Pathogenic factors of *Pseudomonas cepacia* isolates from patients with cystic fibrosis

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Summary. One hundred and nineteen isolates of *Pseudomonas cepacia*, 98 of which were from cystic fibrosis (CF) patients and 21 from environmental and other human sources, were examined for biochemical and exo-enzymatic properties that may contribute to the pathogenicity of this bacterium. The following characteristics were demonstrated significantly more frequently in isolates from CF patients than in control isolates: production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginase and trypsin; reduction of nitrate to nitrite; hydrolysis of urea and xanthine; complete haemolysis on bovine red blood cells; cold-sensitive haemolysis on human red blood cells; greening of horse and rabbit red blood cells. The role of these factors in the pulmonary disease associated with cystic fibrosis is not clear. However, several factors which have been reported previously as being associated with pathogenic processes with other bacteria have now been described in *P. cepacia*. Additional factors not previously reported as "pathogenicity factors" are also described.

Introduction

*Pseudomonas cepacia* has become an important pathogen in patients with cystic fibrosis (CF). In 1980, Rosenstein and Hall reported a case of pneumonia and septicaemia caused by *P. cepacia* in a 17-year-old CF patient. This case is of interest because it typifies the syndrome of rapid pulmonary deterioration frequently accompanied by bacteraemia in CF patients colonised by *P. cepacia*. Following this case report, other CF centres evaluated their experiences with *P. cepacia* colonisation. In Toronto, patient colonisation rates with this organism had increased from 10% in 1971 to 18% in 1981 while the rate of colonisation with *P. aeruginosa* had remained constant over the same period. In the same study, the presence of *P. cepacia* correlated with a greater impairment of pulmonary function than did *P. aeruginosa* colonisation. A CF centre in Cleveland demonstrated a doubling in *P. cepacia* colonisation from 1978 to 1983 and the proportion of patients from which *P. cepacia* was isolated at autopsy increased sixfold.

Certain characteristics of *P. cepacia* may explain the frequency of recovery from CF patients. *P. cepacia* has minimal growth requirements and can survive in hostile environments such as distilled water and disinfectants for extended periods. The organism has been associated with nosocomial outbreaks and has been recovered from pulmonary equipment. The virulence of *P. cepacia* in animals has been examined in two studies. In one, *P. cepacia* recovered from contaminated disinfectant was lethal to mice only at doses > 10⁹ cfu/ml. In a second study, a *P. cepacia* isolate from a CF patient was found to colonise burned skin in a mouse infection model for at least 3 weeks without tissue invasion or mortality. In this same model, *P. aeruginosa* was invasive and caused deaths within 2 days.

The finding of long term survival in hostile environments and the ability to colonise damaged tissue may explain the acquisition and persistence of *P. cepacia* in CF patients, but it does not explain the role played by this organism in the pulmonary disease of these patients. In the present study we examined two populations of *P. cepacia*—isolates...
from patients with CF and from other sources—for the production of a wide variety of enzymes and potential pathogenicity factors that may play a role in the pulmonary disease associated with \( P. \) *cepacia* in CF patients.

**Materials and methods**

**Strains, test conditions, and reagents**

One hundred and nineteen \( P. \) *cepacia* isolates were studied. Of these, 98 were respiratory isolates from CF patients—83 from patients seen at the Cystic Fibrosis Center, St Christopher’s Hospital for Children and 13 isolates from other CF centres in Cleveland, OH, USA (3), Toronto, Canada (3), Seattle, WA, USA (3), Calgary, Ontario, Canada (2), Columbia University (1) and Yale-New Haven, CT, USA (1). Primary identification of each CF isolate from St Christopher’s Hospital for Children was performed in the clinical microbiology laboratory. These isolates were then frozen in litmus milk and held at \(-70^\circ\)C. The other 21 isolates were from environmental sources and other human infections (control isolates). The number and source of this subset of organisms were as follows: American Type Culture Collection (ATCC) environmental (10), ATCC human infection (7), Philadelphia environmental (3) and Philadelphia human infection (1).

Exo-enzymatic plate assays and biochemical tests were incubated at 37°C for 48 h, then held at 25°C for another 5 days. Biochemical results were recorded at 24 and 48 h and 7 days, and exo-enzymatic results were recorded at 48 and 72 h and 7 days, unless otherwise stated. The API-ZYM panel (Analytab Products, Inc., Plainview, NY, USA) was also used to screen for specific enzymatic activity. Quality control was performed on all assays with known positive and negative organisms.

Media for these assays was purchased from Difco (Detroit, MI, USA), BBL (Cockeysville, MD, USA), Sacks Farms (Evans City, PA, USA), Remel (Lenexa, KA, USA), Sigma Chemical Co. (St Louis, MO, USA), Flow Laboratories, Inc. (McLean, VA, USA), Scott Laboratories, Inc. (Carson, CA, USA), and Analytab Products (Plainview, NY, USA). All forms of media were prepared and used according to the manufacturers’ instructions.

**Biochemical tests**

Biochemical tests were performed in accordance with established methods. The tests included: citrate utilisation on Simmons citrate agar, growth on cetrimide agar, growth at 42°C, growth on PC agar (Scott), nitrate reduction, urea hydrolysis, aesculin hydrolysis, production of catalase and oxidase. Catalase and oxidase activities were evaluated after incubation at 37°C for 48 h on heated blood agar plates. Nitrate reduction was tested after incubation for 48 h at 37°C.

**Tests with amino acids and nucleic acids**

Determination of amino-acid decarboxylation or dihydroxylation was performed by Greenwood’s method for non-fermentative organisms. The amino acids tested were arginine, ornithine and lysine. The organisms were grown at 37°C in broth containing glucose 0·5%, \( \text{KH}_2\text{PO}_4 \) 5% and amino acid 0·5%. After 24 h, one drop of 10N NaOH was added followed by 1 ml of ninhydrin (Sigma N-4876) 0·1% in chloroform. Development of a purple colour in the chloroform layer at 5 min was positive. Phenylalalanine deaminase activity was determined after incubation at 37°C for 24 h by flooding the medium with ferric chloride. Leucine, valine and cystine aminopeptidases were tested with the API-ZYM panel and were elevated according to manufacturer’s instructions. Activity in xanthine and tyrosine were assayed by clearing of the media (Remel) around the inoculum. DNAase activity was demonstrated by a zone of clearing around the inoculum when the DNAase plate (Difco) was flooded with 1N HCl after incubation for 72 h. RNAase test agar contained RNA (Sigma R-7125 from Baker’s Yeast) 0·2% in Brain Heart Infusion (BHI) agar base. A test was considered positive if, after incubation at 37°C for 5 days, flooding with 1N HCl produced a zone of clearing.

**Tests with lipids**

Several methods were used to screen for lipolytic activity. Lecithinase activity was detected by growth on (Remel) BHI agar with egg yolk 10%. Esterase was detected by growth on BHI agar containing Tween 80 (Sigma P-1754) 1%. C4 esterase, C8 esterase/lipase, C14 lipase, acid and alkaline phosphatases and phosphohydrolase were detected in the API-ZYM panel.

**Haemolysin**

Haemolytic activity was determined on five types of blood agar: sheep (BBL), bovine, rabbit and human (Sacks Farms). Plates were incubated at 37°C for 48 h and then at 0°C for a further 24 h. Results were recorded at 24, 48 and 72 h and reported as no haemolysis, greening of the media or complete haemolysis.

**Tests with proteins and polysaccharides**

Enzymatic activity against cartilage (Sigma C-5268), collagen (Sigma C-9879) and elastin (Sigma E-1625) was determined by incorporation of substrate 2% in BHI agar. Caseinase activity was determined on BHI with skimmed milk (Remel, casein) 50% and agar 1%. Albuminase was detected by growth on BHI agar with Bovine Serum Albumin (Sigma A-4503) 8%. Gelatinase activity was evaluated by liquefaction of gelatin (Difco) 12% in BHI broth. Digestion of algin was observed in the algin-charcoal media of von Riesen (yeast extract, Difco, 0·5%; Algin, Sigma A-7128, 1%; broomothymol blue 0·002%; charcoal 0·1%). Amylase was detected in BHI agar with starch 2%. Mucinase activity was tested...
on BHI agar with hog gastric mucin (Sigma M-2378) 0.28%. After incubation for 7 days, the medium was flooded with calcium chloride 1%; mucinase-positive strains produced clear zones around the inoculum.

The other enzymes detected in the API-ZYM panel were: trypsin, chymotrypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, α-mannosidase, α-fucosidase and N-acetyl-β-glucosaminidase.

Cytotoxicity

Cytotoxicity was evaluated by the method of McKevitt and Woods in which CHO cells and Vero cells were grown to monolayers in RPMI 1640 medium (Flow Laboratories, Inc.) supplemented with fetal calf serum 10% and gentamicin 0.08%. The cells were resuspended in fresh growth medium and incubated for 24 h at 37°C in CO₂ 5%. Filter-sterilised supernate (100 μl) from P. cepacia cultures grown in RPMI 1640 for 48 h at 37°C were pipetted on to the cells, together with 100 μl of fresh growth medium. Cells were observed for cytopathic effects after 24 and 48 h and 5 days at 37°C in CO₂ 5%.

Statistical analysis

Statistical analysis of all results was performed with Fisher’s Exact test to compare the proportion of positive tests in each group. Significance was indicated by p values ≤0.05.

Results

Test results recorded as positive on or before the final reading were considered positive and are presented as such in the following sections and tables.

Biochemical properties

The biochemical properties of P. cepacia are summarised in table I. In these biochemical tests, the CF isolates gave positive results for ≥90% of the isolates for four tests—catalase production, growth at 42°C, growth on PC agar, and nitrate reduction. With the control isolates, ≥90% gave positive results in two tests—citrate utilisation and urea hydrolysis. Statistically significant differences between CF isolates and controls were noted for catalase production, urea hydrolysis and nitrate reduction.

Tests with amino acids and nucleic acids

The results of the amino acid and nucleic acid assays are summarised in table II. The CF and control isolates gave positive results with ≥90% of isolates only for tyrosine and leucine aminopeptidases. With the control isolates, ≥90% gave positive results for RΝAse. Statistically significant differences were noted with ornithine decarboxylase, xanthine hydrolysis and valine aminopeptidase.

Tests with lipids

The results of the lipid assays are shown in table III. With isolates of P. cepacia from both CF patients and controls, ≥90% gave positive results
Table III. Comparison of lipolytic activity of isolates of *P. cepacia* from CF patients and controls

<table>
<thead>
<tr>
<th>Property</th>
<th>Number (%) of isolates that gave positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CF (98)</td>
</tr>
<tr>
<td>Lipase (Tween 80)</td>
<td>74 (76)</td>
</tr>
<tr>
<td>Lecithinase (egg yolk)</td>
<td>68 (69)</td>
</tr>
<tr>
<td>C4 esterase</td>
<td>95 (97)</td>
</tr>
<tr>
<td>C8 esterase/lipase</td>
<td>98 (100)</td>
</tr>
<tr>
<td>C14 lipase</td>
<td>35 (36)</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>96 (98)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>86 (88)</td>
</tr>
<tr>
<td>Phosphohydrolase</td>
<td>89 (91)</td>
</tr>
</tbody>
</table>

for the C4 and C8 esterases, acid phosphatase and phosphohydrolase. With the control isolates, ≥ 90% also gave positive results for alkaline phosphatase. A statistically significant difference was seen for the C14 lipase only.

Haemolysis

Some of the *P. cepacia* strains had an effect on some or all of the five types of red blood cells. This activity was shown as complete lysis of the red blood cells, cold-dependent lysis and greening of the red blood cells. Complete, non-temperature-dependent lysis was exhibited in 3–6% of the cystic isolates and 5–20% of the control isolates. Cold-sensitive haemolysis was noted for 2–18% of CF isolates and 0–20% of control isolates. Greening of the red blood cells was detected in 14–25% of the CF isolates and 0–10% of the control isolates. Statistically significant differences were noted for complete haemolysis on bovine red blood cells, cold-sensitive haemolysis on human red blood cells and greening on horse and rabbit red blood cells. All haemolytic activity is shown in table IV.

Tests with proteins and polysaccharides

None of the tests for proteolytic or saccharolytic enzymes included in this study demonstrated ≥ 90% positive results with CF or control isolates. All results are shown in table V. Statistically significant differences were found for alginase and trypsin.

Cytotoxicity

None of the CF nor the control isolates showed any cytotoxicity for CHO or Vero cells.

Discussion

*P. cepacia* colonisation in CF patients has been identified as a potentially significant factor in the pulmonary disease process in these patients.¹⁻³ One explanation for this association is that this organism may play an active role through proteolytic, saccharolytic or lipolytic activity. Such factors have been well characterised in *P. aeruginosa*.¹⁵ The proteases and elastases of *P. aeruginosa* have been shown to injure pulmonary tissue directly.¹⁸ The report by Marks¹⁸ cites that “intratracheal administrations of highly purified *P. aeruginosa* proteases

Table IV. Comparison of haemolytic activity by 97 CF and 20 control isolates of *P. cepacia* against various red blood cell species

<table>
<thead>
<tr>
<th>Cell species</th>
<th>Horse</th>
<th>Human</th>
<th>Bovine</th>
<th>Rabbit</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete haemolysis</td>
<td>3 (3)</td>
<td>2 (2)</td>
<td>3 (3)</td>
<td>6 (6)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Cold-sensitive haemolysis</td>
<td>1 (18)</td>
<td>4 (4)</td>
<td>7 (7)</td>
<td>5 (5)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Greening</td>
<td>24 (25)</td>
<td>20 (21)</td>
<td>20 (21)</td>
<td>22 (23)</td>
<td>14 (14)</td>
</tr>
</tbody>
</table>

Percentages may exceed 100% due to the expression of multiple haemolytic activities. Significant differences (p < 0.05): lysis of bovine erythrocytes (p = 0.016), cold sensitive lysis of human erythrocytes (p = 0.030), greening of horse (p = 0.006) and rabbit (p = 0.010) erythrocytes.
Table V. Comparison of proteolytic and saccharolytic activity by CF and control isolates of P. cepacia

<table>
<thead>
<tr>
<th>Property</th>
<th>Number (%) of isolates that gave positive results</th>
<th>CF (98)</th>
<th>Control (21)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caseinase</td>
<td></td>
<td>79 (81)</td>
<td>15 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>Gelatinase</td>
<td></td>
<td>38 (39)</td>
<td>11 (52)</td>
<td>NS</td>
</tr>
<tr>
<td>Alginase</td>
<td></td>
<td>16 (16)</td>
<td>10 (48)</td>
<td>0.003</td>
</tr>
<tr>
<td>Mucinase</td>
<td></td>
<td>9 (9)</td>
<td>3 (14)</td>
<td>NS</td>
</tr>
<tr>
<td>Albuminase</td>
<td></td>
<td>5 (5)</td>
<td>1 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td>4 (4)</td>
<td>5 (24)</td>
<td>0.009</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td></td>
<td>3 (3)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td></td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td></td>
<td>7 (7)</td>
<td>4 (19)</td>
<td>NS</td>
</tr>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td></td>
<td>41 (42)</td>
<td>13 (62)</td>
<td>NS</td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td></td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

None of the isolates gave positive results in tests for α-mannosidase, α- and β-galactosidase, β-glucuronidase, cartilage, collagenase, elastase and amylase.

in rabbits elicits extensive, grossly observable lung damage". Also noted by Marks was similar pulmonary toxicity by elastase following intranasal administration in mice.

A few investigators have studied bacterial products that may be associated with the pathogenicity of this organism; however, no clear associations have been reported. Therefore, this project was undertaken to establish the extracellular factors of P. cepacia that may be involved in the pulmonary disease process of CF and to delineate areas for further investigation.

Recently, Lonon and associates have reported experiments on the lipolytic activity of P. cepacia in tests with egg yolk agar, four different Tweens (20, 40, 60 and 80) and a chromogenic substrate for phospholipase C. These researchers detected no phospholipase C activity with egg yolk, but activity was observed with the other substrates. The present study supports and expands on their findings.

Nakazawa and associates have recently reported the characterisation of a haemolysin produced by P. cepacia with activity against sheep erythrocytes. They showed that c. 4% of the P. cepacia strains tested produced complete haemolysis of sheep red blood cells, which is consistent with our findings. We examined erythrocytes from five sources and found that the percentages of isolates haemolytic for bovine, rabbit, human and horse red blood cells differed from the percentages found with sheep red blood cells. It is likely that differences in the composition of the cell-membranes of the different erythrocytes account for the different activities. Furthermore, when the haemolytic activity of P. cepacia was examined for related phenomena, a larger number of isolates demonstrated either greening of the red blood cells or cold-sensitive haemolysis (see table IV). The differences in host range as well as the complex combination of phenomena make it especially difficult to evaluate the role of haemolytic activity in the pathogenicity of P. cepacia.

The apparent lack of activity for a number of substrates does not necessarily show that the organism does not possess the appropriate enzymes. One explanation for the apparent lack of proteolytic or saccharolytic activity maybe the availability of iron. Virulence of P. aeruginosa in animal models, as well as amounts of extracellular enzymes produced in culture, have been shown to be affected by the concentration of iron in the growth media.

Consistent with the report by McKevitt and Woods, none of the P. cepacia isolates was cytotoxic.

In addition to a number of potentially pathogenic factors produced by all strains of P. cepacia, we found several significant differences in the expression of biochemical reactions or the production of exo-enzymes by P. cepacia isolates from CF patients when compared to control isolates. These included: production of catalase, ornithine decarboxylase, valine aminopeptidase, C14 lipase, alginate and trypsin; reduction of nitrate to nitrite; hydrolysis of urea and xanthine; complete haemolysis on bovine red blood cells; cold sensitive haemolysis on human red blood cells; and greening of horse and rabbit red blood cells.

The role of any of these factors in the disease associated with respiratory colonisation or infection in CF patients is not clear, but the present study has provided a number of avenues for future research. Using a variation on the chronic respiratory infection model in rats developed by Cash et al., the role of specific biochemical reactions or enzyme production in the pathogenesis of chronic infection with P. cepacia may be determined.

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REFERENCES