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Clinical Laboratory Differentiation of *Legionellaceae* Family Members with Pigment Production and Fluorescence on Media Supplemented with Aromatic Substrates

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A systematic study of pigment production (browning) and fluorescence (extracellular yellow-green and intracellular blue-white) by nine *Legionellaceae* species was performed. A total of 56 strains representing *Tatlockia micdadei* (Pittsburgh pneumonia agent), *Legionella pneumophila*, *Legionella jordanis*, *Legionella longbeachae*, *Legionella oakridgensis*, *Legionella wadsworthii*, *Fluoribacter bozemanae*, *Fluoribacter gormanii*, and *Fluoribacter dumoffii* could be separated on media supplemented with tyrosine plus cystine, 3,4-diaminobenzoic acid, 3,5-diaminobenzoic acid, and 3-aminotyrosine. Parallel testing by hippurate hydrolysis and the bromocresol purple spot test enabled the identification of *Legionellaceae* species 24 to 72 h after primary isolation. This schema may be a practical alternative to species-specific antisera methods (slide agglutination or direct immunofluorescence) in the identification of members of the family *Legionellaceae*.

Classical methods of bacteria identification, such as carbohydrate fermentations or substrate utilization with pH changes, are not useful when working with the members of the family *Legionellaceae*. Traditional tests such as oxidase, catalase, and gelatinase give variable results (2, 6). Laboratory identification test procedures have been limited because of the specific growth requirements and minimal biochemical activity of the *Legionellaceae*. We previously reported the use of the bromocresol purple (BCP) dye solution (BCP spot test) to differentiate *Tatlockia micdadei* (*Legionella micdadei*, Pittsburgh pneumonia agent) from other *Legionellaceae* (6). We also showed that dye-containing media could differentiate *T. micdadei* from other *Legionellaceae* species on the basis of colonial coloration (10). Hébert showed that the hippurate hydrolysis test is positive for *Legionella pneumophila* and negative for all other *Legionellaceae* (7).

Pigment production is a proven aid for the identification and classification of microorganisms. Pigment production by members of the *Legionellaceae* family can be visually determined in at least three forms: (i) browning, in which the organisms secrete a brown melanin-like pigment into media containing L-tyrosine or L-phenylalanine (1); (ii) extracellular fluorescence, in which the organisms secrete a compound into agar media that fluoresces yellow or yellow-green when exposed to long-wave UV light (5); and (iii) intracellular fluorescence, in which the colonies of the organisms exhibit a blue-white or dull-yellow fluorescence when exposed to UV light (3). The first and second forms listed above are examples of diffusible, extracellular pigments produced by the organism; the browning and yellow-green fluorescence are seen in media that are in immediate proximity to the organism. The third form listed above is an example of nondiffusible, intracellular pigment; the blue-white or dull-yellow fluorescence is seen within the organism.

In this paper, we present a systematic study to separate the nine species of *Legionellaceae* by using pigmentation and fluorescence on the following substrates: tyrosine, tyrosine plus cystine, 3,4-diaminobenzoic acid (3,4-DABA) and 3,5-diaminobenzoic acid (3,5-DABA), and 3-aminotyrosine.

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MATERIALS AND METHODS

Base agar, substrates, and test media preparation. (i) Agar base preparation. Charcoal-free, buffered yeast extract agar supplemented with *N*-(2 acetamido)-2-aminoethanesulfonic acid buffer (10 g), L-cysteine monohydrochloride (0.4 g), and ferric pyrophosphate (0.25 g/liter adjusted to pH 6.9 ± 0.05) (BYEA) was prepared as previously described (9). Sterile agar base medium was dispensed into sterile bottles in 100-ml portions and stored at 2 to 8°C for no longer than 7 days before its use.

(ii) Substrates and test media preparation. L-Tyrosine, L-cystine, 3,5-DABA, 3,4-DABA, and 3-aminotyrosine were used as substrates. Tyrosine was dissolved in 1 N KOH (0.05 g/1.5 ml), cystine was dissolved in 1 N HCl (0.05 g/1.3 ml), and 3-aminotyrosine was dissolved in water (0.02 g/5 ml) and placed in a 35 to 37°C water bath for 15 min. 3,5-DABA and the 3,4-DABA were dissolved in water (0.05 g/5 ml) and heated to between 80 and 100°C. All substrates were sterilized by membrane filtration. Stored bottles of sterile BYEA base were melted in a boiling water bath and cooled to 50 to 55°C. The filter-sterilized substrates were added singly to 100 ml of BYEA with a final medium concentration of 0.5 g/liter, except 3-aminotyrosine, which was 0.2 g/liter. Tyrosine and cystine were also tested in combination, both at 0.5 g/liter. All substrate media and the BYEA base were tested as whole plates, quadrant plates, and slanted medium in screw-capped tubes.

(iii) Bacterial strains and testing procedure. Table 1 lists the 56 patient and environmental isolates of the *Legionellaceae* used. All strains were grown on standard *N*-(2-acetamido)-2-aminoethanesulfonic acid-buffered charcoal-yeast extract agar (9) from frozen stock cultures for 24 to 48 h and inoculated directly onto plates and slants of substrate and nonsubstrate BYEA base media by using a bacteriological

TABLE 1. Strains of *Legionellaceae* used in this study

Species and strain[a]	No. of isolates	Serogroup
L. pneumophila		
ATCC[b] 33152 (Philadelphia-1)	1	1
ATCC 33153 (Knoxville-1)	1	1
Clinical	9	1
Environmental	7	1
ATCC 33154 (Togus-2)	1	2
CDC[c] (Atlanta 1-2-4)	3	2
ATCC 33155 (Bloomington-2)	1	3
ATCC 33156 (Los Angeles-1)	1	4
Environmental	1	4
ATCC 33216 (Dallas-1E)	1	5
Environmental	2	5
CDC (Chicago-2)	1	6
CDC (Houston-1)	1	6
Clinical	2	6
Environmental	1	6
L. longbeachae		
ATCC 33462 (Longbeach-4)	1	1
ATCC 33484 (Tucker-1)	1	2
L. jordanis		
ATCC 33623 (BL-540)	1	
L. oakridgensis		
ATCC 33761 (OR-10)	1	
L. wadsworthii		
ATCC 33877 (Wadworth 81-716A)	1	
T. micdadei		
ATCC 33218 (TATLOCK)	1	
Clinical	9	
Environmental	3	
F. bozemanae		
ATCC 33217 (WIGA)	1	
CDC (Mi-15)	1	
F. dumoffii		
ATCC 33279 (NY-23)	1	
ATCC 33343 (TEX-KL)	1	
F. gormanii		
ATCC 33297 (LS-13)	1	

[a] *L. pneumophila* and *T. micdadei* strains listed as clincial or environmental were isolated at the Veterans Administration Medical Center and confirmed by using direct fluorescent-antibody serogroup conjugates purchased from the Centers for Disease Control.
[b] ATCC, Obtained from the American Type Culture Collection.
[c] CDC, Obtained from the Centers for Disease Control.

loop to obtain confluent growth. One set of uninoculated substrate media and BYEA base medium was always incubated in parallel with inoculated test media and BYEA base medium for color comparisons. Plates and tubes (loosely capped and tightly capped) were incubated for 3 days at 35 to 37°C in air.

(iv) BCP spot test. The BCP spot test was performed as previously described (6). One drop of a 0.04% BCP dye solution was placed onto a piece of Whatman no. 1 filter paper or its equivalent, and a generous portion of growth from an agar culture was rubbed into the wet area of the paper in a circular motion. The appearance of an aquamarine color within 30 s was defined as a positive reaction, whereas the appearance of a yellow to dull-green color was considered a negative reaction.

Definitions. (i) Browning. Browning was recorded as positive if a brown discoloration was visible on the substrate agar medium being used. The browning produced had to be greater than any browning produced on substrate-free BYEA base medium before a positive result was assigned. Since nonspecific browning due to chemical breakdown of the 3,4-DABA, 3,5-DABA, and 3-aminotyrosine occurred over a 3-day period, a positive result was recorded only if browning produced on media by bacterial growth exceeded that produced on uninoculated media.

(ii) Fluorescence. A Wood lamp (long-wave, UV light) was used in the dark to detect the extracellular yellow or yellow-green fluorescence excreted into the agar medium. The lack of extracellular fluorescence was manifested as a blue glow derived from the light source. Fluorescence of the growth (intracellular) was recorded as the color of the colony surface when it was exposed to the Wood lamp in the dark.

(iii) Growth. Growth was recorded as good (confluent bacterial growth covering the entire streak) or sparse (growth on the streak but not confluent growth).

RESULTS

Results for each substrate tested are listed below and summarized in Table 2 and Fig. 1 through 5 for the nine *Legionellaceae* species. We noted several observations common for all substrate media: (i) blue-white intracellular fluorescence was produced only by *F. bozemanae*, *F. gormanii*, and *F. dumoffii* (Fig. 1 and 4); (ii) when slants were tightly capped, browning and extracellular fluorescence was reduced or absent when compared with that on slants with loosened caps; (iii) quadrant plates demonstrated greater browning and extracellular fluorescence than did whole plates since diffusion was confined to the compartments; (iv) surface growth of *F. bozemanae* and *F. gormanii* was white, whereas that of *F. dumoffii* was gray (Fig. 5).

Tyrosine and cystine. Browning was produced on tyrosine and tyrosine-plus-cystine media by all species except *L. wadsworthii* (Table 2). Browning, if produced by a *T. micdadei* strain, was merely a trace amount. *L. pneumophila*, *L. longbeachae*, and *F. bozemanae* produced trace amounts of both browning and extracellular fluorescence on cystine alone as well as on unsupplemented base agar. Tyrosine plates exposed to a Wood lamp showed a dull-green fluorescence for strains which produced any amount of browning. The addition of cystine to tyrosine medium markedly enhanced fluorescence, which appeared as bright yellow or yellow-green. A trace amount of yellow fluorescence was seen on the tyrosine-plus-cystine combination for *L. wadsworthii* but was not seen on tyrosine alone. Figure 1 compares the extracellular fluorescence seen on tyrosine only to that on tyrosine plus cystine. The intensity of extracellular fluorescence was directly related to the degree of browning and was exemplified by *L. longbeachae* serogroup 2, which produced both greater browning and greater fluorescence than did *L. longbeachae*, serogroup 1. Growth by all species was good on the tyrosine-plus-cystine medium.

3,5-DABA. *F. gormanii*, *F. dumoffii*, and *L. longbeachae* produced a dark-brown pigment on 3,5-DABA, whereas all other species were negative or produced only trace amounts (Table 2 and Fig. 2). Extracellular fluorescence could not be detected owing to the green autofluorescence of the 3,5-DABA substrate; blocking of the autofluorescence was observed when browning was produced. Growth by all species was good.

TABLE 2. Browning and fluorescent reactions for nine *Legionellaceae* species with four substrate media after 1 to 3 days of incubation

Species (no. tested)	Browning with following substrate[a]:				Extracellular[b] fluorescence with following substrates[a]:				Intracellular[c] fluorescence with any substrate
	T + C	3,5-DABA[d]	3,4-DABA	3-Amino-tyrosine	T + C	3,5-DA-BA[d]	3,4-DABA	3-Amino-tyrosine	
T. micdadei (13)	0–Trace	0[e]	0	Trace	0–Trace	–	0	Trace	Yellow
L. pneumophila (33)	+[f]	0–Trace	0–Trace	Trace	+	–	0–Trace	Trace	Yellow[g]
L. jordanis (1)	+	Trace	+	+	+	–	+	+	Yellow
L. longbeachae (2)	+[h]	+	0	+	+	–	0	+	Yellow
L. oakridgensis (1)	+	Trace	0	+	+	–	0	+	Yellow
L. wadsworthii (1)	0	0	0	Trace	Trace	–	0	Trace	Yellow
F. bozemanae (2)	+	0	0	Trace	+	–	0	+[i]	Blue-white
F. gormanii (1)	+[j]	+	+	+	+	–	+	+	Blue-white
F. dumoffii (2)	+	+	+	+	+	–	+	+	Blue-white

[a] T + C, Tyrosine plus cystine; 3-AT, 3-aminotyrosine.
[b] Yellow or yellow-green diffusible fluorescence seen when plates were exposed to long-wave UV light.
[c] Nondiffusible (colonial) intracellular fluorescence seen when plates were exposed to long-wave UV light.
[d] The uninoculated 3,5-DABA medium produces a green autofluorescence which prevents detection of "bacterial fluorescence." If browning is produced on 3,5-DABA, the green autofluorescence is blocked.
[e] Figure 2 illustrates 0, trace, and + reactions.
[f] *L. pneumophila* serogroup 6 consistently produced heavier browning than serogroups 1 through 5.
[g] *L. jordanis* produced a yellow-green intracellular fluorescence on 3,5-DABA when exposed to long-wave UV light.
[h] *L. longbeachae* serogroup 2 produced more browning than did *L. longbeachae* serogroup 1.
[i] Fluorescence was distinctly less yellow for *F. bozemanae* than for *F. gormanii* and *F. dumoffii* (Fig. 4).
[j] On all substrates, *F. gormanii* growth was white, and *F. dumoffii* growth was gray (Fig. 5).

3,4-DABA. *F. gormanii*, *F. dumoffii*, and *L. jordanis* produced weak browning and dull-green fluorescence on 3,4-DABA (Table 2). Several *L. pneumophila* strains produced a trace of browning and fluorescence, whereas the other species were negative. Growth on this substrate was generally sparse by all species compared with that on other substrate media. Figure 3 illustrates a positive and negative result for browning.

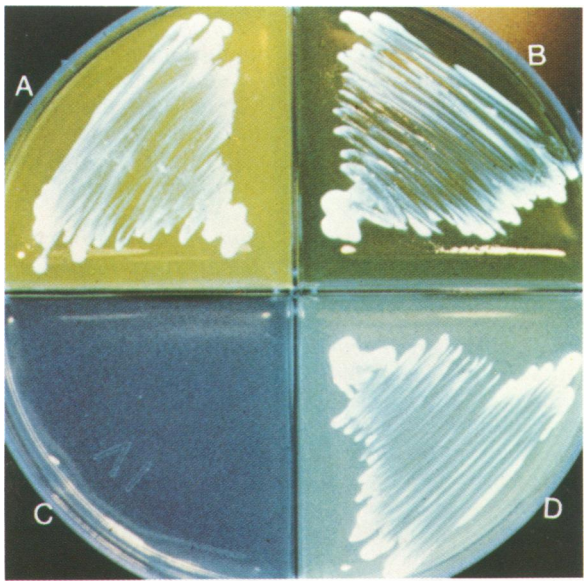

FIG. 1. Demonstration of extracellular and intracellular fluorescence by *F. bozemanae* when viewed in the dark with exposure to long-wave UV light. (A) Tyrosine plus cystine medium. Note bright yellow-green extracellular fluorescence within the medium. (B) Tyrosine only medium. Only a dull-green extracellular fluorescence was seen. (C) Uninoculated, unsupplemented base medium on which no fluorescence was seen. Note the intracellular blue-white fluorescence of *F. bozemanae* on the three inoculated quadrants under long-wave UV light. (D) Unsupplemented base medium. Trace light-green fluorescence was seen.

3-Aminotyrosine. All species produced various amounts of browning and fluorescence (Table 2). *F. dumoffii*, *L. longbeachae*, and *L. jordanis* produced more browning and extracellular fluorescence than did all other species. *F. dumoffii* and *F. gormanii* could easily be differentiated from *F. bozemanae* on this substrate; *F. bozemanae* produced notably less yellow extracellular fluorescence (Fig. 4). Growth of all species was good.

DISCUSSION

The direct fluorescent-antibody stain, gas-liquid chromatography, and DNA homology are the definitive procedures for the identification of members of the family *Legionellaceae*. The direct fluorescent-antibody stain requires a separate conjugate for eight serogroups of *L. pneumophila*, for

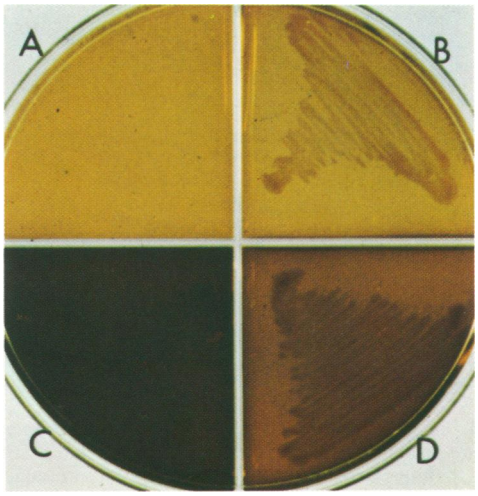

FIG. 2. Browning reactions on 3,5-DABA medium. (A) Uninoculated control. (B) *T. micdadei* was negative for browning. (C) *L. longbeachae* produced heavy browning. (D) *L. jordanis* produced a trace amount.

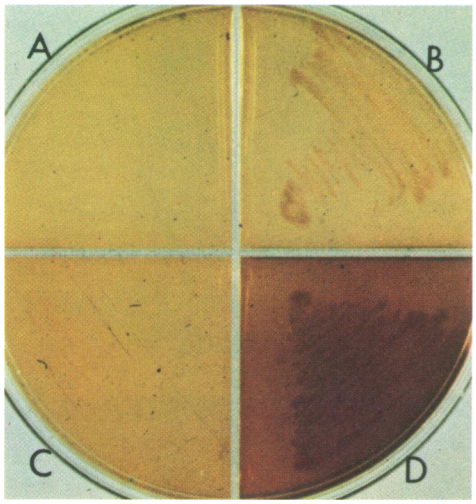

FIG. 3. Browning reactions on 3,4-DABA. (A) Uninoculated control. (B) *T. micdadei* was negative. (C) Growth of *L. longbeachae* was almost completely inhibited on 3,4-DABA. (D) *L. jordanis* produced browning.

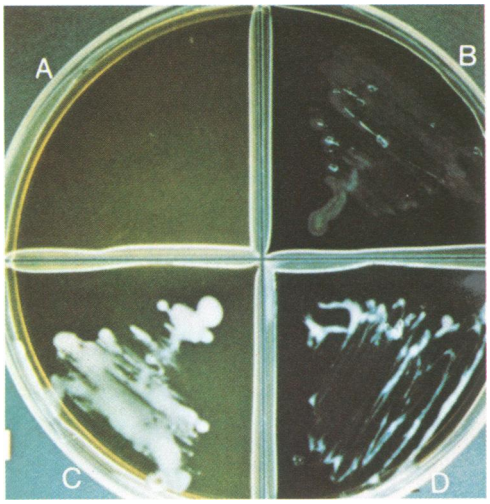

FIG. 5. Colony coloration on 3,5-DABA medium. (A) Uninoculated control. (B) The surface growth of *F. dumoffii* was gray. (C) The surface growth of *F. bozemanae* was milky white. (D) The surface growth of *F. gormanii* was milky white. A dark background was used to enhance the surface density.

two serogroups of *L. longbeachae*, and for seven other species; therefore a dozen or more reagents are now required, many of which are not available commercially. Thus, the clinical microbiologist is in need of easily performed procedures which can screen and identify *Legionellaceae* isolates directly from primarily isolation media or within 24 to 72 h on subculture. We have shown that the use of the substrate media described in this study, in concert with other phenotypic tests, can accurately identify the individual species of the *Legionellaceae*.

Isolates can be screened as possible *Legionellaceae* species by their failure to grow on blood agar, colony morphology, observation of extracellular or intracellular fluorescence, or a positive BCP spot test. Subculture of suspect *Legionellaceae* isolates to plates containing tyrosine plus cystine, 3,4-DABA, and 3,5-DABA and performance of a hippurate hydrolysis test can enable species identification within 24 to 72 h after primary isolation. A tentative schema is shown in Fig. 6.

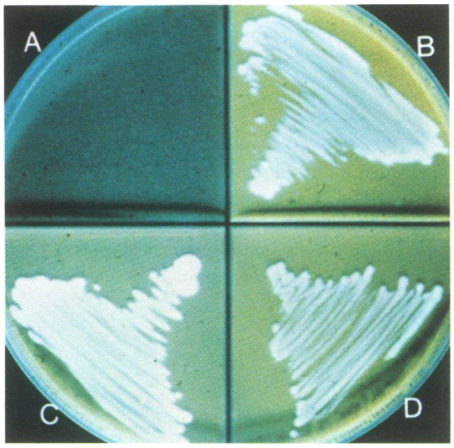

FIG. 4. On 3-aminotyrosine, *F. dumoffii*, *F. gormanii*, and *F. bozemanae* produced yellow-green extracellular fluorescence when exposed to long-wave UV light. (A) Blue fluorescence of the uninoculated medium was the negative control. (B) *F. dumoffii* produced yellow-green extracellular fluorescence. (C) *F. bozemanae* was distinctly less yellow than either *F. dumoffii* (B) or *F. gormanii* (D). Note the characteristic blue-white intracellular fluorescence of all three *Fluoribacter* spp.

We previously reported that *L. pneumophila*, *F. bozemanae*, *F. gormanii*, and *F. dumoffii* were negative with the BCP spot test (6). We now report that *L. longbeachae*, *L. jordanis*, *L. oakridgensis*, and *L. wadsworthii* also give negative results with the BCP spot test. Thus, *T. micdadei* is the only one of the nine species that gives a positive reaction with the BCP spot test, and *L. pneumophila* is the only one which gives a positive reaction with the hippurate hydrolysis test (7). The remaining seven *Legionellaceae* species give negative reactions to both the BCP spot and the hippurate hydrolysis tests (2, 4, 8) and can be further identified by using tyrosine plus cystine, 3,4-DABA, 3,5-DABA, 3-aminotyrosine substrates and colonial characteristics (Table 2 and Fig. 6).

The identification of the six *Legionellaceae* species known to cause human disease (*L. pneumophila*, *L. longbeachae*, *L. wadsworthii*, *T. micdadei*, *F. dumoffii*, and *F. bozemanae*) may now be feasible for the clinical microbiologist. *L. pneumophila* and *T. micdadei* can be differentiated by the hippurate hydrolysis and BCP spot tests, respectively. *Fluoribacter* species can be identified by the presence of blue-white intracellular fluorescence. *F. dumoffii* and *F. bozemanae* can be further differentiated in that *F. dumoffii* produces browning on 3,4-DABA and 3,5-DABA, whereas *F. bozemanae* does not. *L. longbeachae* can be differentiated from *L. wadsworthii* and *L. pneumophila* in that *L. longbeachae* produces browning on 3,5-DABA, whereas *L. wadsworthii* and *L. pneumophila* do not.

Before this system can be adopted, more isolates of *L. longbeachae*, *L. wadsworthii*, and *Fluoribacter* species should be tested to confirm the consistency of browning and fluorescence for individual members of the *Legionellaceae* family. Because reproducibility was a concern, we tested representative strains from each species on three different batches of both base and substrate media. As expected, all species produced variable trace amounts of browning and extracellular fluorescence on the base medium without sub-

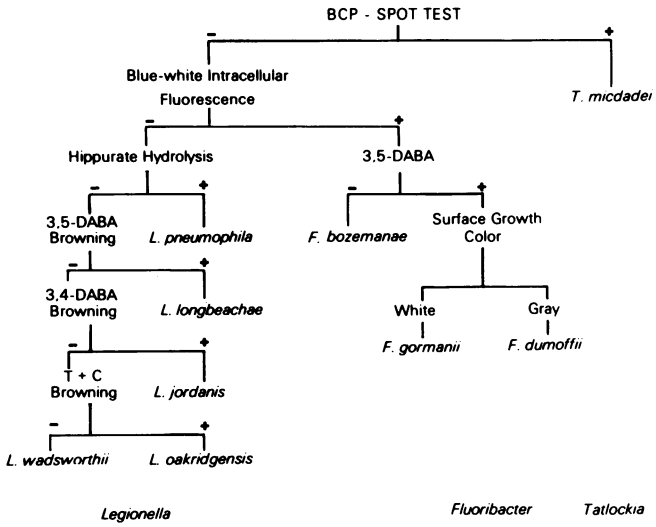

FIG. 6. Identification of nine *Legionellaceae* members by using phenotypic characteristics.

strates; however, this variation posed no problems in interpreting positive reactions on the substrate media. Since yeast extract may contain variable amounts of pigment-producing substrates, positive (e.g., *F. dumoffii*) and negative (unsupplemented medium) controls should be routinely included in test runs for comparison.

As more *Legionellaceae* species, both clinical and environmental, are discovered, the relevance of a schema such as the one proposed in Fig. 6 will increase. Although species-specific antisera (slide agglutination or direct fluorescent-antibody staining) are accurate, their use in the clinical laboratory will become more costly and time consuming as more species are discovered. Phenotypic screening with substrate media such as those described in this paper may prove to be a useful alternative. Undoubtedly, future refinements will make this schema more precise and easier to implement. Finally, Garrity et al. have proposed that the family *Legionellaceae* can be classified into three distinct genera, *Legionella*, *Fluoribacter*, and *Tatlockia*, based on DNA homology studies (6). This study now substantiates the rationality of their classification by using phenotypic characteristics (Fig. 6).

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