Amino Acid Requirements for *Legionella pneumophila* Growth

MARTHA J. TESH AND RICHARD D. MILLER*

Department of Microbiology and Immunology, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40292

Received 7 November 1980/Accepted 30 January 1981

The amino acids L-arginine, L-isoleucine, L-leucine, L-methionine, L-serine, L-threonine, and L-valine were essential for the growth of *Legionella pneumophila* in a chemically defined medium. A partial requirement for L-cysteine (or L-cystine) was also observed. A minimal medium containing only the eight required amino acids supported the growth of this bacterium only if the medium was supplemented with L-glutamic acid. This latter compound was the only amino acid capable of stimulating growth in the eight-amino acid medium.

Initial work concerning the primary isolation of *Legionella pneumophila* has suggested fairly fastidious nutrient requirements, including L-cysteine and ferric iron (2). Since then, several chemically defined media, consisting primarily of amino acids and inorganic salts, have been described which provide adequate growth of this bacterium (3-5) and which have been used to analyze its amino acid requirements. Warren and Miller (5) showed that L-cysteine (or L-cystine), L-serine, and L-methionine were essential for growth in their defined medium. Subsequent work in our laboratory with the same medium (M. J. Tesh and R. D. Miller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D74, p. 50) has identified additional requirements for L-threonine, L-arginine, L-valine, L-leucine, and L-isoleucine. George et al. (3) confirmed these findings but stated that L-tyrosine or L-phenylalanine was also essential for growth in their system. In addition to being required for growth, L-serine and L-threonine appeared to serve as major carbon and energy sources in this latter medium. Using another defined medium, Ristroph and co-workers (J. D. Ristroph, K. W. Hedlund, and S. Gowda, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I42, p. 91) also identified similar essential amino acids but found no requirement for L-leucine, L-isoleucine, L-tyrosine, or L-phenylalanine. The purpose of this investigation was to define more specifically the amino acid requirements for growth of *L. pneumophila* in a chemically defined medium and to develop a minimal defined medium for growth of this bacterium.

MATERIALS AND METHODS

L. pneumophila strains Knoxville-1, Togus-1, Bloomington-2, and Los Angeles-1 were obtained from

R. Weaver, Centers for Disease Control, Atlanta, Ga. All strains were maintained on a medium (GC-FC agar) containing GC medium base (Difco Laboratories, Detroit, Mich.) supplemented with L-cysteine (0.4 g/liter) and soluble ferric pyrophosphate (0.25 g/liter). Cultures were maintained at 37°C on GC-FC agar plates in an atmosphere of 5% CO₂ and transferred weekly. The purity of the cultures was monitored by the following criteria: characteristic colonial morphology and pigmentation on GC-FC agar, lack of growth on tryptic soy agar, and a characteristic Gram stain.

Inocula were prepared from 48- to 72-h cultures grown on GC-FC agar plates. Cells were washed from the plates with sterile saline (0.85%, wt/vol) and washed twice in saline by centrifugation (5,000 × *g* for 15 min) at room temperature, and the sediments were resuspended in sterile saline.

Acid-washed nephelometer flasks (300-ml capacity) containing 20 ml of medium were prepared. Cells were inoculated to give an initial turbidity of 35 Klett units (KU) using a Klett-Summerson colorimeter (660 filter) and incubated at 37°C on a New Brunswick gyratory shaker at 200 rpm. Growth was measured turbidimetrically in KU. Comparative optical density readings were measured on a Beckman Spectronic 20 spectrometer at 660 nm. Due to the production of pigment which interfered with the readings at this wavelength, all measurements were corrected for pigment (total KU minus the KU of the pigment alone).

The complete defined medium (CDM) was prepared by the method of Warren and Miller (5). Concentrations of L-amino acids (micrograms per milliliter) used throughout this study, unless otherwise noted, were as follows: alanine, 500; arginine, 900; asparagine, 150; aspartic acid, 1,000; cysteine, 400; cystine, 75; glutamic acid, 1,650; glutamine, 250; glycine, 1,350; histidine, 300; isoleucine, 555; leucine, 555; lysine, 750; methionine, 300; phenylalanine, 450; proline, 250; serine, 650; threonine, 450; tryptophan, 400; tyrosine, 75; and valine, 600. All media components were dissolved in deionized, distilled water, and the pH was adjusted to 6.9 with 1 N NaOH. Sterilization was

achieved by filtration (Gelman GA-6 Metrical filter, 0.45- μ m pore size).

Amino acids and other organic chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., and were of the highest purity grade offered. Other chemicals were of analytical grade and were obtained from various commercial sources. Soluble ferric pyrophosphate was obtained from M. Suggs, Centers for Disease Control, Atlanta, Ga.

RESULTS

The effect of single amino acid deletions on the growth of *L. pneumophila* strain Knoxville-1 in CDM is shown in Table 1. The single elimination of L-arginine, L-cysteine, L-isoleucine, L-leucine, L-methionine, L-serine, L-threonine, or L-valine resulted in a marked depression or absence of growth. Single elimination of all other amino acids had little or no significant effect on cell growth, although pigment production was occasionally delayed or depressed.

Among the nonessential amino acids, elimination of tyrosine or phenylalanine resulted in

TABLE 1. Effect of single amino acid deletions on growth and pigment of *L. pneumophila* in the complete chemically defined medium

Amino acid deleted	% of:	
	Control growth ^a	Control pigment ^b
Control	100	100
Alanine	96	75
Arginine	0	20
Asparagine	124	66
Aspartic acid	95	120
Cysteine + cystine	47	0
Glutamic acid	95	126
Glutamine	89	70
Glycine	107	87
Histidine	102	92
Isoleucine	0	16
Leucine	0	22
Lysine	126	150
Methionine	0	18
Phenylalanine	89	43
Proline	94	53
Serine	0	18
Threonine	0	21
Tryptophan	90	89
Tyrosine	83	47
Valine	0	12

^a Percentage of the turbidity in CDM (control culture) after 48 h of incubation. This period of incubation was chosen to achieve maximum cell density in the control culture.

^b Percentage of the pigment in CDM (control culture) after 140 h of incubation. This period of incubation was chosen to achieve maximum pigment production. An extended incubation period was necessary due to the recognized kinetics of pigment production by *L. pneumophila* (5).

depressed pigment production, as previously reported (1). Elimination of proline, and to a lesser extent alanine, asparagine, or glutamine, also resulted in depressed pigment production without a corresponding effect on growth. Interestingly, a small amount of pigment was generally produced in those flasks where no growth was observed (i.e., lacking an essential amino acid). The exception was the medium lacking cysteine (plus cystine), where there was no detectable pigment despite growth corresponding to 47% of the control culture.

Figure 1 illustrates the growth kinetics of *L. pneumophila* strain Knoxville-1 in CDM and in complete medium minus threonine (essential amino acid) or aspartic acid (nonessential amino acid). With an initial turbidity of 35 KU (optical density, 0.14), growth was observed after a lag period of 2 to 3 h. In CDM, turbidity continued to increase with a generation time of 6 to 7 h until the culture reached stationary phase at around 40 h. Maximum turbidity (255 corrected KU; optical density, 1.10) was reached between 40 to 48 h, and extended incubation resulted in a slight decline of turbidity. Pigment was first noted in late exponential-early stationary growth, as described by Warren and Miller (5). Similar growth kinetics were observed in CDM lacking the nonessential amino acid aspartic acid. In contrast, CDM lacking threonine

FIG. 1. Growth kinetics of *L. pneumophila* strain Knoxville-1 in CDM (control) (●), CDM minus aspartic acid (▲), and CDM minus threonine (■).

FIG. 2. Growth kinetics of *L. pneumophila* strain Knoxville-1 in 8AA minimal medium with (●) and without (■) supplemental *L*-glutamic acid.

showed no observable growth during the entire 140-h incubation period. The pH of both CDM and CDM lacking aspartic acid increased during cell growth, reaching 8.1 after 48 h. Similar growth kinetics were observed with CDM minus any other essential or nonessential amino acid with the exception of *L*-cysteine, which showed only a partial requirement (47% of control growth).

Additional amino acid deletions were performed to develop a minimal defined medium. A medium consisting solely of the eight essential amino acids (as described above) could not support the growth of *L. pneumophila* strain Knoxville-1, even when the concentrations of the amino acids were increased two- or threefold (data not shown). This result suggested that an additional amino acid(s) may be required in this medium, perhaps as an energy source.

To investigate this possibility, each remaining nonessential amino acid was added singly to the eight essential amino acids (8AA medium; concentrations as specified for CDM). Growth was observed only with the addition of glutamic acid. Figure 2 illustrates the growth kinetics of *L. pneumophila* strain Knoxville-1 in the 8AA medium with and without glutamic acid. After an extended lag period of 15 to 20 h, the culture grew exponentially with a generation time of 15 h and reached a maximum turbidity of 130 KU (optical density, 0.52). No pigment was noted at any time during the growth cycle. The pH of

this medium increased with cell growth, reaching 8.0 at 60 h. Similar results were obtained with *L. pneumophila* strains Togus-1, Bloomington-2, and Los Angeles-1.

Continuous passage of *L. pneumophila* in the 8AA plus glutamic acid medium was accomplished with strain Knoxville-1 (data not shown). As the culture entered stationary phase, a sample was transferred into a flask of fresh medium to give an initial turbidity of 35 KU. The growth kinetics of the second flask were similar to those of the first. Cultures were passed continuously in this manner for three transfers, with similar growth kinetics being obtained each time.

DISCUSSION

Attempts have been made by several investigators to define the amino acid requirements of *L. pneumophila*. These investigations, with their similar yet distinct findings, suggest that variations in the cultural conditions generate differences in the observed nutritional requirements of this organism. The addition or deletion of various nutrients might stimulate the induction of enzyme systems which bypass certain metabolic steps to utilize the materials at hand. Thus, absolute essential requirements are often difficult to obtain. Although similarities in the growth requirements of *L. pneumophila* are emerging, the differences obtained by other investigators can only be explained through the examination of the cultural conditions and growth media utilized in each study.

In this investigation, we have attempted to define the essential amino acid requirements for *L. pneumophila* by using the chemically defined medium of Warren and Miller (5). This medium, consisting of inorganic salts and amino acids, is not hampered by complex incubation procedures or complicated constituent preparation. Single amino acid deletion studies indicated that, of the 21 amino acids comprising CDM, 7 (*L*-arginine, *L*-serine, *L*-threonine, *L*-valine, *L*-methionine, *L*-leucine, and *L*-isoleucine) were found to be absolutely required for growth of the organism. Media lacking any of these substances failed to support growth, although limited amounts of pigment were produced in some cases. Defined medium lacking *L*-cysteine (an amino acid reported to be essential in previous reports) gave partial growth (47%) when compared with controls. Although partial growth was observed in this latter medium, there was no pigment produced at any time during the growth cycle. This partial growth has been noted by Warren and Miller (5) and may be due to the limited utilization of available *L*-methionine (or other biosynthetically related amino acids) in

the medium for synthesis of L-cysteine. No growth was noted when L-cysteine was deleted from the minimal (8AA plus glutamate) medium.

Pigment production in all media was comparable to that in controls, with the exception of media lacking L-phenylalanine, L-tyrosine, L-proline, or L-cysteine and, to a lesser extent, L-alanine, L-asparagine, or L-glutamine. The role of L-phenylalanine and L-tyrosine in the synthesis of this melanin-like pigment has been addressed by Baine et al. (1). However, the reason for the decreased pigment production in media lacking these other amino acids is unknown. Medium lacking cysteine (plus cystine) gave diminished growth, which could account for some decrease in pigment. However, all other defined media shown in Table 1 gave detectable pigment, even those media in which there was a complete absence of cell growth.

George et al. (3) have noted that, in addition to the eight amino acids identified in our study, there is an additional requirement for L-phenylalanine or L-tyrosine. In our study, single elimination of either of these had only a marginal effect on growth (89 and 83% of control growth, respectively), although pigment production was suppressed as described above. Their system, based on a defined medium of Pine et al. (4), differs considerably from the system of Warren and Miller (5), which may account for the additional requirements. The medium used by George et al. (3) contained (in addition to inorganic salts and amino acids) several vitamins, cofactors, coenzymes, α -ketoglutaric acid, pyruvate, and soluble starch. Cultural conditions were also different; notably, the stock cultures were maintained on charcoal-yeast extract agar, sealed culture tubes were used, and dessiccants and internal CO₂ sources were different. All of these could play a role in varying the metabolic potential of the organism and thus vary the growth requirements. It is noteworthy that, although our medium had fewer constituents, the study by George et al. (3) demonstrated a greater number of amino acid requirements.

A report by Ristroph et al. (Abstr. Ann. Meet. Am. Soc. Microbiol. 1980, 142, p. 91) noted similar amino acid requirements. As in our study, they found no requirement for L-tyrosine or L-phenylalanine, but they also detected no requirement for L-leucine and L-isoleucine. This lack of a requirement for these two amino acids may be due to the concentration of amino acids in the medium or the composition of their medium, which consisted of inorganic salts, 18 amino acids, rhamnose, and choline. Studies in our laboratory have demonstrated that *L. pneumo-*

phila undergoes approximately one doubling in this medium when the medium is deficient in leucine or isoleucine. However, when rhamnose and choline were omitted, there was an absolute requirement for these two amino acids (unpublished data).

The second purpose of this investigation was to perfect a minimal medium for growth of *L. pneumophila*. A medium consisting of the 8AA plus L-glutamic acid was developed, and it supported the growth of four strains of *L. pneumophila*. The slow generation time and total cell yield indicated that this medium was nutritionally deficient, compared with CDM. Slow generation times in a minimal medium were also observed in the report by George et al. (3). However, the continuous passage of *L. pneumophila* in our minimal medium demonstrated that this organism was capable of synthesizing all of the essential cellular products necessary for growth and division with only the nine amino acids present in the medium. Glutamic acid was essential for growth in this medium. Other amino acids reported to be potential energy sources for *L. pneumophila* (i.e., serine, threonine, histidine, tryptophan, or tyrosine) could not replace glutamic acid, even at concentrations as high as 0.1%. In most bacteria, glutamic acid plays a central role in the metabolism of amino acids and the intracellular distribution of nitrogen. This amino acid is not required for growth of *L. pneumophila* in the presence of the other 20 amino acids. But in a medium depleted to only eight amino acids, the addition of an easily metabolized amino acid would be crucial to the generation of energy as well as to the synthesis of the other amino acids and cellular constituents. Based on data published recently, Weiss et al. (6) concluded that glutamate is utilized as an energy source and is an important metabolite for the nutrition of *L. pneumophila*. Determination of the exact role of other amino acids in the nutrition of *L. pneumophila* will be the subject of future investigations.

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