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Effects of Growth Rate and Nutrient Limitation on Virulence Factor Production in *Burkholderia cepacia*

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The influence of growth rate and oxygen availability on siderophore, protease, and lipase production in *Burkholderia cepacia* was assessed for cells grown in a chemostat under iron limitation. Whereas siderophore and protease production increased with growth rate and oxygen yet decreased under oxygen depletion, lipase production demonstrated the opposite trend.

Burkholderia cepacia, once thought to be only a phytopathogen, is now recognized as a major opportunistic pathogen of human disease (14). Although *B. cepacia* emerged as an important etiological agent in nosocomial infection, *B. cepacia* is now considered a particularly problematic pathogen in patients with fibrocystic lung disease (CF) owing to its increasing association with fatal pulmonary infections (6, 16). Formerly known as *Pseudomonas cepacia* and belonging to *Pseudomonas* RNA homology group II, the organism was recently renamed *B. cepacia*, forming the type species of the new genus *Burkholderia* (15).

Clinically, *B. cepacia* colonization of CF patients can be symptomless, or it can be associated with a slow decline in lung function. More alarmingly, approximately 20% of patients who have been mildly affected by their disease unexpectedly succumb to a rapid and fatal deterioration in pulmonary function accompanied by fever, necrotizing pneumonia, and in some cases, bacteremia (16, 38). This third syndrome is not observed with other CF pathogens and is central to the concern over *B. cepacia* infections among the CF population. In virtually all instances, however, isolates of *B. cepacia* are extremely resistant to a wide range of antibiotics, both in vitro and in vivo, and to the bactericidal action of serum in vivo (2, 6). As such, the variations in clinical outcome are thought to be due to the underlying clinical condition of the lung prior to infection or alternatively to infections by different strains of *B. cepacia* (16).

Whereas certain strains of *B. cepacia* are particularly transmissible (11, 20), there is no evidence at present to suggest that some strains are more virulent than others. Indeed, relatively little is known concerning the virulence factors of this organism. Although *B. cepacia* has been shown to produce hemolysins, lipase, protease (8, 27), exopolysaccharide (1, 30), and iron-chelating siderophores (5, 28, 33, 34), their role in pulmonary disease of CF patients remains to be elucidated. This lack of understanding may in part result from the methods employed in the study of such pathogenic determinants.

Growth rate is known to be an important factor in microbial pathogenicity and will contribute to the outcome of an infection (4, 32). During infections, bacterial growth rates appear to vary from slow to fast, depending upon the site and stage of infection and the concentration and availability of essential growth-limiting nutrients. In many bacterial infections, including those of the CF lung, iron is often not readily available to the microorganism (12). Hence, the ability to obtain iron is considered an important virulence factor, and in response to iron deprivation, many microorganisms modify their physiology in order to obtain sufficient levels of iron for growth. Such changes may result in low growth rates which subsequently affect virulence factor production. These changes have been mimicked in vitro through the use of iron-restricted batch culture (21, 25, 39). However, in vivo, nutrients as well as or other than iron may be growth limiting (4, 32). Given that abnormally viscous mucus is overproduced in the lungs of CF patients (17), along with copious amounts of bacterial exopolysaccharide (23), it is highly likely that oxygen gradients will develop. As such, bacteria trapped within CF sputum may be growing in iron-limited, oxygen-starved conditions. In this respect, the use of closed growth systems such as batch culture to mirror bacterial infections can be physiologically misleading, since only the starting conditions can be defined accurately (3). By contrast, chemostat culture offers many advantages over batch culture in the study of microbial responses to various growth rates. The use of continuous culture enables either the specific growth rate to be varied by changing the dilution rate while maintaining the cells in a constant physical and chemical environment or a constant specific growth rate to be maintained while physicochemical parameters of growth, such as pH, temperature, or nutrient availability, are varied (9, 13).

Because of the increased reporting of *B. cepacia* infections among the CF population and their associated rapid mortality rates, there is a need to examine factors affecting the production of extracellular virulence determinants. In this report, we describe the influence of growth rate and oxygen availability under iron limitation on lipase, protease, and siderophore production. In the oxygen-depleted chemostat, the oxygen concentration was lowered to a value close to but above the concentration which would be rate limiting.

B. cepacia NCTC 10661 was used throughout this study. Continuous cultures were established, at 37°C, utilizing 50-ml, all-glass chemostats (10) and a chemically defined minimal salts medium (CDM) in which iron was the growth-limiting nutrient. The composition of CDM was as follows (in grams per liter): NaCl, 0.175; $MgSO_4$ $7H_2O$, 0.789; KCl, 0.224; $(NH_4)_2SO_4$, 0.396; K_2HPO_4, 0.205; morpholinepropanesulfonic acid (MOPS), 10.47; and glucose, 10; its pH was 7.4. Growth in batch culture using this medium results in an optical density at 470 nm (OD_{470}) of 2.0. Iron-limited, oxygen-depleted cultures were established by reducing the level of aeration by 50%, corresponding to a theoretical OD_{470} maximum of 0.5. For each experiment, the chemostat was allowed to equilibrate to steady state by passing a minimum of five com-

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plete changes of medium through the growth vessel. Batch cultures (20 ml) were grown to mid-exponential phase in iron-depleted CDM at 37°C with shaking at 250 rpm in 100-ml Erlenmeyer flasks. Samples removed directly from the growth chamber of the chemostat were assayed for siderophore (31), protease (40), and lipase (22) activities following removal of cells by centrifugation (10,000 × *g*, 10 min). Viable counts were performed in triplicate with predried nutrient agar plates and the samples from which the supernatant fluids had been obtained, enabling virulence factor production to be related to a unit cell number (10^6 cells).

Accumulated evidence suggests that the in vivo characteristics of *B. cepacia* are different from those observed in vitro (6, 19). As such, since it is well documented that growth rate is a known modulator of microbial structure and physiology, as is the nature of the nutrient controlling growth rate (3, 4, 7), it is important that evaluation of virulence factor production in bacterial pathogens is performed under conditions which represent as closely as possible their in vivo environment. In this respect, *B. cepacia* is very likely to be found growing slowly under iron limitation in the lungs of CF patients where oxygen gradients are likely to occur with increasing depth within the sputum. Hence, the results presented in Fig. 1, 2, and 3 illustrate the effect of specific growth rate under iron-limited, oxygen-replete and iron-limited, oxygen-depleted conditions on siderophore, protease, and lipase production, respectively, in *B. cepacia*. Although it is theoretically possible with *B. cepacia* in this CDM, possessing a doubling time (t_d) of approximately 3 h, to achieve a dilution rate of 0.2 h^{-1} corresponding to a maximum specific growth rate, in practice the chemostat was stable only up to dilution rates of 0.15 h^{-1} before washout occurred (24). Consequently, the growth rates studied varied from 0.025 to 0.15 h^{-1}. The growth rates chosen are representative of bacterial growth rates in vivo at different stages of infection. In chronic CF infections, bacterial doubling times have been estimated to be in the order of 24 to 40 h (3, 32). In this manner, a low growth rate of 0.025 h^{-1} (t_d, ca. 28 h) corresponds to chronic infection while a higher growth rate of 0.15 h^{-1} (t_d, ca. 4.6 h) is associated with cells seeking to establish an infection (3). Thus, through the use of a nutrient (iron)-limited CDM, the range of growth rates produced will be much slower than those obtained using nutrient-rich media and will provide a more accurate indication of *B. cepacia* physiology relevant to the CF lung.

Unlike batch cultures, in continuous culture, secondary metabolites and extracellular products are subject to constant dilution by the addition of fresh medium. At steady states, production of such factors by the resident population equals their rate of removal or dilution. In order to make direct comparisons between the different growth rates studied, the units for each virulence factor were expressed as productivities (units of activity per 10^6 cells $hour^{-1}$) relative to the resident population in the chemostat.

The effect of growth rate on siderophore production is shown in Fig. 1. Siderophore production was quantified according to absorbance at OD_{630}. Whereas production increased significantly with increases in growth rate for cultures grown under conditions of oxygen excess, productivity was growth rate independent under conditions of oxygen depletion. Iron chelation at lower rates of growth (0.025 to 0.09 h^{-1}) was greater for oxygen-depleted cells than for those grown under oxygen-replete conditions. At growth rates in excess of 0.09 h^{-1}, oxygen-replete cells showed greater siderophore productivity than cells grown under oxygen depletion. Hence, the results presented in Fig. 1 demonstrate siderophore production in *B. cepacia* to be growth rate as well as oxygen level

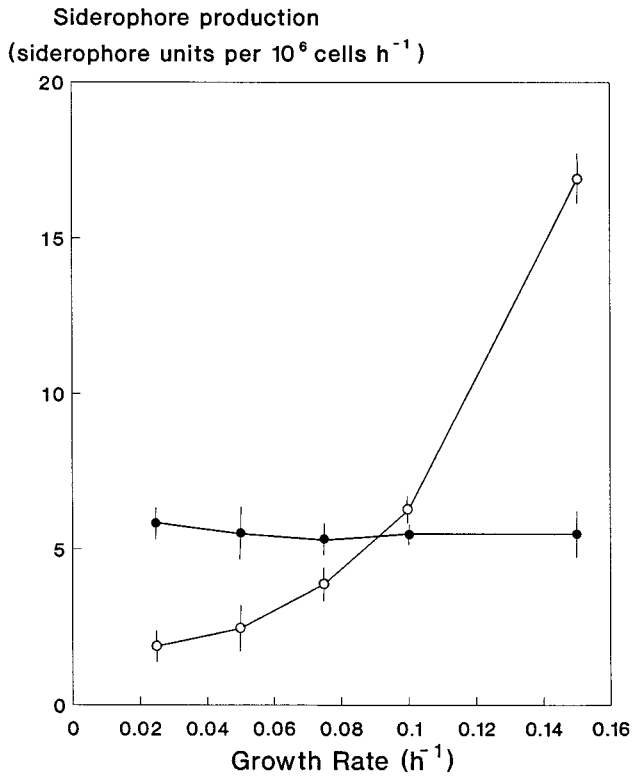

FIG. 1. Effect of specific growth rate upon the production of siderophores by *B. cepacia* in an iron-limited, chemically defined simple salts medium. Siderophore content was expressed as productivity under oxygen-replete (○) or oxygen-depleted (●) conditions.

dependent. A similar dependence of siderophore production on growth rate has been reported for *Klebsiella pneumoniae* (21) where under various levels of iron availability in continuous culture, siderophore levels increased with growth rate. At low rates of growth, the iron requirement of *B. cepacia* may be relatively low such that a basal level may provide sufficient iron for the cells. In contrast, a greater requirement of iron at higher rates of growth was reflected in a derepression of the siderophore-mediated iron uptake mechanism. Indeed, such levels, particularly at the highest growth rate measured (0.15 h^{-1}), approaching the maximum specific growth rate, may well be in excess of the cell's requirements.

The iron chelation assay employed provides only a measure of the total iron-binding capacity of the culture supernatants and is not necessarily indicative of the production of siderophores. Although the results might imply induction of a high-affinity uptake mechanism for iron-associated siderophores at higher growth rates where it is likely the cell's iron requirements are greater, they might also indicate the production of weak iron chelators such as phosphate or organic acids (7). While the iron uptake mechanism elaborated by *B. cepacia* 10661 in this study was not identified, iron-regulated outer membrane proteins were produced in all instances of iron deprivation (data not shown). *B. cepacia* is known to express at least four siderophore-mediated iron transport systems, each possessing a different affinity for iron (5, 28, 33, 34, 36). Pyochelin appears to be the major iron chelator (35), although there has as yet been no clearly ascribed role for it in pathogenicity, since many clinical isolates are in fact pyochelin negative (29). Furthermore, both cepabactin and azurechelin can be secreted simultaneously to pyochelin. In summary, siderophore produc-

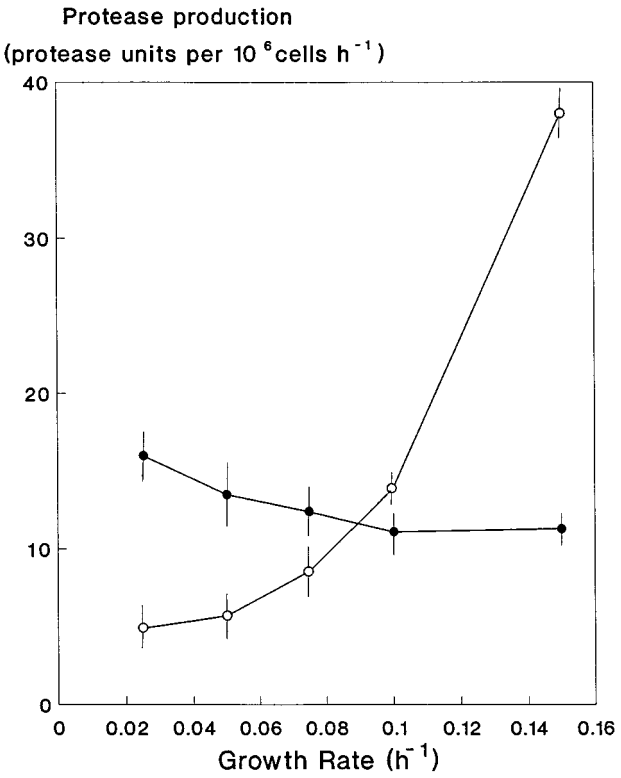

FIG. 2. Effect of specific growth rate upon the production of protease by *B. cepacia* in an iron-limited, chemically defined simple salts medium. Protease content was expressed as productivity under oxygen-replete (○) or oxygen-depleted (●) conditions.

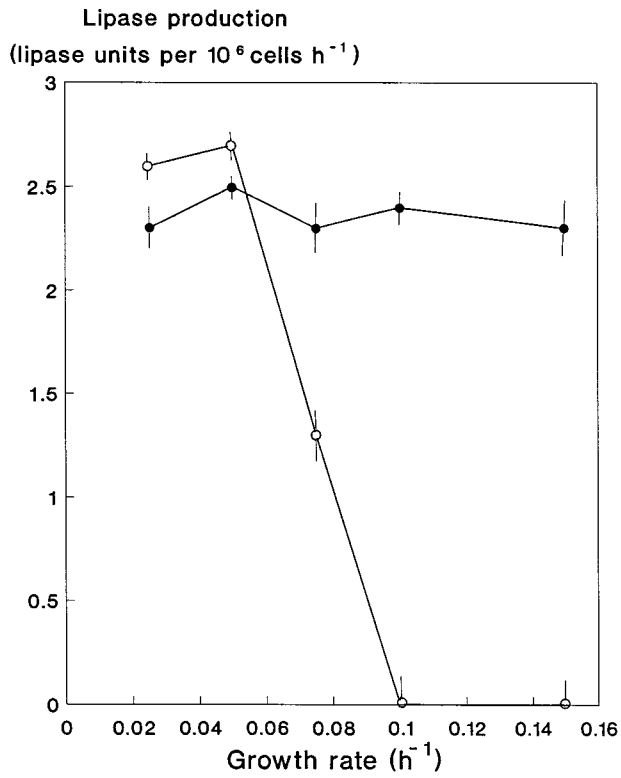

FIG. 3. Effect of specific growth rate upon the production of lipase by *B. cepacia* in an iron-limited, chemically defined simple salts medium. Lipase content was expressed as productivity under oxygen-replete (○) or oxygen-depleted (●) conditions.

tion by *B. cepacia* infections of the CF lung are complexed not only by the synthesis of four or more different types of iron chelators but are also markedly affected by cell density, growth rate, and the nutrient status of the surrounding microenvironment.

Extracellular protease productivity (Fig. 2), calculated as OD_{280} increase in hydrolysis of heat-denatured casein per unit cell number over a 1-h incubation period, increased significantly with increases in growth rate for oxygen-replete cultures but decreased slightly with growth rate under conditions of oxygen depletion. At low rates of growth (0.025 to 0.09 h^{-1}), protease production was greater under conditions of oxygen stress. Thereafter, at higher growth rates, production was substantially greater for cells under oxygen-replete conditions than in cells grown under oxygen restriction. Although *B. cepacia* has recently been shown to produce two extracellular proteases (18), this study simply examined changes in gross protease activity with alterations in the growth environment. As such, iron chelator and protease production appear from the above data to be coinduced. This might be the case in vivo if a function of the protease were to liberate iron from the naturally occurring iron carriers transferrin and lactoferrin (12). The 34-kDa extracellular protein of *B. cepacia* possesses antigenic properties similar to those of *P. aeruginosa* elastase and is capable of cleaving collagen and causing proteinaceous exudation of polymorphonuclear leucocytes but unable to degrade immunoglobulins G, M, and A (26). The ability to combat cell-mediated host defense mechanisms is critical to the microorganism when seeking to establish an infection.

The results shown in Fig. 3 illustrate the relationship between specific growth rate and lipase production in *B. cepacia* with oxygen availability. Using Tween 80 as substrate, 1 U of lipase activity is represented by 0.01 OD_{400} unit. Under oxygen-replete conditions, productivity was nonlinearly related to growth, with production being maximal for cells growing at a specific growth rate of 0.05 h^{-1}. At higher rates of growth (>0.05 h^{-1}), lipase production decreased rapidly to almost negligible amounts at a specific growth rate of 0.1 h^{-1}. By contrast, lipase productivity demonstrated a slight growth rate dependency under conditions of oxygen depletion, with production increasing with growth rate. Moreover, at growth rates in excess of 0.05 h^{-1}, lipase production was substantially greater in oxygen-deprived cultures than in the oxygen-replete counterparts. These results demonstrate that lipase production in *B. cepacia* is also significantly affected by growth rate and oxygen availability. However, because the responses to growth rate and oxygen availability are opposite those observed for both siderophore and protease activities, lipase production may be regulated in a manner different than that of siderophore and protease production. Indeed, lipase has been shown to be an important virulence factor in *B. cepacia*, increasing in the presence of sublethal concentrations of antibiotics (25) and adversely affecting the phagocytic function of rat pulmonary alveolar macrophages in a dose-dependent manner (37). In the presence of lipase, engulfment of *B. cepacia* by alveolar macrophages was significantly reduced (37). As such, production of lipase may help *B. cepacia* to evade the mammalian host defense system, particularly when seeking to establish an infection. In this respect, the increased levels of lipase activity detected at the higher rates of growth under conditions of oxygen stress may accurately reflect events occurring in vivo.

In conclusion, the evidence presented clearly indicates that extracellular virulence factor production in *B. cepacia* is affected by growth rate and nutrient availability. Although the basal medium components and limiting nutrient were identical, changes in the concentration of oxygen resulted in significant changes in the expression of siderophore, protease, and lipase production. Moreover, these results indicate that lipase production in *B. cepacia* is regulated differently than siderophore and protease production is. Such changes in extracellular virulence factor production will substantially alter the phenotype expressed in vivo following growth of *B. cepacia* in oxygen-deprived microenvironments of the mucus-laden CF lung.

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