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Relationship between Oxygen and Siderophore Synthesis in *Pseudomonas aeruginosa*

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Abstract. *Pseudomonas aeruginosa* strain PAO1, growing in low-iron medium, produces two siderophores, pyochelin and pyoverdin, in massive bursts as the culture shifts from logarithmic phase to stationary phase. Two medium components, oxygen and iron, prolonged the logarithmic phase when they were added to the medium. Oxygen and iron appeared to be in demand during this period because, as heme synthesis increased in response to the low oxygen concentration in the medium, a situation resulting from the high density of bacteria present in the medium during late log phase, the iron content of the bacteria decreased. These phenomena resulted in the production of massive amounts of siderophores late in the log phase to supply iron for the increased heme synthesis.

Pseudomonas aeruginosa possesses a complex array of cytochromes which participate in oxygen-dependent respiration [1, 10, 12]. The heme components of these cytochromes must account for a portion of the iron content of the bacteria and the iron demand that has characterized this bacterium [18]. In order to obtain extracellular iron to meet this demand, bacteria produce iron chelators called siderophores [13]. *Pseudomonas aeruginosa* produces and utilizes two siderophores, pyochelin [4, 5] and pyoverdin [3, 19]. These siderophores are released from cells, bind iron, and the resultant ferrisiderophores are substrates for high-affinity iron transport [2, 3]. A large burst of siderophore synthesis has been noticed in iron-deficient medium in the later portion of the logarithmic phase of growth. This report describes this burst of siderophore synthesis in relation to the oxygen concentration, iron accumulation from the culture medium, and to the iron demand for heme synthesis under conditions of low aeration.

Materials and Methods

Culture conditions and bacteria. Succinate minimal medium (SMM) [4] consisted of 20 m*M* succinate, 40 m*M* NH_4Cl, 0.4 m*M* $MgCl_2$, 5 m*M* K_2SO_4, and 5 m*M* potassium phosphate buffer. Glucose minimal medium (GMM) consisted of the same ingredients except that 20 m*M* glucose replaced the succinate. Casamino acids (CAA) medium consisted of 0.5% Casamino acids and 0.4 m*M* $MgCl_2$. These media were autoclaved and supplemented prior to inoculation to yield the following final concentrations: 5 m*M* potassium phosphate buffer (pH 7.4), 1 μM $MnCl_2$, 1 μM $CaSl_4$, 1 μM $ZnCl_2$, 1 μM $FeCl_3$, and 5 m*M* morpholinorpopane sulfonate (MOPS) buffer (pH 7.4). Cultures were either 1-liter quantities in 2.8-liter Fernbach flasks or 7-liter quantities in a 9-liter culture bottle. Flasks were shaken at 200 rpm, and the bottle was stirred with a magnetic stir bar and aerated by an air stream directed against the bar. Incubations were conducted at 37°C.

Assay of bacterial products, components, and metabolism. Medium was inoculated with between 10^3 and 10^6 bacteria/ml and was stirred with a Teflon-coated stir bar to yield a vortex which extended from the surface of the medium to the stirring bar. Samples of 3 ml were withdrawn at intervals for determining the absorbance at 600 nm in order to estimate bacterial growth. Another 2-ml sample was taken for the determination of pyoverdin. This aliquot was centrifuged to remove bacteria and was diluted sufficiently in 0.05 *M* Tris–HCl buffer, pH 7.4, to be on the linear portion of the concentration curve during the measurement of the fluorescence at 460 while exciting at 400 nm with an Aminco spectrofluorometer. The fluorometer was calibrated with quinine sulfate. An additional 20 ml of the culture was removed, acidified to pH 2.5 with 1 *M* HCl, and was extracted with ethyl acetate. Pyochelin concentrations in these extracts were determined by fluorescence [4].

Bacterial cytochromes were determined by collecting 200-ml quantities of medium, harvesting the bacteria by centrifugation, and extracting the hemochromes from the pellets with acetic acid and ethyl acetate as described by Falk [7]. Pyridine (0.075 *M*) was added to make the pyridine hemochromes, and NaOH (0.25 *M*) was added just prior to measurement of the dithionite-reduced (crystals) minus $K_3Fe(CN)_6$-oxidized (0.05 ml of 3×10^{-3} *M*) spectra with a Cary 15 spectrophotometer.

Dissolved oxygen in the culture medium was assayed by

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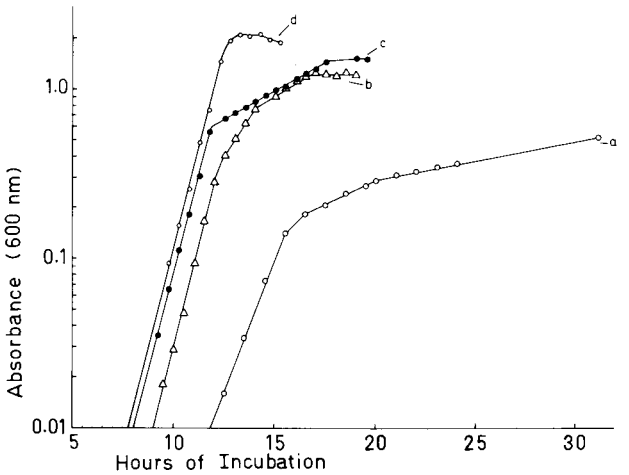

Fig. 1. Effects of iron on the appearance of the growth curve in SMM containing no added $FeCl_3$ (a), 0.1 μM $FeCl_3$ (b), 1 μM $FeCl_3$ (c), and 10 μM $FeCl_3$ (d). Washed bacteria were inoculated into 1-liter quantities of medium in Fernbach flasks at 10^3 cfu/ml, the flasks were shaken at 200 rpm, and growth was measured by absorbance at 600 nm.

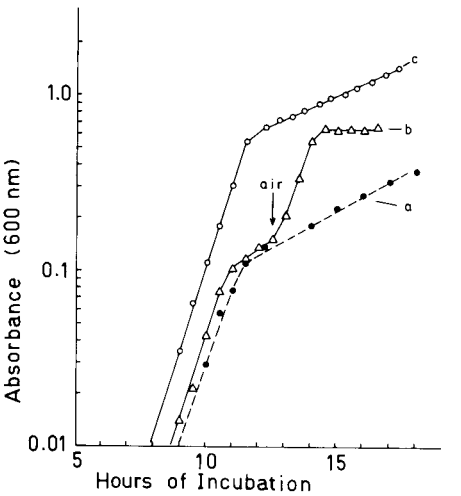

Fig. 2. Effect of aeration on the appearance of the growth curve in SMM containing 0.1 μM $FeCl_3$ receiving aeration from an air stream of 50 ml/min which was directed against a stirring bar, curve a. An increase in the aeration rate to 1000 ml/min resulted in the increased growth at the arrow (air), curve b. A consistent air stream of 1000 ml/min resulted in curve c. Bacteria were inoculated as in Fig. 1.

placing a YSI oxygen electrode 1 cm below the surface of the medium halfway between the middle of the vortex and the wall of the bottle. Oxygen uptake rates by bacteria were measured [15] by removing 100-ml quantities of culture medium at various times and harvesting the bacteria by centrifugation. The bacteria were washed once with distilled water and resuspended in potassium phosphate buffer (4 mM, pH 7.4) to an absorbance of 0.2 for uptake measurements.

Methods for determining mitochondrial iron were employed [6] with sonication in two 15-s bursts (250 W, Heat Systems) to expose the bacterial contents to the assay reagents. Iron accumulation by bacteria during growth was measured by incubating 50-ml quantities of SMM containing 0.01 μCi $^{55}FeCl_3$/ml which had been inoculated with 10^6 bacteria/ml in 250-ml Erlenmeyer flasks; 1-ml aliquots were removed at various times and poured through 0.45-μm pore-size filters which were under vacuum. The bacteria trapped on the membranes were washed with 5 ml of 1% thioglycolate to remove iron precipitates, then with 5 ml distilled water, and were finally dried and measured for accumulated iron as described previously [2].

Results

Effects of iron and oxygen on the growth curve. *Pseudomonas aeruginosa* strain PAO1 displayed slow growth in SMM which had no added iron (curve a, Fig. 1). The growth curve appeared to assume a slower rate of logarithmic growth at approximately 15 h of incubation instead of entering the stationary phase. Determinations of the viable numbers of bacteria also revealed the onset of a slower, logarithmic growth curve at the same time registered by absorbance measurements. Bacterial growth was stimulated, and the second, slower growth rate was modified by successive additions of iron (Fig. 1). The same phenomena were observed in CAA medium and GMM.

Other minerals added in place of iron had no effects (data not shown), but increasing the aeration rate did affect the second rate of growth. A stream of air was regulated to 50 ml/min to a culture containing 0.1 μM $FeCl_3$, and growth curve a in Fig. 2 was obtained. An identical experiment was conducted until the culture entered the slower growth rate, at which point the air flow rate was increased to 1000 ml/min. The growth curve immediately assumed the original log rate (arrow, curve b, Fig. 2). Curve c in Fig. 2 demonstrates the appearance of the growth curve when the culture was routinely aerated at 1000 ml/min. Oxygen could reproduce the effects of air, but could only be used at flow rates of approximately 5.0 ml/min. If oxygen were bubbled at faster rates, 50 ml/min, the bacteria died immediately. Nitrogen or carbon dioxide gases caused a cessation of growth when sparged into the bottle.

Correlation of oxygen consumption and siderophore production. Strain PAO1 was grown in 7 liters of SMM containing 1 μM $FeCl_3$ and aerated at 100 ml/min. Aliquots of 50–100 ml of culture were removed at times to test the concentrations of siderophores

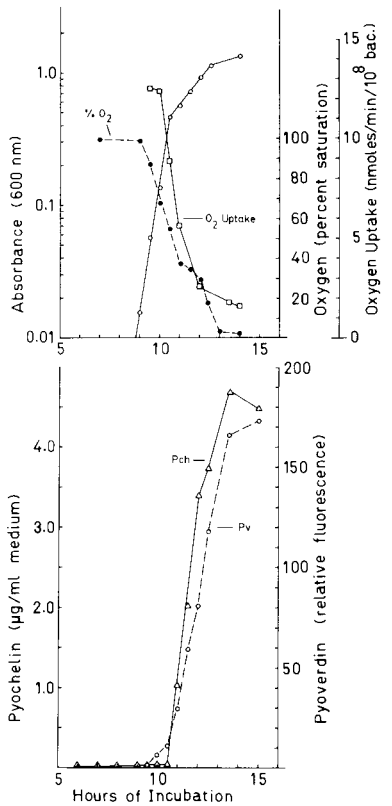

Fig. 3. Bacterial growth (open circles, top panel) in 7 liters of SMM containing 0.1 μM $FeCl_3$ aerated with 100 ml/min was measured by absorbance at 600 nm in relation to the oxygen concentration in the medium (%O_2) and the oxygen uptake rate by a suspension of 10^8 cfu/ml of washed cells taken from the growth medium at each time point. Siderophore synthesis (bottom panel) was measured by the fluorescence of pyoverdin in dilutions of growth media (Pv) and by the fluorescence of pyochelin which appeared in ethyl acetate extracts of growth media and subsequently in the extracts from silica scraped from spots where pyochelin should migrate on thin-layer plates (Pch).

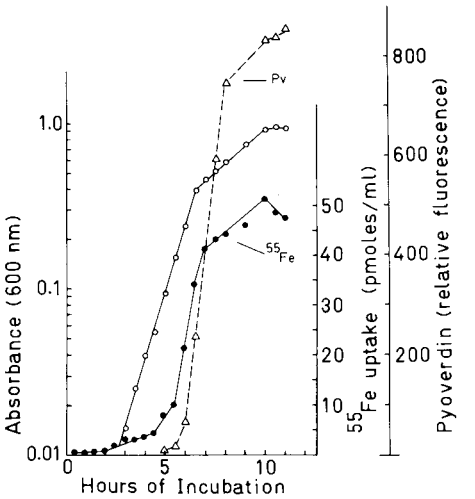

Fig. 4. Iron accumulation (^{55}Fe) was measured as the amount of radioactivity trapped with bacteria, not as precipitated iron because it resisted solubilization with 1% thioglycolate washes, on 0.45-μm pore-size filters from SMM containing 0.1 μM $FeCl_3$ and 0.01 μCi/ml ^{55}Fe. Bacterial growth (open circles) was measured by absorbance at 600 nm for cells inoculated at 10^6 cfu/ml in 100 of SMM in a 250-ml Erlenmeyer flask which was shaken at 200 rpm. Siderophore synthesis was measured by the fluorescence of pyroverdin (Pv) in dilutions of the growth medium.

produced and oxygen uptake rates by the bacteria. The bacteria demonstrated the second, slower growth rate (open circles, top of Fig. 3) which had been observed previously. The maximum synthesis of both pyoverdin (Pv) and pyochelin (Pch) occurred during the onset of the slower rate of growth (bottom of Fig. 3). This was at the same time that the oxygen concentration in the medium was falling (%O_2, top of Fig. 3). Respiration rates by bacteria harvested from the medium also appeared to be decreasing with the growth rate (O_2 uptake, top of Fig. 3). These data suggested that siderophores were produced in massive amounts during a shift in the growth rate that coincided with a depletion of oxygen from the growth medium and an apparent alteration in the respiratory mechanism of the bacteria.

Iron accumulation and siderophore synthesis. Iron accumulation by bacteria during growth was studied by inoculating bacteria at 10^6 cfu/ml into SMM containing 0.1 μM $FeCl_3$ and 0.01 μCi $^{55}FeCl_3$ per milliliter. The onset of maximal siderophore production (Pv, Fig. 4), which occurred during the onset of the slower rate of logarithmic growth (open circles, Fig. 4), coincided with an increase in the iron accumulation by the bacteria (^{55}Fe, Fig. 4).

Siderophore and heme synthesis. When siderophore synthesis was studied in CAA medium containing 1 μM $FeCl_3$, a procedure designed to allow greater bacterial harvests for the determination of heme contents, there was an onset of growth at 8 h (A600, top of Fig. 5) and a staggered synthesis of pyoverdin (Pv, top of Fig. 5), presumably owing to the elevated iron content of the medium. Although it has been reproducibly demonstrated that bacterial iron accumulation increases with siderophore production (as in Fig. 4), the concentration of bacterial iron per mg protein decreased during the slower phase of growth (iron, bottom of Fig. 5). Both hemochrome b and hemochrome c were extracted and measured as pyridine complexes to estimate the cytochrome contents. Hemochrome c (and hemochrome b, data not shown) increased in concentra-

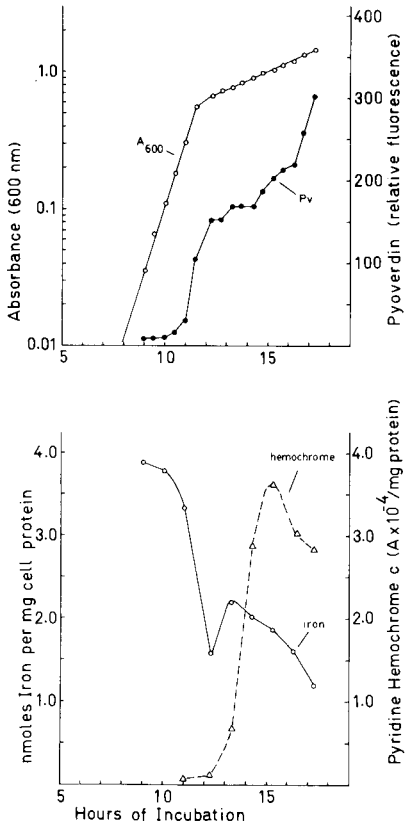

Fig. 5. Heme synthesis was measured in relation to bacterial growth (top panel, A600) in 7 liters of SMM containing 1 μM $FeCl_3$ being aerated at 100 ml/min and to siderophore synthesis (top panel, Pv) measured by the fluorescence of diluted medium. Heme content (bottom panel, hemochrome) was measured as the pyridine hemochrome c extracted from cells harvested at times of incubation in relation to the iron content of the bacteria, which was measured as the ferrous-bathophenanthroline complexes appearing in isoamyl alcohol extracts of sonicated cells (iron).

tion (hemochrome, bottom of Fig. 5) at the same time as the cellular iron content was decreasing.

Discussion

Prior to this study, it had been anticipated that siderophore synthesis would continue throughout lag and log phases so that there would be a steady accumulation of siderophores to support growth. However, the bursts of siderophore synthesis by *P. aeruginosa* late in log phase suggested that there was a critical bacterial demand for iron in this phase of growth. This burst of siderophore synthesis also coincided with a decrease in the growth rate. The slower growth rate often appeared to be linear by both absorbance and viable count measurements (Figs. 1 and 2), but it is possible that this phenomenon represents the entry of the cultures into stationary phase. Subsequent investigations indicated that essential factors became growth limiting in the later periods of the log phase. This growth-limiting situation was abrogated by the addition of iron to the medium (Fig. 1) or by increasing the aeration (Fig. 2). Oxygen could replace air to relieve the growth limitation, but oxygen also appeared to be toxic. The sensitivity of cultures to oxygen may have been due to a lack of the necessary enzymes that protect bacteria from the free radicals generated during respiration [8]. The sudden shift in oxygen concentration may not have allowed sufficient time for synthesis of the protective enzymes. The early cessation of growth noticed after the onset of sparging with air (curve b versus curve c, Fig. 2) may also have been due to the same oxygen toxicity.

A measurement of the oxygen concentration during the growth curve (top of Fig. 3) showed that it dropped precipitously at high bacterial densities during the slower rate of growth and during the burst of siderophore synthesis (bottom of Fig. 3). The coincidence of slower growth with the decreasing oxygen concentration, the increased siderophore synthesis, and the increased iron accumulation (Fig. 4) suggested that the levels of oxygen and iron may have resulted in a metabolic crisis for the bacteria. During the shift to slower growth rate there was a fourfold reduction in the level of bacterial iron per mg protein (Fig. 5), but a tenfold increase in heme concentration. Although a direct examination of bacterial cytochrome content was attempted, we were unable to quantitate cytochromes directly. Therefore, hemochromes were extracted from bacterial pellets [7, 16] and measured by spectrophotometry without the problems of light scattering by bacterial cells or membranes.

It is critical to note that other investigators, using a variety of techniques, have measured the same phenomenon; the cytochrome content of bacteria increases during periods of oxygen depletion. White [20] described the increase in cytochrome synthesis during the log to stationary phase shift of *Haemophilus parainfluenzae*. Lenhoff [11] described a similar phenomenon in *Pseudomonas fluorescens* when he discovered a relation between the oxygen and iron concentrations in media and the amounts of pyoverdin (fluorescein) and cytochrome synthesis. The same relation between oxygen and cytochrome content has been described in more detail in *Escherichia coli* [14] and *Pseudomonas putida* [17]. In addition to the concentrations, changes in the types of cytochromes and Km

values of the oxidases have been noted during these respiratory alterations brought on by oxygen deprivation [9]. This phenomenon may also have been observed during the present study as an alteration in the oxygen uptake rate per 10^8 bacteria (top of Fig. 3).

The coincidences of several physiological phenomena found in this study suggest that the bursts of siderophore synthesis (Figs. 3–5) occur in response to the iron demand for cytochrome synthesis during conditions of oxygen deprivation. We are continuing to study the control of siderophore synthesis by both iron and oxygen, using mutants defective in the control of siderophore synthesis.

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