Characterization of Hemolysin in Extracellular Products of *Pseudomonas cepacia*

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*Pseudomonas cepacia* is recognized as an opportunistic pathogen in immunocompromised patients. We screened 120 strains of *P. cepacia* isolated from clinical specimens for production of extracellular products. About 70% of these strains produced lipase, protease, and lecitinase, but only 4% produced hemolysin. A hemolysin produced by *P. cepacia* JN106 was characterized. The hemolysin was most active against human erythrocytes. Horse, sheep, chicken, and rabbit erythrocytes were also susceptible. The hemolysin was heat labile and was inhibited by sterols but was not activated by 2-mercaptoethanol and dithiothreitol. Four hemolysin-negative mutants obtained by N-methyl-N'-nitro-N-nitrosoguanidine treatment produced the other extracellular products. A 58-kilobase-pair plasmid found in the parent strain was also found in the mutant strains, suggesting that the hemolysin gene resides on the chromosome.

In the genus *Pseudomonas*, *Pseudomonas cepacia* has the most versatile ability to catabolize various organic compounds (24). It is highly resistant to many antibiotics and disinfectants and has been isolated with increasing frequency from clinical specimens and hospital environments (3, 6, 20, 27). The species is recognized as an opportunistic pathogen and is associated with various types of nosocomial infections (4, 7, 22, 27). In addition, *P. cepacia* now complicates cystic fibrosis (13).

There are few reports on the virulence factors of *P. cepacia*. McKevitt and Woods (19) have reported the production of virulence factors by 48 strains of *P. cepacia* isolated from patients with cystic fibrosis. A majority of strains produced protease and lipase, and about one-half of the strains produced smooth lipopolysaccharide. On the other hand, none of the strains produced elastase, cytotoxins, or ADP-phosphoribosyl transferase.

This study was initiated to determine the virulence factors of *P. cepacia*. One hundred and twenty strains isolated from clinical specimens were screened for extracellular products. In addition, a hemolysin produced by one of the strains was characterized.

**MATERIALS AND METHODS**

**Bacterial strains.** One hundred and four strains of *P. cepacia* isolated from clinical specimens in Juntendo University Hospital, Tokyo, Japan, from 1983 to 1984 were generously provided by T. Oguri and J. Igari. Among 79 strains of known origin, 15 strains were isolated from sputum, 12 strains from pus, 11 strains from urine, and 9 strains from cerebrospinal fluid; and 4 strains each were from blood, catheters, and drain tubes. Other strains were provided by S. Oiye, Yamaguchi University Hospital, Ube, Yamaguchi, Japan, and E. Yabuuchi, Gifu University, Gifu, Japan. Identification of strains was confirmed by biochemical tests (9). Strains were stored in sterile glycerol solution (10% [wt/vol]) at −80°C.

**Detection of extracellular products.** Protease (28), elastase (23), and lecitinase (8) were determined by plate assays; lipase production was determined by Tween 80 hydrolysis (26); and hemolysin production was determined by using plates containing 7.5% defibrinated sheep blood in heart infusion agar (Eiken Co. Ltd., Tokyo, Japan). Results were determined after incubation at 28°C for 48 h.

**Preparation of crude hemolysin.** Cells were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) or dialyzed of brain heart infusion broth (Eiken Co.) for about 48 h at 28°C with shaking. When the A660 of the culture reached 1.5, the culture supernatant was obtained by centrifugation (9,000 × g, 10 min). Crude hemolysin was prepared by ammonium sulfate fractionation and by taking the fraction between 20 and 60% saturation. This fraction was then dialyzed against 10 mM Tris hydrochloride (pH 7.4). The preparations of culture supernatant and crude hemolysin were stored at −80°C until use. Protein concentrations were determined by the method described by Lowry et al. (18).

**Hemolysin assay.** The hemolysin assay mixture contained 10 mM Tris hydrochloride (pH 7.4)−160 mM NaCl (hemolysin assay buffer), 2% suspensions of sheep erythrocytes that had been washed with saline, and an appropriate volume of sample containing hemolysin in a total volume of 2 ml. Control experiments for spontaneous lysis or complete lysis were carried out without hemolysin and with 0.2% sodium dodecyl sulfate, respectively. Reaction mixtures were incubated at 37°C for 10 min and chilled on ice for 2 min to stop the reaction. The lysed erythrocytes were removed by centrifugation at 1,500 × g for 2 min, and the A530 of the supernatant was determined. The activity resulting in 50% hemolysis of 2 ml of 2% sheep erythrocyte suspensions was defined as 1 hemolytic unit (HU).

**Studies on effects of various reagents on hemolysin.** The culture supernatant of strain JN106 (8 HU/ml) was preincubated at 0°C for 2 h with N-ethylmaleimide (1 mM), p-chloromercuribenzoate (1 mM), 2-mercaptoethanol (1 mM), dithiothreitol (1 mM), or disodium EDTA (1 mM and 10 mM); and the residual activity was determined. The effect of CaCl2 on hemolysin was determined by the addition of 1 or 10 mM CaCl2 to the assay mixture. Sterols were dissolved in ethanol to make a 5 mM solution which was serially diluted with hemolysin assay buffer just before use. A small volume of crude hemolysin was added to 1 ml of sterol solution and...
incubated at 0°C for 30 min. Then, the hemolysin activity was determined under standard assay conditions.

**Mutagenesis.** An overnight culture (4 ml) of *P. cepacia* JN106 was incubated with 12 ml of fresh nutrient broth at 28°C for 4 h with shaking. Cells were harvested by centrifugation, washed once with 0.1 M citrate buffer (pH 5.0), and suspended in 8 ml of the same buffer. N-Methyl-N, N′-nitro-N-nitrosoguanidine (NTG; 2 ml) was added to a final concentration of 1 mg/ml, followed by incubation at 28°C for 90 min without shaking. To screen hemolysin-negative mutants, cells were washed twice with carbon source-free M9 medium (5) and spread on sheep blood-agar plates, which were incubated at 28°C. About 0.5% of the cells remained viable after NTG treatment.

**Isolation of plasmid DNA and digestion with restriction enzyme.** Plasmid DNA was isolated by the method of Kado and Liu (15). DNA was subjected to 0.7% agarose gel electrophoresis in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid [pH 8.0]) at a constant 100 V. Gels were stained with ethidium bromide (1 μg/ml) and photographed. Digestion of plasmid DNA with BamHI (Takara Shuzo Co., Kyoto, Japan) was performed by the method of the supplier.

**RESULTS**

**Detection of extracellular products.** One hundred and twenty clinical isolates of *P. cepacia* were screened for the production of protease, lecinthinase, lipase, hemolysin, and elastase (Table 1). The majority of strains produced protease, lecinthinase, and lipase. Only 3 strains produced hemolysin, as indicated by a clear zone of hemolysis around the colonies on sheep blood-agar plates. All the hemolysin-producing strains also produced protease, lecinthinase, and lipase. None of the strains produced elastase. Among 120 strains, 30 strains did not produce any of the extracellular products described above.

**Characterization of hemolysin produced by *P. cepacia* JN106.** Because hemolysins produced by various bacteria are known to be involved in bacterial virulence, we characterized the *P. cepacia* hemolysin produced by strain JN106.

**TABLE 1. Extracellular products produced by 120 clinical isolates of *P. cepacia***

<table>
<thead>
<tr>
<th>Extracellular product</th>
<th>No. of positive strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease</td>
<td>83 (69)</td>
</tr>
<tr>
<td>Lecithinase</td>
<td>80 (67)</td>
</tr>
<tr>
<td>Lipase</td>
<td>88 (73)</td>
</tr>
<tr>
<td>Hemolysin</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Elastase</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Extracellular products were determined by plate assays, as described in the text.*

**TABLE 2. Sensitivity of erythrocytes from various animal species to strain JN106 hemolysin***

<table>
<thead>
<tr>
<th>Source of erythrocytes</th>
<th>Relative sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>195</td>
</tr>
<tr>
<td>Horse</td>
<td>142</td>
</tr>
<tr>
<td>Sheep</td>
<td>100</td>
</tr>
<tr>
<td>Chicken</td>
<td>97</td>
</tr>
<tr>
<td>Rabbit</td>
<td>77</td>
</tr>
</tbody>
</table>

*Culture supernatant (8.8 HU/ml) was used.*

**TABLE 3. Heat stability of strain JN106 hemolysin***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100°C, 5 min</td>
<td>3.2</td>
</tr>
<tr>
<td>100°C, 20 min</td>
<td>1.8</td>
</tr>
<tr>
<td>56°C, 30 min</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* A total of 0.1 ml of crude hemolysin (10 HU) was dissolved in hemolysin assay buffer and incubated under various conditions.
tions of sterols to give 50% inhibition were variable, depending on the hemolysin preparations. No inhibition was observed by 50 μM dehydroepiandrosteron, pregnenolone, or estradiol (data not shown).

Isolation of hemolysin-negative mutants of JN106. P. cepacia JN106 produced protease, lecithinase, and lipase, in addition to hemolysin. To establish that the hemolysin activity was not associated with the other extracellular products, hemolysin-negative mutants were isolated after NTG treatment. By screening 1,500 clones, 4 hemolysin-negative mutants were obtained (Table 4). The mutant JN106 did not produce any hemolysin, as judged by plate or tube assay, by using a sample of the 20 to 60% ammonium sulfate fraction containing 0.25 mg of protein. In contrast, the same ammonium sulfate fraction of the parent strain (crude hemolysin) had approximately 40 HU/mg of protein. Other mutants appeared to be slightly leaky, producing a narrow hemolytic zone on plates after incubation for 4 days at 28°C. Other extracellular products were produced normally by the mutants, except for mutant JN1075, which did not produce protease (Table 4). These results indicate that the hemolysin activity of P. cepacia JN106 is not associated with the other extracellular products.

Plasmid analysis of JN106. Strain JN106 was found to carry a plasmid of approximately 58 kilobase pairs, based on the size of restriction fragments produced by digestion with BamHI. All the mutant strains also carried a plasmid of the same size as that carried by the parent strain. It was assumed that the plasmid was not involved in the hemolysin synthesis of JN106.

DISCUSSION

The results of the survey of extracellular products of 120 strains of P. cepacia described in this report coincided well with those of McKevitt and Woods (19), who screened for extracellular products of 48 strains of P. cepacia isolated from patients with cystic fibrosis. It appears, therefore, that the described pattern of extracellular products (Table 1) may represent the pattern of P. cepacia populations of clinical origin. Gonzalez and Vidaver (11) reported that onion maceration tests and pectolytic activity at low pHs were positive in strains of plant origin, whereas these activities were negative or minimal in strains of clinical origin.

Hemolysin is considered to be one of the virulence factors of several bacteria such as Streptococcus pyogenes (1), Staphylococcus aureus (14), Vibrio parahaemolyticus (21), Vibrio vulnificus (16), Escherichia coli (29), and Pseudomonas aeruginosa (17). The heat-labile hemolysin produced by P. aeruginosa is phospholipase C, which catalyzes the hydrolysis of phosphatidylcholine (lecithin) to phosphorylcholine and diacylglycerol (2). The hemolysin-producing strain P. cepacia JN106 also produced lecithinase, as determined by the egg yolk reaction. Association of the hemolytic activity with lecithinase is unlikely, because hemolysin-negative mutants still produced lecithinase.

The hemolysin produced by strain JN106 was inhibited by several sterols. Cholesterol and 7-dehydrocholesterol showed the strongest inhibition of the hemolysin. The inhibition pattern of P. cepacia hemolysin by sterols was very similar to that of streptolysin O, a representative of thiol-activated and cholesterol-binding cytolsins (25). In contrast to streptolysin O, however, the P. cepacia hemolysin was not activated by 2-mercaptoethanol or dithiothreitol. Hemolysin produced by V. vulnificus is also not affected by 2-mercaptoethanol or dithiothreitol, although it is inhibited by cholesterol (12).

Results of many studies have shown that the genetic determinants for certain virulence factors are carried by plasmids. For example, E. coli strains of animal origin carry a hemolysin gene on a plasmid (10). We analyzed strain JN106 and found that it contains a 58-kilobase-pair plasmid. The hemolysin determinant, however, is unlikely to reside on the plasmid, because hemolysin-negative mutants still carry the plasmid.

ACKNOWLEDGMENTS

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LITERATURE CITED


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**TABLE 4.** Properties of hemolysin-negative mutants of strain JN106

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysin</th>
<th>Protease</th>
<th>Lecithinase</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>JN106 (wild type)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JN1066</td>
<td>+ ±</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JN1067</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JN1070</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>JN1075</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Extracellular products were determined as described in the text.
* Slight hemolysin was found after incubation for 4 days.
17. Liu, P. V. 1974. Extracellular toxins of Pseudomonas aerugi-

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