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Amino Acid Requirements of *Legionella pneumophila*

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The amino acids required for growth and as energy sources by 10 strains of *Legionella pneumophila* were determined by using a chemically defined medium. All strains required arginine, cysteine, isoleucine, leucine, threonine, valine, methionine, and phenylalanine or tyrosine. Most strains (7 of 10) required serine, and two strains had to be supplied proline before growth could be established. All 10 strains used serine and, to a lesser extent, threonine as the sole sources of carbon and energy. The Y serine calculated was 94.9 ± 8.5 g (dry weight) of cells/mol of serine. Assuming that the value of Y adenosine 5'-triphosphate is 10.5, these results indicate that oxidative catabolism of 1 mol of serine yielded approximately 9 mol of adenosine 5'-triphosphate. This high yield suggests that although serine was the major source of carbon, other amino acids may also be metabolized.

The first bacteriological medium used to grow *Legionella pneumophila* was Mueller-Hinton agar supplemented with 1% IsoVitaleX and 1% hemoglobin (6). This medium supported growth of the organism but, because of its complexity, furnished limited information on the metabolic requirements of the bacterium. While formulating this medium, investigators found that cysteine was an absolute requirement for growth and that soluble ferric pyrophosphate was stimulatory. The functions of these factors in this complex medium were not defined.

During the development of a chemically defined medium for the growth of *L. pneumophila* (10), investigators found that cysteine was a required amino acid for growth and that amino acids, in general, played a major role in the growth metabolism of *L. pneumophila*. No organic substrate tested other than amino acids supported growth, and serine served as the primary substrate for energy production. In the current investigation, we examined 10 strains of *L. pneumophila* to define more clearly the role of amino acids as sources of energy and as specific requirements for growth. The primary purpose was to further describe these organisms for taxonomical purposes and to determine whether or not the four serotypes represented by these strains could be further delineated by their amino acid requirements.

MATERIALS AND METHODS

Strains and inoculum. Amino acid requirements were determined for 10 strains of *L. pneumophila* which included the four recognized serogroups. These strains were Philadelphia 1 and 2, Bellingham, Flint 1, Miami Beach, Detroit 1, and Olda from serogroup 1; Togus, serogroup 2; Bloomington 2, serogroup 3; and Los Angeles 1, serogroup 4. All strains were obtained from W. B. Cherry, Bacteriology Division, Center for Disease Control. Cultures were maintained by transferring stocks biweekly to fresh charcoal yeast extract (CYE) agar slants (5). Inocula for various experiments were prepared from 48-h cultures grown on CYE agar slants. Cells from these slants were washed from the surface with sterile distilled water, washed once in water, and suspended in 1 ml of distilled water. When 0.1 ml of this inoculum was added to 5 ml of media in an 18- by 150-mm test tube, the absorbance was, as determined by a Beckman model B spectrophotometer, 0.15 to 0.2 at 660 nm. The relationship between absorbance and dry weight was published earlier (10).

Glassware. Glassware was machine washed at a temperature of 180°F (82.2°C) at a pH of 11 to 11.5. There are 8 wash cycles, 9 tap-water rinses and 3 demineralized water rinses. All items were dry sterilized for 2 h at 356°F (162.2°C).

Media. Chemically defined medium (10) containing 0.05% soluble starch (Baker, Potato, soluble for iodometry) was used for all experiments. Cultures were maintained on CYE agar (5).

Cultural conditions. Agar cultures were incubated in a moist candle jar at 37°C. Liquid cultures were grown in tightly sealed, rubber-stoppered 18- by 150-mm tubes. CO_2 was generated within the sealed tubes by using absorbent cotton with 0.3 ml of 10% Na_2CO_3 and 0.3 ml of 1 M KH_2PO_4; rubber stoppers were then inserted (12). The tubes were placed on a rotary shaker at 70 rpm at 37°C.

Amino acid studies. Amino acids required for growth were determined by inoculating with washed inoculum duplicate tubes of defined medium deficient in one amino acid. Duplicate tubes containing all amino acids recommended (10) were inoculated as growth controls. Determinations were made for the 10 strains and 20 amino acids listed in Table 2. Lack of growth after 48 h indicated that the missing amino acid was required for growth of that particular strain.

The use of amino acids as an energy source was measured by growing 10 strains of *L. pneumophila* in chemically defined media containing the amino acid to be tested at 0.1% concentrations. The concentra-

tions of other amino acids, with the exception of cysteine, were reduced to 0.001%. Cysteine was present at 0.05% concentrations in all media for reasons discussed previously (10). This 0.001% amino acid concentration was sufficient to allow only limited growth of the strains tested, with the increase in absorbance reaching 0.03 to 0.3, depending upon the strain tested. Increased levels of growth above that observed on 0.001% amino acid concentrations were considered to be evidence that the particular strain could use the tested amino acid as an energy source. When the amino acids required for growth and energy were determined, a chemically defined medium containing only those amino acids was prepared and inoculated with 10 strains of *L. pneumophila*, and each strain was transferred five times. Growth on this medium was taken as confirmation that all required amino acids had been determined.

Serological procedures. Purity of cultures was ascertained by Gram stain and by staining with fluorescein isothiocyanate (FITC) conjugates prepared against the four serogroups of *L. pneumophila* (3). These conjugates were obtained from the Biological Products Division, Center for Disease Control. Cells were also observed with medium dark-phase and dark-field optics.

RESULTS

Amino acids used as a source of carbon and energy. The use of a particular amino acid (0.1% concentration) was indicated by an increment of growth over that observed on the same basal medium containing all 20 amino acids, each at 0.001% concentrations. Only two amino acids, serine and threonine, produced levels of growth greater than those of the controls. The increment of growth produced by the 10 strains when 0.1% threonine was substituted for an equal weight of serine ranged from 60.4% for Togus to 21.6% for Bloomington 2 (Table 1).

TABLE 1. *Comparison of growth obtained on 0.1% serine versus that obtained on 0.1% threonine*[a]

Strain	Cell yield (mg/ml [dry wt])			
	Growth control[b]	0.1% Serine	0.1% Threonine	(Threonine cell yield/serine cell yield) × 100
Miami Beach	0.320	1.220	0.580	47.5
Bellingham	0.270	1.130	0.620	54.9
Philadelphia 2	0.100	1.060	0.370	34.9
Togus	0.300	1.010	0.610	60.4
Philadelphia 1	0.200	0.950	0.510	53.7
Detroit 1	0.140	0.940	0.220	23.4
Bloomington 2	0.110	0.880	0.190	21.6
Flint 1	0.120	0.640	0.210	32.8
Olda	0.090	0.335	0.160	47.8
Los Angeles 1	0.030	0.330	0.190	57.6

[a] Comparison of 10 strains of *L. pneumophila*.

[b] Growth obtained on a mixture of 20 amino acids (see Table 2), each at 0.001% concentration, after 48 h at 37°C.

Maximum cell yields were obtained by adding 0.1% or greater amounts of serine (Fig. 1).

Amino acids required for growth. The amino acid requirements of 10 strains, which include the four serogroups of *L. pneumophila*, are summarized in Table 2. The requirement for arginine, cysteine, isoleucine, leucine, threonine, valine, methionine, and phenylalanine or tyrosine was absolute in that no growth occurred if any one of these amino acids was deleted from the defined medium. Tyrosine and phenylalanine could substitute for each other.

Serine was an absolute requirement for 7 of 10 strains when grown on a defined medium deficient in serine but containing 0.1% threonine as a source of carbon and energy. Togus, Bellingham, and Miami Beach strains did not have an absolute requirement for serine but produced cell yields that were only 83, 75, and 57%, respectively, of those obtained when 0.01% serine was present in the same medium. Subsequent transfers on serine-deficient media did not alter the cell yield for these strains. Proline was an absolute requirement of two strains—Flint 1 and Olda. Omitting this amino acid did not affect the growth of the other eight strains.

Methionine was found to be an absolute growth requirement for all 10 strains tested. However, both Philadelphia 1 and Olda, each in one of two duplicate tubes, grew in the absence of methionine after a 72-h lag period. The Philadelphia 1 culture did not grow when transferred to fresh medium, but the Olda culture, when

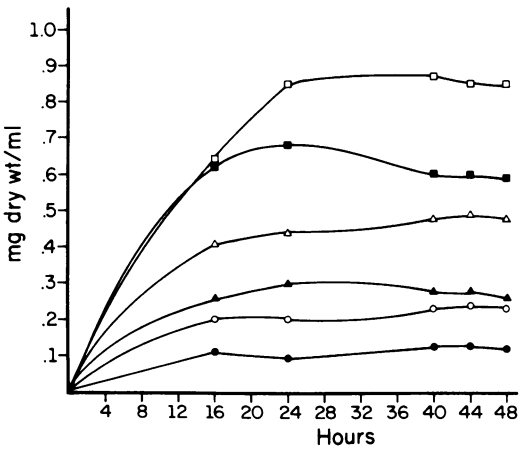

FIG. 1. *Effect of increasing amounts of serine on the growth rate and cell yield of L. pneumophila. The basal medium was the chemically defined medium; CO_2 seals were used, and tubes were incubated at 37°C and shaken at 70 rpm. This figure represents the growth rate of the Philadelphia 1 strain under the conditions listed. Symbols: □, 0.1%; ■, 0.05%; △, 0.025%; ▲, 0.01%; ○, 0.005%; ●, 0.001%.*

TABLE 2. *Amino acids required for growth by 10 strains of L. pneumophila*

Amino acid	Growth of strain[a]									
	Philadelphia 1	Philadelphia 2	Togus	Bloomington 2	Bellingham	Flint 1	Detroit 1	Miami Beach	Olda	Los Angeles 1
Arginine	+	+	+	+	+	+	+	+	+	+
Cysteine	+	+	+	+	+	+	+	+	+	+
Isoleucine	+	+	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+	+	+
Threonine	+	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+	+	+
Methionine	+	+[b]	+	+	+	+	+	+	+	+
Phenylalanine[c] / Tyrosine	+	+	+	+	+	+	+	+	+	+
Serine	+	+	±	+	±	+	+	±	+	+
Proline	−	−	−	−	−	+	−	−	+	−
Alanine	−	−	−	−	−	−	−	−	−	−
Aspartic acid	−	−	−	−	−	−	−	−	−	−
Glutamic acid	−	−	−	−	−	−	−	−	−	−
Glutamine	−	−	−	−	−	−	−	−	−	−
Glycine	−	−	−	−	−	−	−	−	−	−
Histidine	−	−	−	−	−	−	−	−	−	−
Lysine	−	−	−	−	−	−	−	−	−	−
Tryptophan	−	−	−	−	−	−	−	−	−	−
Asparagine	−	−	−	−	−	−	−	−	−	−

[a] +, No growth occurred in the absence of the amino acid tested; −, growth occurred equal to growth control observed in the absence of the amino acid tested; ±, growth occurred in the absence of the amino acid but at a lower level than control growth.
[b] Growth occurred in one of two tubes after 72 h; subculture to methionine-deficient media gave rapid growth.
[c] Either phenylalanine or tyrosine must be present for growth. Each may substitute for the other.

transferred to fresh methionine-deficient medium, grew as well as it did on medium containing methionine. The Met^+ strain of Olda (Olda Met^+) produced light-orange colonies when grown on CYE agar that were not present in the parent strain or any other strain of *L. pneumophila*. Examination of this strain by Gram stain, dark-field microscopy, and examination of its cultural and colony characteristics gave no indication of contamination. Immediately after it was isolated, this culture stained 4+, as expected with anti-group 1 *L. pneumophila* FITC-labeled rabbit globulin. Upon subsequent transfers on CYE agar the culture no longer stained with serotype-specific or polyvalent (serogroups 1, 2, 3, 4) FITC-labeled rabbit globulin. These results were interpreted to mean that a Met^+ mutant of these two strains had been selected and that, upon subsequent transfer, the Olda Met^+ strain had lost its type-specific antigen.

Growth of *L. pneumophila* on a minimal chemically defined medium. The results of the above experiments described a minimal amino acid requirement for several strains of *L. pneumophila*. The following experiments were done to determine (i) whether or not a minimal amino acid medium would support growth in sequential transfer and (ii) whether or not there were any differences in maintenance among the various serotypes. A medium was prepared containing only the required amino acids and inoculated with the 10 strains of *L. pneumophila*. Serine (0.1%) was added as a source of carbon and energy. All other amino acids were at 0.01% concentrations. The first passage on this medium gave growth levels which were generally 80 to 90% of those obtained on the complete defined medium containing all 20 amino acids (Table 3). On subsequent transfers of the strains on the same medium, the level of growth of 7 of 10 isolates was essentially equal on either medium. However, the results clearly showed that the minimal amino acid medium did not adequately support growth of all strains of serotype 1, with Detroit 1, Miami Beach, and Olda showing definite reduction of growth by passage 3. Growth of Detroit 1 and Olda could not be initiated after passages 2 and 3, respectively. Serotypes 2 (Togus) and 3 (Bloomington 2) showed consistent growth in the three transfers, but serotype 4 (Los Angeles 1) did not grow after passage 1. The poor growth observed for these strains may be due to a partial requirement for one of the omitted amino acids. An attempt was made to enhance growth by adding one or more additional amino acids. One amino acid was added to the minimal medium, then the same amino acid and one other were added, then the same two amino acids and a third were added, and so on, until a medium containing all 20 amino acids was prepared. An increment of growth was observed upon the addition of each amino acid (Fig. 2), indicating that each amino acid made a contribution to achieving maximum

TABLE 3. *Growth of L. pneumophila on a minimal chemically defined medium*[a]

Strain	ΔAbsorbance[b] after:					
	Passage 1		Passage 2		Passage 3	
	Complete	Minimal	Complete	Minimal	Complete	Minimal
Philadelphia 1	0.660	0.550	1.230	1.125	1.215	0.950
Philadelphia 2	1.085	0.785	0.985	0.770	1.010	0.905
Togus	1.040	0.690	0.975	1.000	0.940	0.855
Bloomington 2	0.570	1.295	0.550	0.540	0.755	0.715
Bellingham	1.115	0.890	1.200	1.066	1.230	1.190
Flint 1	0.935	0.795	1.095	1.100	1.160	0.930
Detroit 1	0.850	0.620	0.680	0.574	0.695	DNG[c]
Miami Beach	1.090	0.835	1.130	1.040	0.590	0.620
Olda	1.005	0.710	0.725	0.565	0.535	0.110
Los Angeles 1	0.670	0.640	DNG	DNG		

[a] Minimal in respect to amino acids necessary for growth.
[b] Absorbance measured at 660 nm.
[c] DNG, Did not grow.

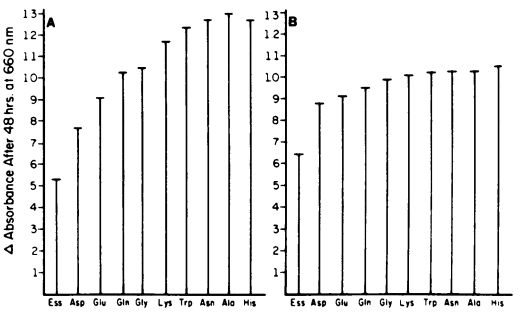

FIG. 2. *Growth response of passage 1 of Detroit 1 (A) and Los Angeles 1 (B) strains to other amino acids added in an cumulative manner. A chemically defined basal medium which contained only the required amino acids was used. Conditions were as described in the legend of Fig. 1.*

growth. The minimal medium and one amino acid gave the same increment of growth, regardless of which amino acid was added. The addition of a quantity of one amino acid (lysine) equal to the combined concentration of all nine additional amino acids did not give maximum growth. Thus, it would appear that the increased growth observed upon adding each nonessential amino acid was not due to an increased buffering capacity of the medium. It is obvious that the chemically defined medium is not adequate for strain Los Angeles 1; how the combined addition of those nonessential amino acids stimulated growth of Detroit 1 or Los Angeles 1 is not known.

DISCUSSION

The use of amino acids as the major source of energy and carbon appears to be a relatively uncommon type of metabolism among bacteria. Mutants of *Escherichia coli* K-12 constitutive for D-serine deaminase synthesis, which use D-serine as the sole nitrogen and carbon source, have been isolated (2). A wild strain of *Serratia marcescens* used threonine as its sole carbon and nitrogen source when grown on a minimal medium; it did so through a high threonine dehydrogenase activity (7). *Peptococcus* species can use protein decomposition products (peptones, amino acids) as their sole energy source (11). All of these bacteria can grow on these amino acid substrates, but on minimal media they also have the metabolic processes to use other carbohydrates and nitrogenous compounds as sources of nitrogen, carbon, and energy. *L. pneumophila* has not been found to use any substrate other than serine and threonine for satisfying its energy requirements. The extent to which these amino acids are used as carbon sources as opposed to energy sources cannot be ascertained without tracer experiments to determine the products formed.

Although it was not the primary purpose of these experiments, the data relating growth response to the serine concentration were examined in regard to molar growth yields per mole of serine (8, 9). Difficulties of such determinations under aerobic conditions are well recognized, and the considerations in regard to nitrogenous substrates are even less secure (8). Nevertheless, such calculations can give some insight into the relationship of the metabolic function of the compound to the growth of the organism. When the value for dry weight (milligrams per milliliter) of cells was plotted versus that for serine (milligrams per milliliter) (Fig. 1), a straight line was not obtained; instead, the positive portion of a parabolic function was observed between 0.001 and 0.05% serine. At con-

centrations about 0.1%, the system was virtually saturated with serine, and no further increments of growth were obtained with increased serine. Obviously, therefore, serine functioned in a manner more complex than that prescribed for a simple energy-growth relationship first defined by Bauchop and Elsden (1), and the tenet of a straight-line relationship required for such calculations was not fulfilled. Part of the nonlinearity may be because the serine is also required for growth and other amino acids (e.g., threonine) are also sources of energy. Nevertheless, molar growth yields were calculated by using the essentially linear portion of the plot obtained when 0.1-, 0.25-, and 0.5-mg/ml amounts of serine were added. The yield (Y) of serine calculated was 94.9 ± 8.5 g (dry weight) of cells/mol of serine. Assuming that the adenosine 5'-triphosphate yield (Y_{ATP}) = 10.5 (8), these results indicate that oxidative catabolism of 1 mol of serine yielded approximately 9 mol of ATP. Similar calculations using the Yav e- (9) and assuming that the serine was equivalent to glyceric or pyruvic acid (av e- = 10) showed that the Yav e- calculated was 9.5 g (dry weight) of cells/av e-. The calculated values for ATP yields and Yav e- were approximately two to three times higher than those reported for other organisms, the latter being 3 to 4 mol of ATP and 3.17 g/av e-, respectively (9). High yields of 9 mol of ATP/mol of glucose have been reported under aerobic conditions of continuous culture with *E. coli* (4); this value is approximately half the yield calculated for *L. pneumophila*, assuming that two pyruvates were generated per mole of glucose. Although the function of serine is complex metabolically, the cell yields observed still indicate that serine was used as a primary source of energy, generating high levels of ATP. This occurs, presumably, through pyruvate, as judged by the release of ammonia as described previously (10). However, the high cell yields observed probably reflect the use of other amino acids as well. A number of amino acids were found to be absolute growth requirements (see Table 2), but there was no homogeneity in the amino acid requirements of all strains tested. Further, the required amino acids alone did not give levels of growth equivalent to those of media containing all 20 amino acids for the Los Angeles 1, Olda, or Detroit 1 strain. The addition of nonessential amino acids to the minimal medium gave an increment of growth in response to each amino acid (Fig. 2). This result was interpreted to mean that, even though it could synthesize these amino acids, the bacterium did not have to do so when these amino acids were added to the medium, and therefore the energy that would have been expended for the synthesis of these compounds would now be available for other uses. Another interpretation is that these amino acids are also used as a minor source of carbon or energy or both.

These results clearly show that these 10 strains form a tight and novel taxonomical group as defined by their amino acid requirements for growth and energy. The lack of homogeneity in the amino acids required by the strains tested still prevents the formulation of a general minimal amino acid medium, since further variations would no doubt occur as new strains are examined. In addition, the minimal amino acid medium does not maintain adequate growth for Los Angeles 1, Detroit 2, and Olda in continuous transfer. Although the amino acid requirements for growth do not contribute to the delineation of one serotype from another, they do appear to define the genus *Legionella* in a characteristic manner and they might characterize strains which cannot be readily identified by other means. Thus, the Olda Met^+ culture, which lost its surface antigen specifically identifying it by the direct fluorescent-antibody procedure, nevertheless retained both its ability to grow in the absence of carbohydrate and its unusual amino acid requirements.

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