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Lineage specific recombination and positive selection in coding and intragenic regions contributed to evolution of the main *Listeria monocytogenes* virulence gene cluster

Renato H. Orsi¹, Steven B. Maron¹, Kendra K. Nightingale^{1,2}, Morganne Jerome³, Helen Tabor³, and Martin Wiedmann^{1*}

¹Department of Food Science, Cornell University, Ithaca, NY

²Department of Animal Science, Colorado State University, Ft. Collins, CO

³National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba

Abstract

The major virulence cluster of *Listeria monocytogenes* harbors six virulence genes that encode proteins critical for the intracellular life cycle of this human and animal pathogen. In this study, we determined the sequence (8,709 nt) of the virulence gene cluster (including the six main virulence genes) in 40 *L. monocytogenes* isolates from different source populations (human clinical cases, animal clinical cases, foods, and natural environments). An alignment of the full length cluster as well as individual gene alignments and alignments of intragenic regions were used for phylogenetic, recombination, and positive selection analyses. Initial phylogenetic analyses showed that the sequences represented two main clusters, consistent with previously defined *L. monocytogenes* phylogenetic lineages. The 40 sequences represented 25 distinct allelic types and the overall alignment included 592 polymorphic sites. Overall, our data show that (i) virulence genes in the main *L. monocytogenes* virulence gene cluster include highly conserved genes (i.e., *hly*, *prfA*) as well as diverse genes that appear to have evolved by positive selection (*mpl*, *actA*, *plcA*), (ii) recombination has played an important role in the evolution of the virulence gene cluster, but is limited to lineage II isolates, and (iii) the promoter region driving the transcription of virulence genes transcribed early in intracellular infection (i.e., *hly*, *plcA*) has evolved by positive selection. The genes and intragenic regions in the *L. monocytogenes* virulence gene cluster thus have evolved independently, despite their close physical linkage, likely reflecting distinct selective pressures associated with expression and function of the proteins encoded in this region.

1. Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen that can cause gastroenteritis as well as invasive disease in humans and animals (Vazquez-Boland et al., 2001). Human listeriosis is typically foodborne and *L. monocytogenes* can be found in many different environments, including food processing plants, farms, and urban and natural environments (Lappi et al., 2004; Sauders et al., 2006). A number of virulence genes important

*Corresponding author: Cornell University, Department of Food Science, 412B Stocking Hall, Ithaca, NY 14853, Phone: 607-254-2838, Fax 607-254-4868, Email: mw16@cornell.edu.

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in the intracellular life cycle of *L. monocytogenes* have been identified. Six key *L. monocytogenes* virulence genes (i.e., *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) that are critical for the intracellular life cycle, including vacuolar escape and cell-to-cell spread, are located in a virulence gene island, which is often referred to as the “*prfA* virulence gene cluster (pVGC)” (Ward et al., 2004) or the “*Listeria* pathogenicity island 1 (LIPI-1)” (Vazquez-Boland et al., 2001). *hly* encodes listeriolysin O (LLO), which facilitates *L. monocytogenes* escape from the host cell vacuole. *plcA* and *plcB* encode the two phospholipases, (PI-PLC and PC-PLC), which also contribute to disruption of the host cell vacuole membrane (Gaillard et al., 1987; Smith et al., 1995), while *mpl* encodes a zinc metalloproteinase needed to activate PC-PLC. *actA* encodes the ActA protein, which is responsible for host actin accumulation and motility. *prfA*, which can be transcribed from at least three different promoters (Camilli et al., 1993; Freitag et al., 1993), encodes the transcriptional factor PrfA; PrfA positively regulates transcription of the other genes in the virulence cluster as well as of some other virulence genes (e.g., *inlA*, *inlB*) (Chakraborty et al., 1992; Domann et al., 1992; Leimeister-Wachter et al., 1990; Scortti et al., 2007). PrfA-dependent transcription of virulence genes appears to be hierarchical, such that virulence genes needed early in the intracellular life cycle (e.g. *hly*, *plcA*) are more rapidly activated than genes required later (e.g., *actA*) (Sheehan et al., 1995). While *hly* and *plcA* are transcribed in opposite directions using a shared PrfA binding site that overlaps with the -35 site for both promoters, *hly* can also be transcribed from a second putatively PrfA-independent promoter (Mengaud et al., 1989). A PrfA-dependent promoter upstream of *mpl* generate a monocistronic *mpl* transcript as well as a polycistronic *mpl-actA-plcB* transcript, while another PrfA-dependent promoter upstream of *actA* can generate an *actA* as well as an *actA-plcB* transcript (reviewed in Kreft and Vazquez-Boland, 2001). In addition to the coding sequences, the *prfA* virulence gene cluster thus includes intergenic regions with complex regulatory elements that are needed for virulence of *L. monocytogenes*.

L. monocytogenes is a diverse organism with a structured population that includes at least three phylogenetic lineages (Rasmussen et al., 1995). While two lineages (I and II) are common, lineage III appears to be uncommon (Jeffers et al., 2001; Nightingale et al., 2004; Sauders et al., 2006). Although isolates from all lineages seem to be capable of causing disease, epidemiological and phenotypic differences among these lineages have been observed. Lineage II strains, although the most prevalent in foods, are underrepresented among human sporadic listeriosis cases and are seldom associated with human listeriosis outbreaks (De Cesare et al., 2007; Gray et al., 2004; Jeffers et al., 2001; McLauchlin et al., 2004; Norton et al., 2001). Conversely, lineage I isolates, and in particular lineage I serotype 4b strains, have been associated with most human outbreaks and the majority of human listeriosis cases in most countries despite their lower prevalence in foods (McLauchlin et al., 2004). These epidemiological findings suggested that all or some lineage I strains could be more virulent than lineage II strains, a hypothesis that has been supported by some *in vitro* assays, including tissue culture assays that found that lineage I isolates, on average, seem to form more, and larger plaques in mouse L cells (Gray et al., 2004; Norton et al., 2001; Wiedmann et al., 1997). Moreover, several lineage II isolates were shown to carry nonsense or frameshift mutations in virulence genes (particularly in *inlA*), which may lead to virulence attenuation (Rousseaux et al., 2004; Nightingale et al., 2005a; Orsi et al., 2007; Roche et al., 2005; Velge et al., 2007).

The goal of this study was to use evolutionary analyses (including positive selection and recombination analyses) of both coding and intergenic regions in the *prfA* cluster to improve our understanding of the evolution and adaptation of this virulence gene cluster in *L. monocytogenes*, using isolates representing different lineages and obtained from different source population (i.e., human and animal clinical cases, foods, and natural environments). While one study (Ward et al., 2004) has previously used DNA sequence data for the *prfA* cluster for a phylogenetic analyses, comprehensive analyses on the contributions of recombination

and positive selection to the evolution of this virulence gene cluster have been missing so far. In particular, positive selection analyses have only been reported for a fragment of one virulence gene (i.e., *actA*) in the *prfA* cluster (Nightingale et al., 2005b) and no positive selection analyses have been reported for the non-coding and regulatory regions in the *prfA* cluster.

2. Methods

2.1. *Listeria monocytogenes* isolates

The 40 *L. monocytogenes* isolates used here were selected from a larger set of 132 isolates as previously described (Orsi et al., 2007; Tsai et al., 2006); DNA sequences for selected internalin genes had previously been reported and analyzed for these isolates (Orsi et al., 2007; Tsai et al., 2006). Briefly, the original set of 132 isolates represented isolates from four source populations, including human clinical cases (n = 60), animal clinical cases (n = 30), foods (n = 30), and pristine environment (n = 12); these isolates had been obtained from two previous studies, which provide details on these isolates (Nightingale et al., 2005b; Sauders et al., 2006). For each of the four source populations, ten isolates were randomly selected (using a random number table) to yield the set of 40 isolates used here (Supplemental Table 1; all supplemental are available at http://www.foodscience.cornell.edu/cals/foodsci/research/labs/wiedmann/links/orsi_2008.cfm). Serotypes were previously reported for 30 isolates (Nightingale et al., 2005b) and were determined for 10 isolates using the methods previously described (Nadon et al., 2001); serotype data were used to determine clustering of serotypes within a lineage.

2.2. DNA amplification and sequencing

The *L. monocytogenes* virulence gene cluster (see Figure 1 for a schematic) was amplified using a total of eight separate PCRs (see Supplemental Table 2 for primers). PCR products were purified using the Qiaquick Purification kit (Qiagen) and sequenced using PCR primers and internal primers (Supplemental Table 2); sequencing was performed (at the Biotechnology Resource Center, Cornell University) using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequences were proofread and assembled using Seqman (Lasergene). The sequences used for the full virulence gene cluster alignment have been deposited in GenBank (accession numbers EU372018 to EU372057).

2.3. Descriptive analyses

Clustal W implemented in Megalign (Lasergene) was used to create alignments of each gene and of the whole virulence cluster. DNASP version 4.0 (Rozas and Rozas, 1999) was used to assess the number of allelic types, number of polymorphic sites (S), number of nonsynonymous (NNS) and synonymous substitutions (NSS), and nucleotide diversity (π) in each alignment.

2.4. Phylogenetic analysis

The model of DNA substitution that best fit each data was identified using MODELTEST (Posada and Crandall, 1998) and was used to create phylogenetic trees using the maximum likelihood method implemented in PAUP (<http://paup.csit.fsu.edu/>). The phylogeny for the full virulence gene cluster was rooted using the *L. ivanovii* subsp. *londoniensis* NRRL 33021 sequence (GenBank accession number AY510073) previously published (Ward et al., 2004). Support for each branch was assessed by the bootstrap procedure in PAUP (100 replicates).

2.5. Positive selection analyses of coding regions

Alignments and trees generated for each of the six coding regions within the virulence gene cluster were used to assess whether a given gene had evolved by positive selection; only the

sequences for the 40 *L. monocytogenes* isolates were used for these analyses. Trees were generated using the maximum likelihood method as described above. Positive selection in coding regions can be assessed by comparing the rate of nonsynonymous substitutions (d_N) to the rate of synonymous substitutions (d_S) in that region. By assuming that the rate of synonymous mutation is constant and not targeted by natural selection, a $d_N/d_S > 1$ indicates that nonsynonymous mutations were more likely to be fixed in the population than synonymous mutations suggesting positive selection of nonsynonymous mutations. While some studies use an estimation of a single average d_N/d_S for a whole gene to identify genes under positive selection, the approaches implemented “Phylogenetic Analysis using Maximum Likelihood” (PAML) provide an advancement over these methods by allowing for detection of positive selection at specific sites and in specific lineages (Lefebvre and Stanhope, 2007; Yang et al., 2000; Zhang et al., 2005). In PAML, three null models, in which positive selection is not allowed, and three alternative models, in which positive selection is allowed, can be compared using a Likelihood Ratio Test (LRT) to assess whether the alternative model is significantly better than the null model. If an alternative model is accepted over the null model, one can conclude that a given gene evolved by positive selection (Yang et al., 2000). Specifically, the null models M0, M1a, and M7 are compared to the alternative models M3, M2a, and M8, respectively. M0 assumes only one class of ω along the sequence whereas M3 assumes three classes of ω ; the comparison between these two models can be used to determine whether selection differed along a given sequence (suggesting, for example, that regions in a gene differ in their selection). M1a assumes two classes of codon sites each with a different value of ω (i.e., $\omega_0 < 1$ and $\omega_1 = 1$), while M2a assumes one additional class of sites (i.e., ω_2) with an $\omega > 1$. Similarly, M7 assumes ten classes of sites with ω following a beta distribution that approximate a continuous distribution constrained to values between 0 and 1, while M8 assumes an extra class of sites with $\omega_1 > 1$. Hence, comparisons between M1a and M2a as well as comparisons between M7 and M8 assess whether a gene has evolved by positive selection; acceptance of an alternative model (i.e., M2a or M8) suggests that a given gene evolved by positive selection. However, the test between M7 and M8 is less conservative than the test between M1a and M2a as models M7 and M8 do not assume a class of sites with $\omega = 1$. A Bayes Empirical Bayes (BEB) approach implemented in PAML was used to further identify specific codon sites with a significant posterior probability of having evolved by positive selection.

In addition to the tests described above, branch-site models as described by Zhang et al. (2005) were used to assess whether a given gene evolved by positive selection within specific branches in a tree; we specifically tested branches within *L. monocytogenes* lineage I or II for positive selection in each gene. Similar to the tests described above, the branch-site test relies on the comparison of a null model with an alternative model. While both the null model and the alternative model have four site classes, only the alternative model allows positive selection (i.e., ω classes with $\omega > 1$) in the branch(es) of interest (Zhang et al., 2005). Therefore, if the alternative model is accepted over the null model, one can conclude that a given gene evolved under positive selection in the branch(es) of interest. A BEB approach was also used to identify specific codon sites evolving by positive selection in the branches of interest. All analyses for assessing evolution by positive selection were carried out using PAML version 3.15.

2.6. Positive selection analyses of the non-coding regions

Six separate alignments (each including a given gene and its upstream and downstream non-coding regions) were used to construct phylogenetic trees; these alignments and phylogenetic trees were then used to identify non-coding fragments that have evolved by positive selection using the program EvoNC (Wong and Nielsen, 2004). Like PAML, EvoNC assumes that synonymous mutations are neutral and happen in a constant rate within the coding fragment. Hence, calculating the ratio of the estimated nucleotide substitution rate in the non-coding

region to the estimated synonymous substitution rate in the coding region ratio (this ratio is designated as ζ) provides a numerical approach to assess whether a non-coding region has evolved by positive selection. Three models implemented in EvoNC can be used in two different tests to determine whether a non-coding region is under positive selection; the neutral model assumes two classes of nucleotide sites with $\zeta_0 < 1$ and $\zeta_1 = 1$; the two-category model assumes two classes with $\zeta_0 < 1$ and $\zeta_1 \geq 1$; and the three-category model assumes three classes of nucleotides with: $\zeta_0 < 1$, $\zeta_1 = 1$, and $\zeta_2 > 1$. The test comparing the neutral model and the three-category model is called Test 1; Test 1 is more conservative than Test 2, which compares the neutral model and the two-category model (Wong and Nielsen, 2004).

2.7. Recombination analyses

The Sawyer's test implemented in GENECONV (<http://www.math.wustl.edu/~sawyer>) was initially used to identify recombinant fragments within the virulence cluster, using the default settings of the program. As previously described (Nightingale et al., 2005b), GENECONV fragments that shared the same 5' or 3' ends were considered to represent the same recombination event.

Clonal Frame v1.1 (Didelot and Falush, 2007) was used to identify isolates that have recently imported external DNA fragments in the virulence cluster. Two independent runs of the program were used to estimate a 95% consensus tree. Branches supported by the tree were analyzed for recombination. Only recombination events with probability higher than 90% were considered. The program was run with 100,000 burn-in iteration and 100,000 sampling iteration.

3. Results

3.1. Descriptive analysis

The alignment of the virulence gene cluster for the 40 *L. monocytogenes* isolates was 8,709 bp long with an average G + C content of 36.3 %. A total of 7,984 sites were monomorphic; 133 sites in the alignment represented indels (insertions or deletions). Overall, 592 polymorphic sites (S) were identified (excluding the sites with indels), including 117 singletons (i.e., a polymorphic sites with substitution in only one isolate) and 475 informative sites (451 and 24 sites with two and three nucleotide variants, respectively). The 40 sequences represented 25 unique virulence gene cluster alleles. The overall nucleotide diversity (π) was estimated as 0.0246; π was significantly higher for lineage II isolates (21 isolates; $\pi_{II} = 0.0064$) than lineage I isolates (18 isolates, $\pi_I = 0.0030$) (Table 1). Lineage I and II isolates shared nine substitutions whereas 267 were fixed in either lineage I or II.

Descriptive analyses of the six genes in the virulence gene cluster showed considerable variation in nucleotide diversity and other parameters among these genes (Table 1). *actA* was the most diverse gene ($\pi = 0.0383$) with a total of 184 polymorphic sites and 197 substitutions (excluding polymorphic sites in the indel regions). Thirteen polymorphic sites had three different nucleotides. Twelve isolates (11 in lineage I and 1 in lineage II) showed a single large deletion of 105 nucleotide in *actA* (representing the majority of the indel sites observed in the virulence gene cluster), representing a previously reported deletion (Smith et al., 1996; Wiedmann et al., 1997; Moriishi et al., 1998; Jacquet et al., 2002) of one of the four Proline-Rich Repeats (PRR) in ActA. On average, lineage I isolates showed significantly lower ($P < 0.0001$) nucleotide diversity in *actA* ($\pi_I = 0.0052$) as compared to lineage II isolates ($\pi_{II} = 0.0149$) (Table 1). *prfA* was the most conserved gene ($\pi = 0.0143$) with only 25 substitutions, including only two nonsynonymous substitutions; one of these substitutions (K197N) is present in one lineage II isolate (FSL S4–304) and the lineage III isolate (FSL F2–695). While *prfA*

and *mpl* showed higher nucleotide diversity (π) in lineage I isolates, all other genes in the virulence gene cluster showed higher nucleotide diversity in lineage II isolates (Table 1).

3.2. Phylogenetic analysis

The maximum likelihood tree constructed using the complete virulence gene cluster sequence showed two main clusters (Fig. 2). Isolate grouping into these clusters is consistent with previous lineage classification of these isolates (Nightingale et al., 2005b; Orsi et al., 2007; Saunders et al., 2006; Tsai et al., 2006) and also consistent with serotype classification (i.e., serotype 1/2a and 1/2c isolates group into lineage II and serotypes 1/2b, 3b and 4b cluster into lineage I) (Nadon et al., 2001). The one serotype 4a, lineage III isolate included in our isolate set was clearly separate from the lineage I and II clusters. Long branch lengths were observed among lineage II isolates but not among lineage I isolates. Lineage I isolates formed two main clusters, one containing 1/2b isolates (83% bootstrap support) and one containing 4b isolates (84% bootstrap support; Fig. 2), even though 1/2b and 4b isolates sometimes clustered together, consistent with previous reports (Call et al., 2003; Ward et al., 2004; Nightingale et al., 2005b). Most lineage II isolates belonged to serotype 1/2a, one lineage II isolate (FSL S6-072) could not be serotyped unambiguously and belongs to either serotype 1/2a or 3a; for this isolate the H antigen was identified as AB and the O antigen was identified as II, despite repeated serotyping attempts this isolate reacted neither with the O:IV or the O:I antibodies indicating that this isolate is either serotype 3a or 1/2a. The virulence gene cluster sequences for all three serotype 1/2c isolates (lineage II; all three isolated from foods) were identical to each other, consistent with previous studies that found 1/2c isolates to be highly clonal (Zhang et al., 2004; Orsi et al., 2007), and were also indistinguishable from three serotype 1/2a isolates obtained from the pristine environment (allelic type 18, Supplemental Table 1). Clustering of serotype 1/2a and 1/2c isolates has also been observed previously (Mereghetti et al., 2002; Zhang et al., 2004; Orsi et al., 2007) and suggests that serotype 1/2c emerged from a serotype 1/2a ancestor.

3.3. Positive selection analysis of the coding regions

Three of the six coding regions in the virulence gene cluster (i.e., *mpl*, *plcA*, and *actA*) showed evidence for positive selection. For all three genes M0 was rejected in favor of the alternative model M3 (Table 2), suggesting that selection is not homogeneous across these genes. For *mpl* and *plcA*, the branch-specific analyses showed evidence for positive selection among branches within lineage I and II, respectively (Table 2). For *mpl*, 1.5% of sites were classified into the site category for positively selected sites ($\omega = 7.28$) in the branch specific model for lineage I; one *mpl* aa site (aa 103) showed a posterior probability > 95% of being under positive selection (Table 2). For *plcA*, 3.8% of sites were classified into the site category for positively selected sites (the ω for this site category was estimated to be 2.82) in the branch-specific model for lineage II; no *plcA* sites were identified as having a posterior probability > 95% of being under positive selection.

For *actA*, both overall tests (i.e., M1a vs M2a and M7 vs. M8, Table 2) showed evidence for positive selection (Table 2). In addition, the branch-specific analyses for *actA* showed evidence for positive selection among lineage II branches (Table 2). M8 estimated that 9.6% of the sites evolved by positive selection ($\omega = 2.40$) and identified six aa sites (aa 112, 123, 291, 449, 461 and 522) with posterior probabilities > 95% of being under positive selection. The more conservative M2a model estimated that 7.7% of *actA* aa sites evolved by positive selection ($\omega = 2.55$) and identified one aa site (aa 461) with posterior probability > 95% of being under positive selection. In the branch specific analysis, 2.5% of the sites were estimated to have evolved by positive selection ($\omega = 3.59$), but no sites presented a posterior probability > 95% of being under positive selection. ActA aa site 498, which had previously been reported, using a different isolate set, to be under positive selection (Roberts and Wiedmann, 2006) was

classified into the positively selected sites, although with a posterior probability $< 95\%$ (i.e., 63%).

Among lineage II isolates, the nucleotide diversity among nonsynonymous (π_{NNS}) and synonymous (π_{NSS}) sites were higher for the genes identified as being under positive selection in this lineage, i.e., *plcA* and *actA* (Table 1), suggesting that *plcA* and *actA* evolved faster than the other virulence genes in this lineage. Moreover, *actA* showed the highest π_{NNS} among all isolates (0.0226) while *prfA* and *hly* showed the lowest π_{NNS} among all isolates (Table 1).

3.4. Positive selection and polymorphism analysis for non-coding regions

Analysis in non-coding regions was performed separately using each gene including the upstream and downstream non-coding regions, except that no sequence downstream of *prfA* (i.e., on the 5' end of the virulence cluster) and only 47 nucleotides downstream of *plcB* (i.e., on the 3' end of the virulence gene cluster) were available for analyses. Both, the analysis with *plcA* and the analysis with *hly* (representing two adjacent genes), found significant evidence for positive selection in the non-coding areas; five out of the six sites identified as being under positive selection (with a posterior probability $> 95\%$; Table 3) fall in the intergenic region between *plcA* and *hly*, which was included in both analyses (as this region is located 5' of both of these genes, which are adjacent to each other and divergently transcribed). One site downstream of *hly* was also identified as being under positive selection (Table 3), but this site was only identified in the analysis with *hly*, but not in the analysis with *mpl* (where this site would be included in the non-coding region upstream of *mpl*). Our analyses thus provide strong evidence for positive selection specifically in the intergenic region between *plcA* and *hly*; this region harbors the promoter sequences for both *hly* and *plcA*, which share a PrfA box and are transcribed in opposite directions. None of the sites under positive selection falls within the PrfA box or a -10 promoter region. However, the nucleotide variation at the positively selected site 92 (Table 3, Fig. 3) generates a putative new -10 site, which matches the consensus -10 for a promoter recognized by the alternative sigma factor σ^{B} ; a search for σ^{B} promoter regions using the genome of the lineage I strain F2365 (Nelson et al., 2004) and a Hidden Markov Model (HMM) constructed from known σ^{B} promoters in *L. monocytogenes* (Raengpradub et al., 2008), identified the putative promoter generated by the nucleotide 92 polymorphism, suggesting that this may indeed represent a σ^{B} -dependent promoter for *hly*, which is present in some lineage I isolates (i.e., 16/18 lineage I isolates sequenced here). In addition, the nucleotide variation at the positively selected site 42 creates a possible -35 region (GTTA, which is present in all lineage I and 10/21 lineage II isolates), which may represent a σ^{B} -dependent promoter combined with a -10 region (GGCAT), even though the spacing between the -35 and -10 region would be unusually short [12 nucleotides rather than the typical 13 to 17 nucleotide spacing for a σ^{B} -dependent promoter (Kazmierczak et al., 2003)].

In addition to the positively selected sites in the *hly-plcA* intergenic region, we also identified a number of polymorphic sites (which were not specifically identified as being under positive selection), which may affect promoter elements. Specifically, we identified three polymorphic sites within the -35 region (GGGACA) of the putatively PrfA-independent *hly* P1 promoter (Mengaud et al., 1989) (Fig. 3); one mutation in the lineage III isolate FSL F2-695 changes this -35 region to GAGACA, while one mutation in the lineage II isolate FSL F2-515 changes this region to AGGACA. In three other lineage II isolates (FSL S4-304, FSL F2-663, and FSL E1-123), the *hly* P1 promoter -35 region is AGGATA and thus differs by two nucleotides from P1 promoter -35 region of most other *L. monocytogenes* isolates in our study.

3.5. Recombination analysis

Initial GENECONV analyses identified 91 unique fragments, which could be grouped into 26 independent events (i.e., events with distinct 5' and 3' breakpoints). *actA* showed the most

recombination events, while *prfA* showed the least recombination events (Supplemental Table 3).

We subsequently used ClonalFrame to further assess recombination events and to identify the recipient strains in the recombination events. ClonalFrame detected no recombination events where lineage I isolates served as recipients, while it detected 25 events where lineage II isolates served as recipients in a recombination event (Fig. 4; Table 4); these events occurred in 18 branches (in each of five branches two recombination events; in one branch [branch F; Table 4] three events occurred). Recombination events were identified in isolates from all four source populations (natural environment, foods, animal and human clinical cases). While ClonalFrame identified a number of recombination events in the single lineage III isolate (FSL F2–695) and in the ancestral branch between lineage II and lineage I/III, these data are not reported as only a single highly divergent lineage III isolate was included in our data. The majority of the recombination events seem to fall within the coding sequences of *actA* (11 events) and *plcA* (5 events); four recombination events fall in the *hly-plcA* intergenic region (Fig. 1 and Fig. 4; Table 4).

4. Discussion

We determined and analyzed the DNA sequences for six virulence genes and intergenic regions in the *prfA* cluster in 40 *L. monocytogenes* isolates representing diverse source populations. Initial phylogenetic analyses confirmed that the 40 sequences group into two main clusters representing the two common *L. monocytogenes* lineages that have first been described by Piffaretti et al. (1989) and subsequently been confirmed by phylogenetic analyses based on various virulence and housekeeping genes (Nightingale et al., 2005b; Orsi et al., 2007; Tsai et al., 2006) as well as a previous phylogenetic study of the *prfA* virulence gene cluster (Ward et al., 2004); these two main clusters have also been defined by all other subtyping studies that included cluster analyses, including PFGE and ribotyping studies (as reviewed by Wiedmann, 2002). Subsequent positive selection and recombination analyses showed that (i) virulence genes in the main *L. monocytogenes* virulence gene cluster includes highly conserved genes (i.e., *hly*, *prfA*) as well as diverse genes that appear to have evolved by positive selection (*mpl*, *actA*, *plcB*), (ii) recombination has played an important role in the evolution of the virulence gene cluster, but is limited to lineage II isolates, and (iii) the promoter region driving the transcription of virulence genes transcribed early in intracellular infection (i.e., *hly*, *plcA*) appears to have evolved by positive selection and recombination.

4.1. Virulence genes in the main *L. monocytogenes* virulence gene cluster include highly conserved genes (i.e., *hly*, *prfA*) as well as diverse genes that appear to have evolved by positive selection (*mpl*, *actA*, *plcB*)

The genetic diversity for the six genes in the virulence cluster ranged from 0.0143 (*prfA*) to 0.0383 (*actA*) with an average diversity for the whole virulence gene cluster of 0.0246. Using the same 40 isolates, similar diversity was previously observed for another virulence gene, *inlA* (0.02134) (Orsi et al., 2007), while other internalin genes found in most or all of these isolates (i.e., *inlB*, *inlC*, *inlC2*, *inlD*, *inlE*) (Tsai et al., 2006) showed a wider range of diversity (0.0167 to 0.0702) with some highly diverse genes (e.g., *inlE*; $\pi = 0.0702$). Nucleotide diversity within *actA* was not limited to single nucleotide polymorphisms, but also included a previously reported (Wiedmann et al., 1997; Moriishi et al., 1998; Jacquet et al., 2002) deletion of a fragment encoding a 35 aa Proline-Rich Repeats (PRR) in a number of lineage I isolates. This deletion yields an ActA protein with three rather than four PRRs; while Smith et al. (1996) previously reported a linear relationship between the number of PRRs and the rate of bacterial movement in the host cell cytoplasm, the effects of PRR variation on virulence (Smith et al., 1996) and in natural strains remain unclear (Roberts and Wiedmann, 2006).

The three most diverse genes in the virulence gene island (i.e., *plcA*, *actA* and *mpl*) also showed evidence for positive selection in their coding regions, although with different lineage-specific patterns of positive selection. While positive selection in *plcA* and *mpl* were restricted to lineage II and lineage I isolates, respectively, positive selection in *actA* was identified in the overall analysis, suggesting that this gene evolved by positive selection in different branches. Interestingly, ActA region C (aa 50–126), which is necessary for continuous actin filament elongation (Lasa et al., 1997), was very diverse with ten polymorphic sites, including two sites (aa 112 and 123) that were identified by M8 as being under positive selection and where three amino acid variants were observed in each site. Another site identified as being under positive selection (aa 291) falls within the first PRR domain. On the other hand, the site with highest probability of being under positive selection (aa 461) as well as other two sites (aa 449 and 522) fall in regions with no described function. Overall, these findings suggest that *actA* may be adapting to certain functions in addition to cell-to-cell spread [e.g., a role in host cell invasion (Suarez et al., 2001)] that require specific aa sequences in regions that have not yet been assigned specific biological functions. This hypothesis is consistent with experimental data, which showed that an isogenic mutation in a positively selected site in ActA did not affect cell-to-cell spread phenotype (Roberts and Wiedmann, 2006).

Although *mpl* showed evidence for positive selection and considerable nucleotide and aa diversity, the Mpl aa 84–92 region, which has been identified as a CTL epitope in mice (Busch et al., 1997) was conserved in all isolates analyzed, possibly because this region may represent the active domain of Mpl. Similarly, the major murine CTL epitope region in LLO (aa 91 and 99) (Pamer et al., 1991; Vijn and Pamer, 1997) was conserved among all 40 isolates, possibly because conservation of this region appears to be important for virulence (Lety et al., 2006). These findings illustrate conservation of certain sequence features, including some that may be expected to be under selection for diversification. Overall, the analyses reported provide important new data on specific aa sites under positive selection in *mpl*, *actA*, and *plcA*; identification of these sites provides an opportunity for mutational analyses of specific polymorphic sites in order to probe the contributions of diversification in these virulence genes to niche adaptation in *L. monocytogenes*.

4.2. Recombination has played an important role in the evolution of the virulence gene cluster, but is limited to lineage II isolates

Interestingly, our data suggest that recombination events within the virulence gene cluster commonly involve lineage II strains as recipients, but rarely involve lineage I isolates as recipients (recombination in lineage III isolates was not characterized as only a single lineage III isolate was included in our isolate set). These findings are in agreement with previous studies that have shown considerable recombination in other virulence genes, such as *inlA*, *inlC*, *inlC2*, *inlD*, *inlE*, *inlF* and *inlG* among lineage II isolates (Orsi et al., 2007; Tsai et al., 2006), with recombination generally more frequently in lineage II than lineage I isolates. Similar observations (i.e., more common recombination among lineage II strains than among lineage I strains) have also been reported for *L. monocytogenes* housekeeping genes (Meinersmann et al., 2004). Recombination thus appears to be critical for the diversification of lineage II isolates. While lineage II has generally been reported as showing considerably higher nucleotide diversity than lineage I isolates, our data suggest that this higher diversity in lineage II strains may be largely due to recombination. Specifically, this hypothesis is supported by the observation that the two virulence genes with no evidence for recombination (i.e., *prfA* and *mpl*) show higher nucleotide diversity among lineage I strains than among lineage II strains. Similarly, *sigB*, the only gene which showed no evidence for recombination among 120 *L. monocytogenes* isolates in previous study, showed identical nucleotide diversity among lineage I and II isolates (Nightingale et al., 2005b). Additional evolutionary analyses in *L. monocytogenes* genes with no evidence for recombination will be necessary to further

characterize the evolutionary history of lineages I and II and to test the current hypothesis that only lineage I has experienced a recent bottleneck (Meinersmann et al., 2004). Our data reported here specifically suggest as a possible alternative hypothesis that lineage II simply diversified more rapidly, e.g., through more frequent recombination than lineage I. Possible reasons for more common recombination in lineage II may involve molecular (e.g. different restriction systems in each lineage), epidemiological (e.g. abundance of different isolates in environments where horizontal gene transfer might be facilitated) or evolutionary (e.g. different selective pressures to maintain recombinant fragments in the population) factors.

4.3. The promoter region driving the transcription of virulence genes transcribed early in intracellular infection (i.e., *hly*, *plcA*) appears to have evolved by positive selection and recombination

While analyses for positive selection in coding regions are increasingly recognized as a powerful tool for identifying functionally important genes or residues within a gene, few studies have used emerging evolutionary analyses tools (Wong and Nielsen, 2004) to identify intergenic regions under positive selection, including the specific nucleotide sites that may be under positive selection. Interestingly, the positive selection analyses on non-coding regions in the *L. monocytogenes* virulence gene cluster provided initial evidence that the intergenic region between *hly* and *plcA* includes nucleotide sites that are under positive selection; Clonalframe also provided evidence for recombination events in this region. While the PrfA binding site in this region (as well as the other PrfA binding sites upstream of *prfA*, *mpl*, and *actA*) was perfectly conserved, a nucleotide change in at least one of the positively selected sites in *hly* and *plcA* intergenic region (nucleotide 92, Figure 3) appears to generate a putative σ^B -dependent promoter upstream of *hly*; this mutation was only found in lineage I isolates. While there is no evidence that the transcription of *hly* is σ^B -dependent in a lineage II strain (Chaturongakul and Boor, 2006), no data are currently available on σ^B -dependent *hly* transcription in lineage I strains. Future experimental validation of our findings is thus still necessary. Diversification of virulence gene regulation among *L. monocytogenes* lineages I and II has recently been demonstrated by comparative transcriptome analyses though (Severino et al., 2007). These analyses (Severino et al., 2007) also reported evidence for differences in transcription of σ^B - and σ^B -dependent genes among lineages, supporting the potential importance of our finding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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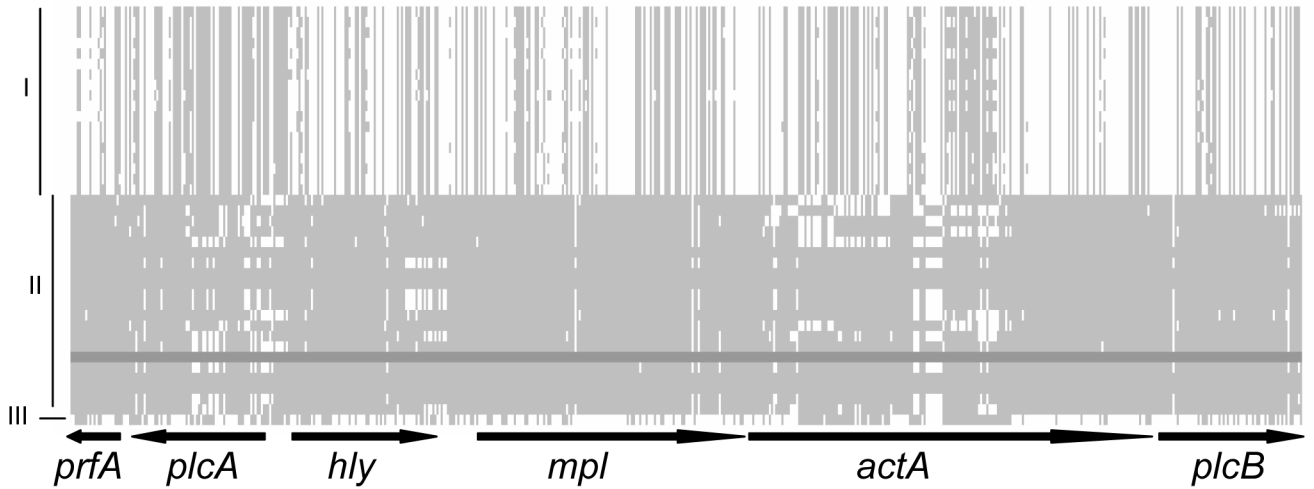


Figure 1.

Alignment of polymorphic sites in the virulence cluster. Gaps are not considered. Lineages are labeled as I, II and III. A lineage II isolate (FSL S4–497) was used as reference (dark gray shade). Lightly shaded areas represent nucleotides in other isolates that match the nucleotide present in FSL S4–497 for a given site. Regions in white within lineage II sequences may represent recombination events. Schematic of the coding regions are shown below the alignment. The lengths of the coding regions are proportional to the number of polymorphic sites within each coding region.

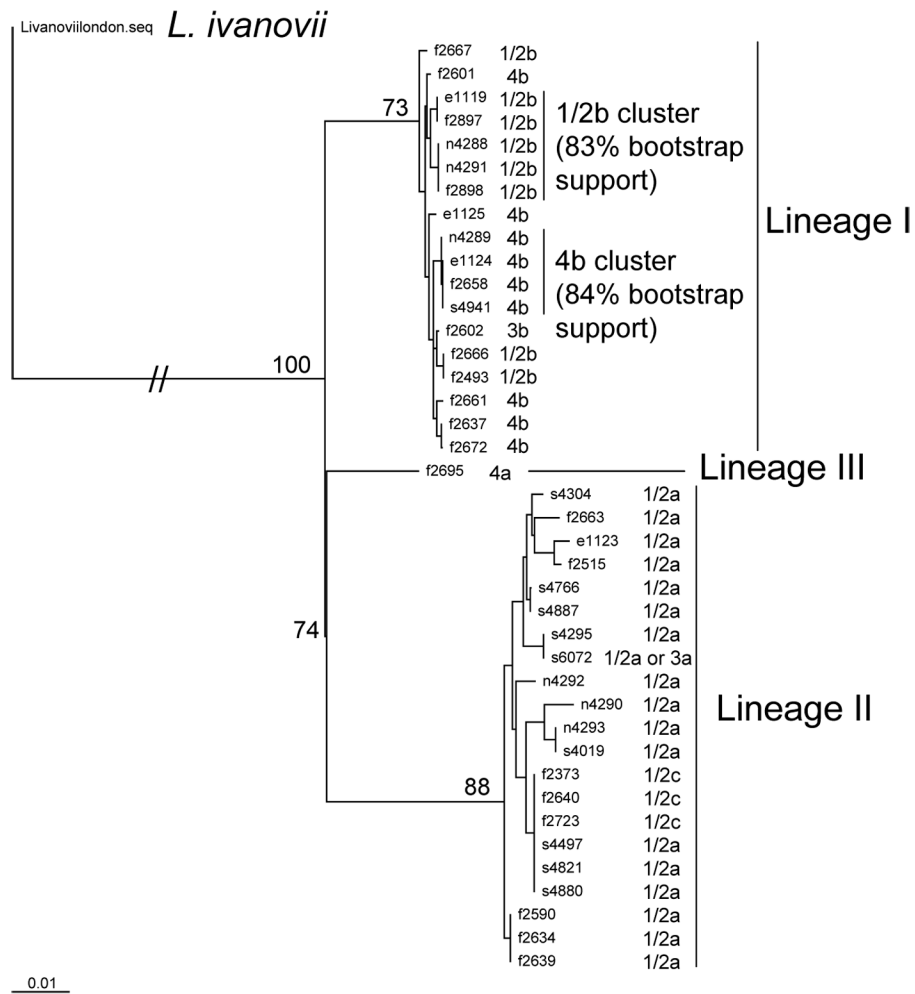


Figure 2. Maximum likelihood tree of the virulence gene cluster for the 40 *L. monocytogenes* isolates in the study. The virulence gene cluster of a *L. ivanovii* subspecies *londoniensis* (NRRL 33021) was used as outgroup. Bootstrap values are shown at the branches that define the lineage of the isolates.

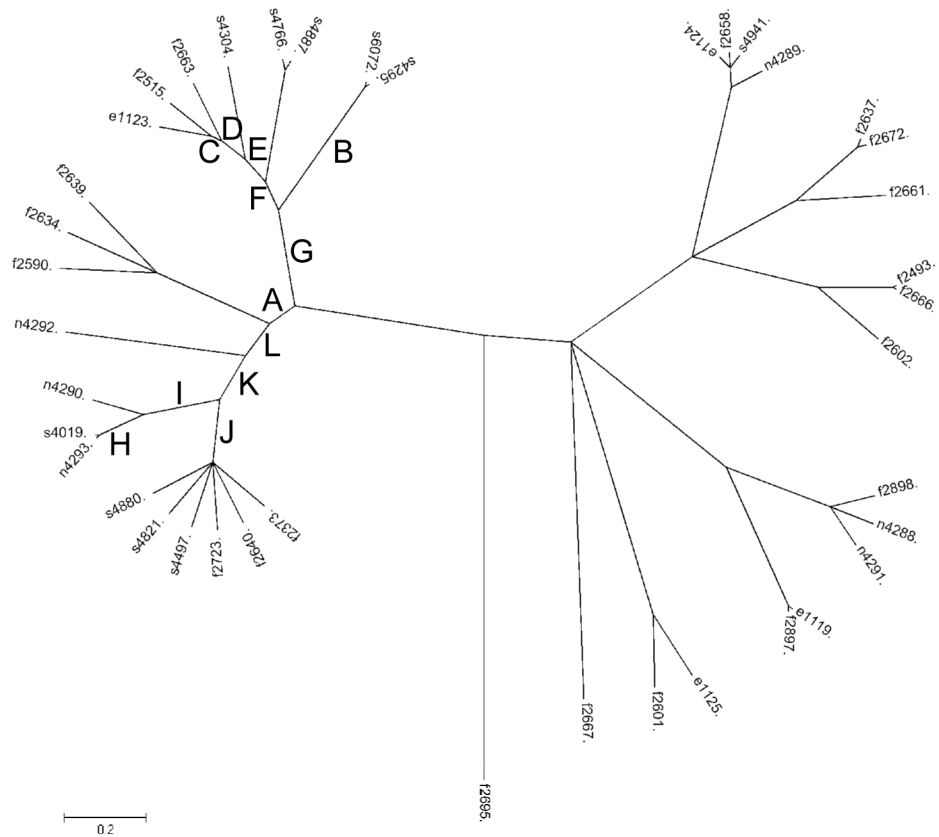


Figure 4. Phylogeny of the 40 *L. monocytogenes* isolates inferred by ClonalFrame based on the virulence cluster sequence data. The tree represents a 95 % consensus between two independent runs of the program with the same initial parameters. In order to construct the tree, ClonalFrame takes into account the possibility of recombination. Internal branches where recombination events were identified are marked with letters (see Table 4 for details regarding the recombination events).

Table 1
Descriptive analysis of the virulence gene cluster nt sequence among 40 *L. monocytogenes* isolates.

Fragment (length in nt)	H ^b	S ^c	PIS ^d	Substitutions	NSS ^e	NNS ^f	π^g	π (NSS)	π (NNS)
Cluster (8709), all	25	592	475	616	NA	NA	0.0246	NA	NA
Lineage I	12	75	64	75	NA	NA	0.0030	NA	NA
Lineage II	12	178	147	181	NA	NA	0.0064	NA	NA
<i>prfA</i> (711), all	9	25	23	25	23	2	0.0143	0.0684	0.0003
Lineage I	4	6	6	6	6	0	0.0028	0.0129	0
Lineage II	4	3	0	3	1	2	0.0004	0.0006	0.0003
<i>pLcA</i> (951), all	18	66	55	67	50	17	0.0211	0.0729	0.0072
Lineage I	8	7	6	7	5	2	0.0028	0.0098	0.0009
Lineage II	9	35	33	35	25	10	0.0138	0.0431	0.0056
<i>hty</i> (1587), all	19	71	55	73	67	6	0.0160	0.0685	0.0020
Lineage I	8	8	7	8	7	1	0.0017	0.0061	0.0004
Lineage II	10	23	20	23	20	3	0.0042	0.0159	0.0008
<i>mpl</i> (1530), all	18	126	91	130	100	30	0.0293	0.1084	0.0096
Lineage I	10	19	13	19	14	5	0.0035	0.0114	0.0013
Lineage II	7	7	6	7	4	3	0.0015	0.0036	0.0009
<i>accA</i> (1917), all	20	184	161	197	98	90	0.0383	0.0977	0.0226
Lineage I	9	24	23	24	13	10	0.0052	0.0123	0.0030
Lineage II	10	82	74	85	47	35	0.0149	0.0371	0.0090
<i>pLcB</i> (867), all	11	62	46	64	45	19	0.0254	0.0868	0.0097
Lineage I	5	5	5	5	2	3	0.0017	0.0035	0.0012
Lineage II	5	13	3	13	7	6	0.0023	0.0054	0.0015

^a the nt positions for the six coding regions in the alignment include: *prfA*, 1 – 711 (located in the opposite strand); *pLcA*, 988 – 1938 (located in the opposite strand); *hty*, 2181 – 3767; *mpl*, 4105 – 5634; *accA*, 5837 – 7753; *pLcB*, 7793 – 8659; data are shown for all 40 isolates as well as for lineage I and II separately (18 and 21 isolates, respectively; the data for the one lineage III isolate are not shown separately)

^b H: number of haplotypes

^c S: number of polymorphic sites (excluding polymorphic sites within indels)

^d PIS: number of parsimonious informative sites;

^e NSS: number of synonymous substitutions;

^f NNS: number of nonsynonymous substitutions;

^g π = nucleotide diversity (average pairwise differences per site); the π values for lineage I and II strains (for the whole cluster as well as for all genes) are statistically significantly different ($P < 0.0001$, T test).

Table 2

Positive selection in coding regions.

LRT and parameters	<i>prfA</i>	<i>plcA</i>	<i>hly</i>	<i>mpl</i>	<i>actA</i>	<i>plcB</i>
M0 vs M3 LRT^d	6.5852	52.3129 [*]	5.0478	18.3828 [*]	153.0975 [*]	0.0
ω^b	1.20	2.12	0.61	1.05	2.39	0.105
p^c	0.0169	0.0696	0.0347	0.0917	0.0978	0.3034
M1a vs M2a LRT^d	0.02886	5.2502	0.0	0.0019	12.3971 [*]	0.0005
ω^b	1.20	2.12	1.00	1.04	2.55	1.00
p^c	0.0169	0.0696	0.0109	0.0917	0.0771	0.00
aa sites under pos. selection^d	-	-	-	-	461 (97%)	-
M7 vs M8 LRT^d	2.1510	5.2529	0.7376	0.0097	13.7596 [*]	0.0
ω^b	1.20	2.12	1.00	1.07	2.40	1.00
p^c	0.0169	0.0696	0.0175	0.0882	0.0955	0.00
aa sites under pos. selection^d	-	aa 119 (95%)	-	-	aa 112 (97%)	-
					aa 123 (96%)	
					aa 291 (97%)	
					aa 449 (97%)	
					aa 461 (99%)	
					aa 522 (95%)	
Lineage I LRT^d	NA ^e	0.0	0.0	6.2440 [*]	1.4834	0.0
ω^b	NA ^e	1.00	1.00	7.28	6.77	1.00
p^c	NA ^e	0.00	0.0056	0.0151	0.174	0.00
aa sites under pos. selection^d	NA ^e	-	-	aa 103 (98%)	-	-
Lineage II LRT^d	0.0	4.6409 [*]	1.1946	0.0001	5.0142 [*]	0.0
ω^b	1.00	2.82	3.95	1.00	3.59	1.00
p^c	0.00	0.0381	0.0044	0.0802	0.0245	0.2064
aa sites under pos. selection^d	-	-	-	-	-	-

^aThis row reports the results for the likelihood ratio test (LRT) for different models (e.g., M0 versus M3; lineage specific tests are indicated as "Lineage I LRT" and "Lineage II LRT"); the value shown represents the likelihood ratio between the two models;

^{*} indicates that the null model can be rejected in favor of the alternative model ($P < 0.05$; significance is calculated using a χ^2 -distribution with degrees of freedom equals the difference in the number of parameters estimated for the alternative model in comparison to the null model)

^b $\omega = dN/dS$ for the site category that allows ω to be larger than 1 (i.e., ω_2 in M2, M3, and the branch specific models; ω in M8)

^c p = the proportion of sites under positive selection

^d aa site identified by Bayes Empirical Bayes (BEB) as having probability > 95% of being under positive selection are shown; posterior probabilities are given in parenthesis; "-"; indicates that no sites showed probability $\geq 95\%$ of having evolved by positive selection

^e NA, not assessed because no amino acid changes were observed within lineage I in *prfA*

Table 3

Positive selection in non-coding regions.

Alignment ^a	LRT M2 vs M1 ^b	LRT M3 vs M1 ^b	Zeta(ζ)	Frequency	Sites under positive selection ^c
<i>plcA</i>	16.63 [*]	16.55 [*]	16.24	0.01	42 ^{***} ; 53 ^{**} ; 180 ^{**}
<i>hly</i>	10.93 [*]	10.92 [*]	5.70	0.10	42 [*] ; 53 [*] ; 92 [*] ; 195 [*] ; 410 [*]
<i>mpl</i>	0.13	0.12	1.49	0.14	-
<i>actA</i>	0.14	0.13	1.48	0.11	-
<i>plcB</i>	0.90	0.86	3.58	0.14	-

^a Alignments contained the coding regions for the listed genes as well as the noncoding regions upstream and downstream of each gene (e.g. the *plcA* alignment contained the intergenic region between *plcA* and *prfA*, the *plcA* coding region, and the intergenic region between *plcA* and *hly*). See Fig. 1 for the relative position of the genes in the virulence gene cluster

^b These two columns report the results for the likelihood ratio test (LRT) for different models (M2 versus M1 [test 2]; M3 versus M1 [test 1]); the value shown represents the likelihood ratio between the two models;

^{*} indicates that the null model can be rejected in favor to the alternative model ($P < 0.05$)

^c Sites with posterior probability > 95% of being under positive selection as estimated by M3; “-” indicates that no sites were identified as evolving by positive selection;

^{*} and ^{**} indicates sites identified with posterior probabilities > 95% and > 99%, respectively. Sites 42, 53, 92, 180, and 195 are located between *plcA* and *hly* (see Fig. 3); site 410 is located between *hly* and *mpl* (i.e., 168 bp downstream of the *hly* stop codon)

Table 4

Recombination events identified by ClonalFrame.

Branch ^a	Lineage of recipient ^b	Region ^c
A	II	<i>plcA</i> ↔ <i>plcA</i>
B	II	<i>actA</i> ↔ <i>actA</i>
FSL S4–304	II	<i>actA</i> ↔ <i>actA</i>
FSL F2–663	II	<i>actA</i> ↔ <i>actA</i>
FSL F2–515	II	<i>plcA</i> ↔ <i>plcA</i> ; <i>actA</i> ↔ <i>actA</i>
FSL E1–123	II	<i>actA</i> ↔ <i>actA</i>
C	II	<i>actA</i> ↔ <i>actA</i>
D	II	<i>plcA</i> ↔ <i>plcA</i>
E	II	<i>plcA-hly</i> ↔ <i>plcA-hly</i>
F	II	<i>plcA-hly</i> ↔ <i>plcA-hly</i> ; <i>hly</i> ↔ <i>hly-mpl</i>
G	II	<i>plcA</i> ↔ <i>plcA-hly</i>
FSL N4–292	II	<i>actA</i> ↔ <i>actA</i>
FSL N4–290	II	<i>actA</i> ↔ <i>actA</i> ; <i>plcB</i> ↔ <i>plcB</i>
H	II	<i>actA</i> ↔ <i>actA</i>
I	II	<i>actA</i> ↔ <i>actA</i>
J	II	<i>plcA</i> ↔ <i>plcA</i>
K	II	<i>plcA-hly</i> ↔ <i>plcA-hly</i> ; <i>actA</i> ↔ <i>actA</i>
L	II	<i>hly</i> ↔ <i>hly-mpl</i>

^aFor internal branches, letters are shown (A to L; corresponding to the branch labels in Fig. 4); for external branches, the isolate name is shown (e.g., FSL S4–304 indicates that the recombination event was identified in the branch leading to the FSL S4–304 sequence).

^bIndicates the lineage of the recipient sequence.

^c“gene-gene” designations denote intergenic regions (e.g., *plcA-hly* denotes the intergenic region between *plcA* and *hly*); “↔” indicates the extent of the recombinant fragment (e.g., in branch G, one event occurred with the recombinant fragment extending from the coding region of *plcA* to the intergenic region between *plcA* and *hly*). Regions are approximate and were determined based on Clonalframe outputs.