Growth of *Legionella* spp. under conditions of iron restriction

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Summary. The growth inhibiting activity of transferrins, citrate, 2-2' dipyridyl and desferrioxamine methanesulphonate towards *Legionella* spp. and their serogroups was investigated. The inhibitory activity of all these compounds depended upon the iron-free state of the molecules and was abolished by saturation with iron. No bactericidal effect by transferrins was observed at concentrations up to four times the minimal bacteriostatic concentration. No interaction of transferrins with the legionella cell surface was detected by direct or indirect fluorescence assay, or by dialysis culture experiments in which transferrin was separated from the bacterial cells. The demonstration of a siderophore-like activity in supernates of iron-deficient legionella cultures may account for the ability of *Legionella* spp. to multiply in conditions of iron restriction.

Introduction

Iron is an essential element for all forms of life, being an irreplaceable component of many enzymes, particularly those connected with respiratory chains.^{1,2} The acquisition of iron by micro-organisms, in competition with the host transferrins (serum transferrin, lactoferrin, and ovotransferrin), is fundamental to the production of infection by pathogenic bacteria.^{3–5} Because of the extreme insolubility of ferric iron at neutral pH, and of the low availability of free iron in the body fluids,⁶ most micro-organisms synthesise iron-chelating compounds called siderophores, which are excreted into the medium and taken up by cells as siderophore-Fe(III) complexes via specific receptors.⁷ In a few micro-organisms, e.g., *Neisseria* spp., *Bordetella pertussis* and *Haemophilus influenzae*, iron acquisition has been shown to take place by direct uptake from the transferrins of the host, and not by means of siderophores.^{8–12} Conflicting results have been reported on the production of siderophores by *Legionella* spp. Warren and Miller¹³ stated that *L. pneumophila* produces at least two different types of catechol-like siderophore when cultured in an iron-deficient chemically defined medium, but Reeves *et al.*¹⁴ did not detect any production of hydroxamate or catechol-like siderophore by various species and serogroups of *Legionella*. Moreover it has been shown that *L. pneumophila* was significantly

inhibited by different iron chelators,^{15,16} and was sensitive to the bactericidal effect of both lactoferrin and serum transferrin.^{15,17}

Here we report a study of the sensitivity of different *Legionella* spp. and serogroups to chemical and biological iron chelators and of the possible mechanisms of iron acquisition by these bacteria.

Materials and methods

Bacterial strains

The type strains of various *Legionella* spp. and serogroups were used: *L. pneumophila* serogroup 1, strain Philadelphia 1; serogroup 2, strain Togus 1; serogroup 3, strain Bloomington 2; strain Los Angeles 1, formerly described as serogroup 4; strain Dallas 1E, formerly described as serogroup 5; serogroup 6, strain Chicago 2; serogroup 7, strain Chicago 8; serogroup 8, strain Concord 3; serogroup 9, strain IN-23-G1-C2; serogroup 10, strain Leiden 1; *L. micdadei* strain Tatlock; *L. bozemanii* serogroup 2, strain Toronto 3; *L. dumoffii*, strain NY/23; *L. jordanis*, strain B/540; *L. oakridgensis*, strain OR-10. An additional clinical isolate of *L. pneumophila* serogroup 6, strain Monza 3/1386,¹⁸ which was subcultured only twice after isolation, was used for some assays. All type strains were stored as stock cultures at –70°C in skimmed milk, and maintained on Buffered Charcoal-Yeast Extract Agar with α -ketoglutarate 0.1% (BCYE- α -agar, Oxoid).¹⁹ The recent clinical isolate of *L.*

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pneumophila serogroup 6 was stored at -70°C in skimmed milk, and subcultured once for use.

Media and culture conditions

Buffered Yeast Extract (BYE) broth was prepared with yeast extract (Difco) 10 g/L supplemented with ACES buffer (Sigma) 10 g/L, pH 7.0, L-cysteine hydrochloride (BDH Chemicals Ltd, Poole) 400 mg/L and α -ketoglutarate (Sigma) 1 g/L. A liquid chemically defined medium (CDM) was prepared according to Reeves *et al.*¹⁴ The iron concentration, as determined by atomic absorption spectrophotometry, was $27\text{ }\mu\text{M}$ in BYE broth and $20\text{ }\mu\text{M}$ in CDM. In experiments for siderophore detection, the iron concentration in both media was reduced by omitting ferrous sulphate from the CDM and by treating the BYE broth with the iron-chelating resin Chelex 100 (Biorad Laboratories, Richmond, Ca, USA) 5 mg/ml, for 24 h at 4°C . In the case of the latter treatment it must be considered that other ions, such as Mg (II), Ca (II), Cu (II), Mn (II) etc., in addition to iron might have been removed. Plate counts were performed on BCYE- α -agar. A growth temperature of 36°C was used for cultures in both liquid and solid media; plates were incubated in air in a moist atmosphere.

Transferrins

Apo-transferrin (Tf) from human serum and apo-lactoferrin (Lf) from human colostrum were obtained from Sigma; apo-ovotransferrin (Otf) was kindly supplied by the Department of Biochemistry of the University of Rome "La Sapienza". The purity of Tf and Lf was checked by discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis,²⁰ and the preparations of Otf were characterised by electrophoresis, analytical ultracentrifugation and iron-binding capacity.^{21, 22} In control experiments, iron-saturated transferrins²³ were also used. Fluorescein-conjugated Otf was prepared by us with fluorescein-isothiocyanate (Isomer IV; Sigma).²⁴ For the experiments, transferrins were prepared by dilution of a $266\text{ }\mu\text{M}$ stock solution in phosphate-buffered saline (PBS) containing 50 mM sodium bicarbonate (pH 7.0) to generate iron-binding function and sterilised by membrane filtration (Millipore SpA, Rome, Italy), $0.45\text{-}\mu\text{m}$ pore size. The antibacterial activity of transferrin preparations was controlled in all the experiments with a sensitive strain (803) of *Escherichia coli* K-12.²³

Chelating agents

2-2' Dipyriddy (Sigma), sodium citrate (Merck, Darmstadt, FRG) and desferrioxamine methanesulphonate (DSF) (Desferal; Ciba-Geigy, Basel, Switzerland) were used as chemical chelators. Stock aqueous solutions, at 10 times the required final concentration, were sterilised by membrane filtration (Millipore), $0.45\text{-}\mu\text{m}$ pore size.

Growth inhibition assay

L. pneumophila strains were tested for their sensitivity to iron chelators in microtitration plates. Serial two-fold dilutions of the chelator were made in BYE broth and in CDM. The inoculum was prepared from a 96-h culture in iron-free CDM and consisted of $c. 10^4$ cfu in $200\text{ }\mu\text{l}$ of CDM/well. For other *Legionella* spp., which grew very poorly in CDM, the growth inhibition assay was performed in BYE broth only. For this purpose, 72-h cultures on BCYE- α -agar were washed twice in sterile distilled, deionised water, being held at 36°C for 1 h on each occasion before centrifugation, and then adjusted to the concentration indicated above before use as inocula. The iron-binding agents were added just before the bacterial suspensions and the microtitration plates were incubated at 36°C with moderate agitation. Experiments were performed in triplicate. The activity of chelators was expressed as the minimal concentration inhibiting bacterial growth after incubation for 72 h (A_{620} of the cultures <0.100).

Bactericidal activity assay

The tests were performed in duplicate in microtitration plates with the following strains: *L. pneumophila* serogroups 1 and 8 (reference strains); *L. pneumophila* serogroup 6, strain Monza 3/1386; *L. dumoffii* (a non-*pneumophila* species). Cultures grown for 72 h on BCYE agar were twice washed in sterile distilled, deionised water, being held at 36°C for 1 h on each occasion before centrifugation and resuspension to a density of 4 (McFarland turbidity standard; $c. 10^9$ cfu/ml). Doubling dilutions of the stock transferrin solutions (Tf, Lf, Otf) were prepared in sterile distilled, deionised water to give final dilutions from 106.66 to $6.66\text{ }\mu\text{M}$. The inoculum was then added to a final concentration of 10^5 cfu/ml. The number of viable cells was checked by plating dilutions of the bacterial suspensions immediately and after incubation at 36°C for 2 h.

Fluorescent staining assays

Direct fluorescence (DF) or indirect immunofluorescence (IF) assays were performed to detect a possible interaction between transferrins and the bacterial surface. Experiments were performed with *L. pneumophila* serogroups 1, 4 and 8, *L. dumoffii*, *L. micdadei*, and *L. oakridgensis* in suspension or fixed by different methods and satisfactory observations were obtained as follows. *Legionella* cells grown on BCYE- α -agar were washed once, resuspended in PBS, pH 7.2, and distributed on multiwell microscope slides (500–600 organisms/field). After heat-fixation, slides were immersed in formalin 10% for 10 min at room temperature, washed and air-dried. The DF assay was performed for Otf. A solution of fluorescein isothiocyanate-conjugated apo-Otf (FITC-Otf), 2 mg/ml, was

dropped on to the slides before incubation at 36°C for up to 3 h in a moist chamber. After washing with PBS, slides were mounted with buffered glycerol, pH 9.0, and examined under UV light. The IF assay was performed for Tf and Lf. Slides were prepared and fixed as described above; transferrins (1 mg/ml; 5- μ l volumes) were distributed into the wells and the slides were incubated for up to 3 h at 36°C. The preparations were then rinsed with PBS, placed in a PBS bath for 5 min, and air-dried. Tf goat antiserum and Lf rabbit antiserum (Sigma) were distributed into the appropriate wells (5- μ l volumes of a 1:8 dilution), incubated for 2 h at 36°C, and washed twice with PBS. FITC-anti-goat immunoglobulin (Cappel Organon Teknika Corp., PA, USA; dilution 1:10) and FITC-anti-rabbit immunoglobulin (Wellcome Research Laboratories, Beckenham; dilution 1:50) were then used for staining the preparations. After incubation for 30 min at 36°C the slides were washed, mounted with buffered glycerol, pH 9.0, and observed under UV light. As a positive control, the sensitive *E. coli* strain 803 was used in DF experiments.

Dialysis cultures

L. pneumophila serogroup 1 and the recent clinical isolate of *L. pneumophila* serogroup 6 were used in experiments with apo- and iron-saturated Otf. Either 1 ml of 266- μ M Otf in 50 mM sodium bicarbonate, pH 7.2, was added directly to 9 ml of CDM, or 1 ml of 266 μ M Otf was enclosed in a dialysis bag and immersed in a tube (180 mm $\times$ 20 mm, iron-free) containing 9 ml of CDM to separate it from culture medium. Tubes were inoculated with 100- μ l volumes of bacterial suspensions (c. 10^8 cfu/ml) in iron-free CDM.

Siderophore assay

Tests for siderophore detection were performed on culture supernates of all *L. pneumophila* strains grown in "iron-free" CDM (Fe concentration 0.05 ppm, determined by atomic absorption spectrophotometry) with or without 100 μ M ferrous sulphate. Bacteria from a 96-h culture in CDM were washed twice in distilled, deionised water as described above, and 5×10^5 cfu were used to inoculate 200 ml of medium either in 250-ml or 500-ml Erlenmeyer flasks. Cultures were grown in aerated conditions with vigorous agitation of the 500-ml flasks at 250 rpm in a G25 orbital shaker (New Brunswick) and in non-aerated static cultures in sealed 250-ml flasks. *Legionella* spp. other than *L. pneumophila* were grown in Chelex 100-treated BYE broth (Fe concentration 0.33 ppm). At 24-h intervals samples of culture were examined for optical density at 620 nm and for siderophore activity in the supernate. The presence of catechol-like siderophores was identified by the Arnow assay²⁵ with 2,3-dihydroxybenzoic acid as standard; enterochelin produced by *E. coli* 803 was used as a positive control. For the determination of

hydroxamate-like siderophores a modification of the Csáky test²⁶ proposed by Gibson and Magrath²⁷ was employed; aerobactin produced by *Enterobacter aerogenes* 62-1 was used as a positive control. In addition, iron-binding compounds were detected by the method proposed by Schwyn and Neilands.²⁸ In all tests for siderophores the uninoculated medium was used as a control. Glassware was washed in 0.1 M HCl, and rinsed with double-distilled water. To express siderophore activity, serial dilutions of DSF were used as standards.

Results

Sensitivity of *Legionella* spp. to iron chelators

Serial two-fold dilutions of chelating agents with different affinities for iron were used in the following concentration ranges: sodium citrate 136–4.25 mM, 2-2' dipyridyl and DSF 512–16 μ M, and transferrins (Tf, Lf, Otf) 53.33–1.66 μ M. In this test, *L. pneumophila* strains were grown in CDM and other *Legionella* spp. were grown in BYE broth.

Results obtained after incubation for 72 h are reported in the table. Little variation was found between strains in their sensitivity to sodium citrate, 2-2' dipyridyl and transferrins. Only minor differences were apparent between *L. pneumophila* and other *Legionella* spp. except with DSF. The differences seen with this substance may have been due to the complex

Table. Minimal inhibitory concentrations of transferrins and chelators for *L. pneumophila* strains and other *Legionella* spp.

<i>Legionella</i> spp. and strains	Minimal inhibitory concentration* (μ M) of					
	transferrin			chelator		
	Tf	Lf	Otf	Citrate† (10^3)	DSF	DPD
<i>L. pneumophila</i>						
Philadelphia 1	26.6	13.3	26.6	8.5	32	128
Togus 1	26.6	13.3	13.3	8.5	64	128
Bloomington 2	13.3	13.3	13.3	17.0	64	256
Los Angeles 1	26.6	13.3	13.3	17.0	32	256
Dallas 1E	26.6	13.3	13.3	17.0	64	256
Chicago 2	26.6	26.6	26.6	17.0	64	256
Chicago 8	26.6	13.3	26.6	8.5	32	256
Concord 3	26.6	13.3	26.6	8.5	64	256
IN-23-G1-C2	13.3	13.3	13.3	8.5	32	128
Leiden 1	26.6	13.3	26.6	17.0	32	256
<i>L. bozemanii</i>	53.3	26.6	26.6	34.0	512	256
<i>L. dumoffii</i>	26.6	13.3	26.6	34.0	512	256
<i>L. jordanis</i>	26.6	13.3	26.6	68.0	512	256
<i>L. oakridgensis</i>	26.6	13.3	26.6	34.0	512	256
<i>L. micdadei</i>	53.3	26.6	53.3	34.0	512	256

* Minimal inhibitory concentrations gave $A_{620} < 0.100$ after incubation of the culture at 36°C for 72 h.

† Citrate as $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$.

DSF, desferrioxamine methanesulphonate; DPD, 2-2' dipyridyl.

and ill-defined composition of the BYE broth used for the non-*pneumophila* species, which grew very poorly in CDM. When all the *L. pneumophila* strains were grown in BYE broth they gave DSF inhibition values similar to those of other *Legionella* spp. (data not shown).

The inhibitory activity of the chelators and transferrins was completely suppressed by pre-incubation with equimolar amounts of ferric chloride before adding the bacteria.

To determine whether the inhibitory activity of transferrins was solely bacteriostatic or in part bactericidal, a bactericidal activity assay was performed with some strains of *Legionella*. The bacteria were suspended in sterile distilled, deionised water and kept in contact with transferrins at concentrations of 106.66–6.66 μM for 2 h. No bactericidal activity was detected for concentrations at least four times as great as the minimal inhibitory concentrations reported in the table. The bacteria employed in all growth

inhibition assays, including tests for bactericidal activity, underwent prolonged (2 h) washing in sterile distilled, deionised water to avoid the carry-over of small quantities of iron from the cultures from which they were derived.

"Interaction tests" with dialysis cultures and fluorescent staining

These experiments were performed to determine whether transferrins act on legionellae only as chelating agents, i.e., without any interaction with the bacterial surface, or require direct contact with the cells for their antibacterial action. The experiments were performed with legionellae either kept in contact with Otf or separated from it by a dialysis membrane; 53.3 μM Otf produced the same degree of growth inhibition, regardless of whether it was in direct contact or was separated from the bacterial cells by a dialysis membrane. Under both conditions, iron-

Figure. Growth of *L. pneumophila* Philadelphia 1 and production of siderophore-like activity in static (A) and aerated (B) cultures. $\circ$, A_{620} of culture in iron-free CDM; $\bullet$, A_{620} of culture in CDM supplemented with 100 μM iron; $\square$, siderophore activity, corresponding to ($\pm$) μMDSF , in iron-free CDM culture supernates. $\blacksquare$, Siderophore activity in supernate of culture in CDM supplemented with 100 μM iron (mean values from duplicate tests). Data shown from one of three separate experiments.

saturated Otf caused no inhibition and permitted growth similar to that of the control culture in CDM without Otf.

The lack of interaction between transferrins and the legionella surface was confirmed by DF and IF assays; negative results in tests for the presence of transferrins on the bacterial surface were obtained with bacteria both in suspension and fixed by different methods.

Production of siderophores

Siderophore activity was investigated in both static and aerated cultures of different strains of *L. pneumophila*. The figure shows the growth curves of *L. pneumophila* strain Philadelphia 1 in static and aerated conditions, and the siderophore activity measured by the assay of Schwyn and Neilands.²⁸ Parallel cultures were set up in "iron-free" CDM (0.05 ppm of iron) and in CDM supplemented with 100 μ M iron, and growth was monitored for up to 10 days. The results show that, with aeration, growth was much more luxuriant in iron-supplemented than in iron-free culture medium, whereas in static cultures the converse was true. It is possible that oxygen toxicity is enhanced during iron-deficient culture, when the activity of various oxygen detoxifying iron-containing enzymes has declined.²

With or without aeration, siderophore activity appeared in the cultures after incubation for 3–4 days, i.e., during the logarithmic growth phase, and reached its maximum at the stationary phase; maximum activity was consistently higher in static than in aerated cultures. Supplementation of CDM with 100 μ M iron completely suppressed synthesis of the substances responsible for the positive reaction in the Schwyn and Neilands test.²⁸ Siderophore activity was produced by all eight *L. pneumophila* serogroups examined; however, for some strains, aeration of the culture did not favour bacterial growth and this prevented the demonstration of siderophore activity. However, activity was demonstrated in supernates of cultures of *L. micdadei*, *L. bozemanii*, *L. dumoffii*, *L. jordanis* and *L. oakridgensis* grown for 6 days in Chelex 100-treated BYE broth; it was abolished by the addition of 100 μ M iron to the growth medium. It must be pointed out that, for all samples that gave positive results in the Schwyn and Neilands test,²⁸ the Arnow test²⁵ for catechols and the Csáky assay²⁶ for hydroxamates gave negative results.

Discussion

The results demonstrate that iron-binding compounds (transferrins, and chemical and siderophore-like chelators) inhibit the growth of various *Legionella* spp. and serogroups. Because no standard protocol for the assessment of the activity of iron-binding compounds has been established, the experiments were

made in CDM for *L. pneumophila*, and in BYE broth for non-*pneumophila* species. The iron concentration in CDM and BYE broth corresponded to 20 μ M and 27 μ M respectively. The growth-inhibiting activity of the chelators tested depended on the abolition of their iron-free state by saturation with Fe(III). The effect of all the chelators was bacteriostatic within wide concentration ranges but not bactericidal. Thus, the results conflict with previous reports,^{15, 17} which suggested that transferrins (Tf and Lf) and other iron chelators such as 1-10 phenanthroline and desferrioxamine B had bactericidal activity. In our in-vitro test, we did not observe any bactericidal effect of chelators that included Lf, Tf and Otf. However, this result does not exclude the possibility that there may be a bactericidal effect *in vivo* when transferrins in the apo or partly saturated form interact with other host defence proteins such as antibodies (IgA) and complement. It must also be borne in mind that any possible short-term bactericidal action of transferrins could not be simply ascribed to the iron-chelating activity but would also depend on direct interaction with the microbial surface.^{17, 29, 30} In the present experiments, the observed lack of bactericidal activity of transferrins was supported by the results of DF and IF assays, which did not show any binding of transferrins (Tf, Lf and Otf) to the cell surface of legionellae. The experiments with dialysis membrane separation showed that the bacteriostatic activity of transferrin was the same, regardless of whether it was free in the culture medium or separated from the bacteria. For micro-organisms interacting directly with transferrin, the antimicrobial activity is enhanced by direct contact and dramatically reduced by separation from the bacterial cells. Our results indicate that transferrins do not interact directly with the legionella surface. The transferrin preparations were free from iron and contaminating protein.

The capacity of *Legionella* spp. to grow under iron-deficient conditions, e.g., those occurring in the presence of sub-bacteriostatic amounts of chelators, has been related to siderophore-like activity. In the experiments described, all *L. pneumophila* serogroups tested, as well as other *Legionella* spp., produced substances with siderophore-like activity when cultured under conditions of iron restriction; this activity was completely suppressed in iron-rich cultures.

The siderophore-like activity of *Legionella* spp. has been demonstrated by the siderophore-specific universal chemical assay of Schwyn and Neilands.²⁸ Negative reactions with the Arnow test²⁵ and the Csáky test excluded catechols and hydroxamates,²⁶ respectively, as the siderophore-like compounds. However, several siderophores are known to be neither hydroxamates nor catechols.^{28, 31} Previous conflicting results^{13, 14} should be reconsidered in the light of the methods used at the time. Further studies are required to determine the chemical and biological properties of the substances responsible for siderophore-like activity in *Legionella* spp. These substances, like microbial

siderophores in general, were produced only in the absence of iron. The concentration of iron in CDM was too low (0.05 ppm) to allow acquisition by a low affinity system; therefore, it may be argued that the growth of *L. pneumophila* in this medium occurred only by means of a high affinity system.

The production of siderophores in response to iron restriction is necessary for the growth of most microorganisms; this may be taken to include *Legionella* spp., in view of their iron requirements and the aerophilic nature of their metabolism.^{16,32} *Legionella* spp. occur in natural aquatic environments, at a pH close to neutrality (5.4–8.1)³³, that contain extremely low amounts of soluble trace metals and are unable to support survival and growth in the absence of any iron-solubilising agent. In legionella infection, hypoferraemia and hypertransferrinaemia have been shown to act as host defence mechanisms.³⁴

In conclusion, *Legionella* spp., like most microorganisms, can grow under conditions of iron restriction by synthesising chelators; they also show an overall sensitivity to iron-binding agents similar to that observed for several gram-negative bacteria.^{22, 23}

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