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Influence of Iron-Limited Continuous Culture on Physiology and Virulence of *Legionella pneumophila*

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A virulent strain of *Legionella pneumophila* serogroup 1, subgroup Pontiac, was grown in continuous culture at a constant growth rate under iron-replete and iron-limited conditions. Iron limitation was achieved by the removal of ferrous sulfate and hemin from the chemically defined medium. Residual contaminating iron, 0.45 μM, was sufficient to support iron-limited growth. Typical iron-replete cultures metabolized 3.3 μM iron. Serine provided the principal source of carbon and energy for both cultures, although iron-replete cultures also depleted a number of other amino acids. There was a 40% decrease in culture biomass under iron-restricted conditions. Iron limitation did not significantly affect carbohydrate metabolism, with the molar growth yield for carbon (Y_{carbon}) comparable for both cultures. However, under iron-limited conditions a sixfold increase in Y_{iron} correlated with a significant decrease in the iron content of the biomass, as the culture utilized the available iron more efficiently. Highly pleomorphic iron-replete cultures became uniform cultures of short fine rods when adapted to iron-deficient conditions. In addition to the morphological and physiological changes, iron limitation had a critical effect on culture virulence. The virulence of this strain was significantly ($P < 0.05$) reduced when the culture was subjected to iron-limited conditions. This phenomenon was reversible, with a significant increase in culture virulence upon reversion to iron-replete conditions. When compared in an in vitro macrophage assay, the number of culturable avirulent iron-limited cells located intracellularly after infection was significantly lower than for the virulent replete and control cultures. These results further support the role of environmental parameters in regulating the virulence of *L. pneumophila*.

Iron plays a key role in microbial pathogenesis and physiology through its participation in diverse biological processes, such as cellular respiration and transcriptional regulation (34, 54). Although the fourth most abundant element in nature, it is usually present in very low concentrations as a freely soluble ferric ion. Spontaneous oxidation leads to the formation of insoluble ferric hydroxides and oxyhydroxides in aqueous environments at neutral pH. Iron availability is also restricted in vertebrates to concentrations usually below 10^{-18} M (22), which is too low to satisfy normal bacterial requirements; for example, gram-negative bacteria usually need 0.3 to 1.8 μM iron for growth (53). In humans most iron is located intracellularly in ferritin or heme, while the small amount of extracellular iron is tightly bound to the iron-binding and transport glycoproteins transferrin and lactoferrin. Furthermore, the human host responds to infection by reducing the amount of available iron, through the increased synthesis and release of lactoferrin by neutrophils, and ferritin in the liver (35). Therefore, microorganisms are constantly confronted by severe iron-restricted conditions both in vitro and in vivo.

In order to survive and compete in these iron-restricted environments, many microorganisms have developed specific mechanisms for iron acquisition, the components of which are considered to be important virulence factors. The most common specific iron uptake system involves the synthesis of low-molecular-weight, high-affinity iron chelators called siderophores, which are capable of solubilizing iron and transporting it to the bacterial cell (23). Alternatively, some pathogenic bacteria express specific cell surface receptors which obtain iron by interacting directly with iron-loaded transferrin or lactoferrin (45). The concentration of free iron in the environment participates in regulating the expression of these iron uptake mechanisms and other virulence determinants in many pathogens. For example, in *Escherichia coli* coordinated regulation of several genes encoding virulence determinants is achieved via the regulatory locus *fur* (ferric uptake regulator), which codes for an iron-responsive regulatory protein. Under iron-restricted conditions the *fur* regulon is derepressed, allowing transcription of several virulence loci to proceed (24). Similar iron-responsive regulatory systems have been identified in a number of pathogens, including *Vibrio cholerae* (20), *Neisseria meningitidis* (50), *Yersinia* spp. (46), and *Pseudomonas aeruginosa* (40). These regulatory networks allow pathogens to sense and respond to a dynamic host environment during infection.

The importance of iron for the intracellular and extracellular growth of the respiratory pathogen *Legionella pneumophila* is well documented (6, 12, 18, 42). This facultative intracellular bacterium must acquire iron both in its natural aquatic habitat as an intracellular parasite of protozoa and within the human host, where it invades and multiplies within monocytes and alveolar macrophages, with the potential of causing Legionnaires' disease. Numerous studies have demonstrated the inhibitory effect of iron chelators and serum iron-binding proteins on *Legionella* cultures (4, 6, 41). Furthermore, in vitro studies by Byrd and Horwitz (6) have indicated that gamma interferon-activated monocytes restrict the intracellular growth of *L. pneumophila* by reducing the availability of intracellular iron.

However, little is known about the ability of *L. pneumophila* to acquire iron either intracellularly or extracellularly, and the influence of iron limitation on the physiology and virulence of

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this pathogen is also poorly understood. Studies by Byrd and Horwitz (6) have suggested that the intracellular labile pool is the primary source of iron within alveolar monocytes. A number of *L. pneumophila* iron-containing proteins have been identified (33), and Johnson et al. (29) have demonstrated that a periplasmic ferric reductase may participate in iron acquisition. Reeves et al. (43) concluded, when studying legionellae grown in iron-limited batch culture, that legionellae do not produce commonly recognized siderophores. Furthermore, investigations by Barker et al. (2) using cells grown within amoebae and in iron-deficient batch culture failed to detect the induction of specific membrane-associated iron uptake systems. Interestingly, Hickey and Cianciotto (25) recently cloned and characterized a *fur* gene from *L. pneumophila*, which suggests that iron may participate in the regulation of gene expression. Continuous culture, which overcomes the inherent problems of batch culture and allows the effects of growth rate and environmental parameters to be separated (38), has so far not been employed to study the influence of iron limitation on the virulence and physiology of legionellae.

In this study we used continuous culture to grow a virulent strain of *L. pneumophila* in a controlled iron-limited environment. This enabled investigations on the influence of iron limitation as may be encountered in nature on the virulence and physiology of this pathogen.

MATERIALS AND METHODS

Strain and culture. A virulent clinical isolate of *L. pneumophila* serogroup 1, subgroup Pontiac, strain Corby (28), was grown in chemostat culture as described previously by Mauchline et al. (31). In brief, the 1-liter culture vessel, constructed of glass and titanium, contained 500 ml of ACES-buffered chemically defined (ABCD) medium (37). The medium was inoculated from 72-h buffered charcoal yeast extract (BCYE) agar (11) cultures, which were grown from stock cultures stored at −70°C. The oxygen concentration was maintained at 0.31 mg liter^{-1} (equivalent to 4.5% [vol/vol] air saturation at 37°C) through feedback control of the agitation rate, while the temperature and pH of the culture were controlled at 37°C and 6.9, respectively. All parameters were continuously monitored and controlled with the aid of an Anglicon Microlab Fermentation Control System (Brighton Systems, Newhaven, United Kingdom) linked to the relevant probes immersed in the culture. Steady-state growth was achieved at a dilution rate of 0.08 h^{-1}, equivalent to a mean generation time of 8.7 h. Optical density at 540 nm and biomass concentration were used to assess steady-state growth. For iron-limited growth, the iron sources, ferrous sulfate and hemin, were omitted from the ABCD medium, while the nonferrous design of the chemostat avoided problems of iron leaching from the stainless steel components of conventional fermentation systems. The low concentration of contaminating iron contributed by other medium components was sufficient to support iron-limited growth. All cultures were allowed to achieve steady state under iron-replete conditions before a switch over to iron limitation and finally reversion back to iron-replete conditions.

Culture analyses. Cultures were routinely sampled for optical density (540 nm) and cell dry weight monitoring. The turbidity of culture samples was measured at 540 nm in a UV-260 spectrophotometer (Shimadzu Co., Kyoto, Japan). The biomass concentration of samples was determined by filtering 10 ml of formolized culture (1% [vol/vol] formaldehyde solution) through a preweighed, predried, 0.2-μm-pore-size nylon membrane filter. Filtered samples were rinsed with 10 ml of distilled water before being dried in a microwave oven and reweighed. Total viable counts were performed by plating serially diluted culture samples on BCYE agar and incubating them at 37°C. Culture purity was checked by plating neat samples onto BCYE agar and control BCYE agar deficient in cysteine. Steady-state culture samples were collected on ice, and biomass was separated by centrifugation. All glassware and centrifuge tubes used for collection and storage of iron-limited cultures were acid washed by soaking in 6 M hydrochloric acid overnight and rinsing with distilled, deionized water. Aliquots of supernatant, sterilized by passage through 0.2-μm-pore-size cellulose acetate filters, and cell paste samples were stored at −40°C for analysis.

Amino acids in the clarified culture supernatants and uninoculated culture medium were analyzed with a 4400 dedicated amino acid analyzer (LKB, Cambridge, United Kingdom). Separation was achieved by ion-exchange chromatography with postcolumn ninhydrin reaction and detection at 440 and 570 nm. Cations were determined by flame atomic absorption spectroscopy, with the exception of iron, which was assayed by the more sensitive electrothermal furnace atomic absorption spectroscopy. Ammonia was assayed by the Berthelot reaction (21). To determine the iron content of biomass, washed cell paste dried at 120°C to a constant weight was resuspended in deionized water and analyzed by electrothermal atomic absorption spectroscopy, with an ashing stage to remove organic material.

Culture morphology was monitored by differential interference contrast microscopy (44). Culture samples were fixed in 1% (vol/vol) formaldehyde solution before a dry smear was prepared on a glass slide and viewed by episcopic differential interference contrast microscopy. Culture samples were also indirectly immunolabelled with *L. pneumophila* serogroup 1-specific polyclonal antiserum (9) and visualized by episcopic UV illumination.

Virulence testing. Culture virulence was assessed using the guinea pig aerosol model as developed and described by Baskerville et al. (3) and Fitzgeorge et al. (16). Steady-state culture samples were used to generate fine-particle aerosol challenges for guinea pigs. Iron-limited cultures were concentrated 10-fold by centrifugation and resuspended in sterile distilled water before aerosolization. Groups of eight animals were exposed to neat and 2.5-fold serially diluted samples, facilitating subsequent determination of the fifty percent lethal dose (LD_{50}). The retained dose was quantitated by performing viable counts on lung macerates from guinea pigs receiving the maximum dose and sacrificed immediately after challenge. Six doses of iron-replete and three doses of iron-limited cultures were administered. LD_{50} values and their 95% confidence intervals were calculated by the method described by Finney (14) for determining the fiducial limits of the LD_{50}.

In vitro macrophages assay. The uptake and multiplication of iron-limited and -replete cultures in guinea pig alveolar macrophages were examined and compared using an in vitro assay (15, 51). Guinea pig alveolar macrophages, obtained by lung lavage, were used to form monolayer cultures with 5×10^5 macrophages per well in a microtiter plate. Eagle's minimum essential medium (Imperial Laboratories, Andover, United Kingdom) supplemented with sodium bicarbonate (10 mM), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (20 mM), L-glutamine (2 mM), and 5% (vol/vol) normal autologous guinea pig serum was used as the culture medium. The monolayers were inoculated with an aliquot of bacterial suspension containing 5×10^7 CFU in culture medium and incubated at 37°C for 2 h. All nonphagocytosed bacteria were killed by treatment with gentamicin at 50 mg/liter for 2 h and removed by washing the monolayer three times with culture medium. The uptake of bacteria into the macrophages was assessed by immediately lysing duplicate cultures with digitonin (0.8%, wt/vol) and enumerating the culturable bacteria via growth on BCYE agar. The remaining cultures were incubated in culture medium at 37°C, and duplicate wells were harvested at 24 and 48 h. Intracellular growth was assessed by determining the total number of culturable legionellae present in both the culture medium as a result of host cell rupture and in the remaining intact macrophages following digitonin lysis. Control reactions were included for the Corby virulent (CV) stock strain and an avirulent variant, "CAC," obtained by repeated subculture on BCYE-α agar (27). Stock suspensions of these control cultures harvested from BCYE agar and stored at 4°C were used as inocula.

RESULTS

L. pneumophila Corby grew well in ABCD medium, achieving steady-state iron-replete growth characteristics comparable to those reported by Mauchline et al. (31). Optimum growth was achieved at an oxygen concentration equivalent to 0.31 mg liter^{-1}, with serine providing the principal source of carbon and energy. Steady-state chemostat cultures were established under conditions of carbon limitation and iron excess before conversion to iron limitation. The modified iron-limited medium contained trace amounts of contaminating iron, normally 0.3 to 1.0 μM, which was found to be sufficient to support steady-state growth at a reduced culture density. Conversion to iron-limited conditions resulted in a loss of culture pigmentation within 24 h, with the culture becoming creamy white. The culture density remained stable for 8 to 10 generations while the iron concentration remained in excess, after which point the culture density dropped by at least 50%, reaching a new steady state within 10 generations from the onset of iron limitation.

Morphology. Adaptation to the iron-restricted environment resulted in changes in cell morphology (Fig. 1). Iron-replete cultures were characteristically pleomorphic with cells ranging from 2 to 40 μm in length, while iron-limited cultures became uniform with short rods 1 to 3 μm in length. Cultures regained their pleomorphic characteristics when returned to iron-replete conditions. Both cultures were nonflagellated when viewed by both differential interference contrast and electron microscopy. Colony morphology on BCYE plates and reaction

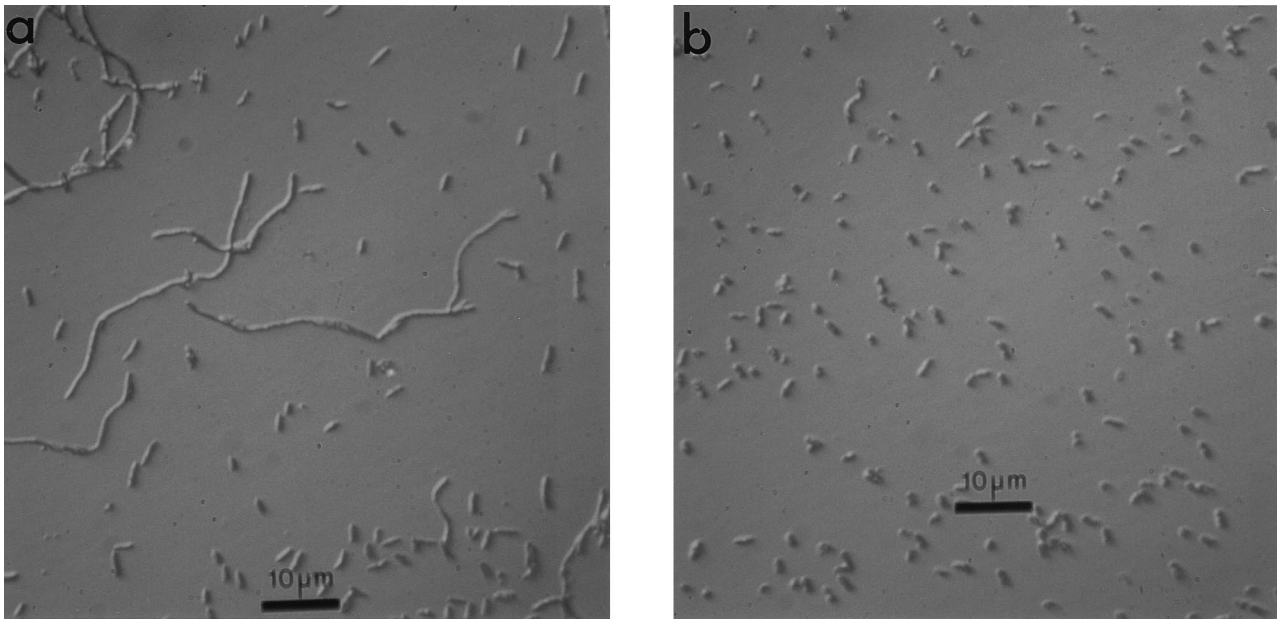

FIG. 1. *L. pneumophila* viewed by differential interference contrast microscopy. (a) Iron-replete cultures exhibiting pleomorphism, with both long and short cells; (b) iron-limited, uniform cultures of short fine rods.

with serogroup 1-specific polyclonal antiserum were similar for both iron-limited and -replete cultures.

Nutrient utilization. Amino acid analysis of iron-replete culture filtrates confirmed the earlier observation of George et al. (19) and Mauchline et al. (31) that serine provides the main source of carbon and energy (Table 1). In this growth environment tyrosine has been shown to be the growth-limiting nutrient (31), even though a number of other amino acids are depleted from the culture medium. Metabolism of 17.2 mM serine was accompanied by the production of 16.2 mM ammonia, indicating a stoichiometric relationship close to the theoretical value of 1:1 for serine metabolism and ammonia production. The presence of cystine in iron-replete media and culture supernatants demonstrates that oxidation of cysteine catalyzed by iron has occurred. Essential trace elements, including iron, remained in excess (Table 1).

In iron-limited cultures, the majority of amino acids, including tyrosine and serine, remained in excess, with only proline being completely depleted. There was no evidence of cysteine oxidation under iron-limited conditions. A decrease was observed in the metabolism of sulfur-containing amino acids, in particular methionine, which may suggest a decrease in the synthesis of iron-sulfur proteins. The metabolism of 14 mM serine was associated with the production of 9.5 mM ammonia, which represents a decrease in ammonia production in relation to serine metabolism. Iron-limited-culture supernatants still contain a low concentration of iron, approximately 0.1 μM. Titrating ferrous sulfate back into iron-limited culture medium while monitoring the growth response confirmed iron as the growth-limiting nutrient and demonstrated that 3.3 μM iron is required to support iron-replete growth. Other important trace elements were found to remain in excess.

Growth yield. Table 2 lists the growth characteristics calculated from pooled analyses of representative iron-limited and -replete cultures. *L. pneumophila* grew well in ABCD medium, with values for culture optical density at 540 nm, biomass, and viable count comparable to those reported previously (31). Decreasing the iron concentration caused a dramatic drop in culture density, biomass, and total viable count, with a 40% decrease in the cell dry weight of iron-limited cultures. The molar growth yield for serine (Y_{serine}) was also reduced. However, it is more accurate to account for the metabolism of other amino acids, particularly in iron-replete culture, by calculating the molar growth yield for carbon metabolized, Y_{carbon}. No significant differences were found between iron-limited and -replete cultures for values of Y_{carbon}, indicating no major loss in the efficiency of carbon metabolism. Under iron limitation, Y_{iron} increased sixfold, suggesting increased efficiency of iron metabolism for the maintenance of essential cellular components and functions. This correlates with a 54% decrease in the iron content of iron-limited-culture biomass.

Virulence and intracellular growth. LD_{50} values for iron-limited and -replete cultures of *L. pneumophila* demonstrate that iron-replete cultures are significantly ($P < 0.05$) more virulent than the same culture grown under iron-limited conditions (Table 3). Aerosol challenge of guinea pigs with iron-replete culture produced death, with an LD_{50} of 3.79 $\log_{10}$ (Table 3). However, when the culture was converted to iron limitation, no deaths were observed at the maximum challenge of 5.8 $\log_{10}$, indicating that the culture had become less virulent for guinea pigs. When the culture was converted back to iron-replete conditions, there was a statistically significant ($P < 0.05$) increase in virulence, with an LD_{50} value comparable to that recorded prior to iron limitation obtained (Table 3). This LD_{50} value was slightly higher than that reported for the replete culture prior to iron limitation. However, the difference was not significant. Mauchline et al. (32) observed a similar event during temperature studies. Two repeat culture and virulence experiments have confirmed that virulence modulation in response to iron limitation is a reproducible phenomenon.

The in vitro macrophage assay was used to compare the uptake and multiplication of virulent iron-replete and avirulent iron-limited cells in cultured guinea pig alveolar macrophages (Fig. 2). Data represent the mean of six determinations from three separate assays, each performed in duplicate. Results were compared with those for virulent and avirulent control

TABLE 1. Concentration of nutrients in unused ABCD medium and in iron-limited and -replete culture filtrates

Nutrient	Concn (mM) of nutrient[a]		
	Unused medium	Iron replete	Iron limited
Alanine	1.14	0.68	1.6
Arginine	0.54	ND	0.19
Aspartic acid	0.52	0.36	0.35
Cysteine	0.34 (0.6)[b]	0.30	0.69
Cystine	0.22 (ND[c])	0.13	ND
Glutamic acid	1.17	0.28	0.76
Glycine	0.94	0.65	0.96
Histidine	0.32	ND	0.21
Isoleucine	0.62	ND	0.26
Leucine	0.63	ND	0.15
Lysine	0.41	ND	0.2
Methionine	0.62	0.04	0.52
Phenylalanine	0.54	ND	0.36
Proline	0.9	ND	ND
Serine	17.45	0.28	3.6
Threonine	0.53	ND	0.50
Tryptophan	0.35	0.26	0.39
Tyrosine	0.23	ND	0.17
Valine	0.83	ND	0.5
Iron[d]	150.6 (0.45)	147.3	0.13
Potassium	27.85	36.83	37.92
Zinc	0.07	0.07	0.07
Ammonium	6.3	22.5	15.67

[a] Values for unused medium represent the means of seven samples, and filtrate figures are the means of four samples. All standard deviations were less than 18% of the means.
[b] Values in parentheses are for iron-limited medium.
[c] ND, not detected (<0.04 mM).
[d] Iron expressed as micromolar concentrations.

cultures. In this model system there was good uptake of the virulent iron-replete culture (Fig. 2), although the uptake frequency was slightly lower than that of the control cultures. By contrast, the numbers of culturable iron-limited cells located intracellularly after phagocytosis was significantly lower. The difference between the numbers of culturable iron-limited and -replete cells located intracellularly after phagocytosis was statistically significant ($P < 0.01$) when tested by the unpaired *t* test. However, both test cultures proceeded to multiply rapidly, with a slightly greater initial growth rate apparent for the iron-limited culture. The behavior of both control cultures was consistent with previous results reported by Tully et al. (51) for these strains. Corby virulent is characterized by good infection frequency and rapid intracellular multiplication. While the "CAC" variant also demonstrates good uptake frequency, it is unable to multiply intracellularly.

TABLE 2. Growth characteristics of iron-limited and -replete cultures of *L. pneumophila*

Characteristic	Iron replete	Iron limited
Optical density at 540 nm	3.49	1.62
Biomass (g of dry wt liter^{-1})	1.38[a]	0.82[a]
CFU ml^{-1} (10^9)	5.38 ± 0.56[b]	1.71 ± 0.26[b]
Y_{serine} (g mol^{-1})[c]	80.2	59.5
Y_{carbon} (g mol^{-1})[c,d]	14.9	13.6
Iron metabolized (μmol liter^{-1})	3.3	0.32
Y_{iron} (10^5 g mol^{-1})[c]	4.2	26
Iron content (μg g^{-1} [dry wt])	94	44

[a] Biomass values represent the means of five samples. Standard deviation, less than 15%.
[b] Mean ± standard error of the mean.
[c] Y_x, the molar growth yield, represents the biomass yield in grams (dry weight) per mole of substrate metabolized.
[d] Calculated from amino acid analysis.

TABLE 3. LD_{50} values and their 95% confidence intervals obtained for *L. pneumophila* grown in chemostat culture under iron-limited and -replete conditions

Culture conditions	$\log_{10}$ LD_{50}	95% confidence interval of LD_{50}[a]
Iron replete	3.79	3.45–4.13
Iron limited	>5.80[b]	NA[c]
Iron replete[d]	4.18	3.96–4.39

[a] Ninety-five percent fiducial limits for the LD_{50} were calculated by the method of Finney (14).
[b] No deaths occurred at the maximum challenge.
[c] NA, not appropriate.
[d] The culture was returned to iron-replete conditions after being grown under iron-deficient conditions.

DISCUSSION

The nonferrous design of the culture system, together with the omission of iron from the defined medium, provided a suitable iron-limited environment for the continuous culture of *L. pneumophila*. This facilitated a detailed study on the influence of iron deficiency on the physiology and virulence of this pathogen.

In agreement with the results of other workers, iron was found to be a critical nutrient for the growth of this microorganism, with 3.3 μM iron required for optimum growth. However, previous reports on the iron requirements of *L. pneumophila* vary considerably. Reeves et al. (42) observed that the concentration of metals required for optimum growth varied with strain, with a Philadelphia 1 strain needing 20 μM iron for optimum growth in batch culture. The findings of the present study are in closer agreement with those of Johnson et al. (29), who found that 3 μM iron supported the growth of a virulent strain of *L. pneumophila.*

Growing *L. pneumophila* in an iron-restricted environment had a significant effect on cellular morphology. Pleomorphic iron-replete cultures became uniform cultures of short fine rods in response to iron limitation. These morphological changes were reversible, with the iron-limited cultures reverting to pleomorphic forms when iron was added back to the medium. Alterations in cellular morphology in response to changes in the growth environment have been reported previously. Employing a similar culture system, Mauchline et al. (31) observed uniform cultures of short fine rods at 24°C which became highly pleomorphic at 37°C. Pine et al. (36) also reported changes in cell morphology as a function of culture conditions when *L. pneumophila* was grown in batch culture, with substrate limitation resulting in the formation of coccally shaped cells.

As demonstrated previously, serine provides the principal source of carbon and energy for the growth of *L. pneumophila* (31). The nonstoichiometric relationship between serine metabolism and ammonia production under iron-limited conditions suggests that serine is also being incorporated into biomass. However, when iron is in excess, the serine is almost depleted. In order to maintain growth, other amino acids are either metabolized for energy or incorporated directly into the biomass, resulting in the depletion of a number of amino acids, with tyrosine becoming the growth-limiting nutrient (31). Previous investigations into the nutritional requirements of *L.*

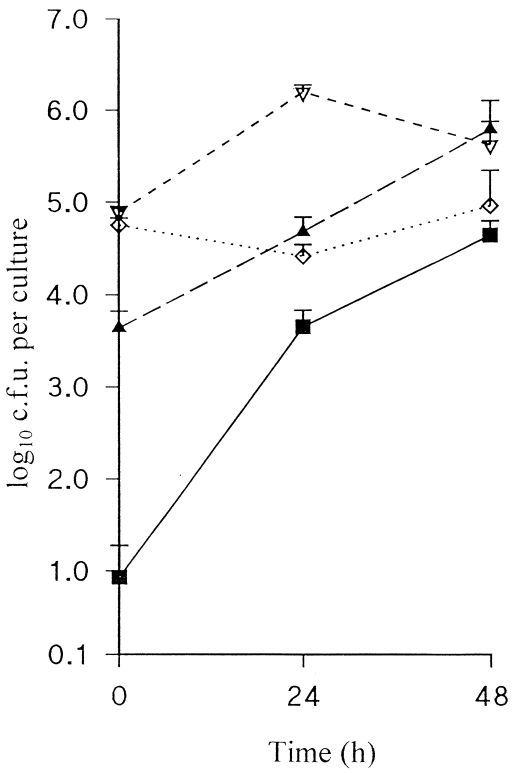

FIG. 2. Infection and multiplication of *L. pneumophila* Corby within cultured alveolar macrophages. ■, iron limited; ▲, iron replete; ▽, Corby virulent control; ◇, Corby avirulent control. Data represent the means of six determinations + standard errors of the means.

pneumophila have confirmed that a number of amino acids may act as an energy source (19, 49). Under iron-limited conditions only a minor decrease was observed in the molar growth yield for carbon metabolized, Y_{carbon}, which correlated with a slightly lower efficiency of biomass production. In contrast, the molar growth yield for iron, Y_{iron}, increased dramatically for iron-limited cultures. This suggests that legionellae, in common with other microorganisms, contain a complex regulatory network which allows them to control their metabolism, structure, and function in order to utilize the available iron more efficiently while maintaining essential cellular functions. Increased metabolic efficiency is also supported by the observation that the iron content of iron-limited biomass is reduced by approximately 50%. These observations are in agreement with the views of Tempest and Neijssel (48) in relation to microbial adaptation to nutrient-deficient environments.

In addition to the morphological and physiological response to iron limitation, this study clearly demonstrates that iron availability has a dramatic effect on the virulence of *L. pneumophila*. Iron-limited cultures were found to be significantly less virulent than iron-replete cultures. Chemostats provide a strong selective pressure which often encourages irreversible genotypic alterations, producing mutants more suitable to the imposed environment (39). Under these circumstances the virulent parent phenotype would be lost and could not be reestablished. However, the present work demonstrates that the virulent phenotype could be reestablished by switching the iron-limited avirulent culture back to iron-replete conditions, suggesting that avirulence is the result of phenotypic modulation, as opposed to the selection of an altered genotype.

These results are complemented by the in vitro macrophage assay, which demonstrates a significantly lower number of culturable avirulent iron-limited cells located within alveolar macrophages after infection, in comparison with the virulent replete and control cultures. However, the small number of intracellular iron-limited cells proceed to multiply rapidly after phagocytosis, demonstrating that they are not defective in their ability to overcome host cell antimicrobial mechanisms. A separate assay employing in vivo infection confirmed these differences between the numbers of iron-limited and -replete cells recovered after infection (data not shown). Therefore, reduced phagocytosis and/or reduced survival after uptake appears to be a critical factor affecting the virulence of this pathogen. This avirulent iron-limited phenotype differs considerably from that of the avirulent "CAC" control culture, which although able to enter and survive within mononuclear phagocytes, is unable to multiply intracellularly. Horwitz (26) also characterized an avirulent mutant of *L. pneumophila* which was capable of surviving but not multiplying within human monocytes, because of an inability to inhibit phagosome-lysosome fusion. Although the nature of *L. pneumophila* virulence is believed to be multifactorial, so far only one virulence determinant has been identified. The 24-kDa Mip (macrophage infectivity potentiator) surface protein is required for optimal intracellular infection and for full virulence in the guinea pig model (7). The 29-kDa major outer membrane protein also participates in phagocytosis by acting as a complement (C3) receptor; however, its role in pathogenesis is unclear because of its expression on both virulent and avirulent cells (10). Further studies are necessary to investigate the molecular basis for avirulence conversion, as observed in this study.

The theory that numerous environmental factors may participate in regulating the virulence of *L. pneumophila* is supported by the observations of this study and those reported by Mauchline et al. (32), who demonstrated the role of growth temperature in the reversible induction of *L. pneumophila* virulence. As occurs with many bacterial pathogens, different environmental stimuli may control the expression of different genetic programs, with virulence representing the cumulative action of numerous response regulons, which govern the products of many genes (24). Indeed this notion is further supported by the demonstration of a global response to the intracellular environment of the macrophage (1). These results are in contrast to the role of iron in regulating the virulence of many other pathogens such as *E. coli*, *V. cholerae*, and *N. meningitidis*, in which in the absence of iron the transcription of virulence determinants including iron acquisition mechanisms is induced (5, 24, 30).

The occurrence of this distinct phenomenon in *L. pneumophila* is supported by previous observations. Firstly, *L. pneumophila* is a natural inhabitant of aquatic environments, where it has been suggested to multiply primarily as an intracellular parasite of protozoa (13, 52). Outside the protozoan host, legionellae may have to survive a range of stressful physical and chemical conditions, including low nutrient availability, for long periods of time (17, 47). Under these conditions downregulation of virulence determinants and other nonessential cellular components may be a survival mechanism helping to conserve vital carbon and energy reserves. Secondly, intracellular growth is iron dependent and can be inhibited by iron chelators (6, 18). These observations imply that when confronted by iron limitation intracellularly, *L. pneumophila* is unable to elaborate an effective repertoire of virulence determinants to combat iron restriction and, therefore, is rendered avirulent. Conversely, an adequate supply of iron is essential for the growth and virulence of this pathogen. An alternative

explanation for this phenomenon is that the regulatory effect of iron is coupled to other environmental parameters such as temperature or oxygen, which help to coordinate virulence.

On the basis of the observations of this study, it appears necessary for legionellae to encounter an iron-rich environment prior to aerosolization to induce the expression of a virulent phenotype. One of the few niches in aquatic habitats that may fulfil this requirement is the intraprotozoal environment. Although little is known about the nature of this environment, the ability of *L. pneumophila* to multiply to high cell densities suggests that it provides a relatively rich source of nutrients in comparison with the extracellular environment (2, 13). Indeed it has been proposed that adaptation to intracellular growth within protozoa may precondition legionellae for infection and survival within human phagocytic cells. This hypothesis has been supported by the apparent involvement of similar genes and mechanisms in the infection of macrophages and protozoa (8). Furthermore, phenotypic changes in response to intra-amoebic growth have been demonstrated (2). It is also possible that the increased concentration of nutrients encountered in the sediment of water systems and cooling tower ponds, together with leached metal ions from water pipes, could participate in inducing the virulence of *L. pneumophila.*

In conclusion, this study clearly demonstrates the critical role of iron in modulating the physiology and virulence of *L. pneumophila* and further supports the theory that multiple environmental factors participate in the coordinated regulation of the physiology and virulence of this pathogen.

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