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Growth of Legionnaires Disease Bacterium (*Legionella pneumophila*) in Chemically Defined Medium

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A chemically defined medium containing 21 amino acids and inorganic salts was developed which supported the growth of four isolates of Legionnaires disease bacterium (*Legionella pneumophila*). Growth in liquid defined medium at 37°C with shaking approximated the generation time and growth kinetics observed for growth in complex media. After a 3-h lag, the culture grew exponentially with a generation time of 6 h and reached a maximum optical density of 230 Klett units (170 Klett units corrected for pigment). A soluble brown pigment was first observed as the culture entered late exponential to early stationary phase of growth. Morphologically, *L. pneumophila* grew in the liquid defined medium with extensive filamentation and numerous intracellular lipid granuoles. L-Serine, L-methionine, and L-cysteine were required for optimum growth. The latter amino acid could be replaced by L-cystine or reduced glutathione but not by D-cysteine, thiomalate, thioglycollate, or 2-mercaptoethanol. Ferric iron was needed for maximum growth, but supplemental iron was not an essential growth requirement. Carbohydrates (i.e., glucose) or organic acids did not stimulate growth. In fact, pyruvate, acetate, and citrate all gave varying degrees of inhibition (69, 37, and 0% of control growth, respectively).

Preliminary characterization of the Legionnaires disease bacterium (*Legionella pneumophila*) has suggested rather fastidious nutrient requirements (7, 10). *L. pneumophila* is unable to grow on many standard bacteriological media such as blood agar or unenriched chocolate agar and was first grown on Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX enrichment (7). The growth requirements of this organism have been studied in more depth recently by Feeley et al. (5); however, their results were limited by the complex nature of their media. In the present communication we describe the growth kinetics and nutrient requirements of *L. pneumophila* in a chemically defined medium. This medium should aid future investigations into the physiology, metabolism, and genetics of this unique pathogen.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* strains Knoxville; Philadelphia (no. 2); Bellingham, Wash. (D9422); and California (E1115) were obtained from R. Weaver, Center for Disease Control, Atlanta, Ga. The medium used for routine maintenance of the cultures (referred to as GC-FC agar) contained GC medium base (Difco) 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 slants in an atmosphere of 5% CO_2 and transferred weekly. Stock cultures of all strains were also stored at −70°C in tryptic soy broth (Difco) containing 20% (vol/vol) glycerol. The purity of all cultures was routinely monitored and was based on characteristic growth and pigment production, microscopic morphology, and the absence of growth on blood agar or brain heart infusion agar.

Media and growth conditions. The complex liquid medium (GC-FC liquid medium) used throughout this study for the growth of *L. pneumophila* contained the following, per liter: proteose peptone no. 3 (Difco), 15 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; NaCl, 5 g; L-cysteine·$HCl·H_2O$, 0.4 g; soluble ferric pyrophosphate, 0.25 g; and $NaHCO_3$, 0.042 g. The pH of the medium was adjusted to 6.9 by addition of 1 N NaOH, and the medium was sterilized by filtration through a 0.45-μm filter.

Inocula were prepared from cultures grown for 72 h on GC-FC agar slants. Cells were washed from four slants with a sterile buffer salts solution (SBSS [pH 6.9]; inorganic salts concentrations as listed in Table 1). Cells were removed by centrifugation at 1,200 × *g* for 15 min at room temperature, washed once, and suspended in 10 ml of SBSS. Microscopic examination of these cells revealed mainly single rods with only an occasional filament.

Acid-washed nephelometer flasks (300-ml flasks containing 20 ml of medium) were inoculated with

TABLE 1. *Composition of the chemically defined medium*

Component	Final concn (μg/ml)
L-Alanine	500
L-Arginine·HCl	900
L-Asparagine	150
L-Aspartic acid	1,000
L-Cysteine·HCl·H_2O	400
L-Cystine[a]	75
L-Glutamic acid	1,650
L-Glutamine	250
Glycine	1,350
L-Histidine·HCl	300
L-Isoleucine	555
L-Leucine	555
L-Lysine·HCl	750
L-Methionine	300
L-Phenylalanine	450
L-Proline	250
L-Serine	650
L-Threonine	450
L-Tryptophan	400
L-Tyrosine	75
L-Valine	600
KH_2PO_4	1,000
K_2HPO_4	4,000
NaCl	5,000
$MgSO_4·7H_2O$	10
$CaCl_2·2H_2O$	10
$(NH_4)_2SO_4$	2,000
$Fe_4(P_2O_7)_3$ (soluble)	250
$NaHCO_3$[b]	420
Agar[c]	18,000

[a] L-Cystine was first dissolved in a small volume of 1 N HCl.

[b] $NaHCO_3$ was used only for liquid media.

[c] For solid media, agar (Difco) was prepared double strength, autoclaved, and added to an equal volume of double-strength liquid medium.

sufficient cells to obtain an initial turbidity of approximately 30 Klett units (KU; ca. 5×10^7 colony-forming units per ml) and incubated at 37°C on a New Brunswick gyratory shaker at 200 rpm.

For all solid media incubation was at 37°C in a moist atmosphere containing 5% CO_2.

Preparation of the chemically defined medium. The composition of our chemically defined medium is listed in Table 1. The components were added to deionized, distilled water, the pH was adjusted to 6.9 with 1 N NaOH, and the medium sterilized by filtration through a 0.45-μm filter.

Soluble ferric pyrophosphate was obtained from M. Suggs, Center for Disease Control, Atlanta, Ga. Amino acids and other organic chemicals used in the defined medium were from Sigma Chemical Co. (St. Louis, Mo.) and were the highest quality available. All other chemicals were of analytical grade and obtained from standard commercial sources.

Miscellaneous measurements. Cell growth and pigment production were measured turbidimetrically with a Klett-Summerson colorimeter at 660 nm. Because the soluble brown pigment contributed to the total turbidity at this wavelength, the culture growth was also reported as a corrected turbidity which was determined from the total KU minus the KU of pigment (growth supernatant).

Viable cell counts were performed with SBSS as a diluent and plated on GC-FC agar. Colonies were counted after incubation for 72 h.

The presence of free sulfhydryl groups was determined with the use of Ellman reagent [5,5-dithiobis(2-nitro-benzoic acid); DTNB]. Three milliliters of sample was added to 0.1 ml of DTNB solution (40 mg of DTNB in 1.0 ml of sodium phosphate buffer, pH 8). After 15 min at room temperature, the absorbance was read at 410 nm. L-Cysteine was used as standard.

Ferric iron was determined colorimetrically by reaction with thiocyanate and extraction with amyl alcohol as described by Mueller and Miller (8).

Intracellular lipid granuoles were detected by staining with Sudan black B as described by Weaver (10).

RESULTS

Growth in complex liquid media. The growth kinetics of *L. pneumophila* strain Knoxville in liquid GC-FC medium are shown in Fig. 1. With an initial turbidity of 30 KU, no lag period was observed and the cultures grew exponentially with a generation time of approximately 6 h based on turbidity (4 h based on viable cell counts). This slow generation time correlated with the slow growth of this organism on agar media (7, 10). A soluble brown pigment was first observed in the medium when the

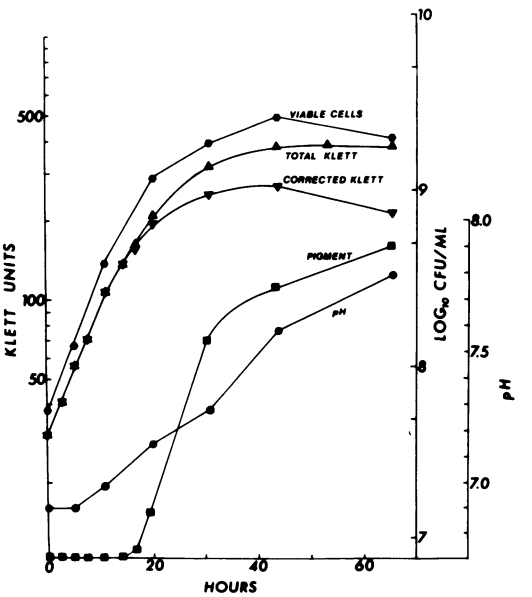

FIG. 1. *Kinetics of growth, pigment production, and pH of L. pneumophila strain Knoxville in GC-FC complex medium. Corrected KU were calculated from the total KU minus the KU of the pigment (growth supernatant). CFU, colony-forming units.*

culture reached 150 KU (7.5×10^8 colony-forming units per ml) and the observed turbidity continued to increase for another 24 h to a final value of 380 KU. Viable cell counts and corrected KU values (total KU minus the KU of the pigment) indicated that the production of pigment was initiated during late exponential to early stationary phase of growth. Maximum cell yield of 270 KU (corrected) or 2.5×10^9 colony-forming units per ml was obtained by 45 h. Extended incubation for 68 h resulted in a decline in the corrected KU and a corresponding decline in the viable cell count. Incubation beyond 68 h resulted in a continued decline in the viable cell count (data not shown). The pH of the medium increased continuously during growth from 6.9 initially to 7.8 after 68 h. The above growth kinetics were typical of all four strains of *L. pneumophila* tested.

The growth of *L. pneumophila* was influenced by the concentration of proteose peptone no. 3 in the GC-FC medium. Although the generation time was unaltered, total cell yield and pigment production were proportionally less at 10 or 5 g/liter, whereas a doubling of the concentration to 30 g/liter gave slightly better cell yield and pigment (data not shown).

Growth in defined liquid media. The chemically defined medium used in these studies contained only amino acids and inorganic salts as listed in Table 1. The growth kinetics of *L. pneumophila* strain Knoxville in this defined medium are illustrated in Fig. 2. In general, the growth kinetics were similar to that shown for the GC-FC complex medium. After a short lag of approximately 3 h (with a drop in KU), the culture grew exponentially with a generation time of 6 h. The brown pigment again appeared as the culture reached late exponential to early stationary phase (140 KU), and the observed turbidity continued to increase to a maximum of 230 KU by 38 h. A decline in the corrected KU was again observed by 68 h. However, the final cell yield (170 corrected KU) and pigment produced (80 KU) were significantly less than that observed in the GC-FC complex medium. Increasing the amino acid concentrations beyond that listed in Table 1 did not increase the growth yield. Pigment production could be stimulated by increasing the concentration of L-tyrosine; however, no concomitant increase in growth yield was observed (data not shown).

An interesting feature of the growth of *L. pneumophila* in our defined medium was the cellular morphology which was observed. Although the inoculum was primarily single cells, the culture grew with extensive filamentation in the defined medium. This fact led to our inability to obtain accurate, representative viable cell counts in this medium. Such a problem was not encountered in the GC-FC medium where the organisms retained their single-cell morphology. In addition to the filamentous growth, the defined medium-grown organisms contained numerous intracellular lipid granuoles which were seen only occasionally in cells grown in the complex medium.

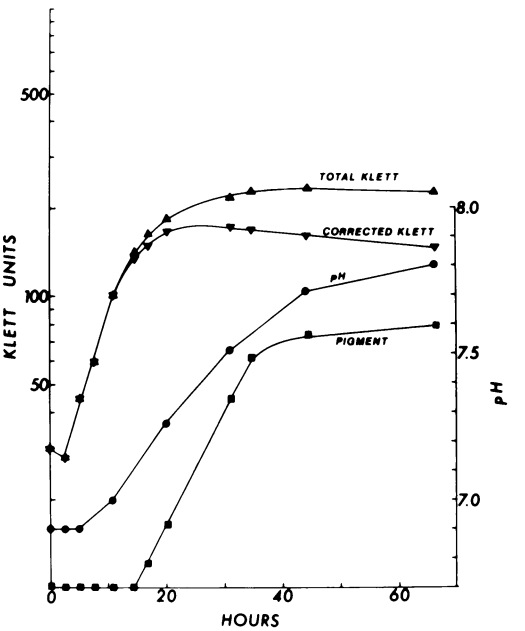

FIG. 2. *Kinetics of growth, pigment production, and pH of L. pneumophila strain Knoxville in a chemically defined medium.*

All four strains of *L. pneumophila* could be transferred continually on our solid defined medium as described in Table 1.

Growth requirements for L-cysteine, L-serine, and L-methionine. Initial reports on the growth requirements of *L. pneumophila* indicated a requirement of L-cysteine (5). L-Serine and L-methionine have also been reported to be required for growth (L. Pine, personal communication). These three amino acids were required for growth in our defined medium (Table 2). Whereas the requirement for L-serine and L-methionine was absolute, a slight amount of growth (31% of the control) was obtained in a medium lacking L-cysteine (and L-cystine). The requirement for L-cysteine could be replaced by 0L-cystine or reduced glutathione, but not by D-cysteine, thiomalate, thioglycollate, or 2-mercaptoethanol. The latter compound was, in fact, inhibitory to growth. No contaminating L-cysteine (based on assay for free sulfhydryl groups)

TABLE 2. *Growth requirements for* L-*serine,* L-*methionine, and* L-*cysteine in a defined medium*

Composition of the medium[a]					% of control growth[d]
L-Serine[b]	L-Methionine[b]	L-Cysteine[b]	L-Cystine[c]	Other additions	
+	+	+	−		100
−	+	+	−		0
+	−	+	−		0
+	+	−	−		31
+	+	−	+		100
+	+	−	−	Glutathione (1,200 μg/ml)	100
+	+	−	−	D-Cysteine (400 μg/ml)	38
+	+	−	−	Thiomalate (400 μg/ml)	34
+	+	−	−	Thioglycollate (600 μg/ml)	31
+	+	−	−	2-Mercaptoethanol (280 μg/ml)	0

[a] Defined medium as listed in Table 1 except for those amino acids listed below.
[b] Concentrations as listed in Table 1.
[c] Final concentration of 300 μg/ml.
[d] Percent of maximum cell yield (corrected turbidity) in the complete defined medium.

was detected in the defined medium containing only L-cystine.

Growth requirement for iron. A requirement for ferric iron or hemoglobin for growth of *L. pneumophila* has been reported (5, 10). We examined the iron requirement for growth of *L. pneumophila* strain Knoxville in our defined liquid medium. As shown in Fig. 3, the omission of iron from the medium resulted in a slight increase in the lag period with the generation time increased and the total cell yield somewhat depressed. Analysis of the unsupplemented (iron-deficient) defined medium for contaminating iron indicated levels which were below the sensitivity of the assay employed (<1.5 μM). Similar growth responses were obtained with other strains of *L. pneumophila*.

Effects of vitamins, cofactors, purines, pyrimidines, and alternate carbon sources on growth. Preliminary results indicated that *L. pneumophila* could be grown in GC-FC complex medium without additional supplements beyond ferric iron and L-cysteine. In developing our defined medium, a number of vitamins, cofactors, purines, and pyrimidines (alone or in combinations) were subsequently tested and were shown to have no effect on the growth of this organism. These compounds and the concentrations tested are listed in Table 3. Addition of yeast extract (0.02% final concentration) to our defined medium stimulated growth significantly (data not shown). The growth-promoting compound in yeast extract has not been identified.

The effect of supplemental carbon sources on the growth of *L. pneumophila* in liquid defined medium is shown in Table 4. At concentrations of 0.5% (wt/vol), glucose, succinate and α-ketoglutarate were without effect, whereas pyruvate, acetate, and citrate all gave varying degrees of inhibition (69, 37, and 0% of the control growth, respectively). The pH of these media all increased during growth and was typical of the control medium.

DISCUSSION

The results of the present study have indicated that the nutrient requirements of *L. pneumophila* are not particularly complex Good

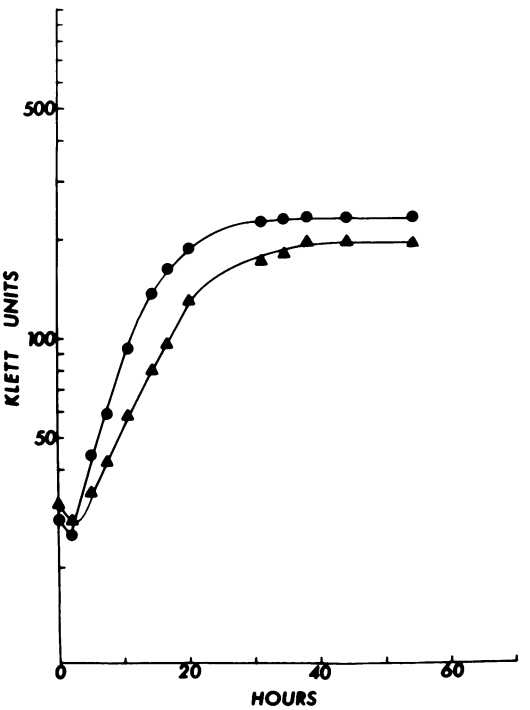

FIG. 3. *Growth of L. pneumophila strain Knoxville in a chemically defined medium with* (●) *and without* (▲) *supplemental ferric pyrophosphate.*

TABLE 3. *Vitamins, cofactors, purines, and pyrimidines which had no influence on the growth of L. pneumophila*

Compound	Concn tested (μg/ml)
Thiamine·HCl	0.03, 0.05, 2.0
Biotin	0.003, 0.04, 0.043
Pantothenic acid	1.9
p-Aminobenzoic acid	0.13, 0.26
Folic acid	0.13
Pyridoxine	0.06
Cobalamin	0.1, 0.20
Nicotinic acid	2.54
Nicotinamide	2.8
Nicotinamide adenine dinucleotide	2.5, 5.0
Riboflavin	0.20
Flavin mononucleotide	0.20
Inositol	50
Cocarboxylase	1.0, 2.0
Adenine	10, 20
Guanine	0.3, 0.6
Cytosine	10
Thymine	10
Uracil	10
Hypoxanthine	5.0

TABLE 4. *Effect of supplemental carbon sources on the growth of L. pneumophila in a defined medium*

Compound[a]	% of control growth[b]
Glucose	100
Pyruvate[c]	69
Acetate[c]	37
Citrate[c]	0
α-Ketoglutarate[c]	100
Succinate[c]	100

[a] All compounds were added at a final concentration of 0.5% (wt/vol).

[b] Percent of maximum cell yield (corrected turbidity) in the control medium (Table 1).

[c] Sodium salt.

growth was obtained in liquid or solid media containing only 21 amino acids and inorganic salts. An absolute requirement for each of these amino acids was not determined, although our results indicated that L-cysteine, L-serine, and L-methionine were required for growth, and the optimum concentrations of these amino acids were determined. In all our experiments, the critical factor for obtaining good growth appeared to be adequate concentrations of amino acids in the medium. For example, the defined medium of Catlin (2) for growth of *Neisseria gonorrhoeae* supported only poor growth of *L. pneumophila* until the amino acid concentrations were increased, some by as much as 20-fold or more (unpublished data). Growth was especially responsive to the level of L-serine in the medium. It has been suggested (L. Pine, personal communication) that this amino acid is used as a source of energy by this organism, and our results are not out of line with this conclusion.

The requirement for high levels of L-cysteine could be interpreted as a need for a reducing compound in the medium; however, our results do not support this interpretation. The requirement for L-cysteine could be replaced by its oxidized form, L-cystine, but not by D-cysteine or other reducing compounds. Curiously, supplemental L-serine and L-methionine were not needed in the GC-FC complex medium. Apparently, the levels of these amino acids in the complex medium were sufficient to support the growth of this organism. The requirement for these three metabolically interrelated amino acids suggests possible defects in the incorporation and utilization of inorganic sulfur by this organism.

Aside from the amino acid requirements, *L. pneumophila* appeared to have the biosynthetic capabilities to synthesize all the required vitamins, coenzymes, and nucleotides. Although we tested only a few concentrations of these compounds, the concentrations tested matched those indigenous to proteose peptone no. 3 (Difco literature), present in vitamin enrichments such as IsoVitaleX (Baltimore Biological Laboratories), or have been commonly used for the growth of other microorganisms. *L. pneumophila* also does not ferment any known carbohydrates (10). However, since the organism is reported to be an obligate aerobe (10), utilization of carbohydrates would proceed via oxidative pathways. Results of our study indicate only that carbohydrates and organic acids do not stimulate growth in either complex or defined medium. However, preliminary results in our laboratory (unpublished data) have shown that these carbon compounds also did not stimulate oxygen uptake in washed cell suspensions of *L. pneumophila*. Several of the organic acids (pyruvate, acetate, and citrate) were inhibitory to growth. The reasons for this inhibition are not clear, although it is possible that the inhibition by citrate was due to its ability to chelate iron (9) and make it unavailable to this organism in the chelated form. The importance of supplemental iron for optimum growth would tend to support this contention.

Although supplemental iron was important for maximum growth, iron was not an essential growth requirement. In both complex (unpublished data) and defined medium (Fig. 3), the absence of iron resulted in a slower growth rate and decreased cell yield. We are currently investigating the possibility that growth in low iron results in the synthesis of compounds of an iron

binding and transport system as has been recognized in other bacteria such as the enteric bacteria (9) and *Pseudomonas aeruginosa* (4, 6). It should also be noted that although soluble ferric pyrophosphate was used throughout this study, ferric nitrate could also serve as a source of iron. This compound was not routinely used due to the precipitate formed when added to our growth media.

The production of a soluble brown pigment is characteristic of all strains of *L. pneumophila* isolated to date (1, 10). Our results indicated that the production of this pigment occurred as the culture was entering stationary phase of growth. Pigment production thus appears to be typical of a secondary metabolite, as has been shown for the fluorescein and pyocyanin pigments of *P. aeruginosa* (3, 11). Since the growth rate of *L. pneumophila* was shown to decrease as pigment appeared, the initiation of pigment in liquid or solid medium could be used as an indication of maximum culture viability. It should be noted that the amount of brown pigment produced in our defined medium was influenced by the concentration of L-tyrosine as has been reported by Baine et al. (1).

The filamentous nature of *L. pneumophila* in our defined medium and the extensive intracellular lipid granuoles suggest that these two characteristics may be a response to the more limited nutritional quality of our defined medium. We are currently investigating the relationship between lipid granuole and filament formation and the survival of *L. pneumophila* under starvation conditions.

The defined medium reported here supported the growth of the four strains of *L. pneumophila* tested and should be satisfactory for the needs of most investigators. However, several features of the medium should be considered for future studies. First, no attempt was made to further simplify the medium through the deletion of nonessential amino acids. Second, we were unable to match the total cell yield obtained with GC-FC complex medium, either through manipulation of the total amino acid concentrations or through addition of a number of defined supplements as listed in Table 3. Yet addition of small amounts of yeast extract significantly increased the cell yield. These results suggest that improvements in our defined medium may be forthcoming, although the exact components of the yeast extract responsible for the stimulation of cell yield have not been determined.

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