus formed could then potentially protect anaerobic bacteria. The kinetics of oxygen consumption would be of key importance in ensuring gradient development over such short distances. Future studies will examine the mechanisms by which the obligately anaerobic bacteria might survive the conditions of aeration.

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