

Original Article

The Roles of the Quorum-Sensing System in the Release of Extracellular DNA, Lipopolysaccharide, and Membrane Vesicles from *Pseudomonas aeruginosa*

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SUMMARY: Biofilms play an important role in the establishment of chronic infection caused by *Pseudomonas aeruginosa*. It has been suggested that membrane vesicles (MVs) are released into the surrounding medium during normal growth and might supply the bacterial extracellular DNA that is required for early biofilm formation, as MVs released from the bacterial outer membrane are suspected to be the source of extracellular DNA. MVs possess lipopolysaccharide (LPS), extracellular DNA, and several hydrolytic enzymes. It is well known that the quorum-sensing (QS) system is important in controlling virulence factors in *P. aeruginosa* and biofilm formation. In the current study, we investigated extracellular LPS and DNA in the supernatants of culture solutions from PAO1, the wild-type *P. aeruginosa*, and those of QS mutants. As compared to that of *las* QS mutants, the amount of LPS and DNA released was significantly higher in PAO1 and in *las* QS mutants complemented with *N*-(3-oxododecanoyl) homoserine lactone. Our study indicated that the QS is among the regulators involved in the release of extracellular DNA and LPS. It is possible that these extracellular components are supplied from MVs. Investigation of the mechanism of biofilm formation is of particular interest, as it may be useful for designing treatments for severe *P. aeruginosa* infection.

INTRODUCTION

Pseudomonas aeruginosa is an important bacterial pathogen in patients with severe burn injury or chronic pulmonary diseases such as cystic fibrosis (CF) and diffuse panbronchiolitis (DPB) (1). It is well documented that *P. aeruginosa* quorum-sensing (QS) signal molecules, which are referred to as autoinducers, play an important role in the differentiation process (2). The QS system is a well known and important system that controls virulence factors in *P. aeruginosa* (3). *P. aeruginosa* has two QS systems, *las* and *rhl*. In the *las* system, the autoinducer synthase, LasI, directs the synthesis of *N*-(3-oxododecanoyl) homoserine lactone (C₁₂-HSL), which then triggers the transcriptional activator, LasR. The *las* system also autoregulates *lasI*, which leads to the production of more C₁₂-HSL. Similarly, the *rhl* system is comprised of the transcriptional activator RhlR and the RhlI autoinducer synthase, which synthesizes *N*-butyryl homoserine lactone (C₄-HSL). The *rhl* system also autoregulates *RhlI*, which leads to the production of additional C₄-HSL. Pearson et al. examined the pathogenicity of *P. aeruginosa* and reported that rhamnolipid production and elastolysis were reduced in both the $\Delta lasI$ single and $\Delta lasI \Delta RhlI$ double mutants (4). In addition, defects in the production of elastase, LasA protease, rhamnolipid, and pyocyanin have been noted in the *rhlI*

mutant (5). Biofilm formation, on the other hand, has been reported to be controlled by the QS and plays an important role in the establishment of chronic infection (2). However, the mechanism of biofilm formation and its correlation with the QS remains unclear. During normal growth, *P. aeruginosa* blebs off membrane vesicles (MVs) into the culture medium (6,7). Natural MVs are known to possess lipopolysaccharide (LPS), extracellular DNA, and several hydrolytic enzymes, such as protease, phospholipase C (PLC), and peptidoglycan hydrolase (8,9). MVs play a predatory role in the natural ecosystem, as the parent bacteria release them in order to lyse surrounding cells, including both Gram-positive and Gram-negative bacteria. Via this predatory process, MVs help to increase the amount of nutrients available to the parent strain (10). It has been suggested that MVs possibly supply bacterial DNA, which is required for early biofilm formation (11). In the current report, we demonstrate the release of MVs by *P. aeruginosa* during the early stationary phase, and we also examine the relationship between QS and MV production.

MATERIALS AND METHODS

Bacterial strains and growth conditions: The wild-type *P. aeruginosa*, PAO1, and its isogenic QS mutants, PAO-JP2 (*lasI*-/*rhlI*-), PAO-JP1 (*lasI*-), and PDO100 (*rhlI*-), were kindly provided by Professor Iglewski (University of Rochester School of Medicine and Dentistry, Rochester, N.Y., USA) for use in this study. It has been previously demonstrated that PAO-JP1 and PAO-JP2 are deficient in the production of C₁₂-HSL (4). PAO-JP2 and PDO100 are deficient in

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the production of C₄-HSL (5). All bacterial strains were grown in trypticase soy broth (TSB) to the early stationary phase at 37°C, with shaking at 250 rpm. The broth supernatants were collected every 2 h. We added either 10 μM of C₁₂-HSL or 5 μM of C₄-HSL prior to incubation, when mutants co-cultured with autoinducers.

Supernatants preparation: To investigate the amount of LPS and DNA in the supernatant, bacterial strains with or without autoinducers were cultured in 50 ml of TSB for 0, 2, and 4 h (early stationary growth phase, OD₆₆₀ = 1.1) at 37°C with shaking at 250 rpm. Cells were removed from the suspension by centrifugation at 1,500 × g for 10 min, and then were filtered through a membrane (pore size, 0.22 μm). To isolate and quantify the MVs, PAO1, PAO-JP1, PDO100, and PAO-JP2 were cultured in 50 ml of TSB for 4 h (early stationary growth phase, OD₆₆₀ = 1.1) at 37°C with shaking at 250 rpm. Cells were removed from the suspension by centrifugation at 750 × g for 5 min, and were filtered through a 0.22-μm pore membrane in order to remove the residual cells.

LPS analysis: The LPS content in the culture supernatant samples was determined by thiobarbituric acid assay (12). A standard curve was prepared using purified LPS obtained from *P. aeruginosa* serotype 10 (Sigma, Tokyo, Japan). The LPS analysis was performed on 25-μl supernatant samples. Samples were placed in screw-cap tubes, after which 25 μl of 0.5 N H₂SO₄ was added. The mixture was hydrolyzed at 100°C for 15 min and cooled to room temperature. The mixture was further acidified by the addition of 25 μl of H₅IO₆, and the samples were incubated for 10 min at room temperature. Sodium arsenite (2% in 0.5 N HCl) was added to the mixtures, followed by the addition of 400 μl of thiobarbituric acid (pH 2). The samples were then placed in a 100°C heating block for 10 min, and were then cooled to room temperature, after which, 0.75 μl of butanol (5% in concentrated HCl) was added. The tubes were centrifuged at 200 × g for 5 min, with the absorbance of each sample measured at 548 nm.

Western blotting: Subsequently, 0.9 ml of the supernatant samples with 0.1 ml of 100% trichloroacetate were then added to a screw-cap tube, and the mixture was kept at room temperature for 1 h, followed by centrifugation at 6,500 × g for 3 min. Finally, 500 μl of acetone was added to samples in order to wash out the tube. All tubes prepared in the manner were then air-dried at room temperature, and the protein pellets were separated out by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (3–8% Tris-Acetate; Invitrogen, Tokyo, Japan) prior to transfer of the samples to nitrocellulose. The samples were then reacted with a monoclonal antibody (mAb) to *P. aeruginosa* serotype 5 LPS (Rougier Bio-Tech, Montreal, Canada), with detection performed using an HRP-conjugated anti-mouse antibody (Sigma).

DNA analysis: To the supernatant samples (5 ml), 2M Tris-HCl (pH 7.5), 500 mM EDTA (pH 8.0), and 10% SDS (final concentrations: 10 mM, 5 mM, and 0.1%, respectively) were added, and then the samples were mixed with 2.5 ml of chloroform and 2.5 ml buffer-saturated phenol, followed by vortexing. Mixtures were centrifuged at 6,500 × g for 10 min, and 3 ml of each supernatant was collected, followed by the addition of 1/10 volume of 3 M sodium acetate (pH 7.4) and 2 volumes of 99% ethanol in order to precipitate the DNA. The mixtures were kept at –20°C for 30 min, and then were centrifuged at 6,500 × g for 10 min. Next, 70% ethanol was

added to the pellets, followed by centrifugation at 6,500 × g for 10 min. The pellets were air-dried at room temperature, with quantification of the DNA performed using a previously described assay (9). A standard curve was prepared using Salmon Testes DNA (Sigma). To each purified DNA sample, 3 ml of fluorescent dye (100 μg/ml, bisbenzimidazole, H33258) was added. The fluorescence was measured in a RF-5300 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan) with excitation and emission wavelengths set at 350 and 455 nm, respectively (slit width, 10 nm).

Isolation and quantification of MVs: MVs were recovered from supernatants filtrated through a membrane with a pore size of 0.22 μm using ultracentrifugation at 75,000 × g for 4.5 h at 4°C in a CP-56G rotor (Hitachi, Tokyo, Japan). We observed the vesicle pellets deposited at the bottom of the centrifuge tubes by transmission electron microscopy (TEM) (JEM-1200EX; JEOL, Tokyo, Japan).

RESULTS

LPS analysis by thiobarbituric acid assay: The amount of LPS released from these strains into the culture supernatant during growth was measured in order to determine the effect of the QS on the release of LPS into the supernatant. As shown in Table 1, there was a significant difference observed between PAO1 and *las* QS mutants (PAO-JP1 and PAO-JP2) with regard to the amount of LPS in the supernatant, especially in the early stationary phase ($P < 0.001$). Additionally, as compared to naïve PAO-JP2, the LPS release was significantly higher for both PAO-JP1 and PAO-JP2 exposed to C₁₂-HSL (Fig. 1).

SDS-PAGE analysis of supernatants: SDS-PAGE analysis of the supernatants from *P. aeruginosa* clearly demonstrated the presence of LPS in the PAO1 samples during

Table 1. The time dependent increase of extracellular LPS analyzed by thiobarbituric acid assay

Strain	Extracellular LPS (μg/ml) (mean ± SD)		
	0 h	2 h	4 h
PAO1	1.21 ± 0.82	12.75 ± 1.65	60.23 ± 4.23
PAO-JP1	1.18 ± 0.21	10.20 ± 1.23	22.22 ± 2.43
PDO100	1.12 ± 2.72	11.53 ± 2.01	48.92 ± 4.32
PAO-JP2	1.11 ± 3.25	9.92 ± 1.62	19.18 ± 2.45

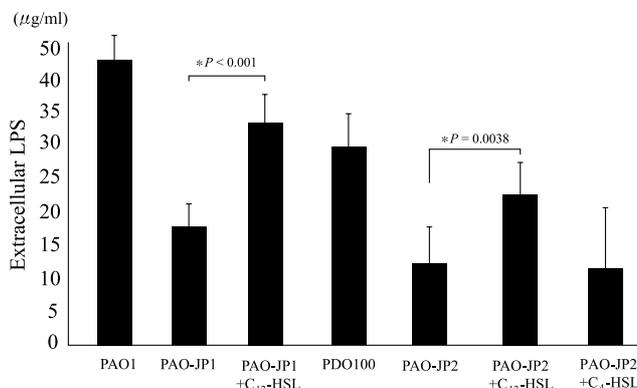


Fig. 1. The amounts of LPS into the supernatants in the early stationary phase with *P. aeruginosa* stains co-cultured with or without autoinducers. The amounts of extracellular LPS were induced with C₁₂-HSL in PAO-JP1 and PAO-JP2, whereas no induction was noted for the PAO-JP2 cultures exposed to C₄-HSL.

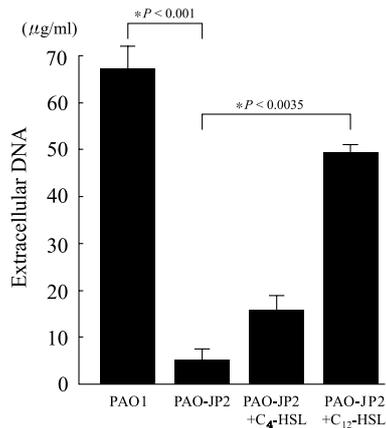


Fig. 2. The amounts of extracellular DNA in the early stationary phase with *P. aeruginosa* strains co-cultured with or without autoinducers. The amounts of extracellular DNA were induced with C₁₂-HSL in PAO-JP2, whereas no induction was noted for the PAO-JP2 co-cultured with C₄-HSL.

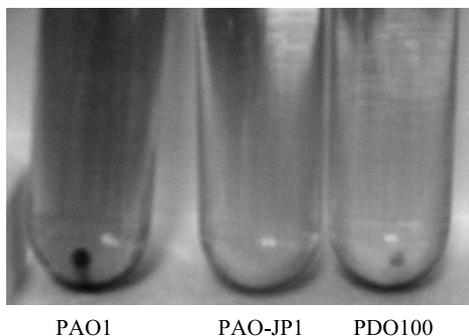


Fig. 3. Precipitates after ultracentrifugation with the *P. aeruginosa* culture supernatants. After ultracentrifugation, pellets were observed in the PAO1 and PDO100.

the early stationary phase. Western blot analysis showed no reaction for PAO-JP2 when it was exposed to a mAb against *P. aeruginosa* serotype O5 (data not shown). However, a reaction to the mAb was detected in the PAO-JP2 samples cultured with C₁₂-HSL. This band was not detected when C₄-HSL was used instead of C₁₂-HSL. These results indicate that when the *las* QS is activated, higher levels of extracellular LPS are observed.

Fluorometric quantification of DNA in the supernatants: Fluorometric quantification of DNA was carried out in order to determine and compare the amounts of extracellular DNA released from two *P. aeruginosa* strains into the supernatants. As shown in Fig. 2, the amount of DNA released into the culture medium was higher for PAO1 cultures during the early stationary phase than for cultures of PAO-JP2. Furthermore, the amount of DNA in the supernatants increased when PAO-JP2 was co-cultured with C₁₂-HSL. Thus, it appears that the QS, and in particular the *las* QS, might control extracellular DNA levels.

Transmission electron microscopy (TEM): After ultracentrifugation of the membrane-filtrated culture supernatants, pellets were observed from the PAO1 and the PDO100 cultures, but not from the PAO-JP1 or PAO-JP2 cultures (data not shown) (Fig. 3). After careful aspiration, the pellets were examined by TEM. As seen in Fig. 4, many bilayer membrane vesicles with diameters between 50 and 150 nm were observed, which indicates that the *Pseudomonas* QS controlled the release of MVs into the culture medium.

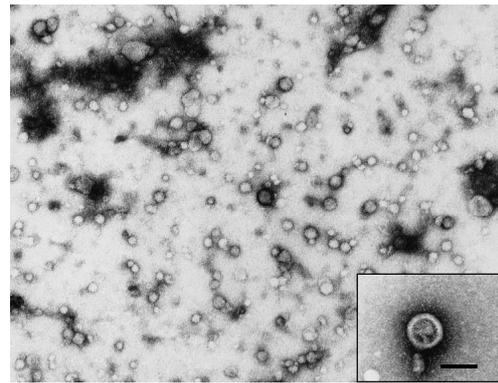


Fig. 4. Electron micrograph of the precipitate after ultracentrifugation with the PAO1 culture supernatants. Many membrane vesicles were observed. Bar = 100 nm.

DISCUSSION

During normal growth, *P. aeruginosa* releases MVs into the culture medium (6,7). These MVs naturally possess several important virulence factors. In addition, the MVs of *P. aeruginosa* have been found to be enriched with LPS from the cell surface (13). It has also been demonstrated that discrete enzymes such as alkaline phosphatase, PLC, and protease, which are important components in the pathogenesis of *P. aeruginosa* infection, are packaged into MVs (8,9). On the other hand, it has been previously reported that there is an association between extracellular DNA and biofilm formation, which plays an important role in the establishment of chronic infection caused by *P. aeruginosa* (11,14-16). Biofilms are structured communities of cells that are enclosed within a self-produced hydrated polymeric matrix that can adhere to an inert or living surface (17). Biofilms of mixed bacterial communities such as *P. aeruginosa* are able to form thick layers that consist of differentiated mushroom structures (3). A previous report has suggested that since MVs released from the bacterial outer membrane are the suspected source of extracellular DNA, such MVs might supply the bacterial extracellular DNA required for early biofilm formation (9,18). The presence of DNA in membrane blebs has been reported in a few Gram-negative bacteria, including *P. aeruginosa*. Additionally, it has been reported that biofilm formation is controlled by the QS (14,19) and that the *las* QS controls the early stages of static biofilm formation, i.e., periods in which cells adhere to surfaces and form microcolonies (20). QS systems are critical regulators of the expression of virulence factors in various organisms. The two major QS components are *las* and *rhl*, which are regulated by their corresponding autoinducers, C₁₂-HSL and C₄-HSL, respectively (3). In addition, it has been reported that 2-heptyl-3-hydroxy-4-quinoline (Pseudomonas quinolone signal [PQS]) is part of an additional QS that has been identified in *P. aeruginosa*, and that *las* QS is required for the production of PQS (21). Mashburn and Whiteley reported that PQS is responsible for mediating the packaging of autoinducers into membrane vesicles, which are used to traffic the molecule to neighboring cells (22). In order to identify the relationship between the QS and the release of extracellular DNA and LPS, we measured the amounts of DNA and LPS in the supernatants of the culture media from two *P. aeruginosa* strains, PAO1 (wild-type) and PAO-JP2 (QS mutant). To enhance the role of the QS, we added C₁₂-HSL and C₄-HSL to the cultures. To estimate the amount of LPS present and to

investigate the possible role of the QS in the promotion of extracellular LPS, we performed a thiobarbituric acid assay and Western blot analysis (Table 1, Fig. 1). There were obvious reactions in the case of PAO1, but no reactions to the *P. aeruginosa* antibody were noted in the Western blot analysis of the PAO-JP2 culture medium supernatants (data not shown). As expected, when they had been incubated with C₁₂-HSL, the supernatants of the PAO-JP2 cultures reacted with this antibody, although less strongly than was observed in the case of the PAO1 culture media supernatants. This finding is suggestive of a relationship between the amount of extracellular LPS and the *las* QS. Our results also indicated that the pattern depicted in Table 1 and Fig. 1 possessed a trend seen in the extracellular DNA quantification experiment (Fig. 2). In contrast, Kievit et al. used a whole-cell lysis method to determine the expression of *P. aeruginosa* during the early stages of static biofilm formation (observations were obtained after 18 h of incubation) and reported that the QS does not appear to regulate LPS production (20). However, our data indicate that the release of extracellular LPS in the supernatant was controlled by QS at an early stage of growth of *P. aeruginosa* (4 h). Additionally, as shown Figs. 3 and 4, the results also suggested that the *las* QS controls the release of MVs into the culture medium. Mashburn and Whiteley recently reported that PQS is required and sufficient for the formation of MVs. These data, taken together, indicate that the *las* QS mainly regulates MV production by PQS activation (22).

In conclusion, in Gram-negative biofilms such as those formed by *P. aeruginosa*, it remains unclear what is responsible for prompting the release of extracellular DNA. Our data suggest that during the early stationary phase of *P. aeruginosa*, the *las* QS plays an important role in the production of extracellular DNA and LPS. The source of these extracellular components might be MVs, as the *las* QS possesses the capacity to control the amount of MVs released. Further investigation of the mechanism of biofilm formation will be necessary and of particular interest, as the results may be useful in the design of new approaches to treating severe *P. aeruginosa* infection.

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REFERENCES

- Hurley, J.C. (1992): Antibiotic-induced release of endotoxin: a reappraisal. *Clin. Infect. Dis.*, 15, 840-854.
- Davies, D.G., Parsek, M.R., Pearson, J.P., et al. (1998): The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, 280, 295-298.
- Passador, L., Cook, J.M., Gambello, M.J., et al. (1993): Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science*, 260, 1127-1130.
- Pearson, J.P., Pesci, E.C. and Iglewski, B.H. (1997): Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in the control of elastase and rhamnolipid biosynthesis genes. *J. Bacteriol.*, 179, 5756-5767.
- Brint, J.M. and Ohman, D.E. (1995): Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.*, 177, 7155-7163.
- Dorward, D.W., Garon, C.F. and Judd, C.F. (1989): Export and intercellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. *J. Bacteriol.*, 171, 2499-2505.
- Kadurugamuwa, J.L. and Beveridge, T.J. (1997): Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. *J. Antimicrob. Chemother.*, 40, 615-621.
- Kadurugamuwa, J.L. and Beveridge, T.J. (1996): Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. *J. Bacteriol.*, 178, 2767-2774.
- Kadurugamuwa, J.L. and Beveridge, T.J. (1995): Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J. Bacteriol.*, 177, 3998-4008.
- Li, Z., Clarke, A.J. and Beveridge, T.J. (1998): Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. *J. Bacteriol.*, 180, 5478-5483.
- Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., et al. (2002): Extracellular DNA required for bacterial biofilm formation. *Science*, 295, 1487.
- Osborn, M.J., Gander, J.E., Parisi, E., et al. (1972): Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J. Biol. Chem.*, 247, 3962-3972.
- Beveridge, T.J. (1981): Ultrastructure, chemistry, and function of the bacterial wall. *Int. Rev. Cytol.*, 72, 229-317.
- Allesen-Holm, M., Barken, K.B., Yang, L., et al. (2006): A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol. Microbiol.*, 59, 1114-1128.
- Spoering, A.L. and Gilmore, M.S. (2006): Quorum sensing and DNA release in bacterial biofilms. *Curr. Opin. Microbiol.*, 9, 133-137.
- Yang, L., Barken, K.B., Skindersoe, M.E., et al. (2007): Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology*, 153, 1318-1328.
- Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999): Bacterial biofilms: a common cause of persistent infections. *Science*, 284, 1318-1322.
- Muto, Y. and Goto, S. (1986): Transformation by extracellular DNA produced by *Pseudomonas aeruginosa*. *Microbiol. Immunol.*, 30, 621-628.
- Sakiragi, Y. and Kolter, R. (2007): Quorum-sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *J. Bacteriol.*, 189, 5383-5386.
- De Kievit, T.R., Gillis, R., Marx, S., et al. (2001): Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Appl. Environ. Microbiol.*, 67, 1865-1873.
- Pesci, E.C., Milbank, J.B., Pearson, J.P., et al. (1999): Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA*, 96, 11229-11234.
- Mashburn, L.M. and Whiteley, M. (2005): Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature*, 435, 422-425.