Immunization with 3-oxododecanoyl-L-homoserine lactone–protein conjugate protects mice from lethal *Pseudomonas aeruginosa* lung infection

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Quorum-sensing systems have been reported to play a critical role in the pathogenesis of several bacterial infections. Recent data have demonstrated that *Pseudomonas* *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo- C_{12} -homoserine lactone, 3-oxo- C_{12} -HSL), but not *N*-butanoyl-L-homoserine lactone (C_4 -HSL), induces apoptosis in macrophages and neutrophils. In the present study, the effects of active immunization with 3-oxo- C_{12} -HSL–carrier protein conjugate on acute *P. aeruginosa* lung infection in mice were investigated. Immunization with 3-oxo- C_{12} -HSL–BSA conjugate (subcutaneous, four times, at 2-week intervals) elaborated significant amounts of specific antibody in serum. Control and immunized mice were intranasally challenged with approximately 3×10^6 c.f.u. *P. aeruginosa* PAO1, and survival was then compared. All control mice died by day 2 post bacterial challenge, while 36 % of immunized mice survived to day 4 ($P < 0.05$). Interestingly, bacterial numbers in the lungs did not differ between control and immunized groups, whereas the levels of pulmonary tumour necrosis factor (TNF)- α in the immunized mice were significantly lower than those of control mice ($P < 0.05$). Furthermore, the extractable 3-oxo- C_{12} -HSL levels in serum and lung homogenate were also significantly diminished in the immunized mice. Immune serum completely rescued reduction of cell viability by 3-oxo- C_{12} -HSL-mediated apoptosis in macrophages *in vitro*. These results demonstrated that specific antibody to 3-oxo- C_{12} -HSL plays a protective role in acute *P. aeruginosa* infection, probably through blocking of host inflammatory responses, without altering lung bacterial burden. The present data identify a promising potential vaccine strategy targeting bacterial quorum-sensing molecules, including autoinducers.

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of acute and chronic infections, including sepsis, and wound and pulmonary infections (Richards *et al.*, 1999). This organism is known to produce a variety of virulence factors, such as pigments, proteases and exotoxins. The synthesis of these factors is regulated by

a cell-to-cell signalling mechanism referred to as quorum sensing (Fuqua *et al.*, 1994), which was originally described in *Vibrio fischeri* as a LuxR/LuxI-type system (Kaplan & Greenberg, 1985). LasRI and RhlRI are known to be two major quorum-sensing components in *P. aeruginosa*, and this mechanism enables bacteria to coordinately turn on and turn off genes in a density-dependent manner by the production of small diffusible molecules called autoinducers (Van Delden & Iglewski, 1998). *P. aeruginosa* predominately produces two autoinducers, *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo- C_{12} -HSL) and *N*-butanoyl-L-homoserine lactone (C_4 -HSL) (Pearson *et al.*, 1994, 1995). The expression of autoinducer-regulated virulence factors

Abbreviations: C_4 -HSL, *N*-butanoyl-L-homoserine lactone; HRP, horseradish peroxidase; HSL, homoserine lactone; IL, interleukin; OV, ovalbumin; 3-oxo- C_{12} -HSL, *N*-3-oxododecanoyl-L-homoserine lactone; TNF, tumour necrosis factor.

directly contributes to the colonization and dissemination of the bacteria, and this may determine the course and outcome in individuals infected with *P. aeruginosa* (Miller & Bassler, 2001; Smith & Iglewski, 2003a; Whitehead *et al.*, 2001).

Recent progress in quorum-sensing research has demonstrated that *Pseudomonas* homoserine lactones (HSLs) are not only important in the regulation of bacterial virulence genes but also interact with eukaryotic cells and modulate immune responses (DiMango *et al.*, 1995; Saleh *et al.*, 1999; Telford *et al.*, 1998). Stimulation with 3-oxo-C₁₂-HSL induces production of the chemokine interleukin (IL)-8 in human lung structural cells, such as fibroblasts and bronchial epithelial cells (Smith *et al.*, 2001). More recently, it has been reported that 3-oxo-C₁₂-HSL induces cyclooxygenase-2 and prostaglandin E₂ production in human lung fibroblasts, suggesting a pivotal role for 3-oxo-C₁₂-HSL in inflammation (Smith *et al.*, 2002). On this point, we have reported that *Pseudomonas* 3-oxo-C₁₂-HSL, but not C₄-HSL, induces apoptosis in macrophages and neutrophils (Tateda *et al.*, 2003). These data suggest that autoinducer molecules at the site of infection contribute not only to the expression of bacterial virulence factors, but also to the modulation of host defence systems.

Since the 1960s, a number of experimental vaccines have been developed and tested for the prevention of *P. aeruginosa* infections in animals and patients. A variety of bacterial products (toxins, exopolysaccharide) and cell surface structures (LPS, outer-membrane proteins, flagella, type III secretion apparatus) have been examined for their potential as vaccine targets against *P. aeruginosa* infection (Baumann *et al.*, 2004; Cachia & Hodges, 2003; Mrsny *et al.*, 2002; Thomas *et al.*, 2003). The most promising of the products tested have been vaccines based on LPS as antigens, named Pseudogen and PEV-01. These have proved to be effective in certain individuals, such as patients with burns, cystic fibrosis and malignancies, although the toxicity associated with the lipid A fraction of LPS has prevented approval for routine clinical use of these products (Cryz *et al.*, 1987; Jones *et al.*, 1979; Pier, 1982). Sawa and collaborators have reported a type III secretion apparatus, PcrV, a unique system that injects bacterial toxin into eukaryotic cells, as a new vaccine target, and immunization against PcrV ensures the survival of challenged mice and decreased lung inflammation and injury (Sawa *et al.*, 1999). More recently, the protective efficacy of DNA vaccines against outer-membrane protein F has been reported in a mouse model of chronic lung infection due to *P. aeruginosa* (Price *et al.*, 2002; Staczek *et al.*, 2003). However, there is currently no approved vaccine against *P. aeruginosa* infections in the clinical setting. Recently, several investigators have suggested quorum-sensing systems as a new area to search for novel types of vaccine candidate, although no data have been reported to date (Smith & Iglewski, 2003b; Suga & Smith, 2003). In particular, blocking of bacterial autoinducer molecules by means of active and passive immunization is

believed to be a promising strategy for treatment of *P. aeruginosa* infections.

In the present study, we investigated the effects of active immunization with carrier-conjugated 3-oxo-C₁₂-HSL on the lethality of acute *P. aeruginosa* lung infection in mice. Also, we examined the effects of blocking antibody on bacterial numbers and cytokine levels in the infected lungs.

METHODS

Mice and bacteria used. BALB/c mice were purchased from Charles River Japan. All mice were housed in specific pathogen-free conditions within the animal care facility at the Toho University School of Medicine. Mice were 6–8 weeks old at the start of the experiments. We used *P. aeruginosa* PAO1 strain (a generous gift from B. H. Iglewski, University of Rochester School of Medicine and Dentistry) for pulmonary infection in mice (Tateda *et al.*, 2001).

Preparation of protein-conjugated 3-oxo-C₁₂-HSL. 3-oxo-C₁₂-HSL was chemically synthesized as previously described (Fig. 1; Tateda *et al.*, 2003). The molecule is structurally and functionally identical to those obtained from *P. aeruginosa* cultures. The compound was shown to be pure by HPLC. The absence of detectable levels of endotoxin in preparations was verified by a *Limulus* amoebocyte lysate assay. *N*-(12-(3-Carboxypropionyl)oxy-3-oxo-dodecanoyl) HSL was synthesized as a hapten by the method described previously (Horikawa *et al.*, 2006). The conjugation reaction of the hapten to BSA was carried out by the activated ester method (Hosoda *et al.*, 1979). A mixture of the hapten (26.3 mg), *N*-hydroxysuccinimide (Tokyo-Kasei Kogyo) (8.8 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (water soluble carbodiimide, WSC) (27 mg) in dioxane (0.8 ml) was stirred at 18–24 °C for 6 h. The resulting activated ester solution (274 µl) was diluted with 2 ml 50 mM potassium phosphate buffer, pH 7.4 (KPi), and BSA solution [40 mg BSA (Sigma) in 1 ml KPi] was added. The whole mixture was stirred at 4 °C for 2.5 days. Then, the resulting mixture was subjected to dialysis against saline and stored at –85 °C until use. The hapten was also conjugated with ovalbumin (OV; Sigma) in a similar manner to the immobilized antigen.

Immunization protocol and measurement of specific antibody titre to 3-oxo-C₁₂-HSL. Mice were subcutaneously injected with 3-oxo-C₁₂-HSL–BSA with Freund's complete adjuvant for the priming, and incomplete adjuvant for boost (three times at 2-week intervals). The serum antibody titre to 3-oxo-C₁₂-HSL was examined using the immobilized antigen. Briefly, 96-well microplates were coated with 3-oxo-C₁₂-HSL–OV conjugate overnight. After serial dilutions of serum with PBS, samples were added to wells and incubated overnight at 4 °C. After washing with PBS three times, horseradish peroxidase (HRP)-labelled anti-mouse IgG antibody was added to each well and incubated for 4 h at room temperature.

Fig. 1. Structure of the 3-oxo-C₁₂-HSL–carrier protein conjugate.

Colour-developing substrate was added to the wells after the removal of unbound HRP-labelled antibody by washing with PBS. A_{590} was examined in an auto-reader.

Pulmonary *P. aeruginosa* infection in mice. Bacteria were grown in Mueller–Hinton medium overnight, and diluted to appropriate concentrations in saline. Mice were anaesthetized intramuscularly with 7 mg ketamine and 15 mg xylazine per kilogram of animal, as described previously (Tateda *et al.*, 1996). Bacterial suspension (30 μ l) was administered intranasally, and survival was monitored twice a day for 7 days after infection.

Bacterial number and tumour necrosis factor (TNF)- α in the lungs. At 24 h after infection, the mice were killed by CO₂ asphyxia and blood was collected to obtain serum. Before lung removal, the pulmonary vasculature was perfused with 1 ml saline via the right ventricle. After removal, whole lungs were homogenized in 1.0 ml saline using a tissue homogenizer (Omni International) under a vented hood. Portions of homogenate (10 μ l) were inoculated on Mueller–Hinton agar after serial 1:10 dilutions with saline. The remaining homogenates were stood on ice for 30 min, then centrifuged at 2500 r.p.m. for 10 min. Supernatants were collected, passed through a 0.45 μ m pore-size filter (Kanto Chemical), then stored at -20°C for the assessment of TNF- α and free 3-oxo-C₁₂-HSL. The level of TNF- α in the lung homogenates was determined using an ELISA kit (Duo Set, ELISA development system; R&D Systems), according to the manufacturer's directions.

3-Oxo-C₁₂-HSL levels in serum and lung. The 3-oxo-C₁₂-HSL levels in serum and lung homogenate were determined by ELISA. Briefly, the ELISA system was constructed with the specific anti-3-oxo-C₁₂-HSL antibody prepared in these laboratories with a rabbit, hapten–OV conjugate-coated 96-well microplates and HRP-labelled goat anti-rabbit IgG antibody (Bio-Rad). The substrate for HRP was *o*-phenylenediamine. Ethyl acetate extracts of the serum and supernatant of lung homogenate were subjected to the ELISA. The 3-oxo-C₁₂-HSL extractable by ethyl acetate was mainly in the free fraction rather than in the fraction bound to antibody, and this was confirmed by ultrafiltration using Vivaspin 5000 (Vivascience), which cut off the protein fraction with a molecular mass higher than 5000 Da.

Effects of immune serum on apoptosis of macrophages by 3-oxo-C₁₂-HSL. Macrophage cell line P388D1 was grown in RPMI

medium supplemented with 10% fetal calf serum. Cells were seeded at 10^5 cells per well of a 96-well microplate. Twenty-four hours later, cells were treated with 50 μ M 3-oxo-C₁₂-HSL for induction of apoptosis in the presence of immune or non-immune serum. The viability of cells treated with *Pseudomonas* HSL was measured by using TetraColor ONE (Seikagaku Kogyo), following the manufacturer's instructions (Tateda *et al.*, 2003). At the indicated time points, 10 μ l of this reagent was added to each well and incubated for an additional 4 h. A_{450} was monitored, and the percentage viability of the cells was calculated in comparison with that of control non-treated cells.

Statistical analysis. Statistical significance was determined using the unpaired, two-tailed alternate Welsh *t* test and the nonparametric Mann–Whitney test. Calculations were performed using InStat for Macintosh (GraphPad Software).

RESULTS

Specific antibody titre to 3-oxo-C₁₂-HSL after immunization

After four episodes of boost immunization at 2-week intervals, we examined antibody titres in the serum of mice (no treatment, adjuvant alone and immunized). The serum of mice treated with adjuvant alone showed no increase in antibody titres compared to control mice serum. On the other hand, as expected, immunization with 3-oxo-C₁₂-HSL–BSA conjugate strongly induced specific antibody in mice (Fig. 2). An increase of antibody titre of more than 100 times was demonstrated in the serum of immunized mice.

Survival of immunized and non-immunized mice after intranasal inoculation with *P. aeruginosa*

Immunized and non-immunized mice were intranasally challenged with 3×10^6 c.f.u. *P. aeruginosa* PAO1, and then survival was compared. All non-immunized mice died by day 2 post bacterial challenge, while 36% of immunized

Fig. 2. Antibody titres in sera of control, adjuvant-treated and immunized mice. Mice were treated with adjuvant alone or carrier-protein-conjugated 3-oxo-C₁₂-HSL four times at 2-week intervals, and then serum antibody titres against 3-oxo-C₁₂-HSL were examined by ELISA ($n=5$). Data were expressed as mean $\pm$ SEM.

Fig. 3. Effects of vaccination against 3-oxo-C₁₂-HSL on survival of mice with acute pulmonary *P. aeruginosa* infection. BALB/c mice were immunized with carrier-protein-conjugated 3-oxo-C₁₂-HSL (●), and then *P. aeruginosa* PAO1 (3.0×10^6 c.f.u. per mouse) was introduced intranasally. The survival of control mice is also shown (○). The survival of mice was monitored for 7 days after infection ($n = 11$).

mice survived to day 4 ($P < 0.05$) (Fig. 3). These data suggest that specific antibody against 3-oxo-C₁₂-HSL provides a survival benefit in mice infected with *P. aeruginosa*.

Comparison of pulmonary bacterial numbers and TNF- α in immunized and non-immunized mice

For a better understanding of the mechanisms of increase of survival in immunized mice, we compared pulmonary bacterial numbers and TNF- α levels in immunized and non-immunized mice 24 h after inoculation with bacteria (9.3×10^5 c.f.u. per mouse) (Fig. 4). Interestingly, bacterial numbers in the lungs were not different between the two groups, whereas the levels of TNF- α in the immunized mice were significantly lower than those of control mice ($P < 0.05$). These results suggest that elaboration of specific antibody to 3-oxo-C₁₂-HSL is associated with survival benefit, probably through the blocking of host inflammatory responses, without affecting lung bacterial burden.

Fig. 4. Comparison of pulmonary bacterial numbers and TNF- α levels in immunized and non-immunized mice. Control and immunized mice were intratracheally infected with *P. aeruginosa* PAO1 (9.3×10^5 c.f.u. per mouse), and then bacterial number and TNF- α levels in the lungs of mice were examined 24 h after infection ($n = 5$). Data were expressed as mean $\pm$ SEM. * $P < 0.05$, compared with control.

Comparison of free 3-oxo-C₁₂-HSL levels in serum and lung homogenate in immunized and non-immunized mice

3-oxo-C₁₂-HSL levels in serum and lung homogenate were determined by ELISA. The 3-oxo-C₁₂-HSL extractable by ethyl acetate was mainly in the free fraction rather than in the fraction bound to antibody. As shown in Fig. 5, free 3-oxo-C₁₂-HSL levels in serum and lung homogenates from immunized mice were about one-third and one-tenth those of non-immunized mice, respectively.

Effects of immune serum on apoptosis of macrophages by 3-oxo-C₁₂-HSL

Previously, we have reported that 3-oxo-C₁₂-HSL induces apoptosis in certain cells and cell lines, such as neutrophils and macrophages (Tateda *et al.*, 2003). In a later study, we examined the effects of immune serum on the apoptosis of macrophage cell line P388D1 by 3-oxo-C₁₂-HSL. Although the concentration of autoinducers in the lungs of *P. aeruginosa*-infected patients remains unknown, we observed induction of apoptosis at 12 μ M or higher of 3-oxo-C₁₂-HSL (Tateda *et al.*, 2003). As shown in Fig. 6, 3-oxo-C₁₂-HSL at a concentration of 50 μ M reduced cell viability to approximately 10% that of the control. Although the addition of non-immune serum induced no change in cell viability, immune serum at a concentration of 1% completely restored the reduction of viability effected by 3-oxo-C₁₂-HSL. These results suggest that specific antibody to 3-oxo-C₁₂-HSL inhibits autoinducer-dependent apoptosis in macrophages.

DISCUSSION

These results demonstrated for the first time that specific antibody to 3-oxo-C₁₂-HSL plays a protective role in acute pulmonary *P. aeruginosa* infection. Interestingly, a reduction of pulmonary TNF- α was observed in immunized mice, without affecting bacterial burden in the lungs. Furthermore, the extractable 3-oxo-C₁₂-HSL levels in serum and lung were also reduced in immunized mice, as expected. This finding could be explained as a disturbance of the *las* autoinducing circuit by trapping extracellular

Fig. 5. Comparison of extractable 3-oxo-C₁₂-HSL levels in serum and lung homogenate in immunized and non-immunized mice. Control and immunized mice were intratracheally infected with *P. aeruginosa* PAO1 (9.3×10^5 c.f.u. per mouse), and then the extractable 3-oxo-C₁₂-HSL levels in the serum (black bars) and lung homogenates (white bars) of mice were determined 24 h after infection ($n=5$). Data were expressed as mean $\pm$ SEM.

3-oxo-C₁₂-HSL with the antibody. These data are consistent with previous reports that the autoinducer 3-oxo-C₁₂-HSL is an extracellular molecule that plays a role in the cell-to-cell communication of bacteria and the expression of virulence factors in *P. aeruginosa*. The data also confirmed that the pathogen tightly bound to antibody is unable to exhibit its characteristic pathogenic action. The present results demonstrated the bacterial quorum-sensing system, especially autoinducer molecules, to be a novel

Fig. 6. Effects of immune serum on 3-oxo-C₁₂-HSL-induced apoptosis of macrophages. P388D1 cells were incubated with 3-oxo-C₁₂-HSL (3-oxo-C₁₂, 50 µM) in the presence of immune or non-immune serum (1%) for 24 h, and then the viability of cells was examined by the TetraColor ONE assay ($n=5$). Data were expressed as mean $\pm$ SEM. * $P < 0.05$, compared with control.

candidate for vaccine development against *P. aeruginosa* infection.

Since the molecular mass of 3-oxo-C₁₂-HSL is approximately 300 Da, natural antibodies to this molecule may not be produced in animals and humans, even in individuals infected with *P. aeruginosa*. Therefore, we synthesized carrier protein-conjugated 3-oxo-C₁₂-HSL, in which BSA was bound to the C₁₂ position of the acyl side-chain. Immunization with this molecule led to high titres of specific antibody to 3-oxo-C₁₂-HSL, and this was strongly associated with a survival benefit in mice with acute *P. aeruginosa* lung infection. The position of the conjugation of the carrier protein to 3-oxo-C₁₂-HSL may be important for maximum activity to induce specific antibody and protect mice from lethal infection. In this regard, additional experiments using a variety of techniques (e.g. direct conjugation of carrier protein to the HSL ring) are required to find optimal vaccine products. As an alternative approach to blocking bacterial autoinducers with specific antibodies, the development of mAbs against 3-oxo-C₁₂-HSL is promising, and this is now ongoing in our laboratory.

P. aeruginosa is known to produce a variety of virulence factors, such as exotoxin A, exoenzymes, pigments and LPS, and the expression of the vast majority of these factors is finely regulated through the production of the autoinducers 3-oxo-C₁₂-HSL and C₄-HSL (Van Delden & Iglewski, 1998). Hartl and associates have reported significantly higher levels of proinflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , in bronchoalveolar lavage fluid (BALF) of cystic fibrosis patients if the patients are infected with *P. aeruginosa* (Hartl *et al.*, 2006). Wieland and collaborators have reported the comparative roles of *Pseudomonas* LPS, phospholipase C and exotoxin A in the induction of inflammatory cytokines in the lungs of mice (Wieland *et al.*, 2002). The data demonstrate that LPS and phospholipase C, but not exotoxin A, are strong inducers of TNF- α , IL-1 β and IL-6. In addition, the modulation of host inflammatory and immunological systems by bacterial autoinducers has been a recent topic of quorum-sensing research (Smith & Iglewski, 2003a). In particular, 3-oxo-C₁₂-HSL has been reported to possess a strong immunomodulatory activity, such as the inhibition of lymphocyte proliferation and TNF- α production (Chhabra *et al.*, 2003; Telford *et al.*, 1998). In this regard, we have reported that 3-oxo-C₁₂-HSL specifically induces apoptosis in certain cell types, including macrophages, a major cellular source of TNF- α (Tateda *et al.*, 2003). Indeed, the reduction of viability of P388D1, a macrophage cell line, is induced by 50 µM 3-oxo-C₁₂-HSL, and this effect is completely inhibited in the presence of immune serum. Although the roles and significance of apoptosis in lungs with *P. aeruginosa* infection may be complex, these data suggest that the elaboration of specific antibody against 3-oxo-C₁₂-HSL may have dual effects on TNF- α production in the lungs, probably through the suppression of the expression of bacterial

virulence factors and the modulation of host inflammatory responses.

The *P. aeruginosa* quorum-sensing system is a complex circuit, in which at least two cascades, 3-oxo- C_{12} -HSL-mediated Las and C_4 -HSL-mediated Rhl, are involved (Parsek & Greenberg, 2000; Tateda *et al.*, 2004; Waters & Bassler, 2005). The activation of the Las system mainly induces the production of elastase, while the Rhl system is associated with the induction of other sets of virulence factors, including pigments. These cascades are cross-regulated by a variety of genes and factors. Interestingly, we observed an increase of pigment production in culture supernatants of *P. aeruginosa* when the bacteria were grown in the presence of 3-oxo- C_{12} -HSL-blocking antibody (data not shown). Although we do not know a mechanism for the exaggerated production of pyocyanin in the setting of the blockade of the Las system, one possibility is that the blocking of the Las system cascade may change the balance of Las/Rhl, and that this results in exaggerated expression of the Rhl-mediated virulence factor pyocyanin (Diggle *et al.*, 2002). These data suggest a limitation of a vaccination strategy targeting 3-oxo- C_{12} -HSL, and further support a rationale for total blockage of the bacterial quorum-sensing system, in which at least the Las and Rhl systems should be included.

As is well known, *P. aeruginosa* is a major cause of pulmonary damage and mortality in patients with cystic fibrosis, diffuse panbronchiolitis, and other forms of bronchiectasis (Hoiby, 1994; Wilson & Dowling, 1998). In these lungs, the organism is found in microcolonies or biofilms, which are clusters of bacteria embedded in a matrix of polysaccharide (Lam *et al.*, 1980; Singh *et al.*, 2000). Although the concentration of autoinducers in the lungs of *P. aeruginosa*-infected patients remains unknown, C_4 - and 3-oxo- C_{12} -HSL have been detected in the sputum of these patients (Erickson *et al.*, 2002; Storey *et al.*, 1998). It has been shown that biofilms of *P. aeruginosa* grown *in vitro* can produce approximately 600 μ M 3-oxo- C_{12} -HSL, a concentration that is significantly higher than that earlier measured in planktonic cultures (Charlton *et al.*, 2000). These data demonstrate a role for the quorum-sensing system in diseases of *P. aeruginosa* involving biofilms, and further suggest a necessity to evaluate vaccines against autoinducer molecules in chronic infection models. These projects are ongoing in our laboratory using an agar-bead model of end-bronchial pulmonary infection with a mucoid-type *P. aeruginosa*.

In conclusion, newer vaccine approaches targeting bacterial autoinducer molecules offer additional means to produce an effective immunotherapeutic reagent for this problematic pathogen. Further investigations of the optimal structure, formula and combination of immunogens for maximum induction of protective efficacy, in addition to safety and possible adverse reactions, may be necessary to confirm and construct a new vaccine strategy against *P. aeruginosa* infections.

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