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Bordetella holmesii sp. nov., a New Gram-Negative Species Associated with Septicemia

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CDC nonoxidizer group 2 (NO-2) currently consists of 15 gram-negative, rod-shaped, oxidase-negative, asaccharolytic, brown soluble pigment-producing strains isolated from blood cultures, usually from young adults. On the basis of their cellular fatty acid profiles, NO-2 strains formed a single group that was identical with the profile of *Bordetella avium*. 16S rRNA sequencing of one NO-2 strain and the type strains of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. avium* showed a high degree of homology (≥98% over 1,525 bases). The NO-2 guanine-plus-cytosine content (61.5 to 62.3 mol%) and major ubiquinone analysis (ubiquinone-8) results were both consistent with those for the genus *Bordetella*. DNA relatedness studies (hydroxyapatite method) confirmed a close relatedness between NO-2 and *Bordetella* species and demonstrated that NO-2 strains were a single new species. The name *B. holmesii* sp. nov. is proposed for CDC group NO-2.

CDC nonoxidizer group 2 (NO-2) is the vernacular name given to gram-negative, nonoxidizing, brown soluble pigment-producing rods sent for identification to the Special Bacteriology Reference Laboratory at the Centers for Disease Control and Prevention (CDC). The first of these strains was received in 1983. Since then, 14 additional strains have been received, all of which were isolated from blood cultures obtained, with few exceptions, from young adults. At least four of these strains were isolated from more than one blood culture per patient. Eleven NO-2 strains, including two isolates discovered outside the United States, have been received since 1989, raising the possibility that this group is an emerging pathogen.

In this study we characterized the strains of NO-2 biochemically, by cellular fatty acid (CFA) and ubiquinone analysis, guanine-plus-cytosine (G+C) content, 16S rRNA sequence analysis, and DNA-DNA hybridization. The results indicate that group NO-2 is a single, previously undescribed species whose closest relatives are in the genus *Bordetella*. We propose the name *Bordetella holmesii* for NO-2.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The NO-2 strains and their sources are shown in Table 1. *Bordetella pertussis* ATCC 9797^T, *Bordetella parapertussis* ATCC 15311^T, *Bordetella avium* ATCC 35086^T, and *Bordetella bronchiseptica* ATCC 19395^T were obtained from the American Type Culture Collection, Rockville, Md. In addition, CFA profiles were determined for *B. parapertussis* F6288 and F6289, *B. bronchiseptica* B5821 and G1696, and *B. pertussis* 042, 083, and 087. The *B. parapertussis* and *B. bronchiseptica* strains were submitted to CDC as patient isolates and were identified by the Special Bacteriology Reference Laboratory. The *B. pertussis* strains were obtained from P. Cassiday, Pertussis Laboratory, Division of Bacterial and Mycotic Diseases, CDC. All strains were suspended in defibrinated rabbit blood and stored in a liquid nitrogen freezer until studied. Unless otherwise indicated, all strains except *B. pertussis* were grown on heart infusion agar with 5% rabbit blood (HIA) (BBL Microbiology Systems, Cockeysville, Md.) in a candle jar at 35°C. *B. pertussis* was grown on Regan-Lowe agar (35) (35°C in a candle jar for 4 days) for CFA analysis. To obtain DNA, *B. pertussis* was grown in Stainer-Scholte broth (39) and all other strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). All broth cultures were incubated with shaking at 37°C. To determine the possible effects of media on CFA composition, strains of NO-2, *B. avium*, *B. parapertussis*, and *B. bronchiseptica* were examined for CFA composition after growth on Regan-Lowe agar as well as on HIA.

Biochemical characterization. Biochemical characteristics of NO-2 strains were determined by the methods of the Special Bacteriology Reference Laboratory (4). Test results were read after 48 h and 7 days of incubation at 35°C, except for oxidase and catalase results and optimal growth temperature, which were read at 24 h.

CFA and isoprenoid quinone analysis. Cells were saponified, and the liberated fatty acids were methylated and analyzed by capillary gas-liquid chromatography (30). Identification of fatty acids and determination of double-bond positions in monounsaturated acids were accomplished by gas-liquid chromatography and gas-liquid chromatography–mass spectrometry (30). Isoprenoid quinones were extracted from 100 mg of lyophilized cells and analyzed by reverse-phase high-performance liquid chromatography (29).

16S rRNA sequencing and analysis. The methods used for PCR amplification of ribosomal DNA (rDNA), its sequencing, and analysis of the data are those reported by Daly et al. (6). Template DNA for direct sequencing was produced by enzymatic amplification of 16S rDNA by using the primers fD1 and rD1 described by Weisburg et al. (42). The 10 oligonucleotides used as sequencing primers are those used by Stackebrandt and Charfreitag (38). For comparison studies, 16S rRNA sequences, representing members of the beta, delta, and gamma subdivisions of the *Proteobacteria*, were retrieved electronically (Table 2). With the exception of that for *Oxalobacter formigenes*, sequences were retrieved from the GenBank database (1). The *O. formigenes* sequence was retrieved from the Ribosomal Database Project, University of Illinois–Urbana-Champaign (electronic mail address: RDP.LIFE.UIUC.EDU). Sequences were aligned by using the multisequence alignment program PILEUP (8), and phylogenetic relationships were inferred with version 3.4 of the PHYLIP software package (13). The method of Jukes and Cantor was used to calculate a similarity matrix (23). The dendogram was constructed by the neighbor-joining method of Saitou and Nei (37) using the sequence from *Anacystis nidulans* as the outgroup. The dendogram was further tested by bootstrap analysis (12).

G+C content in DNA. The G+C content was determined spectrophotometrically by thermal denaturation (28), with *Escherichia coli* DNA included as a control.

DNA-DNA hybridization. The preparation and purification of DNA and the conditions used to determine DNA relatedness by the hydroxyapatite method have been described previously (2). DNA from NO-2 strain F5101 was labeled with [^{32}P]dCTP by using a nick translation kit (GIBCO BRL, Gaithersburg, Md.) as described by the manufacturer. Because of the relatively high G+C content of NO-2 DNA, an incubation temperature of 65°C was used for optimal DNA reassociation. Divergence (unpaired bases within hybridized sequences) was estimated to be approximately 1% per degree of decreased thermal stability in a

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TABLE 1. Demographic and clinical information on CDC group NO-2 strains[a]

Strain	Geographic source; yr received	Patient sex, age (yr)[b]	Clinical history
F5101	Buffalo, N.Y.; 1983	M, 37	Mild leukocytosis, cardiomegaly, maximum temperature of 104°F (ca. 40°C)
F6119	Chicago, Ill.; 1984	M, 15	Septic arthritis, sickle cell anemia
F7732	Iowa City, Iowa; 1986	M, 20	Subacute bacterial endocarditis, diabetes
G133	California; 1987	M, 32	Fever, back and upper abdominal pain, possible gall bladder disease, lymphoma
G2910	Farmington, Conn.; 1989	M, 10	Splenectomy, secondary to spherocytosis
G4102	Baltimore, Md.; 1989	F, 22	
G4363	Philadelphia, Pa.; 1990	F, 62	Fever, abdominal pain
G5210	Philadelphia, Pa.; 1990	M, 43	
G5245	Rochester, N.Y.; 1990	M, 21	
G6128	Bethesda, Md.; 1991	M, 17	Splenectomy
G7702	California; 1992	M, 36	Dog bite, Hodgkin's disease
G7851	Denver, Col.; 1992	M	Splenectomy
G7952	Little Rock, Ark; 1992	F, 20	
G8341	Saudi Arabia	F, 32	Endocarditis, previous mitral and atrial valve repair
G8350	Switzerland	F, 24	Fever, hypotension, respiratory insufficiency, postlaparoscopy

[a] All strains were isolated from blood.
[b] M, male; F, female.

heterologous reassociated DNA duplex compared with that of the homologous DNA duplex. Divergence was calculated to the nearest 0.5%.

TABLE 2. Sources and accession numbers for 16S rRNA-rDNA data

Organism	GenBank accession no.	Source or reference
Acinetobacter calcoaceticus NCTC 10292	X74899	NP[a]
Agrobacterium tumefaciens IAM 13129	D12784	NP
Alcaligenes eutrophus ATCC 17697	M32021	NP
Alcaligenes faecalis ATCC 8750	M22508	10
Alcaligenes xylosoxidans ATCC 15173	M22509	10
Anacystis nidulans 6301	X03538	26
Bordetella avium ATCC 35086	U04947	This study
Bordetella bronchiseptica ATCC 19395	U04948	This study
Bordetella parapertussis ATCC 15311	U04949	This study
Bordetella pertussis ATCC 9797	U04950	This study
Chromobacterium violaceum ATCC 12472	M22510	10
Comamonas testosteroni ATCC 11996	M11224	44
Desulfovibrio desulfuricans NCBI 8380	M37316	9
Eikenella corrodens ATCC 23834	M22512	10
Escherichia coli rrB cistron	J01695	3
Iodobacter fluviatile ATCC 33051	M22511	10
Kingella denitrificans ATCC 33394	M22516	10
Kingella kingae ATCC 23330	M22517	10
Kingella oralis CCUG 30450	L06164	10
Methylophilus methylotrophus AS1	M29021	40
Neisseria animalis ATCC 19573	L06172	10
Neisseria canis ATCC 14687	L06170	NP
Neisseria denitrificans ATCC 14686	L06173	NP
Neisseria elongata ATCC 25295	L06171	NP
Neisseria flavescens ATCC 13120	L06168	NP
Neisseria gonorrhoeae NCTC 83785	X07714	36
Neisseria macacae ATCC 33926	L06169	NP
Neisseria polysaccharea ATCC 43768	L06167	NP
Nitrosolobus multiformis C-71	M96401	14
Nitrosomonas europeae C-31	M96399	14
Oxalobacter formigenes[b] ATCC 35274		NP
Pseudomonas cepacia[c] ATCC 25416	M22518	10
Rhodocyclus gelatinosus ATCC 17011	M60682	NP
Rhodocyclus purpureus 6770	M34132	NP
Simonsiella muelleri ATCC 29453	M59071	NP
Spirillum volutans ATCC 19554	M34131	46
Vitreoscilla stercoraria ATCC 15218	L06174	NP
Xanthomonas maltophilia[d] ATCC 13637	M59158	NP
Xylella fastidiosa ATCC 35880	M26601	43

[a] NP, sequence submitted to GenBank as unpublished.
[b] Obtained from the Ribosomal Database Project, University of Illinois–Urbana-Champaign.
[c] Recently proposed name, *Burkholderia cepacia* (45).
[d] Recently proposed name, *Stenotrophomonas maltophilia* (33).

RESULTS

On microscopic observation of 48-h HIA cultures, individual NO-2 cells appeared as gram-negative, predominately small coccoid and short rods with rare longer and wider forms. Growth was slow on all media tested, requiring at least 48 h of incubation before isolated colonies could be observed. On rabbit blood agar at 3 days of incubation, isolated colonies were punctate, semiopaque, convex, and round with complete edges. A zone of browning or greening of the media was observed around colonies of seven strains. All strains produced a brown soluble pigment when grown at 35°C on heart infusion tyrosine agar (4). All strains grew on MacConkey agar, although three strains required between 3 and 7 days of incubation to produce visible growth. All strains grew at 35°C, and 11 of 15 strains grew at 25°C. Other variable tests included catalase production (7 of 15 strains produced a weak reaction), H_2S production as detected by lead acetate paper (9 of 15 strains produced a weak reaction), reactivity in litmus milk (5 of 15 strains produced alkalinization, and 1 strain reduced the indicator without alkalinization), and growth in nutrient broth with 0% NaCl (six strains produced visible growth within 2 days of incubation, eight strains produced visible growth between 3 and 7 days of incubation, and 1 strain failed to produce visible growth at 7 days of incubation). All strains were negative for the following tests: growth at 42°C; acid production from glucose, xylose, mannitol, lactose, sucrose, and maltose; motility; oxidase; urease; nitrate reduction; nitrite reduction; indole production; citrate; acid production in the slant or butt of triple sugar iron agar; H_2S production in triple sugar iron agar; gelatin hydrolysis; esculin hydrolysis; lysine decarboxylase; arginine dihydrolase; ornithine decarboxylase; growth on *Salmonella-Shigella* agar; growth on cetrimide agar; and growth in nutrient broth with 6% NaCl.

The CFA compositions of selected strains of NO-2, *B. avium*, *B. parapertussis*, *B. bronchiseptica*, and *B. pertussis* are presented in Table 3. For strains grown on both HIA and Regan-Lowe agar, no medium-related differences in CFA profiles were observed. The CFA compositions of eight NO-2 strains tested were essentially identical to one another and to the CFA profile of *B. avium*. Both NO-2 and *B. avium* CFA profiles were characterized by major amounts (30 to 41%) of hexadecanoate ($C_{16:0}$) and cycloheptadecanoate ($C_{17:0cyc}$),

TABLE 3. CFA composition of CDC group NO-2, *B. avium*, *B. parapertussis*, *B. bronchiseptica*, and *B. pertussis*

Fatty acid[a]	%Total fatty acids[b]				
	CDC group NO-2 (8)[c]	*B. avium* (1)	*B. parapertussis* (2)	*B. bronchiseptica* (2)	*B. pertussis* (3)
3-OH-$C_{10:0}$	—[d]	—	2	—	4
$C_{12:0}$	—	T[e]	—	2	—
2-OH-$C_{12:0}$	4	2	—	4	—
3-OH-$C_{12:0}$	—	—	—	—	1
$C_{14:0}$	1	1	6	6	5
$C_{15:0}$	—	1	1	—	—
2-OH-$C_{14:0}$	4	3	—	—	—
3-OH-$C_{14:0}$	5	4	1	5	9
$C_{16:1w7c}$	2	3	6	27	40
$C_{16:0}$	41	40	40	35	32
$C_{17:0cyc}$	30	34	35	14	—
$C_{17:0}$	—	2	3	—	—
$C_{18:2}$	2	—	1	1	1
$C_{18:1w9c}$	1	—	—	—	1
$C_{18:1w7c}$	—	1	—	2	—
$C_{18:0}$	7	5	5	2	8
$C_{i\text{-}19:0}$	—	T	—	—	—
$C_{19:0cyc11\text{-}12}$	—	T	—	—	—
$C_{19:0cyc9\text{-}10}$	1	—	—	—	—

[a] The number before the colon is the number of carbon atoms and the number after the colon is the number of double bonds; w, double-bond position from the hydrocarbon end of the chain; c, *cis* isomer; i, methyl group at the penultimate (iso-) carbon atom; cyc, cyclopropane ring; 2-OH-, hydroxyl group at the 2 position; 3-OH-, hydroxyl group at the 3 position.
[b] Values are arithmetic means. Numbers in parentheses are numbers of strains.
[c] Strains G4363, G5210, G5245, G6128, G7702, G7952, G8341, and G8350.
[d] —, not detected.
[e] T, 0.4 to 0.8%.

moderate amounts (5 to 7%) of octadecanoate ($C_{18:0}$), small amounts (2 to 5%) of straight-chain hydroxy acids (2-OH-$C_{12:0}$, 2-OH-$C_{14:0}$, and 3-OH-$C_{14:0}$), and only traces to small amounts (1 to 3%) of tetradecanoate ($C_{14:0}$) and *cis*-9-hexadecenoate ($C_{16:1w7c}$). The apparent small differences in CFA composition between these two organisms ($C_{17:0}$, $C_{18:2}$, $C_{18:1w9c}$, $C_{18:1w7c}$, and $C_{19:0cyc9–10}$) are not sufficient to distinguish NO-2 from *B. avium*, since the relative amounts of these acids varied among the NO-2 strains tested. The CFA profile of NO-2 and *B. avium* differed from that of *B. parapertussis* by the absence of 3-OH-$C_{10:0}$ (2%), the presence of 2-OH-$C_{12:0}$ (2 to 4%) and 2-OH-$C_{14:0}$ (3 to 4%), smaller amounts of $C_{14:0}$ (1 versus 6%) and $C_{16:1w7c}$ (2 versus 6%), and larger amounts of 3-OH-$C_{14:0}$ (4 to 5 versus 1%). The CFA profile of NO-2 and *B. avium* differed from that of *B. bronchiseptica* by smaller amounts of $C_{12:0}$ (0 to T versus 2%), $C_{14:0}$ (1 versus 6%), and $C_{16:1w7c}$ (2 versus 27%), the presence of 2-OH-$C_{14:0}$ (3 to 4%), and larger amounts of $C_{18:0}$ (6 to 7 versus 2%).

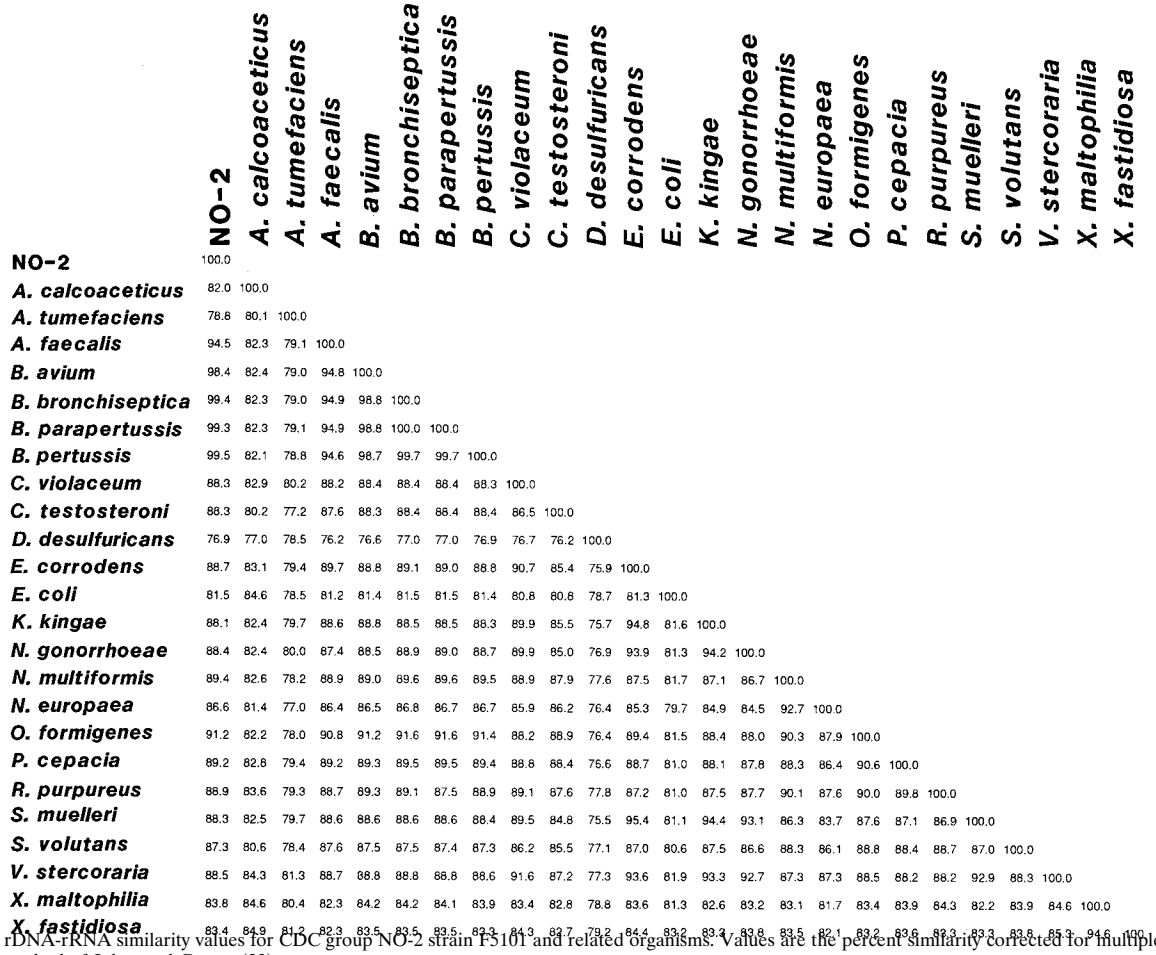
A significant difference in the CFA profile of *B. pertussis* compared with those of other organisms listed in Table 3 was the absence of $C_{17:0cyc}$. This acid was present in moderate to large amounts (14 to 35%) in all other organisms when grown on either HIA or Regan-Lowe agar. *B. pertussis* also differed from NO-2 and *B. avium* by the presence of 3-OH-$C_{10:0}$ (4%) and 3-OH-$C_{12:0}$ (1%), the absence of 2-OH-$C_{12:0}$ and 2-OH-$C_{14:0}$, and larger amounts of $C_{14:0}$ (5 versus 1%), 3-OH-$C_{14:0}$ (9 versus 4 to 5%), and $C_{16:1w7c}$ (40 versus 2%). Thus, on the basis of CFA composition NO-2 and *B. avium* are indistinguishable from each other but differ from other *Bordetella* species (Table 3). The overall CFA profiles of NO-2 and *B. avium* are most similar to those of *Alcaligenes faecalis* and *Alcaligenes xylosoxidans*, except for smaller amounts of $C_{16:1w7c}$ (2 versus 20%) (28a). Strains of both NO-2 and *B. parapertussis* contained ubiquinone-8 as the major isoprenoid quinone as determined by high-performance liquid chromatographic analysis.

Biochemical tests that differentiate NO-2 from *Bordetella* species and other phenotypically similar bacteria are listed in Table 4. The lack of oxidase activity and the production of a brown soluble pigment differentiate NO-2 from *B. pertussis*, *B. bronchiseptica*, and *B. avium*. The lack of urease activity differentiates NO-2 from *B. parapertussis*. Other phenotypically similar bacteria include asaccharolytic *Acinetobacter* species and CDC group NO-1 (15). Like NO-2, these organisms are usually oxidase negative and do not utilize carbohydrates; however, they usually do not produce a brown soluble pigment, and group NO-1 reduces nitrate. Rarely occurring asaccharolytic *Acinetobacter* species that produce a brown soluble pigment can be differentiated from NO-2 by the *Acinetobacter* transformation assay and CFA analysis (4, 31). *Brucella canis*

TABLE 4. Differentiation of CDC group NO-2 from *Bordetella* species and other phenotypically similar pathogenic bacteria

Test	% of following strains (no.) positive[a]								
	CDC group NO-2 (15)	*B. pertussis* (51)	*B. parapertussis* (7)	*B. bronchiseptica* (85)	*B. avium* (2)	*Brucella canis* (28)	*X. maltophilia* (228)	Asaccharolytic *Acinetobacter* species (253)	CDC group NO-1 (17)
Growth on MacConkey agar	80 [100]	0	100	100	100	12 [41]	100	90 [97]	5 [15]
Oxidase	0	94	0	100	100	72	0	0	0
Urease	0	0	100	99	0	100	3 [15]	5 [9]	5
Nitrate reduction	0	NT[b]	0	92	0	100	39	3	100
Motility	0	NT	0	100	100	0	100	0	0
Brown soluble pigment	100	0	100	0	0	NT	98	13	0
Beta-like hemolysis	0	81	100	12	50	0	1	29	0
Acinetobacter transformation	0	NT	NT	NT	NT	NT	NT	100	0

[a] With the exception of data for CDC group NO-2 and *B. avium*, data were previously published (4, 15). Results are at 48 h [7 days] of incubation.
[b] NT, not tested.



	NO-2	A. calcoaceticus	A. tumefaciens	A. faecalis	B. avium	B. bronchiseptica	B. parapertussis	B. pertussis	C. violaceum	C. testosteroni	D. desulfuricans	E. corrodens	E. coli	K. kingae	N. gonorrhoeae	N. multiformis	N. europaea	O. formigenes	P. cepacia	R. purpureus	S. muelleri	S. volutans	V. stercoraria	X. maltophilia	X. fastidiosa
NO-2	100.0																								
A. calcoaceticus	82.0	100.0																							
A. tumefaciens	78.8	80.1	100.0																						
A. faecalis	94.5	82.3	79.1	100.0																					
B. avium	98.4	82.4	79.0	94.8	100.0																				
B. bronchiseptica	99.4	82.3	79.0	94.9	98.8	100.0																			
B. parapertussis	99.3	82.3	79.1	94.9	98.8	100.0	100.0																		
B. pertussis	99.5	82.1	78.8	94.6	98.7	99.7	99.7	100.0																	
C. violaceum	88.3	82.9	80.2	88.2	88.4	88.4	88.4	88.3	100.0																
C. testosteroni	88.3	80.2	77.2	87.6	88.3	88.4	88.4	88.4	86.5	100.0															
D. desulfuricans	76.9	77.0	78.5	76.2	76.6	77.0	77.0	76.9	76.7	76.2	100.0														
E. corrodens	88.7	83.1	79.4	89.7	88.8	89.1	89.0	88.8	90.7	85.4	75.9	100.0													
E. coli	81.5	84.6	78.5	81.2	81.4	81.5	81.5	81.4	80.8	80.8	78.7	81.3	100.0												
K. kingae	88.1	82.4	79.7	88.6	88.8	88.5	88.5	88.3	89.9	85.5	75.7	94.8	81.6	100.0											
N. gonorrhoeae	88.4	82.4	80.0	87.4	88.5	88.9	89.0	88.7	89.9	85.0	76.9	93.9	81.3	94.2	100.0										
N. multiformis	89.4	82.6	78.2	88.9	89.0	89.6	89.6	89.5	88.9	87.9	77.6	87.5	81.7	87.1	86.7	100.0									
N. europaea	86.6	81.4	77.0	86.4	86.5	86.8	86.7	86.7	85.9	86.2	76.4	85.3	79.7	84.9	84.5	92.7	100.0								
O. formigenes	91.2	82.2	78.0	90.8	91.2	91.6	91.6	91.4	88.2	88.9	76.4	89.4	81.5	88.4	88.0	90.3	87.9	100.0							
P. cepacia	89.2	82.8	79.4	89.2	89.3	89.5	89.5	89.4	88.8	88.4	76.6	88.7	81.0	88.1	87.8	88.3	86.4	90.6	100.0						
R. purpureus	88.9	83.6	79.3	88.7	89.3	89.1	87.5	88.9	89.1	87.6	77.8	87.2	81.0	87.5	87.7	90.1	87.6	90.0	89.8	100.0					
S. muelleri	88.3	82.5	79.7	88.6	88.6	88.6	88.6	88.4	89.5	84.8	75.5	95.4	81.1	94.4	93.1	86.3	83.7	87.6	87.1	86.9	100.0				
S. volutans	87.3	80.6	78.4	87.6	87.5	87.5	87.4	87.3	86.2	85.5	77.1	87.0	80.6	87.5	86.6	88.3	86.1	88.8	88.4	88.7	87.0	100.0			
V. stercoraria	88.5	84.3	81.3	88.7	88.8	88.8	88.8	88.6	91.6	87.2	77.3	93.6	81.9	93.3	92.7	87.3	87.3	88.5	88.2	88.2	92.9	88.3	100.0		
X. maltophilia	83.8	84.6	80.4	82.3	84.2	84.2	84.1	83.9	83.4	82.8	78.8	83.6	81.3	82.6	83.2	83.1	81.7	83.4	83.9	84.3	82.2	83.9	84.6	100.0	
X. fastidiosa	83.4	84.9	81.2	82.3	83.5	83.5	83.5	83.3	84.3	82.7	79.2	84.4	83.2	83.4	83.8	83.5	82.1	83.2	83.6	83.3	83.3	83.6	85.3	94.6	100

FIG. 1. 16S rDNA-rRNA similarity values for CDC group NO-2 strain F5101 and related organisms. Values are the percent similarity corrected for multiple base changes by the method of Jukes and Canter (23).

and *Xanthomonas maltophilia* (recently proposed name, *Stenotrophomonas maltophilia* [33]) are weak glucose oxidizers that may grow on MacConkey agar and produce a negative oxidase reaction. The urease and nitrate tests differentiate *B. canis* and the motility test differentiates *X. maltophilia* from NO-2. CFA analysis also differentiates *Brucella*, *Xanthomonas*, and *Acinetobacter* spp. and group NO-1 from NO-2 (31).

A 1,525-base sequence corresponding to bases 6 to 1540 of the *E. coli* 16S sequence (3) was determined for NO-2 strain F5101 and the type strains of *B. parapertussis*, *B. pertussis*, *B. bronchiseptica*, and *B. avium*. These sequences have been submitted to the GenBank database, and their accession numbers are listed in Table 2. Over the region sequenced, there were 15 differences between F5101 and *B. pertussis*, 18 differences with *B. parapertussis*, 20 differences with *B. bronchiseptica*, and 35 differences with *B. avium*. The differences appear to be randomly distributed.

A sequence similarity matrix based on edited 16S sequences from members of the beta, delta, and gamma subdivisions of the *Proteobacteria* was constructed. Representative results of the matrix are shown in Fig. 1. The sequence from strain F5101 was 99.5% similar to the *B. pertussis* sequence, 99.4% similar to the *B. bronchiseptica* sequence, 99.3% similar to the *B. parapertussis* sequence, and 98.4% similar to the *B. avium* sequence. Other 16S sequences that were greater than 90% similar to F5101 include *A. xylosoxidans* (97.3% similarity), *A. faecalis* (94.5% similarity), and *O. formigenes* (91.2%). All other sequences examined were less than 90% similar to F5101.

The similarity matrix was used to construct a dendrogram showing the phylogenetic order of the organisms on the basis of their edited 16S sequences. As shown in Fig. 2, strain F5101 formed a distinct cluster with the *Bordetella* species and was on a common line of descent with the genus *Alcaligenes*, another genus in the beta-2 subdivision of *Proteobacteria*. When a bootstrap analysis of the 40 aligned sequences was performed, the cluster of NO-2, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* occurred in 100 of 100 trees. In 29% of the trees, the branching order within the *Bordetella* cluster changed, but the members in the cluster remained the same, as seen in Fig. 3.

The G+C content of DNA was determined for five NO-2 strains and for the type strains of *B. avium*, *B. parapertussis*, and *B. pertussis* (Table 5). All NO-2 strains studied yielded G+C contents from 61.5 to 62.3 mol%. The respective type strains of the species of *Bordetella* yielded G+C contents from 60.2 to 66.7 mol%.

Labeled DNA from NO-2 strain F5101 was hybridized with unlabeled DNAs from 12 other NO-2 strains and from the type strains of *B. avium*, *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis* (Table 5). Relatedness among NO-2 strains was 89 to 100% (average, 96%). Divergence within related se-

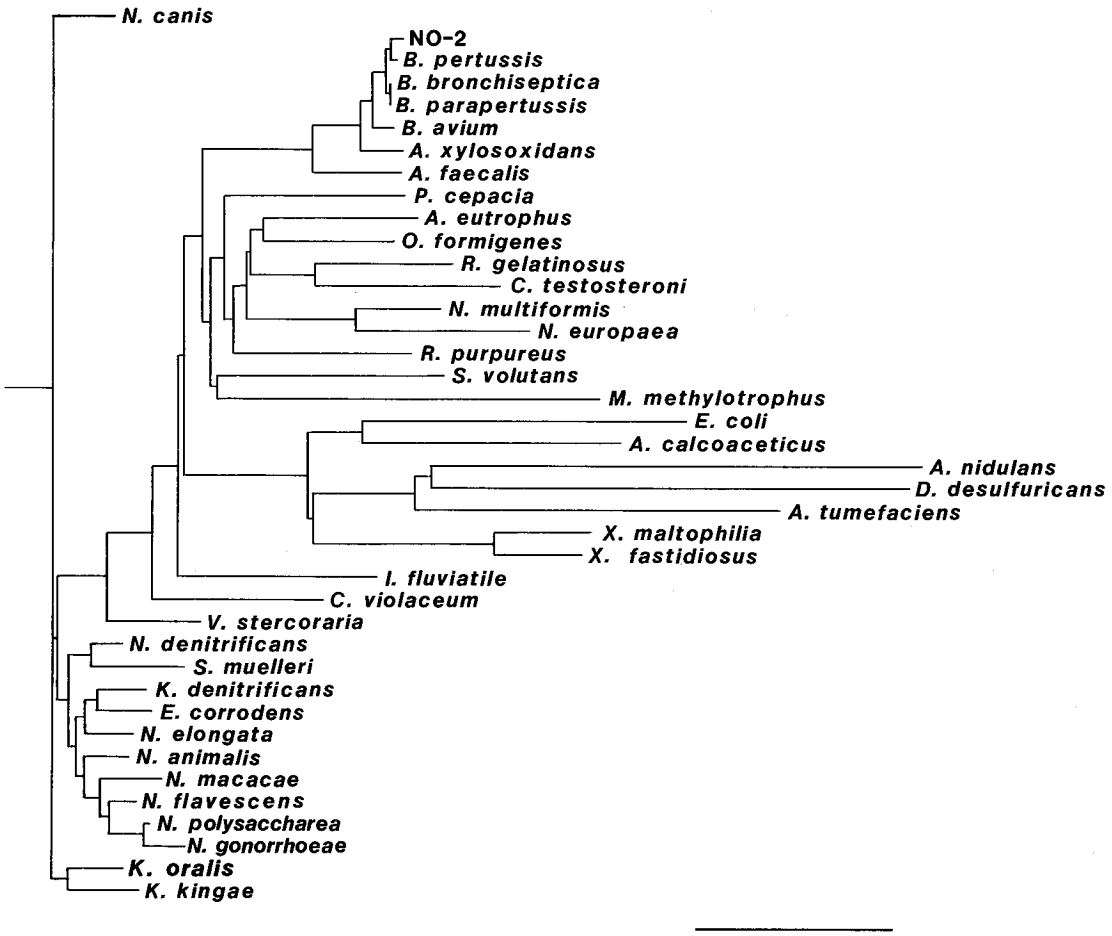


FIG. 2. Phylogenetic neighbor-joining dendrogram for alignment of 1,318-nucleotide section of 16S rDNA from CDC F5101 and species listed in Table 2. The bar represents a 5% difference in nucleotide sequence.

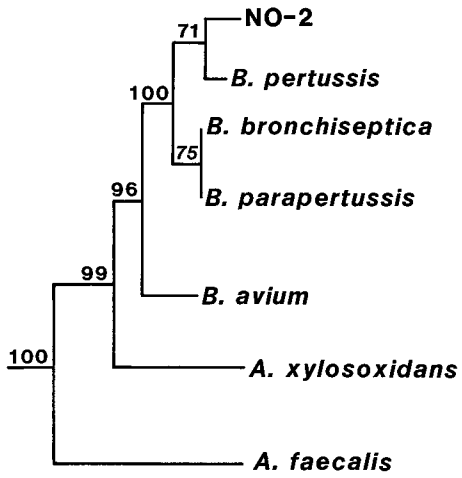


FIG. 3. Bootstrap analysis of the *Bordetella-Alcaligenes* 16S rRNA cluster. Numbers at the branch points represent the number of times the arrangement appeared in 100 randomly generated trees.

quences was 0 to 6% (average, 1.5%). Strain F5101 was 24 to 34% related to type strains of the four *Bordetella* species, with 13 to 14% divergence within the related sequences.

DISCUSSION

CDC group NO-2 currently consists of 15 strains of fastidious gram-negative bacteria isolated from human blood cultures. Isolates were obtained from nine states in the United States, Saudi Arabia, and Switzerland. At least four of these strains were obtained from two or more blood cultures per patient. Little clinical information is available on the patients; however, seven had underlying conditions (sickle cell anemia, diabetes, lymphoma, Hodgkin's disease, and prior splenectomies) that may have enhanced their susceptibilities to infection. Two other patients (associated with strains G8341 and G8350) may have developed postoperative NO-2 infections.

NO-2 strains are very similar biochemically and are closely related genetically. Our studies indicate that this group belongs in the genus *Bordetella*. They exhibit a CFA profile essentially identical to that observed with *B. avium* and contain ubiquinone-8, which is the major isoprenoid quinone of other *Bordetella* species (5). The 16S rRNA sequence of NO-2 shows a high level of homology with 16S rRNA sequences of the respective type strains of the species of *Bordetella*. The placement of NO-2 within the genus *Bordetella* is also supported by

TABLE 5. Relatedness of labeled DNA of CDC group NO-2 strain F5101 to other NO-2 strains and to type strains of *Bordetella* species

Source of unlabeled DNA	G+C content (mol%)	%Binding at 65°C	D^a
F5101	61.9	100	0.0
G7952		100	0.0
G6128	61.8	99	0.5
G7851		98	4.0
G5210	61.5	97	0.0
G133		97	0.0
G7702		97	2.0
F7732	62.1	96	1.5
G2910	62.3	95	0.0
G4363		95	1.0
G5245		94	0.0
G4102		93	6.0
F6119		89	0.0
B. avium ATCC 35086	60.2	34	14.0
B. parapertussis ATCC 15311	66.4	30	14.0
B. pertussis ATCC 9797	66.7	28	13.0
B. bronchiseptica ATCC 19395		24	13.0

[a] *D*, divergence, decrease in thermal stability (in degrees Celsius) of heterologous DNA duplexes compared with that of homologous DNA duplexes.

its G+C content of 61 to 62 mol%, similar to those of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (7).

Our DNA-DNA hybridization results confirm that all NO-2 strains tested are highly related (average of 96%) and are a single species, according to the longstanding species definition used in our laboratory and recommended by Wayne et al. (41). Relatedness of NO-2 to *Bordetella* species is 24 to 34%, higher than the 10 to 19% relatedness observed between *B. avium* and strains of other *Bordetella* species (2a), and supports assignment of NO-2 to this genus.

NO-2 is the first *Bordetella* species that is not associated with respiratory infections. It is the fifth named species (nomenspecies) in the genus, although we are of the opinion that only *B. pertussis* and *B. avium* are true species as defined genetically (strains whose DNAs are 70% or more related, with not more than 5% divergence within related sequences) (41). Since there is some controversy, species designation within the genus *Bordetella* merits some discussion.

Kloos et al. demonstrated that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are not separable by DNA hybridization (25). We obtained similar results (2a). The conclusion that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* are a single genospecies is also supported by the results of multilocus enzyme electrophoresis (32), rRNA-DNA hybridization (7), envelope proteins (11), and interspecies transformation studies (27).

Khattak and Matthews recently reported that restriction fragment patterns of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were species specific (a given pattern was present in only one of these three nomenspecies) (24). They concluded that restriction fragment length polymorphism, as demonstrated by pulsed-field gel electrophoresis, "reflects the overall organization of the bacterial genome and may for some species be a better indicator of clonal origin and genetic relatedness than data from other types of nucleic acid homology studies."

Although pulsed-field gel electrophoretic analysis of DNA restriction fragments may be more sensitive than other methods in determining clonal and overall genetic relatedness, this does not mean that it is a suitable criterion for creating or defining species. An effective species definition must apply to all species (41) and preferably be quantifiable, as is the case with the species definition based on DNA relatedness. A species definition based on DNA restriction patterns does not seem to be quantifiable: how many bands need be different; how close should the patterns be? Of more importance, what basis will be used to determine how many different patterns should be included in any given species? The number of restriction patterns observed within a species will have to be linked to another characteristic, or all species definitions will be completely subjective.

We propose that NO-2 be named *B. holmesii* and formally describe this new species below.

Description of *B. holmesii* sp. nov. *Bordetella holmesii* (holmes′i.i. N.L. gen. n. *holmesii*), named in honor of Barry Holmes, for his substantial contributions to the characterization, classification, and identification of unusual pathogenic and opportunistic bacteria (16–22). This species conforms to the description of the family *Alcaligenaceae* (7) and to the description of the genus *Bordetella* (34). The bacteria are gram-negative small coccoid and short rods, with medium-width longer rods occasionally observed. They are asaccharolytic, oxidase negative, nonmotile, and fastidious, and they produce a brown soluble pigment. Growth occurs on MacConkey agar at between 3 and 7 days of incubation at 35°C. Additional characteristics of this species are given in Results. The G+C content is 61.5 to 62.3 mol%. It has been found only in the blood of humans.

Description of the type strain. The type strain of *B. holmesii* is CDC F5101 (ATCC 51541). It shares all of the characteristics of the species. The G+C content is 61.9 mol%. The sequence of its 16S rRNA was deposited in the GenBank database under accession number U04820. It was isolated from the blood of a 37-year-old man in Buffalo, N.Y.

REFERENCES

1. **Bilofsky, H. S., and C. Burks.** 1988. The GenBank genetic sequence data bank. Nucleic Acids Res. **16:**1861–1864.
2. **Brenner, D. J., A. C. McWhorter, J. K. Leete Knutson, and A. G. Steigerwalt.** 1982. *Escherichia vulneris*, a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. **15:**1133–1140.

2a. **Brenner, D. J., and A. G. Steigerwalt.** Unpublished data.

3. **Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller.** 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. **148:**107–127.
4. **Clark, W. A., D. G. Hollis, R. E. Weaver, and P. Riley.** 1984. Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria. Centers for Disease Control, Atlanta.
5. **Collins, M. D., and D. Jones.** 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. Microbiol. Rev. **45:**316–354.
6. **Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor.** 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. **31:**872–881.
7. **De Ley, J., P. Segers, K. Kersters, W. Mannheim, and A. Lievens.** 1986. Intra- and intergeneric similarities of the *Bordetella* ribosomal ribonucleic acid cistrons: proposal for a new family, *Alcaligenaceae*. Int. J. Syst. Bacteriol. **36:**405–414.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Devereux, R., S.-H. He, C. L. Doyle, S. Orkland, J. LeGall, and W. B. Whitman.** 1990. Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. J. Bacteriol. **172:**3609–3619.
10. **Dewhirst, F. E., B. J. Paster, and P. L. Bright.** 1989. *Chromobacterium*, *Eikenella*, *Kingella*, *Neisseria*, *Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.). Int. J. Syst. Bacteriol. **39:**258–266.
11. **Ezzell, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark.** 1981. Phase-shift markers in *Bordetella*: alterations in envelope proteins. J. Infect. Dis. **143:**562–569.
12. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.

13. **Felsenstein, J.** 1989. PHYLIP–phylogeny inference package (version 3.2). Cladistics **5:**164–166.
14. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **139:**1147–1153.
15. **Hollis, D. G., C. W. Moss, M. I. Daneshvar, L. Meadows, J. Jordan, and B. Hill.** 1993. Characterization of Centers for Disease Control group NO-1, a fastidious, nonoxidative, gram-negative organism associated with dog and cat bites. J. Clin. Microbiol. **31:**746–748.
16. **Holmes, B., M. Costas, S. L. W. On, P. Vandamme, E. Falsen, and K. Kersters.** 1993. *Neisseria weaveri* sp. nov. (formerly group M-5), from dog bite wounds of humans. Int. J. Syst. Bacteriol. **43:**687–693.
17. **Holmes, B., R. J. Owen, and T. A. McMeekin.** 1984. Genus *Flavobacterium* Bergey, Harrison, Breed, Hammer, and Huntoon 1923, 97^{AL}, p. 353–361. *In* N. R. Kreig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
18. **Holmes, B., R. J. Owen, A. G. Steigerwalt, and D. J. Brenner.** 1984. *Flavobacterium gleum*, a new species found in human clinical specimens. Int. J. Syst. Bacteriol. **34:**21–25.
19. **Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner.** 1986. *Weeksella zoohelcum* sp. nov. (formerly group IIj), from human clinical specimens. Syst. Appl. Microbiol. **8:**191–196.
20. **Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner.** 1986. *Weeksella virosa* gen. nov., sp. nov. (formerly group IIf), found in human clinical specimens. Syst. Appl. Microbiol. **8:**185–190.
21. **Holmes, B., A. G. Steigerwalt, R. E. Weaver, and D. J. Brenner.** 1987. *Chryseomonas luteola* comb. nov. and *Flavimonas oryzihabitans* gen. nov., comb. nov., *Pseudomonas*-like species from human clinical specimens and formerly known, respectively, as groups Ve-1 and Ve-2. Int. J. Syst. Bacteriol. **37:**245–250.
22. **Holmes, B., R. E. Weaver, A. G. Steigerwalt, and D. J. Brenner.** 1988. A taxonomic study of *Flavobacterium spiritivorum* and *Sphingobacterium mizutae*: proposal of *Flavobacterium yabuuchiae* sp. nov. and *Flavobacterium mizutaii* comb. nov. Int. J. Syst. Bacteriol. **38:**348–353.
23. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism, vol. 3. Academic Press, Inc., New York.
24. **Khattak, M. N., and R. C. Matthews.** 1993. Genetic relatedness of *Bordetella* species as determined by macrorestriction digests resolved by pulsed-field gel electrophoresis. Int. J. Syst. Bacteriol. **43:**659–664.
25. **Kloos, W. E., N. Mohapatra, W. J. Dobrogosz, J. W. Ezzell, and C. R. Manclark.** 1981. Deoxyribonucleotide sequence relationships among *Bordetella* species. Int. J. Syst. Bacteriol. **31:**173–176.
26. **Kumano, M., N. Tomioka, K. Shinozaki, and M. Sugiura.** 1986. Analysis of the promoter region in the rRNA operon from a blue-green alga, *Anacystis nidulans* 6301. Mol. Gen. Genet. **202:**173–178.
27. **Manclark, C. R., and J. C. Hill (ed.).** 1979. International Symposium on Pertussis. U.S. Department of Health, Education, and Welfare publication no. 79-1839. National Institutes of Health, Washington, D.C.
28. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5:**109–118.

28a. **Moss, C. W.** Unpublished observation.

29. **Moss, C. W., and G. O. Guerrant.** 1983. Separation of bacterial ubiquinones by reverse-phase high-performance liquid chromatography. J. Clin. Microbiol. **18:**15–17.
30. **Moss, C. W., and M. A. Lambert-Fair.** 1989. Location of double bonds in monounsaturated fatty acids of *Campylobacter cryaerophila* with dimethyl disulfide derivatives and combined gas chromatography-mass spectrometry. J. Clin. Microbiol. **27:**1467–1470.
31. **Moss, C. W., P. L. Wallace, D. G. Hollis, and R. E. Weaver.** 1988. Cultural and chemical characterization of CDC groups EO-2, M-5, and M-6, *Moraxella* (*Moraxella*) species, *Oligella urethralis*, *Acinetobacter* species, and *Psychrobacter immobilis*. J. Clin. Microbiol. **26:**484–492.
32. **Musser, J. M., E. L. Herlett, M. S. Peppler, and R. K. Selander.** 1986. Genetic diversity and relationships in populations of *Bordetella* spp. J. Bacteriol. **166:**230–237.
33. **Palleroni, N. J., and J. F. Bradbury.** 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. Int. J. Syst. Bacteriol. **43:**606–609.
34. **Pittman, M.** 1984. Genus *Bordetella* Moreno-López 1952, 178^{AL}, p. 388–393. *In* N. R. Kreig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
35. **Regan, J., and F. Lowe.** 1977. Enrichment medium for the isolation of *Bordetella*. J. Clin. Microbiol. **6:**303–309.
36. **Rossau, R., L. Heyndrickx, and H. van Heuverswyn.** 1988. Nucleotide sequence of a 16S ribosomal RNA gene from *Neisseria gonorrhoeae*. Nucleic Acids Res. **16:**6227.
37. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
38. **Stackebrandt, E., and O. Charfreitag.** 1990. Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii* specific oligonucleotide probe. J. Gen. Microbiol. **136:**37–43.
39. **Stainer, D., and M. Scholte.** 1971. A simple chemical defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
40. **Tsuji, K., H. C. Tsien, R. S. Hanson, S. R. DePalma, R. Scholtz, and S. LaRoche.** 1989. 16S ribosomal RNA sequence analysis for determination of phylogenetic relationships among methylotrophs. J. Gen. Microbiol. **136:**1–10.
41. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463–464.
42. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
43. **Wells, J. M., B. C. Raju, H.-Y. Hung, W. G. Weisburg, L. Madelco-Paul, and D. J. Brenner.** 1987. *Xylella fastidiosa* gen. nov., sp. nov.: gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. Int. J. Syst. Bacteriol. **37:**136–143.
44. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
45. **Yabuuchi, E., K. Yoshimasa, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36:**1251–1275.
46. **Yang, D., Y. Oyaizu, H. Oyaizu, G. J. Olsen, and C. R. Woese.** 1985. Mitochondrial origins. Proc. Natl. Acad. Sci. USA **82:**4443–4447.