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# Induction of Interleukin-1 from Murine Peritoneal Macrophages by *Pseudomonas aeruginosa* Exotoxin A

MICHAEL L. MISFELDT,* PAUL K. LEGAARD, SAMUEL E. HOWELL, MILTON H. FORNELLA, AND ROY D. LEGRAND

*Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212*

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***Pseudomonas* exotoxin A, an ADP-ribosylating toxin produced by *Pseudomonas aeruginosa*, has been shown to stimulate the proliferation of murine thymocytes, which requires the participation of accessory cells. This requirement for accessory cells can be replaced by supernatant from adherent peritoneal exudate cells that have been stimulated with exotoxin A. Antibody to exotoxin A inhibits the induction of the thymocyte mitogenic activity from adherent peritoneal macrophages. However, antibody to exotoxin A had no effect on the thymocyte proliferation if the antibody was added to supernatant which contained thymocyte mitogenic activity. The thymocyte mitogenic activity was associated with a protein or protein complex with a molecular mass of >10,000 daltons. D10 bioassays indicated the presence of interleukin-1 (IL-1) in the supernatant. Antibody to IL-1 inhibited the ability of supernatant to induce thymocytes to proliferate. Therefore, these data suggest that *Pseudomonas* exotoxin A can stimulate the production of IL-1 from adherent peritoneal cells, which induces murine thymocytes to proliferate.**

A number of bacterial products, including bacterial toxins, have been shown to act as T-cell mitogens for mouse and human lymphocytes (1–6, 12–14, 17, 19, 22; M. L. Misfeldt, Clin. Immunol. Newsl. **9**:21–24, 1988). The ability of these molecules to induce lymphocyte proliferation is dependent on the presence of accessory cells (6, 12–14, 17, 19, 22). For staphylococcal enterotoxin B and toxic shock syndrome toxin 1, accessory cells are required for presentation of the molecule but not for processing (14, 17). In contrast, *Mycoplasma arthritidis* supernatant, a known T-cell mitogen, requires accessory cells for both processing and presentation in order to induce T-cell proliferation (3). Furthermore, a recent report indicates that cytokine(s) produced by the accessory cells may play a role in the *M. arthritidis* supernatant stimulation of T lymphocytes (2).

Our laboratory has been examining the immunomodulatory activity of a bacterial toxin, *Pseudomonas aeruginosa* exotoxin A (8–11, 16). *Pseudomonas* exotoxin A, an ADP-ribosylating toxin, has been shown by our laboratory to enhance the ability of athymic nude splenocytes to respond to thymus-dependent antigens (8, 15). In addition, we have observed that exotoxin A induces the lymphoproliferation of athymic nude splenocytes (11, 16). We observed that immunoglobulin-negative and $Ia^-$ cells in athymic splenocytes were induced to proliferate by *Pseudomonas* exotoxin A and that this proliferation could be enhanced by the addition of nude peritoneal exudate cells (PEC) (11). Furthermore, recent studies have shown that exotoxin A can induce the proliferation of murine thymocytes, which requires the participation of accessory cells (16).

In the present study, we have examined the role of accessory cells in the exotoxin A-induced murine thymocyte proliferation. Our results clearly indicate that exotoxin A induces interleukin-1 (IL-1) from adherent murine peritoneal macrophages.

* Corresponding author.

## MATERIALS AND METHODS

**Mice.** NFR/NUmm (*H-2$^q$*) mice were derived from brother and sister breeding pairs maintained in the animal care facilities at the University of Missouri-Columbia School of Medicine from breeding stock originally obtained from Carl Hansen, National Institutes of Health. Mice were maintained in autoclaved microisolator cages containing autoclaved bedding and were fed autoclaved mouse chow and acidified water ad libitum. Mice were serologically monitored for antibody titers against mouse pathogens by the University of Missouri-Columbia Research Animal Diagnostic and Investigative Laboratory.

**Reagents.** *P. aeruginosa* exotoxin A was purchased from the Swiss Serum and Vaccine Institute, Berne, Switzerland. Recombinant IL-1α (rIL-1α) and polyclonal rabbit anti-rIL-1α were generously provided by Hoffmann-La Roche Inc., Nutley, N.J. Antibody to exotoxin A was purchased from List Biological Laboratories, Inc., Campbell, Calif.

**Thymocytes.** Thymocytes were harvested from 6- to 9-week-old mice. Mice were euthanized, and an incision was made with scissors up the sternum, exposing the thoracic cavity. Thymuses were removed, and the cells were teased into single-cell suspensions with a cell selector (Bellco Glass, Inc., Vineland, N.J.). Cells were collected, washed, and suspended in RPMI 1640 medium supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, 10% fetal bovine serum, 2-mercaptoethanol, and gentamicin (complete medium).

**PEC.** PEC were harvested from each mouse by injecting 7 to 10 ml of RPMI 1640 medium into the peritoneal cavity, massaging the abdomen, and removing the fluid.

**Production of supernatant from PEC.** PEC were collected, erythrocytes were lysed, and the remaining cells were washed and suspended in RPMI 1640 medium. Cells were seeded into 48-well cluster plates (Costar, Cambridge, Mass.) at a concentration of $5 \times 10^5$ cells per well. Cells stimulated with or without 50 ng of exotoxin A were incubated for up to 48 h, and supernatants were collected. Additional wells were incubated overnight to allow the cells

to adhere to plastic. The nonadherent cells were removed with a pipette and placed into separate wells. The wells containing the adherent PEC were washed with medium, the medium was removed, and fresh complete medium was added. Exotoxin A at a concentration of 50 ng per well was added to half of the wells, and the plates were incubated for up to an additional 48 h, at which time supernatants were collected and frozen at −70°C for later analysis.

**Proliferative response cultures.** Thymocytes were seeded at a density of $5 \times 10^6$ cells in a 48-well cluster plate in the presence or absence of $5 \times 10^5$ PEC or 100 μl of PEC supernatant per well. The volume of medium was kept constant at 600 μl per well. The cells were incubated at 37°C in 5% $CO_2$ and 100% relative humidity for 60 h, at which time 1 μCi of [$H^3$]thymidine ([$^3$H]TdR) per well was added. The cells were collected with a cell harvestor (Skatron, Inc., Sterling, Va.) after an additional incubation period of 12 h, and [$^3$H]TdR uptake was determined by dissolving the glass fiber disks in ScintiVerse II (Fisher Scientific Co., Pittsburgh, Pa.) and counting in a 1900 CA liquid scintillation analyzer (Packard Instrument Co., Inc., Chicago, Ill.). Each treatment was performed in triplicate, and each experiment was performed multiple times.

**D10 assay.** D10.G4.1, a mouse helper T-cell clone (15), was stimulated to proliferate with mitomycin C-treated spleen cells from AKR/J mice along with 100 μg of conalbumin per ml. The cells were cultured for at least 10 days before use in an assay. The cells were maintained in Click's medium containing 10% fetal calf serum, L-glutamine, sodium pyruvate, nonessential amino acids, 50 mM 2-mercaptoethanol, antibiotics, and 10% rat concanavalin A (ConA) supernatant containing 20 mg of α-methyl-D-mannoside per ml. The D10 cells were incubated in the medium described above minus ConA supernatant overnight to ensure low IL-2 receptor expression and a resting state. Cells were washed extensively in Click's medium, and the cell concentration was adjusted to $2 \times 10^5$ cells per ml. A 100-μl sample of cells in Click's medium containing 2.5 μg of ConA per ml and 100 μl of supernatants were added to wells of a 96-well cluster plate. Plates were incubated in a $CO_2$ incubator and pulsed with [$^3$H]TdR for the last 12 to 16 h of the 65-h assay. The cells were collected with a Skatron cell harvestor, and the amount of [$^3$H]TdR uptake was determined.

## RESULTS

**Requirement for accessory cells for toxin-induced thymocyte proliferation.** We had previously observed that *Pseudomonas* exotoxin A could induce lymphoproliferation which was dependent on accessory cells (10, 16). Since exotoxin A induced the proliferation of a non-B-cell population in athymic nude splenocytes (10), we examined exotoxin A for its ability to induce murine thymocytes to proliferate (Fig. 1). Exotoxin A was unable to stimulate murine thymocytes or PEC to proliferate. However, when PEC were added to murine thymocytes, exotoxin A was able to stimulate a 20-fold increase in [$^3$H]TdR uptake, suggesting that both PEC and thymocytes were required for the toxin-induced proliferation. Treatment of PEC with mitomycin C had no effect on the exotoxin A-induced murine thymocyte proliferation (unpublished observations). These results suggest that PEC division was not required for the toxin-induced proliferation and that the thymocytes were the proliferating cells, possibly in response to monokines produced by the PEC.

**Replacement of PEC with supernatant from toxin-stimulated PEC.** To determine whether cytokines were involved in the toxin-induced thymocyte proliferation, PEC were stimulated with exotoxin A, and the resulting 24-h supernatant was added directly to the thymocytes. Supernatant from PEC stimulated with exotoxin A could induce murine thymocytes to proliferate (Fig. 2). In addition, PEC allowed to attach to plastic tissue cultures dishes overnight were able to produce a monokine(s) that could induce murine thymocytes to proliferate when the adherent cells were stimulated with exotoxin A. In contrast, supernatant from nonadherent PEC stimulated with exotoxin A failed to induce thymocytes to proliferate. These results indicate that adherent PEC, probably macrophages, are producing the thymocyte mitogenic activity.

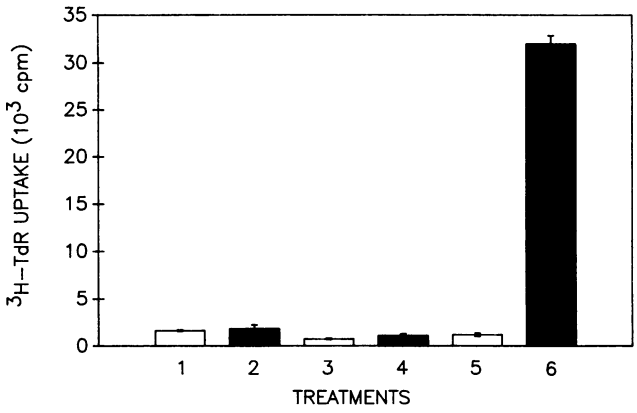


FIG. 1. Requirement for accessory cells in exotoxin A-induced proliferation of murine thymocytes. Each bar represents the mean counts per minute ± standard error of the mean of three cultures per group. Cell treatments: 1 and 2, PEC; 3 and 4, thymocytes; 5 and 6, PEC plus thymocytes. Symbols: □, medium; ■, 50 ng of exotoxin A.

**Antibody to exotoxin A fails to inhibit thymocyte mitogenic activity in supernatant.** In order to rule out the possibility that residual exotoxin A was stimulating the murine thymocytes to proliferate, we performed the experiment whose

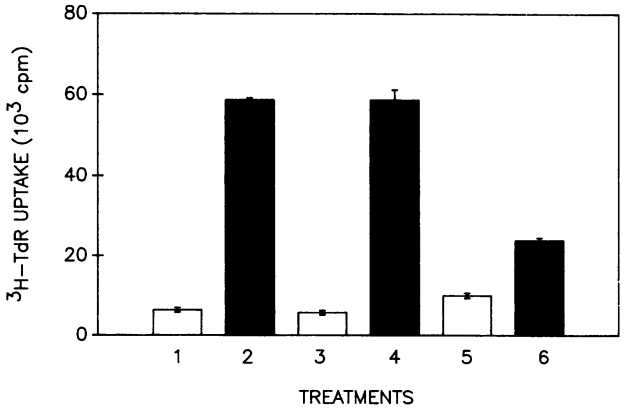


FIG. 2. Supernatant from toxin-stimulated PEC can induce thymocyte proliferation. Each bar represents the mean counts per minute ± standard error of the mean of three cultures per group. Cell treatments: 1 and 2, supernatant from PEC added to thymocytes; 3 and 4, supernatant from adherent PEC added to thymocytes; 5 and 6, supernatant from nonadherent PEC added to thymocytes. Symbols: □, PEC stimulated with medium; ■, PEC stimulated with 50 ng of exotoxin A.

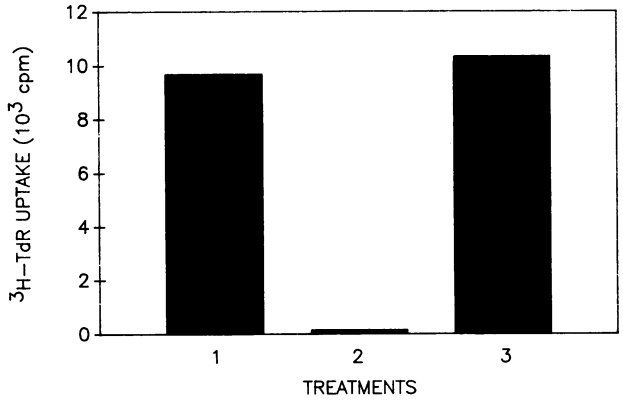


FIG. 3. Effect of goat anti-exotoxin A antibody on thymocyte mitogenic activity. Each bar represents the change in counts per minute of cultures stimulated with exotoxin A minus the counts per minute for unstimulated cultures. Cell treatments: 1, untreated supernatant; 2, supernatant from cultures to which antibody was added prior to exotoxin A stimultaion of PEC; 3, supernatant from cultures in which antibody was added to supernatant from PEC stimulated with exotoxin A.

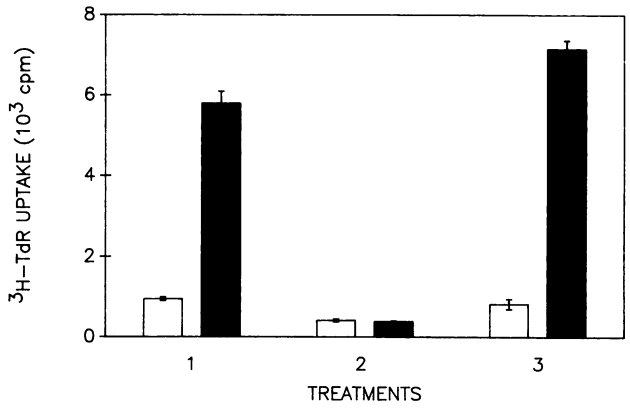


FIG. 4. Molecular mass characterization of thymocyte mitogenic activity. Each bar represents the mean counts per minute ± standard error of the mean of three cultures per group. Cell treatments: 1, unfractionated sypernatant; 2, <10-kilodalton protein; 3, >10-kilodalton protein. Symbols: □, PEC stimulated with medium; ■, PEC stimulated with 50 ng of exotoxin A.

results are shown in Fig. 3. PEC were stimulated with exotoxin A in the presence or absence of a goat antibody reactive with exotoxin A. The supernatants from these cells were collected at 24 h and examined for the ability to induce thymocytes to proliferate. Furthermore, antibody to exotoxin A was incubated with a sample of the supernatant from toxin-stimulated PEC prior to its addition to thymocytes. Antibody to exotoxin A abrogated the ability of toxin to stimulate the production of a thymocyte mitogenic factor from the PEC (Fig. 3). In contrast, supernatant from PEC stimulated by toxin could induce thymocytes to proliferate even in the presence of antibody to the toxin. These results indicate that the thymocyte mitogenic activity associated with supernatant from PEC stimulated with exotoxin A is not due to residual exotoxin A in the supernatant.

**Characterization of supernatant activity.** The supernatant activity was found to be trypsin sensitive (unpublished observations), indicating that the active moiety in the supernatant was protein in nature. In order to determine an approximate molecular mass for this active protein moiety, we centrifuged supernatants through Centricon 10 microconcentrators (Amicon Corp., Danvers, Mass.). The effluents and eluates were assayed for thymocyte mitogenic activity (Fig. 4). The only thymocyte mitogenic activity observed was found associated with a protein(s) that had a molecular mass greater than 10 kilodaltons. These results indicate that exotoxin A induces a >10,000-dalton molecule(s) which contains thymocyte mitogenic activity.

**Supernatant from PEC stimulated by toxin supports D10.G4.1 growth.** Since the active fraction was associated with a molecule(s) of >10,000 daltons, we examined supernatant from toxin-stimulated PEC for the presence of IL-1. Supernatant was assayed for IL-1 activity on the D10.G4.1 cell line, a cloned helper T-cell line which requires IL-1 for growth (Fig. 5). D10.G4.1 cells incubated with ConA and no IL-1 showed little proliferation. When IL-1 was added to wells containing D10.G4.1 and ConA, we observed an increase in proliferation of approximately 24-fold. Supernatants from PEC which were incubated with medium showed some ability to induce D10.G4.1 cells to proliferate. However, when supernatants from PEC stimulated by exotoxin A or supernatants from adherent PEC stimulated by exotoxin A were added to D10.G4.1 cells, we observed a 12-fold increase over proliferation with medium supernatants. Furthermore, antibody to IL-1 inhibited the ability of rIL-1α and the supernatant from adherent PEC stimulated by exotoxin A to induce D10.G4.1 cells to proliferate. Thus, these results suggest that IL-1 is present in the supernatant of adherent PEC stimulated with exotoxin A.

**Antibody to IL-1 inhibits supernatant activity.** The D10 bioassay indicated that IL-1 was present in supernatant from PEC or adherent PEC that had been stimulated with exotoxin A. Therefore, in order to determine whether IL-1 was responsible for the thymocyte mitogenic activity associated with the supernatant, we incubated supernatant with a polyclonal rabbit antibody to rIL-1α prior to addition of the supernatant to thymocytes. The antibody to rIL-1α inhibited the ability of the supernatant from exotoxin A-stimulated

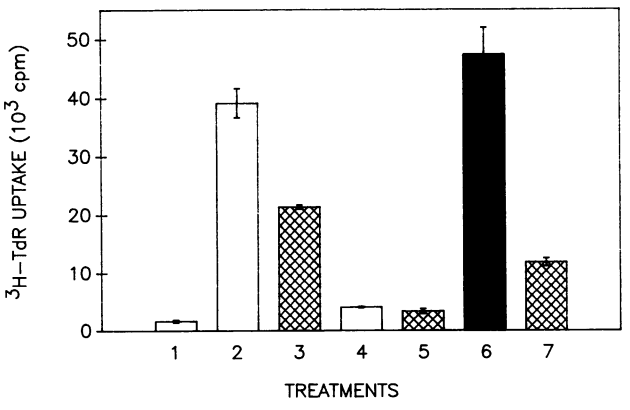


FIG. 5. D10.G4.1 bioassay. Each bar represents the mean counts per minute ± standard error of the mean of three cultures per group. D10.G4.1 cell treatments: 1, 2.5 μg of ConA per ml; 2 and 3, 2.5 μg of ConA per ml plus 0.5 U of rIL-1α; 4 and 5, ConA plus 1:10 dilution of supernatant from PEC stimulated with medium; 6 and 7, ConA plus 1:10 dilution of supernatant from PEC stimulated with 50 ng of exotoxin A. Symbols: ■, supernatant from PEC stimulated with 50 ng of exotoxin A; ▩, antibody to IL-1 added to supernatant prior to addition of supernatant to D10 cells; □, supernatant from PEC stimulated with medium.

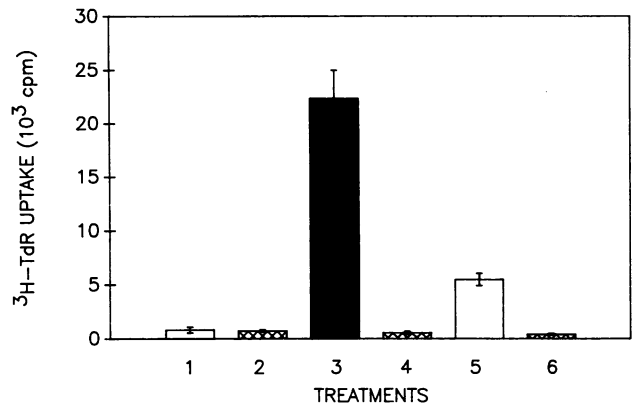


FIG. 6. Antibody to IL-1 inhibits supernatant activity. Each bar represents the mean counts per minute ± standard error of the mean of three cultures per group. Cell treatments: 1 and 2, supernatant from PEC stimulated with medium added to thymocytes; 3 and 4, supernatant from PEC stimulated with 50 ng of exotoxin A added to thymocytes; 5 and 6, 1 U of rIL-1α added to thymocytes. Symbols: ■, PEC stimulated with 50 ng of exotoxin A; ▩, rabbit antibody added to rIL-1α; □, PEC stimulated with medium.

PEC to induce thymocyte proliferation (Fig. 6). These results suggest that IL-1 plays a role in exotoxin A-induced murine thymocyte proliferation.

## DISCUSSION

The data presented here show that *Pseudomonas* exotoxin A, an ADP-ribosylating toxin produced by *P. aeruginosa*, can induce murine thymocytes to proliferate and that the thymocyte proliferation requires the presence of accessory cells or an accessory cell signal(s) (Fig. 1 and 2). Accessory cells are required for T-cell mitogenesis induced by lectins, such as ConA or phytohemagglutinin, and bacterial products, such as staphylococcal enterotoxins and *M. arthritidis* supernatant (20, 21).

In this report, we describe the ability of *Pseudomonas* exotoxin A to stimulate the production of a cytokine(s) from adherent peritoneal macrophages which can induce murine thymocytes to proliferate (Fig. 2). Nonadherent PEC stimulated by toxin produced very little thymocyte mitogenic activity. Furthermore, when the nonadherent PEC were subjected to additional adherence to plastic, the remaining thymocyte mitogenic activity was eliminated (unpublished observations). Since exotoxin A could be found in the supernatant, we ruled out the possibility that intact residual toxin was directly stimulating the thymocytes by incubating the supernatants with a goat antibody reactive with exotoxin A. The thymocyte mitogenic activity associated with the supernatant was shown not to be dependent on residual exotoxin A, as the antibody failed to inhibit the ability of the supernatant to induce thymocyte proliferation (Fig. 3). In contrast, if the antibody was added prior to toxin addition to PEC, no thymocyte mitogenic activity was observed. These results indicate that exotoxin A must interact with PEC in order for PEC to produce the mitogenic activity. Recently we have shown that exotoxin A requires processing by PEC in order to stimulate the production of mitogenic activity (P. K. Legaard and M. L. Misfeldt, submitted for publication). Finally, we have shown that thymocyte mitogenic activity is associated with a protein molecule(s) of >10,000 kilodaltons. Thymocyte mitogenic activity was shown to require IL-1, since IL-1 was present in the supernatant from toxin-stimulated PEC as determined by the D10 bioassay and since antibody to IL-1 inhibited the thymocyte mitogenic activity of the supernatant (Fig. 5 and 6). Therefore, IL-1 produced by PEC stimulated with exotoxin A could induce murine thymocytes to proliferate. The IL-1 could be functioning as the sole signal for thymocyte proliferation, or it may induce other cytokines, such as IL-6, which could participate in the thymocyte proliferation (8). Another possibility is that IL-1 functions as a cosignal with some as-yet-unidentified molecule. Experiments to delineate these possibilities are currently being studied in the laboratory.

A number of bacterial products have been shown to act as T-lymphocyte mitogens, including staphylococcal toxins and *M. arthritidis* supernatant (2–6, 12–14, 17, 19, 22). Both staphylococcal toxins and *M. arthritidis* supernatant may mimic the murine T-cell proliferative responses to Mls loci differences (12, 13, 17) because they stimulate a significant percentage of T cells to proliferate at concentrations several magnitudes lower than conventional T-cell mitogens such as phytohemagglutinin or ConA do (13). Therefore, both staphylococcal toxins and *M. arthritidis* supernatant have been termed superantigens, since they stimulate a large population of T lymphocytes to proliferate and they require major histocompatibility complex class II antigen-presenting cells to induce T-cell proliferation (2, 3, 6, 12–14, 17, 22). However, these bacterial superantigens may require different functions from accessory cells. The staphylococcal toxins require accessory cells for presentation of the toxin (6, 14, 17, 19). In contrast, *M. arthritidis* supernatant requires accessory cells for processing and presentation an accessory cell-derived cytokine(s) (2, 3). Therefore, the experimental results described in this study suggest that *Pseudomonas* exotoxin A may require accessory cells in a manner similar to that observed for *M. arthritidis* supernatant. Thus, both *Pseudomonas* exotoxin A and *M. arthritidis* supernatant may be representative of a subset of superantigens which can induce T-lymphocyte proliferation and which requires accessory cells or an accessory cell signal(s) such as IL-1. Finally, this report shows that *Pseudomonas* exotoxin A induces IL-1, which may play a role in the pathogenesis of *P. aeruginosa*.

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