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Isolation and Characterization of Auxotrophic Mutants of *Legionella pneumophila* That Fail To Multiply in Human Monocytes

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Attempts to isolate auxotrophic mutants of *Legionella pneumophila* have been hampered by the complex nutritional composition of the media used to cultivate this organism. We developed a semidefined medium, designated CAA, to facilitate the isolation and characterization of *Legionella* auxotrophs. Unlike previously described chemically defined media for this organism, *L. pneumophila* formed colonies on CAA agar. Using this medium, we isolated several independent tryptophan auxotrophs of strain Philadelphia-1 after ethyl methanesulfonate mutagenesis and penicillin enrichment. Trimethoprim selection was used to isolate several independent thymidine-requiring mutants of the same strain. The thymidine auxotrophs exhibited a marked decrease in viability when they were deprived of thymidine. The results of monocyte infection experiments with both the tryptophan and thymidine auxotrophs indicated that the thymidine auxotrophs were incapable of intracellular survival or multiplication. In contrast, the tryptophan auxotrophs grew well in monocyte cultures. The isolation of additional auxotrophic mutants will facilitate the study of the nutritional requirements of *L. pneumophila* for growth in human mononuclear phagocytes.

Legionella pneumophila, the causative agent of Legionnaires disease, is a facultative intracellular pathogen capable of multiplying in human alveolar macrophages (5) and monocytes (16). *L. pneumophila* evades the antimicrobial defenses of mononuclear phagocytes because *L. pneumophila*-containing phagosomes fail to fuse with lysosomes (13). This enables the organism to grow exponentially within the phagosome of the infected cell, ultimately leading to its destruction. The bacterial virulence determinants that enable *L. pneumophila* to grow in mononuclear phagocytic cells are unknown at the present time.

Several studies have demonstrated that bacterial metabolism may be an important factor in intracellular survival and growth (3, 8, 11). Recently, Fields et al. (8) showed that a variety of auxotrophic mutants of *Salmonella typhimurium* had a reduced ability compared with that of the wild type to survive in mouse macrophages. The isolation of auxotrophic mutants of *L. pneumophila* could be very helpful in determining the nutritional requirements of this organism for intracellular metabolism and growth. Also, these mutants could be very useful for studies of the physiology and genetic organization of *Legionella*.

Attempts to isolate *Legionella* auxotrophs have been hampered by the complex nutritional composition of the media routinely used to cultivate this organism. Despite these difficulties, Dreyfus and Iglewski (6) reported the isolation of a Thy$^-$ derivative of *L. pneumophila*, Knoxville-1. The ability of the mutant to multiply in phagocytes was not reported (6). Although several chemically defined liquid media have been formulated for *L. pneumophila* (20, 21, 25), they have not been useful for the isolation of auxotrophs due to the lack of efficient colony formation by *L. pneumophila* on agar plates made with these media (Richard D. Miller, University of Louisville, Louisville, Ky., personal communication).

In the present study, we formulated a semidefined medium, designated CAA, that we used to isolate and characterize several tryptophan and thymidine auxotrophs of *L. pneumophila* Philadelphia-1. Results of monocyte infection experiments with these mutants showed that thymidine auxotrophy interfered with the intracellular growth of *L. pneumophila*.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* Philadelphia-1 was obtained from Marcus A. Horwitz, School of Medicine, University of California, Los Angeles, Calif. *L. pneumophila* CS1 was isolated as a spontaneous streptomycin-resistant mutant of strain Philadelphia-1. Strain HS1 is a spontaneous rifampin-resistant mutant of strain Philadelphia-1. These drug-resistant derivatives have been used to facilitate genetic crosses that will be described in another report. Neither of these antibiotics is the drug of choice for the treatment of Legionnaires disease. ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal yeast extract agar (ABCYE) was used for the routine cultivation of these strains (7). Liquid cultures were prepared in albumin yeast extract (AYE) medium (9). Cultures were routinely grown at 37°C. Frozen stocks of wild-type and mutant strains were prepared by mixing 2 volumes of overnight AYE cultures with 1 volume of sterile 50% (vol/vol) glycerol and freezing at −80°C.

Preparation of CAA medium. The composition of CAA medium is listed in Table 1. To facilitate the preparation of CAA, Casamino Acids (Difco Laboratories, Detroit, Mich.) were sterilized by autoclaving and stored as a 20% (wt/vol) stock solution. Inorganic salts, excluding calcium chloride and ferric nitrate, were prepared and stored as a 50× stock solution. Calcium chloride was prepared as a separate 1,000× stock solution. Ferric nitrate was prepared immediately prior to use. CAA medium was routinely prepared by the addition of all components except ferric nitrate to glass distilled water. The pH of the medium was adjusted to 6.9 with 10 N KOH. The ferric nitrate solution (1 mg/ml) was prepared separately and added to the medium. The completed medium was sterilized by filtration through a 0.2-μm-

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TABLE 1. Composition of CAA medium

Component	mg/liter
Casamino Acids	5,000
ACES buffer	10,000
L-Cysteine	400
NaCl	50
NH_4Cl	316
$MgSO_4 \cdot 7H_2O$	70
$CaCl_2$	40
$Fe(NO_3)_3 \cdot 9H_2O$	50
Starch[a]	5,000
Agar[a]	15,000

[a] Added when solid medium was required.

pore-size filter (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.).

For solid media, agar and starch (Sigma Chemical Co., St. Louis, Mo.) were prepared at double strength, autoclaved, and added to a equal volume of sterile, double-strength CAA medium. In this instance, the ferric nitrate solution was prepared separately, filtered through a 0.2-μm-pore-size filter, and added to the CAA-agar mixture.

In some experiments, CAA agar was prepared by autoclaving the basal medium (without L-cysteine and ferric nitrate) plus starch and agar together. L-Cysteine and ferric nitrate were added as filter-sterilized solutions to the autoclaved mixture immediately prior to use.

Casamino Acids contain insufficient tryptophan or thymidine to support the growth of tryptophan or thymidine auxotrophs.

Measurement of cell growth in CAA medium. Inocula for the measurement of cell growth in CAA medium were prepared in the following manner. AYE-grown organisms were inoculated into CAA medium (1:5 dilution) and grown for 24 to 48 h at 37°C on a rotary shaker. Cells were removed by centrifugation, washed once, and suspended in 2 ml of CAA medium. Sidearm flasks (300 ml) containing 8 to 10 ml of CAA medium were inoculated with this suspension and grown at 37°C on a shaking water bath. Cell growth was measured turbidimetrically with a Klett-Summerson colorimeter and a no. 20 green filter (500 to 570 nm). Klett values were not corrected for the brown pigment produced by strain Philadelphia-1 at high cell densities. At various times, viable cell counts were determined by diluting samples in M63 salts (18) and plating the diluted samples onto ABCYE agar. Plates were incubated for 72 h, and the number of CFU per milliliter was determined.

Ethyl methanesulfonate mutagenesis for the isolation of tryptophan auxotrophs. *L. pneumophila* Philadelphia-1 was mutagenized with ethyl methanesulfonate (EMS) by the method of Miller (18). Briefly, washed, overnight AYE cultures of strain Philadelphia-1 were incubated in 2 ml of CAA basal salts solution containing 0.2 M Tris (pH 7.5) and 30 μl of EMS for 1.5 h at 37°C with gentle shaking. Mutagenized cultures were washed several times with CAA basal salts solution and suspended in CAA supplemented with L-tryptophan (CAATP; 100 μg/ml). The CAATP culture was incubated overnight with shaking at 37°C to allow expression of EMS-induced mutations. After incubation, a portion of the CAATP culture was removed, washed in M63 salts solution, and suspended in CAA broth. The CAA culture was grown to the early log phase on a shaking water bath at 37°C and was enriched for tryptophan auxotrophs by the penicillin enrichment technique described by Miller (18), except that carbenicillin (100 μg/ml) was used instead of penicillin. Carbenicillin-treated cultures were washed with distilled water and suspended in AYE broth and incubated for 48 h at 37°C with shaking. Serial dilutions of these cultures were made in M63 salts solution and plated onto CAATP agar plates. These plates were incubated at 37°C for 5 to 7 days. Putative tryptophan auxotrophs were identified by their inability to grow after replica plating of the CAATP master plates onto CAA agar. Mutants were picked, purified twice on CAATP plates, and checked for their inability to grow on CAA agar without added tryptophan.

Prototrophic revertants of these mutants were selected by plating portions of overnight AYE cultures of the auxotrophs on unsupplemented CAA agar. Revertants were picked from these plates, purified twice on ABCYE agar, and retested for their ability to grow on unsupplemented CAA.

Trimethoprim selection of thymidine auxotrophs. Trimethoprim selection (18) was used to isolate thymidine auxotrophs of strains CS1 and HS1. Wild-type bacteria are unable to grow in the presence of trimethoprim, an inhibitor of dihydrofolate reductase. Thy^- mutants can grow in the presence of the drug when supplied with thymidine (18). This selection has been used to isolate many thymidine mutants. Unless otherwise specified, thymidine (Sigma) was added to all media at a final concentration of 100 μg/ml. Several well-isolated colonies of strains CS1 and HS1 were grown overnight at 37°C in AYE broth supplemented with thymidine. A 0.1-ml portion of these cultures was spread onto CAA plates containing trimethoprim (10 μg/ml) and thymidine (150 μg/ml) and incubated at 37°C. After incubation for 6 to 7 days, trimethoprim-resistant colonies were picked and purified twice on ABCYE agar plus thymidine (ABCYETM). These isolates were tested for growth at 37°C on plates containing CAA and CAA plus thymidine (CAATM). Thymidine auxotrophs were identified by their inability to grow on CAA medium without added thymidine. Prototrophic revertants of these mutants were selected as described above. However, it was necessary to add thymidine to AYE broth to facilitate the growth of the thymidine auxotrophs in this medium. Cultures grown in the presence of thymidine were washed twice in M63 salts solution before they were plated onto CAA agar.

Growth curve experiments. The nutritional requirements of putative tryptophan and thymidine auxotrophs were confirmed by measuring the growth of several of these mutants in CAA broth and CAA broth supplemented with either L-tryptophan or thymidine at final concentrations of 100 μg/ml. Inocula were prepared from mutant or wild-type bacteria grown overnight at 37°C in 2 to 4 ml of appropriately supplemented CAA broth. Cells were pelleted, washed twice, and suspended in M63 salts solution. Sidearm flasks (300 ml) containing 8 to 10 ml of CAA, CAATM, or CAATP liquid medium were inoculated with the appropriate strain and grown at 37°C on a shaking water bath. Cell growth was measured turbidimetrically with a Klett-Summerson colorimeter as described above.

Isolation and cultivation of peripheral blood monocytes. Monocytes were prepared from the blood of a healthy donor with no previous history of Legionnaires disease. Usually, 100 ml of blood was collected in anticoagulant and divided into 4 equal portions. Each sample was diluted 1:1 with 6% dextran (Sigma), which was prepared in 0.154 M NaCl. The blood-dextran mixtures were kept at room temperature for approximately 1 h, until a sharp interface appeared between the lower erythrocyte layer and the upper plasma layer. The plasma layers were removed, combined, and centrifuged at 200 × *g* for 10 min at room temperature. The resultant

supernatant fluid was discarded, and the leukocyte-rich pellet was carefully suspended in approximately 40 ml of 0.154 M NaCl. To obtain the mononuclear cell fraction, this suspension was divided into two 20-ml portions and centrifuged on top of 6 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) at 400 × *g* for 30 min at room temperature. The layer containing the mononuclear cell fraction was removed and diluted 1:2 in RPMI 1640 medium (KC Biological, Lenexa, Kans.), and the mononuclear cells were collected by centrifugation at 400 × *g* for 10 min at 4°C. The mononuclear cells were washed two times in RPMI 1640 medium and collected by centrifugation at 150 × *g* for 10 min at 4°C. The cells were suspended in 2 to 3 ml of RPMI 1640 medium–10% normal human serum, counted in a hemacytometer, and adjusted to 6×10^6 mononuclear cells per ml in the same medium. The cells were greater than 99% viable, as assessed by trypan blue exclusion. Cells (0.25 ml) were added to dish wells (Linbro; Becton Dickinson Labware, Lincoln Park, N.J.), usually 16 wells per plate, to give 1.6×10^6 mononuclear cells per well. The dishes were incubated at 37°C for 2 h in 95% CO_2 atmosphere, to allow adherent cells to attach in the wells. Following incubation, the nonadherent cells were removed by washing each well two times with RPMI 1640 medium. After the final wash, 0.5 ml of RPMI 1640 medium–25% normal human serum was added to each well, and the dishes were incubated at 37°C in a 5% CO_2 atmosphere.

Infection of monocyte cultures. The *L. pneumophila* strains used to infect monocyte cultures were grown on ABCYE or ABCYETM agar for 72 h at 37°C. In some experiments, strains were grown on CAA plates for 5 to 7 days at 37°C. After growth, bacteria were harvested from the plates, washed, and suspended in 2 ml of RPMI 1640 medium. Each preparation was divided into 0.2-ml portions and kept at −70°C. The CFU per milliliter were determined for each strain prior to freezing. Periodically, samples were thawed and checked for viability. There was no appreciable decrease in the CFU per milliliter for any of the strains over the course of this investigation.

Infection of monocyte monolayers was accomplished by adding 5.0×10^3 to 1.0×10^4 CFU of *L. pneumophila* to wells that contained approximately 1×10^6 monocytes. The infected monolayers were incubated in 5% CO_2 for 4 days. At daily intervals, a 25-μl sample was removed from each well, appropriately diluted in RPMI 1640 medium, and plated onto ABCYE or ABCYETM agar. The plates were incubated at 37°C for 72 h and then counted for CFU. Previous studies (5, 16) have shown that *L. pneumophila* does not grow in RPMI 1640 medium containing normal human serum (16). We verified that this is indeed the case for the *Legionella* strains used in this study (data not shown). Thus, any increase in CFU over time represents the intracellular growth of *L. pneumophila* in monocytes. All experiments were repeated at least two times, and all samples within a given experiment were tested in triplicate.

RESULTS

Growth of strain Philadelphia-1 in CAA medium. A representative growth curve of strain Philadelphia-1 is shown in Fig. 1. Strain Philadelphia-1 grew exponentially in CAA medium, with a generation time of approximately 6 to 8 h. A soluble brown pigment appeared in this medium as the culture reached the late-exponential to early-stationary phase (approx. 200 Klett units). Other investigators (20, 21, 25) have also reported the production of this pigment in media used to cultivate *L. pneumophila*. The observed turbidity of these cultures continued to increase to a maximum of 400 to 500 Klett units (5.0×10^8 to 8.0×10^8 CFU/ml) by 40 h. Microscopic examination of logarithmic-phase cultures revealed a mixture of short and elongated rods interspersed with occasional filamentous forms. Organisms grown on solid CAA medium exhibited a similar morphology.

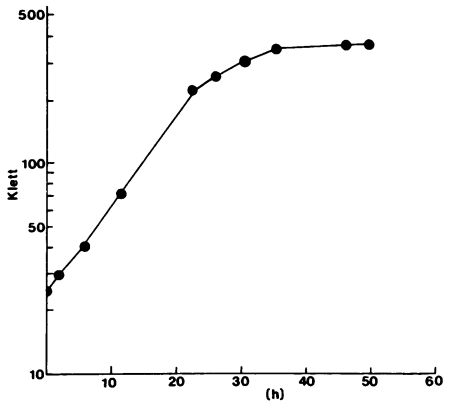

FIG. 1. Growth kinetics of *L. pneumophila* Philadelphia-1 in CAA broth.

Strain Philadelphia-1 had an efficiency of plating of approximately 60% on CAA agar as compared with that on ABCYE medium. Colonies appeared on CAA plates after 5 to 7 days of incubation at 37°C and 7 to 10 days at 30°C. CAA agar plates prepared without starch failed to support the growth of strain Philadelphia-1. Also, this strain exhibited a very low efficiency of plating (approximately 2%) when grown on CAA agar plates prepared with autoclaved basal medium as compared with that when grown on the same plates prepared with filter-sterilized basal medium. For this reason, CAA agar made with filter-sterilized basal medium was used throughout this investigation.

Since CAA medium does not contain L-tryptophan or thymidine, the ability of strain Philadelphia-1 to grow in CAA medium indicated that this organism is prototrophic for these compounds. Thus, we reasoned that we should be able to isolate thymidine and tryptophan auxotrophs of this organism.

Isolation of tryptophan auxotrophs. EMS mutagenesis of strain Philadelphia-1 enabled us to isolate several independent tryptophan auxotrophs using CAA medium. These mutants failed to grow on CAA agar but grew on CAATP and ABCYE agar. Two of these mutants, JC1 and JC2, were chosen for further study. Both JC1 (Fig. 2A) and JC2 (data not shown) were unable to grow in CAA broth without added tryptophan. The growth rate of these mutants in CAATP was similar to that of the wild type. The frequency of spontaneous reversion to tryptophan prototrophy for JC1 was determined to be 2.2×10^{-7}, and that for JC2 was 1.0×10^{-9}.

Isolation of thymidine auxotrophs. Trimethoprim selection has been used to isolate thymidine auxotrophs from a variety of gram-negative bacteria (18, 19). Using CAA medium and trimethoprim selection, we isolated several independent thymidine auxotrophs of strains CS1 and HS1. These mutants did not grow on CAA agar but grew on CAATM. Also, due to limiting amounts of thymidine in yeast extract, these auxotrophs did not form colonies on ABCYE agar. The addition of thymidine to ABCYE agar enabled these mutants to form colonies on this medium. Again, two mutants, CS140

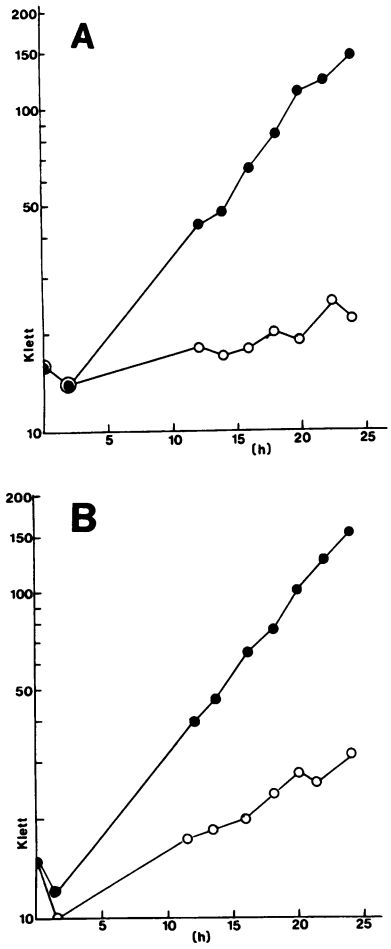

FIG. 2. Confirmation of the nutritional requirements of auxotrophic mutants JC1 and CS140. (A) JC1. Symbols: ○, CAA broth; ●, CAATP. (B) CS140. Symbols: ○, CAA broth; ●, CAATM.

and CS193, were selected for further study. Both CS140 (Fig. 2B) and CS193 (data not shown) grew slowly in CAA broth without added thymidine. As in the case of the tryptophan mutants, the growth rates of these mutants in CAATM were similar to that of the wild type. Neither CS140 nor CS193 formed detectable colonies on solid CAA medium lacking thymidine. The frequency of spontaneous reversion to thymidine prototrophy for CS140 was 2.7×10^{-9}, and for CS193 it was 1.1×10^{-8}.

Infection of monocytes with CAA-grown organisms. *L. pneumophila* Philadelphia-1 cultivated on ABCYE agar grows well in human monocytes (16). Although CAA medium proved to be very valuable for the isolation of auxotrophic mutants, we were interested in determining whether growth on CAA medium affected the ability of this organism to grow in human monocytes. To test whether growth on CAA agar affects the ability of *L. pneumophila* to infect or grow in monocytes, AYE-grown organisms were plated onto ABCYE and CAA agar and incubated at 37°C. The bacteria were prepared as described above and were used to infect monocyte cultures. The results of these experiments indicated that CAA-grown organisms grow as well as ABCYE-grown organisms in monocytes (data not shown).

In contrast to the results obtained with growth on ABCYE agar, serial passage of *L. pneumophila* on certain media, such as Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.), has been reported to result in the loss of virulence of this organism (4, 17). Interestingly, *L. pneumophila* retains its virulence for guinea pigs and embryonated hen eggs after two passages in this medium. However, by the fifth passage the organism becomes avirulent in both of these animal models (4) and it loses its ability to grow in human monocytes (14). In light of these observations, we were interested in determining whether repeated passage of strain Philadelphia-1 on CAA agar affects the ability of this organism to grow in monocytes. To test this idea, strain Philadelphia-1 was sequentially streaked for single colonies on CAA agar at 37°C 10 times. Each time, a single colony was picked, restreaked onto CAA agar, grown in AYE broth at 37°C, and frozen at −70°C. Samples representing each passage on CAA were grown on ABCYE and CAA agar and tested in monocytes as described above. Ten serial passages of strain Philadelphia-1 on CAA agar did not affect its ability to multiply in monocytes (Fig. 3).

Infection of monocytes with auxotrophs. Several investigations (3, 8) have shown that auxotrophic mutations interfere with the ability of certain facultative intracellular pathogens to grow or survive in host phagocytic cells. Therefore, we tested the tryptophan and thymidine mutants, as well as prototrophic revertants, for the inability to grow in monocytes.

Tryptophan auxotrophs JC1 and JC2 and revertants CS169 and CS165 grew in monocytes at approximately the same rate as strain Philadelphia-1 (data not shown). In contrast, thymidine auxotrophs CS140 and CS193 failed to grow in monocytes (Fig. 4). Moreover, there was a 100-fold reduction in the CFU per milliliter exhibited by these auxotrophs over the time course of the experiments. This suggests that these mutants are unable to survive in monocytes. Prototrophic revertants, strains CS161 and CS221, grew as well as Philadelphia-1 did in these experiments. These results suggest that thymidine auxotrophy interfers with the ability of strain Philadelphia-1 to grow in monocytes.

Effect of thymidine deprivation on thymidine auxotrophs. In

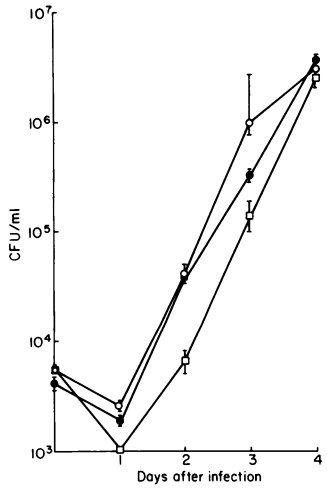

FIG. 3. Infection of monocyte cultures with strain Philadelphia-1 after serial passage on CAA agar. Symbols: □, strain Philadelphia-1 not passaged on CAA and grown on ABCYE prior to infection; ○, strain Philadelphia-1 passaged 10 times on CAA agar and grown on ABCYE prior to infection; ●, strain Philadelphia-1 passaged 10 times on CAA agar and grown on CAA agar prior to infection. Each point represents the mean ± standard error of three separate monocyte cultures.

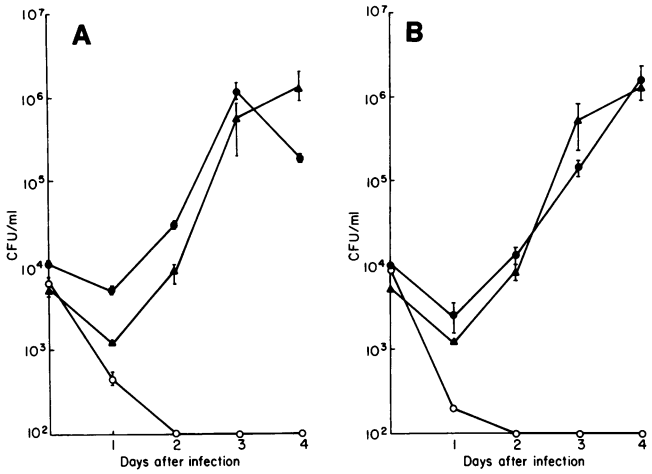

FIG. 4. Infection of monocyte cultures with thymidine auxotrophs CS140 and CS193 and prototrophic revertants CS161 and CS221. (A) Symbols: ▲, strain Philadelphia-1; ○, strain CS140; ●, strain CS161. (B) Symbols: ▲, strain Philadelphia-1; ○, strain CS193; ●, strain CS221. Strain Philadelphia-1 was included in these experiments as a positive control. Each point represents the mean ± standard error for three separate monocyte cultures. The lowest detectable number of viable bacteria permitted by the assay employed in these experiment was 100 bacteria per ml. The values presented for strains CS140 and CS193 on days 2, 3, and 4 postinfection actually represent fewer than 100 bacteria per ml.

Escherichia coli (1, 2) and several other bacterial species (10, 19, 23, 24), thymidine auxotrophs die when they are deprived of thymidine or thymine. For this reason, we were interested in determining the effect of thymidine deprivation on strains CS140 and CS193. In these experiments, each of the strains was grown to the mid-logarithmic phase in CAATM at 37°C on a rotary shaker. Cells were removed from each culture by centrifugation, washed two times in M63 salts solution, and suspended to their original volume in CAA medium. Each cell suspension was divided in half and distributed to two sidearm flasks. Thymidine was added to one of the flasks at a final concentration of 100 μg/ml. The flasks were incubated at 37°C on a shaking water bath. At various times, samples were removed from each of the flasks, appropriately diluted in M63 salts, and plated onto ABCYETM. These plates were incubated for 72 h at 37°C, and the CFU per milliliter was determined.

Both thymidine auxotrophs exhibited a marked reduction in viability following 24 h of incubation in unsupplemented CAA medium. Cell death was first observed for both strains after 3 h of incubation in this medium. Over the 24-h incubation period, strain CS140 exhibited a 10-fold reduction in CFU per milliliter, whereas strain CS193 exhibited a 550-fold decrease in viable counts. Corresponding control cultures of strains CS140 and CS193 grown in CAATM showed an increase in CFU per milliliter over the same incubation period. These results indicate that thymidine auxotrophs of strain Philadelphia-1 die when they are deprived of thymidine in vitro.

Infection of monocyte cultures supplemented with thymidine. The inability of our thymidine auxotrophs to survive or multiply in monocyte cultures may have resulted from insufficient intracellular levels of available thymidine in the monocytes. This seemed reasonable since RPMI 1640 medium does not contain thymidine. Thus, the addition of thymidine to our monocyte cultures might be able to elevate the intracellular pool of thymidine and enable the thymidine auxotrophs to grow. To test this hypothesis, we performed monocyte infection experiments in which thymidine was added to monocyte cultures prior to infection with strains CS140 and CS193. Monocyte cultures that did not receive thymidine were also infected with these strains and served as negative controls. Prototrophic revertants, strains CS161 and CS221, were used to infect both supplemented and unsupplemented monocyte cultures and served as positive controls.

The addition of thymidine (100 μg/ml) to monocyte cultures restored the ability of the auxotrophs to grow (Fig. 5). However, the growth of these mutants was substantially less than that exhibited by their corresponding prototrophic revertants. The addition of thymidine at final concentrations greater than 100 μg/ml had no additional effect on the growth of the auxotrophs in monocytes (data not shown). These results indicate that exogenous thymidine is available to the auxotrophs during intracellular growth.

DISCUSSION

A relationship between bacterial metabolism and intracellular survival and growth has been described for several facultative intracellular pathogens (3, 8, 11). One approach used to study this relationship involves auxotrophic mutants. For example, strains of *S. typhimurium* that are auxotrophic for purines, pyrimidines, histidine, and aromatic amino acids have a reduced ability to survive in mouse macrophages in vitro and are avirulent in mice (8). Purine auxotrophs of *Yersinia pestis* are also avirulent (3). The avirulence of particular auxotrophs can be explained by the unavailability of required nutrients in serum or host tissues. In the absence of essential nutrients, these mutants cannot grow and/or synthesize virulence determinants, which may be necessary for intracellular survival or growth.

The ability of *L. pneumophila* to multiply in mononuclear phagocytes indicates that this organism has developed strategies to acquire essential nutrients in vivo. In an attempt to define those bacterial factors that facilitate the intracellular

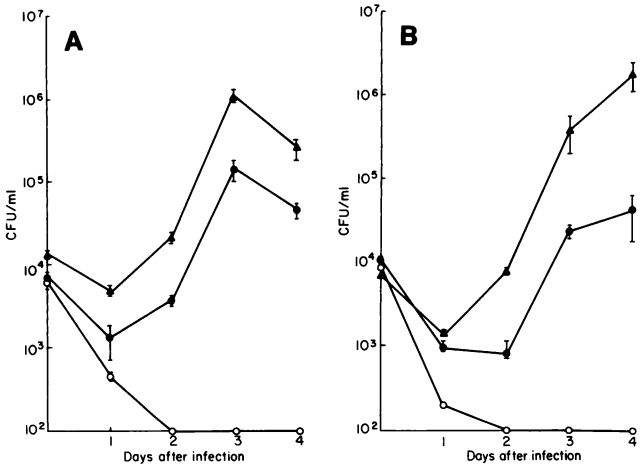

FIG. 5. Growth of strains CS140 and CS193 in monocyte cultures supplemented with thymidine. In these experiments thymidine was added to monocyte cultures at a final concentration of 100 μg/ml immediately prior to infection. (A) Symbols: ○, CS140, no thymidine; ●, CS140 plus thymidine; ▲, CS161 plus thymidine. (B) Symbols: ○, CS193, no thymidine; ●, CS193 plus thymidine; ▲, CS221 plus thymidine. Each point represents the mean ± standard error of three separate monocyte cultures.

growth of *L. pneumophila*, we developed CAA medium to isolate and characterize auxotrophic mutants of this organism. Using these mutants, we sought to gain a better understanding of the nutritional requirements of *L. pneumophila* for growth in mononuclear phagocytes.

CAA medium is composed of Casamino Acids, ACES buffer, L-cysteine, and inorganic salts (Table 1). This medium does not contain purines, pyrimidines, L-tryptophan, fatty acids, or diaminopimelic acid. The ability of strain Philadelphia-1 as well as strains Knoxville and Bloomington (data not shown) to grow in CAA indicates that *L. pneumophila* is prototrophic for these compounds. These results are in agreement with the findings of Warren and Miller (25) and Ristroph et al. (21), who reported that *L. pneumophila* can grow in chemically defined medium devoid of nucleotides, vitamins, and coenzymes. Recently, we grew strain Philadelphia-1 in CAA broth prepared with vitamin-free Casamino Acids (unpublished data). However, the final cell yield of *L. pneumophila* grown in this medium was substantially reduced compared with that when CAA medium made with conventional Casamino Acids was used.

An important feature of CAA medium is the ability of *L. pneumophila* to form colonies on CAA agar. This represents an important advantage of CAA over previously described defined media, since agar plates prepared with these media failed to support efficent colony formation of *L. pneumophila* (Richard Miller, personal communication). Another important feature of CAA medium is that repeated subculture of strain Philadelphia-1 did not affect the ability of this organism to infect or grow in monocytes. Both of these features enabled us to successfully isolate the auxotrophs used in this study and to evaluate their ability to grow in monocytes.

An examination of the nutritional composition of CAA suggests that we should be able to isolate additional *Legionella* auxotrophic mutants. Indeed, during the course of our investigation we isolated several auxotrophs following EMS mutagenesis of strain Philadelphia-1 that grew well on ABCYE agar but that failed to grow on CAA, CAATM, or CAATP. We are currently determining the nutritional requirements of these mutants.

The tryptophan auxotrophs isolated in this study grew in monocyte cultures. This result was not unexpected since there is tryptophan (5 μg/ml) in the RPMI 1640 medium used to cultivate the monocytes. The most likely explanation for these observations is that the tryptophan in the growth medium fulfilled the intracellular growth requirement of these auxotrophs, enabling them to grow in monocytes. Therefore, it is difficult to comment on the relationship between tryptophan auxotrophy and the intracellular growth of *L. pneumophila*.

Unlike the tryptophan auxotrophs, two independently isolated thymidine auxotrophs of strain Philadelphia-1 failed to multiply in monocytes (Fig. 4). Moreover, these mutants were shown to have a diminished capacity for intracellular survival. The ability of prototrophic revertants of these mutants to grow well in monocyte cultures indicated that the lack of intracellular survival and growth exhibited by the auxotrophs was due to thymidine auxotrophy.

The addition of thymidine to monocyte cultures enabled the thymidine auxotrophs to survive and multiply. However, these mutants did not grow as well as their corresponding prototrophic revertants did in thymidine-supplemented monocyte cultures. It is not clear from the results of our experiments why the thymidine supplement did not restore the ability of the auxotrophs to grow to wild-type levels. One possible explanation is that the mutants are unable to efficiently transport or assimilate thymidine intracellularly. Under these conditions the mutants would still be able to grow, but at a reduced rate. Nonetheless, our results suggest that thymidine limitation may have been responsible for the inability of these auxotrophs to grow in monocytes.

The growth of *L. pneumophila* in mononuclear phagocytes is contingent upon the inhibition of phagosome-lysosome fusion (13). This creates a suitable environment within the phagosome for the multiplication of this organism. As mentioned earlier, the bacterial factor(s) that mediate the inhibition of phagosome-lysosome fusion is unknown. However, all of the available evidence suggests that living, metabolically active *L. pneumophila* is necessary to inhibit fusion (12, 13, 15, 16). On the basis of these observations, we can provide several possible explanations for the inability of our thymidine auxotrophs to survive or grow in monocytes.

The thymidine auxotrophs were phagocytosed in the same fashion as wild-type *L. pneumophila*. These mutants were initially able to inhibit fusion by utilizing their internal thymidine pools for growth. However, due to limiting amounts of thymidine in the monocyte cytoplasm, the auxotrophs became starved for thymidine and stopped growing. As mentioned above, in the absence of bacterial growth, phagosome-lysosome fusion proceeded, resulting in the destruction of these mutants. An alternate explanation makes use of our observation that the thymidine auxotrophs died when they were deprived of thymidine in vitro. Again, the mutants were phagocytosed and were initially able to inhibit fusion. However, in this instance, the auxotrophs died as a result of thymidine deprivation rather than from an inability to inhibit fusion. The decrease in viability exhibited by these mutants in our thymidine deprivation experiments is sufficient to account for the cell death exhibited by the auxotrophs in our monocyte infection experiments (Fig. 5). It is not unreasonable to assume that a combination of both of these mechanisms can account for the inability of these auxotrophs to survive or grow in monocytes. Finally, it is possible that the auxotrophs are less able to attach to or enter monocytes as efficiently as wild-type cells are. In the absence of a suitable intracellular environment, *L. pneumophila* cannot multiply under the tissue culture conditions used in this study (16). Additional experiments, including an electron microscopic examination of monocytes infected with our thymidine auxotrophs, are necessary to distinguish between these possibilities.

The results of this study demonstrate that CAA medium is useful for the isolation and characterization of auxotrophic mutants of *L. pneumophila*. Although there are limitations on the type of auxotrophs which can be isolated, it is clear that auxotrophic mutants will be important in future studies concerned with the pathogenesis, physiology, and genetic organization of *L. pneumophila*.

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LITERATURE CITED

1. **Alikhanian, S. I., T. S. Iljina, E. S. Kaliaeva, S. V. Kameneva, and V. V. Sukhodolec.** 1965. Mutants of *Escherichia coli* K12 lacking thymine. Nature (London) **206**:848–849.
2. **Alikahanian, S. I., T. S. Iljina, E. S. Kaliaeva, S. V. Kemeneva, and V. V. Sukhodolec.** 1966. A genetic study of thymineless

mutants of *E. coli* K12. Genet. Res. **8**:83–100.
3. **Burrows, T. W.** 1955. The basis of virulence for mice of *Pasteurella pestis*, p. 152–175. *In* J. W. Howie and A. J. O'He (ed.), Mechanisms of microbial pathogenicity. Fifth Symposium of the Society for General Microbiology. Society for General Microbiology, Cambridge, England.
4. **Elliott, J. A., and W. Johnson.** 1982. Virulence conversion of *Legionella pneumophila* serogroup 1 by passage in guinea pigs and embryonated eggs. Infect. Immun. **35**:943–946.
5. **Elliott, J. A., and W. C. Winn.** 1986. Treatment of alveolar macrophages with cytochalasin D inhibits uptake and subsequent growth of *Legionella pneumophila*. Infect. Immun. **51**:31–36.
6. **Dreyfus, L. A., and B. H. Iglewski.** 1985. Conjugation-mediated genetic exchange in *Legionella pneumophila*. J. Bacteriol. **161**: 80–84.
7. **Feeley, J. C., R. J. Gibson, G. W. Gorman, N. C. Langford, J. K. Rasheed, D. C. Makel, and W. B. Baine.** 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. **10**:437–441.
8. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83**:5189–5193.
9. **Gabay, J. E., and M. A. Horwitz.** 1985. Isolation and characterization of the cytoplasmic and outer membranes of the Legionnaire's disease bacterium (*Legionella pneumophila*). J. Exp. Med. **161**:409–422.
10. **Harrison, A. P.** 1965. Thymine incorporation and metabolism by various classes of thymine-less bacteria. J. Gen. Microbiol. **41**: 321–333.
11. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291**:238–239.
12. **Horwitz, M. A.** 1983. Formation of a novel phagosome by the Legionnaire's disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158**:1319–1331.
13. **Horwitz, M. A.** 1983. The Legionnaire's disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158**:2108–2126.
14. **Horwitz, M. A.** 1987. Characterization of mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. J. Exp. Med. **166**:1310–1328.
15. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99**:1936–1943.
16. **Horwitz, M. A., and S. C. Silverstein.** 1980. Legionnaire's disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. **60**:441–450.
17. **McDade, J. E., and C. C. Shepard.** 1979. Virulent to avirulent conversion of Legionnaire's disease bacterium (*Legionella pneumophila*)—its effect on isolation techniques. J. Infect. Dis. **139**:707–711.
18. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 218–234. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **O'Donovan, G. A., and J. Neuhard.** 1970. Pyrimidine metabolism in microorganisms. Bacteriol. Rev. **34**:278–343.
20. **Pine, L., J. R. George, M. W. Reeves, and W. K. Harrell.** 1979. Development of a chemically defined medium for the growth of *Legionella pneumophila*. J. Clin. Microbiol. **9**:615–626.
21. **Ristroph, J. D., K. W. Hedlund, and S. Gowda.** 1981. Chemically defined medium for *Legionella pneumophila* growth. J. Clin. Microbiol. **13**:115–119.
22. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 219. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Smith, D. W., and P. C. Hanwalt.** 1968. Macromolecular synthesis and thymineless death in *Mycoplasma laidlawii* B. J. Bacteriol. **96**:2066–2076.
24. **Wachsmann, J. T., S. Kemp, and L. Hogg.** 1964. Thymineless death in *Bacillus megaterium*. J. Bacteriol. **87**:1079–1086.
25. **Warren, W. J., and R. D. Miller.** 1979. Growth of Legionnaire's disease bacterium (*Legionella pneumophila*) in chemically defined medium. J. Clin. Microbiol. **10**:50–55.