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Production and sensitivity of bacteriocin-like activity among *Porphyromonas gingivalis*, *Prevotella intermedia* and *Pr. nigrescens* strains isolated from periodontal sites

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The production of and sensitivity to bacteriocin-like activity among 44 strains of black-pigmented anaerobes isolated from periodontal sites were evaluated by both an overlay and an agar diffusion method. The species studied were *Porphyromonas gingivalis*, *Prevotella intermedia* and the closely related species *Pr. nigrescens*. *Pr. intermedia* strains (90%) produced bacteriocin-like activity against *Pr. nigrescens* and all *Pr. nigrescens* were active against *Pr. intermedia*. Both species showed a high degree of activity against *P. gingivalis*, whereas only one *P. gingivalis* strain produced bacteriocin-like activity against either of the other two species. Both *Pr. nigrescens* and *Pr. intermedia* showed some activity (40% and 20%, respectively) against other strains of the same species. Such bacteriocin production might be expected to influence the distribution of these black-pigmented species *in vivo*. Of 224 periodontal sites sampled, only 2.6% yielded mixed cultures of black-pigmented species and of these only one strain, a *P. gingivalis* isolate, produced bacteriocin-like activity against any of the other strains isolated from these sites. These data support the concept that local production of bacteriocin-like activity *in vivo* may contribute to the selection of the black-pigmented bacterial profile in subgingival sites.

Introduction

Black-pigmenting, oral anaerobic bacteria have been associated with destructive forms of inflammatory periodontal disease. Of these bacteria, *Porphyromonas gingivalis* has been isolated frequently from advancing lesions and is thought to be the most virulent species [1]. *Prevotella intermedia* has also been found frequently in diseased sites of patients with chronic adult periodontitis, acute necrotising ulcerative gingivitis and pregnancy gingivitis [2]. However, some of the strains previously reported as *Pr. intermedia* may well have belonged to the closely related but newly described species, *Pr. nigrescens*. These two species can be separated by serotyping [3–5], DNA–DNA hybridisation [6], 16S-RNA analysis [7], RAPD profiles [8] or total protein profiles on SDS-polyacrylamide gels [9, 10]. Although recent studies with these methods have shown *Pr. nigrescens* to be present at both

clinically diseased and healthy subgingival sites, the view is now generally held that the organism is associated with health rather than disease [9, 11, 12]. In contrast, *P. gingivalis* is rarely found in the oral cavity of healthy subjects [9, 13].

Despite black-pigmented anaerobes being found frequently in periodontal infections, recent studies have indicated that mixed populations of these organisms are relatively uncommon, suggesting that they may be incompatible [9, 14–16]. Such incompatibility may be due either to competition between species for essential nutrients or to the production of aggressive compounds, such as bacteriocins. Similar incompatibility, which is due to bacteriocin production [17], has been described among strains of another putative periodontal pathogen, *Actinobacillus actinomycetemcomitans*, and between this and various commensal oral species [18].

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Only limited information is available concerning bacteriocin production and sensitivity among black-pigmented anaerobes. A proteinaceous bacteriocin-like

compound, called melanocin, has been isolated from a black-pigmented '*Bacteroides*' strain – '*B*'. (now *Pr.*) *melaninogenicus*(a) [19] – and two separate bacteriocins have been isolated from '*B*'. (*Pr.*) *intermedius* (a), one with activity against '*B*'. (*P.*) *gingivalis* and one against '*B*'. (*Pr.*) *intermedius*(a) [20, 21]. More recently, Hohne *et al.* [22] studied the bacteriocin production and activity of numerous black-pigmenting strains isolated from periodontal sites and showed that *Pr. intermedia* was the dominant effector species. However, only a few indicator strains were used in the study and there is no information available on bacteriocin production by the new species, *Pr. nigrescens*.

The aim of this study was to assess the production of and sensitivity to bacteriocin-like activity among clinical isolates of *P. gingivalis*, *Pr. intermedia* and *Pr. nigrescens* and to determine whether such activity could explain the apparent incompatibility between black-pigmented anaerobes in periodontal sites.

Materials and methods

Bacteria

Forty-eight strains (4 reference strains and 44 clinical isolates) were examined for their bacteriocin production and sensitivity, comprising *P. gingivalis* (13), *Pr. intermedia* (14) and *Pr. nigrescens* (21). The four reference strains used were *P. gingivalis* W50, *Pr. intermedia* ATCC 25611 and *Pr. nigrescens* ATCC 25261 and MH11; the latter was provided by H.N. Shah, National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London.

Clinical strains were isolated from subgingival plaque obtained from diseased and healthy sites of 46 adult patients with a clinical diagnosis of adult periodontitis as described previously [9]. Samples were obtained with sterile paper points placed subgingivally for 10 s and cultured on Fastidious Anaerobe Agar (FAA; Lab M) supplemented with yeast extract 0.5% and horse blood (Oxoid) 8% in an atmosphere of CO₂ 10%, H₂ 10%, N₂ 80% in an anaerobic jar for 7–14 days. Collection and processing of samples were completed within 1 h. Several colonies that were either uniformly black or showing the beginning of brown pigmentation were selected from each specimen and identified to species level by the methods described previously [9, 23].

Bacteriocin production

Bacteriocin-like activity was assessed by an agar overlay technique and by an agar diffusion method. In the overlay method, based on that originally described by Fredericq [24], several possible producer strains were stab-cultured on plates of FAA, anaerobi-

cally for 24–48 h. The plates were then exposed to UV light overnight under aerobic conditions to stop growth and all strains were re-streaked to check for their survival. Indicator strains were grown anaerobically for 24–48 h in BHI broth supplemented with haemin 5 µg/ml, yeast extract 0.5% and vitamin K 1 µg/ml. Cell suspensions (0.5 ml adjusted to A₅₅₀ 1.5) were each added to 5 ml of molten FAA and immediately poured on top of the plates of the producer strains. These were then incubated for 24 h at 37°C in anaerobic conditions and examined for the presence of zones of inhibition around the inoculae of the producer strains.

In the agar diffusion method, modified from that of Riley and Mee [25], filter membranes (9 cm diameter, 0.45 µm pore size; Schleicher and Schulle) were sterilised by exposure to UV light (30 min each side) and floated on molten FAA supplemented with horse blood 8% in petri dishes; after setting, the plates were dried. The total surface of each membrane was then inoculated with growth from a young plate culture of a possible producer strain and incubated at 37°C anaerobically. The membrane, with adherent growth, was then carefully removed with forceps and a thin layer of molten FAA plus blood (5 ml) was pipetted on to the surface to replace any nutrient loss from the agar. After drying, the plates were inoculated with 5-µl of suspensions of indicator strains; up to 10 strains were placed on a single plate. Plates were then incubated anaerobically for up to 7 days and examined for growth of indicators. As a control in both methods, the producer strain was also included as an indicator strain.

Results and discussion

Initial tests with a 'cross-streak' method showed that most strains failed to grow on areas of agar that had previously supported growth of the homologous strain. This suggested that the original inoculum had significantly depleted the nutrients in the surface layer of the agar and that this lack of growth might be confused with bacteriocin production. To overcome this problem, two approaches were taken. In the first, organisms were suspended in a suitable nutrient agar overlay on the killed growth of the producer strains, while in the second approach, all growth of the original strain was removed and the plate was then overlaid with a thin layer of the same medium before strains were inoculated as spots. Both approaches gave satisfactory and similar results and are illustrated in Figs. 1 and 2.

Production of bacteriocin-like activity by strains of *P. gingivalis*, *Pr. intermedia* and *Pr. nigrescens* isolated as single species from clinical sites is shown in Table 1. The strains were tested against a selection of eight indicator strains comprising at least one reference strain and a range of clinical isolates chosen at

Fig. 1. Agar plate showing bacteriocin production by *Pr. intermedia* (1–3) and *Pr. nigrescens* (4–5) screened by incorporation of a *P. gingivalis* indicator strain in an agar overlay. Strains 6–8 are not active.

random. Occasional strains were lost during the period of the study, which explains why not all strains were tested against all indicators. None of the *P. gingivalis* isolates tested produced activity against any *Pr. intermedia*, *Pr. nigrescens* or the other strains of *P. gingivalis*. This finding is similar to that reported by Hohne *et al.* [18] who showed that only a few strains of *P. gingivalis* showed activity against *P. endodontalis* and *P. asaccharolytica* and apparently none against *Pr. intermedia* strains. *Pr. nigrescens* was not tested in that study. In contrast to *P. gingivalis*, most *Pr. intermedia* and *Pr. nigrescens* strains produced bacteriocin-like activity; 90% of *Pr. intermedia* strains showed activity against *Pr. nigrescens* strains and all *Pr. nigrescens* strains were active against *Pr. intermedia*. The majority (85%) of *Pr. nigrescens* strains and 91% of *Pr. intermedia* strains inhibited the *P. gingivalis* indicator strains. Furthermore, 20% of *Pr. intermedia* and 40% of *Pr. nigrescens* strains showed bacteriocin-like activity against other strains of the homologous species. These data show that production of bacteriocin-like activity is common among this group of black-pigmented anaerobes and is, therefore, in general agreement with the findings of other workers [19–22].

No attempt has been made in this study to establish that the inhibitory activity detected conformed to the criteria defining bacteriocins [26] because this would require purification of the active factor. However, the activity detected showed a limited spectrum, as do bacteriocins and it did not appear to be due to

diffusible metabolites, such as acids, because the pH of the agar under the growth of a number of strains was approximately 7.5.

The relevance of the bacteriocin-like activity described above for the ecology of black-pigmented anaerobes is currently unclear. However, if bacteriocin production *in vivo* is significant, mixtures of these three black-pigmented organisms in periodontal sites might be expected to be rare. Indeed, of the 224 periodontal sites sampled in 46 patients in this study, only six sites (2.7%) yielded a mixture of black-pigmented species and all except one of these were from diseased periodontal sites. This finding is in accordance with those of others [9, 14–16], although one report differs from the findings of the present study [27]. This exception may have been due either to the method used to collect the specimens (e.g., paper point *versus* curette) or to the fact that, in the present study, only up to 13 colonies of black-pigmenting organisms from each sample were speciated and so organisms present in low numbers may have been missed. The mixtures obtained comprised *P. gingivalis* + *Pr. intermedia* (one site), *P. gingivalis* + *Pr. nigrescens* (two sites), *P. gingivalis* + *P. denticola* (one site), *Pr. intermedia* + *Pr. nigrescens* (one site) and *Pr. nigrescens* + *Pr. denticola* (one site). All the *Pr. nigrescens*, *P. gingivalis* and *Pr. intermedia* isolates were examined for production of bacteriocin-like activity against each other and with the exception of one *P. gingivalis* strain, none of the isolates produced inhibitory activity against any other strain.

Fig. 2. (a) Control agar plate showing growth of *Pr. nigrescens* indicator strains 1–8 and **(b)** test plate showing bacteriocin sensitivity of strains 1, 2, 7 and 8 as illustrated by lack of growth. The *Pr. nigrescens* producer strain had been grown on a membrane filter on the agar surface, which was removed before inoculation of the indicator strains.

Although the numbers are small, this finding would support the concept that bacteriocin production by *Pr. intermedia* and *Pr. nigrescens* is a significant factor in determining their distribution in periodontal sites. Nonetheless, as *P. gingivalis* was not found to produce bacteriocin activity *in vitro*, the question arises as to why any sites should harbour high levels of *P. gingivalis* but no significant numbers of *Pr. intermedia* or *Pr. nigrescens*, as was found in 8.5% of sites by Teanpaisan *et al.* [23]. Perhaps this can be explained on the basis of the time at which species become established relative to each other, their nutrient

requirements and the environment prevailing at that time. For example, *P. gingivalis* is known to have a higher affinity for haemin than *Pr. intermedia* [28] and so if *P. gingivalis* is present at a site first where haemin is available, any *Pr. intermedia* cells subsequently attempting to colonise might not be able to obtain enough haemin to proliferate sufficiently to generate a bacteriocin challenge. Consequently, although not producing bacteriocin activity, *P. gingivalis* may become the dominant black-pigmented species at some sites by virtue of its metabolic activity. In contrast, at sites where *Pr. intermedia*

Table 1. Bacteriocin-like activity among *P. gingivalis*, *Pr. intermedia* and *Pr. nigrescens* strains isolated as the sole black-pigmented species from periodontal sites

Indicator strains*	Number of producer strains/number of strains tested		
	<i>P. gingivalis</i>	<i>Pr. intermedia</i>	<i>Pr. nigrescens</i>
<i>P. gingivalis</i>			
W50	0/10	11/12	11/13
strain 1	0/10	11/12	11/13
strain 2	0/10	11/12	11/13
strain 3	0/10	11/12	11/13
strain 4	0/10	11/12	11/13
strain 5	0/10	11/12	11/13
strain 6	0/10	11/12	11/13
strain 7	0/10	11/12	11/13
<i>Pr. intermedia</i>			
25611	0/10	2/10	18/18
strain 1	0/10	2/10	18/18
strain 2	0/10	2/10	18/18
strain 3	0/10	2/10	18/18
strain 4	0/10	2/10	18/18
strain 5	0/10	2/10	18/18
strain 6	0/10	2/10	18/18
strain 7	0/10	2/10	18/18
<i>Pr. nigrescens</i>			
25261	0/10	9/10	4/10
MH 11	0/10	9/10	4/10
strain 1	0/10	9/10	4/10
strain 2	0/10	9/10	4/10
strain 3	0/10	9/10	4/10
strain 4	0/10	9/10	4/10
strain 5	0/10	9/10	4/10
strain 6	0/10	9/10	4/10

*Indicator organisms labelled as 'strain 1, 2, 3', etc. are clinical isolates; others are reference strains.

begins to grow first, production of bacteriocin-like activity could limit the proliferation of newly colonising *P. gingivalis* cells and consequently reduce the competition for nutrients faced by the resident *Pr. intermedia* strains. At present, such an explanation is speculative and will require further work, perhaps with mixed chemostat cultures of organisms, to establish exactly how these three species interact so that one becomes dominant.

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