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Biochemical Identification of Citrobacteria in the Clinical Laboratory

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We biochemically identified 235 *Citrobacter* strains to the species level on the basis of the recently proposed taxonomic changes of Brenner et al. (D. J. Brenner, P. A. D. Grimont, A. G. Steigerwalt, G. R. Fanning, E. Ageron, and C. F. Riddle, Int. J. Syst. Bacteriol. 43:645–658, 1993). *Citrobacter* isolates were initially identified as *C. koseri* or as members of the *C. freundii* complex or *C. amalonaticus* group on the basis of indole production, formation of H_2S, malonate utilization, and acid production from D-arabitol and adonitol. On the basis of the results of these tests, 68% of the *Citrobacter* strains were identified as members of the *C. freundii* complex, 25% were *C. koseri*, and 8% were members of the *C. amalonaticus* group. By using a 15-test system recently proposed by Brenner et al. (D. J. Brenner, P. A. D. Grimont, A. G. Steigerwalt, G. R. Fanning, E. Ageron, and C. F. Riddle, Int. J. Syst. Bacteriol. 43:645–658, 1993) to help identify new species in the *C. freundii* complex and *C. amalonaticus* group, 81% of the *C. freundii* complex strains and 100% of the *C. amalonaticus* strains could be definitively assigned to one of the previously established or recently designated species or hybridization groups of the genus *Citrobacter*. Within the *C. freundii* complex, *C. freundii* predominated overall (37%), followed by *C. youngae* (24%), *C. braakii* (13%), and *C. werkmanii* (6%). Only one strain each of *C. sedlakii* and *Citrobacter* DNA group 11 was identified in this study. Among *C. amalonaticus* complex members, all were identified as *C. amalonaticus* with the singular exception of one fecal isolate of *C. farmeri*. *C. freundii* and *C. koseri* were the two *Citrobacter* species most commonly (80 of 93 [86%]) isolated from extraintestinal sources (genitourinary tract, wounds, blood).

Members of the genus *Citrobacter* are gram-negative, oxidase-negative bacilli that usually utilize citrate as a sole carbon source and are motile by means of peritrichous flagella; two additional properties of citrobacteria that help to distinguish them from other members of the family *Enterobacteriaceae* are their failure to produce acetylmethylcarbinol (Voges-Proskauer) and the lack of lysine decarboxylase (10). In humans, citrobacters cause significant morbidity and mortality and cause a variety of infectious processes ranging from urinary tract and wound infections (including pyomyositis) to more invasive diseases, including septicemia and neonatal meningitis (3, 6, 8). Although unconfirmed, citrobacters have also been implicated as an occasional cause of gastroenteritis, particularly in infants and young children (9).

Although the genus was initially described in 1932, both the composition and names used for species in the genus have been the subject of controversy. *Bergey's Manual of Systematic Bacteriology* lists three *Citrobacter* species, namely, *C. freundii*, *C. diversus*, and *C. amalonaticus* (10). However, in 1974, Crosa et al. (2) showed by DNA-DNA hybridization at 60°C that the DNA relatedness of strains identified as either *C. freundii* or *C. amalonaticus* varied over a considerable range (ca. 50 to 65%), suggesting the possibility that additional *Citrobacter* species might exist. In addition, although *C. diversus* appears to be fairly homogeneous on a molecular basis, the correct species designation of this organism has been challenged (7).

Recently, Brenner and colleagues (1) proposed dramatic revisions for species status within the genus on the basis of DNA relatedness studies performed at 60 and 75°C. The results of these DNA-DNA hybridization experiments indicate that a previously recognized biogroup of *C. amalonaticus* (biogroup 1) deserves species status, and therefore was named *Citrobacter farmeri*, and that the *C. freundii* complex is actually composed of at least eight distinct DNA hybridization groups (genomospecies). Within the *C. freundii* complex, four new species were named (*Citrobacter youngae*, *Citrobacter braakii*, *Citrobacter werkmanii*, and *Citrobacter sedlakii*) and DNA hybridization groups 9, 10, and 11 were identified but left unnamed because of the lack of a sufficient number of strains within each. All 11 of the designated species could be identified by using either classical biochemical methods or carbon substrate assimilation profiles. Since the feasibility of identifying citrobacteria (to the species level) with this new scheme in the clinical laboratory is presently unknown, as are the clinical frequency and distribution, we have characterized a large collection of citrobacteria by biochemical methods by using these recently described definitions (1).

MATERIALS AND METHODS

Nomenclature. Throughout this article, we use the term "*C. freundii*" to refer solely to *C. freundii* DNA hybridization 1 (DNA group 1); we use the term "*C. freundii* complex" to refer to a larger group of strains now known to be composed of eight or more DNA hybridization groups. The species designation *C. koseri* is used in place of *C. diversus* throughout the text since a recent ruling by the Judicial Commission of the International Committee on Systematic Bacteriology has stated in Opinion 67 that *C. diversus* is a nomen dubium (12).

Bacterial strains. A total of 235 *Citrobacter* strains were studied in this investigation; 124 strains originally identified as *Citrobacter* spp. were retested for this study and were from the reference collection (1971 to 1992) of the Microbial Diseases Laboratory (MDL). All strains were of human origin, with the exception of one monkey and one water isolate. The remaining 111 *Citrobacter* strains were primarily received as consecutive,

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nonselected isolates from different individuals over a 2- to 3-month period. The majority of these strains were kindly provided by Peter C. Appelbaum (University Hospital, Hershey, Pa.), Amy M. Carnahan (Ann Arundel Medical Center, Annapolis, Md.), Harry P. Dalton (Medical College of Virginia, Richmond), Larry D. Gray (Bethesda North Hospital, Cincinnati, Ohio), Josephine A. Morello (University of Chicago Medical Center, Chicago, Ill.), Timothy L. Overman (VA Medical Center, Lexington, Ky.), Victoria Velculescu (Kaiser Regional Laboratory, Berkeley, Calif.), and Mary York (University of California Medical Center, San Francisco, Calif.). All citrobacteria were maintained as working cultures on motility deeps at ambient temperature during the course of this investigation. All studies were performed at 35°C unless otherwise specified.

Preliminary identification. Citrobacteria were identified as either *C. koseri*, belonging to the *C. amalonaticus* group, or belonging to the *C. freundii* complex on the basis of the following tests: indole production in tryptone broth, H_2S production, malonate utilization, and acid production from adonitol and D-arabitol (5). H_2S production was detected on both triple sugar iron (TSI) slants and in gelatin-cysteine-thiosulfate tubes as previously described (11); H_2S formation on TSI was used as the standard method. All reactions were recorded daily, with final readings at 72 h. No changes in reactions were observed past 72 h. Strains identified as *C. amalonaticus* complex members were further characterized on the basis of the fermentation of sucrose, raffinose, α-methyl-D-glucoside, and melibiose and the ability to utilize Simmons citrate (1). Strains of *C. amalonaticus* that fermented these four sugars and were citrate negative were identified as *C. farmeri*, formerly known as *C. amalonaticus* biogroup 1 (4).

Identification of individual species within the *C. freundii* complex. Strains belonging to the *C. freundii* complex were identified to the species level by biochemical methods on the basis of the results of Brenner et al. (1), who found 15 tests to be useful in separating *Citrobacter* genomospecies. These tests included indole production; citrate, acetate, and malonate utilization; arginine dihydrolase and ornithine decarboxylase activities; motility; urease production; esculin hydrolysis; and acid production from sucrose, dulcitol, melibiose, raffinose, α-methyl-D-glucoside, and salicin. All tests were performed by conventional methods. Sugar fermentation was performed in Acumedia (Acumedia Manufacturers, Inc., Baltimore, Md.) with Andrade's indicator. Esculin hydrolysis was performed on bile esculin agar slants (Difco, Detroit, Mich.). Tests were read daily, and final results were recorded at 7 days. *C. freundii* ATCC 8090^T and *C. youngae* ATCC 29935^T (CDC 460-61) served as controls for biochemical studies.

On the basis of the results from these 15 tests, a biochemical profile of each named and unnamed species within the *C. freundii* complex was generated from Tables 2 and 4 of Brenner et al. (1). Biochemical properties for each genomospecies that were ≥85% positive or negative at day 7 of that study (1) were respectively scored as + or − for that given genomospecies (Table 1); biochemical tests that yielded between 15 and 85% positivity for individual species were listed as variable (V). Such an analysis yielded between 11 and 14 key differential tests for each of the named genomospecies (*C. freundii*, *C. youngae*, *C. braakii*, *C. werkmanii*, and *C. sedlakii* and the three unnamed species). Biochemical results for individual strains were then matched against each of these characteristic profiles in order to identify isolates. For the five named species within the *C. freundii* complex, patterns that were identically matched or that deviated in only one phenotypic property from the idealized profile were identified as belonging to that species.

TABLE 1. Differential biochemical tests useful in distinguishing *C. freundii* complex members[a]

Test	Test result for[b]:				
	C. freundii	*C. youngae*	*C. braakii*	*C. werkmanii*	*C. sedlakii*
Indole	V	−	V	−	+
Citrate	+	+	+	+	+
Urease	V	V	V	V	+
Arginine dihydrolase	+	+	+	+	+
Ornithine decarboxylase	−	−	+	−	+
Motility	+	+	+	+	+
Sucrose	+	V	−	−	−
Dulcitol	−	+	V	−	+
Raffinose	+	−	−	−	−
α-Methyl-D-glucoside	V	−	V	−	−
Melibiose	+	−	+	−	+
Salicin	−	−	−	−	V
Esculin	−	−	−	−	V
Acetate	V	V	+	+	V
Malonate	−	−	−	+	+

[a] Data are for named species only; all data are derived at day 7 from Tables 2 and 4 of reference 1.

[b] +, 85 to 100% positive; −, 85 to 100% negative; V, variable (15 to 85% positive).

For the unnamed species (genomospecies 9, 10, and 11), since only three strains were found within each of these groups in the study by Brenner et al. (1), an ideal match was required for species identification. The results of a preliminary characterization of the citrobacterial strains used in this study are presented in Table 2.

RESULTS

Of 238 cultures originally submitted as citrobacteria, 235 were identified by biochemical properties as members of the genus *Citrobacter*. With the six-test system shown in Table 2, each citrobacter isolate could be assigned to one particular species or complex on the basis of the results of tests for indole production, H_2S formation on TSI, fermentation of adonitol and D-arabitol, and malonate utilization. Members of the *C. freundii* complex were the most frequently observed strains in the two collections, accounting for 68% of all strains charac-

TABLE 2. Preliminary characterization of citrobacterial strains used in this study

Test	No. of strains (% positive) of[a]:		
	C. freundii complex (*n* = 159)	*C. koseri* (*n* = 58)	*C. amalonaticus* group (*n* = 18)
Indole	5 (3)	58 (100)	18 (100)
H_2S production on:			
TSI	158 (99)	0 (0)	0 (0)
Gelatin-cysteine-thiosulfate	158 (99)	57 (98)	4 (22)
Acid from:			
Adonitol	0 (0)	58 (100)	0 (0)
D-Arabitol	0 (0)	58 (100)	0 (0)
Malonate	23 (14)	58 (100)	0 (0)

[a] Results were derived after a 72-h incubation.

TABLE 3. Biochemical identification of species within the *C. freundii* complex

Identification	No. (% distribution of strains) identified from:		Total (%)
	MDL strains (n = 75)	Multicenter isolates (n = 87)	
C. freundii	7 (9)	53 (62)	60 (37)
C. youngae	26 (35)	13 (15)	39 (24)
C. braakii	14 (19)	7 (8)	21 (13)
C. werkmanii	8 (11)	1 (1)	9 (6)
C. sedlakii	1 (1)	0 (0)	1 (<1)
DNA group 11	1 (1)	0 (0)	1 (<1)
Citrobacter species	18 (24)	13 (15)	31 (19)

terized, followed by *C. koseri* (25%) and *C. amalonaticus* (8%). Although neither *C. koseri* nor *C. amalonaticus* produced H_2S in TSI slants at 24 h, almost all of the *C. koseri* strains did produce H_2S in gelatin-cysteine-thiosulfate medium, usually after only 1 day of incubation. Even on TSI, a majority of *C. koseri* strains produced a thumbnail of H_2S at the interface between the slant and butt after prolonged incubation (usually 6 to 7 days). Only 14 of the 18 strains of *C. amalonaticus* appear to be H_2S negative in both media. Fermentation of adonitol and D-arabitol was associated only with *C. koseri*, while malonate utilization was chiefly but not invariably associated with *C. koseri*. Further characterization of *C. amalonaticus* strains revealed only one *C. farmeri* isolate, which was from a fecal specimen submitted to our laboratory in 1985. This strain fermented sucrose, raffinose, and melibiose and was α-methyl-D-glucoside negative at 1 day and citrate positive at 2 days (confirmed by the Centers for Disease Control as *C. amalonaticus* biogroup 1).

The 162 strains of the *C. freundii* complex were identified to the species level by using a 15-test format (Table 1). On the basis of the criteria described above, 81% of the strains tested could be assigned to one of the recently named species of this complex or to one of the three unnamed DNA groups identified by Brenner et al. (1). The frequency distribution of species within the *C. freundii* complex, however, differed dramatically by submitter (Table 3). *C. youngae* was the most common species identified from a collection of 75 strains submitted to the MDL over a 21-year period. In contrast, from isolates submitted from nine different laboratories throughout the United States, *C. freundii* predominated, accounting for almost two-thirds of the 87 strains analyzed. Overall, *C. freundii* was the most commonly identified species within the complex, followed by *C. youngae*, *C. braakii*, and *C. werkmanii*. Only one strain each of *C. sedlakii* and genomospecies 11 was identified from the 162 strains tested; no isolates resembling genomospecies 9 or 10 were found. Of the 130 strains identified to the species level (excluding DNA genomospecies 11), 64% overall yielded perfect biotypes while the remaining 36% deviated in one phenotype from the ideal pattern. Of the four most commonly identified species, *C. braakii* strains most often gave a perfect biotype (81%), while *C. werkmanii* strains did so the least often (56%). It is possible that these strains may belong to yet undefined *Citrobacter* species. The most common phenotypic variation associated with *C. freundii* was dulcitol positivity, while with *C. youngae* phenotypic variation involved malonate utilization. Of 31 unidentified members of the *C. freundii* complex, 5 resembled *C. freundii* (e.g., sucrose, raffinose, and melibiose positive), 6 were *C. braakii*-like, and one appeared to be *C. youngae*, although each of these strains deviated by at least two or more tests from the ideal pattern. Tests most commonly in disagreement with idealized phenotypes included esculin and salicin reactions. The remaining 19 unidentified citrobacters fell into three groups. The most common of these latter patterns was called "nonreactive" in that most or all of the sugars tested were not fermented and the arginine dihydrolase and ornithine decarboxylase reactions were often negative (n = 9); these strains, however, were citrate and H_2S positive and Voges-Proskauer and lysine decarboxylase negative. A second reactive group consisted of isolates that fermented most sugars (n = 7). A final heterogeneous group (n = 3) did not fit either of these patterns.

TABLE 4. Biochemical properties of species within the *C. freundii* complex found in this study

Test	% Positive at day 7				
	C. freundii (n = 60)	*C. youngae* (n = 39)	*C. braakii* (n = 21)	*C. werkmanii* (n = 9)	*C. sedlakii* (n = 1)
Indole	2	0	0	0	100
Citrate	100	100	100	100	100
Arginine dihydrolase	100	97	95	100	100
Ornithine decarboxylase	2	8	100	0	100
Sucrose	100	15	0	11	0
Dulcitol	12	92	52	0	100
Raffinose	100	0	0	0	0
α-Methyl-D-glucoside	5	0	67	0	0
Esculin	0	3	0	0	100
Salicin	12	3	15	0	100
Melibiose	100	0	100	0	100
Urea	98	92	95	100	100
Motility	93	97	100	100	100
Acetate	100	100	100	100	100
Malonate	2	15	0	56	100

Most (80 to 100%) biochemical tests used to identify 73 routine clinical isolates in the *C. freundii* complex were positive within 24 to 48 h of incubation. Exceptions were α-methyl-D-glucoside and salicin fermentation, in which incubation periods of >48 h were required for a majority of isolates to become positive. Salicin in particular produced very weak reactions with a number of citrobacters that sometimes were difficult to interpret. Many salicin-positive strains fermented this compound only after prolonged incubation (5 to 7 days). The composite biochemical properties for 130 strains identified to the species level are listed in Table 4. Particularly ornithine decarboxylase activity and the fermentation of sucrose, dulcitol, raffinose, and melibiose were found to be the most discriminatory tests. All 130 of these strains were citrate and acetate positive, and most were motile and arginine dihydrolase and urease positive. Only 1 indole-positive strain of *C. freundii* was observed in this collection of 130 strains.

Finally, we looked at the distribution of citrobacteria by site of isolation (Table 5). As previously reported, *C. koseri* and *C. amalonaticus* were distinctly associated with specific body sites; 47% of all *C. koseri* organisms were recovered from the genitourinary tract (64% from genitourinary tract and wounds), while 94% of the *C. amalonaticus* organisms were isolated from feces; the one nonfecal isolate of *C. amalonaticus* was recovered from lung tissue. For species within the *C. freundii* complex, the gastrointestinal tract was the most common site from which each genomospecies was isolated, with the exception of *C. freundii*, which was most frequently recov-

TABLE 5. Distribution of identifiable *Citrobacter* species by isolation site[a]

Species	No. from the following isolation site:					
	Feces	Genitourinary tract	Respiratory system	Blood or cerebrospinal fluid	Wound[b]	Miscellaneous[c]
C. koseri	7	27	7	6	10	1
C. amalonaticus	16	0	1	0	0	0
C. farmeri	1	0	0	0	0	0
C. freundii	16	23	4	2	12	3
C. youngae	29	6	0	2	0	2
C. braakii	14	3	1	0	1	1
C. werkmanii	8	0	0	0	1	0
C. sedlakii	1	0	0	0	0	0

[a] Includes strains from the MDL and other laboratories.
[b] Includes abscesses.
[c] Bile (n = 3), peritoneal fluid (n = 1), ear (n = 1), gall bladder (n = 1), catheter tip (n = 1).

ered from genitourinary specimens. Seventy-four percent of all *C. freundii* strains were recovered from extraintestinal body sites (Table 5); this is in contrast to 30% of *C. braakii* strains, 27% of all *C. youngae* isolates, and 11% of *C. werkmanii* isolates. Only *C. freundii* and *C. youngae* were recovered from blood (twice each); *C. youngae* was also recovered, along with a *Leminorella* sp., from the gall bladder of a 60-year-old woman. In addition to urine, *C. braakii* was recovered in pure culture from the peritoneal fluid of a 22-year-old woman who presented with abdominal pain, nausea, and vomiting, and underwent a subsequent appendectomy. The only nonfecal isolate of *C. werkmanii* identified came from a penile lesion.

DISCUSSION

In 1993, Brenner et al. (1) proposed some sweeping changes regarding the genus *Citrobacter* on the basis of the results of DNA-DNA hybridization. The major change proposed was in the *C. freundii* complex, for which four new named species and three unnamed species were defined. Each of these new species can usually be identified biochemically with conventional tests. From available clinical information previously presented (1) and the results of this investigation (Table 5), most species of the complex appear to be pathogenic for humans, with *C. youngae* being the most common of the new species identified.

In the present investigation, we used the biochemical criteria of Brenner et al. (1) to determine both the species distribution of citrobacteria and the feasibility of identifying members of the *C. freundii* complex in the laboratory with existing biochemical tests. Although results for strains submitted to the MDL paralleled the Centers for Disease Control's finding that *C. youngae* was the most common species of the *C. freundii* complex observed, results from the analysis of multicenter-submitted strains and overall composite results indicated that *C. freundii* was the predominant species seen. The differences in species distribution between reference and routine isolates characterized in the present investigation may be related to how multicenter strains were selected (consecutive, unselected, time interval) or alternatively may be related to strain characteristics—many of the *Citrobacter* strains in the MDL collection were originally submitted for identification because of cross-reactions with *Salmonella* antisera. However, regardless of differences between these two studies in strain selection, *C. freundii* appears to be more common clinically than originally suspected (1), since it accounted for 37% of all *C. freundii* complex strains in our study compared with 14% in the previous investigation (1). *C. freundii* was the most common species identified at all body sites except feces. In gastrointestinal specimens submitted to the MDL, *C. freundii* ranked fourth behind *C. youngae*, *C. braakii*, and *C. werkmanii*, while in routine fecal isolates (multicenter) it ranked first. Overall, *C. freundii*, *C. youngae*, and *C. braakii* accounted for 92% of all *C. freundii* complex isolates identified to the species level. Of the later five species (and *C. farmeri*), only *C. werkmanii* (6%) was identified with any regularity.

Using biochemical tests suggested by Brenner et al. (1), we were able to identify >80% of all citrobacteria belonging to the *C. freundii* complex to the species level. Final identification in these instances required either a perfect biotype or a biotype that deviated in a single phenotypic property. If one accepts final identification to include strains with more than a single characteristic deviating from the idealized profile yet with phenotypic properties indicative of a single genomospecies, then 88% of all complex strains could be identified to the genomospecies level. However, there still exists a sizable (ca. 10%) group of isolates which did not resemble any of the eight published genomospecies residing in the *C. freundii* complex. This suggests either more extensive biochemical variation within defined species or the presence of other unrecognized genomospecies within the *C. freundii* complex.

A four-test system is proposed to identify the most frequently encountered species currently residing in the *C. freundii* complex (Table 6). The tests include ornithine decarboxylase activity and acid production from sucrose, dulcitol, and melibiose. The tests were chosen on the basis of species frequency (Table 3), rapidity of a positive reaction, and discriminatory value (Table 4). By conventional assay, most results will be available in 24 h; whether similar results will be

TABLE 6. Simplified biochemical scheme for identification of the more frequently encountered members of the *C. freundii* complex

Test	Test result (%) for[a]:				
	C. freundii	*C. youngae*	*C. braakii*	*C. werkmanii*	*C. sedlakii*
Ornithine decarboxylase	− (2)	− (0)	+ (0)	− (0)	+ (100)
Sucrose	+ (100)	− (15)	− (0)	− (11)	− (0)
Dulcitol	− (12)	+ (92)	V (52)	− (0)	+ (100)
Melibiose	+ (100)	− (0)	+ (100)	− (0)	+ (100)
Indole[b]	− (2)	− (0)	− (0)	− (0)	+ (100)

[a] +, 85 to 100% positive; −, 85 to 100% negative; V, variable (15 to 85% positive).
[b] Indole results are taken from the initial screening reactions (Table 2).

generated with commercial microidentification systems remains to be determined.

The genus *Citrobacter*, as previously defined prior to recent taxonomic changes, included the three distinct species *C. freundii*, *C. koseri*, and *C. amalonaticus* (10). *C. koseri* is often pathogenic for humans, causing urinary tract infections and, on occasion, fulminant neonatal meningitis (8, 13). In contrast, *C. amalonaticus* is most commonly isolated from feces. Its role in human infections is not clear. The *C. freundii* complex is by far the most common group isolated from clinical material. The results by Brenner et al. (1) and the present study indicate that most of the newly described *Citrobacter* species are human pathogens, since *C. freundii*, *C. youngae*, *C. werkmanii*, and *C. selakii* in addition to genomospecies 11 have all been isolated from blood and *C. freundii*, *C. braakii*, and *C. youngae* have been isolated from urine, both of which are normally sterile body sites. Although large differences in the site of isolation of species within the *C. freundii* complex were not observed (Table 5), genomospecies 1 (*C. freundii*) appeared to be the species most commonly isolated from extraintestinal sites such as wounds. Whether differences in the disease spectrum and pathogenicity of these redefined species will emerge in a fashion similar to that of *C. koseri* will await further clinical and laboratory investigations.

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