Nucleic Acid Homologies of Some Vancomycin-Resistant *Leuconostocs* and Description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomesenteroides* sp. nov.

JOHN A. E. FARROW,¹ RICHARD R. FACKLAM,² AND MATTHEW D. COLLINS^{1*}

Department of Microbiology, Agricultural Food Research Council Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT, United Kingdom,¹ and Centers for Disease Control, Atlanta, Georgia 30333²

Deoxyribonucleic acid-deoxyribonucleic acid hybridizations were performed with 31 vancomycin-resistant gram-positive cocci isolated from human sources. A total of 14 human isolates exhibited high levels of homology with *Leuconostoc mesenteroides* subsp. *mesenteroides* NCDO 523^T (T = type strain), whereas 2 strains were genetically closely related to *Leuconostoc lactis* NCDO 533^T. Nine human isolates formed a tight homology group with an atypical *L. mesenteroides* strain (strain NCIB 3351), whereas two isolates (together with four strains from food) were found to be highly related to *Leuconostoc* sp. strain NCDO 768^T. Two new species, *Leuconostoc citreum* and *Leuconostoc pseudomesenteroides*, are proposed for these homology groups. The type strains of *L. citreum* and *L. pseudomesenteroides* are strains NCDO 1837 and NCDO 768, respectively.

Vancomycin is a glycopeptide antibiotic that is used as a therapeutic agent for gram-positive coccal infections. Recently, there have been a number of reports of vancomycin-resistant "leuconostocs" from clinical sources (1–3, 10, 11). *Leuconostocs* are normally found living in association with plant material and are common in dairy and other food products. *Leuconostocs* are generally considered nonpathogenic for humans and animals (6). The assignment of vancomycin-resistant clinical isolates to the genus *Leuconostoc* is based entirely on simple morphological and physiological tests. However, it is recognized that it is extremely difficult to reliably distinguish leuconostocs from some gram-positive cocci and certain lactobacilli (6). For example, the separation of leuconostocs from some heterofermentative lactobacilli (e.g., *Lactobacillus confusus* and *Lactobacillus viridescens*) is often based solely on Gram-stained films from broth cultures (leuconostocs are more coccoid than rodlike, and lactobacilli are more rodlike than coccoid). To our knowledge there has been no definitive identification of leuconostocs from human sources by using modern molecular techniques. Therefore, in this study 31 vancomycin-resistant cocci isolated from clinical sources were examined by using deoxyribonucleic acid (DNA)-DNA molecular hybridization in an attempt to determine their generic and specific status.

MATERIALS AND METHODS

Strains. Details of the test strains and their sources are given in Table 1.

DNA base composition and DNA-DNA hybridization. Cells were grown in MRS broth at 25°C and were harvested in late exponential phase by centrifugation. DNA was prepared by using a modification (4) of the method of Garvie (5). DNA base composition was estimated by thermal denaturation in standard saline citrate, using DNA from *Escherichia coli* K-12 NCDO 1984 (guanine-plus-cytosine [G+C] content, 51.5 mol%) as the standard. DNA-DNA hybridizations were performed under optimum conditions (25°C below the melting temperature) by using the membrane filter method described previously (7). The washing procedure of Garvie (5) was modified to include one wash consisting of 10 ml of 0.2

× standard saline citrate per filter to reduce nonspecific binding.

Biochemical tests. Biochemical tests were performed by using the API 50CH and API 10E systems (API-Biomérieux Ltd., Basingstoke, United Kingdom) according to the instructions of the manufacturer. Test preparations were incubated at 30°C, and readings were made after 24 and 48 h. Pigment production was observed in wet packed cells harvested from MRS broth by centrifugation.

RESULTS AND DISCUSSION

The DNA base compositions of the test strains are shown in Table 1. The clinical isolates had G+C contents which ranged from ca. 36 to 43 mol%. These values are within the accepted G+C content range (ca. 37 to 45 mol%) for the genus *Leuconostoc* (8, 9). The results of the DNA-DNA hybridization experiments are shown in Table 2. The clinical isolates fell into four groups on the basis of DNA hybridization results. A total of 14 human isolates exhibited high homology levels (ca. 60 to 100%) with the type strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* (strain NCDO 523), whereas 2 strains were found to be genomically closely related to *Leuconostoc lactis* NCDO 533^T (T = type strain). Nine of the clinical strains formed a relatively homogeneous group which showed a high degree of relatedness (ca. 80 to 100%) with an atypical *Leuconostoc mesenteroides* strain (strain NCIB 3351). Of the remaining human isolates, two strains were found to be closely related to *Leuconostoc* sp. strain NCDO 768^T. Four other strains isolated from food and designated *Leuconostoc mesenteroides* were also related to this group. Members of the strain NCIB 3351 and NCDO 768^T homology groups were genetically distinct from each other and from all other *Leuconostoc* species. If 70% homology is taken as the border line or guideline for species differentiation, the DNA results clearly demonstrate that the two homology groups warrant separate specific status. The name *Leuconostoc citreum* sp. nov. is proposed for strains belonging to the strain NCIB 3351 group, and the name *Leuconostoc pseudomesenteroides* sp. nov. is proposed for the strain NCDO 768^T group. The differential biochemical characteristics of *Leuconostoc citreum*, *Leuconostoc pseudomesenteroides*, and other *Leuconostoc* species are shown in Table 3. *Leuconostoc citreum*

* Corresponding author.

TABLE 1. Strain histories and DNA base compositions of the test strains

Strain	Source and/or comments	G+C content (mol%)
<i>Leuconostoc citreum</i>		
NCDO 1837 ^T	Honey dew of rye ear; received as <i>Leuconostoc mesenteroides</i>	39.8
NCIB 3351	Canned tomatoes; received as <i>Leuconostoc mesenteroides</i>	40.3
1562-84	Blood	39.4
71-86	Blood	38.0
192-86	Blood	39.4
1352-86	Blood	39.5
2985-86	Blood	ND ^a
3228-86	Blood, cerebrospinal fluid	40.3
133-87	Blood	38.6
1363-87	Medical source unknown	ND
2028-87	Blood	39.2
<i>Leuconostoc lactis</i>		
NCDO 533 ^T	ATCC 19256, DSM 20202	44.7
NCDO 2037	Received from T. Cogan	43.1
644-88	Blood	ND
606-88	Blood	ND
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> (phenotype)		
NCDO 543 ^T	Dried starter powder; ATCC 19254, DSM 20346	38.7
NCDO 183	Received as <i>Leuconostoc dextranicum</i>	32.3
NCDO 769	Swedish fermented milk	37.8
NCDO 812	Received as <i>Leuconostoc dextranicum</i> ATCC 8082; NCIB 8189	38.0
NCDO 828	The Netherlands	39.3
NCDO 829	The Netherlands	37.8
NCDO 887	Received as <i>Leuconostoc dextranicum</i>	36.4
NCDO 1226	Cheese starter	38.8
NCDO 1388	Received from P. R. Elliker	38.8
NCDO 1589	Sauerkraut; received as <i>Leuconostoc dextranicum</i>	37.1
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> (phenotype)		
NCDO 523 ^T	Olives; ATCC 8293, DSM 20343	37.9
NCDO 520	Silage	35.2
NCDO 527	<i>Leuconostoc</i> sp.	36.7
NCDO 529 ^T	<i>Leuconostoc dextranicum</i> (type strain)	38.5
NCDO 540	Produces dextran	35.6
NCDO 547	Raw milk	34.0
NCDO 551	Fermenting beans; received as <i>Leuconostoc dextranicum</i> ; ATCC 8086, NCIB 8029	35.9
NCDO 797	ATCC 9135, DSM 20241	36.4
NCDO 800	Slimy sugar syrup; received as <i>Leuconostoc dextranicum</i>	34.1
NCDO 861	Cheese, The Netherlands; received as <i>Leuconostoc dextranicum</i>	37.3
NCDO 877	Received from J. Naylor	38.0
NCDO 888	Received from J. Naylor	37.2
NCDO 2028	Beetroot silage	37.3
1190-77	Wound	39.1
738-78	Blood	36.5
872-78	Cerebrospinal fluid	37.0
1304-80	Blood	ND
1305-80	Blood	ND

Continued

TABLE 1—Continued

Strain	Source and/or comments	G+C content (mol%)
1007-81	Wound	36.9
1008-81	Medical, unknown	37.0
1055-82	Urine	ND
1591-82	Blood	37.5
1336-83	Blood	37.5
1661-85	Blood	37.5
225-86	Blood	ND
2906-87	Blood	36.3
128-88	Blood	36.7
SML 41	Meat; received from G. Shaw	36.7
SML 50	Meat; received from G. Shaw	37.1
<i>Leuconostoc para-mesenteroides</i>		
NCDO 803 ^T	ATCC 33313, DSM 20288	39.3
NCDO 871	Silage	38.6
<i>Leuconostoc pseudomesenteroides</i>		
NCDO 768 ^T	Received as <i>Leuconostoc mesenteroides</i> ATCC 12991, DSM 20193	39.3
NCDO 882	Received from J. Naylor	38.1
NCDO 1590	Received as <i>Leuconostoc dextranicum</i> NCIB 3740	40.6
NCDO 2053	Starter, Switzerland	38.6
NCDO 2069	Received as <i>Leuconostoc mesenteroides</i> NRRL B1416	40.8
2187-81	Blood	40.0
1296-87	Blood	38.7
<i>Leuconostoc</i> species		
NCDO 1562	Canker in hardwood	35.9
780-86	Lung	38.0
1882-86	Blood	36.9
2325-87	Blood	42.5
2850-87	Blood	ND
H18	Meat; received from G. Shaw	37.8
L19	Meat; received from G. Shaw	38.6
1A21	Meat; received from G. Shaw	37.1

^a ND, Not determined.

produces a lemon yellow pigment and may be readily distinguished from *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc pseudomesenteroides* because it does not produce acid from ribose. Strains of *Leuconostoc citreum* are also α - and β -D-galactosidase and β -D-xylosidase negative, whereas *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc pseudomesenteroides* give positive reactions. In contrast, differentiation between *Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc pseudomesenteroides* is difficult. *Leuconostoc mesenteroides* subsp. *mesenteroides* produces acid from mannitol but not from starch after 7 days of incubation, whereas *Leuconostoc pseudomesenteroides* strains give the opposite reactions. In addition, *Leuconostoc pseudomesenteroides* is L-isoleucine arylamidase and L-proline arylamidase negative. *Leuconostoc citreum* and *Leuconostoc pseudomesenteroides* are easily distinguished from all other *Leuconostoc* species (Table 3).

Description of *Leuconostoc citreum* sp. nov. *Leuconostoc citreum* (cit're.um. M.L.adj. *citreum*, lemon colored). Spherical or lenticular cells (ca. 0.5 to 0.7 by 0.7 to 1.2 μ m) that occur usually in pairs or short chains. Gram positive. Nonmotile. Most strains produce a lemon yellow pigment. Grows at 10 and 30°C; some strains grow at 37°C but not at 40°C. Acid is produced from N-acetylglucosamine, esculin,

TABLE 2. DNA-DNA homologies of *Leuconostoc* species

Strain	% Homology with [³ H]DNA from:					
	<i>Leuconostoc citreum</i> NCIB 3351	<i>Leuconostoc lactis</i> NCDO 533 ^T	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>		<i>Leuconostoc pseudomesenteroides</i> NCDO 768 ^T	<i>Leuconostoc paramesenteroides</i> NCDO 803 ^T
			NCDO 523 ^T	NCDO 520		
<i>Leuconostoc citreum</i>						
NCDO 1837 ^T	91		21		27	
NCIB 3351	100	23	23	25	38	10
1562-84	101		26		38	16
71-86	102		28		34	
192-86	104		25		45	20
1352-86	100		22		33	16
2985-86	81		23		30	16
3228-86	100		30		54	17
133-87	100		27		47	20
1363-87	94		29		37	16
2028-87	102		26		56	17
<i>Leuconostoc lactis</i>						
NCDO 533 ^T		100		29		12
NCDO 2037	52	81	21	41	28	
644-88	52	99	24	44	29	14
606-88	53	97	16		28	
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> (phenotype)						
NCDO 523 ^T	27	7	100	62	43	14
NCDO 527			77		45	
NCDO 540			108		52	
NCDO 547			75		24	
NCDO 551			94		24	
NCDO 797			79		35	
NCDO 861			87		34	
NCDO 877			92		62	
NCDO 888			83		63	
NCDO 2028			86		43	
1190-77			73		45	
738-78			94		38	
1304-80			92		39	
1305-80			80		33	
1007-81			76		53	
1008-81			85		43	
1591-82			75		43	
128-87			75		36	
2906-87			82		38	
SML 41			86		36	
NCDO 520	40	12	55	100	33	
NCDO 800	48		62	73	29	
865-78	49	13	60	117	35	14
872-78	47	15	58	97	29	
1055-82	56		66	110	50	16
1336-83	53		70	100	44	
1661-85			69	106	44	
SML 50	52		60	111	24	
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> (phenotype)						
NCDO 534 ^T	38	17	66	98	33	
NCDO 183	45			82	27	
NCDO 887	45	14		77	27	10
NCDO 1226	48		58	91	31	
NCDO 1388			72	79	30	
NCDO 1589			93		37	
NCDO 529	54		100		39	
NCDO 769			101		49	
NCDO 812			91		28	
NCDO 828			106		32	
NCDO 829			90		50	
<i>Leuconostoc pseudomesenteroides</i>						
NCDO 768 ^T	51	13	18	28	100	9
NCDO 882	44		23		90	

Continued on following page

TABLE 2—Continued

Strain	% Homology with [³ H]DNA from:				
	<i>Leuconostoc</i> <i>citreum</i> NCIB 3351	<i>Leuconostoc</i> <i>lactis</i> NCDO 533 ^T	<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>		<i>Leuconostoc</i> <i>pseudomesenteroides</i> NCDO 768 ^T
			NCDO 523 ^T	NCDO 520	<i>Leuconostoc</i> <i>paramesenteroides</i> NCDO 803 ^T
NCDO 1590	55		33		100
NCDO 2053			38		100
NCDO 2069	51		31	50	70
2187-81			46		100
1596-87			32		103
<i>Leuconostoc paramesenteroides</i>					
NCDO 803 ^T	22	8	9	13	14
NCDO 871	31		10		30
<i>Leuconostoc</i> sp.					
1A 21	46		24		53
H18	52	17	20	24	43
L19	50	19	22	32	37
NCDO 1562	44	10	31	42	31
780-86	40		10	20	16
1882-86	54		43	36	51
2325-87	34		26	30	24
2850-87	49			37	

D-fructose, glucose, D-mannose, maltose, α-methyl-D-glucoside, sucrose, trehalose, and D-turanose. The vast majority of strains produce acid from amygdalin, arbutin, L-arabinose, cellobiose, β-gentiobiose, gluconate, D-mannitol, and salicin. A few strains produce acid from 2-keto-glu-

conate, galactose, and D-xylose. Acid is not produced from adonitol, D-arabitol, L-arabitol, D-arabinose, dulcitol, erythritol, D-fucose, L-fucose, glycerol, glycogen, inositol, inulin, 5-keto-gluconate, lactose, D-lyxose, melezitose, melibiose, α-methyl-D-mannoside, β-methyl-xyloside, raffinose, ribose,

TABLE 3. Differential characteristics of *Leuconostoc* species

Characteristic	<i>Leuconostoc</i> <i>citreum</i> (n = 11) ^a	<i>Leuconostoc</i> <i>lactis</i> (n = 4)	<i>Leuconostoc</i> <i>mesenteroides</i> subsp. <i>mesenteroides</i> (n = 30)	<i>Leuconostoc</i> <i>mesenteroides</i> subsp. <i>cremoris</i> (n = 14)	<i>Leuconostoc</i> <i>paramesenteroides</i> (n = 3)	<i>Leuconostoc</i> <i>pseudomesenteroides</i> (n = 7)
Acid produced from ^b :						
L-Arabinose	91 ^c	25	83	0	100	71
Ribose	0	0	84	28	100	100
D-Xylose	18	0	96	29	0	100
D-Galactose	18	100	87	86	100	86
D-Fructose	100	100	100	43	100	100
D-Mannose	100	100	100	43	100	100
D-Mannitol	55	0	18 (97) ^d	7 (14)	0 (100)	0 (14)
α-Methyl-D-glucoside	100	0	94	21	100	86
Amygdalin	82	0	60	0	0	29
Arbutin	91	0	66	0	0	14
Esculin	100	0	97	7	33	57
Salicin	91	0	93	0	33	43
Cellobiose	91	0	73	0	33	71
Maltose	100	100	100	21	100	100
Lactose	0	100	53	65	0	58
Melibiose	9	75	97	29	100	86
Sucrose	100	100	100	50	100	71
Trehalose	100	50	100	43	100	100
Raffinose	0	75	77	21	33	86
Starch	0	0	0	0	0	14 (76)
β-Gentiobiose	91	0	70	7	33	58
D-Turanose	100	0	87	7	100	71
Gluconate	91	0	57	7	100	57
2-Keto-gluconate	45 (63)	0	3	0	33	14 (43)
5-Keto-gluconate	0	0	0 (12)	0	0	14 (43)
Lemon yellow pigment ^e	91	0	7	14	0	14

^a n is the number of strains tested.

^b Tests were performed by using the API 50CH system; readings were determined at 48 h.

^c Percentage of strains positive.

^d Values in parentheses refer to readings at 7 days.

^e Pigment detected in centrifuged cell pellet from MRS broth.

L-rhamnose, sorbitol, L-sorbose, starch, D-tagatose, xylitol, or L-xylose. Arginine dihydrolase, β -D-xylosidase, and urease negative. The G+C content of the DNA ranges from 38.0 to 40.3 mol% (thermal denaturation method). Isolated from food and clinical sources. The type strain is strain NCDO 1837. In most respects the description of the type strain corresponds to the description of the species. The type strain produces acid from amygdalin, L-arabinose, arbutin, cellobiose, β -gentiobiose, gluconate, mannitol, and salicin. Acid is not produced from melibiose, raffinose, or D-xylose.

Description of *Leuconostoc pseudomesenteroides* sp. nov. *Leuconostoc pseudomesenteroides* (pseu.do.me.sen.ter.oi'des. Gr.adj. *pseudos*, false; Gr.n. *mesenterium*, the mesentery; Gr.n. *oides*, form, shape; M.L.adj. *pseudomesenteroides*, not the true *mesenteroides*). Spherical or lenticular cells (ca. 0.5 to 0.7 by 0.7 to 1.2 μ m) that occur usually in pairs or short chains. Gram positive. Nonmotile. Most strains are nonpigmented. Grows at 10 and 37°C. Acid is produced from N-acetylglucosamine, D-fructose, D-glucose, maltose, D-mannose, melibiose, α -methyl-D-glucoside, raffinose, ribose, trehalose, and D-xylose. Most strains produce acid from L-arabinose, cellobiose, esculin, galactose, β -gentiobiose, lactose, sucrose, and D-turanose. A few strains produce acid from amygdalin, arbutin, 2-keto-gluconate, 5-keto-gluconate, and mannitol (after 7 days). Acid is not produced from adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, glycerol, inositol, inulin, D-lyxose, melezitose, α -methyl-D-mannoside, L-rhamnose, L-sorbose, sorbitol, D-tagatose, L-xylose, or xylitol. Arginine dehydrolase, urease, L-isoleucine arylamidase, and L-proline arylamidase negative. The G+C content of the DNA ranges from 38.1 to 40.8 mol% (thermal denaturation method). Isolated from dairy sources, food, and clinical sources. The type strain is strain NCDO 768. In most respects the description of the type strain resembles the description of the species. The type strain produces acid from L-arabinose, cellobiose, 2-keto-gluconate, 5-keto-glu-

conate, galactose, β -gentiobiose, gluconate, and lactose. Acid is not produced from amygdalin, arbutin, mannitol (after 7 days), salicin, or starch.

LITERATURE CITED

1. Colman, G., and A. Efstratiou. 1987. Vancomycin-resistant leuconostocs, lactobacilli and now pediococci. *J. Hosp. Infect.* **10**:1-3.
2. Coovadia, Y. M., Z. Solwa, and J. Van Den Ende. 1987. Meningitis caused by vancomycin-resistant *Leuconostoc* sp. *J. Clin. Microbiol.* **25**:1784-1785.
3. Coovadia, Y. M., Z. Solwa, and J. Van Den Ende. 1988. Potential pathogenicity of *Leuconostoc*. *Lancet* **i**:3-7.
4. Farrow, J. A. E., D. Jones, B. A. Phillips, and M. D. Collins. 1983. Taxonomic studies on some group D streptococci. *J. Gen. Microbiol.* **129**:1423-1432.
5. Garvie, E. I. 1976. Hybridization between the deoxyribonucleic acids of some strains of heterofermentative lactic acid bacteria. *Int. J. Syst. Bacteriol.* **26**:116-122.
6. Garvie, E. I. 1986. Genus *Leuconostoc*, p. 1071-1075. In P.H.A. Sneath (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. The Williams & Wilkins Co., Baltimore.
7. Garvie, E. I., J. A. E. Farrow, and B. A. Phillips. 1981. A taxonomic study of some strains of streptococci which grow at 10°C but not at 45°C, including *Streptococcus lactis* and *Streptococcus cremoris*. *Zentralbl. Bakteriol. Parasitenkd. Infektionsskr. Hyg. Abt. 1 Orig. Reihe C* **2**:151-165.
8. Garvie, E. I., V. Zezula, and V. A. Hill. 1974. Guanine plus cytosine content of the deoxyribonucleic acids of the leuconostocs and some heterofermentative lactobacilli. *Int. J. Syst. Bacteriol.* **24**:248-251.
9. Hontebryrie, M., and F. Gasser. 1977. Deoxyribonucleic acid homologies in the genus *Leuconostoc*. *Int. J. Syst. Bacteriol.* **27**:9-14.
10. Horowitz, H. W., S. Handwerger, K. G. Van Horn, and G. P. Wormser. 1987. *Leuconostoc*, an emerging vancomycin-resistant pathogen. *Lancet* **ii**:1329-1330.
11. Isenberg, H. D., E. M. Vellozzi, J. Shapiro, and L. G. Rubin. 1988. Clinical laboratory challenges in the recognition of *Leuconostoc* spp. *J. Clin. Microbiol.* **26**:479-483.