Congo red binding by Porphyromonas gingivalis is mediated by a 66 kDa outer-membrane protein

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Congo red was bound from solution by strains of Porphyromonas gingivalis including W50, HG189, HG184, NCTC 11834, Bg 381, WPH35, the slower brown pigmenting colonial variant W50/BR1, and the avirulent mutant W50/BE1, and by Porphyromonas endodontalis HG370 and Porphyromonas asaccharolytica B537. SDS-PAGE of whole cells of all species examined displayed a 66 kDa Congo-red-binding component which was also detected in the outer membranes of P. gingivalis W50 grown in the chemostat under both haemin limitation and haemin excess, and which corresponded to a Coomassie-blue-stained band of the same mobility. Pretreatment of haemin-excess batch-grown cells of P. gingivalis W50 with polymyxin B, which binds to lipid A, did not inhibit binding, whilst binding was enhanced in the presence of 2 M ammonium sulphate, suggesting the involvement of non-specific hydrophobic interactions. Binding was also reduced by pretreatment with trypsin and papain, and by 8-anilino-1-naphthalenesulphonic acid, which binds to hydrophobic amino acids. The 66 kDa binding component was sensitive to proteinase K digestion, and loss of Congo red staining of this band correlated with the quantitative reduction in Congo red binding by whole cells. These data, and our previous work, show that Congo red and iron protoporphyrin IX (haemin) are bound to different outer-membrane components, and that Congo red binding may be of little value as a marker to detect virulent strains of P. gingivalis or those expressing haemin-binding proteins.

Keywords: Porphyromonas gingivalis, ligand binding, Congo red, haemin

INTRODUCTION

Cellular binding of the sulphonated diazo dye Congo red is associated with virulence in a number of Gram-negative human pathogens including Actinobacillus (Haemophilus) pleuropneumoniae (Deneer & Potter, 1989), Shigella flexneri (Daskaleros & Payne, 1987; Stugard et al., 1989), enteroinvasive Escherichia coli (Stugard et al., 1989) and the fish pathogen Aeromonas salmonicida (Kay et al., 1985).

In all the above organisms, the Congo-red-binding phenotype also correlates with the expression of outer-membrane haemin-binding proteins (Deneer & Potter, 1989; Daskaleros & Payne, 1987; Kay et al., 1985). In S. flexneri and enteroinvasive E. coli (Stugard et al., 1989), haemin and Congo red bind to a plasmid-encoded 101 kDa outer-membrane protein, whilst cells of Actinobacillus pleuropneumoniae, when maintained under iron restriction, express 105 and 76 kDa outer-membrane proteins, and concomitantly display enhanced binding of both Congo red and haemin (Deneer & Potter, 1989). Virulent (A*) cells of Aeromonas salmonicida possess a surface layer composed of a 50 kDa A protein which binds haemin, while isogenic A- avirulent mutants, which lack the A layer, fail to bind both Congo red and haemin (Ishiguro et al., 1981; Kay et al., 1981). Congo red agar has also been employed as a solid medium for the detection of virulent strains of Aeromonas salmonicida (Ishiguro et al., 1985), Yersinia pestis (Jackson & Burrows, 1956; Surgalla & Beesley, 1969) and Y. enterocolitica (Pripic et al., 1983). Recently, Scott et al. (1993) demonstrated that the binding of both haemin and Congo red to Treponema denticola is mediated by a 47 kDa protein located in the outer membrane, and that these ligands can compete for this binding component.

Abbreviations: e-ACA, e-aminocaproic acid; ANSA, 8-anilino-1-naphthalenesulphonic acid; NEM, N-ethylmaleimide.
Iron protoporphyrin IX (commonly referred to as haemin or haem depending upon the oxidation state of the iron atom) is an essential nutrient for the periodontopathogen Porphyromonas gingivalis (Gibbons & MacDonald, 1960; Bramanti & Holt, 1991). It has also been reported that P. gingivalis can utilize non-haemin iron sources, including ferrous and ferric ions (Bramanti & Holt, 1991) and transferrin (Inoshita et al., 1991). Protoporphyrin IX, the iron-free precursor of haemin, can also support the growth of P. gingivalis when sufficient inorganic iron is provided (Wyss, 1992; Minas et al., 1993). The virulence and pathogenicity of P. gingivalis are modulated by the levels of haemin in the growth environment (McKee et al., 1986; Marsh et al., 1994). Whole cells of P. gingivalis can bind haemin (Smalley et al., 1991, 1993, 1994; Carman et al., 1990; Genco et al., 1994) via the lipid A fraction of lipopolysaccharide (Grenier, 1991). In addition, novel outer-membrane proteins are expressed under haemin limitation (Bramanti & Holt, 1990, 1992a, b; Papaioannou et al., 1991), some of which are haemin-binding proteins (Bramanti & Holt, 1993; Smalley et al., 1993).

Although the growth of members of the Porphyromonas group in vitro is inhibited to varying degrees by Congo red (Mayrand et al., 1984) it is not known whether P. gingivalis binds this dye. Preliminary studies in our laboratory have shown that P. gingivalis cells stain red after 4-5 d when grown on nutrient agar containing serum and Congo red, but this staining is lost slowly after further incubation. In this study we report the presence of a Congo-red-binding protein in the outer membrane of P. gingivalis W50, and in whole cells of a number of other P. gingivalis strains including an avirulent variant W50/BE1, and in whole cells of P. asaccharolytica B537 and P. endodontalis HG370.

METHODS

Bacterial strains and growth conditions. P. gingivalis strains W50, HG184, HG189, NCTC 11834, WPH35, W83, Bg 381, the brown-pigmenting variant W50/BR1 (McKee et al., 1988), and P. asaccharolytica B537 (originally obtained from Dr H. N. Shah, The Eastman Dental Institute, London, UK) were grown in batch culture under haemin excess in Schaedler anaerobe broth (Oxoid) for 3 d and harvested at the end of the exponential phase of growth as described previously (Smalley & Birss, 1987). P. endodontalis HG370 was obtained from Dr T. J. M. van Steenbergen, Amsterdam, The Netherlands. Because of poor growth in liquid culture it was grown on blood-agar plates as satellite colonies around a growth of Staphylococcus aureus NCTC 6571. The cells were washed once in 10 mM phosphate-buffered 0.14 M NaCl, pH 7.4, and once in deionized water, and freeze-dried. P. gingivalis W50 was also grown in a chemostat under conditions of haemin limitation and haemin excess in a complex medium as described previously (Smalley et al., 1991). The avirulent, slow-pigmenting beige variant W50/BE1 was also grown under conditions of haemin excess in the chemostat as above.

Outer membrane preparation. The outer membrane fraction was recovered from washed cells by a modification of the EDTA-shearing method of Mansheim & Kasper (1977) as described previously (Smalley & Birss, 1987). Briefly, cells were incubated in 0.01 M EDTA in phosphate-buffered saline (PBS), pH 7.3, containing the protease inhibitors phenylethylthio-

sulphonyl fluoride (PMSE), N-ethylmaleimide (NEM) and e-aminocaproic acid (EACA), each at a concentration of 1 mM, for 30 min at 37 °C, and forced through a 25 gauge hypodermic needle twice. The sheared cells were then pelleted by centrifugation at 10000 g for 30 min at 4 °C, and the supernatant buffer containing the outer membrane fraction was dialysed against PBS buffer with the above protease inhibitors for 4 h, and then against deionized water for a further 4 g to remove buffer salts. The total outer membrane fraction was then recovered by freeze-drying.

SDS-PAGE. This was carried out on 10 % (w/v) polyacrylamide gels by the method of Laemmli (1970) as described previously (Smalley et al., 1993). Densitometric scans of Coomassie-blue- and Congo-red-stained bands in the gels were made with a 2220 laser densitometer (Pharmacia-LKB).

Identification of Congo-red-binding components. In order to visualize Congo-red-binding components in SDS-polyacrylamide gels, freeze-dried cells, at a concentration of 8 mg ml⁻¹, were suspended in 0.14 M NaCl, 0.1 M Tris/HCl (NaCl/Tris), pH 7.4, containing 0.1 mM Congo red, incubated for 30 min at 37 °C, and centrifuged at 13000 g for 10 min at 20 °C. The cells were washed twice in the above buffer to remove unbound Congo red, and solubilized at 37 °C for 1 h in electrophoresis sample application buffer containing 1 % (w/v) SDS, 2 M urea, 50 mM dithiothreitol and the protease inhibitors PMSE, NEM, EACA and EDTA, each at 1 mM. Congo-red-binding components in the gel were observed directly as red/pink bands. Parallel cell samples were electrophoresed simultaneously and the gels were stained for protein using Coomassie blue R-250 (Merck) in 50 % methanol, 7 % acetic acid, 43 % water.

Congo red binding assay. The quantitative measurement of binding of Congo red to whole cells was carried out in NaCl/Tris, pH 7.4, at a cell concentration of 4 mg (dry weight) ml⁻¹; cells were incubated with 0.1 M Congo red for 30 min. After centrifugation at 13000 g for 10 min to pellet the cells, the A488 of the supernatant buffer containing unbound ligand was measured (Kay et al., 1985) using an MR 700 Dynatech microtestplate reader. The quantity (nmol) of ligand bound per 4 mg dried cells in 1 ml was calculated subtractively from the amount of the residual, free Congo red in the supernatant. Controls were included to correct for the presence of unsedimented cells in the supernatant, and for any absorbance changes occurring in the standard concentrations of Congo red during incubation. Each assay was performed in quadruplicate.

Congo red binding to cells after various pretreatments. To identify the components responsible for Congo red binding, whole cells of strain W50 grown in haemin-excess batch culture (at a concentration of 4 mg ml⁻¹) were subjected to various pretreatments as described by Scott et al. (1993). These included (a) papain in NaCl/Tris pH 6.5, containing 1:3 mM cysteine hydrochloride; (b) pepsin in 10 mM HCl; (c) trypsin in NaCl/Tris, pH 8.5; and (d) proteinase K in NaCl/Tris, pH 7.4. All the above proteases were obtained from Sigma, and used at a concentration of 40 μg ml⁻¹ (at an enzyme:protein substrate ratio of 1:25), except proteinase K, which was obtained from Boehringer Mannheim, and used at a concentration of 40 μg ml⁻¹ (enzyme:protein ratio 1:50). Incubations were carried out for 120 min with constant mixing at 37 °C, and the cells were centrifuged at 13000 g for 10 min, and washed and recentrifuged twice in NaCl/Tris, pH 7.4, containing PMSE, NEM, EDTA and E-ACA (each at 1 mM) to remove inactive residual protease. In the case of digestion with proteinase K, the incubation mixture was also sampled at various time intervals, the cells exposed to Congo red and
analysed by SDS-PAGE. Cells were also pretreated for 30 min at 37 °C with polymyxin B sulphate (150 μg ml⁻¹; Sigma), and with 8-anilino-1-naphthalenesulphonic acid (ANSA, 150 μg ml⁻¹; Sigma), both in NaCl/Tris, pH 7.4. The cells were centrifuged and treated as above before the extent of Congo red binding was compared to untreated control cells incubated only in the appropriate buffer. To investigate the possible involvement of hydrophobic interactions, Congo red binding was also quantified in the presence of 2 M (NH₄)₂SO₄ (Kay et al., 1985).

**Protein assay.** The protein content of outer membrane and whole cell samples was determined using the Lowry method with bovine serum albumin as standard.

**RESULTS**

**Congo red binding by whole cells**

The cells of all strains of *P. gingivalis*, *P. asaccharobtica* B537 and *P. endodontalis* HG370 stained red after exposure to a 0·1 mM solution of Congo red for 30 min at 37 °C. Congo red could not be removed from the stained cells even after repeated washing with NaCl/Tris buffer. From absorbance measurements of residual ligand in the supernatant buffer, Congo red binding was found to lie in the range 35–79 nmol per 4 mg (dry weight) of cells (Table 1). It was noteworthy that cells of strain W50 grown in the chemostat under haemin limitation and haemin excess bound similar levels of Congo red.

**Identification of Congo-red-binding components**

When outer membranes from chemostat-grown haemin-excess and haemin-limited cells of *P. gingivalis* W50 were exposed to Congo red and electrophoresed on a 10% gel after sample solubilization at 100 °C, Congo red staining in the gel was observed as an intense, single red band with a molecular mass of 66 kDa (Fig. 1a). A minor 66 kDa protein was also observed in gels of the outer membranes of haemin-excess and haemin-limited cells when stained with Coomassie blue (Fig. 1b). The Congo-red-stained 66 kDa component was also present in the outer membranes when solubilized at 37 °C for 1 h, although the Coomassie blue staining of this protein was less intense (results not shown). A similar result was also obtained for whole cells of strain W50 grown under haemin limitation and haemin excess (Fig. 2, tracks G and H). Congo red staining of the 66 kDa component occurred in the presence of 10 mM dithiothreitol and 0·25 mM potassium ferricyanide, i.e. under both reducing and oxidizing conditions (results not shown). The seven other strains of *P. gingivalis*, as well as *P. asaccharolytica* B537 and *P. endodontalis* HG370, also displayed the 66 kDa component when solubilized at 37 °C (Fig. 2) or at 100 °C (results not shown). No other Congo-red-stained components were observed.

**Effect of cellular pretreatments on Congo red binding**

Pretreatment of haemin-excess W50 cells with pepsin had little effect, reducing Congo red binding by only 1·6% from the control value. Papain, trypsin and proteinase K pretreatments reduced binding by 8, 58 and 39% of their respective controls (Table 2). Inclusion of 2 M

### Table 1. Congo red binding by whole cells of *P. gingivalis*, *P. endodontalis* and *P. asaccharolytica*

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Congo red bound (nmol)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td>WPH 35</td>
<td>63·4±2·0</td>
</tr>
<tr>
<td></td>
<td>NCTC 11834</td>
<td>60·8±3·3</td>
</tr>
<tr>
<td></td>
<td>W50/BR1</td>
<td>59·6±2·3</td>
</tr>
<tr>
<td></td>
<td>Bg 381</td>
<td>46·1±1·8</td>
</tr>
<tr>
<td></td>
<td>HG 189</td>
<td>63·9±3·8</td>
</tr>
<tr>
<td></td>
<td>HG 184</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W50 haemin-limited</td>
<td>62·7±2·7</td>
</tr>
<tr>
<td></td>
<td>W50 haemin-excess</td>
<td>57·4±2·5</td>
</tr>
<tr>
<td></td>
<td>W50/BE1</td>
<td>35·3±2·8</td>
</tr>
<tr>
<td><em>P. asaccharolytica</em></td>
<td>B537</td>
<td>79·4±2·3</td>
</tr>
<tr>
<td><em>P. endodontalis</em></td>
<td>HG 370</td>
<td>57·6±1·3</td>
</tr>
</tbody>
</table>

ND, Not determined quantitatively, but Congo red shown to bind by SDS-PAGE.

* Amount of ligand bound per 4 mg (dry weight) cells from 1 ml 0·1 mM Congo red in 0·14 M NaCl, 0·1 M Tris/HCl, pH 7·4. Mean±SD of four replicate determinations.

![Fig. 1](image-url)
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Fig. 2. SDS-PAGE of whole cells of various strains of P. gingivalis, P. endodontalis and P. asaccharolytica after exposure to 0.1 mM Congo red at 37 °C for 30 min in the presence of protease inhibitors. A, strain WPH35; B, NCTC 11834; C, W50/BR1; D, Bg 381; E, HG189; F, HG184; G, haemin-limited W50; H, haemin-excess W50; I, W50/BE1; J, P. endodontalis HG370; K, P. asaccharolytica B537. Protein load was 200 μg per track.

Table 2. Congo red binding by P. gingivalis W50 after various pretreatments

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Congo red bound (nmol)*</th>
<th>Control</th>
<th>After</th>
<th>Change from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinase K</td>
<td>37.1 ± 1.8</td>
<td>14.4 ± 0.9</td>
<td>-39</td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>85.5 ± 1.4</td>
<td>84.1 ± 1.5</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>27.3 ± 0.6</td>
<td>25.1 ± 1.5</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>21.5 ± 0.0</td>
<td>8.9 ± 1.1</td>
<td>-58</td>
<td></td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>48.4 ± 0.9</td>
<td>80.9 ± 1.5</td>
<td>+67</td>
<td></td>
</tr>
<tr>
<td>ANSA</td>
<td>43.6 ± 1.0</td>
<td>35.4 ± 0.6</td>
<td>-19</td>
<td></td>
</tr>
<tr>
<td>2 M (NH₄)₂SO₄†</td>
<td>55.4 ± 2.4</td>
<td>106 ± 2.3</td>
<td>+91</td>
<td></td>
</tr>
</tbody>
</table>

* Amount of ligand bound per 4 mg (dry weight) cells from a fixed concentration of 0.1 mM Congo red. Mean ± SD of four replicate assays.
† Binding assay performed in the presence of ammonium sulphate.

Fig. 3. Effect of proteinase K. (a) Tracks 1 to 8, haemin-excess chemostat-grown cells of strain W50 digested for various time periods (0, 5, 10, 20, 30, 60, 90 and 120 min, respectively) in the presence of proteinase K at 37 °C (see text for details); track 9, the same cells as above but incubated for 120 min in the absence of added protease. After incubation, protease activity in the samples was inhibited by the addition of a cocktail of protease inhibitors, and the cells were exposed to Congo red (0.1 mM) in NaClTris, pH 7.4, for 30 min, and electrophoresed on a 10% (w/v) gel. Samples were solubilized at 37 °C for 30 min in electrophoresis application buffer containing protease inhibitors. Sample load was 400 μg protein per track. (b) As (a), but the gel was stained with Coomassie blue after exposure of the cell samples to Congo red. The bands at Rₓ 0.2 (arrow plus asterisk) were observed at the leading edges of the Congo-red-stained components [as seen in (a), tracks 1 to 5] after the gel was stained with Coomassie blue and destained. Track 10, molecular mass standards.

(NH₄)₂SO₄ in the buffer enhanced Congo red binding by 91% of the control, whilst the pretreatment with ANSA reduced binding by 19%. Pretreatment with polymyxin B sulphate (Table 2) did not abrogate Congo red binding, but led to an increased uptake of ligand (67% above the control value).

Effect of proteinase K on Congo red staining of the 66 kDa component

A limited digestion of haemin-excess-grown W50 cells with proteinase K over a 120 min period, followed by exposure to Congo red, revealed a reduction in the staining intensity of the 66 kDa component (Fig. 3a). This was accompanied by the progressive loss of protein in the same region, and at the top of the separating gel (Fig. 3b). Densitometric integration of a black-and-white photographic transparency of the gel showed that after 120 min incubation with proteinase K (Fig. 3a, track 8), the Congo red staining intensity was reduced to 20% of that for unincubated cells at time = 0 min (Fig. 3a, track 1), and to 39% of non-protease-incubated cells (track 9). A reduction in Congo red staining (equivalent to 50% of incubated control cells) also occurred when cells were incubated for 120 min even without added proteinase K (Fig. 3a, track 9; Fig. 4, bar marked C). This was assumed to occur as a result of autoproteolytic activity, since it was accompanied by a loss of Coomassie blue staining of the 66 kDa band (Fig. 3b, track 9), which was mitigated by
inclusion of the protease inhibitors PMSF, NEM, e-ACA and EDTA at a concentration of 1 mM (results not shown).

In order to quantify any changes in the Congo red staining or to the protein components during the progressive digestion with proteinase K, the electrophoresis cell sample protein load in the above experiment was set at 400 μg. This increase in sample load resulted in an apparent increase in the electrophoretic mobility of the Congo-red-stained component and the appearance of a band of \( R_p \approx 0.2 \) at its leading edge after staining of the gel with Coomassie blue and destaining (Fig. 3b). This band was not seen in cell samples which had not been exposed to Congo red and was not considered to be attributable to the presence of other Congo-red-staining components since no other proteins were observed in this region of the gel at this (and higher) protein loads.

**DISCUSSION**

In this study we have demonstrated that Congo red binding to several strains of both *P. gingivalis*, and to both haemin-excess and haemin-limited cells of strain W50, was mediated by a minor 66 kDa outer-membrane protein. This component was also present in the slower-pigmenting beige (avirulent) and brown colonial variants, W50/BE1 and W50/BR1, respectively. Preliminary studies revealed that a 66 kDa Congo-red-stained component was also present in *P. endodontalis* HG370 and *P. asaccharolytica* B537, indicating that this phenotype may be common to the genus *Porphyromonas*.

The lack of any inhibitory effect on Congo red binding to whole cells after pretreatment with polymyxin B would rule out the possibility of an involvement of the lipid A moiety of lipopolysaccharide. However, as with cells of *Treponema denticola* (Scott et al., 1993), polymyxin B pretreatment lead to an increase in Congo red binding. Polymyxin B is a cationic antibiotic comprising a diamino-butyr-ic-acid-containing ring, and a methylcoctan-1-oi acid side chain through which it binds to lipid A. It is possible that increased Congo red binding results from an interaction of its negatively charged sulphonic acid groups with the cationic ring structure of lipid-A-bound polymyxin, or that the membrane-disruptive effect of polymyxin B leads to greater accessibility of the ligand to the Congo-red-binding component. In the presence of 2 M ammonium sulphate, which is considered to enhance non-specific hydrophobic interactions (Kay et al., 1985), Congo red binding was elevated 91 % above control values. Such an increase has also been observed for Congo red binding to A+ cells of *Aeromonas salmonicida* (Kay et al., 1985), where it is thought to result in the amplification of binding by the stacking of Congo red micelles. This may partly explain the increase we observed in the anodal migration of the Congo-red-stained component when the electrophoresis cell sample load was increased, as seen in Fig. 3. We would speculate that, since this dye is sulphonated, the stacking of Congo red molecules to the Congo-red–protein complex might increase its negative charge and hence the electrophoretic mobility.

Pretreatment of whole cells with the proteases trypsin, papain and proteinase K, and with ANSA (which binds to hydrophobic amino acid residues) decreased binding, indicating that the 66 kDa component was a protein. Limited proteolysis with proteinase K concomitantly abrogated both Congo red and Coomassie blue staining of this component, and this loss of staining correlated quantitatively with the reduction in Congo red binding to whole cells, indicating that the 66 kDa protein was solely responsible for binding. It is noteworthy that loss of staining and Congo red binding also occurred when whole cells were incubated in the absence of added protease. These effects could be mitigated by protease inhibitors, indicating that the Congo red binding protein was degraded by endogenous cellular proteases. This phenomenon would explain the gradual loss of red staining of strain W50 after 4–5 d growth on Congo red agar, which is in contrast to the increase in the black pigmentation of colonies due to haem accumulation when grown on blood agar.

Unlike *T. denticola* (Scott et al., 1993) and *Aeromonas salmonicida* (Kay et al., 1985), in which Congo red and iron protoporphyrin binding is mediated by the same hydrophobic outer-membrane proteins, this study has shown that these ligands are bound by different components in *P. gingivalis*. We have demonstrated the presence of major (32 kDa) and minor (51, 53, 56 and 61 kDa) haemin-binding proteins in outer membranes (solubilized for electrophoresis without heating) of *P. gingivalis* grown in the chemostat under haemin limitation (Smalley et al., 1993), whilst Bramanti & Holt (1993) found a 39 kDa
(unheated; 26 kDa, heated) haemin-binding outer-membrane protein in the same strain, but grown in batch cultures through progressively lower levels of haemin to simulate haemin limitation. Genco et al. (1994) quantified Congo red binding in P. gingivalis after growth in batch culture under haemin-excess and haemin-depleted conditions. These workers found that cells grown under haemin excess bound more Congo red than those from conditions of haemin limitation, and that preincubation of haemin-depleted cells with haemin increased subsequent Congo red binding. From these data, Genco et al. (1994) postulated that haemin and Congo red binding are closely linked and that the haemin-inducible component must be activated for binding to occur. In the present study, however, in contrast to haemin-binding proteins, we have categorically demonstrated that the 66 kDa Congo-red-binding protein was present in both haemin-limited and haemin-excess cells. Moreover, haemin-excess and haemin-limited cells bound similar levels of Congo red. It would thus appear that Congo red binding is not induced by haemin and that the binding of these two ligands, and their ‘receptors’, may be unrelated.

In other organisms which display Congo red binding and which express haemin-binding proteins, either constitutively or in response to low environmental levels of iron protoporphyrin IX, the cellular binding of haemin can be inhibited by Congo red, and both these ligands appear to bind to the same cell surface components (Stugard et al., 1989; Kay et al., 1985; Scott et al., 1993). It is not known whether these two ligands show any affinity for each other’s binding sites in P. gingivalis. Bramanti & Holt (1993) have reported that Congo red can inhibit haemin uptake into batch-culture-grown haemin-starved cells of P. gingivalis W50, although it was not reported whether haemin-excess-grown cells behaved similarly, or to which components haemin was bound.

Evidence from affinity binding studies points to the existence of two iron protoporphyrin IX binding sites in both haemin-limited and haemin-excess-grown P. gingivalis under oxidizing conditions (Smalley et al., 1994), although ferroprotoporphyrin IX (haem) binding is inhibited by Congo red and its presence was haemin-independent. Although haemin-excess and haemin-limited W50 bound similar amounts of Congo red, it is not known whether quantitative differences exist in binding at high and low redox potentials.

All the P. gingivalis strains, and the single strains of P. endodontalis and P. asaccharolytica examined in this study, expressed the 66 kDa binding protein, but we are unaware of any other biological functions of this outer-membrane component. On a semi-quantitative basis, as judged from the SDS-PAGE, there was no difference in the Congo red staining intensity of the 66 kDa component for the slower-pigmenting avirulent strain W50/BE1, or for W50/BR1, although W50/BE1 bound less Congo red than strain W50 (at one fixed ligand concentration). We are currently examining these strains for any quantitative differences in Congo red binding affinity. Since cells of strain W50/BE1 (McKee et al., 1988) and of haemin-limited strain W50 (McKee et al., 1986) are of attenuated virulence, but still bind Congo red, it would appear that unlike T. denticola (Scott et al., 1993), Aeromonas salmonicida (Kay et al., 1985), Yersinia pestis (Surgalla & Beesley, 1969) and Y. enterocolitica (Prpic et al., 1983), Congo red binding may be of little value as a marker to detect strains of P. gingivalis which bind haemin and are virulent, or which display haemin-binding proteins.

Conclusions

Congo red binds to strains of P. gingivalis, via a 66 kDa outer-membrane protein which is distinct from haemin-binding proteins. This protein accounts quantitatively for Congo red binding, which is probably mediated hydrophobically. Its expression is iron protoporphyrin IX (haemin) independent. Congo red binds to cells of an avirulent strain of P. gingivalis and to cells of a virulent strain whose virulence is attenuated through growth under haemin limitation. Congo red binding by P. gingivalis may be of little use as a virulence marker or for detecting cells expressing haemin-binding proteins. The 66 kDa Congo-red-binding component was also observed in whole cells of one strain of P. endodontalis and P. asaccharolytica, indicating that this may be a phenotype common to the genus Porphyromonas.

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