INFECTION AND IMMUNITY, May 1996, p. 1873–1875 Vol. 64, No. 5
0019-9567/96/$04.00+0

Absence of Siderophore-Like Activity in *Legionella pneumophila* Supernatants

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Received 27 December 1995/Returned for modification 29 January 1996/Accepted 13 February 1996

Conflicting reports have been given as to the existence of a *Legionella pneumophila* siderophore. Hence, we rigorously examined the reported siderophore-like activity using the chrome azurol S indicator. Although chrome azurol S reactivity was detected in supernatants, control experiments indicated that it was due to cysteine in the media. When bacteria were cultured in media without cysteine, no siderophores were detected.

The mechanisms by which *Legionella pneumophila*, the agent of Legionnaires' disease, acquires iron from its environment have yet to be fully characterized (1, 8, 10, 11, 13, 15). In particular, the suggestion of the existence of a *Legionella* siderophore has been controversial. Using the Arnow and Csaky assays, an earlier study demonstrated that this facultative intracellular pathogen does not excrete phenolate or hydroxamate siderophores (17). However, when the chrome azurol S (CAS) indicator was employed to test *L. pneumophila* culture supernatants, a siderophore-like activity was detected (6). The CAS dye changes from a dark blue to a yellow color when ferric iron is chelated, allowing for a colorimetric determination of iron binding independent of molecular structure (2, 7, 18). Taken together, these data suggested that *L. pneumophila* excretes a novel siderophore or iron-binding protein. We recently isolated streptonigrin-resistant, iron chelator-sensitive *L. pneumophila* mutants and had hoped to test them for defects in CAS reactivity and other iron acquisition functions (16). Consequently, we first sought to characterize the CAS-reactive substance produced by wild-type *L. pneumophila.*

For this study, we used the virulent serogroup 1 strain 130b (4). Prior to testing of its CAS reactivity, the strain was grown in a guinea pig and samples were passaged three to five times on buffered charcoal-yeast extract agar (3). The liquid CAS reaction was performed as previously described (18) with legionellae that were grown in a chemically defined medium (CDM) without added iron at 37°C (17). More specifically, a washed, overnight CDM culture was used to inoculate CDM containing a low level of iron to an optical density at 660 nm of 0.1 and then, over the next 9 to 12 days, a portion of the supernatant was recovered and reacted with the CAS dye for 15 min. As a control, uninoculated CDM was incubated and tested for CAS reactivity. With the exception of the CAS dye (Aldrich Chemical Co., Inc., Milwaukee, Wis.), key chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Initially, we confirmed that a siderophore-like activity was present in supernatants from static (Fig. 1) and shaking cultures (see below) (6). However, the previously unreported level of CAS reactivity associated with CDM was rather striking. Although the activity level of the medium was the same as that of the supernatants at time zero, it declined dramatically over time (Fig. 1). A similar trend was evident with *L. pneumophila* Philadelphia I (data not shown). Since the CAS reactivity level of the culture supernatant was nearly constant over time, *L. pneumophila* strains appeared to be excreting increasing amounts of an iron chelator.

Since CDM does not contain substances known to interfere with this assay (12, 17), we tested each of its components for CAS reactivity at their respective final concentrations. Of all the reagents, only L-cysteine reacted with the CAS dye. This iron-binding activity depended directly upon the concentration of cysteine, with as little as 5 μg/ml causing a change in CAS color. CDM lacking cysteine did not react with the iron-dye complex. To confirm that the CAS reactivity of CDM was due to cysteine, we determined whether the loss of CAS reactivity over time correlated with a drop in cysteine levels. Thus, we measured the concentration of cysteine in static media with an acidic ninhydrin reagent (5). Of the 500 μg of cysteine per ml initially present in CDM, only 4% remained after 12 days of incubation at 37°C. One explanation for the loss of cysteine could be its oxidation to cystine, a compound which does not react with ninhydrin (5). Indeed, when the incubated media were treated with dithiothreitol to reduce any oxidized cysteine, almost 100% of the original cysteine level was recorded. Consequently, the gradual decrease in the CAS reactivity level of CDM was a result of the oxidation of cysteine. Given this unexpected experience with cysteine, other investigators seeking to identify iron chelators with the CAS dye should be wary of interference even from defined media.

We considered the possibility that *L. pneumophila* might actively maintain cysteine in its reduced, iron-binding form. Utilizing the ninhydrin reagent, we detected almost 50% of the added cysteine remaining in the supernatants of 12-day-static cultures. *L. pneumophila* might be modulating the redox status of this amino acid by lowering the rate of cysteine oxidation or by expressing a disulfide reductase (see below). In any case, the increased amount of cysteine in the supernatants was clearly contributing to the observed siderophore-like activity.

To test whether *L. pneumophila* supernatants contained a CAS-reactive substance besides cysteine, we subjected them to various treatments and fractionations. The CAS reactivity of the supernatant disappeared after dialysis in a 1-kDa membrane against water, showed no effect from boiling for 10 min, and could not be extracted into either chloroform or 2-butanol. Since cysteine dissolved in CDM shared these characteristics, we then analyzed both the CDM and the supernatants by paper chromatography, using the CAS dye to visualize iron-binding activities (12). The culture supernatant gave a CAS-reactive spot that migrated in the same manner as the CAS-reactive spot given by CDM (R_f = 0.35). As expected, CDM lacking cysteine did not yield a reactive spot. Although these data

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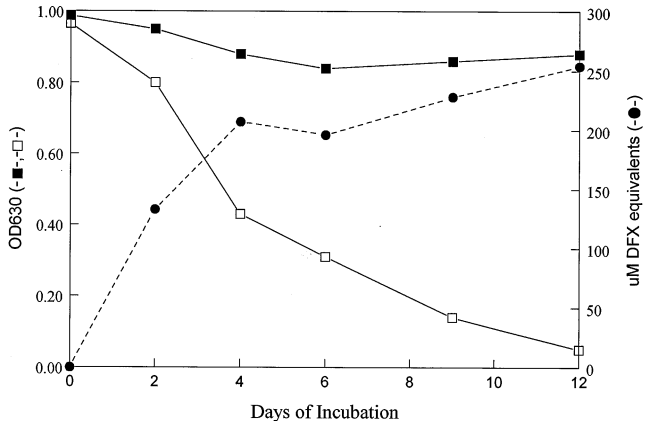

FIG. 1. CAS reactivity levels of *L. pneumophila* 130b supernatants and CDM. At the indicated times, the culture supernatant (filled squares) and the medium control (open squares) were reacted with the CAS indicator dye, and the changes in optical density at 630 nm (OD630) were recorded. The siderophore-like activity level of the supernatant (filled circles, dashed line) was calculated by subtracting the amount of control medium at each time point and was expressed as micromolar equivalents of the iron chelator deferoxamine (DFX). This same experiment was performed two additional times, with similar results (data not shown).

eliminated the prospect of a *L. pneumophila* proteinaceous chelator and raised doubts about the existence of a unique siderophore, they did not exclude the possibility that the supernatants contained an iron-binding compound that comigrates with cysteine.

To establish whether *L. pneumophila* excretes a siderophore-like compound, we tested the CAS reactivities of supernatants derived from bacteria grown in the absence of cysteine. Hence, we replaced the cysteine in CDM with cystine and/or glutathione (19), since they did not react with the CAS dye. Given that the expression level of siderophores in other species is higher under aerobic conditions (12), we employed shaking cultures for this experiment. Cultures containing cystine and glutathione grew comparably to those with cysteine, while the glutathione-only culture had a prolonged lag period (Fig. 2A). Analysis of the CAS reactivities of these culture supernatants, however, revealed no significant iron-binding activity (Fig. 2B). These observations indicate that all CAS reactivity in CDM cultures is due to cysteine.

The absence of CAS reactivity in the noncysteine cultures also suggests that *L. pneumophila* cannot generate cysteine from cystine. Indeed, when these cultures were reacted with ninhydrin, no cysteine was detected. Thus, *L. pneumophila* does not express a cysteine disulfide reductase, and the high cysteine levels in CDM cultures are likely a repercussion of bacterial uptake of ferric iron, a known catalyst of cysteine oxidation (9, 14).

In summary, we conclude that *L. pneumophila* does not excrete a novel siderophore or iron-binding compound under standard extracellular growth conditions. Without this ability, the bacterium must depend upon the diffusion of iron carriers to its cell surface. The identification of the membrane-associated iron acquisition systems of *L. pneumophila* may be achieved through the study of mutants such as those that have reduced abilities to grow on media with low levels of iron (16).

This work was supported by Public Health Service grant AI34937 from the NIH.

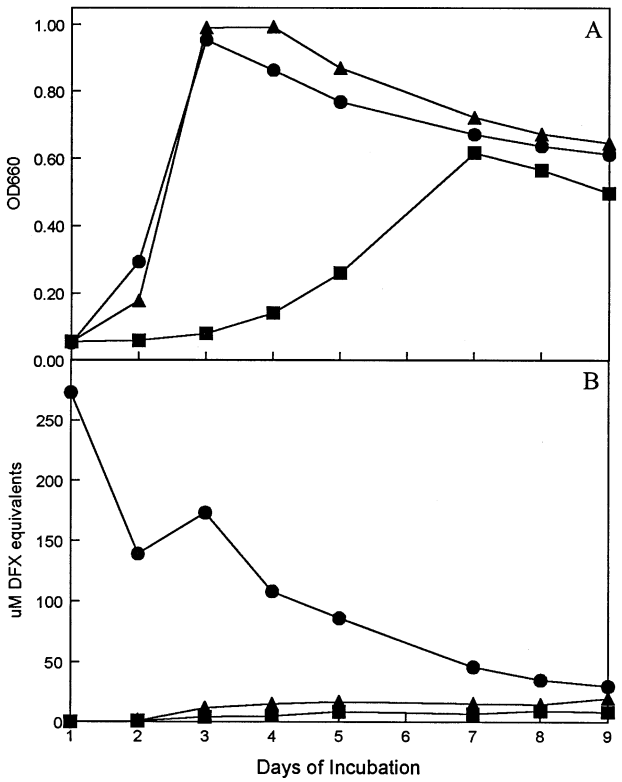

FIG. 2. Absence of siderophore-like activity in CDM cultures without cysteine. *L. pneumophila* was grown for 9 days in CDM containing either cysteine and glutathione (circles), cystine and glutathione (triangles), or glutathione alone (squares). Each day, 1 ml was removed from each culture and used to measure cell growth (the optical density at 660 nm [OD660] in panel A) and CAS reactivity (micromolar deferoxamine [DFX] equivalents in panel B). In two additional experiments, bacteria grown in CDM containing glutathione alone did not exhibit CAS reactivity at 7 and 12 days postinoculation.

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Editor: B. I. Eisenstein