Iron deficiency leads to inhibition of oxygen transfer and enhanced formation of virulence factors in cultures of *Pseudomonas aeruginosa* PAO1

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**Abbreviations:** CF, cystic fibrosis; \(k_a\), volumetric oxygen transfer coefficient; \(pO_2\), dissolved oxygen tension; TEM, transmission electron microscopy.

*Pseudomonas aeruginosa* PAO1 was recently found to exhibit two remarkable physiological responses to oxidative stress: (1) a strong reduction in the efficiency of oxygen transfer from the gas phase into the liquid phase, thus causing oxygen limitation in the culture and (2) formation of a clear polysaccharide capsule on the cell surface. In this work, it has been shown that the iron concentration in the culture plays a crucial role in evoking these phenomena. The physiological responses of two *P. aeruginosa* PAO1 isolates (NCCB 2452 and ATCC 15692) were examined in growth media with varied iron concentrations. In a computer-controlled bioreactor cultivation system for controlled dissolved oxygen tension (\(pO_2\)), a strong correlation between the exhaustion of iron and the onset of oxygen limitation was observed. The oxygen transfer rate of the culture, characterized by the volumetric oxygen transfer coefficient, \(k_a\), significantly decreased under iron-limited conditions. The formation of alginate and capsule was more strongly affected by iron concentration than by oxygen concentration. The reduction of the oxygen transfer rate and the subsequent oxygen limitation triggered by iron deficiency may represent a new and efficient way for *P. aeruginosa* PAO1 to adapt to growth conditions of iron limitation. Furthermore, the secretion of proteins into the culture medium was strongly enhanced by iron limitation. The formation of the virulence factor elastase and the iron chelators pyoverdine and pyochelin also significantly increased under iron-limited conditions. These results have implications for lung infection of cystic fibrosis patients by *P. aeruginosa* in view of the prevalence of iron limitation at the site of infection and the respiratory failure leading to death.

**INTRODUCTION**

*Pseudomonas aeruginosa* is an opportunistic pathogen responsible for frequent nosocomial and burn infections. It is the common cause of pulmonary infection in patients suffering from cystic fibrosis (CF). During pulmonary infection, *P. aeruginosa* is subjected to intense oxidative stress due to the production of reactive oxygen intermediates such as superoxide (\(O_2^-\)) and hydrogen peroxide (\(H_2O_2\)) by phagocytic cells of the host. In addition, *P. aeruginosa* is an obligate aerobe that may be exposed to endogenous oxidative stress due to the metabolism of \(O_2\). Several mechanisms have been proposed in the literature for the defence of *P. aeruginosa* against oxidative intermediates (Mathee et al., 1999; Hassett et al., 1999; Stewart et al., 2000; Valente et al., 2000). An efficient way to scavenge reactive oxygen intermediates, which is unique to *P. aeruginosa*, is the formation of the exopolysaccharide alginate. Alginate can form a capsule on the cell surface and represents a physical barrier to oxygen transfer into the cell. Several enzymic antioxidant defence systems, including superoxide dismutase, catalase and peroxidase, have been described for both free-living planktonic cells and cells in biofilm (Elkins, 1999; Hassett et al., 1999; Stewart et al., 2000). Interestingly, *P. aeruginosa* may increase the formation of the hydroxyl radical (OH) by interaction of a siderophore (i.e. pyochelin), which is secreted by the cell, with oxygen, thereby causing tissue damage and inflammation, but not apparently contributing to its own destruction (Britigan et al., 1998; Ratledge & Dover, 2000).

In a previous paper, we showed that *P. aeruginosa* PAO1 can strongly reduce the rate of \(O_2\) transfer from the gas phase into the culture, causing oxygen limitation and simply blocking the supply of oxygen for the formation of reactive oxygen intermediates (Sabra et al., 2002). Under these oxygen-limited or microaerobic conditions, *P. aeruginosa* PAO1 itself grew effectively and appeared to be more pathogenic (Sabra et al., 2002). This possibly represents a new and more efficient defence strategy of *P. aeruginosa* PAO1 against oxidants. However, little is known about the...
factors causing the blockage of oxygen transfer in the *P. aeruginosa* culture.

It is known that iron and iron-containing proteins play important roles in the growth and pathogenesis of *P. aeruginosa*, especially in its defence against oxidative stress (Vasil & Ochsner, 1999). Many proteins involved in respiration (e.g. ferredoxins and other iron– sulphur proteins) and degradation of H₂O₂ and O₂⁻ (e.g. haem catalase), iron superoxide dismutase and peroxidase) require iron for functionality. However, iron in an aerobic environment exists mainly in the form of Fe³⁺ which is extremely insoluble at neutral pH. Thus, increased oxygen tension in the culture can reduce the availability of iron. This was considered as the main reason why aerobic bioprocesses normally require a much higher concentration of usable iron compared to microaerophilic or anaerobic processes (Andrews, 1998; Vasil & Ochsner, 1999). In this connection it is worth mentioning that in the lung, which normally has a highly oxygenated environment, the iron concentration is very low (Griffiths et al., 1988; Stintzi et al., 1998).

Low-iron solubility, together with the process of withholding iron from infecting bacteria by the host through iron complexing with proteins such as transferrin and lactoferrin, is an important strategy in host defence (Griffiths et al., 1988; Stintzi et al., 1998; Ratledge & Dover, 2000). However, the interplay between high oxygenation and low-iron concentration, and its implications for pathogen–host interactions, have not been studied to our knowledge.

The current study was undertaken to examine whether iron concentration in the growth medium influences the transfer rate of oxygen in *P. aeruginosa* cultures and how the physiology of *P. aeruginosa* is affected under these conditions.

**METHODS**

**Bacterial strains and growth conditions.** Two *P. aeruginosa* PAO1 isolates (NCCB 2452 and ATCC 15692), obtained from the Netherlands Culture Collection of Bacteria and American Type Culture Collection, respectively, were used in this study. Cells were cultivated in a modified glucose minimal medium described previously (Sabra et al., 2002) with replete (7 mg l⁻¹) or low (0.6 mg l⁻¹) concentrations of FeSO₄·7H₂O, respectively. Seed cultures were prepared in medium A without iron (Mian et al., 1978). Batch cultivations with control of the dissolved oxygen tension (pO₂) were carried out in a computer-controlled bioreactor as described previously (Sabra et al., 2002). The volumetric oxygen transfer coefficient, kₐO₂, a key parameter to characterize the oxygen transfer rate from the gas phase to the liquid phase, was determined by the static method of gassing-out as described by Stanbury & Whitaker (1987).

**Electron microscopy.** Transmission electron microscopy (TEM) was used to detect the possible presence of polysaccharide capsule on the surface of cells grown in iron-rich and iron-limited media. Cells were taken directly from bioreactor cultures controlled at a pO₂ of 10% air saturation. Embedding and ultrathin sectioning of *P. aeruginosa* were as described previously for Azotobacter vinelandii (Sabra et al., 2000).

**Biochemical analysis.** The total extracellular protein in cell-free supernatants was determined by the method of Lowry. Elastase activity was determined in a spectrophotometric assay using Elastin-Congo red (Sigma) as substrate as described by Kessler et al. (1993). Siderophores (pyoverdine and pyochelin) were measured with a microtitre plate fluorometer (MFX Microtiter Plate Fluorometer; Dynex Technologies). Fluorescence was determined by exciting the culture supernatant at 400 nm for pyoverdine and 355 nm for pyochelin; the emission was measured at 460 nm for pyoverdine and 440 nm for pyochelin (McMorran et al., 2001; Ankenbauer et al., 1985). Biomass dry weight was determined gravimetrically as described previously (Sabra et al., 2000).

**Determination of iron concentration.** The concentration of iron in culture supernatants was determined as Fe²⁺ or Fe³⁺ by spectrophotometric assay using iron test kits (Merck). Briefly, Fe²⁺ in the sample was reacted with 1,10-phenanthroline to form a red complex that was determined photometrically. Fe³⁺ was first reduced to Fe²⁺ by ascorbic acid and the total amount of Fe²⁺ was measured as above. The detection limit of iron concentration was given as 0.01 mg l⁻¹ for the test kits.

**RESULTS**

**Iron deficiency leads to a reduction in oxygen transfer in *P. aeruginosa* cultures**

To examine the possible effects of iron, we cultivated two *P. aeruginosa* PAO1 isolates (NCCB 2452 and ATCC 15692) in a defined minimal medium with different iron concentrations at a preset pO₂ of 10% air saturation. The cell growth, pO₂ profile, inlet flow rate of pure O₂ in the aeration gas mixture (O₂+N₂ at a constant total aeration flow rate of 1 l min⁻¹) and the residual iron concentration in these cultures are depicted in Fig. 1 and Fig. 2. With the iron-rich medium the residual iron concentration remained above 0.4 mg l⁻¹ (NCCB 2452) and 0.2 mg l⁻¹ (ATCC 15692) till the end of cultivation periods, while complete iron deficiency was observed in the late exponential phase of the cultures with low-iron medium. Fig. 2 also shows the concentration profiles of Fe²⁺ and Fe³⁺ in the culture of ATCC 15692. The iron source mainly existed in form of Fe²⁺. The concentrations of both Fe²⁺ and Fe³⁺ decreased, with the less soluble Fe³⁺ being unexpectedly exhausted at the end of cultivation in cultures both with iron-rich and with low-iron media.

Interestingly, the iron deficiency was accompanied by a sharp decrease in pO₂ and a significant increase in the portion of pure oxygen in the aeration gas (Figs 1b, 2b). In the late exponential phase of these cultures, pO₂ dropped to zero and oxygen limitation prevailed despite aeration with 100% oxygen. Therefore, control of pO₂ at 10% air saturation throughout cultivation was not possible under these conditions. A similar oxygen limitation phenomenon was observed in our previous study with *P. aeruginosa* PAO1 NCCB 2452 grown in a low-iron medium with a different pO₂ stress (Sabra et al., 2002).

Both *P. aeruginosa* PAO1 NCCB 2452 and ATCC 15692 grew somewhat faster in the iron-rich medium than in the low-iron medium. Growth in the low-iron medium showed
**Iron deficiency and blockage of oxygen transfer**

Fig. 1. The control of \( pO_2 \) and growth parameters in cultures of *P. aeruginosa* PAO1 NCCB 2452 grown in (a) iron-rich medium and (b) low-iron medium. ●, biomass; —, \( pO_2 \); △, inlet flow rate of \( O_2 \); □, total iron concentration.

Fig. 2. The control of \( pO_2 \) and growth parameters in cultures of *P. aeruginosa* PAO1 ATCC 15692 grown in (a) iron-rich medium and (b) low-iron medium. ●, biomass; —, \( pO_2 \); △, inlet flow rate of \( O_2 \); ■, \( Fe^{2+} \) concentration; ▲, \( Fe^{3+} \) concentration.
a relatively long lag phase (3–5 h), whereas almost no lag phase was observed in the iron-rich medium. However, the biomass concentrations reached in both media are comparable, indicating an effective utilization of iron in the cultures grown in the low-iron medium. Calculation of the specific growth rates (μ) during the different phases of the low-iron culture revealed a higher growth rate during the period of iron deficiency than in the iron non-deprived period, indicating an effective adaptation of the organism to the low-iron or iron-deficient conditions. For example, the growth rate of strain ATCC 15692 increased from 0·026 h⁻¹ in the first 5 h of cultivation, to 0·07 h⁻¹ in the 7–11 h period, to 0·16 h⁻¹ during the period of iron deficiency (about 13–16 h, Fig. 2b). The μ max reached in the low-iron culture was the same as the μ max (0·16 h⁻¹) of this strain reached in the iron-rich medium, although the former had a very slow growth rate in the initial period of cultivation compared to the iron-rich culture. A similar effect on the growth rate was observed with strain NCCB 2452, where the μ max reached in the low-iron medium was 0·30 h⁻¹, somewhat lower than the μ max reached in the iron-rich medium (0·40 h⁻¹).

The oxygen limitation observed in Figs 1(b) and 2(b) was not caused by a high oxygen consumption rate of the cells (Geckil et al., 2001; Sabra et al., 2002). In fact, the biomass concentrations in these cultures (<1·6 g l⁻¹; see Fig. 1b for NCCB 2452) were not high. For strain ATCC 15692, the biomass concentration during the oxygen limitation period in the low-iron culture (Fig. 2b) was even lower than that in the late exponential phase of the iron-rich culture (Fig. 2a). The measurement of k L a in cell-free supernatants from NCCB 2452 cultures under different iron concentrations revealed the reason for oxygen limitation in the low-iron culture. As depicted in Fig. 3, both cultures had a relatively low k L a value after inoculation with the iron-exhausted seed culture which had poor oxygen transfer properties. The k L a value increased during the first few hours of cultivation in both iron-rich and low-iron cultures. Whereas the increase of k L a continued in the culture with sufficient iron (Fig. 3a), the k L a value of the low-iron culture levelled off and significantly decreased after about 9 h of cultivation (Fig. 3b), namely at about the same time as the onset of iron depletion (Fig. 1b). The reduction of the k L a value was much more drastic in our previous study with strain NCCB 2452 grown under different preset p O 2 values (Sabra et al., 2002). This decrease in oxygen transfer efficiency from the gas to the liquid phase could explain why the p O 2 value reached zero even though the O 2 content in the inlet gas was increased to 100% oxygen under iron-deficient conditions (Figs 1b and 2b). This study clearly shows that the reduction of the oxygen transfer rate is mediated by conditions of iron deficiency.

**Occurrence of polysaccharide capsule on the cell surface is caused by iron deficiency**

Using TEM of ultrathin sections of negatively stained cells we studied the effect of iron concentration on morphological changes of *P. aeruginosa* by comparing PAO1 cells grown under iron-replete conditions (Fig. 4) with those grown under iron-limited conditions and the same, controlled

![Fig. 3](image-url)  
*Fig. 3.* k L a in water and culture supernatant of *P. aeruginosa* PAO1 NCCB 2452 grown in (a) iron-rich medium and (b) low-iron medium. ○, k L a; , p O 2 .
pO2 value (10% air saturation). The occurrence of polysaccharide (alginate) capsule on the cell surface was reported previously and attributed to the physiological response of PAO1 to oxidative stress (Sabra et al., 2002). In this work, we found that cells from iron-rich culture do not possess such a polysaccharide capsule (Fig. 4) even though O2 stress conditions prevail. This is consistent with the measurement of alginate concentration in culture supernatants. In the supernatant of the iron-limited culture (Sabra et al., 2002) alginate concentrations in the range of 0.1–0.3 g l−1 were found for pO2 ranges of 1–50% air saturation. In the supernatant of iron-rich cultures reported in this work a negligible alginate concentration, as low as 0.01–0.03 g l−1, was detected. Thus, iron deficiency seems to be a more dominant factor in triggering the formation of alginate and alginate capsule on the surface of PAO1 cells.

**Release of extracellular proteins and virulence factors under iron-limited and iron-replete conditions**

The release of extracellular proteins in cultures with different iron concentrations was measured and the results are shown in Fig. 5. The iron-rich culture consumed the carbon source faster and thus had a shorter cultivation time. Considering the rate of concentration change it can be stated that protein secretion was considerably enhanced under iron-limited conditions. In the iron-rich cultures the protein concentration was in the range of 0.3–0.5 g l−1, whereas it increased up to 0.7–0.8 g l−1 in the low-iron cultures during the iron limitation period. From the corresponding changes of concentrations of biomass and extracellular proteins during the cultivation we can calculate the protein yield, Yp/X [g protein (g biomass)−1] under different conditions. In the previous study (Sabra et al., 2002) we showed that Yp/X can be significantly affected by pO2. To avoid the influence of pO2 we compared the Yp/X values for cultures with low and replete iron only for the cultivation period in which pO2 was controlled at 10% air saturation. The Yp/X value of the strain NCCB 2452 was increased from 0.1 g g−1 in the replete iron culture to 1.12 g g−1 in the low-iron culture. Strain ATCC 15692 also showed a more than 10-fold increase (from 0.16 g g−1 to 1.69 g g−1) of extracellular protein yield in the low-iron culture. The increased protein secretion was not due to lysis of cells under these conditions. In fact, the strains grew better under low-iron conditions as can be ascertained in Figs 1 and 2.

*P. aeruginosa* secretes a number of extracellular proteins as virulence factors. The synthesis of many of them is known to be affected by the concentration of iron (Vasil & Ochsner, 1999; Forsberg & Bullen, 1972; Cox, 1986). In this work, the activity of elastase was measured as an example of these virulence factors in culture supernatants of both strains grown in low-iron and iron-rich medium. As shown in Fig. 6, a more than twofold increase in specific elastase activity was recorded under iron-limited growth conditions for each of the strains. Since total protein secretion was also increased, the total elastase activity increased more markedly, especially as a function of the biomass under low-iron conditions (see inset in Fig. 6).

Siderophores are another type of virulence factor produced by *P. aeruginosa*, the regulation of which is known to be sensitive to iron (Vasil & Ochsner, 1999). We measured the production of two typical siderophores, i.e. pyoverdine and pyochelin, by cultures under different iron conditions. As shown in Fig. 7, the iron-limited culture showed up to a fourfold increase of specific pyoverdine production for the two *P. aeruginosa* isolates studied. Total pyoverdine is a strong function of biomass concentration. The trends are quite similar to those of the elastase activities (Fig. 6). Pyochelin also showed the same patterns of change as pyoverdine for each of the strains, though its levels were about 10 times lower than those of pyoverdine (data not shown).

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**Fig. 5.** Protein secretion in batch cultures of *P. aeruginosa* PAO1 ATCC 15692 (a) and NCCB 2452 (b) grown in low-iron (■, △) and iron-rich (○, ▽) media. The time points for the occurrence of iron limitation (→) and oxygen limitation (→) are indicated. ■, ○, extracellular protein concentration; △, ▽, biomass.

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**DISCUSSION**

Since iron acquisition is important for the infection and survival of pathogens in their hosts in general, and the CF patient lung normally represents an environment with a very low iron concentration in particular, the responses of *P. aeruginosa* to iron limitation have been the object of extensive studies in the past (for a review and recent work see Vasil & Ochsner, 1999; Andrews, 1998; Ratledge & Dover, 2000; Frederick et al., 2001; Cowart, 2002; Schalk et al., 2002). Different physiological responses and mechanisms of iron acquisition have been reported for *P. aeruginosa* and other pathogens to scavenge iron from the environment. These include: (1) production of iron-binding compounds such as siderophores; (2) direct utilization, uptake and enzymic degradation of host iron-binding proteins such as transferrins, lactoferrin and haemoproteins; (3) reduction of the insoluble form of iron (Fe^{3+}) to the more soluble form (Fe^{2+}) by formation of specific enzyme(s); and (4) production of lethal compounds that may eliminate competitors for usable iron resources.

In this work we showed using two *P. aeruginosa* PAO1 isolates that this pathogenic organism can apply a hitherto unreported strategy to deal with iron limitation, namely a significant reduction of the oxygen transfer rate from the gas phase into the liquid phase as reflected by the $k_L a$ value in Fig. 3, thereby causing oxygen-limited or microaerobic conditions in the culture (Figs 1 and 2). These results explain well the phenomenon of oxygen limitation reported in our previous study (Sabra et al., 2002) in which we used the same low-iron medium for cultivating *P. aeruginosa* PAO1 NCCB 2452 under different preset values of $pO_2$. In all the previous cultivations with this strain at different preset $pO_2$ values, a strong oxygen limitation occurred 6–8 h after inoculation despite vigorous aeration of the bioreactor with pure oxygen. The exact mechanism used by *P. aeruginosa* to reduce the oxygen transfer rate is not known. In our previous study we showed that the production of biosurfactants such as rhamnolipid may contribute to this phenomenon (Sabra et al., 2002). Whatever the mechanism is, the physiological consequences of this reduced oxygen transfer rate are obvious. First, the oxygen-limited or microaerobic conditions can greatly increase the...
usability of the remaining iron through the transformation of Fe$^{3+}$ to the more soluble Fe$^{2+}$. Second, the reduced oxygen transfer rate and thus $pO_2$ can better protect the cells from the formation of oxidative radicals, especially under low-iron conditions. It is known that the synthesis and functionality of many enzymes involved in defence against oxidative radicals such as catalase and dismutase require iron (Frederick et al., 2001). Furthermore, under oxygen limitation, the respiration rate is reduced. This in turn can result in a reduction of endogenous generation of oxidative radicals such as H$_2$O$_2$ and O$_3^-$. These favourable conditions may be the reason why P. aeruginosa PAO1 grew even faster under apparently iron-deficient conditions (e.g. cultivation time after 12 h in Fig. 2b, $\mu = 0.16 \text{ h}^{-1}$) than before iron limitation (e.g. cultivation time between 6 and 11 h in Fig. 2b, $\mu = 0.07 \text{ h}^{-1}$). This indicates that P. aeruginosa PAO1 can effectively adapt to environments of iron deficiency. However, it is not known if the improved availability of iron or the reduced formation of oxidative radicals is the main contributor to the improved growth of P. aeruginosa PAO1 under microaerobic conditions. Microaerobic conditions are known to dominate in biofilms, the preferred mode of growth of P. aeruginosa in the lung of CF patients, and in biofouling of different systems (Costerton et al., 1999; Xu et al., 1998). Microaerobic conditions have also been reported to be optimal for the growth of P. aeruginosa on hydrocarbons (Chayabutra & Ju, 2000). The real reason for the improved growth is not clear and deserves more detailed study. This may give useful hints to better combat the infection and contamination by P. aeruginosa.

Oxygen limitation triggered by iron deficiency resulted in a dual limitation in the culture of P. aeruginosa PAO1 in the later phase of cultivation (Figs 1 and 2). Under these conditions P. aeruginosa PAO1 showed a drastic increase in secretion of proteins (Fig. 5). One of these proteins is elastase (Fig. 6). An enhanced activity of elastase at decreased iron concentration has been reported (Bjorn et al., 1979; Brumlik & Storey, 1992; Storey et al., 1992). In host cells, elastase is able to specifically cleave transferrin, an animal iron carrier. Following cleavage of the iron carrier, the iron can be used by the bacterial cells. The increased elastase synthesis has been considered to be one of the strategies of iron acquisition by P. aeruginosa under iron-limited conditions (Wolz et al., 1994). The formation of the two siderophores, e.g. pyoverdine (Fig. 7) and pyochelin which can chelate iron, was also found to increase significantly under iron-limited conditions. This may contribute to the complete consumption of Fe$^{3+}$ in the late phase of cultivation (Fig. 2). Both elastase and siderophores can help P. aeruginosa to remove iron from host sources and enhance growth (Cox, 1982). Under the bioreactor cultivation conditions applied in this study, no iron resource other than FeSO$_4$ was present. It would be of interest to know if the oxygen limitation caused by iron deficiency, which is reported for the first time in this study and suggested as a new strategy for P. aeruginosa to combat iron limitation and oxidative stress, also takes place when iron-containing proteins exist as in the lung of CF patients.

In fact, the environment of the lung of many CF patients is quite similar to the culture conditions applied in this study in at least two important aspects, i.e. low iron concentration and high oxygenation. In this connection there is another important observation in this study (Fig. 4), namely the formation of alginate and the occurrence of a polysaccharide capsule on the cell surface is mainly related to iron limitation rather than to oxidative stress. The latter was previously considered as the main reason for an enhanced formation of an alginate capsule on the surface of PAO1 cells (Sabra et al., 2002). The enhanced formation of alginate may be explained by the iron regulation circuit that is known to be activated under oxygen stress and iron deprivation (Vasil & Ochsner, 1999). In view of the importance of alginate in the pathogenicity (e.g. through the formation of biofilm and avoidance of encounters with phagocyte-derived reactive oxygen intermediates) of P. aeruginosa (Govan & Deretic, 1996; Miller & Britigan, 1997), the finding presented in Fig. 4 represents an important extension of our previous knowledge. The high mortality of CF patients infected by P. aeruginosa is often due to biofilm formation and respiratory failure. Oxygen limitation and the formation of an alginate capsule on the cell surface due to iron deficiency as shown in this work may therefore play an important role and deserve more detailed study.

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