Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments

P. I. Diaz, P. S. Zilm and A. H. Rogers

The authors compared the differences in tolerance to oxygen of the anaerobic periodontopathic bacteria Fusobacterium nucleatum and Porphyromonas gingivalis, and explored the possibility that F. nucleatum might be able to support the growth of P. gingivalis in aerated and CO2-depleted environments. Both micro-organisms were grown as monocultures and in co-culture in the presence and absence of CO2 and under different aerated conditions using a continuous culture system. At steady state, viable counts were performed and the activities of the enzymes superoxide dismutase and NADH oxidase/peroxidase were assayed in P. gingivalis. In co-culture, F. nucleatum was able to support the growth of P. gingivalis in aerated and CO2-depleted environments in which P. gingivalis, as a monoculture, was not able to survive. F. nucleatum not only appeared to have a much higher tolerance to oxygen than P. gingivalis, but a significant increase in its numbers occurred under moderately oxygenated conditions. F. nucleatum might have an additional indirect role in dental plaque maturation, contributing to the reducing conditions necessary for the survival of P. gingivalis and possibly other anaerobes less tolerant to oxygen. Additionally, F. nucleatum is able to generate a capnophilic environment essential for the growth of P. gingivalis.

Keywords: oxidative stress, oral bacteria, anaerobes, interactions, continuous culture

INTRODUCTION

It is well known that elevated oxygen tensions within bacterial cells increase the enzymic and non-enzymic reduction of molecular oxygen to superoxide anions (O2−), which can form, by dismutation, H2O2 and O2. H2O2, in turn, reacts with O2− to form OH• in the presence of iron complexes (Rosen & Klebanoff, 1979). These oxygen species are highly reactive and can cleave nucleic acids and oxidize essential proteins and lipids (Brawn & Fridovich, 1981; Harley et al., 1981). Strictly anaerobic micro-organisms do not possess the antioxidant systems needed to detoxify such reactive oxygen species. However, the susceptibility of anaerobes to oxygen varies even among closely related micro-organisms, and it has been suggested that it correlates with the levels of anti-oxidant enzymes present, superoxide dismutase (SOD) in particular (Park et al., 1992).

Abbreviations: SEM, scanning electron microscopy; SOD, superoxide dismutase.

As the oral cavity is an overtly aerobic environment, it is therefore likely that oral anaerobes encounter residual amounts of oxygen both in the early stages of dental plaque development and in established periodontal pockets (Marquis, 1995). Indeed, periodontal pockets have been reported to possess residual oxygen at one-tenth the level in air-saturated water (which is 0.021 µmol ml−1) (Mettraux et al., 1984) and the average Eh (redox potential) in subgingival plaque appears to be only somewhat negative at about −50 mV (Kenny & Ash, 1969). Moreover, there is evidence in dental plaque of open channels that could deliver oxygen deep into the biofilm (Massol-Deya et al., 1994). Therefore, the survival of anaerobic bacteria in the mouth might be dependent on the specific tolerance to oxygen of each species and on microbial interactions within the community.

Porphyromonas gingivalis and Fusobacterium nucleatum belong to the group of strictly anaerobic bacteria associated with periodontal diseases (Ximenez-Fyvie et al., 2000). Due to its numerous putative virulence
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The effects of F. gingivalis is considered one of the major periodontopathic bacteria (LaMont & Jenkinson, 1998). F. nucleatum is also regarded as a key organism for dental plaque maturation due to its extensive co-aggregating capacity (Kolenbrander et al., 1989). Bradshaw et al. (1998) have suggested that F. nucleatum could be a 'bridge' or 'mediator' of co-aggregation between facultative and obligate anaerobic species and they proposed that this co-aggregation was the mechanism by which strict anaerobes, such as P. gingivalis, survived under aerobic conditions, due to the formation of microenvironments in which the facultative organisms mediated reducing conditions. However, our earlier studies (Diaz et al., 2000) showed that although F. nucleatum is an anaerobe, its capacity to adapt to and reduce an oxygenated environment is extremely high. Therefore, in the present study we explored the possibility that F. nucleatum might be able to protect P. gingivalis and other anaerobic micro-organisms less tolerant to oxygen. Accordingly, our aims were to determine whether F. nucleatum was able to support the growth of P. gingivalis in a continuous aerated co-culture, comparing this co-culture with the tolerance to oxygen of a monoculture of P. gingivalis grown under the same conditions. The levels of anti-oxidant enzymes in P. gingivalis were also assayed in order to compare them with our previously reported levels of the same enzymes in F. nucleatum (Diaz et al., 2000). As preliminary experiments showed that F. nucleatum does not require the external addition of CO₂ for growth in the chemostat, while CO₂ seems to be essential for P. gingivalis, we also explored the possibility that F. nucleatum could supply CO₂ for the growth of P. gingivalis.

METHODS

Micro-organisms and maintenance of the strains. F. nucleatum ATCC 10953 (the type strain) and P. gingivalis W50 (ATCC 53978) were used for all experiments and maintained, short-term, on anaerobic blood agar plates incubated at 37 °C in an atmosphere of N₂/H₂/CO₂ (90:5:5).

Growth conditions. For all experiments, if not otherwise indicated, the micro-organisms were grown under continuous culture conditions in BM medium (Shah et al., 1976) supplemented with 500 μg haemin 1⁻¹. Growth in a 365 ml working-volume chemostat (BioFlo model C30, New Brunswick Scientific) was initiated by inoculating the growth chamber with a 24 h batch culture of the micro-organism(s) grown in the same medium under an atmosphere of N₂/H₂/CO₂ (90:5:5). After 24 h of batch-culture growth in the chemostat vessel, the medium reservoir pump was turned on and the medium flow adjusted to give a dilution rate of D = 0.069 h⁻¹ (t_d = 10 h). The temperature was controlled at 36 °C and the pH maintained at 7.4 by the automatic addition of 2 M KOH. The cultures were sparged with the appropriate gas mixture and the E was constantly monitored, as was dissolved oxygen. Culture purity was checked daily by Gram staining and cell viability was measured, at steady-state, by viable counts. Vortex mixing of co-culture samples, prior to viable counting, was carried out to disperse co-aggregated cells without affecting cell viability. Blood agar plates, with and without 50 μg kanamycin ml⁻¹, were used for viable counts of P. gingivalis and F. nucleatum, respectively. In the kanamycin-containing medium P. gingivalis was recovered with 100% efficiency. Under all conditions, steady state was achieved after about seven generations, as evidenced by sustained stability of the culture E and cell viability. Various culture parameters (see below) were then assayed daily for up to 9 d. Cultures grown under various conditions were also examined by scanning electron microscopy (SEM), as follows. For planktonic-phase analysis, approximately 1 ml of cell culture was removed from the chemostat and 5 μl was placed on a micro glass cover slip and allowed to dry. When formation of biofilms occurred over the chemostat inserts, the chemostat was disassembled and a small amount of the biofilm was mechanically removed with minimal disruption and placed directly onto cover slips. All samples were then placed in fixative solution containing 4% paraformaldehyde, 1:25% glutaraldehyde and 4% sucrose in PBS, followed by post-fixing in 1% OsO₄, and dehydration with increasingly concentrated ethanol solutions. Samples were analysed using a Philips XL30 field emission scanning electron microscope.

Initially, P. gingivalis was grown in a gaseous atmosphere of N₂/O₂ (95:5) and then under the sequentially increased oxygenated conditions N₂/CO₂/O₂ (85:5:10) and N₂/CO₂/O₂ (75:5:20).

To evaluate the CO₂ requirement of the two organisms, each was grown axenically in a N₂/CO₂ (95:5) atmosphere and when steady state had been achieved, CO₂ was excluded from the gas mixture. To determine whether P. gingivalis could survive in a CO₂-depleted environment relying only on the amount of CO₂ produced by its own metabolism, a closed environment was used, rather than the chemostat, in which the gaseous atmosphere is continually replaced. For these experiments, P. gingivalis was grown in batch culture; two anaerobic jars were used for incubation at 37 °C, one gassed with N₂/CO₂ (95:5) and the other with N₂ alone. P. gingivalis cells growing at steady state in continuous culture under an atmosphere of N₂/CO₂ (95:5) were used as the initial inoculum. BM medium was used for all experiments and media were inoculated to produce an initial OD₅₆₀ of 0.45, in a total volume of 50 ml. At late-exponential phase the OD₅₆₀ was recorded, the pH was measured and the appropriate amount of culture, to produce an initial OD₅₆₀ of 0.45, was transferred to fresh medium; these broths were incubated under the appropriate gaseous conditions.

The ability of F. nucleatum to support the growth of P. gingivalis under aerated conditions was determined by growing the bacteria in continuous co-culture under the same gaseous conditions as described above for the P. gingivalis monoculture condition. In a parallel set of experiments CO₂ was excluded from the gas mixture.

Enzyme assays. Since P. gingivalis failed to grow under oxygen concentrations of 10% or more, the activity of anti-oxidant enzymes was assayed in cell-free extracts from the micro-organism grown under anaerobic conditions and under 3% and 6% O₂ in the incoming gas mixture. Extracts were prepared as described previously (Diaz et al., 2000). Protein content was determined using a Coomassie Plus Protein Assay Reagent Kit (Pierce) with bovine serum albumin as a standard. NADH oxidase and NADH peroxidase activities were assayed at 25 °C following the methods of Higuchi (1992). NADH oxidase was assayed by monitoring the oxidation of β-NADH in the reaction mixture (3 ml) at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM β-NADH and cell extract (0.125 mg protein). NADH peroxidase was assayed under anaerobic conditions, achieved...
in a Thunberg-type cuvette, using the same reaction mixture as for NADH oxidase but with the addition of 0.3 mM H₂O₂.

One unit of activity for both enzymes was defined as the amount of extract that catalysed the oxidation of 1 nmol NADH min⁻¹. SOD activity was measured at 550 nm by competitively inhibiting the reduction of cytochrome c at 25 °C, following the methods of McCord & Fridovich (1969). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.01 mM cytochrome c, 0.1 mM xanthine, sufficient volume of xanthine oxidase to produce a constant reduction of cytochrome c at a rate of 0.02 units of absorbance increase min⁻¹ and cell extract (0.125 mg protein). One unit of SOD was defined as the amount of extract that decreased by 50% the rate of reduction of cytochrome c.

**Statistical analysis.** Data were expressed as means ± standard deviations. Differences between means were analysed for statistical significance by Student’s t test.

**RESULTS**

Under the aerated conditions initially tested (10% and 20% O₂), a monoculture of *P. gingivalis* declined according to washout kinetics. However, further experiments showed that *P. gingivalis* was able to maintain steady-state growth in environments containing lower oxygen levels (3% and 6%) and a statistically significant decrease in cell viability occurred as the oxygen concentration was increased. Anti-oxidant enzyme levels for *P. gingivalis* grown in these environments are shown in Table 1.

Also, as seen in Fig. 1, the exclusion of CO₂ from the gas mixture resulted in washout of a monoculture of *P. gingivalis* but not of *F. nucleatum*. It should be noted that NaHCO₃ added to the growth medium did not substitute for CO₂. Furthermore, batch culture experiments under a closed anaerobic atmosphere showed that the CO₂ produced by *P. gingivalis* metabolism was not sufficient to fulfil its growth requirements. As shown in Table 2, the micro-organism did not survive even a single transfer in a CO₂-depleted atmosphere.

In contrast to the low oxygen tolerance of a monoculture of *P. gingivalis*, the organism survived in co-culture with *F. nucleatum* in gaseous environments containing 10% and 20% O₂ (Table 3, Environment 1). Interestingly, a statistically significant increase in the populations of *F. nucleatum* was observed under moderate oxygen levels (10%).

It can also be seen (Table 3, Environment 2) that *F. nucleatum* was able to satisfy the requirements for CO₂ of *P. gingivalis*, as the exclusion of CO₂ from the gas mixture did not have a marked effect on *P. gingivalis* population sizes. This contrasted with the inability of *P. gingivalis* to grow in monoculture without CO₂ (Fig. 1).

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**Table 1. Effect of O₂ on the growth and specific activity of anti-oxidant enzymes of *P. gingivalis***

<table>
<thead>
<tr>
<th>Gas condition†</th>
<th>Eₚ (mV)</th>
<th>Viable counts‡</th>
<th>NADH oxidase§</th>
<th>NADH peroxidase§</th>
<th>SOD§</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂/CO₂ (95:5)</td>
<td>-487</td>
<td>9.79±0.10</td>
<td>8.15±0.72</td>
<td>9.04±0.81</td>
<td>8.55±0.62</td>
</tr>
<tr>
<td>N₂/CO₂/O₂ (92:5:3)</td>
<td>-420</td>
<td>9.36±0.20*</td>
<td>12.77±1.43*</td>
<td>11.12±1.06**</td>
<td>8.45±0.52</td>
</tr>
<tr>
<td>N₂/CO₂/O₂ (89:5:6)</td>
<td>-385</td>
<td>9.15±0.10**</td>
<td>19.83±1.18**</td>
<td>18.11±1.82**</td>
<td>10.67±0.57*</td>
</tr>
</tbody>
</table>

* P < 0.05 for results of anaerobic versus 3% O₂ or 6% O₂.

** P < 0.001 for results of anaerobic versus 3% O₂ or 6% O₂.

† Gas percentages indicate the incoming gas mixture. Dissolved O₂ was not detected in any of the above conditions.

‡ Mean ± sd of log₁₀(c.f.u. ml⁻¹), n = 6.

§ Means ± sd of specific activity [units (mg protein)⁻¹], n = 4.

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**Fig. 1. Effect of CO₂ on the chemostat growth of *F. nucleatum* (△) and *P. gingivalis* (■) as monocultures. The gas phase up until the 12th generation was N₂/CO₂ (95:5); CO₂ was then turned off (arrow).**

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**Table 2. Effect of CO₂ on the batch culture growth of *P. gingivalis***

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>OD₆₅₀</th>
<th>OD₆₅₀ tr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂; CO₂ (95:5)</td>
<td>1.15±0.05 (pH 7.5)</td>
<td>1.25±0.04</td>
</tr>
<tr>
<td>N₂ (100)</td>
<td>1.05±0.05 (pH 7.4)</td>
<td>0.38±0.06</td>
</tr>
</tbody>
</table>

Table 3. Chemostat co-cultures of P. gingivalis and F. nucleatum grown under increasingly oxygenated conditions in CO₂-rich and CO₂-depleted environments

In Environment 1 (n = 5) CO₂ was included in the gas mixture. Anaerobic: N₂/CO₂ (95:5), 10% O₂: N₂/CO₂/O₂ (85:5:10), 20% O₂: N₂/CO₂/O₂ (75:5:20). In Environment 2 (n = 9) CO₂ was excluded from the gas mixture. Anaerobic: N₂ (100). 10% O₂: N₂/O₂ (90:10), 20% O₂: N₂/O₂ (80:20). Dissolved O₂ was not detected in any of the above conditions. Values represent viable counts expressed as means ± standard deviations of log_{10}(c.f.u. ml⁻¹).

<table>
<thead>
<tr>
<th></th>
<th>Anaerobic</th>
<th>10% O₂</th>
<th>20% O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>7.91 ± 0.05</td>
<td>8.52 ± 0.06†</td>
<td>7.76 ± 0.16</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>9.05 ± 0.05</td>
<td>8.16 ± 0.08†</td>
<td>6.38 ± 0.68†</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>7.98 ± 0.18</td>
<td>8.52 ± 0.12†</td>
<td>7.36 ± 0.31††</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>8.00 ± 0.31*</td>
<td>8.10 ± 0.35</td>
<td>5.39 ± 0.79††</td>
</tr>
</tbody>
</table>

* Results are significantly different to Environment 1 in the same gas phase (P < 0.001).
† Results are significantly different to anaerobic conditions in the same Environment (P < 0.001).

Fig. 2. Gram stains of the planktonic phase of a continuous co-culture of P. gingivalis (Pg) and F. nucleatum (Fn) grown under anaerobic conditions (a) and under 20% O₂ (b). Bar, 10 μm.

The co-culture Eₙ was unaffected by the presence or absence of CO₂. Furthermore, the change from anaerobic conditions to 10% O₂ resulted in only a small change in Eₙ values, from about −500 mV to −430 mV. However, the culture Eₙ increased to about −150 mV under 20% oxygen and only under this condition were large planktonic aggregates and biofilms observed around the growth vessel inserts. Daily Gram stains and SEM analysis of the planktonic phase of the culture showed that F. nucleatum cells increased in length as the culture became more oxygen stressed; cells grown under 20% oxygen were at least five times longer than cells grown under anaerobic conditions (Fig. 2). SEM analysis of the biofilms formed under aeration showed that most obvious on the surface of these biofilms were F. nucleatum cells that appeared to form an intricate network; P. gingivalis is difficult to distinguish but its presence was confirmed by viable counts.

Fig. 3. Scanning electron micrograph of a biofilm formed by F. nucleatum and P. gingivalis grown as a continuous co-culture under 20% O₂. The rod-shaped F. nucleatum cells form an intricate network; P. gingivalis is difficult to distinguish but its presence was confirmed by viable counts.

DISCUSSION

Of the 300 to 400 species isolated from the oral cavity, only a small group of micro-organisms, including F. nucleatum and P. gingivalis, is consistently associated with periodontitis (Socransky & Haffajee, 1994). These
two species, as is the case with other periodonto-
pathogens, might colonize the supra- and subgingival
plaques of periodontally healthy individuals for con-
siderable periods of time prior to disease initiation, after
which their levels increase (Ximenez-Fyvie et al., 2000).
It is therefore important to determine how micro-
organisms survive during the different stages of plaque
development and to identify those factors regulating the
ecological shifts that occur in the transition from health
to disease.

Socransky et al. (1998) demonstrated that bacteria in
subgingival plaque exist as microbial complexes. Al-
though P. gingivalis and F. nucleatum were identified as
forming part of different complexes, a close association
was shown to exist between the complexes to which each micro-organism belonged. In fact, the members of the
P. gingivalis complex were rarely found in the
absence of members of the F. nucleatum complex. Our
data provide an explanation for this apparent depen-
dence of P. gingivalis on F. nucleatum, suggesting that
the latter might create the necessary reduced conditions
and supply CO₂ for the survival of the former.

The present study clearly shows that, although F.
nucleatum and P. gingivalis are anaerobes, there is a marked difference in their oxygen tolerance. Our pre-
vious findings showed that F. nucleatum is able to
survive in chemostat cultures sparged with proportions
of oxygen even higher than the oxygen content of air
(Diaz et al., 2000), while the present findings show that
P. gingivalis is not able to survive, in the same system,
when the gas phase of the culture contains more than
6% oxygen. It should be noted that the ability to
metabolize oxygen by a culture of F. nucleatum or P.
 gingivalis may depend upon the cell numbers (deter-
mined by nutrient availability) used in this system, but it
reflects the potential of the organisms to survive oxygen
tensions that might occur in the oral environment.

Interestingly, if the currently detected levels of the anti-
oxidant enzymes in P. gingivalis are compared with our
previous report for F. nucleatum (Diaz et al., 2000), the
major differences occur in NADH oxidase activity.
Under anaerobic conditions, F. nucleatum produced 190
units of activity (mg protein⁻¹, while P. gingivalis
activity was only 8–15 units (mg protein⁻¹, a difference
of more than 20-fold. In contrast, SOD activity in F.
nucleatum was 10 times lower than in P. gingivalis. This
indicates that, in these two micro-organisms, SOD
activity does not correlate with tolerance to oxygen,
which is at variance with previous suggestions for other
species (Park et al., 1992). NADH oxidase/peroxidase
usually exists as a double system, able to metabolize
both molecular oxygen and H₂O₂ (Poole et al., 2000),
while the role of SOD is the detoxification of O₂⁻ with
the formation of H₂O₂ (McCord & Fridovich, 1969). There-
fore, high levels of SOD without correspondingly
high levels of NADH oxidase/peroxidase activities
would seem to be required by anaerobic micro-organisms because of the generation of large amounts of H₂O₂ that can
not be adequately detoxified, as seems to occur in P.
gingivalis. The negative aspect of the NADH oxidase/
peroxidase systems appears to be that as they are flavin-
based enzymes, the formation of O₂⁻ can be promoted
(Imlay & Fridovich, 1991). Therefore, it seems that an
efficient radical detoxification system in the absence of
catalase, as is the case for F. nucleatum and P. gingivalis,
depends on the delicate balance between these two
anti-oxidant enzymes, although higher proportions of
NADH oxidase/peroxidase seem more beneficial for F.
nucleatum.

The present results clearly show that F. nucleatum is
able to support the growth of P. gingivalis under aerated
conditions in which the latter cannot survive as a
monoculture. Indeed, the fact that the populations of F.
nucleatum increase under oxygen stress indicates its
capacity to survive in natural environments that might
be partially oxygenated, in contrast to the low tolerance
to oxygen of P. gingivalis. Thus, these results suggest
that the capacity of F. nucleatum to protect P. gingivalis
from oxidative damage might be one of the reasons why
there seems to be a close in vivo association between
these two micro-organisms (Socransky et al., 1998).
Additionally, this study shows that the growth of F.
nucleatum does not require a capnophilic environment,
whereas CO₂ seems to be essential for P. gingivalis. The
fact that P. gingivalis survived in co-culture with F.
nucleatum without the addition of CO₂ indicates that
the latter is able to satisfy the CO₂ requirement of the
former. This might constitute another metabolic in-
teraction that explains the close association between
the pair. Other possible interactions between the two micro-
organisms include the presence of proteolytic enzymes
in P. gingivalis that cleave proteins into peptides and
therefore increase the energy sources for F. nucleatum,
which does not possess high endopeptidase activity
(Grenier, 1994).

The formation of biofilms, as observed in this study,
might be a strategy used by F. nucleatum to overcome
high oxidative stress, possibly because of the more
reduced microenvironments inside the biofilm, which
could in turn benefit P. gingivalis. The intricate networks
formed by F. nucleatum in these biofilms are not
surprising, considering the shape of the micro-organism,
which increases its length in an oxygenated environ-
ment.

It is worth noting that haem-limiting conditions were
used in this study for the growth of P. gingivalis. This
choice was made in order to avoid the possible protective
effect of haem in excess under oxidative stress conditions,
as it has been suggested that the binding of haem
dimers to the surface of P. gingivalis would serve as a
catalase-like buffer system (Smalley et al., 2000). The
contribution of this mechanism to the ability of P.
gingivalis to overcome oxygen stress could be deter-
mined by growing the organism under haemin-excess
conditions. In this context, the study by Bradshaw et al.
(1998), showing that P. gingivalis numbers were greatly
reduced when F. nucleatum was omitted from a 10-
member oral bacterial consortium subjected to aerated
conditions, was conducted under haemin-excess conditions.

In conclusion, additional to the known direct potential involvement of F. nucleatum in the disease process (Han et al., 2000; Yoshimura et al., 1997), this study suggests that F. nucleatum could have an important indirect role in the aetiology of periodontal diseases by supporting the growth of P. gingivalis and possibly other oral anaerobes in oxygenated and CO₂-depleted environments. Protection against the deleterious effects of oxygen might be important both in the early stages of plaque development and in periodontal pockets, where micro-organisms have to face the constant presence of residual oxygen levels.

From an ecological point of view, the identification of micro-organisms that, like F. nucleatum, support the growth of other periodontopathogens is important because control of such species might radically alter the pathogenic ecosystem.

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