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Development of the BIOLOG Substrate Utilization System for Identification of *Legionella* spp.

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The genus *Legionella* consists of 51 serogroups comprising 34 species. Biochemical reactions and cell wall fatty acid and quinone analyses may confirm that an isolate is a *Legionella* sp. and indicate to which species it belongs, but DNA hybridization studies have been necessary for a definitive identification. Recently, the commercially available BIOLOG identification system has offered a standardized, easily reproducible system of substrate metabolism by bacteria resuspended in multiwell plates. A tetrazolium dye acts as an electron acceptor during the oxidation of the wide range of substrates and forms an irreversible, highly colored formazan when reduced. The 95 substrate wells are read rapidly with a conventional plate reader, and the results are downloaded for comparison with a computer data base, allowing quick identification. The BIOLOG system's ability to test more diverse classes of substrates, including amino acids, peptides, carboxylic acids, and carbohydrates, was used in this study to establish a new data base and identify the asaccharolytic *Legionella* spp. In particular, *Legionella pneumophila* behaved as a microaerophile, and the fastest, most diverse metabolic activities occurred after the development of a low-oxygen incubation environment. Alternatively, bacteria could be successfully incubated in air when their concentration was double that recommended by the manufacturer. Similar results were obtained by using either Page's amoebal saline or distilled water as the resuspending and incubation medium. Type strains did not cross-identify with any of the strains already in the manufacturer's data base. The results indicate that this modified system has value in being able to identify *Legionella* isolates to the species level.

The outbreak of acute febrile respiratory illness among participants of the American Legion Convention in Philadelphia in 1976 and the subsequent investigation led to the isolation of a new bacterium, the Legionnaires' disease bacterium (8). It was formally classified 2 years later by Brenner et al. (5) as a new species, *Legionella pneumophila*, in the family *Legionellaceae*. The family *Legionellaceae* consists of a single genus, although others (*Tatlockia* and *Fluoribacter*) have been proposed (9). The 34 species that make up the genus *Legionella* have all been defined by DNA hybridization (3). Although the genus is genetically heterogeneous, it was considered by Brenner et al. (4) a good and practical phenotypic genus. Identification of legionellae at the species level is difficult; indeed, some species can only be identified by DNA hybridization studies. However, in practice, when phenotypic characteristics and tests are used in conjunction with serological methods, most species can be correctly identified.

Both serological and the present phenotypic tests have several limitations. Many species and serogroups have common antigens; this problem can be overcome by absorption but this causes its own problems. Firstly, it is labor intensive. Secondly, the description of new species requires reevaluation and possibly additional absorption procedures. Finally, antisera are evaluated against very few representative strains of each serogroup, but antigenic variation may be large. A similar criticism of the phenotypic characteristics presently used can be made, since they have resulted from the study of relatively few strains.

Several phenotypic tests are useful in identifying legionellae to the genus level and, in some cases, to the species level. These are the requirement for L-cysteine and ferric iron, colony morphology, Gram stain characteristics, catalase reaction, flagellar staining, and the absence of nitrate reduction. In addition, others, including hippurate hydrolysis, autofluorescence, pigment production, and gelatinase production, are of use in identifying species of legionellae (10). Unfortunately, legionellae are metabolically inactive with respect to the most commonly used biochemical tests, which largely rely on acid formation from carbohydrates and produce transient color changes.

By contrast, the recently available BIOLOG identification system offers a standardized, easily reproducible system with a computer data base for bacterial identification that incorporates a much wider range of substrates (2). This system employs a redox dye, tetrazolium violet, as an indicator of substrate utilization. The cells' metabolism of the test substrate results in the formation of NADH, which, in order to be reoxidized, passes electrons to an electron transport chain. The tetrazolium dye has a redox potential suitable to accept these electrons and is irreversibly reduced to a purple formazan. The dye functions independently of the specific structure of the electron transport chain and, therefore, has general application. It is thus able to detect the ability to metabolize any substrate, including amino acids which act as carbon and energy sources for the *Legionellaceae* (22). This makes it of potential use in the identification of the so-called fastidious legionellae, which are considered unable to catabolize carbohydrates. It also provides a convenient and reproducible method of screening a large number of strains for their utilization of an extensive panel of substrates. The protocol described by the manufacturer was modified in several ways to facilitate its use with legionellae, and the modified procedure was optimized. In particular, *L. pneumophila* behaved as a microaerophile,

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and the quickest reactions and the most diverse metabolic activities occurred during incubation in a low-oxygen atmosphere. Type strains did not cross-identify with any of the strains already in the manufacturer's data base, indicating that this modified system has value in being able to identify legionellae to the species level.

MATERIALS AND METHODS

Strains and cultivation. The strains used were all laboratory stocks which were maintained on glass beads at −70°C. They were all originally obtained from the United Kingdom National Collection of Type Cultures, Colindale, London, United Kingdom. The cultures used were single strains of *L. pneumophila* serogroups 1 through 14 (excluding 4 and 9) and eight type strains of other *Legionella* spp. Prior to inoculation of the BIOLOG plate, all isolates were grown on buffered charcoal-yeast extract (BCYE) agar (21) at 37°C for 72 h. Since the BIOLOG system requires metabolically active cells, it is considered necessary to use cultures that are as young as possible. It was possible to get sufficient growth on BCYE plates after 24 h when they were inoculated heavily from a primary plate, itself inoculated from the freezer stock. When these young cultures were used to inoculate the BIOLOG plates, it was found that slightly more substrates were utilized. However, it was difficult to obtain sufficient growth for some of the strains, particularly those not belonging to *L. pneumophila*; in some instances, the growth on three BCYE plates was barely adequate to provide a sufficient inoculum, especially when the higher cell concentration (described later) was used. Since it appeared that there was little to gain by this extra step, and to allow the development of a standard protocol (with a common incubation time) for the identification of all *Legionella* spp., BCYE plates were incubated at 37°C for 72 h to provide inocula for the identification of legionellae.

BIOLOG inoculation and incubation. Cell suspensions were made as described in the manufacturer's manual. This requires a cell density in a turbidity range set by using the milk of magnesia standards supplied by BIOLOG at that time, which was equivalent to A_{590} of 0.5 to 0.65, as measured on an SP 500 spectrophotometer (Pye Unicam, Cambridge, United Kingdom). (These turbidity standards are no longer recommended by BIOLOG since they were found to lack consistency. The standards now supplied by BIOLOG have A_{590} of 0.34 to 0.39.) In an effort to use a uniform cell density throughout the experiments, an inoculum in the range of 0.55 to 0.6 was used (approximately 1.3×10^9 to 1.7×10^9 CFU ml^{-1}), except that Page's amoebal saline (PAS) (20) was used in place of 0.85% (wt/vol) NaCl, as this concentration of NaCl has been reported to inhibit growth of several legionella strains (1). Bacterial suspensions used to inoculate the BIOLOG plates were streaked onto BCYE agar minus L-cysteine to test the purity, since legionellae cannot grow without cysteine. The BIOLOG multiwell plates were inoculated as described in the BIOLOG User's Manual (Biolog Inc., Hayward, Calif.). Multiwell plates were incubated at 37°C in either air or a low-oxygen atmosphere, since it is known that legionellae are sensitive to low levels of hydrogen peroxide and superoxide radicals (12, 15). This was achieved by evacuating an anaerobic jar, bleeding in 0.2 bar of air, and filling the remaining volume with an anaerobic gas mixture (95% N_2–5% CO_2).

Metabolic profiles and data base comparison. A purple formazan formed in those wells in which the substrate was metabolized, and the color development in the multiwell plates was read by eye at 24-h intervals up to 72 h. Alternatively, the multiwell plates were read automatically in a Metertech Microplate Reader (Atlas Bioscan, Bognor Regis, United Kingdom) with a filter cutoff of 600 nm. The 95 substrate wells were read against a substrate blank well. The BIOLOG manufacturer recommends for a positive result a minimum reading of 40% of the highest positive substrate response, but experience led to the selection of a minimum reading of 20%. The substrate utilization profile of each strain was recorded and used to construct a specific *Legionella* data base. The files in this data base were compared against, and used to identify, subsequent known strains and replicate experiments of the same previously identified strains. The data were also compared with the currently available BIOLOG data base of environmental and medical bacteria. Additional software which generated spreadsheets allowing easier comparisons of substrate utilization between strains was written in the laboratory.

Purification and analysis of putative polyhydroxybutyrate from *L. pneumophila*. Lyophilized *L. pneumophila* cells were extracted with boiling chloroform, and the extract was purified by being washed with volumes of ice-cold absolute ethanol and diethyl ether. The purified material was investigated by ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and the resultant ^{13}C NMR spectrum was compared with that of an *Alcaligenes* sp. polyhydroxybutyrate (PHB) (Sigma, Poole, United Kingdom). Gas chromatography-mass spectrometry was used to analyze a methanolysate of the material.

RESULTS

Genus metabolism. An important attribute of the BIOLOG system is that recovered bacteria do not need to grow subsequently in the assay; they have only to be metabolically active to reduce the tetrazolium violet. Legionellae, however, have been shown to be sensitive to hydrogen peroxide and superoxide (12, 15), and we have demonstrated previously that a strain of *L. pneumophila* serogroup 1 behaved as a microaerophile when growing in laboratory media (27). This physiological response was found to affect the rate of formazan formation in the BIOLOG system when strains were incubated in air; only β-hydroxybutyrate and methylpyruvate were recorded as giving positive reactions within 24 h for the various *L. pneumophila* serogroups. In all subsequent experiments, these substrates were consistently the most rapidly metabolized, as indicated by the formation of a deeper-color reaction than that produced by any of the other compounds in the panel. After 72 h of incubation, more positive results were observed by either the eye or using the microplate reader.

When the multiwell plates were incubated aerobically, some of the color reactions were not visible after 24 h and became apparent only after longer incubation. For example, incubating *L. pneumophila* serogroup 1 NCTC 11192 at the manufacturer's recommended concentration in the multiwell plates took 72 h in air to give 15 positive reactions. This number is considered by the manufacturer to be rather low for an accurate resolution of species; also 72 h of incubation is considered by the manufacturer to be unacceptably long, as the multiwell plates are not packaged aseptically and, thus, spurious positive results may occur with prolonged incubation. A possible explanation for this low level of metabolic activity is that *Legionella* spp. are sensitive to low levels of hydrogen peroxide and superoxide radicals (12, 14);

also, we have found that *L. pneumophila* appears to be microaerophilic in laboratory media since it grows best in our continuous culture system when the dissolved oxygen tension is strictly controlled to 4% of air saturation (16). Consequently, resuspended legionellae were incubated in the BIOLOG system in a low-oxygen environment by degassing anaerobic jars and regassing with 0.2 bar of air and then with 0.8 bar of 5% (vol/vol) carbon dioxide in nitrogen. This gave a final oxygen concentration of approximately 4% (vol/vol). Multiwell plates incubated in this environment gave definite reactions within only 24 h, which, although weaker, were consistent with results obtained during the 72 h of incubation in air. However, there were now 20 positive and 2 variable reactions. An extra seven substrates gave positive reactions, including Tween 40 (polyoxyethylenesorbitan monopalmitate), Tween 80 (polyoxyethylenesorbitan monooleate), monomethyl succinate, acetate, L-aspartate, glycyl-L-aspartate, and urocanate; two substrates, succinate and γ-aminobutyrate, gave variable reactions in replicate samples. Only L-alanylglycine was no longer metabolized.

Representatives of other *L. pneumophila* serogroups and a range of type strains of other *Legionella* spp. were investigated by this modified BIOLOG procedure. All of the *L. pneumophila* strains investigated were similarly affected by the concentration of oxygen in air and gave the largest number of positive results in a 4% (vol/vol) oxygen environment. The serogroup 7 strain was the least reactive of the *L. pneumophila* strains tested. Nevertheless, even this strain metabolized 15 substrates, which gave sufficient resolution, in this study, to differentiate it to the species level. All of the *L. pneumophila* strains consistently metabolized Tween 40, Tween 80, methylpyruvate, β-hydroxybutyrate, α-ketobutyrate, α-ketovalerate, alaninamide, L-alanine, L-glutamate, glycyl-L-glutamate, L-leucine, L-proline, L-serine, L-threonine, and urocanate (Table 1). The ability to utilize Tween 40 and Tween 80 suggests that *L. pneumophila* possesses esterase activity similar to that reported by Muller (18) and Nolte et al. (19), except that our data suggest that the strains we investigated are able to split esters of higher fatty acids, as indicated by the metabolism of Tween 40 and Tween 80. Interestingly, although urocanate is an intermediate in histidine metabolism, only one of the strains tested, the serogroup 5 strain (NCTC 11405), was able to metabolize histidine within the 24-h incubation period. The strains NCTC 12000 and NCTC 11984, which belong to serogroups 10 and 13, respectively, took 48 h in 4% (vol/vol) oxygen to metabolize histidine.

Similar metabolic profiles were noted for the other *Legionella* spp., except that they generally gave fewer positive reactions than did the *L. pneumophila* strains. The most metabolically versatile isolate was the type strain of *L. longbeachae*, which gave 18 positive reactions (Table 1). By contrast, *L. hackeliae* gave only seven positive reactions. Of the 95 substrates in the BIOLOG panel, 26 different substrates were metabolized by one or more *Legionella* species, with all the strains tested metabolizing methylpyruvate, β-hydroxybutyrate, and L-proline. The majority of the species incubated in 4% (vol/vol) oxygen metabolized the following within 24 h: Tween 40 (except *L. rubrilucens*), α-ketobutyrate (except *L. israelensis*), α-ketovalerate (except *L. hackeliae* and *L. micdadei*), alaninamide (except *L. hackeliae*), L-asparagine (except *L. hackeliae* and *L. micdadei*), L-glutamate (except *L. micdadei*), L-serine (except *L. dumoffii* and *L. hackeliae*), and L-threonine (except *L. dumoffii* and *L. rubrilucens*). Unlike the *L. pneumophila* strains, none of the species metabolized acetate, propionate, aspartate, glycyl-L-aspartate, histidine, L-ornithine, or γ-aminobutyrate. Only *L. feeleii* metabolized glycyl-L-glutamate, *L. longbeachae* metabolized succinate, *L. bozemanii* and *L. longbeachae* metabolized L-leucine, and *L. bozemanii*, *L. israelensis*, and *L. longbeachae* metabolized monomethyl succinate. Although the BIOLOG panel of substrates contains a number of sugars, e.g., glucose, fructose, galactose, and maltose, none of the strains tested reacted positively with any of them. This is not unexpected since previous reports indicate that legionellae have low levels of glucose uptake and catabolism (13, 25). Although Weiss et al. (26) reported that the Philadelphia 2 strain of *L. pneumophila* utilized glucose-1-phosphate, none of the strains in this study showed any indication of utilizing either glucose-1-phosphate or glucose-6-phosphate; however, this may be due to insufficient reducing power being generated to cause detectable reduction of the tetrazolium dye.

TABLE 1. Substrate metabolism profiles of *Legionella* spp. incubated in 4% (vol/vol) oxygen at 37°C for 24 h with Page's amoebal saline as diluent

Substrate	Profile of *Legionella*[a]:								
	bozemanii	*dumoffii*	*feeleii*	*hackeliae*	*israelensis*	*longbeachae*	*micdadei*	*pneumophila*	*rubrilucens*
Tween 40	+[b]	+	+	+	V	+	+	+	
Tween 80	+	+				+	+	+	
Methylpyruvate	+	+	+	+	+	+	+	+	+
Monomethyl succinate	+				+	+		V	
Acetate								V	
β-Hydroxybutyrate	+	+	+	+	+	+	+	+	+
α-Ketobutyrate	+	+	+	+		+	+	+	+
α-Ketovalerate	+	+	+		+	+		+	+
Propionate								V	
Succinate						+		+	
Alaninamide	+	+	+		+	+	+	+	+
L-Alanine	+		+		+	+	V	+	
L-Alanylglycine	+					+		V	
L-Asparagine	+	+	+		+	+		V	V
L-Aspartate								V	
L-Glutamate	+	+	+	+	+	+		+	+
Glycyl-L-aspartate								V	
Glycyl-L-glutamate			+					+	
L-Histidine								V	
L-Leucine	+					+		+	
L-Ornithine								+	
L-Proline	+	+	+	+	+	+	+	+	+
L-Serine	+		+		+	+	+	+	V
L-Threonine	V		+	+	+	+	+	+	
γ-Aminobutyrate								V	
Urocanate	+	+			+	+		+	+

[a] *L. bozemanii* (NCTC 11368); *L. dumoffii* (NCTC 11370); *L. feeleii* (NCTC 12022); *L. hackeliae* serogroup 1 (NCTC 11979); *L. israelensis* (NCTC 12010); *L. longbeachae* serogroup 1 (NCTC 11477); *L. micdadei* (NCTC 11371); pooled results for *L. pneumophila* serogroups 1 (NCTC 11192), 2 (NCTC 11230), 3 (NCTC 11232), 5 (NCTC 11405), 6 (NCTC 11287), 7 (NCTC 11984), 8 (NCTC 11985), 10 (NCTC 12000), 11 (NCTC 12179), 12 (NCTC 12180), 13 (NCTC 12181), and 14 (NCTC 12174); *L. rubrilucens* (NCTC 11987).

[b] + and V denote positive and variable reactions, respectively, in replicate samples.

Specificity and sensitivity. The metabolic profiles of all of the strains, which had been incubated in 4% (vol/vol) oxygen for 24 h, were compared with the manufacturer's data base and then entered into a new *Legionella* computer data base. None of the legionellae tested were identified as closely

matching any other of the 434 strains of bacteria in the BIOLOG data base, indicating that the profiles obtained were specific to this genus. Environmental isolates which had been provisionally identified by serology as *Legionella* spp. (kindly provided by John Kurtz, Oxford, United Kingdom) were typed by using our modified BIOLOG system. The results were compared with a combination of the manufacturer's data base and our *Legionella* data base. The identification that we obtained agreed with that of Kurtz to the species level. Environmental isolates of *Pseudomonas fluorescens*, *P. paucimobilis*, *P. vesicularis*, *Klebsiella* spp., *Alcaligenes* sp., *Achromobacter* sp., and other water isolates were not identified as closely matching any of the *Legionella* profiles.

Effect of increased biomass on metabolism. One possible means of encouraging growth and metabolism of microaerophilic bacteria is to increase their cell mass so that the higher respiration rate helps to maintain a lower oxygen concentration diffusing into static cultures. When the concentration of the *L. pneumophila* serogroup 1 strain was doubled (approximately 2.3×10^9 to 3.0×10^9 CFU ml^{-1}) and the multiwell plates were incubated in air, 24 substrates were metabolized and gave positive results within 24 h. A similar effect was noted with *L. micdadei*, which metabolized 18 substrates compared with only 9 at the prescribed concentration.

Simplification of diluent. The previous experiments described data obtained with legionellae resuspended in PAS, since the physiological saline diluent recommended by the manufacturer, which has a sodium concentration of 146 mM, may be inhibitory to some strains. However, PAS is low in sodium (4 mM) and has been shown to provide a good diluent and transport medium for legionellae (14). In an attempt to simplify this diluent, which also contains 1 mM K^+, 2 mM phosphate, 27 μM Ca^{2+}, and 16 μM Mg^{2+}, legionellae were resuspended and incubated in deionized water. This was carried out with bacterial suspensions at double the concentration described in the manufacturer's manual. By this method, results with deionized water as the diluent were similar to those obtained by using PAS.

When the latter two modifications were used, the profiles obtained for the strains investigated were similar to those recorded in the initial experiments with a low-oxygen incubation and PAS as the diluent. Sufficient numbers of positive reactions could be obtained within 24 h by using distilled water as the diluent and a normal aerobic atmosphere to allow accurate identification of the strains tested. Thus, the modified protocol was considerably simplified for routine laboratory use.

Identification of putative PHB. The ^{13}C NMR spectrum of the material extracted from *L. pneumophila* was consistent with that of PHB and was identical to that of authentic PHB from an *Alcaligenes* sp. Analysis of a methanol lysate of the purified material by gas chromatography-mass spectrometry demonstrated that 3-hydroxybutyrate was the predominant hydroxyalkanoic acid monomer of the material. These data indicate that the material extracted from *L. pneumophila* was PHB.

DISCUSSION

The results obtained in this study suggest that when this modified procedure is employed, the BIOLOG bacterial identification system has the ability to identify legionellae at least to the species level. Also, the results obtained so far suggest that both the specificity and sensitivity are good. In order to create stable data bases, it will be necessary to characterize multiple strains from all known species. Although our data base is derived from reaction data for only a small number of previously identified type strains, we have shown that it is now possible with this modified system to identify legionellae by phenotypic means, and we have obtained accurate identification of nontype strains, which had previously been identified by serology, by using this system. With this modified system, which provides a simple, rapid, and standardized procedure, it is now possible to address the points that Brenner (3) made in his review of the classification of *Legionellaceae*: "Representative numbers of strains from all species must be systematically studied by all the phenotypic methods available . . . to determine whether a means of phenotypically speciating legionellae is achievable" and if so to decide if there are valid phenotypic grounds for more than one genus in the family *Legionellaceae*.

The *Legionellaceae* have previously been considered to be fastidious because of their inability to metabolize carbohydrates or to grow on a range of routine laboratory media. The present study showed, as was expected, that the legionellae investigated were most metabolically active towards amino acids and their derivatives in the BIOLOG panel of substrates, results which agree with previous work on the metabolism of this genus (7, 13, 22, 25), and were, in addition, able to metabolize a range of carboxylic acids and their derivatives, including acetate, propionate, succinate, monomethyl succinate, and methylpyruvate. Our results agreed with those of Tesh et al. (25), who found that the addition of pyruvate, succinate, and acetate stimulated oxygen uptake by suspensions of washed cells of *L. pneumophila* but that α-ketoglutarate did not. Although cell extracts of *L. pneumophila* have α-ketoglutarate dehydrogenase activity (11, 13, 25), whole cells fail to oxidize this substrate to any appreciable level (13, 25). This led Tesh et al. (25) to postulate that *L. pneumophila* was unable to transport this compound. Our results seem to support this, as we found no metabolism of α-ketoglutarate, although the other keto-acids in the panel were metabolized by almost all of the strains investigated. All the strains tested metabolized methylpyruvate and β-hydroxybutyrate, and most metabolized Tween 40 and Tween 80, suggesting that they possesses esterase activity, as reported previously (18, 19). However, this may not be due to specific esterase activity but may be due to protease activity (24). Specific aminopeptidase activities for *L. pneumophila* have been reported by Muller (18); in our study, we found that several dipeptides were metabolized by legionellae.

Many researchers have reported the presence of electron translucent granules in *Legionella* spp. The appearance and staining properties of these granules have led the authors of these reports to suggest that the inclusions resemble PHB granules (6, 23). Our finding that *L. pneumophila* contained PHB provides direct evidence for PHB accumulation by legionellae (17). Since all the strains investigated in this study metabolized the monomer, β-hydroxybutyrate, PHB may play a significant role as a carbon and energy storage compound for survival in oligotrophic environments.

Since slightly more substrates were utilized when the BIOLOG plates were inoculated with cultures grown on BCYE for 24 h, use of a shorter incubation time for the inoculum may be advisable when the system is being used to investigate the physiology of strains. In the interest of standardization and simplicity, however, inoculum grown for 72 h seems suitable when the system is used to identify isolates. Incubating the test plates in a low-oxygen atmo-

sphere or at high cell density allows much more rapid development of positive tests and, thus, facilitates identification after only 24 h of incubation. To increase the ability of the system to differentiate between *Legionella* spp., it may be desirable to increase the number of substrates which can be potentially used by a *Legionella* isolate. Since legionellae are asaccharolytic, the replacement of the carbohydrate substrates with substrates that can be utilized by these bacteria should increase the system's resolving power for *Legionellaceae*.

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