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Intermediary Metabolism in *Legionella pneumophila*: Utilization of Amino Acids and Other Compounds as Energy Sources

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The utilization of amino acids and other compounds as carbon and energy sources by *Legionella pneumophila* was examined. Based on the stimulation of oxygen consumption in washed-cell suspensions, glutamate, serine, threonine, and tyrosine were the only amino acids which were utilized as energy sources. Other stimulators of oxygen uptake were lactate, pyruvate, acetate, fumarate, and succinate. Citrate was a good stimulator only when the bacteria were grown in the presence of the substrate. Radiolabeling studies showed that [^{14}C]glutamate was rapidly metabolized, with the label distributed evenly in all cell fractions. [^{14}C]pyruvate and [^{14}C]acetate were incorporated into the lipid-containing cell fraction, whereas glucose and glycerol were found in both the lipid- and polysaccharide-containing cell fractions. Radiorespirometry of differentially labeled [^{14}C]glucose indicated that this compound was metabolized primarily by the pentose phosphate and Entner-Doudoroff pathways rather than by the glycolytic pathway.

The nutritional requirements of *Legionella pneumophila* have been the subject of numerous investigations (2, 4, 9–12), including some in our laboratory (14, 17). The independent development of three chemically defined media for growing this organism (9, 11, 17) conclusively showed that amino acids were the primary nutrients required as carbon and energy sources. There have been a few reports on slight stimulation of growth by compounds such as α-ketoglutarate (1, 9) and rhamnose plus choline (11); however, most carbohydrates, organic acids, or other metabolic intermediates generally have little effect on the growth of *L. pneumophila* in complex or defined medium (4, 11, 17). In fact, citrate and a few other compounds can inhibit growth under certain conditions (4, 11, 17).

Working with the results of these early studies, several investigators attempted to define more precisely the primary carbon and energy sources used by this organism. George et al. (4) studied the role of individual amino acids in stimulating cell yield and concluded that serine and threonine were the major energy sources during growth of *L. pneumophila* cells in their defined medium. Weiss et al. (19) determined the release of $^{14}CO_2$ when washed cells were incubated in the presence of radiolabeled substrates. The results of these radiorespirometric experiments indicated that glutamate (and, to a lesser extent, glutamine) was metabolized most actively and appeared to be a major energy source. Glucose, acetate, and succinate were utilized slowly, suggesting a more biosynthetic role for these compounds. Serine and threonine were not examined in this study.

The utilization of glucose by *L. pneumophila*, as noted by Weiss et al. (19) was not reported previously, but it is consistent with the negative glucose fermentations reported by others (18) since metabolism would proceed by oxidative pathways. Weiss et al. (19) also performed a limited respirometric investigation into the pathways of glucose metabolism and concluded that the Entner-Doudoroff or pentose phosphate pathway or both, rather than the glycolytic pathway, played the major role(s) in the metabolism of glucose.

The purpose of the present investigation was to reexamine the utilization of serine, threonine, glutamate, glucose, and other potential energy sources by *L. pneumophila* based on their stimulation of oxygen uptake and their incorporation into various cellular fractions.

MATERIALS AND METHODS

Bacteria. *L. pneumophila* Knoxville-1 (serogroup 1) was obtained from R. Weaver, Centers for Disease Control, Atlanta, Ga. Cultures were maintained by continuous passage on GC-FC agar (17) plates and incubated at 37°C in an atmosphere of 5% CO_2.

Media and growth conditions. The chemically defined liquid medium used in these studies was that described by Warren and Miller (17). This medium contains 18 amino acids and inorganic salts and has a final pH of 6.9. The complex liquid medium was the GC-FC medium (17), containing the following (per liter): proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.) 15 g; K_2HPO_4, 4 g; and KH_2PO_4, 1 g. After being autoclaved, the medium was supplemented with L-cysteine (0.4 g/liter) and soluble ferric pyrophosphate (0.25 g/liter). The final pH of the medium was 6.9.

Unless otherwise stated, cultures were grown in 1-liter Erlenmeyer flasks containing 300 ml of liquid medium. Inocula were prepared from cultures grown on GC-FC agar plates. Cells were washed off the plate with sterile distilled water, washed twice, and inoculated to give an initial turbidity of 35 Klett units with a Klett-Summerson colorimeter (660 filter). The flasks were incubated at 37°C on a New Brunswick gyratory shaker at 200 rpm. Growth was monitored turbidimetrically in Klett units.

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

[*U*-^{14}C]glucose (specific activity, 138 mCi/mmol), [1-^{14}C]glucose (specific activity, 80 mCi/mmol), [2-^{14}C]glucose (specific activity, 4 mCi/mmol), [3,4-^{14}C]glucose (specific activity, 13.7 mCi/mmol), [6-^{14}C]glucose (specific activity, 50 mCi/mmol), L-[*U*-^{14}C]glutamic acid (specific activity, 200 mCi/mmol), and sodium [2-^{14}C]pyruvate (specific activity, 20 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. L-[*U*-^{14}C]leucine (specific activity, 300 mCi/mmol), [*U*-^{14}C]glycerol (specific activity, 16 mCi/mmol), and sodium [1,2-^{14}C]acetate (specific activity, 55.5 mCi/mmol) were obtained from International Chemical and Nuclear Corporation, Irvine, Calif.

Oxygen uptake. Oxygen utilization was measured with a biological oxygen monitor (model 53; Yellow Springs Instruments, Yellow Springs, Ohio). Cells grown for 48 to 72 h on GC-FC agar plates were removed and inoculated into either GC-FC complex liquid medium or into defined medium. Cells were harvested by centrifugation after 15 to 18 h of incubation, washed twice in sterile water, and suspended in 0.85% (wt/vol) saline. Cell suspensions (approx. 1 mg [dry weight] of cells per ml) were held at 37°C until used.

A 3-ml amount of the cell suspension was placed in the oxygen electrode chamber and oxygenated for 3 min. The test substrate (0.1 ml) was added after a steady rate of endogenous respiration was established. The concentrations used were based on those concentrations that gave optimal results. All substrates were adjusted to a pH of 7.0 with 1 N NaOH before use. Increases or decreases in the rate of endogenous respiration were determined and corrected for the dry weight of the sample used. Values are expressed as either a percentage of the endogenous rate or as microliters of O_2 consumed per minute per milligram (dry weight) of cells.

Oxygen uptake studies of actively growing cultures were performed to determine the optimal sampling times. Samples (3 ml) were removed from the culture flasks, which contained either complex or defined medium, and placed directly into the oxygen electrode chambers. The rate of oxygen uptake was then measured. No additional substrates were added in these studies.

Radiorespirometry. Glucose metabolism pathways were determined by the radiorespirometric procedures of Wang (16) as modified by Morse et al. (7). Cultures were harvested during the exponential phase of growth, centrifuged at 27,000 × *g* for 10 min, and suspended in GC-FC medium without glucose to a final concentration of 0.8 to 0.9 mg (dry weight) per ml. The cell suspension (25 ml) was added to each radiorespirometer vessel. These previously described vessels (8) consisted of 125-ml Erlenmeyer flasks with side arms. Cold carrier glucose (69.4 μmol) and the specifically labeled [^{14}C]glucose were added to the side arms. At zero time the material in the side arms was tipped in and $^{14}CO_2$ collection began, with samples collected every 60 min for 6 h.

Cell fractionations. Cultures were grown in 20 ml of defined medium containing 10 μCi of radioactive substrate plus 0.5% (wt/vol) unlabeled substrate. Cells were harvested after 12 to 15 h of incubation and combined with 2 liters of cells grown in the same medium without radiolabel. Cells were washed twice by centrifugation (10,000 × *g* for 15 min), and the pellets were analyzed by a standard sequential extraction procedure as described by Sutherland and Wilkinson (13).

Triplicate samples (0.1 ml) taken from each supernatant and residue were placed in 15 ml of Tritosol scintillation fluid (3) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Miscellaneous measurements. Protein was determined by the method of Lowry et al. (6) with bovine serum albumin as the standard. Dry weights were determined by centrifuging samples at 15,000 × *g* for 15 min, suspending the cells in distilled water, and drying them to constant weight in tared aluminum dishes in an oven at 80°C.

RESULTS

Oxygen uptake during growth of *L. pneumophila*. Previous studies showed that the viability of *L. pneumophila* cultures can decrease significantly as they enter the stationary phase (11, 17). For the present study we required cells that had optimum metabolic activity. Thus, we removed *L. pneumophila* cells at different times during the growth cycle in both complex and defined media and used the rate of oxygen utilization as a measure of metabolic activity. As shown in Fig. 1, the rate of oxygen utilization (microliters of O_2 per minute) paralleled the culture turbidity, reaching a maximum at the same time as the culture entered the stationary phase of growth. However, when the data were corrected for the dry weight of the cells, it became obvious that the maximum rate of oxygen utilization per bacterium occurred at 15 to 18 h, corresponding to the mid- to late-exponential

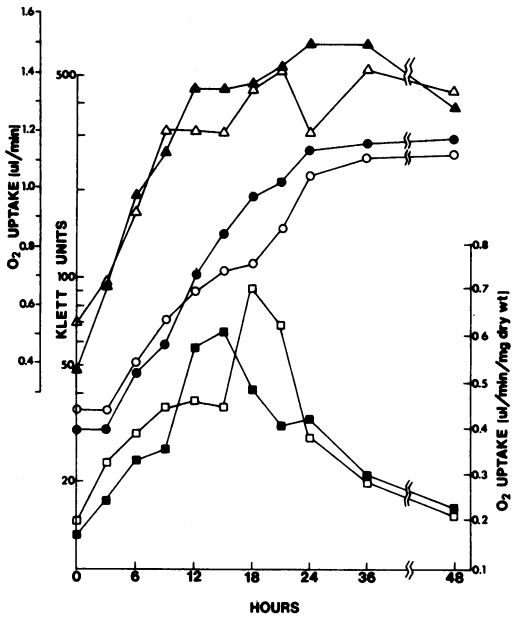

FIG. 1. Kinetics of growth and oxygen utilization by actively growing cultures of *L. pneumophila* in complex and defined media. Symbols: open symbols, defined medium; closed symbols, complex medium; ●, ○, turbidity (Klett units); ▲, △, total oxygen consumed per minute; ■, □, total oxygen consumed per minute per milligram (dry weight) of cells.

phase of growth. After this time, the rate of oxygen uptake per milligram of dry weight decreased, owing perhaps to the accumulation of dead or metabolically less active cells. Because of these results, *L. pneumophila* cells to be used for the oxygen uptake experiments described below were routinely harvested at 15 to 18 h of incubation.

Substrate-mediated stimulation of oxygen uptake with washed cells. An evaluation of various compounds as potential energy sources was based on their ability to stimulate oxygen consumption by a suspension of washed cells of *L. pneumophila*. In all these experiments, washed cells had an endogenous rate of oxygen utilization (0.2 to 0.4 μl of O_2 per min per mg [dry weight] of cells), owing to the metabolism of intracellular pools. The addition of an oxidizable substrate to this system would result in an increase in the rate of oxygen consumption.

The amino acids serine and glutamate were the best stimulators of oxygen uptake, regardless of whether the cells were grown in defined or complex medium (Fig. 2). Threonine was a good stimulator of oxygen uptake in cells grown in defined but not in complex medium. Tyrosine, on the other hand, stimulated only cells grown in complex medium. None of the other common amino acids stimulated either of the cell populations (data not shown).

Pyruvate, acetate, fumarate, succinate, and lactate all stimulated oxygen uptake, although this varied according to the medium in which the cells were initially grown. Although pyruvate was oxidized by cells grown in either complex or defined medium, the utilization of the other compounds was growth medium dependent. Acetate and fumarate only stimulated cells grown in defined medium, and succinate and lactate only stimulated cells grown in complex medium. Glucose, glycerol, α-ketoglutarate, and citrate did not stimulate cells grown in either of the media.

One possible explanation for the lack of stimulation may have been the lack of induction of enzymes involved in the transport or metabolism of the particular compound. In most cases, however, growth in the presence of the substrate did not alter the response of the cells to that compound. The exceptions were citrate and succinate, which stimulated oxygen uptake by cells grown in either defined or complex medium to which substrate was added (data not shown).

Incorporation of radiolabeled substrates during growth. Other measures of the utilization of a particular ^{14}C-labeled compound are the incorporation of radioactivity into various cellular fractions and the release of $^{14}CO_2$. We first determined the incorporation of various ^{14}C-labeled compounds into cellular components

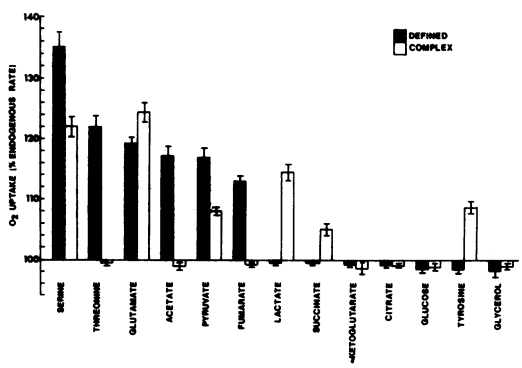

FIG. 2. Graphic comparison of the effects of substrate addition on oxygen uptake by washed cells of *L. pneumophila* Knoxville-1 grown in defined or complex medium. The rate of endogenous oxygen uptake was generally 0.2 to 0.4 μl of O_2 per min per mg (dry weight) of cells. The final concentration of each substrate (based on the concentration that gave the best stimulation) was: serine, 6 mM; threonine, 5 mM; glutamate, 5 mM; acetate, 40 mM; pyruvate, 60 mM; fumarate, 20 mM; lactate, 35 mM; succinate, 20 mM; α-ketoglutarate, 4 mM; citrate, 13 mM; glucose, 18 mM; tyrosine, 0.4 mM; and glycerol, 36 mM. The data represent the mean ± standard error for 10 trials.

TABLE 1. Incorporation of labeled substrates during growth of *L. pneumophila* in defined medium[a]

Substrate	Radioactivity incorporated (%)	Radioactivity in soluble fractions (% of total recovered)					Recovery (%)
		Cold TCA[b] soluble	Methanol/ chloroform soluble	Hot TCA soluble	Phenol soluble	Residue	
[*U*-^{14}C]glutamate	4.5	24	29	16	21	9	99
[2-^{14}C]pyruvate	1.9	9	87	4	3	11	114
[*U*-^{14}C]glucose	0.6	13	51	7	10	13	94
[*U*-^{14}C]glycerol	0.8	16	39	9.5	9.5	27	101
[1,2-^{14}C]acetate	2.95	9	72	5	4	9	99
[*U*-^{14}C]leucine	6.0	5	30	4	59	2	100

[a] All cultures were grown in the presence of the label plus 0.5% unlabeled substrate for 12 to 15 h.
[b] TCA, Trichloroacetic acid.

during the growth of *L. pneumophila* cells in defined medium. The carbon from radiolabeled glutamate was distributed throughout the cell, indicating its use in a variety of biosynthetic reactions (Table 1). Of the total radioactivity recovered, 29% was incorporated into lipids, 21% into proteins, 16% into nucleic acids, and 9% into miscellaneous polysaccharides (i.e., residue), with 24% remaining in low-molecular-weight compounds. Carbon from pyruvate and acetate was incorporated primarily into the lipid-containing cell fraction (87% of total pyruvate and 72% of total acetate incorporated). This is consistent with their use in fatty acid biosynthesis via acetyl coenzyme A. Radiolabeled glucose and glycerol were also incorporated into cellular components, with 51% of the glucose and 39% of the glycerol incorporated present in the lipid-containing cell fraction, whereas 13 and 27%, respectively, were incorporated into the residue fraction. Leucine was primarily incorporated into the protein-containing cell fraction (59%), consistent with its role being primarily in protein synthesis, although 30% was also detected in the lipid-containing cell fraction. Adenine and uridine (data not shown) were incorporated primarily into nucleic acid fractions as expected.

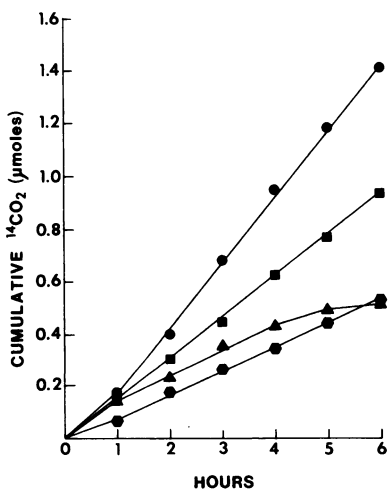

FIG. 3. Radiorespirometric patterns for the utilization of glucose during growth of *L. pneumophila* Knoxville-1. Flasks contained 20 mg (dry weight) of cells and 69.4 μmol of glucose. Symbols: ●, [1-^{14}C]glucose; ■, [2-^{14}C]glucose; ◆, [3,4-^{14}C]glucose; ▲, [6-^{14}C]glucose.

Pathways of glucose metabolism. The pathways of glucose catabolism were examined by radiorespirometry. Figure 3 shows the kinetics of $^{14}CO_2$ released from specifically labeled glucose by growing cells of *L. pneumophila*. The differential rates of $^{14}CO_2$ evolution were C1 > C2 > C6 > C3,4. The rate of $^{14}CO_2$ release was slow, indicating that glucose was not rapidly metabolized. The inventory of ^{14}C in these experiments is shown in Table 2, and the recoveries were within acceptable limits. The lack of equivalence in the amount of CO_2 released from the C-1 and C-6 atoms of glucose suggests that catabolism of glucose did not occur via the glycolytic pathway, but rather by the pentose phosphate and Entner-Doudoroff pathways. This conclusion is supported by the data (Table 2) comparing the amount of glucose incorporated and the amount released as CO_2. It is evident that the C-1 and C-6 atoms of glucose were used differently. Whereas the latter was preferentially incorporated into cellular components, C-1 was used to a greater extent in the formation of CO_2.

DISCUSSION

The chemically defined medium described for growing *L. pneumophila* cells contains primarily amino acids and inorganic salts (9, 11, 17). However, the role of each amino acid in the overall metabolism of *L. pneumophila* has received only limited attention. Based on the stimulation of oxygen consumption, the present study showed that glutamate, serine, threonine,

TABLE 2. Utilization of [^{14}C]glucose by growing cells of *L. pneumophila* Knoxville-1

Substrate	Radioactive inventory[a]			Total ^{14}C recovery (%)	Ratio[b] (C-CO_2)
	CO_2 formed (%)	C incorporated (%)	Medium (%)		
[1-^{14}C]glucose	0.6	2.0	97.4	99.4	3.64
[2-^{14}C]glucose	0.4	2.6	97.0	99.3	6.98
[3,4-^{14}C]glucose	0.2	2.6	97.2	92.7	11.39
[6-^{14}C]glucose	0.2	3.0	96.8	95.4	14.48

[a] Determined at the end of the experiment.
[b] The ratio of C incorporated to CO_2 formed was calculated from the nanomoles of C incorporated and CO_2 formed per milligram (dry weight) per hour. Flasks containing 28.25 mg (dry weight) of cells and 10 mM substrate were incubated at 37°C for 6 h.

and tyrosine play important roles as energy sources for this organism.

Our finding of a key metabolic role for glutamate confirms the earlier findings of Weiss et al. (19), who showed by radiorespirometric procedures that glutamate appeared to be rapidly metabolized as an energy source during growth of *L. pneumophila* cells. Our data also showed that glutamate plays an important biosynthetic role, as evidenced by the distribution of radiolabeled carbon from [^{14}C]glutamate into various cellular fractions. In addition, the central role that glutamate plays was shown in a different manner by Tesh and Miller (14). This amino acid was the only compound which permitted growth of *L. pneumophila* cells when added to a minimal medium containing just the eight essential amino acids.

An interesting contrast to these results was the work of Pine et al. (9) and George et al. (4), who found no evidence that glutamate was used as an energy source in their defined medium. Rather, serine, threonine, and perhaps tyrosine served in this role. Our results confirm the importance of these amino acids in the oxidative metabolism of *L. pneumophila* and provide a possible explanation for the findings of these other investigators. The stimulation of oxygen uptake by threonine and tyrosine depended on the medium used to grow the cells. It is possible that with glutamate as well, the chemical nature of the defined medium and the growth conditions employed were sufficiently different from those used for the present study to repress the enzymes involved in the transport or metabolism of this compound.

Whether any of these amino acids can serve as the sole energy source has not been determined. But since all strains of *L. pneumophila* appear to be naturally auxotrophic for both serine and threonine (9, 11, 17), and considering the important biosynthetic role that glutamate plays, it is reasonable to assume that several amino acids would contribute to overall energy production during growth in any medium.

In addition to amino acids, several other carbon sources were shown to stimulate oxygen uptake, including pyruvate, lactate, acetate, succinate, fumarate, and citrate. Recent results (unpublished data) suggested that oxaloacetate and malate also show some stimulation. All of these compounds are either intermediates in the tricarboxylic acid cycle or are closely linked, as with pyruvate and acetate. It would appear that *L. pneumophila* uses the tricarboxylic acid cycle for energy and that any intermediate of the cycle, or a compound which can be fed rapidly into the cycle, would be a potential energy source. The inability of α-ketoglutarate to stimulate oxygen uptake was probably caused by a defect in the ability of the cells to transport this compound. Although the catabolism of citrate and succinate appears to require inducible enzymes, the inability of other compounds (i.e., lactate, acetate, fumarate) to stimulate oxygen uptake in cells grown in one medium but not another could not be explained by this type of regulation.

The radiolabeling studies (Table 1) showed that the tricarboxylic acid cycle is also important in cellular biosynthesis in *L. pneumophila*. Glutamate was rapidly catabolized, with the carbon skeleton distributed to many other molecules within the cell. Interestingly, pyruvate and acetate, which stimulated oxygen uptake, were primarily incorporated into the lipid-containing cell fraction. This finding is consistent with the work of Weiss et al. (19), who reported that acetate was used preferentially in a biosynthetic role.

Glucose and glycerol are apparently metabolized more slowly, so that they never have a significant effect on oxygen utilization. Their incorporation into lipid and carbohydrate supports the conclusion that they are used primarily for biosynthesis. Nevertheless, it is clear that glucose is metabolized by *L. pneumophila*, and the pathways of glucose metabolism were investigated in the present study. The lack of equivalence in the amount of $^{14}CO_2$ released from C-1 and C-6 suggested that the glycolytic pathway was not involved to any great extent. Although we are aware of the inherent difficulties involved

in estimating concurrent pathways (5), our CO_2 yield data (Fig. 3) and the equations of Wang (16) indicated that approximately 60% of the glucose was metabolized by the pentose phosphate pathway and approximately 40% by the Entner-Doudoroff pathway. These results are in agreement with the findings of Weiss et al. (19). Definitive conclusions about the existence of these pathways must await the results of actual enzyme analysis.

The results of the present study were derived with a single, laboratory-adapted strain of *L. pneumophila*. Although it may be argued that our results may not be representative of all *L. pneumophila* strains, previous findings with this organism indicated that the metabolism of different strains is similar in several respects: (i) overall growth rates in a variety of liquid media (12); (ii) amino acid requirements in defined media (4, 9, 11, 14, 17); (iii) the ability to grow in a minimal defined medium (4, 14); (iv) the lack of *N*-acetylglutamate synthetase in the arginine biosynthetic pathway (Tesh and Miller, submitted for publication); (v) the requirement for magnesium and potassium (15); and (vi) the metabolism of glucose, glutamate, acetate, and succinate (19). In addition, the study by Weiss et al. (19) detected no difference in the metabolism of these compounds between strains of *L. pneumophila* grown in chicken embryo yolk sacs and those strains grown continuously on agar media. Thus, although *L. pneumophila* cells growing in vivo may express a type of metabolism unique to that environment, it is likely that the metabolic potential of all strains of *L. pneumophila* is similar.

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