***Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes**

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Regions of the genes encoding flagellin (*flaA*), the invasive associated protein (*iap*), listeriolysin O (*hly*) and 23S rRNA were sequenced for a range of *Listeria monocytogenes* isolates of different origin and serotypes. Several nucleotide sequence variations were found in the *flaA*, *iap* and *hly* genes. No differences were found for the rRNA genes, but our approach does not exclude the existence of differences between single copies of these genes. Based on the sequence differences, the *L. monocytogenes* strains can be divided into three distinct sequence types. Further, the presence of only a small number of sequence differences within each group indicates a strong degree of conservation within the groups. There was a complete correspondence among the groups of strains formed according to the analysis of the *flaA*, *iap* and *hly* genes, and the grouping correlates with serotype, pulsed field gel electrophoretic and multilocus enzyme electrophoretic data. Analysis of the region encoding the threonine-asparagine repeat units in the *iap* gene revealed some striking features. Sequence type 1 strains were found to have 16–17 repeats, sequence type 2 strains had 16–20 repeats whereas the two sequence type 3 strains analysed had only 11 repeats. Furthermore, within a 19 bp segment there was a 37% difference between the sequences of type 1 and 2 strains and that segment was absent in type 3 strains. Within the threonine-asparagine repeat region the nucleotide differences gave rise to four amino acid changes; however, all were changes among the three amino acids present in the repeat structure indicating a strong selective pressure on the composition of this region.

Keywords: clonality, evolutionary lines, flagellin gene, haemolysin gene, invasive associated protein gene

INTRODUCTION

The Gram-positive bacterium *Listeria monocytogenes* is widespread in the environment and may invade humans and animals where it behaves as a facultative intracellular pathogen (Seelinger & Jones, 1986). From the port of

entrance, listeriosis can develop into severe invasive diseases, including septicaemia and meningitis, primarily in immunocompromized individuals, neonates and foetuses (McLauchlin, 1987). Over the last decade there have been several food-associated outbreaks of listeriosis (Fleming *et al.*, 1985; Linnan *et al.*, 1988), and *L. monocytogenes* is now recognized as an important food-borne pathogen.

Several molecular methods have been employed for the characterization of *L. monocytogenes* strains. Using multilocus enzyme electrophoresis (MLEE), Bibb *et al.* (1990) found that 390 isolates of 6 different serotypes could be grouped into two distinct clusters of electrophoretic types

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Abbreviations: MLEE, multilocus enzyme electrophoresis; RAPD, random amplified polymorphic DNA.

The EMBL accession numbers for the sequences reported in this paper are listed in Table 1.

and that the clusters coincided with the flagellar types b (ABC H-antigens) and non-b (AB or BD H-antigens) serovars, respectively. Similar results were obtained by Boerlin *et al.* (1991) and Piffaretti *et al.* (1989) using the same technique. A somewhat different pattern was found by Tabouret *et al.* (1992). Analysing surface proteins by SDS-PAGE, they identified two clusters which correlated with the O-antigen groups, 1/2 serovars and 4 serovars, respectively. Brosch *et al.* (1991) and Buchreiser *et al.* (1991) also found a distinction between serotype 1/2 and serotype 4 isolates using PFGE. However, in a recent study with 176 isolates, PFGE could separate the isolates according to flagellar antigens (Brosch *et al.*, 1994).

A new method for strain differentiation is profiling by random amplified polymorphic DNA (RAPD). Using this method on strains of different *L. monocytogenes* serotypes Mazurier & Wernars (1992) showed that some strains from different serotypes exhibited identical RAPD profiles. However, the RAPD profiles could, in general, differentiate between the isolates according to the flagellar antigens. Only a single profile was shared among isolates with H-antigens ABC and isolates with other combinations of H-antigens. Furthermore, Vines *et al.* (1992) have shown that RFLP is able to subdivide *L. monocytogenes* into two distinct groups.

The most precise analytical tool for characterization is DNA sequence analysis, and PCR has now made the characterization of gene segments from a large number of isolates feasible. Using this approach we have previously found that *L. monocytogenes* isolates can be grouped into two major types according to the nucleotide sequence of a segment of the listeriolysin gene, *bly*. Furthermore, we found a correspondence between this grouping and flagellar antigens (Rasmussen *et al.*, 1991).

To investigate whether the pattern of distinct DNA sequence types also exists outside the *bly* gene, we have in this report analysed parts of the flagellin gene, *flaA*, isolated by Dons *et al.* (1992), and the invasive associated protein gene, *iap*, (Köhler *et al.*, 1990), as well as the 23S rRNA genes. The *flaA* gene encodes the polypeptide subunits of the flagella filament, and it has been suggested that the invasive associated protein is associated with the invasion of non-professional phagocytic cells (Kuhn & Goebel, 1989). Moreover, in search of additional sequence types, we have extended the analysis of the *bly* gene segment to include a total of 77 isolates.

METHODS

Bacterial strains and growth conditions. A total of 77 *L. monocytogenes* strains were included in this study (Table 1). Of these strains, 48 were human clinical isolates, and 24 strains were of different animal or environmental (manure, fodder, etc.) origins. The remaining strains were culture collection isolates of unknown origin. There were 44 strains of serotype 1/2, one isolate was serotype 3, and 32 were serotype 4, 4a or 4b. Bacteria were propagated in brain heart infusion broth overnight at 30 °C without shaking.

Oligonucleotides. Synthetic oligonucleotides were prepared on a Cyclone DNA Synthesizer according to the manufacturer's

instructions (Biosearch). The following primers were used: for the *bly* gene: LM4 (5'-CAGTTGCAAGCGCTTGGAGT-3'); LM5 (5'-CCTCCAGAGTGATCGATGTT-3'); LM6 (5'-TTGTTTCAATATATTTCTGAGT-3'); for the *iap* gene: LM20 (5'-GAGAAATCTGTTAGCGCAACTTGG-3'); LM21 (5'-CCATTACCAACGTAGATACCACG-3'); LM22 (5'-CAGAGATTCTTGTAAGTGC-3'); for the *flaA* gene: P1 (5'-GATTGTTCCAGCAGAAAGAC-3'); P3 (5'-AAATGAGATATAATAAAGC-3'); P4 (5'-GCGACTCATACCAGCACC-3'); for the 23S rRNA gene: 23-1104f (5'-AGAAAGCGTAATAGCTCAC-3'); 23-1608r (5'-CCACCTGTGTCGTTT-3'). LM4, LM5, LM20, LM21, P3, P4, 23-1104f and 23-1608r were used as amplification primers, and LM6, LM21, LM22, P1 and 23-1608r were used for sequencing. LM4-LM6 were selected according to Rasmussen *et al.* (1991); LM20-LM22 were selected from Köhler *et al.* (1990); P1, P3 and P4 were selected from Dons *et al.* (1992), and 23-1104f and 23-1608r were selected according to Lane (1991).

Asymmetric PCR and DNA sequencing. Asymmetric PCR and DNA sequencing were performed as described previously (Rasmussen *et al.*, 1991). DNA sequences were aligned using the PC Gene Screen Device, version 5.03 (IntelliGenetics).

RESULTS AND DISCUSSION

The flagellin gene

A 180 bp region of the *flaA* gene was sequenced in 27 strains (Table 1). The analysed region covers 125 bp of the N-terminal conserved region (position 623-747) and 55 bp of the variable region (position 748-803) according to the sequence of Dons *et al.* (1992).

In the N-terminal conserved region, which is assumed to be involved in flagellin export as well as flagellar assembly and shape (Jones & Aizawa, 1991; Macnab, 1992), four general nucleotide differences which separated the strains into three distinct sequence types were found (Table 2). A total of 22 of the strains had previously been included in the analysis of the *bly* gene (Rasmussen *et al.*, 1991), and all strains of the *bly* sequence type 1 grouped in the same *flaA* sequence type. Likewise, all strains of the *bly* sequence type 2 grouped in the same *flaA* sequence type, and the two *flaA* types were consequently assigned the corresponding numbers. The third sequence type included two serotype 4a strains which were not analysed in the former study on *bly*. In the 125 bp of the N-terminal region they differed from the two other sequence types at one and four positions, respectively. None of the general nucleotide differences gave rise to an amino acid change. Furthermore, in this region two strains were found to have strain-specific differences (Table 3).

The central region, which shows considerable variability among species in both length and amino acid composition, is believed to be responsible for the antigenic variation of the flagellar filament (Jones & Aizawa, 1991). It was therefore surprising to find only a single strain-specific difference (Table 3) and no general differences in the 55 bp region analysed. For a smaller number of strains an additional 60 bp region at the opposite end of the variable region was sequenced (data not shown), and a single general nucleotide difference between sequence types 1 and 2 was found. This base difference gives rise to an

amino acid change, a glutamic acid in sequence type 1 strains is replaced with a glutamine in type 2 strains. Complete flagellin sequences have been obtained for three *Escherichia coli* serovars (Schoenhals & Whitfeld, 1993) and 10 *Salmonella* g... series serovars (Masten & Joys, 1993). For both species a much greater variation with several amino acid changes was found in the variable region. A direct comparison to *L. monocytogenes* is, however, difficult, as the variable regions of *E. coli* and *Salmonella* are approximately 200 amino acids longer (Dons *et al.*, 1992).

The invasive associated protein gene

The *iap* gene of strain EGD (serotype 1/2a) is 1452 nucleotides long and has a central part of 400 bp which is specific for *L. monocytogenes*. In this region there is an extended repeat segment which in the EGD strain consists of 19 threonine-asparagine (TN) repeat units separated by two blocks of 3 amino acids each (the ANK and PSK motifs) (Köhler *et al.*, 1990) (Fig. 1). Sequence analysis in this study of the repeat unit and flanking regions showed that the pattern of three DNA sequence types also exists for the *iap* gene (Table 1). Furthermore, full correspondence among the groups of strains formed according to the sequence analysis of each of the *hly* (Rasmussen *et al.*, 1991), *flaA* and *iap* genes was found. Consequently, in the following the term 'sequence type' is used as a general term with no specific gene reference. In the description of specific features below, the segment of the *iap* gene investigated is divided into three regions according to structural features of the gene.

The region upstream of the repeat region, position 1315–1373 (Fig. 1), was analysed for five sequence type 1, three sequence type 2, and one sequence type 3 strains. All the type 2 strains had a sequence identical to that of EGD, whereas all type 1 strains differed at three positions (Fig. 1). The differences at position 1358 and 1359 are within the same codon, changing it from an alanine in type 2 strains to an arginine in type 1 strains. The sequence of the type 3 isolate was identical to that of EGD except for an insertion of six nucleotides at position 1360 (Fig. 1).

A total of 26 strains were analysed in a second region, position 1374–1586, containing the repeat units (Fig. 1). From direct sequence alignment it was obvious that there were variations in the number of repeat units among the strains. The serotype 4a strains both had 11 repeat units, sequence type 1 strains had 16 or 17 repeats, whereas sequence type 2 strains had from 16 to 20 repeats (Fig. 2, Table 4). Our data are in accordance with the observations by Bubert *et al.* (1992), who found 11 repeats in both a serotype 4a and a serotype 4c isolate and 16 and 19 repeats, respectively, in two different serotype 1/2a isolates.

The sequence with 20 repeats was found in a serotype 1/2c strain (Fig. 2, Type I), and the extra repeat compared to the EGD strain had the sequence 5'-ACAAAT-3', which is identical to the sequence of the first 5 repeats downstream of the region encoding the ANK motif (position 1394–1423, Fig. 1). The alignment of the

sequences from the 26 strains showed that the differences in length were confined primarily to this part of the repeat region with identical repeats. This finding supports the notion of Köhler *et al.* (1990) that the repeat region has apparently been generated by tandem multiplication of the basic unit ACAAAT. It is further substantiated by the pattern of nucleotide differences in this region. All sequence type 1 strains were found to differ from the sequence of the EGD strain in having a G instead of an A at the third position in all the threonine codons (positions 1414 and 1420 for strains with 16 repeats, and additionally at position 1408 for strains with 17 repeats). Apparently this A to G change occurred before the multiplications of the TN repeats in the sequence type 1 strains. Also, the serotype 4a strains were found to have a G at the relevant position (position 1420), and four out of the eight sequence type 2 strains had a G at the third position of the first threonine codon after the ANK motif (Table 3).

Atypical repeat structures were found only for the serotype 4a strains and a serotype 1/2a strain which lacks a repeat after the single asparagine between the ANK and PSK motifs (Fig. 2, Type VII). Moreover, it is noteworthy that all four serotype 1/2 strains of sequence type 1 had 17 repeats, whereas all 12 serotype 4/4b strains of sequence type 1 had 16 repeats (Table 4).

There were several other general nucleotide differences in the TN repeat region. There appears to be a hot spot for mutations just downstream of the PSK motif. In this part, which is missing in the serotype 4a strains, a total of seven nucleotide differences were found between the sequence type 1 and sequence type 2 strains within a 19 bp region (Fig. 1). This corresponds to a difference of 37% in that region. The differences at positions 1443, 1472 and 1478/1480 all cause amino acid changes, but only changes among asparagine, threonine and serine (Fig. 2). The serotype 4a strain had 11 nucleotide differences to the EGD strain in addition to the A/G difference at position 1420. These differences gave rise to only one amino acid change, at positions 1496/1498, where a serine in EGD is replaced with a threonine. It is noteworthy that all the amino acid changes within the repeat region are among the three amino acids already present in the repeats: threonine, asparagine and serine. It indicates a strong selective pressure on the amino acid composition of the region. Within the repeat region, no strain-specific nucleotide differences were found within the groups of the sequence type 1 or the sequence type 3 strains, respectively, whereas a few strain-specific differences were found among the sequence type 2 strains (Table 3).

In the 79 bp region downstream of the TN repeat region (position 1508–1586, Fig. 1), two general differences between sequence type 1 and sequence type 2 strains as well as two others between sequence type 2 and sequence type 3 strains were found (Fig. 1). Furthermore, the sequences of the type 1 strains were found to differ at position 1549, where 8 out of 16 strains had a G and the rest had an A. There may be a correlation between this nucleotide difference and the number of repeats as all the strains with a G at this position had 16 repeats. Within the

Table 1. *L. monocytogenes* strains used in this study and their sequence type

Analysis of a specific gene segment is shown by the presence of the corresponding EMBL accession number.

Strain	Source or reference*	Serotype†	Sequence type	Analysed genes			
				<i>hly</i>	<i>flaA</i>	<i>iap</i>	23S rRNA
1542‡	SS	4	1	X85816			
3391‡	SS	4b	1	X85817	X86993		
4521‡	SS	4	1	X85818	X86994		X85883
5223	SS	4	1	X85819		X85860	X85884
5626	SS	4	1	X85820	X86995		X85885
7595	SS	4	1	X85854		X85863	X85886
7751	SS	4	1	X85821		X85861	X85887
8160	SS	4	1	X85822		X85857	X85888
8309‡	SS	1	1	X85823	X86996		X85889
8819‡	SS	4b	1	X85815	X86992	X85864	X85890
9050	SS	4	1	X85824		X85862	X85891
9618	SS	4	1	X85825		X85858	X85892
9709	SS	1	1	X85826		X85867	X85893
10165	SS	4	1	X85827		X85859	
10511	SS	1	1	X85828		X85868	
11539	SS	1	1	X85829			
11984	SS	4	1	X85855		X85865	
12067‡	SS	4b	1	X85830	X86997	X85856	X85882
12352	SS	4	1	X85831			
12621	SS	4	1	X85832			
4789/85‡	SS	1/2b	1	X85833	X86998		
604/86‡	SS	4	1	X85834	X86999	X85866	
12664/86‡	SS	4	1	X85835			X85894
391/88‡	SS	4b	1	X85836			
7382/88‡	SS	4b	1	X85837			
429/89‡	SS	4b	1	X85838			
12705/89	SS	1	1	X85839		X85869	
13408/89‡	SS	4b	1	X85840	X87000		
14614/89‡	SS	4b	1	X85841			
14676/89	SS	1	1	X85842		X85870	
15632/89‡	SS	4	1	X85843			
H10‡	ICB	4b	1	X85844	X87001	X85936	
652‡	ICB	4b	1	X85845	X87002		X85895
Ep2‡	ICB	4b	1	X85846	X87003		X85896
245-1‡	RVAU	1	1	X85847			
315-5‡	RVAU	1	1	X85848			
85F/1	RVAU	4	1	X85849			
1169F1.2‡	RVAU	4	1	X85850	X87004		
R33	RVAU	1	1	X85851			
T31‡	RVAU	1	1	X85852	X87005		X85897
444‡	CC	4b	1	X85853			
5001‡	SS	1/2a	2	X85924	X86989	X85879	
5700	SS	1	2	X85925			X85898
7785	SS	1	2	X85909		X85877	X85899
9495	SS	1	2	X85926			X85900
10472	SS	1	2	X85910			
10585	SS	1	2	X85927			
11049	SS	1	2	X85928			
11463	SS	1	2	X85911			
11464	SS	1	2	X85912			
11819	SS	1	2	X85929			
65500	SS	1	2	X85930	X86980	X85939	X85901

Table 1. (cont.)

Strain	Source or reference*	Serotype†	Sequence type	Analysed genes			
				<i>hly</i>	<i>flaA</i>	<i>iap</i>	23S rRNA
4772/83‡	SS	1/2	2	X85931	X86981		
14387/89	SS	1	2	X85932			
5.1.1	RVAU	1	2	X85933			
12.1.1‡	RVAU	1	2	X85934			
18.1.2	RVAU	1	2	X85913			
45.1.1	RVAU	1	2	X85940			
57.1.1	RVAU	1	2	X85941			
64.1.1‡	RVAU	1	2	X85942	X86982	X85875	
103-5‡	RVAU	1	2	X85943	X86983		X85902
202.2.3	RVAU	1	2	X85914			
204.2.3‡	RVAU	1	2	X85915	X86984		X85903
206.1.1	RVAU	1	2	X85935			
2947	RVAU	1	2	X85916			
1156F1.4‡	RVAU	1	2	X85917			
B46	RVAU	1	2	X85944			
K26‡	RVAU	1	2	X85908	X86979	X85876	X85904
K69‡	RVAU	1	2	X85945	X86985		X85905
441‡	CC	1/2c	2	X85918		X85938	
442‡	CC	3	2	X85919			
15526‡	CC	1/2a	2	X85920	X86986	X85878	X85906
NCTC7973‡	NCTC	1/2a	2	X85921	X86987		
443	CC	4a	3	X85813	X86991	X85880	X85907
ATCC19114	ATCC	4a	3	X85814	X86990	X85881	X85937
LO28	IP	1/2c	2	IP*	X86988		
EGD	Ref.	1/2a	2	Ref.*		Ref.*	

* Sources: SS, W. Frederiksen and P. Gerner-Schmidt, Statens Seruminstitut, Copenhagen, Denmark; ATCC, American Type Culture Collection; CC, Culture Collection, University of Gothenburg, Gothenburg, Sweden; NCTC, National Collection of Type Cultures, Colindale, UK; RVAU, B. Nørnung, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark; ICB, J.-C. Piffaretti, Instituto Cantonale Bacteriologico, Lugano, Switzerland (obtained via B. Nørnung); IP, P. Cossart, Institut Pasteur, Paris, France: the sequence of the *hly* gene is from Mengaud *et al.* (1988); Ref., the sequences of the *hly* and *iap* gene segments are from Doman & Chakraborty (1989) and Köhler *et al.* (1990), respectively.

† Flagellar antigens have not been determined for all isolates.

‡ The nucleotide sequence of the *hly* gene was determined by Rasmussen *et al.* (1991).

type 2 strains, a few strain-specific differences were found (Table 3).

Finally, a 99 bp region (position 1587–1685, not shown in Fig. 1) downstream of the *L. monocytogenes* specific region of *iap* was sequenced for six strains of type 1 and one type 2 strain. All sequences in this region were identical to that of strain EGD.

The 23S rRNA gene

The 230 bp region from positions 1350 to 1580 of the *L. monocytogenes* 23S rRNA gene (Thompson *et al.*, 1992) was sequenced in 27 strains (16 type 1, nine type 2, and two type 3 strains). This region was selected because it contains some of the more variable parts (V9 and V10) of the rRNA operon (Lane, 1991). By our approach, where we obtained an average sequence for the six copies (Michel &

Cossart, 1992) of the rRNA operon, we did not find any differences between or within the sequence types. This does not exclude, however, that in the analysed region there are differences within single copies of the rRNA genes, as such differences would not be detected by our approach. Recently, Czajka *et al.* (1993) sequenced most of the 16S rRNA gene of a limited number of *L. monocytogenes* strains by an approach similar to ours and they did find differences in the V2 region between four serotype 1/2a isolates and also between these and a single serotype 4b isolate. This shows that intraspecies differences do occur in the rRNA genes.

Compared with the sequence reported by Thompson *et al.* (1992), all strains analysed in this study were found to have two additional Cs at position 1411. This difference may be explained by the fact that they sequenced a single, cloned rDNA copy.

Table 2. General nucleotide differences among the three DNA sequence types in the 180 bp region of the *flaA* gene and the 150 bp region of the *hly* gene analysed

Position	Nucleotide in sequence type:		
	1	2	3
<i>flaA</i>*			
648	C	T	C
666	C	T	C
711	T	A	T
735	C	C	T
	1	2†	3
<i>hly</i>†			
1773	T	A	T
1776	C	T	T
1806	C	C	T
1809	T	C	C
1827	A	C/A	A
1845	T	C	T
1857	C	T	C
1860	G	A	G
1884	C	T	T

* The analysed region is from position 622 to 803 [numbering according to Dons *et al.* (1992)].

† The analysed region is from position 1740 to 1890 [numbering according to Mengaud *et al.* (1988)].

‡ The *hly* sequence type 2 includes the two subtypes, 2A and 2B, described in Rasmussen *et al.* (1991).

The listeriolysin gene

To clarify whether the *hly* gene also exists in three types and to search for more possible variants, a 150 bp region of the gene was analysed for 39 strains in addition to the 36 strains analysed in our previous study (Rasmussen *et al.*, 1991). As for the *flaA* and the *iap* genes, the two serotype 4a strains did form a third separate sequence type (Table 2). All other strains were found to have a sequence of one of the two other sequence types previously observed (Rasmussen *et al.*, 1991). Furthermore, we did not find deviations from the full correspondence among the groups of strains formed according to the *hly* gene and the two other genes. Three strains had strain-specific nucleotide differences at various positions (Table 3).

Evolutionary lines of *L. monocytogenes*

This study confirms our earlier work (Rasmussen *et al.*, 1991) which showed that the *hly* gene of *L. monocytogenes* exists in distinct, well-conserved types. However, in addition to the two types previously found, a third type containing serotype 4a strains was identified. Moreover, an identical pattern was found for the *flaA* and the *iap*

Table 3. Strain-specific nucleotide differences in the *flaA*, *iap* and *hly* genes

The normal nucleotides and deduced amino acids, if the differences cause a change, are shown in parentheses.

Gene	Isolate	Position*	Nucleotide	Amino acid change
<i>flaA</i>	5001	690	T (C)	—
	443	622	G (C)	Glu (Gln)
	1028	758	T (C)	Ile (Thr)
<i>iap</i>	5001	1414	G (A)	—
		1513	C (T)	—
	7785	1408	G (A)	—
	11463	1513	C (T)	—
		1525	T (C)	—
		1546	C (T)	—
	65500	1396	G (A)	—
		1402	T (A)	—
	15526	1408	G (A)	—
<i>hly</i>		1441	T (A)	—
	7595	1764	G (A)	—
	11984	1789	C (G)	Gln (Glu)
	206.1.1	1778	T (C)	Phe (Ser)

* The positions of the *flaA*, *iap* and *hly* genes are according to Dons *et al.* (1992), Köhler *et al.* (1990) and Mengaud *et al.* (1988), respectively.

genes and there was full correspondence among the grouping of strains according to the three genes analysed. As the *hly* and *iap* genes are located 30–1225 kbp apart on the genetic map of the *L. monocytogenes* genome (Michel & Cossart, 1992), and the *flaA* gene is not located adjacent to the two other genes (data not shown), it is most likely that the pattern of at least three distinct types is a characteristic feature of a larger part of the *L. monocytogenes* genome, conceivably the complete chromosome. Thus, there may be three or possibly more distinct evolutionary lines of *L. monocytogenes*. The presence of distinct evolutionary lines is substantiated by MLEE (Bibb *et al.*, 1990; Piffaretti *et al.*, 1989), RFLP (Vines *et al.*, 1992), and RAPD profiling (Mazurier & Wernars, 1992) data, but with these methods, however, only two evolutionary lines have been indicated so far. Our data further indicate that recombination among *L. monocytogenes* strains of different evolutionary lines is rare.

Serotype 4a strains of sequence type 3 have been characterized in only a few earlier studies using other techniques. In the MLEE study by Piffaretti *et al.* (1989), the single serotype 4a isolate included grouped together with 4b isolates, whereas Mazurier & Wernars (1992) found that all five serotype 4a isolates included in their RAPD analysis formed a separate group. It has further been found that the *lmaA* probe which is highly specific for *L. monocytogenes* does not hybridize to serotype 4a isolates (Notermans *et al.*, 1989). These data support the

Q Q T A P K A P T E A A K P A (L) (H) P
 Type 2 CAACAAACAGCACCTAAAGCACCAACAGAAGCTGCAAAACCAGCT-----CCT 1363
 Type 1 CAACAAACAGCACCTAAAGCACCAACAGAAGCTGCAAAACCAGCT-----CCC
 Type 3 CAACAAACAGCACCTAAAGCACCAACAGAAGCTGCAAAACCAGCTCTGCACCT

 V
 A P S T N T N **A N K** T N T N T N T N
 Type 2 GCACCATCTACAAACACAAATGCTAATAAAACAAATACAAATACAAAT 1417
 Type 1 GCACCATCTACAAACACAAATGCTAATAAA-----ACGAAT
 Type 3 GCACCATCTACAAATACAAATGCTAATAAA-----

T N T N N T N T N T **P S K** N T N T N
 Type 2 ACAAATACAAACAAATACTAATAACAAATACACCATCTAAAAATACAAATACAAAC 1471
 Type 1 ACGAATACAAACAAATACTAATAACAGTACACCATCTAAAAATACAAATACAAAT
 Type 3 ACGAATACAAATACAAATACAAATACAGTACACCATCTAAAAAC-----

S N T N T N T N S N T N A N Q G S S
 Type 2 TCAAAATACAACTACGAATACAACTCAAATACGAATGCTAATCAAGGTTCTTCC 1525
 Type 1 ACAAATCAAACTACGAATACAACTCAAATACGAATGCTAATCAAGGTTCTTCT
 Type 3 -----ACAAATACAAACACGAACAAATGCTAATCAAGGTTCTTCC

N N N S N S S A S A I I A E A Q K H
 Type 2 AACAAATACAGCAATTCAAGTGCAAGTGCTATTATTGCTGAAGCTCAAAAACAC 1579
 Type 1 AACAAATACAGCAATTCAAGTGCGAGTGCTATTATTGCTGAAGCTCAAAAACAC
 Type 3 AACAAATACAGCAATTCAAGTGCAAGTGCTATTATTGCGAGAAGCTCAAAAACAC

Type 2 CTTGGAAAAGCTT
 Type 1 CTTGGAAAAGCTT
 Type 3 CTTGGAA

Fig. 1. Nucleotide sequences of sequence type 1 (12067), type 2 (EGD) and type 3 (443) strains from position 1316–1586 of the *iap* gene. This part covers the regions 1 and 2 in the analysis of the gene. Differences are indicated by asterisks. For the type 3 strain, 443, the sequence of the four TN repeats distal to the PSK motif is tentatively located to obtain the least number of nucleotide sequence differences. The amino acid sequence of EGD is shown in single-letter code above the nucleotide sequences. The amino acids shown in parentheses are deduced from the sequence of isolate 443. Dashes indicate alignment gaps. The repeat region is in bold, the ANK and PSK motifs are in boxes, and the separation between regions 1 and 2 is indicated by ∇.

Type I	TN	TN	ANK	TN	TN	TN	TN	TN	TN	N	TN	TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	20 repeats	
Type II	TN	TN	ANK		TN	TN	TN	TN	TN	N	TN	TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	19 repeats	
Type III	TN	TN	ANK			TN	TN	TN	TN	N	TN	TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	18 repeats	
Type IV	TN	TN	ANK				TN	TN	TN	N	TN	TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	17 repeats	
Type V	TN	TN	ANK					TN	TN	TN	N	TN	TS	T	PSK	N	TN	TN	TS	TN	TN	SN	TN	17 repeats	
Type VI	TN	TN	ANK						TN	TN	TN	N	TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	16 repeats
Type VII	TN	TN	ANK					TN	TN	TN	N		TN	T	PSK	N	TN	TN	SN	TN	TN	TN	SN	TN	16 repeats
Type VIII	TN	TN	ANK						TN	TN	TN	N	TN	TS	T	PSK	N	TN	TN	TS	TN	TN	SN	TN	16 repeats
Type IX	TN	TN	ANK							TN	TN	N	TN	TS	T	PSK	N					TN	TN	TN	11 repeats

Fig. 2. Types of TN repeat structures found in the polypeptide sequence deduced from the *iap* genes of the 26 isolates analysed (cf. Table 4). The types are divided according to the number, arrangements and composition of repeats. One letter amino acid codes are used.

hypothesis that serotype 4a isolates form a distinct homogeneous group. It is uncertain whether other less frequent serotypes not included in this study will group in one of the three identified DNA sequence types or form additional separate groups.

Based on the isolates for which the flagellar antigens have been determined, there is a correlation between sequence types and flagellar antigens. Sequence type 1 isolates are of serotype b (ABC H-antigens), whereas sequence type 2 isolates are of serotypes a or c (AB or BD H-antigens, respectively), the only exception being the two sequence type 3 isolates of serotype 4a. In accordance with the

correlation between sequence types and flagellar antigens, all the human clinical isolates, which most likely are of flagellar b types (McLauchlin, 1987), group in sequence type 1, and the serotype 1/2 isolates which may be expected to be of different flagellar serotypes fall into both sequence types 1 and 2. The groups of the sequence types 1 and 2 thus correspond to groupings obtained by MLEE (Bibb *et al.*, 1990), RAPD profiling (Mazurier & Wernars, 1992), RFLP (Vines *et al.*, 1992) and PFGE (Brosch *et al.*, 1994).

A remarkably strong degree of conservation was found within each sequence type. At only two positions, in the

Table 4. Correlation between number of TN repeats in the *iap* gene and sequence type. Repeat type is according to Fig. 2.

Repeat type	No. of repeats	No. of strains analysed	Serotype*	Sequence type
I	20	1	1/2c	2
II	19	1	1/2	2
II	19	1	1/2a	2
III	18	2	1/2	2
IV	17	1	1/2	2
V	17	4	1/2	1
VI	16	1	1/2	2
VII	16	1	1/2	2
VIII	16	9	4	1
VIII	16	3	4b	1
IX	11	2	4a	3

* For some strains the flagellar antigens were not determined.

iap gene for sequence type 1 strains and in the *bly* gene for sequence type 2 strains, were intra-sequence-type differences common to several strains found. More strikingly, in the total of approximately 23 kb sequenced in this study, as few as 16 strain-specific nucleotide differences (Table 3), corresponding to 0.69‰, were found. The degree of strain-specific differences varied among the sequence types, being 0.15‰ for type 1 (13.0 kb analysed), 0.87‰ for type 2 (9.1 kb analysed), and 0.87‰ for type 3 (1.1 kb analysed). This variation among the sequence types is consistent with the MLEE data of Bibb *et al.* (1990), who found a higher genetic variability in ETGB which corresponds to the sequence type 2 than in ETGA.

In conclusion, *L. monocytogenes* has been shown to exist in at least three well-conserved evolutionary lines with a strong degree of conservation within those lines. The majority of the isolates group into two of the sequence types for which there is a correlation with flagellar antigens as well as grouping based on MLEE, RAPD, RFLP and PFGE.

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