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Production of hydrolytic enzymes by oral isolates of *Eikenella corrodens*

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Abstract *Eikenella corrodens* isolates from periodontally healthy subjects and adult periodontitis patients were compared for their ability to produce a range of potential virulence factors All were positive for proline aminopeptidase, thiol-dependent haemolysin and esterase activities Low or negative activities were found against casein, phospholipid, lipid, collagen, aminophosphate, phosphate under acid or alkaline conditions, and eleven other amino acid substrates tested In oral infections, the haemolytic activity of *E corrodens* could be amplified in the reduced environment of the periodontal pocket and damage host cells Proline aminopeptidase may act against proline residues in collagen, immunoglobulins and complement proteins

Key words Hydrolase, *Eikenella corrodens*, Proline aminopeptidase, Haemolysin

Introduction

Eikenella corrodens is gaining recognition as an important opportunistic pathogen It has been isolated from various sites of infection, either in mixed culture or as the sole infecting organism, including abscesses, osteomyelitis, chorioamnionitis, septic arthritis, meningitis and endocarditis [1] In addition, *E corrodens* is regarded as an important cause of infection following human and animal bite wounds [2] In the mouth, numerous studies implicate *E corrodens* as one of the organisms involved in diseases of the periodontal tissues [1] *E corrodens* was recently shown, by the use of species-specific monoclonal antibodies and DNA probes, to be the most prevalent suspected pathogen detected in subgingival plaque from adults with periodontal disease [3,4] This organism should thus be considered an important contributor to tissue destruction in these complex disease processes However, information regarding virulence factors that may influence the capacity of *E corrodens* to cause tissue damage remains sparse

We have examined the capacity of a number of *E corrodens* isolates, from periodontally healthy subjects and adult periodontitis patients, to produce a range of hydrolytic enzymes This was

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undertaken to identify particular factors that may have a role in periodontitis, and to determine whether isolates from disease are distinct with regard to the production of such factors

Materials and Methods

Bacterial isolates and culture media

Twenty *E corrodens* isolates, all from different subjects, were included in the present study Ten were previously obtained from the supragingival plaque of periodontally healthy adults working at The London Hospital Medical College, UK (Group 1) A further 10 originated from deep (> 5 mm) periodontal pockets of adults attending for chronic periodontitis treatment at The Dental Institute, The London Hospital Medical College, UK (Group 2) National Collection of Type Cultures (NCTC) 10596 and 10647 strains, both originally isolated from sputum samples, were included for comparative purposes All isolates were stored on 'microbank' beads (Pro-Lab Diagnostics) at −70°C until required A medium (Basal medium, BM1) was developed for liquid culture of *E corrodens* The medium contained proteose peptone (Difco, 2 0% w/v), yeast extract (Difco, 0 5% w/v), cysteine hydrochloride (0 05% w/v), haemin (0 001% w/v), menadione (0 00005% w/v) and potassium nitrate (0 2% w/v) Growth in this medium was measured by culture absorbance at 540 nm, and was related to dry weight of cells by reference to a calibration curve Bacterial dry weights were measured according to Greenman et al [5] Solid medium (BM2) contained trypticase soy agar (Becton Dickinson, 4 0% w/v) and all the above ingredients except proteose peptone Unless otherwise stated, cultures were grown under anaerobic conditions (80% N_2 10% CO_2 10% H_2) at 37°C for 72 h

Screening of isolates

Collagenase activity was examined by the method of Krepel et al [6] using bovine type I collagen added to BM1 medium Caseinase activity, from isolates grown in BM1 medium, was measured by the azocasein digestion method of Millet [7] Phospholipase activity was detected using egg yolk emulsion [8] added to BM2 medium at a concentration of 5% v/v Blood agar base (Oxoid CM271) with either defibrinated horse or sheep blood (5% v/v) was used to screen for haemolytic activity The isolates were plated as streaks and then incubated in air and anaerobically for 7 days The semi-quantitative ZYM and ID32 A biochemical test systems (API Systems, France) were also used to screen isolates Suspensions containing 0 3 mg bacterial dry weight per ml were used in these assays

Quantification of proline aminopeptidase activity

Levels of proline aminopeptidase were determined with cultures grown in BM1 medium Enzyme activity was measured using the synthetic chromogenic substrate L-proline *p*-nitroanilide (0 25% w/v in 0 1 M Tris/HCl buffer, pH 7 5), which, upon hydrolysis, produces the coloured end-product *p*-nitroaniline ($\epsilon = 8800\ M^{-1}\ cm^{-1}$) A 0 2 ml volume of either culture supernatant or cell suspension (cells washed and re-suspended in buffer) was added to 0 5 ml of substrate and 2 3 ml of buffer, and incubated at 37°C for 1 h The *p*-nitroaniline liberated was measured at a wavelength of 410 nm

Quantification of haemolytic activity

Isolates were grown in BM1 medium in air and anaerobically A spectrophotometric assay with horse erythrocytes [9] was used to quantify haemolytic activity Washed (×3) erythrocyte suspensions (5% v/v in phosphate-buffered saline) were prepared and 0 5 ml of this suspension was added to an equal volume of culture supernatant or cell suspension (cells washed and re-suspended in buffer) in sealed Eppendorf tubes The reaction mixture was incubated for 3 h in a water bath at 37°C with frequent inversion of the tubes The reaction was stopped by centrifugation at 13 000 rpm in a bench microfuge for 5 min Released haemoglobin was then measured at 540 nm Assays were also performed in the presence of cysteine hydrochloride, 15 μl of a 1 M solution was added to 0 5 ml of culture supernatant or cell suspension These mixtures were incubated for 30 min at 20°C prior to the addition of erythrocytes The readings were corrected for turbidity of the

culture supernatant, and non-specific haemolysis due to BM1 or BM1 plus cysteine HCl

Results

Screening of isolates

All isolates investigated were negative for activity against phospholipid and collagen substrates (Table 1) The activity of *E corrodens* isolates against a casein substrate was either negative or very low (mean level of 34 protease units/mg dry wt) In comparison, a *Porphyromonas gingivalis* control strain liberated approximately 1000 U/mg dry wt

All isolates produced esterase (C4) and esterase lipase (C8) with mean levels of 1 0 and 0 5 μmol substrate hydrolysed/mg dry wt respectively Reactions for trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase and α-fucosidase were negative Phosphoamidase, lipase, acid phosphatase and alkaline phosphatase activities were low ($<0\,25$ μmol substrate hydrolysed/mg dry wt) or negative (Table 1)

Table 1

Hydrolytic enzyme activities of *E corrodens* isolates ($n=10$ per group)

Enzyme activity	No of positive isolates		Reference strains	
	Group 1* (health)	Group 2 (disease)	NCTC 10596	NCTC 10647
azocaseinase	6	9	+	+
trypsin	0	0	–	–
chymotrypsin	0	0	–	–
alkaline phosphatase	10	9	+	+
acid phosphatase	10	10	+	+
phosphoamidase	10	8	+	+
esterase	10	10	+	+
esterase lipase	10	10	+	+
lipase	1	2	–	–
phospholipase	0	0	–	–
collagenase	0	0	–	–

* Isolates in group 1 were obtained from periodontally healthy individuals and isolates in group 2 were obtained from cases of chronic adult periodontitis

Table 2

Aminopeptidase activities of *E corrodens* isolates ($n=10$ per group)

Amino acid(s)	No of positive isolates		Reference strains	
	Group 1* (health)	Group 2 (disease)	NCTC 10596	NCTC 10647
leucine-	5	6	+	+
valine-	2	3	+	–
cystine-	6	8	+	+
arginine-	10	10	+	+
proline-	10	10	+	+
leu-glycine-	9	9	+	–
tyrosine-	7	4	+	+
alanine-	8	6	+	+
glycine-	9	4	+	+
serine-	8	6	+	–
phenylalanine-	0	0	–	–
glutamic acid	0	0	–	–
glu-glutamic acid	0	0	–	–
histidine-	0	0	–	–

* See footnote Table 1

All isolates were significantly positive for proline aminopeptidase activity ($>2\,0$ μmol substrate hydrolysed/mg dry wt) as detected using the micromethod test system Low ($<0\,25$ μmol substrate hydrolysed/mg dry wt) or negative activities were found for leucine, valine, cystine, leucine-glycine, tyrosine, arginine, alanine, glycine, serine, phenylalanine, glutamic acid, glutamic acid-glutamic acid and histidine substrates (Table 2)

Under anaerobic conditions, *E corrodens* isolates demonstrated alpha haemolysis on horse (14/22 isolates) and sheep (22/22 isolates) blood agar, whereas none of the isolates showed any haemolysis on these media when grown in the presence of air Haemolysis on solid media was enhanced by a further incubation at 20°C for 2 h

Proline aminopeptidase levels

The mean levels of proline aminopeptidase activity (μmol *p*-nitroaniline liberated/mg dry wt/min, $\pm$ standard error) for the cell suspensions were not significantly different at 8 43 $\pm$ 1 47 and 10 00 $\pm$ 1 04 for the 2 groups (Table 3) Mean levels for supernatants derived from the same cultures were 3.91 $\pm$ 0 47 and 5 28 $\pm$ 0 66 respectively, and were not significantly different

Table 3

Proline aminopeptidase activity (μmol *p*-nitroaniline/mg dry wt/min) of *E corrodens* washed cell suspensions and supernatants

Group 1 (health) *			Group 2 (disease)		
Isolate	Cells	Supernatant	Isolate	Cells	Supernatant
RA2	2 9	3 5	33EK(L)	13 9	7 1
LF3	8 6	4 7	34G(1)	7 4	6 0
SW5	7 0	3 9	35G(2)	8 3	9 3
HF6	7 2	3 1	36EK	10 8	3 6
Lou8	7 5	2 9	38EK(L)	8 1	3 8
YA9	19 2	3 8	39EK	14 3	4 2
RW10	4 8	2 6	40E	5 8	2 2
Rog11	7 6	4 4	42(1)S	6 0	5 5
HR13	13 3	7 6	45E	11 2	4 3
IT14	6 2	2 6	46E	13 8	6 8
mean	8 4	3 9	mean	10 0	5 3
			NCTC 10596	13 8	5 5
			NCTC 10647	23 4	5 2

* See footnote Table 1

(Table 3) Enzyme levels detected from cells were on average a factor of 2 greater than those detected from culture supernatants Each isolate was assayed 3 times

Haemolysin levels

All culture supernatants were negative for haemolytic activity, when tested against washed erythrocytes in a spectrophotometric assay (each isolate assayed 3 times) However, all isolates demonstrated haemolytic activity when cysteine hydrochloride was included in the assay (Table 4) The mean levels (O D units at 540 nm/mg dry wt/h, $\pm$ standard error) for the 2 groups of isolates were not significantly different at 0 28 $\pm$ 0 07 and 0 27 $\pm$ 0 10 respectively (each isolate assayed 3 times) No significant haemolytic activity was detected using washed cell suspensions

The two NCTC strains were not markedly different in their enzyme profiles when compared to the test isolates (Tables 1–4)

Table 4

Haemolytic activities of *E corrodens* isolates (O D units at 540 nm/mg dry wt/h)

Group 1 (health) *		Group 2 (disease)	
isolate	activity	isolate	activity
RA2	0 21	33EK(L)	0 05
LF3	0 44	34G(1)	0 50
SW5	0 34	35G(2)	0 04
HF6	0 55	36EK	0 22
Lou8	0 62	38EK(L)	0 35
YA9	0 03	39EK	0 04
RW10	0 16	40E	0 01
Rog11	0 04	42(1)S	0 20
HR13	0 10	45E	0 32
IT14	0 27	46E	1 01
mean	0 28	mean	0 27
		NCTC 10596	0 36
		NCTC 10647	0 31

* See footnote Table 1

Discussion

In the case of adult periodontitis, it is known that a single periodontal pocket will sometimes yield more than one clonal type of *E corrodens* [10], which possibly could include both virulent and avirulent variants The absence of clear differences in the enzyme profiles between the two groups of isolates in this study perhaps suggest that other factors, as yet unidentified, may be the key virulence determinants Alternatively, it is possible that strains from both healthy and periodontal disease sites are of comparable virulence, and that all strains possess the required virulence determinants Any differences in effect may represent the different physiological state of the cells due to the different micro environmental conditions which exist in healthy or diseased sites If this is the case, all *E corrodens* isolates would be

capable of establishing infections given the appropriate host predisposing conditions

Although a range of hydrolytic capabilities was demonstrated, it remains uncertain as to the importance of individual factors in disease High levels of extracellular proline aminopeptidase and a thiol-dependent haemolysin were found Extracellular products of this nature may be central to pathogenicity, as indicated for a number of virulent organisms [11,12]

Reduced conditions were necessary for *E corrodens* haemolytic activity, this finding may be related to those found within the periodontal pocket, where activity may be amplified considerably in this reduced environment and so damage erythrocytes and other host cells All isolates were negative for activity against lecithin, suggesting that the haemolysin does not fall into the phospholipase category of membrane-damaging haemolysins Activity was not inhibited by cholesterol (Allaker, unpublished observations), indicating that the haemolysin does not belong to the thiol-activated group of cytolysins [13] It has been suggested that ester bond hydrolysis may play a role in cytolysis [13], the haemolysin of *E corrodens* may therefore possess esterase activity as demonstrated in this study Further studies with purified preparations are required to define the relationship between these activities

The proline aminopeptidase activity may be related to the nutritional requirements of *E corrodens*, its need to modify local environmental conditions, or as protection against host immune mechanisms For these functions it is significant that collagen (a major component of periodontal ligament and alveolar bone), the hinge region of immunoglobulins and certain complement proteins are all rich in proline and could possibly act as substrates Indeed Chen and Wilson [14] have shown that complement-mediated killing constitutes an important component of host defence against *E corrodens*, and that such killing is critically dependent upon the presence of bactericidal antibody *E corrodens* is unusual in that production of this enzyme is not a characteristic of most other putative periodontal pathogens [15] The catalytic activity of a proline aminopeptidase enzyme is to cleave N-terminal proline residues from peptides This bond is less susceptible to the action of aminopeptidases of broad specificity because proline residues confer structural constraints on the peptide [16] The proline aminopeptidase from *E corrodens* may thus have an important role in the removal of exposed proline residues, perhaps in conjunction with other microbial and host peptidases, in the periodontal environment

Further studies with defined mutants, deficient in peptidase and haemolysin activities, are needed to help elucidate the role of these products in disease

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