Abstract:
The present invention provides compositions and methods for reducing and/or inhibiting biofilm formation using a pseudan inclusion body.

Title: USE OF PSEUDAN AND PSEUDAN INCLUSION BODIES
USE OF PSEUDAN AND PSEUDAN INCLUSION BODIES

This application claims the benefit of U.S. Provisional Application Serial No. 60/697,388, filed July 8, 2005, the entire disclosure of which is hereby incorporated by reference.

SUMMARY OF THE INVENTION

A biofilm is a population or a community of microorganisms adhering to environmental surfaces in the form of multicellular aggregates. Although biofilms can be made up of a single species of microorganisms, in the natural setting, they often involve a mixture of species. Biofilms can form in almost any hydrated environment that has the proper nutrient conditions, and can develop on a wide variety of abiotic hydrophobic and hydrophilic surfaces, including glass, metals, and plastics. Biofilms also readily form on biotic surfaces including human skin and epithelial cells. Generally, surface material does not strongly affect biofilm growth. Examples of bacterial biofilms are chronic P. aeruginosa infections in the lungs of cystic fibrosis patients, oral microbes on teeth, the "slime" layer on the surface of submerged objects in aquatic environments, biofouling of water supply, sewage, and oil pipelines, and bacterial colonization of plant surfaces. A detailed review of biofilm structure and architecture is provided by Dunne et al. (Clinical Microbiology Reviews, vol. 15, pages 155-166, 2002).

It is now appreciated that the formation and maintenance of structured multicellular communities critically depends upon the production of extracellular substances that, in conglomerate, constitute an extracellular matrix. Genetic studies using biofilm-generating systems have revealed many of the extracellular components of biofilms, and have helped to define the various components of biofilms. Initiation of biofilm formation is characterized by the interaction of cells with a surface or interface as well as with each other. Once enough cells have aggregated, the biofilm begins to mature the final architecture of the community is guided by the extracellular matrix. Extracellular polysaccharides and proteins have been shown to be key components of the matrix. Other components, such as extracellular DNA, carbohydrate-rich polymers, and dead cells are also thought to
play an important role in the establishment of biofilm structure (Branda et al., Trends in Microbiology, vol. 13, pages 20-26, 2005).

The keystone of biofilm study has been the general recognition that even single species of bacteria, when attached to surfaces and interfaces, express phenotypes that are not seen in liquid culture. For various genera whole cassettes of genes are repressed or de-repressed under the apparent control of touch receptors. Particularly, the 'sessile phenotype' more often displays a reduced susceptibility towards various antibacterial treatments and a more aggressive pathogenesis or corrosion potential than does the free-living 'planktonic' cell. Transition from the sessile to the planktonic depends on the formation of the extracellular polymeric matrix, which not only cements the bacterial cells to the surface but also maintains a spatial arrangement and helps trap many extracellular products and enzymes. The population then becomes quorate and through the mediation and accumulation of cell-cell signals (e.g., N-acyl-homoserine lactones in the case of Gram-negative bacteria) transcriptionally alters the phenotype of member cells. Cross signaling between different species and genera allows complex, multi-functional consortia to become established. For an analysis of the signaling pathways involved in biofilm formation, see Donlan et al., Emerging Infectious Diseases, vol. 8, pages 881-890, 2002. The precise nature of the colonizing species and its relationship with other bacteria determines the nature of the biofilm formed. In dental plaque such interrelationships have been well characterized and similar processes are involved wherever surfaces are exposed to environmental microorganisms.

Methods are known within the art for preventing and/or inhibiting biofilm formation and development. For example, published US patent application No. 2005/0143286 discloses a method for preventing or inhibiting biofilm formation using proteinaceous and non-proteinaceous metal chelators. US 2006/0018945 describes a method of preventing biofilm growth formation on a surface by coating or impregnating the surface with a gallium-containing composition. US 2003/0134783 describes the use of cyclic heptapeptides for inhibiting biofilm formation. US 2006/0030539 describes using compounds that inhibit the formation or
polymerization of actin in order to inhibit biofilm formation. See also US 6,267,979, US 6,086,921, and US 5,688,516.

*Pseudomonas aeruginosa*, a soil and water bacterium, can lead to opportunistic infections in immunocompromised or immunosuppressed patients such as burn patients and patients with cystic fibrosis. As described in published US Application No. 2003/0109548, which is hereby incorporated by reference in its entirety, a study of iron transport and metabolism in *Pseudomonas aeruginosa* (Royt, 1988. Biochim. Biophys. Acta, 939: 493-502) led to the isolation of an iron-chelating siderophore associated with the cytoplasmic membrane of iron-rich cells, although this iron chelator was of unknown structure. US Application No. 2003/0109548 discloses that this iron chelator involved two compounds 4-hydroxy-2-nonylquinoline and 4-hydroxy-2-heptylquinoline shown below:

![4-hydroxy-2-nonylquinoline](image1)

![4-hydroxy-2-heptylquinoline](image2)

Previously isolated from the filtrates of 4 to 6 week old culture of *P. aeruginosa*, the 4-hydroxy-2-nonylquinoline (the C-9 compound) was originally called Pyo Ic and shown to exhibit antibiotic activity (Hays et al., *J. Biol. Chem.*, 159: 725-749, 1945). The name pseudan was applied later (Ritter and Luckner, *Eur. J. Biochem.* 18: 391-400, 1971). The pseudans have been identified as having alkyl chains of C$_7$-C$_1_2$, *e.g.* pseudan IX (Herbert, 1989, 197. The Biosyntheses of Secondary Metabolites. 2nd ed. Chapman and Hall. New York).
US 2003/0109548 further discloses that 4-hydroxy-2-alkylquinolines can be prepared by isolation from natural sources, such as *P. aeruginosa*, using conventional methods, e.g., electrophoresis, mass spectrometry, liquid chromatography, HPLC, thin-layer liquid chromatography, detergent extraction (e.g., non-ionic detergent, Triton X-100, CHAPS, octylglucoside, Igepal CA-630), ethanol extraction, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, lectin chromatography, gel electrophoresis, immuno-purification methodology, etc. A method is described in Royt et al., *Biochim. Biophys. Acta.*, vol. 939, pages 493-502, 1988.

Additionally, US 2003/0109548 discloses that 4-hydroxy-2-alkylquinolines can be prepared synthetically. For example, procedures for the synthesis of 4-hydroxy-2-nonylquinoline are described by Wells, *J. Biol. Chem.*, vol. 196: pages 331-340, 1952; and Somanathan and Smith, J. Heterocyclic Chem., vol. 18: page 1077, 1981. Additional synthetic methods which start with a lower alkyl 4-hydroxyquinolone, such as 4-hydroxy-2-methylquinoline, may also be employed. Reaction of this compound with sodium amide in liquid ammonia solvent gives the dianion where both aromatic substituents are deprotonated. When the dianion is further reacted with a limited amount of an alkyl halide, selective alkylation is achieved at the 2 methyl carbon. The desired 4-hydroxy-2-alkylquinolines can be prepared upon work-up with acid.

In a more recent study on iron transport and metabolism in *Pseudomonas aeruginosa*, the applicants found that a decrease in total cell iron of iron-rich, late logarithmic phase was due to iron efflux which was accompanied by the appearance of aggregated particles (i.e., inclusion bodies) on pellets of centrifuged cells. Inclusion bodies are highly reflective, dense aggregates that function to compartmentalize various substances that are either foreign, toxic, or in excess to the bacterial cell. Inclusion bodies of prokaryotic cells serve various functions (for a detailed review, see Shively et al., *Ann. Rev. Microbiol.*, vol. 28: pages 167-187, 1974). Some inclusion bodies are storage sites for carbon and energy sources. For example, lipid inclusion bodies consisting of poly-β-hydroxybutyric acid or other
polyhydroxyalkanoates, triacylglycerols, and wax esters provide carbon and energy to the cell. Some bacteria derive energy upon oxidizing glycogen or starch found in polysaccharide inclusion bodies or sulfur found in sulfur inclusions bodies, whereas other organisms store inorganic phosphate in inclusions to be used in the synthesis of ATP. Other inclusion bodies carry out more specialized functions such as carbon dioxide fixation during photosynthesis, buoyancy, proper orientation along a magnetic field. A characteristic of prokaryotic inclusion bodies is the absence of a unit membrane defining them. Rather, these structures may be enclosed by a non-unit lipid membrane or by protein. Some inclusion bodies are located in the cytoplasm of the cell, whereas others are found in the periplasmic space.

In general, the above-mentioned inclusion bodies involved in iron transport and metabolism in *Pseudomonas aeruginosa* comprise one or more pseudan species (e.g., the C7 and/or C9 forms), 3,4-dihydroxy-2-heptylquinoline (the *Pseudomonas* quinolone signaling compound, PQS), and one or more forms of iron (e.g., Fe$^{+3}$ and salts thereof). For example, the inclusion bodies can contain iron, pseudan I (4-hydroxy-2-methylquinoline), pseudan VII (4-hydroxy-2-heptylquinoline), pseudan IX (4-hydroxy-2-nonylquinoline), 3,4-dihydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline N-oxide. These aggregated particles were determined to be iron-containing intracellular inclusion bodies which, among other things, affect biofilm formation. Among the quinoline derivatives found in such inclusion bodies pseudan I, VI, and IX were found in relative mole-based amounts of 1.00, 0.99, and 1.10. The 1:1:1 stoichiometry of pseudan I, VII, and IX suggests the complexation ratio of chelators to iron. The size of the inclusion bodies can vary. For example, the size of the inclusion bodies in the cells range from 25 to 50 nm.

These pseudan inclusion bodies were found to result in a decrease in cell aggregation. While not being bound to any particular theory, it is believed rhamnolipids on the surface of *P. aeruginosa* solubilize the inclusion bodies resulting in decrease cell adherence to surfaces.

Thus, according to one aspect of the invention there is provided a method of preventing or inhibiting the growth or development of a biofilm produced by bacteria, comprising contacting the biofilm with an effective amount a pseudan inclusion body.
According to a further aspect of the invention, there is provided a method of preventing or inhibiting the growth or development of a biofilm on a surface, biotic or abiotic, comprising applying to the surface an effective amount of pseudan iron-inclusion bodies, wherein said pseudan inclusion bodies comprise iron, 3,4-dihydroxy-2-heptylquinoline, and at least one pseudan species selected from 4-hydroxy-2-alkylquinolines in which the alkyl group contains 1-12 carbon atoms, e.g., 7-12 carbon atoms.

According to a further aspect of the invention, there is provided a method of preventing or inhibiting the growth of a biofilm on a surface, biotic or abiotic, comprising contacting the surface with an effective amount a pseudan inclusion body which has iron-binding properties.

According to a further aspect of the invention, there is provided a method of preventing or inhibiting the growth of a biofilm on a surface, biotic or abiotic, comprising contacting the surface with an effective amount a pseudan inclusion body which has iron-binding properties, wherein said pseudan inclusion bodies comprise 3,4-dihydroxy-2-heptylquinoline and at least one pseudan species selected from 4-hydroxy-2-alkylquinolines in which the alkyl group contains 1-12 carbon atoms, e.g., 7-12 carbon atoms.

A further aspect of the invention is a composition of isolated pseudan inclusion bodies optionally in combination with at least one carrier, such as a pharmaceutically acceptable carrier. In addition, the composition may further comprise other antibacterial agents. By "isolated" it is meant that the inclusion bodies are essentially free of the components in which they are found with in nature.

When the surface involved is a biotic surface, it is preferable that the inclusion bodies do not include 3,4-dihydroxy-2-heptylquinoline. In particular, the pseudan inclusion bodies used to treat biotic surfaces comprise comprise Fe\(^{3+}\), 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline.
In accordance with the invention, the surface to be treated with the pseudan inclusion bodies can be, for example, the surface of a medical device or a surface used in an industrial situation such as the pipelines in a water treatment plant or system. When the surface is that of a medical device, the inclusion bodies preferably do not include 3,4-dihydroxy-2-heptylquinoline.

Medical devices that can be treated in accordance with the inventive methods include, for example, catheters such as vascular catheters, epidural catheters, peritoneal catheters, and urinary catheters. The device can also be a shunt, a stent (such as a biliary stent, vascular stent or urinary stent), an orthopedic device, a prosthetic device, pacemaker, heart valve, pump, implant, endotracheal device, guidewire, optical or ocular lens, dental device, or surgical device. The device can also be one used for the storage or transportation of biological or medical fluids such as an inhaler, syringe, or ampule.

According to a further aspect of the invention, there is provided a method of treating and/or prevent bacterial infections, comprising contacting a surface, biotic or abiotic, or administering to a patient an anti-bacterial pseudan composition comprising pseudan I (4-hydroxy-2-methylquinoline), pseudan VII (4-hydroxy-2-heptylquinoline), pseudan IX (4-hydroxy-2-nonylquinoline), and optionally Fe^{3+}. Preferably, the pseudan composition contains pseudan I, VI, and IX at a stoichiometric ratio of 1:1:1.

According to a further aspect of the invention, there is provided an anti-bacterial pseudan composition comprising pseudan I (4-hydroxy-2-methylquinoline), pseudan VII (4-hydroxy-2-heptylquinoline), pseudan IX (4-hydroxy-2-nonylquinoline), and optionally Fe^{3+}. Preferably, the pseudan composition contains pseudan I, VI, and IX at a stoichiometric ratio of 1:1:1.

Inclusion bodies useful in the present invention can be collected either from the extracellular milieu or by lysing the cells. In the case of extracellular inclusion bodies routine techniques may be applied in the isolation and purification. For e.g., centrifugation of a 24-hour bacterial culture at 2,987x g for 10 min at 4°C to initially
pellet the bacteria followed by filtering the spent media through a 0.45 µm filter. Ultra-centrifugation of the filtrate at 265,000x g (Ti70 rotor, Beckman Coulter Instruments) for 2 h at 4°C pellets the inclusion bodies. The resultant pellet can then be washed with MOPS buffer and solubilized to isolate its contents or alternatively, stored at -80°C until further experimentation. For purification of the contents, the isolated inclusion bodies are treated with acidified ethyl acetate following the procedure used by others (McGrath et al., *FEMS Microbiol. Letts.*, vol. 230: pages 27-34, 2004) to isolate PQS from spent media, and/or treating the cultures with *P. aeruginosa* rhamnolipid, which is commercially available from Jeneil Biosurfactant Co., Saukville, WI. Solubilization of the inclusion bodies is facilitated by vortexing for 30 min. Various kits and methods may also be employed, either solely or in combination with the above-said method for the extraction of intracellular inclusion bodies. For e.g., Seely et al. (US Patent No. 4,656,255) describes a method for extracting inclusion bodies of *E. coli* for the purification of protein aggregates.

Yet another possible method may rely on the isolation of minute vesicles (about 50 nm in diameter) released from the outer membrane by Gram-negative bacteria (Mashburn et al., *Nature* vol. 437: pages 422-425, 2005). The vesicles consist of a lipid bilayer surrounding an aqueous core and can also transport PQS. In *P. aeruginosa*, PQS is one of at least 55 quinolones and quinolines synthesized by the bacteria, some of which have potent antibacterial activity and signaling properties. The production of these vesicles is regulated by PQS. Fractionation and purification of such vesicles allows for a simple method for the isolation of the contents of the inclusion bodies.

According to a further aspect of the invention, there is provided a method of preventing the growth of a biofilm on a surface, biotic or abiotic, comprising contacting the surface with an effective amount of pseudan inclusion bodies which have iron-binding properties. Any biofilm produced by bacteria can be prevented or reduced and/or inhibited. Although not wishing to be bound by any particular theory, it is believed that the bacterium to be treated preferably are capable of solubilizing the pseudan inclusion bodies. Preferably, the methods are used to inhibit/prevent the growth of biofilms produced by Pseudomonas, such as *Pseudomonas*.
aeruginosa, although the methods can be used against other biofilm-forming bacteria, including E. coli, Staphylococcus, and other gram-positive and gram-negative. Any biofilm can be inhibited in accordance with the present invention, including biofilms that form on artificial surfaces and natural surfaces, such as in lungs (e.g., in cystic fibrosis (CF) patients), burns or other wounds, etc.

The present invention also relates to the use of pseudan-inclusion bodies to treat and/or prevent bacterial infections, comprising: contacting a bacterium with a composition comprising pseudan-inclusion bodies. Any bacteria can be treated, including E.coli, Staphylococcus, Pseudomonas, and other gram-positive and gram-negative. The bacteria can be planktonic, sessile, in a biofilm, in a wound, on a substrate (e.g., a stent or wound drain), etc. The composition can be administered as a liquid, cream, powder, impregnated in a wound dressing, or in any form that is suitable for treating and/or preventing infection.

Again, while not wishing to be bound by any particular theory, in inhibiting the growth of the biofilm it is quite possible that the pseudan inclusion bodies are promoting release of cells from each other and/or from a surface. While this, of course, reduces the biofilm at that location, it also leads to the production of planktonic cells that could to go downstream to colonize at a different location. On the other hand, the planktonic cells, which are outside the polysaccharide matrix of the biofilm, are more susceptible to drug treatments such as treatments with antibiotics and/or antibacterial agents.

When used to treat a biofilm in a patient or prevent the formation of a biofilm in a patient, it would be desirable to remove PQS, 3,4-dihydroxy-2-heptylquinoline, from the pseudan inclusion bodies as the presences of PQS could promote disease caused by P. aeruginosa. One method to do this would be to synthetically prepare the pseudan inclusion bodies using conventional techniques from the pseudans and Fe$^{3+}$. For example, pseudan I, VI, and IX can be combined with Fe$^{3+}$ within a lipid vessel (e.g., a liposome) at a stoichiometry ratio of, for example, 1:1:1.

Biofilms assays can be performed routinely, e.g., by culturing Pseudomonas on polystyrene or other appropriate substrate, and then applying crystal violet to
stain and/or quantitate the biofilm. Quantitative biofilm production assays may comprise measuring bacterial cell turbidity using a microtiter plate reader (Bio-Rad, Richmond, Calif). The assay is carried out at an optical density at 595 nm (OD595) at different time intervals. With *P. aeruginosa*, biofilms form within minutes. Thus, suitable time intervals for *P. aeruginosa* biofilms are within 60-90 min. Alternatively, a fluorescence-based or an enzymatic quantitative assay may be employed for measuring the production of biofilms and/or as a means of screening for clinical isolates (Butterfield et al., *J. Microbiol Methods*, vol. 50, pages 23-32, 2002).

The inclusion bodies can be applied to a surface either alone or as a composition comprising the inclusion bodies and an acceptable carrier. For example, the composition can be applied to the surface by coating or spraying, or the surface can be immersed in a liquid composition. Procedures and techniques for treating surfaces (both biotic and abiotic) with compositions to inhibit and/or prevent biofilms are described in, for example, the published US applications discussed above, such as 2005/0143286 and US 2006/0018945.

When used to treat a patient suffering from a bacterial infection or when used to treat a biofilm in a patient (e.g., a human) (such as a biofilm in the lungs of a human, e.g., a cystic fibrosis patient), the inclusion bodies or pseudan composition (e.g., a combination of pseudan I, pseudan VII, pseudan IX, and optionally Fe+++3) can be used in the form of a composition comprising the inclusion bodies or the pseudans and at least one pharmaceutically acceptable carrier.

The carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not being deleterious to the surface to which they are applied or to the recipient to which they are administered. The composition can further contain conventional excipients used in pharmaceutical formulations. The pharmaceutical acceptable carrier may also provide a controlled release formulation that is capable of slowly releasing a composition of the present invention into a patient. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules,
microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems.

Pharmaceutical compositions include those suitable for oral, rectal, nasal, topical, transdermal, vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration or in a form suitable for administration by inhalation or insufflation (such as an aerosol). Pharmaceutically acceptable carriers are well known in the art and include those materials or vehicles which are not harmful to the individual and are suitable for administering compounds of the present invention to patient (e.g., mammals). Carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material. Examples of suitable pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

When used as a pharmaceutical, the amount of the inclusion bodies or pseudan combination present in the administered composition will vary based on the route of administration, the nature of the condition for which treatment is required, and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or veterinarian. The amount of inclusion bodies to be administered to a patient or applied to a surface can be easily determined by one of skill in the art using routine experimentation.

Pharmaceutical compositions suitable for oral administration may conveniently be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient; as a powder or
granules; as a solution, a suspension or as an emulsion. The active ingredient may also be presented as a bolus, electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrants, or wetting agents. The tablets may be coated according to methods well known in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives.

The inclusion bodies or pseudan combination may also be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, small volume infusion or in multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain additional formulation agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the inclusion bodies can be in powder form, obtained by aseptic isolation of sterile solid or by lyophilisation from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

For topical administration to the epidermis, the pseudan inclusion bodies or pseudan combination may be formulated as ointments, creams or lotions, or as a transdermal patch. Such transdermal patches may contain penetration enhancers such as linalool, carvacrol, thymol, citral, menthol and t-anethole. Ointments and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilizing agents, dispersing agents, suspending agents, thickening agents, or coloring agents.

The pseudan inclusion bodies or pseudan combination can be administered as an aerosol formulation. Suitable for aerosol delivery include powders,
microcapsules, microparticles, liposomes, and nebulized sprays. Aerosol formulations can be administered by, for example, nebulizers and inhalers such as pressurized metered dose inhalers (MDI), dry powder inhalers (DPI), and metered solution devices (MSI).

The entire disclosures of all applications, patents and publications, cited above and below, are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Cell growth, total cell iron, and extractable pseudan in three substrains of *P. aeruginosa*: A) 15692a; B) 15692b; C) 15692d. At each time period, the optical density was determined, and two aliquots of 25 X 109 cells were collected to determine concentrations of total cell iron and extractable pseudan.

FIG. 2. Efflux of $^{55}$FeCl3 from substrain 15692d. A) cell density: B) total cell $^{55}$Fe: C) $^{55}$Fe in 200 µl of spent media before transfer of cells and in the filtrate after transfer of cells.

FIG. 3. A high resolution electrospray mass spectrum of whole inclusion bodies.

FIG. 4. Electron impact mass spectrum of the 23.54 minute peak of the GC-MS of whole, inclusion bodies. The sample was dissolved in methanol.


FIG. 6. Cell growth, total cell iron, and iron in inclusion bodies in A)15692a and B) 15692d cells. Total cell iron in 25 X 109 cells and in washed inclusion bodies was measured after acid hydrolysis.
FIG. 7. Adherence of cells to polystyrene microtiter plate wells and levels of extractable pseudan. The optical density at 600 nm is the average of solubilized crystal violet from three wells after 60 min incubation. Bars represent the range. A) 15692a cells. B) 15692b cells. C) 15692d cells.

FIG. 8. Adherence of A) 5 hr, B) 25 hr, and C) 30 hr 15692b cells to polystyrene microtiter plate wells in the absence (closed bars) and presence of inclusion bodies containing 31 µg iron (light grey bars) and 62 µg (dark-grey bars) iron. The optical density at 600 nm is the average of solubilized crystal violet from three wells after 60 min incubation. Bars represent the range.

EXAMPLES

In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

Bacteria and culture methods. Three substrains of Pseudomonas aeruginosa ATCC 15692 were used in all experiments. These substrains are designated 15692a, 15692b, and 15692d. Cultures were maintained at -70°C in Luria-Bertani broth containing 25% glycerol. Cells were grown in trypticase soy broth (TSB: DIFCO Laboratories), shaking at 100 rpm at 30°C in a New Brunswick rotatory shaker. Typically, cultures were inoculated with logarithmic phase cells to an initial optical density of 0.02 to 0.04. Cells were harvested by centrifugation at 11,950xg for 10 min at 4°C, and washed two times with 60 mM 3-(4-Morpholino) Propane Sulfonic Acid buffer (MOPS buffer), pH 7.2.

Iron and pseudan determination. 25 X 109 cells were harvested in acid-washed centrifuge tubes and washed two times as stated above. To determine total iron concentration, a pellet of cells was acid hydrolyzed with nitric acid/ perchloric acid (1/1 : V/V). Following dilution in 0.1 MTris-HCl buffer, pH 7.8, iron was determined on a Perkin-Elmer 5001 PC Atomic Absorption Spectrophotometer. Total iron concentration consists of intracellular iron, iron in periplasmic inclusion bodies, and
iron in aggregates of inclusion bodies pelleted with cells. The iron in isolated inclusion bodies was also determined after acid hydrolysis as above.

Pseudan VII and IX were extracted from a pellet of cells with ethanol as before (Royt et al., Biochim. Biophys. Acta, vol. 939: pages 493-502, 1988). Here, 25 X10⁹ cells were extracted with 1 ml ethanol for 1 h, followed by centrifuging at 38,720xg for 30 min at 4°C. This extraction procedure was repeated two more times, and the extracts combined. The concentration of pseudan VII and IX was determined spectrophotometrically upon measuring the spectrum of synthesized pseudan IX with a millimolar extinction coefficient of 151.5 mM⁻¹ cm⁻¹ at 231 nm. In this study, extractable pseudan consists of pseudan VII and IX in the pellet of cells, that is the membrane-bound compounds, that which is extractable from periplasmic inclusion bodies, and that which is extractable from extracellular, aggregated inclusion bodies. Efflux experiment. Two flasks each containing 50 ml TSB were inoculated with logarithmic phase cells as described above. Fifty μCi ⁵⁵FeCl₃ (New England Nuclear Corporation, Boston, MA.) was added to one of the flasks. After shaking at 30°C for 24 hours, each culture was centrifuged, and the radiolabeled cells were washed one time with room-temperature 60 mM MOPS buffer. The radiolabeled cells were transferred to the spent media of the unlabeled culture. At designated time intervals, 1 X 10⁹ cells were transferred to a filter, washed two times with MOPS buffer, and the radioactivity counted. Also, 200 μl of the cell-free filtrate, i.e., the spent media, was counted at each time interval.

Collection of extracellular inclusion bodies. A 24 hour culture of 15692d cells was centrifuged at 2,987xg for 10 min at 4°C. The spent media was filtered through a 0.45 μm filter, and the filtrate centrifuged at 265,000xg (Ti70 rotor, Beckman Coulter Instruments) for 2 h at 4°C. The resultant pellet was washed one time with MOPS buffer. Fractionation of the inclusion bodies by thin layer chromatography (TLC). Isolated inclusion bodies were treated with acidified ethyl acetate, following the procedure used by other to isolate the PQS from spent media of P. aeruginosa (McGrath et al., FEMS Microbiol. Letts. Vol. 230: pages 27-34, 2004), and with P. aeruginosa rhamnolipids obtained from Jeneil Biosurfactant Co., Saukville, WI. Solubilization of the inclusion bodies occurred upon vortexing for 30 min. Samples
were spotted on silica gel TLC plates that had been soaked in 5% w/v KH2PO4 and activated for 1 hr at 100°C. Separation of compounds occurred using methylene chloride:acetonitrile:dioxane (17:2:1) (Pesci et al., Proc. Natl. Acad. Sci. U.S.A. vol. 96: pages 11229-11234, 1999).

Electron microscopy.
(i) Transmission electron microscopy (TEM) was performed on pellets of 5 h and 24 h 15692d cells, and on isolated inclusion bodies. Samples were resuspended in 4% formaldehyde/1% glutaraldehyde overnight at 4°C, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Thin (80-100 nm) sections were stained with lead citrate and uranyl acetate and examined in a LEO912 electron microscope (Carl Zeiss SMT, Thornwood, NY).
(ii) Scanning electron microscopy (SEM) was done on 24 hr 15692d cells using a JEOL JSM-840 electron microscope with a LaB6 electron emitter. The SEM was equipped with an energy dispersive x-ray analyzer (EDAX) using a Si(Li) crystal (Princeton Gamma-Tech, Princeton, NJ). The sample was fixed in 1% glutaraldehyde for 2 hr, and dehydrated with acetone.

Attachment assay. An assay for the initiation of biofilm formation was used to determine attachment of cells to wells of a polystyrene microtiter plate (O'Toole et al., Mol. Microbiol., vol. 28: pages 449-46, 1999). Cells (5 X 10^7) in spent media were added to wells of a plate in triplicate. The plates were incubated at 30°C shaking at 35 rpm. At 15 min intervals for 1 hr, media and planktonic cells were removed, and the wells washed 3 times with room-temperature MOPS buffer. 250 µl 0.1% crystal violet was added to the wells for 15 min. The wells were then washed 3 times with MOPS buffer, and the dye in cells adhered to the well surface was solubilized in 95% ethanol. The absorbance at 600 nm was then measured. Controls consisting of spent media treated as above were subtracted from each sample. When mentioned, inclusion bodies resuspended in MOPS buffer at the given concentrations were added to cells in spent media, and the attachment assay carried out. Controls of inclusion bodies resuspended in MOPS and added to spent media were subtracted from these values.
**High resolution electrospray ionization mass spectrometry (ESI MS).** Electrospray ionization mass spectra were obtained on a Waters LCT Premier Time-Of-Flight (TOF) MassSpectrometer. The instrument was operated in positive ion mode at a resolution of 10000. The electrospray capillary voltage was 3 kV and the sample cone voltage was 60 volts. The desolvation temperature was 225°C and the desolvation gas was nitrogen at 300 L/hr. Accurate masses were obtained using the lock spray mode with Leu-Enkephalin as the external reference compound. The ESI mass spectra were obtained via the direct loop injection method.

**RESULTS**

Total cell iron decreases in late logarithmic phase. Upon tracking iron levels in the three substrains of *P. aeruginosa* growing in iron-rich TSB, it was found that total cell iron increased in the early logarithmic phase of growth (Fig. 1). This increase in total cell iron was consistently followed by a decrease as the cell population continued into later logarithmic phase. It is noted in Figure 1 that as total cellular iron decreased, extractable pseudan increased. Substrain 15692a had the greatest quantity of both iron and extractable pseudan, as compared to those quantities produced by substrains 15692b and 15692d. Pseudan was extractable from substrains 15692a and 15692d after approximately 24 hr, whereas pseudan was not extractable from substrate 15692b for approximately 48 hr after culture inoculation, and it is noted that significantly less pseudan was extractable from substrate 15692b than from the other two substrains. The decrease in total cell iron seen with each substrate may be explained by iron efflux from the cell and/or the stoppage of iron uptake as the population of cells increased in number.

To determine if iron efflux occurs from the iron-rich cells, substrain 15692d was radiolabeled with $^{55}$FeCl$_3$, and transferred to $^{55}$Fe-free spent media as described above. At set time periods, cell-associated radioactivity and radioactivity in the spent media were determined (Fig. 2). It is seen that radiolabeled iron accumulated in the cells as cell density increased. Upon transfer of radiolabeled cells to $^{55}$Fe-free spent media, $^{55}$Fe is released from the cells and is recovered in the media. When initially doing such efflux experiments it was noted that cells aggregated when transferred instead to fresh TSB or to MOPS buffer, resulting in erratic results of total cell iron.
Such aggregation suggested that a substance in the spent media served to prevent cell aggregation.

Extracellular aggregates were extracted with rhamnolipid and with acidified ethyl acetate. Thin layer chromatograms of these extracts revealed the appearance of at least four bands, one of which, Rf = 0.55, was fluorescent blue. ESI MS of whole inclusion bodies (Fig. 3) showed a peak of m/z 272.2010 consistent with the C18H26NO formula, or pseudan IX. A peak was also observed at m/z 288.1978 (C18H26NO2) which may be 4-hydroxy-2-nonylquinoline-N-oxide, with establishment of structure via ease of loss of the oxygen atom on the nitrogen (Taylor et al., J. Chromatogr. vol. 664: pages 458-462, 1995). The peak of m/z 270.1862 (C18H24NO formula) is of dehydrated, 4-hydroxy-2-nonylquinoline-N-oxide, and a peak at m/z 244.1724 (C16H22NO) is pseudan VII, and finally, a peak at 260.1667 (C16H22NO2 formula) is 3,4-dihydroxy-2-heptylquinoline. Absent from the spectra is dehydrated, 4-hydroxy-2-heptylquinoline-N-oxide which would have a molecular weight of 242. This absence suggests that the position of the second oxygen atom in the two compounds, i.e., the C7 compound and the C9 compound differs, in that one is a protonated N-oxide, as in the C9 compound, and the other a hydroxyl group. Hence, the peak at 260.1667 is that of 3,4-dihydroxy-2-heptylquinoline. (iii) GC-MS was also carried out using the whole inclusion bodies, and using the blue fluorescent spot eluted from the thin layer chromatogram. The total ion chromatogram (TIC) of the whole inclusion bodies revealed about 20 major peaks. Pseudan VII was identified at 23.54 minutes, and pseudan IX was identified at 23.90, 24.02, and 25.20 minutes. 4-Hydroxy-2-nonylquinoline-N-oxide and dehydrated 4-hydroxy-2-nonylquinoline-N-oxide were also evident. The TIC did not reveal the presence of 3,4-dihydroxy-2-heptylquinoline, but did show the presence of dehydrated 3,4-dihydroxy-2-heptylquinoline. Also present in the TIC at 23.54 minutes as well as in other peaks is 4-hydroxy-2-methylquinoline, or pseudan I. Figure 4 is the mass spectrum of the 23.54 minute peak showing pseudan I, m/z 159. The TIC of the whole inclusion bodies also revealed m/z 56 in several peaks, which may be iron or an organic fragment.

To verify that the inclusion bodies seen inside the cells are indeed iron-containing and hence are the iron containing pelleted aggregates isolated from the
cells, an elemental analysis of intracellular inclusion bodies was compared with that of cells lacking inclusion bodies. Figure 5A shows the back-scattered electron image of 24 hour old 15692d cells, one of which contains a bright area. The elemental analysis of a whole cell itself shows large amounts of carbon and phosphate, and modest amounts of other elements (Fig. 5B). The elemental analysis of the bright area reveals a high concentration of iron in addition to the elements found in the cell itself (Fig. 5C). The two iron peaks identify the types of x-rays produced: the higher energy peak is a K-beta peak in that it represents the x-rays emitted by M-shell electrons going to the K-shell, and the lower energy peak is a K-alpha peak representing x-rays emitted from electrons going from the L-shell to the K-shell.

To determine whether the iron efflux seen in Figure 1 was mediated by the inclusion bodies, each substrain was harvested during a 48 h period including the time of iron efflux seen in Figure 1. At each time period, total iron concentration in the pellet of cells was measured, as was the iron in inclusion bodies isolated from the spent media. It is seen in both 15692a and 15692d cells that as total cell iron increased, the iron of inclusion bodies collected from the spent media also increased. As total cell iron decreased in the 15692a cells, inclusion body iron also decreased. However, this decrease in inclusion body iron with a decrease in total cell iron did not occur in the 15692d cells. In similar studies using the 15692b cells, the iron in inclusion bodies was immeasurable using the conditions described, indicating that this substrain failed to release significant levels of inclusion bodies or that released inclusion bodies were solubilized upon being released.

When doing efflux experiments, it was noted that transfer of washed cells to buffer or to fresh media resulted in cell aggregation. Such aggregation was minimized, however, when washed cells were transferred to spent media that contained inclusion bodies. Therefore, adhesion assays were performed to determine if the inclusion bodies played a role in preventing cell aggregation. Figure 7 shows that with each substrain, an inverse relationship exists between cell adherence and extractable levels of pseudan, in that as the level of extractable pseudan increased with time of growth, cell adherence decreased. Also it is seen that the 15692b cells that produce the least amount of extractable pseudan show the greatest amount of cell adherence. To determine if the inclusion bodies were playing
a role in preventing cell adherence, logarithmic phase 15692b cells, cells from which pseudan is not extractable and which are not producing collectible inclusion bodies, were incubated with inclusion bodies. It is seen in Figure 8 that addition of inclusion bodies to these cells resulted in a decrease in attachment of the cells to the wells.

Attached hereto as an Appendix is a copy of unpublished manuscript describing in further detail the study on iron transport and metabolism in Pseudomonas aeruginosa. The Appendix is hereby incorporated by reference in its entirety.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention and, without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.
APPENDIX

TITLE PAGE

Title: Iron- and 4-hydroxy-2-alkylquinoline-containing periplasmic inclusion bodies of
Pseudomonas aeruginosa: Multifunctional structures

Running title: Periplasmic inclusion bodies of Pseudomonas aeruginosa

Authors:

Paulette W. Royt*, Molecular and Microbiology Department, George Mason University, Fairfax, VA 22030

Robert V. Honeychuck, Chemistry and Biochemistry Department, George Mason University, Fairfax, VA 22030

Ramesh R. Pant, Chemistry and Biochemistry Department, George Mason University, Fairfax, VA 22030

Magnus L. Rogers, Chemistry and Biochemistry Department, George Mason University, Fairfax, VA 22030

Ludmila V. Asher, Division of Pathology, Walter Reed Army Institute of Research, Silver Spring, MD 20910
John R. Lloyd, MDDK, NIH, DHHS, Bethesda, MD 20892

W. E. Carlos, Naval Research Laboratory, Washington, D.C. 20375

Harvey E. Belkin, U.S. Geological Survey, Reston, VA 20192

Swati Patwardhan, Molecular and Microbiology Department, George Mason University, Fairfax, VA 22030

*Corresponding author. Mailing address: Molecular and Microbiology Department, MSN 3El, George Mason University, Fairfax, VA 22030. Phone: (703) 993-1058. Fax: (703) 993-1046. E-mail: proyt@gmu.edu
A study of iron transport and metabolism in *Pseudomonas aeruginosa* showed that a decrease in total cell iron of iron-rich, late logarithmic phase cells was due to iron efflux. This efflux was accompanied by the appearance of aggregated particles on pellets of centrifuged cells. Transmission electron microscopy of iron-rich cells showed inclusion bodies in periplasmic vacuoles, and did not show outer membrane vesicles. Aggregated particles isolated from the spent medium of iron-rich cells contained iron as indicated by atomic absorption spectroscopy and by electron paramagnetic resonance spectroscopy that revealed Fe$^{3+}$. Scanning electron microscopy/energy dispersive x-ray analysis of whole cells revealed the presence of iron-containing particles beneath the surface of the cell, indicating that the isolated aggregates were the intracellular inclusion bodies. Collectively, mass spectroscopy and nuclear magnetic resonance spectroscopy of the isolated inclusion bodies revealed the presence of 3,4 dihydroxy-2-heptylquinoline which is the *Pseudomonas* quinolone signaling compound (PQS); 4-hydroxy-2-heptylquinoline (pseudan VII), an antibacterial compound and a precursor of PQS; 4-hydroxy-2-nonylquinoline (pseudan IX) which is an iron chelator and antibacterial compound; 4-hydroxy-2-methylquinoline (pseudan I), and 4-hydroxy-2-nonylquinoline N-oxide. It is shown that the inclusion bodies prevent cell adherence. The inclusion bodies serve to prevent iron toxicity, limit microbial competitors, activate virulence genes by cell communication, and affect biofikn formation.
INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* is found in a variety of ecological niches including water, soil, and the immunocompromised animal host. The organism's adaptability to different environments is at least partially dependent on its regulation of iron transport and metabolism. In low iron-containing environments, *P. aeruginosa* must sequester needed iron from available iron sources. It does this by the use of two exogenous siderophores, pyoverdin (8) and pyochelin (7), as well as by citrate (7). In the host, *P. aeruginosa* can also sequester iron from heme and heme-containing proteins (27). Here, outer membrane receptors directly recognize, bind, and transport the iron in these exogenous iron-containing sources. In iron replete environments, however, synthesis of exogenous iron-acquisition systems and the membrane-bound receptors is repressed. It is thought that in environments with high iron and oxygen, the ferric ion is reduced to the ferrous ion at the cell surface, allowing the less charged ion to permeate the cell membrane (12). Under these conditions, intracellular levels of iron must be controlled in order to prevent iron toxicity with subsequent cell death. *P. aeruginosa* does store excess iron in the intracellular storage protein bacterioferritin (1). Iron levels could also be controlled by terminating iron uptake, by sequestering intracellular iron in additional storage depots, and/or by releasing iron from the cell, i.e., iron efflux.

Pseudan IX is a novel iron chelator originally isolated from the cytoplasmic membrane of iron-rich *P. aeruginosa* (32). Its structure, determined after HPLC fractionation of ethanol extracts of membranes, is that of 4-hydroxy-2-nonylquinoline (33, Fig. 1). The alkyl chain of pseudan IX serves to embed the chelator into the membrane of the cell, and to form vesicles in
ethanol. Pseudan IX is isolated from the membranes of iron-rich cells as well as from whole, iron-rich cells: it is not extractable from iron-poor cells or membranes of these cells.

Pseudan IX is one of many 4-hydroxy-2-alkylquinolines (HAQs) produced by *P. aeruginosa* (9, 19). Pseudan IX and pseudan VII (4-hydroxy-2-heptylquinoline) were originally isolated in 1945 and shown to have antibacterial activity (13). Later, another group of HAQs was discovered, members of which have an N-oxide group in place of the quinoline nitrogen, and a C7 or a C9 alkyl chain (6). The C7 N-oxide was shown to have antistaphylococcal activity (20). In 1959, 3,4-dihydroxy-2-heptylquinoline, another HAQ, was identified (39), and shown later to be a quorum sensing molecule involved in cell-to-cell signaling (30). Designated the *Pseudomonas* quinolone signal (PQS, 30), this molecule, found in the lungs and sputum of patients with cystic fibrosis (5), ultimately regulates expression of many virulence genes in *P. aeruginosa* (10, 24). As a direct precursor of the PQS, 4-hydroxy-2-heptylquinoline also plays a role in cell-to-cell signaling (9).

Inclusion bodies of prokaryotic cells serve various functions (see ref 37 for a review). Some inclusion bodies are storage sites for carbon and energy sources. For example, lipid inclusion bodies consisting of poly-β-hydroxybutyric acid or other polyhydroxyalkanoates (38), triacylglycerols (21), and wax esters (34) provide carbon and energy to the cell as do polysaccharide inclusion bodies made of glycogen or starch. Some bacteria derive energy upon oxidizing sulfur found in sulfur inclusions bodies, whereas other organisms store inorganic phosphate in inclusions to be used in the synthesis of ATP. Other inclusion bodies carry out more specialized functions in the cell. Carboxysomes, for example, contain ribulose 1,5-
diphosphate carboxylase needed for carbon dioxide fixation during photosynthesis (36). Gas vacuoles keep aquatic bacteria buoyant, whereas magnetosomes, inclusions of iron oxide, serve to orient cells along a magnetic field (2). Characteristic of prokaryotic inclusion bodies is the absence of a unit membrane defining them. Rather, these structures may be enclosed by a nonunit lipid membrane or by protein. Some inclusion bodies are located in the cytoplasm of the cell, whereas others are found in the periplasmic space.

The purpose of this research was to determine the role pseudan VII and IX play in iron metabolism in *P. aeruginosa*. We show here that iron-rich *P. aeruginosa* contains periplasmic inclusion bodies containing iron, pseudan I, pseudan VII and pseudan IX, as well as 4-hydroxy-2-nonylquinoline-N-oxide, and 3,4-dihydroxy-2-heptylquinoline, the PQS. We also show that the inclusion bodies play a role in iron efflux as well as a role in cell adherence. It is apparent from this work that the inclusion bodies serve as vehicles of release of a major signaling compound and its precursor, and hence are involved in quorum sensing and virulence of *P. aeruginosa*. By packaging pseudan VII and pseudan IX the inclusion bodies also are involved in antimicrobial activity of the cell.

**MATERIALS AND METHODS**

**Bacteria and culture methods.** Three substrains of *Pseudomonas aeruginosa* ATCC 15692 were used in all experiments. These substrains are designated 15692a, 15692b, and 15692d. Cultures were maintained at -70°C in Luria-Bertani broth containing 25% glycerol. Cells were
grown in trypticase soy broth (TSB: DIFCO Laboratories), shaking at 100 rpm at 30°C in a New Brunswick rotatory shaker. Typically, cultures were inoculated with logarithmic phase cells to an initial optical density of 0.02 to 0.04. Cells were harvested by centrifugation at 11,950xg for 10 min at 4°C, and washed two times with 60 mM 3-(4-Morpholino) Propane Sulfonic Acid buffer (MOPS buffer), pH 7.2.

**Iron and pseudan determination.** 25 X 10⁹ cells were harvested in acid-washed centrifuge tubes and washed two times as stated above. To determine total iron concentration, a pellet of cells was acid hydrolyzed with nitric acid/perchloric acid (1/1 v/v). Following dilution in 0.1 M Tris-HCl buffer, pH 7.8, iron was determined on a Perkin-Elmer 5001 PC Atomic Absorption Spectrophotometer. Total iron concentration consists of intracellular iron, iron in periplasmic inclusion bodies, and iron in aggregates of inclusion bodies pelleted with cells. The iron in isolated inclusion bodies was also determined after acid hydrolysis as above.

Pseudan VII and IX were extracted from a pellet of cells with ethanol as before (32). Here, 25 X 10⁹ cells were extracted with 1 ml ethanol for 1 h, followed by centrifuging at 38,720xg for 30 min at 4°C. This extraction procedure was repeated two more times, and the extracts combined. The concentration of pseudan VT1 and IX was determined spectrophotometrically upon measuring the spectrum of synthesized pseudan IX with a millimolar extinction coefficient of 15.15 M⁻¹ cm⁻¹ at 231 nm. In this study, extractable pseudan consists of pseudan VII and IX in the pellet of cells, that is the membrane-bound compounds, that which is extractable from periplasmic inclusion bodies, and that which is extractable from extracellular, aggregated inclusion bodies.

**Efflux experiment.** Two flasks each containing 50 ml TSB were inoculated with logarithmic phase cells as described above. Fifty µCi ⁵⁹FeCk (New England Nuclear Corporation, Boston,
MA.) was added to one of the flasks. After shaking at 30°C for 24 hours, each culture was centrifuged, and the radiolabeled cells were washed one time with room-temperature 60 mM MOPS buffer. The radiolabeled cells were transferred to the spent media of the unlabeled culture. At designated time intervals, $1 \times 10^9$ cells were transferred to a filter, washed two times with MOPS buffer, and the radioactivity counted. Also, 200 µl of the cell-free filtrate, i.e., the spent media, was counted at each time interval.

**Collection of extracellular inclusion bodies.** A 24 hour culture of 15692d cells was centrifuged at 2,987xg for 10 min at 4°C. The spent media was filtered through a 0.45 µm filter, and the filtrate centrifuged at 265,000xg (Ti70 rotor, Beckman Coulter Instruments) for 2 h at 4°C. The resultant pellet was washed one time with MOPS buffer.

**Fractionation of the inclusion bodies by thin layer chromatography (TLC).** Isolated inclusion bodies were treated with acidified ethyl acetate, following the procedure used by others to isolate the PQS from spent media of *P. aeruginosa* (23), and with *P. aeruginosa* rhamnolipids obtained from Jeneil Biosurfactant Co., Saukville, WI. Solubilization of the inclusion bodies occurred upon vortexing for 30 min. Samples were spotted on silica gel TLC plates that had been soaked in 5% w/v KH$_2$PO$_4$ and activated for 1 hr at 100°C. Separation of compounds occurred using methylene chloride:acetonitrile:dioxane (17:2:1) (30).

**Electron microscopy.** (i) Transmission electron microscopy (TEM) was performed on pellets of 5 h and 24 h 15692d cells, and on isolated inclusion bodies. Samples were resuspended in 4% formaldehyde/1% glutaraldehyde overnight at 4°C, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Thin (80-100 nm) sections were stained with lead citrate and uranyl acetate and examined in a LEO912 electron microscope (Carl Zeiss SMT, Thornwood, NY). (ii) Scanning electron microscopy (SEM) was done on 24 hr
15692d cells using a JEOL JSM-840 electron microscope with a LaBe electron emitter. The SEM was equipped with an energy dispersive x-ray analyzer (EDAX) using a Si(Li) crystal (Princeton Gamma-Tech, Princeton, NJ). The sample was fixed in 1% glutaraldehyde for 2 hr, and dehydrated with acetone.

**Attachment** assay. An assay for the initiation of biofilm formation was used to determine attachment of cells to wells of a polystyrene microtiter plate (28). Cells (5 X 10^7) in spent media were added to wells of a plate in triplicate. The plates were incubated at 30°C shaking at 35 rpm. At 15 min intervals for 1 h, media and planktonic cells were removed, and the wells washed 3 times with room-temperature MOPS buffer. 250 µl 0.1% crystal violet was added to the wells for 15 min. The wells were then washed 3 times with MOPS buffer, and the dye in cells adhered to the well surface was solubilized in 95% ethanol. The absorbance at 600 nm was then measured. Controls consisting of spent media treated as above were subtracted from each sample. When mentioned, inclusion bodies resuspended in MOPS buffer at the given concentrations were added to cells in spent media, and the attachment assay carried out. Controls of inclusion bodies resuspended in MOPS and added to spent media were subtracted from these values.

**High resolution** electrospray ionization mass spectrometry (ESI MS). Electrospray ionization mass spectra were obtained on a Waters LCT Premier Time-Of-Flight (TOF) Mass Spectrometer. The instrument was operated in positive ion mode at a resolution of 10000. The electrospray capillary voltage was 3 kV and the sample cone voltage was 60 volts. The desolvation temperature was 225 °C and the desolvation gas was nitrogen at 300 L/hr. Accurate masses were obtained using the lock spray mode with Leu-Enkephalin as the external reference compound. The ESI mass spectra were obtained via the direct loop injection method.
Gas chromatography-mass spectrometry (GC-MS). GC-MS was performed on a Hewlett Packard 5890 Series II GC with a Hewlett Packard 5989A Mass Spectrometer. The GC column was a Varian Factor Four Capillary Column Model VF-5ms, with 30 m length, 0.25 mm interior diameter, and 0.25 µm film thickness. The mass spectrometer electron impact voltage was 70 eV.

Electron paramagnetic resonance spectroscopy (EPR). EPR spectra were obtained using a conventional Bruker EMX 9.5 GHz spectrometer operating at room temperature with the sample nominally in the dark, although no attempt was made to exclude room light. Typical operating microwave power was 1 mW.

Nuclear magnetic resonance spectroscopy (NMR). NMR spectra (¹H) were obtained on a Bruker DPX 300 spectrometer at room temperature in the solvents chloroform-d, dimethyl sulfoxide-d₆, or a solution of these two, and were referenced to internal tetramethylsilane (δ = 0 ppm), or to known solvent or compound peaks.
RESULTS

Total cell iron decreases in late logarithmic phase. Upon tracking iron levels in the three substrains of *P. aeruginosa* growing in iron-rich TSB, it was found that total cell iron increased in the early logarithmic phase of growth (Fig. 2). This increase in total cell iron was consistently followed by a decrease as the cell population continued into later logarithmic phase. It is noted in Figure 2 that as total cellular iron decreased, extractable pseudan increased. Substrain 15692a had the greatest quantity of both iron and extractable pseudan, as compared to those quantities produced by substrains 15692b and 15692d. Pseudan was extractable from substrains 15692a and 15692d after approximately 24 hr, whereas pseudan was not extractable from substrate 15692b for approximately 48 h after culture inoculation, and it is noted that significantly less pseudan was extractable from substrate 15692b than from the other two substrains. The decrease in total cell iron seen with each substrate may be explained by iron efflux from the cell and/or the stoppage of iron uptake as the population of cells increased in number.

Iron efflux occurs. To determine if iron efflux occurs from the iron-rich cells, substrate 15692d was radiolabeled with $^{55}$FeCU, and transferred to $^{55}$Fe-free spent media as described above. At set time periods, cell-associated radioactivity and radioactivity in the spent media were determined (Fig. 3). It is seen that radiolabeled iron accumulated in the cells as cell density increased. Upon transfer of radiolabeled cells to $^{55}$Fe-free spent media, $^{55}$Fe is released from the cells and is recovered in the media. When initially doing such efflux experiments it was noted that cells aggregated when transferred instead to fresh TSB or to MOPS buffer, resulting in
erratic results of total cell iron. Such aggregation suggested that a substance in the spent media served to prevent cell aggregation.

Pellets of late logarithmic phase cells reveal the presence of extracellular aggregates.

Earlier it was reported that membranes of iron-rich *P. aeruginosa* were pink (32). After ethanol extraction of these membranes, the membranes were pale and the extract was pink. Similar findings occurred here using whole cells: the pellet of these iron-rich cells was pink, and after ethanol extraction, the pellet was pale by comparison, and the ethanol extract was pink. In Figure 4 are seen the pellets of 5 h and 24 h 15692d cells. Both are visibly pink. The pellet of the older cells, however, contained dark-colored aggregates not seen in the pellet of the early logarithmic phase cells. Since the aggregates were not confluent, it became apparent that the aggregates were extracellular. Pellets of 15692a and 15692b cells were similar in appearance, although pellets of 15692b cells did not have visible aggregates until later in log phase.

Since the extracellular aggregates seen on the pellet were visible to the naked eye, it was obvious that they were larger than the cells themselves, and hence not filterable through a 0.45 μm filter. It was demonstrated, however, that smaller particulate matter was filterable from the spent media of these cells. Ultracentrifugation of filtered spent media resulted in a dark brown pellet (Fig. 5). The UV spectrum (not shown) of an ethanol extract of the pellet was similar to that of purified and synthesized pseudan IX (33).

TLC, ESI MS, GC-MS, NMR, and EPR reveal the chemical composition of aggregates in the pellet, (i) Extracellular aggregates were extracted with rhamnolipid and with acidified ethyl acetate. Thin layer chromatograms of these extracts revealed the appearance of at least four bands (data not shown), one of which, Rf= 0.55, was fluorescent blue, (ü) ESI MS of whole
inclusion bodies (Fig. 6) showed a peak of m/z 272.2010 consistent with the C₁₈H₂₆N₂O₂ formula, or pseudan IX (Fig. 1). A peak was also observed at m/z 288.1978 (C₁₈H₂₆NO₂) which may be 4-hydroxy-2-nonylquinoline-N-oxide, with establishment of structure via ease of loss of the oxygen atom on the nitrogen (40). The peak of m/z 270.1862 (C₁₈H₂₄NO₂) is of dehydrated, 4-hydroxy-2-nonylquinoline-N-oxide, and a peak at m/z 244.1724 (C₁₆H₂₂NO) is pseudan VII, and finally, a peak at 260.1667 (C₁₆H₂₂NO₂ formula) is 3,4-dihydroxy-2-heptylquinoline. Absent from the spectra is dehydrated, 4-hydroxy-2-heptylquinoline-N-oxide which would have a molecular weight of 242. This absence suggests that the position of the second oxygen atom in the two compounds, i.e., the C7 compound and the C9 compound differs, in that one is a protonated N-oxide, as in the C9 compound, and the other a hydroxyl group. Hence, the peak at 260.1667 is that of 3,4-dihydroxy-2-heptylquinoline. (iii) GC-MS was also carried out using the whole inclusion bodies, and using the blue fluorescent spot eluted from the thin layer chromatogram. The total ion chromatogram (TIC) of the whole inclusion bodies revealed about 20 major peaks. Pseudan VII was identified at 23.54 minutes, and pseudan IX was identified at 23.90, 24.02, and 25.20 minutes. 4-Hydroxy-2-nonylquinoline-N-oxide and dehydrated 4-hydroxy-2-nonylquinoline-N-oxide were also evident. The TTC did not reveal the presence of 3,4-dihydroxy-2-heptylquinoline, but did show the presence of dehydrated 3,4-dihydroxy-2-heptylquinoline. Also present in the TIC at 23.54 minutes as well as in other peaks is 4-hydroxy-2-methylquinoline, or pseudan I. Figure 7 is the mass spectrum of the 23.54 minute peak showing pseudan I, m/z 159. The TIC of the whole inclusion bodies also revealed m/z 56 in several peaks, which may be iron or an organic fragment, (iv) The TIC of the blue fluorescent spot eluted from the thin layer chromatogram and dissolved in methylene chloride exhibited one major peak and about 10 minor peaks. The mass spectrum of the most intense peak at 15.26
minutes reveals a C9 compound. A peak at 18.59 minutes is less intense, but has a mass spectrum similar to that of the 15.26 peak, and additionally has a fragment of m/z 241 which corresponds to dehydrated 3,4-dihydroxy-2-heptylquinoline. The TIC of the eluted blue fluorescent spot dissolved in methanol contained peaks of 15.26 and 18.59 minutes as above.

The mass spectrum of the intense 15.26 peak was identical to that of the 15.26 peak above. The mass spectrum of the 18.59 peak showed a peak at m/z 159, the molecular weight of pseudan I. Peaks at 23.17 and 29.63 minutes revealed the presence of a m/z peak of 269, the molecular weight of dehydrated, 4-hydroxy-2-nonylquinoline-N-oxide.

A $^1$H NMR spectrum of whole inclusion bodies reconfirms the presence of pseudan I, pseudan VII, and pseudan IX. The pseudan I assignments are: $\delta$ 2.34 (s, 3 H, CH$_3$ of 1), 6.01 (s, 1 H, H$^3$ of 1), 7.21 (t, $J = 7$ Hz, 1 H, H$^{6or7}$ of 1), 7.48 (m, 1 H, H$^8$ of 1), 7.53 (m, 1 H, H$^{6or7}$ of 1), 8.16 (d, $J = 8$ Hz, 1 H, H$^5$ of 1), 11.35 (br s, 1 H, OH of 1) (Fig. 8). The pseudan Vn and pseudan IX assignments in the spectrum are: $\delta$ 0.85 (t, $J = 6$ Hz, 3 H, CH$_3$ of 2 and 3), 1.28 (m, 10 H, H$^{14}$ of 2 and H$^{1W6}$ of 3), 1.67 (m, 2 H, H$^{10}$ of 2 and 3), 2.56 (t, $J = 8$ Hz, 2 H, H$^9$ of 2 and 3), 6.03 (s, 1 H, H$^3$ of 2 and 3), 7.21 (t, $J = 7$ Hz, 1 H, H$^{6or7}$ of 2 and 3), 7.48 (m, 1 H, H$^8$ of 2 and 3), 7.53 (m, 1 H, H$^{6or7}$ of 2 and 3), 8.16 (d, $J = 8$ Hz, 1 H, H$^5$ of 2 and 3), 11.21 (br s, 1 H, OH of 2 and 3) (Fig. 8). These assignments were made by comparison with literature values for synthetic compounds and spectra done in our laboratory on synthetic compounds (29, 35).

The NMR spectrum exhibits pseudan I, pseudan VII, and pseudan IX in relative molecule-based amounts of 1.00, 0.99, and 1.10. A second $^1$H NMR spectrum was taken of the blue fluorescent spot eluted from the thin layer plate. The spectrum showed broad and sharp peaks indicative of two or more chemical species exchanging at an intermediate rate as might occur if ligands were being attached and detached from Fe$^{3+}$, or alternatively, a paramagnetic metal ion.
attached to and broadening organic ligands. Absence of a peak in the 11 ppm OH region means the absence of hydroxyls: any hydroxyl present, though, may have been deprotonated.

EPR of the inclusion bodies was carried out to determine the oxidation state of the iron. The signal is centered at $g = 4.2$ and has a width of 215 Gauss. This is consistent with the $M_s = \frac{3}{2}$ doublet of $\text{Fe}^{3+}$ in a low symmetry environment which would ideally give $g = 30/7$ (4.3). The line is reasonably symmetric, unlike that expected for a powder pattern. This may suggest that the spin is somewhat delocalized and that the width is due to unresolved hyperfine interactions with surrounding hydrogen atoms. The $g = 4.2$ value puts the *Pseudomonas* compound in the same range as ferrichrome and ferrichrome A from *Ustilago sphaerogena*, and FeCl$_3$ in excess aqueous sodium citrate (11). The EPR work was not done in solution, and as a result the environment around the $\text{Fe}^{3+}$ is not likely to be changed by solvent substitution.

**TEMs reveal periplasmic inclusion bodies.** TEMs of the 5 h and 24 h cells were taken to determine the source of the aggregated, pseudan-containing particles. The aggregates may consist of outer membrane vesicles containing pseudan or instead may be released inclusion bodies containing pseudan. TEMs of the 5 hr culture did not show any unusual cell structures. However, the TEMs of the 24 h culture showed dark inclusion bodies (Fig. 9). Figure 9A shows a cell with multiple inclusion bodies, some of which are inside vacuoles, and a cell with one visible inclusion body. Figures 9B through 9E each show a cell containing one large inclusion body of range 53 to 100 run in diameter. Each inclusion body in Figures 9B through 9E is within a vacuole within a cell. Figure 9B reveals that a cell may have more than one vacuole. Figures 9B and 9C show that these vacuoles are within the periplasm of the cells. In Figure 9D, the inclusion body is seen within a smaller vacuole that is shown inside the large vacuole: in Figure 9E the vacuole that the inclusion body is in appears to contain a smaller vacuole. No outer
membrane vesicles were seen being shed from the cell or detached from the cells in these TEMs. An electron micrograph of the pellet seen in Figure 5 revealed dark aggregates, the individual components of which range 25 to 50 nm in diameter (Fig. 10). Membrane fragments are also seen.

**SEM-EDAX reveals that the intracellular inclusion bodies** contain iron. To verify that the inclusion bodies seen inside the cells are indeed iron-containing and hence are the iron-containing pelleted aggregates isolated from the cells, an elemental analysis of intracellular inclusion bodies was compared with that of cells lacking inclusion bodies. Figure 11A shows the back-scattered electron image of 24 hour old 15692d cells, one of which contains a bright area. The elemental analysis of a whole cell itself shows large amounts of carbon and phosphate, and modest amounts of other elements (Fig. HB). The elemental analysis of the bright area reveals a high concentration of iron in addition to the elements found in the cell itself (Fig. HC). The two iron peaks identify the types of x-rays produced: the higher energy peak is a K-beta peak in that it represents the x-rays emitted by M-shell electrons going to the K-shell, and the lower energy peak is a K-alpha peak representing x-rays emitted from electrons going from the L-shell to the K-shell.

Iron **efflux occurs via the inclusion bodies**. Is the iron efflux seen in Figure 2 mediated by the inclusion bodies? To answer this question, each substrain was harvested during a 48 h period including the time of iron efflux seen in Figure 2. At each time period, total iron concentration in the pellet of cells was measured, as was the iron in inclusion bodies isolated from the spent media. It is seen in both 15692a and 15692d cells that as total cell iron increased, the iron of inclusion bodies collected from the spent media also increased. As total cell iron decreased in the 15692a cells, inclusion body iron also decreased. However, this decrease in inclusion body iron
with a decrease in total cell iron did not occur in the 15692d cells. In similar studies using the 15692b cells, the iron in inclusion bodies was immeasurable using the conditions described, indicating that this substrain failed to release significant levels of inclusion bodies or that released inclusion bodies were solubilized upon being released.

The inclusion bodies prevent cell adherence. When doing efflux experiments, it was noted that transfer of washed cells to buffer or to fresh media resulted in cell aggregation. Such aggregation was minimized, however, when washed cells were transferred to spent media that contained inclusion bodies. We therefore did adhesion assays to determine if the inclusion bodies played a role in preventing cell aggregation. Figure 13 shows that with each substrain, an inverse relationship exists between cell adherence and extractable levels of pseudan, in that as the level of extractable pseudan increased with time of growth, cell adherence decreased. Also it is seen that the 15692b cells that produce the least amount of extractable pseudan show the greatest amount of cell adherence. To determine if the inclusion bodies were playing a role in preventing cell adherence, logarithmic phase 15692b cells, cells from which pseudan is not extractable and which are not producing collectible inclusion bodies, were incubated with inclusion bodies. It is seen in Figure 14 that addition of inclusion bodies to these cells resulted in a decrease in attachment of the cells to the wells.
DISCUSSION

The purpose of this research was to determine the role pseudan VII and IX play in iron transport and metabolism in *P. aeruginosa*. The earlier finding that these compounds are located on the membrane of iron-rich, but not of iron-poor cells suggested that they may play a role in the uptake of iron under iron replete conditions (32). Alternatively, pseudan VII and IX may serve to store iron, keeping excess iron out of the cytoplasm, and by so doing, serve to prevent iron toxicity. When it was learned that total cell iron decreased in late logarithmic phase of iron-rich cells, and that iron efflux was causing this decrease, a role of pseudan VII and IX in iron efflux was investigated.

Upon learning that the dark particulate material in the pellets of the iron-rich cells contained iron and had a UV spectrum similar to that of purified pseudan VII and IX, our attention turned to the chemical analysis of these particles. Iron is present in the isolated inclusion bodies as shown by atomic absorption spectroscopy and EPR which indicates Fe\(^{+3}\). We have shown by NMR and GC-MS that the whole inclusion bodies contain pseudan I, VII, and IX. Pseudan I, m/z of 159, appears multiple times in the GC mass spectra. It is possible that it is a fragment formed in the mass spectrometer as occurs in synthetic samples of pseudan VII and IX (29). However, in the NMR, fragmentation does not occur, and hence, pseudan I is a constituent of the inclusion bodies. Others have previously identified pseudan I in *P. aeruginosa* (40). The 1:1:1 stoichiometry of pseudan I, VII, and IX is suggesting the complexation ratio of chelators to iron. The ESI mass spectrum also shows the presence of pseudan VII and IX. GC-MS of the whole inclusion bodies indicates dehydrated 3,4-dihydroxy-2-heptylquinoline, and ESI MS shows the parent 3,4-dihydroxy-2-heptylquinoline. GC-MS and ESI MS of the whole inclusion bodies show dehydrated 4-hydroxy-2-nonylquinoline-N-oxide, and GC-MS shows 4-
hydroxy-2-nonylquinoline-N-oxide itself. The eluted blue fluorescent spot on the thin layer plate contains the two dehydrated compounds discussed in this paragraph. From these analyzes, we conclude that the inclusion bodies contain iron, pseudan I, pseudan VII, pseudan IX, 4-hydroxy-2-nonylquinoline-N-oxide, and 3,4-dihydroxy-2-heptylquinoline, the PQS. The dehydrated compounds detected are produced in the instruments.

Electron microscopy of mid-logarithmic phase cells revealed dark inclusion bodies in vacuoles of the cells, inclusion bodies absent in the early logarithmic phase cells. Further examination of the TEMs revealed that the inclusion bodies were in the periplasm of the cells. The TEMs did not reveal blebs on the outside of the cells, indicating that under these conditions of growth the cells were not producing outer membrane vesicles as reported by others to occur in *P. aeruginosa* (17, 18, 22, 31). Also, the TEM of the pellet of inclusion bodies showed no unit membrane around the aggregated inclusion bodies. Hence, we conclude that the aggregates are inclusion bodies, and not outer membrane vesicles. The TEM of the inclusion body pellet, however, did show filterable membrane fragments. These membranes may be outer membranes fragmented upon release of the inclusion bodies from the periplasm of the cell, or cytoplasmic membranes from cell lysis that may be occurring. At this time, the method of formation of the inclusion bodies and the role that any membranes may play in such formation are not known. The finding of multiple small inclusion bodies in cells (Fig. 9A) may suggest that the larger inclusion bodies result from the coalescing of the smaller bodies. It is seen that one cell may have more than one periplasmic vacuole (Fig. 9B). The smaller vacuoles found within the larger vacuoles (Fig. 9D and 9E) may be cross sections of vacuoles on the opposite side of the cell invaginated in. However, close examination of these vacuoles does not reveal a membrane with
a bilayer structure, and hence, at this time the origin and composition of these membranous-like
structures is not known. It is also noted that inside the periplasmic vacuoles the inclusion bodies
are larger, 42 to 100 nm, than are the pelleted inclusion bodies of 25 to 50 nm in diameter. Also,
the pelleted inclusion bodies are irregular in shape, unlike the rounded, intracellular inclusion
bodies. Solubilization of the inclusion bodies, as discussed below, may be occurring during the
procedure used to collect the extracellular inclusion bodies. We also note the ring-shaped
structures in the cytoplasm of cells in Figure 9, and question the role of these structures in overall
cell physiology and metabolism.

The elemental analysis of the back-scattering image of 24 hrP. aeruginosa 15692d cells
produced by SEM-EDAX shows a dense iron-containing area below the surface of the cell (Fig.
11). This leads us to conclude that the iron-containing aggregates pelleted from the filtered, spent
media of cells consists of the cellular inclusion bodies. It is apparent from these studies that the
inclusion bodies are released from the cell, and enter the environment of the cell. At this time, it
is not known if the aggregates of inclusion bodies we see in the pelleted cells (Fig. 4) are caused
by the centrifugation of the cells, or if aggregation occurs naturally in the medium. In any case, it
is seen that with population growth, the three substrains of P. aeruginosa differ in 1) total
cellular iron concentration, 2) the time of decrease in total cell iron, and 3) the levels of
extractable pseudan. However, the pattern of the relationship of these parameters is the same in
the three substrains used: as cell density increases, total cell iron increases until mid-logarithmic
phase, and then decreases, and also, as cell density increases, extractable pseudan increases. It is
interesting to note that inclusion bodies are constantly being released from the 15692a and
15692d cells, even during early log phase, even though periplasmic inclusion bodies are not
observable in TEMs at this time. The decrease in inclusion bodies seen late in log phase of the
15692a cells suggests that the inclusion bodies are being solubilized. This is observed visually:
pellets of cells of cultures over 55 hr old, have fewer aggregates of inclusion bodies than do
pellets of younger cells.

It was evident from our studies that the three substrains of cells have different adhesion
properties. Early log phase 15692b cells consistently showed more adhesion to microliter plate
wells than did the other two substrains at the same time of growth phase, leading us to
hypothesize that the inclusion bodies found in the spent medium of substrains 15692a and
15692d either prevent attachment of cells to the wells, or, alternatively, that the inclusion bodies
promote the release of cells from the well after attachment. Addition of inclusion bodies to
15692b cells in spent media did reduce the final number of cells attached to the microtiter plate
wells. Since controls consisting of only inclusion bodies in either MOPS buffer or spent media
did not show significant attachment to the wells, the decrease of adherence by physical
interference is most likely not occurring. Perhaps cell surface rhamnolipid, a surfactant
produced by P. aeruginosa late in growth (16), is solubilizing the inclusion bodies in vivo, and
by so doing, releasing a compound(s) that in turn either decreases adherence, or promotes
detachment of the cells from a surface. Work in our lab has shown that rhamnolipid does indeed
solubilize the inclusion bodies as indicated by thin layer chromatography. It has recently been
reported by others that variants of P. aeruginosa exhibit increased detachment from a biofilm,
and that this detachment requires rhamnolipids (3). It was suggested that the variant produces a
secreted factor that causes detachment of sessile cells (3). The inclusion bodies isolated here may
indeed be that factor. Our results show that young cultures of all three substrains of cells adhere
to well plates, but as cultures go into late logarithmic phase, total cell adherence decreased as
extractable pseudan increased (Fig. 13). The decrease in total cell adherence we see may be the
result of solubilization of inclusion bodies by rhamnolipids with subsequent detachment of cells from the surface of the wells. This would be of advantage to the bacterium: as cell density in a biofilm increased, sessile cells would detach, become planktonic cells, and be free to relocate downstream to initiate a new colony of sessile cells. In any event, our results show that inclusion bodies play a role in biofilm formation by P. aeruginosa in a high-iron environment.

A recent publication showing TEMs of P. aeruginosa PAO1 biofilms reveals cells containing dark inclusions throughout the biofilm profile (14). The possibility exists that those inclusions are indeed the inclusion bodies reported here. As well, the particulate matter found to be an integral part of the exopolymeric substance shown in the TEMs may be released inclusion bodies (14).

The chemical analyses presented here show that the inclusion bodies contain the PQS, and hence play a role in cell-to-cell communication, and consequently, virulence factor regulation. The inclusion bodies also contain pseudan VII (4-hydroxy-2-heptylquinoline) the precursor to PQS. It has been shown by others that 4-hydroxy-2-heptylquinoline is synthesized and released from P. aeruginosa, and then is taken up by nearby cells where it is converted to PQS (9). It was proposed that the monooxygenase that hydroxylates the 4-hydroxy-2-heptylquinoline to form PQS may be periplasmic (9). Localization of the enzyme in the periplasm would give credence to this proposal. However, to enter another cell and be converted to the PQS, the precursor must be released from the inclusion bodies. Others have shown that rhamnolipid stimulates the uptake of hydrophobic compounds by P. aeruginosa (26). It has also been shown that the bioactivity of the PQS is increased by rhamnolipid (4). We suggest again that cell-surface solubilization of the inclusion bodies occurs via the rhamnolipids. Such solubilization would serve to release the precursor of PQS, to be converted to PQS, to be used
with the packaged PQS as a signaling molecule. Solubilization of the inclusion bodies also
results in the release of antimicrobial compound, pseudan VII and IX, and, of course, to recycle
iron. We propose that the decrease in inclusion bodies as seen in Figure 12 is due to
solubilization by rhamnolipids.

It has recently been reported that *P. aeruginosa* strain PA14 packages the PQS as well as
4-hydroxy-2-heptylquinoline and 4-hydroxy-2-nonylquinoline into outer membrane vesicles
(22). Differences in growth conditions, e.g., media components, aeration, and temperature of
growth as well as cell strain, may account for the apparent discrepancy between those findings
and our results reported here. The TEMs of cultures producing inclusion bodies in our study
failed to show the presence of outer membrane vesicles as reported (17, 18, 22, 31). While the
TEM of pelleted aggregates does show some membrane fragments, the preponderance of
material is not membrane-bound.

The question arises as to the source of iron complexed to the pseudans in the periplasmic
space. We know from earlier studies that pseudan VII and pseudan IX are isolated from the
cytoplasmic membrane of the cell, not the outer membrane (32). Is the iron found in the
inclusion bodies of extracellular or intracellular origin? What is the orientation of the pseudans
in the cytoplasmic membrane? If the functional groups face the periplasm, a means of
entrapping exogenous iron after passage through the outer membrane of the cells is evident.
Also, do the chelators in the periplasmic inclusion bodies bind additional iron? Sequestration of
exogenous iron in the periplasm, either by membrane-bound pseudan or by inclusion body-
bound pseudan would keep iron out of the cytoplasm, and hence serve to prevent iron toxicity.
We know from the EPR studies that the iron in the inclusion bodies is oxidized. Under the
aerobic growth conditions used here, extracellular iron is oxidized as well. It is unlikely that this
insoluble form of iron freely passes through the outer membrane of the cells. The possibility
exists that exogenous Fe³⁺ is reduced by the extracellular iron reductase described by others (41),
allowing for the passage of Fe²⁺ through the outer membrane. The periplasmic multicopper
oxidase of *P. aeruginosa* may consequently oxidize the Fe²⁺ to Fe³⁺ (15).

A distinct advantage of the periplasmic location of the inclusion bodies deals with the
release of the inclusion bodies from the cell. The large size of the inclusion bodies precludes the
use of outer membrane components of drug efflux pumps used to release small drugs from gram-
negative bacteria (25). The TEM of the pelleted inclusions bodies shows membrane fragments,
perhaps suggesting that the inclusion bodies are released from the cells upon breaking the outer
membrane. If that is the case, the periplasmic location of the inclusion bodies provides the cell
with a means to release the HAQs and iron without killing the cell.

The iron-containing inclusion bodies identified by this work are unique to prokaryotic
cells. They are not depots of a carbon or energy source, but rather bestow upon the cell several
specialized functions: by serving as vehicles of iron efflux in high iron environments, the
inclusion bodies prevent iron toxicity; by containing antimicrobial compounds, the inclusion
bodies ward off competitors within an environment; by containing a signaling compound as well
as its precursor, the inclusion bodies contribute to population synchrony and virulence; and by
preventing cell adherence, the inclusion bodies allow for biofilm spread. Indeed, these are novel
multifunctional structures.
REFERENCES


FIGURE LEGENDS

FIG. 1 Chemical structures of relevant HAQs.

FIG. 2. Cell growth, total cell iron, and extractable pseudan in three substrains of *P. aeruginosa*: A) 15692a; B) 15692b; C) 15692d. At each time period, the optical density was determined, and two aliquots of 25 X 10^9 cells were collected to determine concentrations of total cell iron and extractable pseudan.

FIG. 3. Efflux of [FeCl₃] from substrain 15692d. A) cell density; B) total cell [Fe]; C) [Fe] in 200 µl of spent media before transfer of cells (•) and in the filtrate after transfer of cells (○).

FIG. 4. Pellets of 15692d cells grown in TSB for A) 5 h and B) 24 h.

FIG. 5. Pellet resulting from the ultracentrifugation (265,000xg/2 h) of filtered spent media of a 24 h culture of *P. aeruginosa* 15692d.

FIG. 6. The high resolution electrospray mass spectrum of whole inclusion bodies.
FIG. 7. Electron impact mass spectrum of the 23.54 minute peak of the GC-MS of whole inclusion bodies. The sample was dissolved in methanol.

FIG. 8. Numbering scheme for the 1H NMR spectra of (1) a methyl quinoline, (2) a heptyl quinoline, and (3) a nonyl quinoline.

FIG. 9. TEMs of 24 h old 15692d cells. Multiple inclusion bodies are seen in one cell in (A). Single inclusion bodies are seen in cells in (B through E). Bars = 200 µm.

FIG. 10. TEM of the pellet seen in Figure 5. Arrows point to membrane fragments. Bar = 200 µm.


FIG. 12. Cell growth, total cell iron, and iron in inclusion bodies in A)15692a and B) 15692d cells. Total cell iron in 25 X 10⁹ cells and in washed inclusion bodies was measured after acid hydrolysis.

FIG. 13. Adherence of cells to polystyrene microtiter plate wells and levels of extractable pseudan. The optical density at 600 nm is the average of solubilized crystal violet from three
FIG. 14. Adherence of A) 5 hr, B) 25 hr, and C) 30 hr 15692b cells to polystyrene microtiter plate wells in the (■■■) absence and presence of inclusion bodies containing (Ezza) 31 µg iron and (Ezzzz) 62 µg iron. The optical density at 600 nm is the average of solubilized crystal violet from three wells after 60 min incubation. Bars represent the range.
n = 0  4-hydroxy-2-methylquinoline
(pseudan I)
n = 6  4-hydroxy-2-heptylquinoline
(pseudan VII)
n = 8  4-hydroxy-2-nonylquinoline
(pseudan IX)

n = 6  4-hydroxy-2-heptylquinoline-N-oxide
n = 8  4-hydroxy-2-nonylquinoline-N-oxide

n = 6  3,4-dihydroxy-2-heptylquinoline
n = 8  3,4-dihydroxy-2-nonylquinoline
We Claim:

1. A method of inhibiting the growth of a biofilm on a surface comprising applying to the surface an effective amount of pseudan iron-inclusion bodies, wherein said pseudan inclusion bodies comprise iron, 3,4-dihydroxy-2-heptylquinoline, and at least one pseudan species selected from 4-hydroxy-2-alkylquinolines in which the alkyl group contains 1-12 carbon atoms.

2. A method according to claim 1, wherein said at least one pseudan species is selected from 4-hydroxy-2-alkylquinolines in which the alkyl group contains 7-12 carbon atoms.

3. A method according to claim 1, wherein said at least one pseudan species is from 4-hydroxy-2-heptylquinoline.

4. A method according to claim 1, wherein said at least one pseudan species is from 4-hydroxy-2-nonyllquinoline.

5. A method according to claim 3, wherein said pseudan iron-inclusion bodies contain 4-hydroxy-2-nonyllquinoline.

6. A method according to claim 1, wherein said pseudan iron-inclusion bodies contain Fe^{3+}, 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, 4-hydroxy-2-nonylquinoline, 3,4-dihydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline N-oxide.

7. A method according to claim 1, wherein said biofilm is a biofilm produced by bacteria of the Pseudomonas genus.

8. A method according to claim 1, wherein said biofilm is a biofilm produced by Pseudomonas aeruginosa.

9. A method of inhibiting the growth of a biofilm on a surface comprising applying to the surface an effective amount of pseudan iron-inclusion bodies,
wherein said pseudan inclusion bodies comprise $\text{Fe}^{3+}$, 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, 4-hydroxy-2-nonylquinoline.

10. A method according to claim 9, wherein said surface is a biotic surface.

11. A method according to claim 9, wherein said pseudan inclusion bodies further comprise 4-hydroxy-2-nonylquinoline N-oxide.

12. A method according to claim 10, said biofilm is in the lungs of a cystic fibrosis patients.

13. A method of preventing the growth of a biofilm on a surface, biotic or abiotic, comprising contacting the surface with an effective amount pseudan inclusion bodies wherein said pseudan inclusion bodies comprise iron, 3,4-dihydroxy-2-heptylquinoline, and at least one pseudan species selected from 4-hydroxy-2-alkylquinoline in which the alkyl group contains 1-12 carbon atoms.

14. A method according to claim 13, wherein said at least one pseudan species is selected from 4-hydroxy-2-alkylquinolines in which the alkyl group contains 7-12 carbon atoms.

15. A method of preventing the growth of a biofilm on a surface comprising applying to the surface an effective amount of pseudan iron-inclusion bodies, wherein said pseudan inclusion bodies comprise $\text{Fe}^{3+}$, 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline.

16. A composition comprising isolated pseudan inclusion bodies optionally in combination with at least one pharmaceutically acceptable carrier, wherein said pseudan inclusion bodies comprise $\text{Fe}^{3+}$, 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline.

17. A composition according to claim 16, wherein said pseudan inclusion bodies further comprise 4-hydroxy-2-nonylquinoline N-oxide.
18. A composition according to claim 16, further comprising at least one additional antibacterial agent.

19. An anti-bacterial pseudan composition comprising pseudan I (4-hydroxy-2-methylquinoline), pseudan VII (4-hydroxy-2-heptylquinoline), pseudan IX (4-hydroxy-2-nonylquinoline), and optionally Fe$^{3+}$.

20. A coated or impregnated medical device comprising a medical device in which at least one surface thereof is coated or impregnated with pseudan inclusion bodies, wherein said pseudan inclusion bodies comprise Fe$^{3+}$, 4-hydroxy-2-methylquinoline, 4-hydroxy-2-heptylquinoline, and 4-hydroxy-2-nonylquinoline.

21. A coated or impregnated medical device comprising a medical device in which at least one surface thereof is coated or impregnated with an anti-bacterial pseudan composition comprising pseudan I (4-hydroxy-2-methylquinoline), pseudan VII (4-hydroxy-2-heptylquinoline), pseudan IX (4-hydroxy-2-nonylquinoline), and optionally Fe$^{3+}$.
FIG. 8.