The influence of exo-enzyme S and proteases on endogenous *Pseudomonas aeruginosa* bacteraemia in mice

Y. HIRAKATA*, N. FURUYA, K. TATEDA, T. MATSUMOTO and K. YAMAGUCHI

*Department of Microbiology, Toho University School of Medicine, Tokyo 143, Japan

**Summary.** The role of *Pseudomonas aeruginosa* exo-enzymes was evaluated in a murine model of endogenous bacteraemia in which the bacteria invaded the bloodstream after oral dosing. Although an elastase mutant PAO-E64 was as virulent as its parent strain PAO1, an exo-enzyme S-deficient mutant, DG1-ExS5 and alkaline protease mutants PAKS-16, PAKS-17, PAKS-19, were less virulent than their parent strains, DG1 and PAKS-1, respectively (p < 0.01). Thus exo-enzyme S and alkaline protease, but not elastase, appear to contribute to the pathogenicity of *P. aeruginosa* in this model.

**Introduction**

*Pseudomonas aeruginosa* is an opportunistic pathogen and frequently causes severe septicaemia, particularly in immunocompromised patients. Among gram-negative bacteria, *P. aeruginosa* septicaemia has the highest mortality rate. Lipopolysaccharide (LPS) is an important virulence factor of many gram-negative pathogens but the LPS of *P. aeruginosa* is not as toxic as that of enterobacteria. Therefore, it is clear that other factors are involved in the virulence of this organism.

*P. aeruginosa* produces a large number of extracellular products that are more toxic than LPS and are important in pathogenesis. We have reported previously that exotoxin A (ETA), which catalyses the transfer of ADP-ribose from NAD to eukaryotic proteins and is the most toxic product of *P. aeruginosa*, is produced in a model of endogenous bacteraemia in mice. Moreover, the lethality for mice of the ETA mutant PAO-PR1 was significantly less than that of the parent strain. We also demonstrated that clinical isolates of *P. aeruginosa* from blood produce large amounts of ETA in vitro and are more lethal for neutropenic mice with endogenous bacteremia than strains isolated from other sources such as sputum and urine. These data indicate that ETA may be an important factor in the development of *P. aeruginosa* bacteraemia and for lethality in mice. However, the role of other extracellular enzymes in septicaemia caused by *P. aeruginosa* is not fully understood. To further understand the pathogenicity of this organism, the contribution of several extracellular enzymes to endogenous bacteraemia with *P. aeruginosa* was examined with enzyme-producing strains and their enzyme-deficient mutants.

**Materials and methods**

**Bacterial strains**

The bacterial strains used in the present study are described in table I. *P. aeruginosa* PAO1, PAO-E64, 388 and 388exs1::Tn112 were kindly provided by Professor B. H. Iglewski, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA. Strain PAO1 has been well characterised and produces most of the recognised virulence factors. Its elastase mutant strain, PAO-E64, obtained by nitrosoguanidine treatment, produces elastase that is antigenically indistinguishable from that of the parent strain. Strain 388 exs1::Tn112 was significantly less lethal for mice than the parent strain. Moreover, the product of the ETA mutant PAO-PR1 was significantly less lethal for mice than the parent strain. We also demonstrated that clinical isolates of *P. aeruginosa* from blood produce large amounts of ETA in vitro and are more lethal for neutropenic mice with endogenous bacteremia than strains isolated from other sources such as sputum and urine. These data indicate that ETA may be an important factor in the development of *P. aeruginosa* bacteraemia and for lethality in mice.
administration of the bacteria. Faecal specimens were
The animals were housed in sterile cages supplied with
drinking water during days 1-4. To facilitate col-

aggregation of the mice with *P. aeruginosa*, daily intra-
peritoneal injections of sodium ampicillin (Vaccillin;
Meiji Seika Kaisha, Tokyo) 200 mg/kg were adminis-
tered on days 1-4 to reduce the normal intestinal flora
of the animal. Mice were then given cyclophosphamide
(Endoxan; Shionogi and Co., Osaka, Japan) 200 mg/kg by intraperitoneal injection on days 5 and 8. At least 20 mice were used in each group and their
survival was monitored until day 18.

Statistical analysis
The χ² test was used to compare survival rates. A
probability level of 5% was accepted as statistically
significant.

Results
The survival kinetics of mice given *P. aeruginosa*
strains orally and treated with cyclophosphamide are
shown in the figure. The mortality of mice given PAO1
orally was 78.8%. These mice died between days 11
and 13 due to a systemic bacteremia caused by the
challenge strain. In the absence of an oral bacterial
challenge, no deaths occurred in mice treated with
cyclophosphamide and ampicillin. Bacterial culture
was negative for *P. aeruginosa* in all control mice killed
at the end of the experiment. In mice given the elastase-
deficient mutant strain PAO-E64, mortality was 80%
and did not differ from that of mice given the parent
strain.

The mortality induced by exo-enzyme S-producing
strain 388 and its exo-enzyme S-deficient mutant
388/ex1::TnI was 20% in both cases. In contrast,
another exo-enzyme S-producing strain, DG1, caused
a high mortality of 80%. The mortality of mice given
its exo-enzyme S-deficient mutant, DG1-ExS5, was
20%, which was significantly lower than that of mice
given the parent strain (table II).

The mortality of mice given the parent strain PAKS-
1 was 70%. On the other hand, the mortalities of mice

Table I. Characteristics of the *P. aeruginosa* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>8</td>
<td>Clinical isolate from a wound; produces most of the recognised virulence factors</td>
</tr>
<tr>
<td>PAO-E64</td>
<td>15</td>
<td>Chemically-induced mutant of PAO1; produces elastase but with reduced enzymic activity</td>
</tr>
<tr>
<td>388</td>
<td>9</td>
<td>Exo-enzyme S producer, clinical isolate from a burn wound; produces no exotoxin A</td>
</tr>
<tr>
<td>388/ex1::TnI</td>
<td>12</td>
<td>Transposon-insertion mutant of 388; produces no exo-enzyme S</td>
</tr>
<tr>
<td>DG1</td>
<td>19</td>
<td>Exo-enzyme S producer; clinical isolate from cystic fibrosis</td>
</tr>
<tr>
<td>DG1-ExS5</td>
<td>20</td>
<td>Transposon-insertion mutant of DG1; produces no exo-enzyme S</td>
</tr>
<tr>
<td>PAKS-1</td>
<td>21</td>
<td>Alkaline protease hyperproducer, clinical isolate from urine</td>
</tr>
<tr>
<td>PAKS-16</td>
<td>21</td>
<td>Chemically-induced mutant of PAKS-1; produces significantly lower amount of alkaline protease, no elastase</td>
</tr>
<tr>
<td>PAKS-17</td>
<td>21</td>
<td>Chemically-induced mutant of PAKS-1; produces significantly lower amount of alkaline protease</td>
</tr>
<tr>
<td>PAKS-19</td>
<td>21</td>
<td>Chemically-induced mutant of PAKS-1; produces lower amount of alkaline protease</td>
</tr>
</tbody>
</table>

*aeruginosa* strains DG114 and DG1-ExS515 were kind
gifts from Professor D. E. Woods, University of
Calgary, Alberta, Canada. Strain DG1 is another well
characterised exo-enzyme S-producing strain, that also
produces ETA and proteases.14 Strain DG1-ExS5 is a
transposon-insertion mutant derived from strain DG1.
It is deficient in exo-enzyme S but otherwise indistiguishable from the parent strain. Strains PAKS-
1, PAKS-16, PAKS-17 and PAKS-1916 were kindly
provided by Dr B. Wretlind, Karolinska Hospital,
Stockholm, Sweden. Strain PAKS-1 is a hyper-
producer of alkaline protease, and, in addition, pro-
duces elastase. PAKS-16, PAKS-17 and PAKS-19 are
mutants of PAKS-1 induced by ethyl methane-
sulphonate treatment and produce only minute
amounts of alkaline protease. Although PAKS-17
produces no elastase, the other mutants produce normal levels of the enzyme.

Animals
Specific-pathogen-free male ddY mice (Japan SLC
Co., Shizuoka, Japan) weighing 20–24 g were used.
The animals were housed in sterile cages supplied with
filter hoods and were fed a sterile diet. They received
sterile distilled water except during the period of oral
administration of the bacteria. Faecal specimens were
obtained before the study and examined to ensure the
absence of *P. aeruginosa*.

Endogenous bacteraemia model
Endogenous bacteraemia was produced as described
previously.37 Bacteria were grown on Trypticase Soy
Agar (BBL Microbiology Systems, Cockeysville, MD,
USA) at 37°C for 18 h, suspended in sterile saline,
0.45% and adjusted to a density of 10⁷/ml by spectrophotometry. The bacterial suspension was given as the
drinking water during days 1–4. To facilitate col-
onisation of the mice with *P. aeruginosa*, daily intra-
peritoneal injections of sodium ampicillin (Vaccillin;
Table II. Mortality of mice given P. aeruginosa exo-enzyme S-producing strains and their mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>388</td>
<td>20</td>
</tr>
<tr>
<td>388ex1::Tn1</td>
<td>20</td>
</tr>
<tr>
<td>DG1</td>
<td>80</td>
</tr>
<tr>
<td>DG1-ExS5</td>
<td>20</td>
</tr>
</tbody>
</table>

Table III. Mortality of mice given P. aeruginosa strain PAKS-1 and its alkaline protease mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAKS-1</td>
<td>70</td>
</tr>
<tr>
<td>PAKS-16</td>
<td>20</td>
</tr>
<tr>
<td>PAKS-17</td>
<td>20</td>
</tr>
<tr>
<td>PAKS-19</td>
<td>0</td>
</tr>
</tbody>
</table>

given its alkaline protease mutants ranged from 0 to 20%, and were all significantly lower than that of mice infected with the parent strain (table III).

Discussion

A number of P. aeruginosa strains can produce various exo-enzymes in vitro, including ETA, exo-enzyme S, alkaline protease, elastase and phospholipase C. These exo-enzymes seem to be important virulence factors in several P. aeruginosa infections. Woods et al. reported that blood isolates of P. aeruginosa produced a large amount of total protease, ETA, and phospholipase in vitro, compared with isolates obtained from other infection sites. Clinical bacteraemia isolates of P. aeruginosa caused higher mortality in mice in our model of endogenous bacteraemia than did strains obtained from other infection sites and our assays of exo-enzymes in the culture supernatants of P. aeruginosa strains were in broad agreement with those of Woods et al. We have also reported that ETA could be detected in murine serum in the endogenous bacteraemia model. Although mice given strain PA01 had a high mortality, strain PAO-PR1, which was chemically-derived from strain PA01 and produces a non-toxic, cross-reactive ETA, showed a significantly lower lethality. Such data indicate that ETA may be an important factor in the occurrence of P. aeruginosa bacteraemia and death of the animal.

Proteases produced by P. aeruginosa may also play a role in the virulence of the organism. Two distinct P. aeruginosa proteases have been isolated and purified. These enzymes, alkaline protease and elastase, are metalloproteases, although their substrate specificities and pH optima are different. P. aeruginosa proteases produce corneal ulcers, necrotic skin lesions and pulmonary haemorrhage. Exo-enzyme S, like ETA, is an ADP-ribosyl transferase produced by P. aeruginosa. Production of exo-enzyme S is fairly common among clinical isolates of P. aeruginosa, especially in strains from burn infections and bacteraemias. Exo-enzyme S is also known as an important virulence factor in a burned mouse model and a chronic rat lung infection model.

The role of these exo-enzymes in the development of endogenous P. aeruginosa bacteraemia is still unclear. Because animals injected directly with a large amount of gram-negative bacteria die of endotoxic shock within a short period, the assessment of other virulence factors in conventional systemic infection models may be difficult. In the infection model used in the present study, each step involved in the production of systemic
bacteraemia mimics the clinical pathophysiology of primary bacteraemia originating in the intestinal microflora.

In the present study, there was little difference in mortality between mice given strain PA01 or its elastase-deficient mutant, PAO-E64. These data suggest that elastase is not an essential factor in endogenous *P. aeruginosa* bacteraemia in mice. When exo-enzyme S-producing strains and their mutants were evaluated, strain 388 exhibited low virulence in spite of its ability to produce exo-enzyme S in *vitro*. This was probably due to a lack of ETA production by this strain. In contrast, strain DG1, which produces both ETA and exo-enzyme S, caused a high mortality whereas that due to its exo-enzyme S-deficient mutant, DG1-ExS5, was significantly lower. These data suggest that exo-enzyme S, like ETA, is an important factor in *P. aeruginosa* bacteraemia and lethality for mice. It is also possible that the ability to produce both ETA and exo-enzyme S is essential for lethality in this model, since DG1-ExS5, which produces ETA but not exo-enzyme S, had a relatively low virulence.

With the alkaline protease mutants of strain PAKS-1, mortality was significantly lower than in mice given the parent strain. These results suggest that alkaline protease is also an important factor in bacteraemia.

In conclusion, we speculate that exo-enzyme S and alkaline protease, but not elastase, affect one or more of the steps involved in colonisation of the mouse intestine by *P. aeruginosa*, in its subsequent invasion of the blood stream, and in its resistance to phagocytosis by Kupffer cells.21

References

6. Ohman DE. *Bacteremia 261*