Optical DNA-DNA Homology in the Genus *Listeria*

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Twenty-three strains of the seven recognized *Listeria* species were studied by using DNA-DNA optical hybridization. The level of error in the data was low. Our results supported the results of Rocourt et al. (J. Rocourt, F. Grimont, P. A. D. Grimont, and H. P. R. Seeliger, *Curr. Microbiol.* 7:383-388, 1982), although there was some overlap between *Listeria monocytogenes* and *Listeria innocua*. We suggest that there may be more than one cluster in the species *L. monocytogenes* or the species may form a large spectrum of relatedness. The level of intraspecies homology in *L. monocytogenes* is very broad, as determined in both this study and other studies.

The members of the genus *Listeria* are gram-positive, nonsporing, motile rods. In the latest edition of *Bergey's Manual of Systematic Bacteriology* (13) Seeliger and Jones list five species (*Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, and *Listeria ivanovii*) and three species incertae sedis (*Listeria murrayi*, *Listeria*

levels of similarity of 81 to 87% (5). The strains of each species cluster at similarity levels of approximately 90%.

In their DNA studies, Rocourt et al. (10) divided what was the *L. monocytogenes* complex into the five species that are recognized today; this division was supported by a small number of biochemical differences (11). However, only six

TABLE 1. Bacterial strains

Species	Serotype	Strain ^a	Source
<i>L. monocytogenes</i>	1/2a	C52 (= NCTC 7973)	Guinea pig mesenteric lymph node
	2	C200 (= NCTC 5348 = ATCC 19112)	Cerebrospinal meningitis
	1/2a	C201 ^T (= NCTC 10357 ^T = ATCC 15313 ^T) ^b	Rabbit
	3a	C202 (= NCTC 5105 = ATCC 19113)	Human
	4b	C203 (= NCTC 4885 = ATCC 19115)	Infant meningitis
	4a	C228 (= NCTC 5214 = ATCC 19114)	Sheep brain
	4c	C231 (= NCTC 4883)	Fowl myocardial disease
<i>L. innocua</i>	6a	C644 ^T (= NCTC 11288 ^T = ATCC 33090 ^T = SLCC 3379 ^T)	Cow brain
	6b	C645 (= SLCC 3479)	
		JS21	Raw chicken
<i>L. welshimeri</i>		JS31	Raw chicken
	6a	C1091 ^T (= NCTC 11857 ^T = ATCC 35897 ^T)	Decaying vegetation
		C1172	
<i>L. seeligeri</i>		C1090 ^T (= NCTC 11856 = SLCC 3954 ^T)	
		C1171	Placenta, ovine abortion
<i>L. ivanovii</i>	5	C1087 ^T (= NCTC 11846 ^T = ATCC 19119 ^T = SLCC 2379 ^T)	Sheep
	5	C659 (= P L242)	Ovine abortion
	5	C663 (= P L72)	
	5	C666 (= P L234)	Ovine abortion
	5	C667 (= P L102B)	
<i>L. grayi</i>		C214 (= SLCC 332/64)	Chinchilla feces
		SLCC 7211	
<i>L. murrayi</i>		C1174 ^T (= NCTC 10812 ^T = ATCC 25401 ^T)	Cornstalks

^a C, University of Leicester Culture Collection; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; SLCC, Special *Listeria* Culture Collection; JS, isolated at University of Leicester; P, Pritchard.

^b T = type strain.

grayi, and *Listeria denitrificans*, which was recently reclassified as *Jonesia denitrificans* [12]). The first five taxa may be termed the *L. monocytogenes* complex.

Rocourt et al. (10) confirmed the low levels of DNA-DNA homology among *L. grayi*, *L. murrayi*, and other strains which were first observed by Stuart and Welshimer (15, 16). However, when numerical taxonomy is used, *L. murrayi* and *L. grayi* group closely with *L. monocytogenes* strains at

reference strains were used by these authors, and the intraspecies range of pairing values within *L. monocytogenes sensu stricto* was considerable (more than 35%).

DNA-DNA pairing values suggest that there is a much greater variation between species than the phenotypic characteristics imply, perhaps because a large portion of the genome is not expressed phenotypically.

The large variation in pairing values within *L. monocytogenes sensu stricto* raises the question of whether there may be two (or more) clusters within the species; alternatively, the strains may form a spectrum in terms of relatedness rather than distinct groups, and this second possibility is

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TABLE 2. Standard deviations obtained from replications of *Listeria* pairing data

Strains	Avg % pairing	n ^a	SD (%)
C52 and C200	67.0	2	2.5
C52 and C231	69.3	3	3.3
C52 and C201	89.4	4	1.0
C52 and C228	56.8	2	1.6
C52 and C644	71.9	7	3.2
C52 and JS31	46.6	3	0.7
C52 and C1090	52.2	3	1.8
C52 and C1091	52.6	5	3.0
C52 and C1172	44.1	2	1.0
C52 and C1087	44.7	3	0.9
C52 and C663	30.4	3	0.6
C52 and C667	36.8	3	1.8
C52 and C659	33.0	3	3.2
C52 and C666	35.7	2	4.3
C52 and SLCC 7211	23.4	4	1.7
C200 and C202	81.5	3	2.5
C200 and C231	73.8	2	2.1
C200 and C1090	55.0	3	0.6
C200 and C663	41.8	2	2.6
C202 and C203	85.1	2	1.5
C203 and C644	62.3	2	2.1
C203 and C663	34.5	2	0.5
C203 and C214	18.9	2	6.2
C231 and C644	44.6	2	3.4
C231 and C1171	53.4	4	2.3
C231 and C667	27.1	2	0.5
C231 and C666	38.4	3	1.2
C228 and JS21	51.2	3	0.6
C228 and C1091	51.7	3	1.6
C644 and C645	94.9	2	4.2
C644 and C1090	50.3	2	0.6
C644 and C1091	49.7	9	2.5
C644 and C1172	45.5	3	0.8
C644 and C663	27.8	3	1.6
C644 and C1174	15.9	2	4.0
C1171 and C645	44.9	4	3.4
JS21 and JS31	95.0	3	1.5
JS31 and C1090	59.7	2	3.5
JS31 and C1091	56.2	2	0.7
JS31 and C1087	38.9	2	0.1
JS31 and C214	24.9	2	2.6
C1090 and C1171	93.5	2	0.4
C1090 and C1091	46.2	3	1.7
C1090 and C1172	53.5	2	0.7
C1090 and C1087	37.3	5	4.4
C1090 and C663	43.0	3	3.1
C1090 and C659	35.4	2	1.8
C1090 and C214	13.6	2	0.2
C1171 and C663	35.9	2	0.4
C667 and C214	11.7	2	2.1
C1091 and C1172	91.6	2	0.5
C1091 and C1087	34.9	2	0.4
C1091 and C659	24.9	3	4.3
C1091 and SLCC 7211	10.5	2	0.6
C1172 and C1087	42.4	2	1.1
C1087 and C659	86.2	2	1.9
C1087 and C1174	14.3	2	0.8
C667 and C659	83.4	3	2.6
C667 and C666	84.9	2	0.1
C667 and SLCC 7211	16.8	2	11.3
C214 and C1174	84.3	2	3.2

^a n, number of replications.

supported by the small number of biochemical differences between strains.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *Listeria* strains used in this study are listed in Table 1. The collection numbers and sources of original isolation are given where they are known. Species identifications were taken from publications by experts on the genus (5, 10). *Listeria* strains were grown in tryptone soya broth (Oxoid) at 35°C and were harvested in the late exponential phase.

DNA extraction. A variation of the method of Marmur (8) was used to extract and purify DNAs.

Bacteria were lysed by incubating them with a lysozyme solution (final concentration, 1 mg ml⁻¹) for 1 h at 37°C, followed by 1 to 2 h of incubation with proteinase K (final concentration, 0.1 mg ml⁻¹) and 2% (wt/vol) sodium dodecyl sulfate. The protein was removed by subjecting preparations to phenol-chloroform extractions, and the DNA was spooled onto a glass rod. RNA was removed by treatment with RNase, followed by proteinase K treatment and phenol-chloroform extractions. The DNA was selectively precipitated with isopropanol, washed with 70% ethanol, and dissolved in 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

DNA diluted to a concentration of 200 to 300 µg ml⁻¹ in 2× SSC was sheared by using a French press to a length of 344 to 514 bp. The sheared DNA was dialyzed overnight at 4°C in 500 volumes of 2× SSC buffer.

DNA-DNA homologies. Purified sheared DNA samples were diluted prior to use with 2× SSC to give an A_{260} 1.55 ± 0.05 (i.e., a concentration of 75 to 80 µg ml⁻¹).

A set of matched semimicro quartz cuvettes equipped with Teflon stoppers were used for the study described below. A volume of 700 µl was used for the homology experiments. A Philips model PU8700 spectrophotometer equipped with a cell programmer and a Peltier heating block was programmed to melt the DNA and follow its renaturation at the optimal renaturation temperature (T_{or}).

The melting temperature (T_m) was determined with the spectrophotometer by increasing the temperature by 1°C min⁻¹ until the DNA was completely single stranded (90 to 96°C depending on the strain). The T_{or} was assumed to be $T_m - 25^\circ\text{C}$ in the same buffer system (4).

Renaturation. Samples were denatured in the spectrophotometer; when the absorbance no longer continued to increase, the temperature was held steady for 5 min to ensure that all of the DNA was single stranded. The temperature was decreased rapidly by setting the programmer to 0°C. When the temperature was $T_{or} + 10^\circ\text{C}$, the programmer was set to the desired renaturation temperature. The whole cooling process took 2.5 to 3 min. The change in absorbance at the T_{or} was followed for 20 to 30 min. The rate of renaturation was determined from the straight-line portion of the graph by using the equation of DeLey et al. (4).

Data were represented by using the unweighted paired-group method of analysis (UPGMA) (14) and principal-coordinate analysis (14).

Errors were estimated by using the methods described by Hartford and Sneath (6).

RESULTS

The average percentage of pairing determined for each pair of strains (from n spectrophotometer runs, where the

TABLE 3. Matrix of DNA-DNA distances between *Listeria* strains, as determined by the optical technique

Species	Strain	DNA-DNA distance from ^a :																		
		Strain C52	Strain C200	Strain C202	Strain C203	Strain C231	Strain C644	Strain C1090	Strain C1171	Strain C1091	Strain C1172	Strain C1087	Strain C663	Strain C667	Strain C659	Strain C214	Strain C1174	Strain C201	Strain C228	Strain JS31
<i>L. monocytogenes</i>	C52	0																		
	C200	33.0	0																	
	C202	29.4	18.5	0																
	C203	33.9	24.8	14.9	0															
<i>L. innocua</i>	C231	30.7	26.2	20.7	28.6	0														
	C644	28.1	48.8	50.3	37.7	55.4	0													
	C1090	47.8	45.0	60.4	56.3	40.2	49.7	0												
	C1171	43.0	49.4	49.7	59.9	46.6	50.7	6.5	0											
<i>L. welshimeri</i>	C1091	47.4	42.6	50.6	44.1	57.7	50.3	53.8	56.4	0										
	C1172	55.9	46.6	54.4	47.7	49.2	54.5	46.5	57.5	8.4	0									
	C1087	55.3	65.4	55.6	70.1	61.8	73.1	62.7	62.8	65.1	57.6	0								
	C663	69.6	58.2	66.4	65.5	58.8	72.2	57.0	64.1	75.7	72.7	30.6	0							
<i>L. ivanovii</i>	C667	63.2	69.0	66.0	63.6	72.9	64.8	61.1	61.4	68.5	63.9	29.3	16.1	0						
	C659	67.0	69.6	65.7	69.6	69.3	69.2	64.6	76.0	75.1	68.0	13.8	41.6	16.6	0					
	C214	88.2	84.3	70.7	81.1	69.9	95.7	86.4	88.3	85.3	93.3	91.1	69.6	71.6	72.5	0				
	C1174	78.4	85.1	95.5	73.7	74.8	84.1	83.1	87.1	75.7	88.6	85.7	91.0	92.0	82.8	15.7	0			
<i>L. grayi</i>	C201	10.6	ND ^b	ND	ND	ND	21.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0		
	C228	43.2	28.4	45.2	ND	ND	ND	52.1	ND	48.3	42.2	67.1	ND	ND	ND	90.2	ND	ND	0	
	JS31	53.4	ND	ND	ND	ND	ND	49.3	ND	43.8	ND	61.1	ND	ND	ND	75.1	92.8	ND	50.2	0
	C645	19.4	44.6	20.9	50.2	45.2	5.1	38.4	55.1	ND	ND	ND	ND	ND	ND	0.0	-3.6	ND	ND	ND
<i>L. innocua</i>	JS21	ND	ND	ND	ND	ND	ND	ND	ND	28.9	ND	ND	ND	ND	ND	87.1	ND	ND	ND	ND
	C666	64.3	ND	ND	ND	61.6	ND	ND	ND	ND	ND	ND	ND	15.1	5.4	ND	ND	ND	ND	ND
	SLCC	76.6	ND	ND	ND	ND	74.8	ND	ND	89.5	ND	82.8	ND	ND	64.1	ND	ND	74.8	ND	ND
	7211																			

^a DNA-DNA distances were determined by subtracting the percentage of pairing from 100%.^b ND, homology not determined.

FIG. 1. UPGMA dendrogram and three-dimensional ordination from principal-coordinate analysis of the complete matrix (16 by 16) (Table 3) of DNA distances. Symbols: ○, *L. monocytogenes*; ●, *L. innocua*; □, *L. ivanovii*; ■, *L. seeligeri*; △, *L. welshimeri*; *, *L. grayi* and *L. murrayi*.

(TRUPCA) on the Leicester University Vax Cluster. The UPGMA and principal-coordinate ordination results from the matrix (16 by 16) of strains are shown in Fig. 1.

The UPGMA showed that all of the strains cluster at a distance of 23. *L. grayi* and *L. murrayi* are easily separated by eye. *L. ivanovii* strains are grouped together; however, the remaining strains appear to be very closely related. Strains C52 and C644 associate before attachment to the rest of the *L. monocytogenes* strains. A single-linkage cluster analysis gave very similar results; the differences from the UPGMA analysis are (i) strains C52 and C644 do not join first and (ii) *L. monocytogenes* strains and *L. innocua* strains are separated, but the branch lengths between the strains of these species are extremely short.

Levels of error in replication experiments were measured as the standard deviations for the DNA-DNA pairing values for each pair of strains (6). Pairing values were divided into 10 bands at 10% intervals, and the errors were averaged over each 10% range. The average error was plotted against the midpoint of the band (Fig. 2). The level of error seemed to be independent of the percentage of pairing, although the levels of error were higher at very low pairing values and high pairing values (more than 90%). There were no zero errors or violating triangles when we used the TRUDNA program (Leicester University Vax Cluster) (6).

DISCUSSION

The range of variation of in levels of DNA relatedness within the species *L. monocytogenes* was very large, as in the study of Rocourt et al. (10), suggesting that there is more than one cluster or a spectrum of relatedness.

number of replications was greater than 1) and the standard deviation are shown in Table 2. A complete matrix (16 by 16) was derived from the data. By subtracting from 100, the matrix of relatedness was converted to a matrix of distances (Table 3), which were used in a principal-coordinate program

FIG. 2. Relationship between error from replications and average percent pairing experiments performed with *Listeria* strains when the optical technique was used. The error was the average standard deviation. Pairing values were divided into 10 bands at 10% intervals, and the results were plotted as the midpoints of the bands, as means (dots), and as 1 standard deviation above and below the mean (vertical lines).

L. grayi and *L. murrayi* appear to be distinct from the other species, although it is not clear from our data whether *L. grayi* can be separated from *L. murrayi*. Some strains of *L. monocytogenes* are more closely related to *L. innocua* strains than to other *L. monocytogenes* strains. Rocourt et al. (10) separated these two species on the basis of DNA homology data, but the *L. monocytogenes* strain that was their only reference strain from this species did not seem to be typical of the species. Strain C52 does not seem to be a particularly typical representative of the species *L. monocytogenes*. Collins et al. (3) compared the 16S rRNA nucleotide sequences of the type strains of *Listeria* species, and a UPGMA dendrogram of their data suggests that *L. monocytogenes* and *L. innocua* come together before they join the other species, although all species are very closely related.

The wide range of values which we obtained supports the hypothesis that there is either a spectrum of relatedness or two or more clusters in the species *L. monocytogenes* sensu stricto, as suggested by work in which multilocus enzyme electrophoresis was used (1, 2, 9).

A few *Listeria* strains were used in both this study and the

study of Rocourt et al. (10), and the pairing values for these strains are shown in Table 4. As shown in other comparisons of DNA-DNA pairing methods, the endonuclease technique is much more stringent than both the filter technique and the optical technique. In this study although the ordination gave results that were broadly similar to the results of Rocourt et al. (10), the values were always higher than the values for similar or identical pairs in the study of Rocourt et al.

Several values below 30% were obtained when this technique was used. Huss et al. (7) found that values below 30% were rarely obtained at the DNA concentration which we used and were meaningless. If this is true, then *L. grayi* and *L. murrayi* may be less closely related to the other five species than is suggested above.

As determined by numerical taxonomy and other chemical relatedness studies, *Listeria* species, especially *L. monocytogenes*, *L. innocua*, *L. welshimeri*, and *L. seeligeri* are very closely related, although *L. ivanovii* is somewhat distinct; DNA pairing data strongly support this conclusion, and there seem to be no strongly defined clusters among the four species.

TABLE 4. Pairing values, as determined by the optical technique and the S1 nuclease technique^a

Strain	% Pairing							
	Strain C52		Strain C644		Strain C1091		Strain C1090	
	S1 nuclease technique	Optical technique	S1 nuclease technique	Optical technique	S1 nuclease technique	Optical technique	S1 nuclease technique	Optical technique
C52	100	100						
C644	53	71.9	100	100				
C1091	42	52.6	46/44 ^b	49.7	100	100		
C1090	24	52.2	24	50.3	28	46.2	100	100

^a Data from reference 10 and Table 2.

^b Pairing values in which DNA from each strain was radiolabelled in turn.

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