Development of a Chemically Defined Liquid Medium for Growth of *Legionella pneumophila*

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Received for publication 13 February 1979

A chemically defined liquid medium has been developed for the study of the physiology and antigen production of the Legionnaires disease bacterium. The medium contains basal salts, vitamins, α -ketoglutaric acid, pyruvate, 0.05% *L*-cysteine, 0.05% glutathione, and a mixture of 20 additional amino acids, each of 0.01% final concentration, except serine, which was at 0.1%. The medium, in shake culture at 37°C with increased CO₂ at pH 6.5, supports the maximum rate of growth, the highest cell yields, and the maximum cell surface antigen as distinguished by specific fluorescein isothiocyanate-conjugated antibody. Studies during the development of this medium showed that CO₂, pyruvate, and α -ketoglutarate strongly stimulated growth; that cysteine and methionine were required for growth; and that serine, threonine, histidine, tyrosine, and tryptophane were energy sources. Glutathione substituted for cysteine, but cystine did not. The organisms did not use glucose and polysaccharides, as judged by cell yields when these carbohydrates were present or absent. The chelators malate, citrate, and ethylenediaminetetraacetic acid totally inhibited growth. Beta-mercaptoethanol, thioglycolate, dithiothreitol, and Tween 80 (0.05%) inhibited growth strongly or completely. Catalase activity was extremely weak or absent. Morphology varied, depending upon conditions and phases of growth. In general, filamentous forms became chains of cigar-shaped bacilli fragmenting to pairs and becoming coccoidal in the late stationary phase of growth. The organism grew at 25, 30, and 37°C. Although they varied in their growth characteristics, 10 isolates were passed for five transfers in the chemically defined broth, giving maximum rates of growth, cell yields, and antigen production.

Recently, Feeley et al. (8) described a primary isolation medium, the Feeley-Gorman (F-G) medium, for the Legionnaires disease bacterium. This bacterium has been named *Legionella pneumophila* by D. J. Brenner and J. E. McDade (Ann. Intern. Med., in press). Although the F-G medium is highly sensitive, it is not satisfactory for use in controlled physiological studies and for determinations of excreted products. *L. pneumophila* has not been cultivated successfully in submerged culture, and no chemically defined liquid or solid medium has been described that satisfactorily supports growth.

In evaluating antibody specific for *L. pneumophila* by the indirect and direct fluorescent antibody tests, it is desirable to have cells of the same physiological state, with uniform antigen content and uniform morphology. A liquid medium is preferred for the production of such cells, because in it cells are uniformly exposed to conditions of the medium and, in shaken culture, can be harvested at specific physiological states during growth. Furthermore, excreted products

are readily available from a liquid culture. In developing a liquid medium suitable for *L. pneumophila*, we first used liquid and agar semisynthetic media containing casein hydrolysate (vitamin-free, acid) and starch as the only undefined constituents (16). We modified these media and developed a completely synthetic liquid medium which permitted the maximum rate of growth, high cell yields, and maximum production of surface antigen. We also obtained a great deal of physiological information. The results of these experiments are reported here.

(This work was presented as an abstract at the International Symposium on Legionnaires' Disease, 13-15 November 1978, at the Center for Disease Control, Atlanta, Ga.)

MATERIALS AND METHODS

Strains and inoculum. In general, the initial studies were done with strain Philadelphia 1, which was isolated from a specimen from a patient who died in the 1976 epidemic in Philadelphia (9). Other strains were used to test and compare the initial and finally

developed media. All strains, obtained from W. B. Cherry, Bacteriology Division, Center for Disease Control, were stored at -70°C . Inocula for various experiments were prepared by transfers to plates or slants of the F-G medium. The subsequently grown cells were washed from the surface with sterile distilled water and stored in thick suspension (approximately 9.0 mg [dry weight] of cells per ml) at -70°C . Media were inoculated by adding 1 to 3 drops of freshly thawed cell suspension (0.05 to 0.15 mg [dry weight] of cells) to the surface of agar plates containing 10 or 15 ml of medium or to 5 to 15 ml of broth in test tubes (18 by 150 mm). In the broth media, this inoculum was sufficient to raise the zero-time suspensions to an optical density (OD) of 0.05 to 0.20 at 660 nm. Relationship of OD to cellular dry weight was determined by drying aliquots of suspended washed cells, killed with 1% Formalin and of known OD, and determining the dry weight after 48 h of incubation at 75°C (Fig. 1). All ODs were measured by using a model B Beckman spectrophotometer at 660 nm.

Media. Media were prepared by adding concentrated solutions, adjusting them to the desired pH at $2\times$ concentration, and then sterilizing them by filtration (0.45- μm filter, Millipore Corp.) The remaining solutions containing starch and oleic acid or agar, or both, were prepared, autoclaved for sterilization, and added to half of the medium while they were hot so that either the complete medium or a medium of 5/4 or greater concentration was obtained. Agar media were tubed at 10 or 15 ml if the medium was complete and at 8 and 12 ml if it was at a 5/4 or greater concentration. These tubes or other basal media were stored at 5°C until used. Before being used, the agar media were melted by steaming for 5 min; if necessary, test materials were added to the melted agar. The melted media were then poured into plates. All plates were used within 16 h. Similarly, culture tubes (18 by 150 mm) were prepared by adding 1 ml of water or of chemical solutions to 4 ml of 5/4-strength basal medium. The fundamental formula for the semisynthetic

medium for *L. pneumophila* (SSLp) is presented in Formula 1. In addition, the F-G medium (8) was used for certain experiments.

Cultural conditions. Agar cultures were incubated in air in moist chambers; in moist candle jars; in tightly sealed chambers dried and depleted of carbon dioxide by sandwiching the agar plates between open petri dishes filled with NaOH pellets; in desiccators with air depleted of carbon dioxide by containing concentrated solutions of sodium hydroxide; or in tightly sealed containers with approximately 4.8% CO_2 in the air. The CO_2 was generated within the sealed container by mixing 2 ml of 10% Na_2CO_3 per liter of internal volume with an excess of 2.5 N sulfuric acid. Growth on agar plates was measured by adding 5 ml of distilled water or 1% Formalin to each plate and removing the cells from the surface of the closed agar plate with a 1-cm wire brad cut from a paper clip. The wire brad was placed on the agar and rotated with a magnetic stirring device. While the cells are removed by the spinning wire brad, the worker is safe from the aerosol, and the procedure does not release agar into the cell suspension (14). The suspensions were drawn off and diluted to an OD of 1.0 or less.

Anaerobic, aerobic, aerobic without CO_2 , or aerobic + increased CO_2 cultural conditions were created in test tubes by using absorbent cotton plugs with combinations of saturated pyrogallol, 20% KOH, 10% Na_2CO_3 , and 1 M KH_2PO_4 ; rubber stoppers were then inserted (18). Virtually all of the broth cultures were incubated with Na_2CO_3 - KH_2PO_4 (CO_2) seals. In general, air with increased CO_2 tension was created by sequentially adding 0.3 ml each of 10% Na_2CO_3 and of 1 M KH_2PO_4 to the cotton plug. The test tubes were placed on a rotary shaker at 70 rpm at 37°C ; ODs were read at intervals as described above.

Chemical, serological, and other procedures. Lactic acid was determined by the procedure of Barker and Summerson (1) and ammonia by the procedure of Chaney and Marbach (4). Amino acids were determined qualitatively and were compared in fresh and fermented medium by the thin-layer chromatographic procedure of Bujard and Mauron (3). Catalase was determined by mixing cells of the various parameters of growth with 1 drop of freshly prepared 3% H_2O_2 on a glass slide and covering it with a slip. The slide was observed for 3 to 5 min and compared to control slides containing uninoculated media, yeast-phase cells of *Histoplasma capsulatum*, vegetative cells of *Flexibacterium flexilis* (ATCC 23079), and *F. flexilis* subsp. *pelliculosus* (ATCC 23098) grown on the basal agar medium (Formula 1). Positive catalase was defined as bubbles forming within 30 s and increasing and streaming to the edge of the cover slip. Catalase of lysed cell extracts was determined by the procedure of Fukui et al. (10), in which F-G-grown cells were used. Cells obtained from various experiments were stained directly with fluorescein isothiocyanate conjugates prepared against the Knoxville strain of *L. pneumophila* (5); sera were obtained from the Biological Products Division, Center for Disease Control. Cells were also observed and photographed with medium dark-phase and dark-field optics. Osmolality of certain of the media was determined by an Osmette (model 2007, Precision Systems, Inc.). The pH of all media was

FIG. 1. Relationship of dry weight to OD (660 nm) of the inoculum of *L. pneumophila*, strain Philadelphia 1, grown on the F-G medium. Most cells were single or doublets of small, cigar-shaped bacilli.

FORMULA 1. *Semisynthetic medium (SSLp) for growth and maintenance of L. pneumophila*^{a,b}**Part I. Casein hydrolysate-vitamin base**

Steps and solutions for preparation of medium (in sequence).

1. Salts (in grams):

KH ₂ PO ₄	8.0
(NH ₄) ₂ SO ₄	8.0
MgSO ₄ ·7H ₂ O	0.86
CaCl ₂ (anhydrous)	0.08
ZnSO ₄ ·7H ₂ O	0.89

Dissolve in 500 ml and make to 1 liter with distilled water; add 250 ml per liter of medium. Bring solution to 400 ml with distilled water before adding other components to minimize formation of precipitate.

2. Casein hydrolysate, 10% acid-hydrolyzed, vitamin-free solution: add 80 ml/liter.

3. Vitamin suspension (in milli-grams)^c:

Inositol	200
Thiamine hydrochloride	200
Calcium pantothenate	200
Riboflavin	200
Nicotinamide	100
Biotin	10

Suspend and make to 1 liter in distilled water; add 10 ml/liter.

4. DL-thioctic acid:

Dissolve 10 mg of DL-thioctic acid in 10 ml of 95% alcohol; store at -20°C; add 0.1 ml/liter.

5. Coenzyme A:

Dissolve 10 mg of coenzyme A in 10 ml of distilled water. Add 2 drops of 0.05% Na₂S·5H₂O solution made in freshly boiled distilled water. Store at -20°C; add 0.1 ml/liter.

6. Solid additions (in grams):

α-Ketoglutaric acid	1.00
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L-Cysteine·hydrochloride	0.50
Glutathione (reduced)	0.50
L-Asparagine	0.10
L-Tryptophane	0.02

7. Minor elements^d (in grams):

Add 1.0 ml of concentrated HCl to 100 ml of distilled water; dissolve each component in the order given before adding second component.

FeSO ₄ ·7H ₂ O	5.70
MnCl ₂ ·6H ₂ O	0.80
Na ₂ MoO ₄ ·2H ₂ O	0.15

Make to 1,000 ml with distilled water; add 10 ml/liter.

8. Adjust pH to 6.3 with 20% KOH.

9. Hemin:

Suspend 200 mg of hemin in 10 ml of distilled water and bring into solution by adding 2 drops of concentrated NH₄OH. Make to 100 ml with distilled water; add 1 ml/liter.

10. Neutralize carefully to pH 6.50 with 20% KOH. Bring volume to 500 ml, and sterilize by filtering through a 0.45-μm membrane filter (Millipore) to give 2×-concentrated basal solution.^e**Part II. Starch, oleic acid, agar**11. Suspend 0.5 g of soluble starch (1)^a in 50 ml of distilled water and pour into 450 ml of boiling distilled water.

12. Suspend 100 mg of oleic acid in 50 ml of distilled water and carefully bring into solution by titrating with NaOH to pH 7.0 with 1 drop of 0.05% phenol red. Warm if necessary. Adjust to final volume of 100 ml; add 1 ml to starch solution.

13. Autoclave starch-oleic acid solution and add to solution prepared in Part I.^f

^a For agar medium, Part I is identical. However, Part II is modified to contain 15 g of agar per liter, 2 g of starch (Eastman, Mallinckrodt, or ACS), and 10 ml of oleic acid solution (10 mg/liter).

^b The medium is to be used in growth conditions including CO₂ seal per tube (or incubation under similar conditions of increased CO₂ tension) and moderate shaking conditions. Good but slower growth may be obtained in static tubes with seals, if the culture tube with 5 to 10 ml of medium is incubated in a near-horizontal position. Increased CO₂ tension on agar plates is not required, but the plates should be incubated in a humidity chamber. To prepare the CO₂ seal, for tubes trim off the cotton plug extending beyond the glass wall. Push this short plug into the tube to within 1 inch below the lip of the tube and place a small wad of absorbent cotton on top of the plug. Add 0.3 ml of 10% Na₂CO₃ to each wad of absorbent cotton. Add 0.3 ml of 1 M KH₂PO₄ to the same spot and close immediately with a rubber stopper. If a series of tubes is to be prepared, the Na₂CO₃ can be added to all, after which KH₂PO₄ solution is added to three tubes before they are sealed with rubber stoppers. To open seal, remove rubber stopper and absorbent cotton, flame tube, and withdraw cotton stopper as desired.

^c Vitamin suspension can be stored at 50°C. A few drops of chloroform are added as preservative. The flask should be shaken and a sample should then be removed from the top.

^d The minor elements can be stored at room temperature. Should oxidation of Fe²⁺ occur, as evidenced by red precipitate, a new solution must be made.

^e As pH approaches 6.5, the solution will turn purple-black, as a cysteine-Fe complex forms. This color disappears after the solution stands. Filtering medium containing a small amount of precipitate has not affected growth.

^f The final volume of the solution prepared in Part II is 500 ml; if concentrated experimental media are desired, the final volume should be less.

determined by a Beckman Zeromatic pH meter standardized with Beckman pH standard buffer (25°C).

RESULTS

Initial experiments. In the initial experiments, we tested four semisynthetic media because they contained the basic ingredients Feeley et al. (8) found to be required for growth and because they were known to support the growth of numerous fastidious organisms. The media were the *H. capsulatum* yeast-phase and mycelial-phase agars (15), the *Actinomyces* broth and agar (17), and the casein hydrolysate-Tween 80 broth (16) for *Streptococcus pyogenes*. No growth was observed with the *Histoplasma* yeast-phase agar or with the streptococcal broth. In an experiment with the *Actinomyces* media, no growth was observed in the deep portions of the semisolid stabs whether the conditions were anaerobic or aerobic; a small amount of growth was observed on the surface under aerobic conditions.

In an experiment with the broth in flasks, in shaking tubes having different amounts of liquid (5 to 15 ml), and under different conditions of aerobiosis induced by incubation with or without shaking, growth was obtained in tubes that had 5 or 10 ml of medium, were incubated with shaking, and had a Na_2CO_3 and acid phosphate (CO_2) seal. Tubes that were incubated stationary with CO_2 seals failed to show significant growth, but when placed on the shaker they showed an increase in OD from 0.19 to 0.77. Tubes incubated stationary without CO_2 seals showed no growth for several days but had final ODs of 0.48 to 0.61 when CO_2 seals were added and the tubes were again incubated with shaking. These results indicated that the organism is a strict aerobe that requires carbon dioxide and ready access to oxygen and is potentially sensitive to excessive aeration.

Since the "mycelial" medium as agar slants gave good surface growth in the absence of CO_2 seals, this medium was further tested as a broth. Good growth was observed with the CO_2 seal and shaking. Because growth was better in the mycelial broth than in the *Actinomyces* broth, the mycelial medium was tested further by using a single strain of the bacterium to determine those factors that influenced growth. Initial studies showed that glucose was not required and that increasing the casein hydrolysate to 0.8% gave maximal cell yields. This modified medium supported seven consecutive transfers of one strain and three consecutive transfers of 13 strains. This modified semisynthetic medium for *L. pneumophila* (SSLp) was the basis for all further experiments; its formula is given in Formula 1.

Growth on agar media. A comparison of growth on the SSLp medium and F-G medium under moist aerobic conditions showed equal growth and cell yields, although the lag period on the SSLp medium was shorter (Fig. 2). The conditions required for growth raised many questions about the function of pH and carbon dioxide, the depletion of oxygen in the candle jar, α -ketoglutaric acid as a source of carbon dioxide, and the effect of pH on cysteine in the medium. In air as compared to incubations in a candle jar (Table 1), at a pH above 6.8, the growth rates definitely decreased, but final cell yields did not. However, 0.1% α -ketoglutaric acid strongly increased the growth rate, and cell yields were substantially higher at pH values above 6.8 (Tables 2 and 3). Increasing the cysteine concentration from 0.1 to 0.2% had no effect in the presence or absence of α -ketoglutaric acid; a brown pigment was produced in those plates containing α -ketoglutaric acid and incubated aerobically (Table 2).

These data do not clearly delineate the function of the factors tested, and a more complete experiment was conducted by using air depleted of CO_2 , air, and air + CO_2 with media having limited casein hydrolysate. The effects of pyruvate and α -ketoglutarate were also determined (Fig. 3). The results obtained in air lay between the extreme results obtained with added and deleted CO_2 ; they are not given in Fig. 3. At pH 7.2, no growth was obtained in air or in air depleted of CO_2 when neither pyruvate nor α -ketoglutaric acid was added. However, if α -ketoglutaric acid or pyruvate was added, growth did occur in the absence of added CO_2 , but the cell yields obtained in their presence were not greater than 33 to 66% of that obtained when CO_2 was added with these acids. This result

FIG. 2. Comparative growth of the *L. pneumophila* on SSLp and F-G media. Each point represents the average of two plates incubated at 37°C in a moist chamber. OD readings represent the values obtained for 10 ml of suspension.

TABLE 1. *Effects of pH, CO₂, and α -ketoglutaric acid on growth on agar surface^a*

Condition	pH	OD at:	
		40 h	120 h
Air	6.6	1.67	1.42
	6.8	1.25	1.16
	7.0	0.97	1.20
	7.2	0.90	1.02
Air plus α -ketoglutarate	6.5	1.43	1.53
	6.6	1.45	1.45
	7.0	1.28	1.30
	7.2	1.31	1.36
Candle jar	6.6	1.83	1.48
	6.8	1.52	1.23
	7.0	1.15	1.21
	7.2	1.06	—
Candle jar plus α -ketoglutarate	6.4	1.62	1.60
	7.0	1.52	—
	7.0	1.05	1.72
	7.2	1.32	2.05

^a Basal medium was *Histoplasma* mycelial agar medium (15) without glucose. Surface growth was removed, and the OD was measured. All OD measurements were normalized to cells per plate per 10 ml of water. Plates were incubated in sealed moist chambers or in candle jars. Zero-time pH values were determined after macerating the agar with a few milliliters of distilled water.

shows clearly that, at this pH, CO₂ was required for growth and that the organic acids could not substitute for it completely. Furthermore, at this pH, the growth rate and cell yields were significantly higher in the presence of pyruvate than in the presence of α -ketoglutarate.

However, at pH 6.5 and in the absence of the organic acids, CO₂ was not required for growth, but growth was greater when it was present. In the absence of CO₂, the cell yields were only 66% of those obtained when the organic acids were present. In the presence of 5% CO₂ and with an extended period of growth, the cell yields reached only 85% of those obtained with either pyruvate or α -ketoglutarate. These results suggest more than one role for carbon dioxide and emphasize that both the CO₂ and organic acids were required for maximum growth. Although stimulation of growth by increased casein hydrolysate might be explained in part by the presence of these organic acids in casein hydrolysate (13), other experiments showed that the amino acids in casein hydrolysate were responsible for the major effect.

Using filter paper disks, saturated with reagent and placed on the agar surface, we observed no effects with succinate, fumarate, lactate, acetate, oxalate, gluconolactone, glucuronic acid, glycerol, gelatin, and cobalamin. Yeast extract, pyruvate, and α -ketoglutaric acid stimu-

TABLE 2. *Effect of pH, cysteine, and α -ketoglutarate on growth on agar surface^a*

Component tested	pH	OD at:		Description
		34 h	65 h	
0.1% cysteine	6.4	0.79	1.60	No pigment
	6.9	0.46	2.22	No pigment
	7.0	0.47	2.36	No pigment
	7.1	0.50	1.64	No pigment
0.1% cysteine plus α -ketoglutarate	6.4	3.30	2.54	Brown pigment formed
	6.9	2.76	3.30	Brown pigment formed
	7.0	2.30	3.26	Brown pigment formed
	7.1	2.00	2.00	Brown pigment formed
0.2% cysteine	6.4	0.73	2.28	No pigment
	6.9	0.45	1.48	No pigment
	7.0	0.39	1.40	No pigment
	7.1	0.40	0.75	No pigment
0.2% cysteine plus α -ketoglutarate	6.4	3.02	2.90	Brown pigment formed
	6.9	2.41	2.82	Brown pigment formed
	7.0	2.28	2.50	Brown pigment formed
	7.1	2.10	ND ^b	

^a Basal medium was *Histoplasma* mycelial agar (15) medium having no glucose but 1.0% casein hydrolysate. Surface growth of two plates was removed, and the OD was measured. All OD measurements normalized to cells per plate per 10 ml of water. Plates were incubated in a moist chamber at 37°C.

^b ND, Not done.

FIG. 3. Effect of CO₂, pyruvate, and α-ketoglutaric acid on the growth of *L. pneumophila* at pH 6.5 and 7.2. Each point represents the average obtained with three plates, each containing 10 ml of SSLp medium modified to contain only 0.4% casein hydrolysate (basal) with or without 0.4% pyruvate (pyr) or α-ketoglutaric acid (α-kg). The plates were incubated in air in a moist chamber, in air + 5% CO₂, or in sealed desiccators containing solutions of 40% NaOH in the bottom. The OD readings obtained were divided by 2 to give values normalized to 5 ml of medium. The values of all of the results obtained in the presence of air (not given) lay between those of the other atmospheric parameters, except that no growth was obtained at pH 7.2 in the absence of pyruvate or α-ketoglutaric acid.

lated growth, whereas citrate strongly inhibited it. The latter finding supported results obtained earlier with the *Histoplasma* yeast-phase agar.

Growth in liquid media, basic observations. In initial experiments with the SSLp medium, there were a significant number of negative results due to the failure of a known viable inoculum to grow in media that had previously supported its growth. More consistent results were obtained when the starting OD of each test culture was at least 0.05 to 0.10. In static, horizontally incubated tubes with CO₂ seals, growth was slower and cell yields were not maximal. Without CO₂ seals, growth was reduced and heavy pigments were associated with the bacterium entering the stationary phase (Table 3). The effect of air in reducing growth was directly correlated with an increase of pH from 6.5 to 7.9, whereas growth under the CO₂ seal was maintained at pH values from 6.35 to 6.45 (Table 3). Control studies showed that the CO₂ seals decreased the initial pH from 6.5 to 6.2 within 48 h. These results suggest that CO₂ was functioning as a buffer to maintain the medium at the

optimal pH observed for growth in agar media; CO₂ may also perform other regulatory functions.

Determination of factors present or added to the SSLp medium which affect growth. Cell yields were proportional to the concentration of casein hydrolysate (Fig. 4). In an experiment with 0.2% casein hydrolysate, the addition of either histidine, threonine, trypto-

TABLE 3. Effect of air and air + carbon dioxide on growth and final pH in shake culture^a

Inoculum (initial OD)	Condition	Final OD	Final pH	Description
0.05	Air + CO ₂	1.16	6.35	No color
0.10	Air + CO ₂	1.13	6.40	No color
0.15	Air + CO ₂	1.12	6.40	No color
0.05	Air	0.75	7.65	Brown pigment
0.10	Air	0.74	7.70	Brown pigment
0.15	Air	0.75	7.75	Brown pigment

^a Medium was the SSLp broth with 1.0% casein. Initial pH was 6.5. Cultures were incubated for 65 h at 37°C and shaken at 70 rpm. Carbon dioxide was generated with 0.3 ml of 10% Na₂CO₃ plus 0.3 ml of 2.5 N H₂SO₄ per tube.

FIG. 4. Effect of casein hydrolysate concentration on growth rates and cell yields of *L. pneumophila*. The basal medium was the SSLp broth without casein hydrolysate; CO₂ seals were used on tubes incubated at 37°C and shaken at 70 rpm.

phane, tyrosine, or serine increased the growth (Fig. 5). Under those conditions, alanine, arginine, aspartic acid, cystine, glutamic acid, isoleucine, leucine, methionine, and valine had no effect; cell yield was slightly increased by glutamine, lysine, and proline. Cell yields due to serine were the same with 0.1 and 0.2% casein hydrolysate, indicating that serine was a source of energy for growth. Pyruvate could not substitute for serine, but other experiments suggested that histidine, glycine, and threonine were also sources of energy. Chromatographic experiments did not delineate the disappearance of any one amino acid during growth in 0.4% casein hydrolysate.

Cysteine was required for growth, and growth was proportional to cysteine concentrations between 0.002 and 0.01% (Fig. 6); higher concen-

FIG. 5. The effect of single amino acids added to the basal SSLp medium containing 0.2% casein hydrolysate. Conditions of growth were those described for Fig. 4.

FIG. 6. Requirement of *L. pneumophila* for cysteine. The basal medium was the SSLp medium with no glutathione; conditions were those described for Fig. 4.

trations gave no increased cell yields and were slightly inhibitory at 0.1 and 0.2%. Cystine did not substitute for cysteine, but, on an equimolar basis, glutathione did substitute for cysteine. Although the initial growth was delayed with unadapted cells, cells adapted to the utilization of glutathione gave equal rates of growth and cell yields (Fig. 7). Under favorable growth conditions, adding 0.12 M dithiothreitol, 2-mercaptoethanol, thioglycolate, and methionine gave moderate to complete inhibition of growth when the medium contained only 0.02% cysteine. Adding ethylenediaminetetraacetic acid at a 0.06% concentration completely inhibited growth.

Adding glucose to the SSLp basal medium had no effect upon growth, although adding 1% glucose (55.5 $\mu\text{mol/ml}$) caused lactate to form in 0.8- to 0.9- $\mu\text{mol/ml}$ amounts; no lactate formed in the absence of glucose. Because no growth increment was observed in the presence of glucose, we conclude that glucose was not used significantly as a source of energy for growth. Oleic acid added in the absence of starch completely inhibited growth at a concentration of 1 $\mu\text{g/ml}$; in the presence of 0.05% starch it was not inhibitory at 10 $\mu\text{g/ml}$. Neither the elimination of starch and oleic acid from the SSLp broth nor the addition of amylose, amylpectin, dextrin,

FIG. 7. Glutathione as a substitute for cysteine; growth of cysteine and glutathione-adapted cells. (●) Strain Philadelphia 1 frozen inoculum growing in the SSLp broth with 0.1% cysteine; (○) strain Philadelphia 1 frozen inoculum growing in the SSLp broth with 0.254% glutathione and no cysteine; (△) glutathione-adapted cells growing in SSLp broth with 0.254% glutathione and no cysteine; (□) glutathione-adapted cells growing in SSLp broth with 0.1% cysteine.

yeast extract, glycogen, bovine albumin (fraction V), or spent growth liquor of *L. pneumophila* had an effect on growth. Adding 0.05% purified Tween 80 instead of starch totally inhibited growth, which explains why growth was not obtained with the streptococcal medium.

In the presence of 0.1% α -ketoglutaric acid, adding 0.4% pyruvate, fumarate, or succinate had no effect on growth, but adding citrate, acetate, and malate totally inhibited growth; lactate was slightly inhibitory. Concentrations of α -ketoglutaric acid and pyruvate could be varied with different concentrations of the casein hydrolysate without affecting growth. Nevertheless, in several experiments where growth was marginal because of limited casein hydrolysate or in a medium which by itself did not support growth, 0.1% pyruvate caused growth to occur. In none of the early experiments with the complete SSLp broth was there an unequivocal increase in cell yields due to adding either α -ketoglutarate or pyruvate. Thus, in these experiments pyruvate and α -ketoglutaric acid appear to stimulate growth without being used for energy.

Complete deletion of all vitamins, oleic acid, and starch resulted in a 90-h delay in growth; if α -ketoglutaric acid was also deleted, no growth occurred under these conditions. These and related experiments suggest that, under the circumstances of total vitamin deletion, the bacterium is sensitive to deletions that would have no effect in the complete SSLp medium.

The overall results presented above show that *L. pneumophila* is a strictly aerobic organism which requires carbon dioxide and is stimulated by α -ketoglutaric acid and pyruvate. It apparently uses these acids very limitedly, if at all, for energy. It is extremely sensitive to chelating agents, does not use glucose, polysaccharides, or other tricarboxylic acid cycle intermediates for growth, and has specific requirements for amino acids not only as nutrients but also as sources of energy for growth. With low pH and with CO₂, no pigment was formed; in air, pigment and ammonia were formed. *L. pneumophila* did not hydrolyze starch as observed with Lugol iodine. Because vigorous shaking seemed to inhibit growth, we tested the cells of the initial agar experiments for catalase directly or later by chemical analyses. Subsequently the cells grown under virtually all of the conditions described above were also tested; all tests were interpreted as being either negative or very weakly positive. Additional tests supported the observations of Weaver (19) that catalase was formed by cells grown on the F-G medium; again, the activities observed were low.

Development of a chemically defined medium. Preliminary studies of the amino acid requirements showed that the casein hydrolysate could be replaced by a mixture of 19 amino acids and the cysteine of the basal medium. In these experiments, methionine was observed to be required for growth. The presence of all the amino acids at 0.01% and cysteine and serine added at 0.1 and 0.4%, respectively, gave maximum growth, which was not further increased by adding casein hydrolysate (Fig. 8A). Adding 0.1% pyruvate, however, gave a small but positive increase (Fig. 8). The results were repeated in experiments in which the concentrations of serine and pyruvate varied individually and together. The conclusion was that enhanced growth could be attributed to pyruvate, its effect with serine being additive rather than synergistic.

A chemically defined medium for the growth of the *L. pneumophila* (CDLp) (Formula 2) was then compared with the SSLp medium for maintenance of growth in five sequential transfers of 10 strains of *L. pneumophila*. These strains were Philadelphia 1, Philadelphia 2, Philadelphia 3, Philadelphia 4, Pontiac, Flint 1, Bellingham, Miami Beach, Detroit 1, and Togus. The CDLp broth, having 0.01% of each of 19 amino acids, supported growth and cell yields of the tested strains to a degree only 0.2 OD units less than that obtained with the SSLp medium (0.8% amino acids). By calculation, the amino acid mix of 0.19% had 2.8 times the cell yield of the same amounts of casein hydrolysate. Thus, some amino acids of the mix were at preferred levels compared to those occurring in casein hydrolysate. Consequently, the medium was supple-

FIG. 8. Effect of casein hydrolysate and pyruvate on the growth of *L. pneumophila* in a chemically defined medium. (A) Growth in the chemically defined medium for *L. pneumophila* (CDLp) with 20 amino acids, 0.1% cysteine, and 0.4% serine. (B) As (A), but with 0.1% pyruvate added.

FORMULA 2. Composition of a chemically defined medium (CDLp) for *L. pneumophila*

1. Basal medium.

- (a) Add:
 250 ml of the 4× salts solution, Formula 1.
 200 ml of the amino acid-organic acid solution described in 2 below.
 10 ml of the vitamin solution, Formula 1.
 0.1 ml of the thiocetic acid solution, Formula 1.
 0.1 ml of the coenzyme A solution, Formula 1.
 0.5 g of L-cysteine hydrochloride
 0.5 g of glutathione (reduced)
 10 ml of the minor element solution, Formula 1.
- (b) Adjust pH to 6.3 with 20% KOH and add 1 ml of the hemin solution, Formula 1.
- (c) Adjust pH to pH 6.5 with 20% KOH, bring the volume to 500 ml with distilled water, and sterilize by filtering through a 0.45-μm membrane filter to give 2×-concentrated medium.

2. Amino acid-organic acid solution.

5 g of serine, 5 g of α-ketoglutaric acid, and 5 g of sodium pyruvate are added to 500 ml of water. Of the following 18 amino acids, 0.5 g of each is also dissolved in the 500 ml of water (L-alanine, L-aspartic acid, L-asparagine [monohydrate], L-arginine [monohydrochloride], L-histidine hydrochloride [monohydrate], L-isoleucine, L-glutamic acid, L-glutamine, glycine, L-leucine, L-lysine [monohydrochloride], L-methionine, L-phenylalanine, L-proline, L-threonine [allo-free], L-tryptophane, L-tyrosine, and L-valine).

3. The 2×-concentrated medium is then brought to final volume with sterile distilled water. For general use, it is recommended that the final medium contain 0.05% soluble starch (Eastman, Mallinckrodt, or Baker). For agar media, 1.0% Ion Agar no. 2 (Oxoid) and 1% soluble starch are recommended.
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mented with serine and pyruvate (Formula 2) to give cell yields and growth responses equivalent to those obtained with the SSLp broth.

Production of surface antigen. In the experiments with the SSLp and CDLp media, the cells of most experiments were examined for morphology and catalase production, and were also stained with the specific fluorescein-conjugated rabbit globulin and compared with cells grown on the F-G medium for the presence of surface antigen. In all cases, the cells grown on the semisynthetic or chemically defined media stained as well as or better than the control cells grown on F-G medium.

General observations. Morphology varied widely under the various conditions of growth (Fig. 9 and 10). In general, growth of the organism on agar covers the surface because of the liquid present; growth appears to begin faster under the condition of high humidity. However, we have not been able to observe spreading or crawling motility. In the cases where plates were incubated between NaOH pellets to absorb CO₂, the pellets also functioned as desiccants and the surfaces were dried, giving discrete colonies with entire edges (Fig. 10). Slower growth observed under these conditions may have been due to depleted CO₂ or a dried surface, or both. Cellular morphology showed progressive changes in the SSLp broth or agar, with large masses of filaments or chains of bacilli in the logarithmic phase breaking into shorter filaments and ultimately forming single and double cigar-shaped cells. With prolonged incubation of limited substrate, cells became coccoid shaped (Fig. 9). The organism grew at 25, 30, and 37°C. The cellular morphology was the same at the two higher temperatures; at the lowest temperature, cells

appeared as fine, small bacilli (Fig. 9).

We have not observed refractile cells or cells which might suggest spores or microcysts. Because of certain strong physiological resemblances of *L. pneumophila* to Fager's groups 3, 12, and 18 (7) of Lewin's strains of flexibacteria (11, 12), we compared the morphologies of *F. flexilis* and *F. flexilis* subsp. *pelliculosis* with that of *L. pneumophila*. Although *L. pneumophila* made terminal "bodies" not unlike the microcysts of *F. flexilis*, these bodies could not be considered microcysts because they were dark under phase optics and were not sufficiently well delineated by cell wall to be considered resting bodies (Fig. 10). Although the two strains of *F. flexilis* grew excellently on the SSLp medium at 25 and 30°C, no growth occurred at 37°C. Growth on this medium produced at certain stages cells morphologically indistinguishable from those of *L. pneumophila*; the colonies were strongly orange with carotenoid pigments; and the formation of microcysts was inhibited. Attempts to induce the Legionnaires disease bacterium to form pigments with and without light were unsuccessful. Although *L. pneumophila* and the species of *Flexibacterium* showed strong morphological and physiological similarities, they were not considered to be of the same genus.

Semisynthetic and chemically defined media had osmolalities of 125 to 200 mosmol. This was low compared to the 280- to 320-mosmol range of other bacterial media. Five media were prepared in which the osmolality was adjusted with NaCl to give a range of 150 to 777 mosmol. Up to 384 mosmol, no differences in growth were observed, but higher osmolalities definitely inhibited growth.

FIG. 9. Morphological aspects of *L. pneumophila*. (A) Three-day-old cells grown in 0.8% casein hydrolysate semisynthetic broth with shaking in air ($\times 1,350$). (B) Four-day-old cells grown in 0.8% casein hydrolysate semisynthetic broth with shaking in air + CO_2 ($\times 1,350$). (C) Four-day-old cells grown in 0.4% casein hydrolysate semisynthetic broth with shaking in air + CO_2 ($\times 1,350$). (D) Cells grown for 7 days at 25°C on semisynthetic agar slants (0.8% casein hydrolysate); incubated in air ($\times 1,350$).

DISCUSSION

Our primary aim was to develop semisynthetic and chemically defined media which would support the maximum growth of *L. pneumophila* on agar and in broth and permit a quantitative

determination of growth responses to added factors. In this, we have succeeded. In addition, we have obtained certain physiological data which verify the results of Weaver (19) and Feeley et al. (8). Production of antigen was of primary concern during these studies, but production

appeared to be constant, showing no change during growth in the various media. Although most of the work was restricted to one strain, results of several experiments in which 10 to 13 strains were used suggest that the media are adequate for basic studies with all strains. With the CDLp medium, definite strain differences were observed not only in rates of growth and maintenance of viability but also in pigment production. Factors influencing the organism's physiology at all stages of growth can be more easily evaluated with a chemically defined liquid medium than with gross organic media.

The data offer some understanding of the

physiology of the organism. In the media described, *L. pneumophila* requires pyruvate or α -ketoglutaric acid and carbon dioxide for maximal growth under diverse conditions; it requires the amino acids cysteine and methionine for growth. Other amino acids appear to serve as sources of energy for growth; these are serine, threonine, histidine, tryptophane, and tyrosine. The organism uses neither glucose nor polysaccharides for growth, nor does it use organic acids as sources of overt energy. Its growth is adversely affected by total deletion of the vitamins that are present; similarly, its growth is totally inhibited by chelating compounds, such as cit-

FIG. 10. Morphological aspects of *L. pneumophila*. (A) Colonies grown for 48 h aerobically on the semisynthetic agar medium. Transmitted light. Magnification, $\times 50$. (B) As (A), $\times 150$. (C) and (D) Terminal microcyst-like bodies of cells grown on the SSLp medium in shake culture, with air + CO_2 . Magnification, $\times 1,500$.

rate, malate, and ethylenediaminetetraacetic acid. Although it does not appear to use starch for growth, its growth is totally inhibited by 1 μ g of oleic acid per ml, yet excellent growth is obtained in the presence of 10 μ g of oleic acid per ml with starch. Therefore, in some cases starch may be recommended for use as a protection against contamination due to fatty acids in cotton plugs or agar or released into the medium by experimental products or by cell lysis. Tween 80 purified by ether extraction did not substitute for starch at the concentration tested (0.05%), since its use totally inhibited growth.

The characteristics of *L. pneumophila*, as described above, and the knowledge of its guanine-cytosine content of 39% (2) strongly suggested that it was taxonomically related to the bacteria showing creeping motility as described by Dworkin (6) and Lewin (11). That *L. pneumophila* was not a *Flexibacterium* was suggested here by the absence of carotenoid pigments, by its ability to grow at 37°C, and by its failure to produce microcysts. *Flexibacterium* was orange, did not grow at 37°C on the SSLp medium, and did produce microcysts. Recently, Brenner et al. (2) showed no DNA homology between members of *Flexibacterium* or *Flavobacterium* and *L. pneumophila*.

The physiological data indicate that certain strains have definite requirements, constitutive or induced, for growth in the SSLp or CDLp medium. Requirements for amino acids in regard to growth and sources of energy have not been determined. Similarly, the role of pyruvate or α -ketoglutarate and carbon dioxide as substrates or in the metabolism of amino acids has not been ascertained. That vitamins may be required for growth is suggested by the longer lag periods observed when they were deleted in toto. That trace elements are required was implied by the strong inhibition consistently observed with 0.1% citrate, 0.1% malate, or 0.06% ethylenediaminetetraacetic acid. Determination of these requirements may have taxonomical implications when correlated with serotype and may form a basis for the development of selective media.

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