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RESEARCH ARTICLE

Comparison of Widely Used *Listeria monocytogenes* Strains EGD, 10403S, and EGD-e Highlights Genomic Differences Underlying Variations in Pathogenicity

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ABSTRACT **For nearly 3 decades, listeriologists and immunologists have used mainly three strains of the same serovar (1/2a) to analyze the virulence of the bacterial pathogen *Listeria monocytogenes*. The genomes of two of these strains, EGD-e and 10403S, were released in 2001 and 2008, respectively. Here we report the genome sequence of the third reference strain, EGD, and extensive genomic and phenotypic comparisons of the three strains. Strikingly, EGD-e is genetically highly distinct from EGD (29,016 single nucleotide polymorphisms [SNPs]) and 10403S (30,296 SNPs), and is more related to serovar 1/2c than 1/2a strains. We also found that while EGD and 10403S strains are genetically very close (317 SNPs), EGD has a point mutation in the transcriptional regulator PrfA (PrfA*), leading to constitutive expression of several major virulence genes. We generated an EGD-e PrfA* mutant and showed that EGD behaves like this strain *in vitro*, with slower growth in broth and higher invasiveness in human cells than those of EGD-e and 10403S. In contrast, bacterial counts in blood, liver, and spleen during infection in mice revealed that EGD and 10403S are less virulent than EGD-e, which is itself less virulent than EGD-e PrfA*. Thus, constitutive expression of PrfA-regulated virulence genes does not appear to provide a significant advantage to the EGD strain during infection *in vivo*, highlighting the fact that *in vitro* invasion assays are not sufficient for evaluating the pathogenic potential of *L. monocytogenes* strains. Together, our results pave the way for deciphering unexplained differences or discrepancies in experiments using different *L. monocytogenes* strains.**

IMPORTANCE **Over the past 3 decades, *Listeria* has become a model organism for host-pathogen interactions, leading to critical discoveries in a broad range of fields, including bacterial gene regulation, cell biology, and bacterial pathophysiology. Scientists studying *Listeria* use primarily three pathogenic strains: EGD, EGD-e, and 10403S. Despite many studies on EGD, it is the only one of the three strains whose genome has not been sequenced. Here we report the sequence of its genome and a series of important genomic and phenotypic differences between the three strains, in particular, a critical mutation in EGD's PrfA, the main regulator of *Listeria* virulence. Our results show that the three strains display differences which may play an important role in the virulence differences observed between the strains. Our findings will be of critical relevance to listeriologists and immunologists who have used or may use *Listeria* as a tool to study the pathophysiology of listeriosis and immune responses.**

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Listeria monocytogenes is a low-GC-content, Gram-positive, rod-shaped bacterium living in a variety of environments, such as soil and decaying vegetation, and can infect animals and humans by means of contaminated food products. The pathogenic properties of *L. monocytogenes* rely on its ability to cross three host barriers (the intestinal, placental, and blood-brain barriers) and

also its ability to enter, replicate, and survive in wide range of human cell types, such as macrophages, epithelial cells, and endothelial cells, thanks to an arsenal of virulence factors. More than 50 virulence factors have been described (1), and the list continuously expands.

During the last three decades, *L. monocytogenes* has emerged as a model organism for the study of host-pathogen interactions (2–5), leading to critical discoveries in a broad range of fields, including virulence factor regulation, cell biology, bacterial adaptation to the host cytosol, and bacterial pathophysiology. In addition, since the pioneering studies of Mackaness (6), *L. monocytogenes* has been widely used as a model to study its interaction with professional phagocytes and host T-cell responses. Remarkably, most of these discoveries have been made using three *L. monocytogenes* strains. These widely used strains are the 10403S, EGD, and EGD-e *L. monocytogenes* strains. The genome of the EGD-e strain was sequenced in 2001 (RefSeq accession number NC_003210 [7]). The sequence and annotation of the 10403S genome have recently been released (NC_017544), as have those of several other strains (8–12). Currently, NCBI's RefSeq database contains 39 *L. monocytogenes* genomes, and this number will probably continue to grow exponentially in the coming years. In this context, the unknown sequence of the extensively used strain EGD remained a gap to fill.

The EGD strain is from the Trudeau Institute (NCTC7973) and derived from the original strain isolated from guinea pigs by E. G. D. Murray et al. in 1926 (13). The name *Listeria monocytogenes* was definitively coined by Pirie (14). Strain EGD was brought back to France by Patrick Berche (see reference 15) in 1982 after a stay at the Trudeau Institute with Robert North. Helmuth Hahn also obtained strain EGD from the Trudeau Institute and gave it to Trinad Chakraborty in 1986 (see reference 16). The two strains from the Trudeau Institute used to be passaged through mice to maintain virulence. When the *Listeria* genome sequencing project was initiated, the European consortium chose to sequence strain EGD, which was retested for its virulence in mice by Trinad Chakraborty and thereafter named EGD-e (where "e" stands for "European" [7]). *L. monocytogenes* 10403S is a streptomycin-resistant (83) derivative of 10403 reported to be isolated from human skin lesions in Bozeman, MT (17).

The three strains belong to serovar 1/2a. The serotyping scheme, based on somatic (O) and flagellar (H) antigens, is the oldest technique used to differentiate *L. monocytogenes* strains (18) and has enabled classification of *L. monocytogenes* in three main lineages (I, II, and III). A subpopulation of lineage III, lineage IIIB, is now called lineage IV (19, 20). Strikingly, a phylogenetic study by multilocus sequence typing (MLST) demonstrated that despite the fact that EGD-e is of serotype 1/2a, it clusters with 1/2c strains and is distantly related to 10403S and EGD (21). Phenotypic differences among the three strains have in the past been observed by listeriologists but not published. However, in different studies that we reported, EGD was used in preference to EGD-e because of its higher invasiveness in human cells (22–27). Nevertheless, until now, no study has been performed to characterize in detail the differences between the three *Listeria* reference strains.

We report here the sequence and the annotation of the genome of *L. monocytogenes* EGD and a genomic and phenotypic comparison of the three laboratory model strains, EGD, EGD-e, and 10403S. A comparison of protein-coding genes and noncoding RNAs shows that even if two of the three strains have nearly the same name (EGD-e and EGD), they differ, with EGD being closer to 10403S and EGD-e being more distant. One major difference is a PrfA mutation found in EGD that induces an overexpression of the PrfA-regulated genes (PrfA*), leading to a higher invasiveness in cultured cells and a difference in virulence in animal models.

RESULTS

Resequencing of the EGD-e genome sequence. Prior to sequencing the *L. monocytogenes* EGD genome, we resequenced the genome of strain EGD-e using the Illumina technique. Only five differences compared to the published sequence were found (Fig. 1A), confirming the high quality of the first published sequence (7, 28). As shown in Data Set S1 in the supplemental material, four of the five differences are in intergenic regions, where no small RNAs (sRNAs) have been identified so far, and only one difference induces an amino acid change, i.e., a glycine to a valine, in Lmo0247, a hypothetical protein.

EGD's genome sequence and its comparison to those of EGD-e and 10403S. The EGD genome was sequenced by the Illumina technique, assembled, annotated, and deposited in the European Nucleotide Archive (ENA) (accession number HG421741). Strain EGD has one chromosome of 2,907,193 bp and no plasmid. This genome is of approximately the same size as that of strain 10403S (2,903,106 bp). The EGD-e genome (7) is 40 kb larger, with a total size of 2,944,528 bp (Table 1).

A single nucleotide polymorphism (SNP) search, using MUMmer (29), comparing all three strains to each other revealed 29,016 SNPs (Table 1) between EGD and EGD-e (Fig. 1A and Data Set S1) and 30,296 SNPs between 10403S and EGD-e. In contrast, only 317 SNPs distinguish EGD from 10403S, indicating that EGD and 10403S are genetically very close. This result is consistent with the reported MLST analysis of EGD and 10403S (21), which shows a high similarity (Fig. 1B) in terms of sequence type (ST) between EGD (ST 12) and 10403S (ST 85), with both strains being classified in clonal complex 7 (CC7), whereas EGD-e belongs to CC9.

We found 2,848 open reading frames (ORFs) in EGD, a number close to the 2,846 ORFs predicted for EGD-e (7) and 2,814 ORFs predicted for 10403S (Table 1). To investigate further the differences between these ORFs, we performed a bidirectional best-hit search (threshold E value, $<1e-4$). As shown in Fig. 2A, more than 95% (2,683) of EGD-e's ORFs are shared by the three strains. EGD, EGD-e, and 10403S have exactly the same number of rRNAs (18 rRNAs) and tRNAs (67 tRNAs). They also have the same low GC content (39%) at the level of the whole genome (Table 1).

Of the 393 ORFs not shared by the three strains, only 8 are common to EGD and EGD-e and not present in 10403S (Data Set S2). EGD and 10403S share 22 ORFs that are absent in EGD-e (Data Set S1), and EGD-e and 10403S share 36 ORFs (Fig. 2A) that are not found in EGD. Of these, 30 come from the A118 prophage, which is integrated into both EGD-e and 10403S, as previously described for EGD-e (7, 30). EGD has 135 ORFs which are not found in the two other strains (Fig. 2A and Data Set S2); 52 are phage proteins, and 50 are hypothetical proteins for which RAST automatic annotation software has found no homolog.

EGD has a prophage different from that of EGD-e and 10403S. Since EGD has 52 specific genes encoding putative phage proteins, we examined whether EGD had an integrated phage. A BLASTN search of each phage gene of the three strains (Fig. S1A)

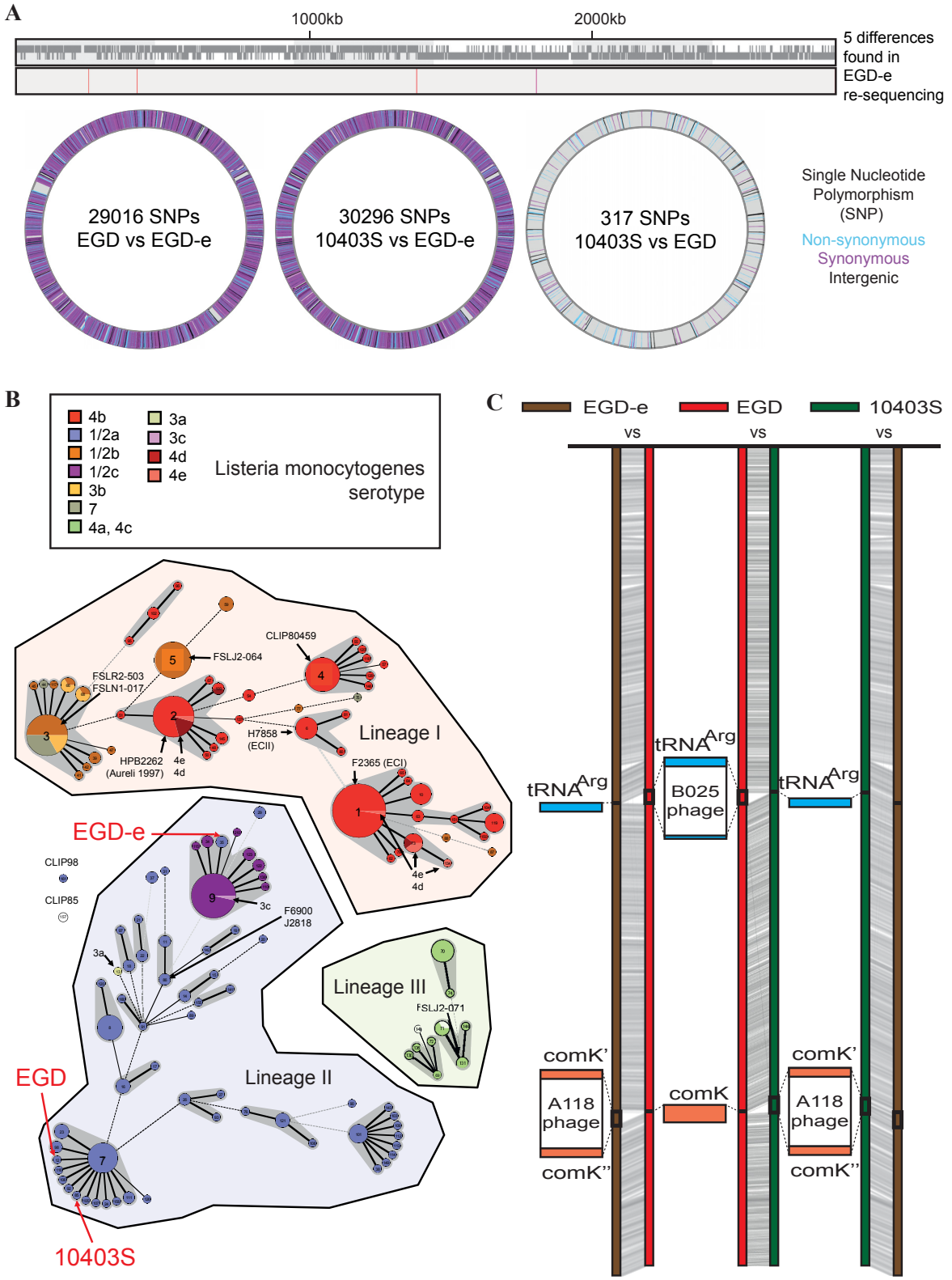

FIG 1 SNPs, synteny, and sequence type analysis of EGD, EGD-e, and 10403S. (A) SNPs among the EGD, 10403S, and EGD-e reference genomes. Purple indicates synonymous changes, blue indicates nonsynonymous changes, and black indicates intergenic changes. (B) Minimum spanning tree analysis of 360 *L. monocytogenes* strains based on MLST (multilocus sequence typing) data (adapted from reference 21). The EGD-e, EGD, and 10403S strains are highlighted in red. (C) Linear synteny view of the three strains. Phage BO25 is integrated into EGD in $tRNA^{Arg}$. Phage A118 is integrated inside the *comK* gene in EGD-e and 10403S. ComK is complete in EGD.

TABLE 1 General properties of EGD-e, EGD, and 10403S sequences and annotations

Listeria monocytogenes strain	Sequence		Annotation						
	Genome size (bp)	No. of SNPs	% G+C content	No. of ORFs	No. of rRNAs	No. of tRNAs	No. of sRNAs	No. of internalins	Prophage (*attB* integration site)
EGD-e	2,944,528	5 between EGD-e (7) and resequenced EGD-e	39	2,846	18	67	154	26	A118 (*comK*)
EGD	2,907,193	29,016 between EGD and EGD-e; 317 between EGD and 10403S	39	2,848	18	67	144	25	B025 ($tRNA^{Arg}$)
10403S	2,903,106	30,296 between 10403S and EGD-e	39	2,814	18	67	145	26	A118 (*comK*)

using 8 sequenced *Listeria* species phage genomes (31) indicated the presence of A118 phage genes in EGD-e and 10403S, integrated into the competence gene *comK* (32), and the presence of B025 phage genes in EGD (Fig. 1C), integrated into the $tRNA^{Arg}$ gene. B025 is found in the first half of the EGD genome (between *LMON_1236* and *LMON_1299*) (Fig. S1B). A118 is integrated in the second half of the EGD-e genome (between *lmo2271* and *lmo2332*) and the 10403S genome (between *LMRG_01560* and *LMRG_01510*).

Conservation of sRNAs. In the past decade, noncoding RNAs in *Listeria* have been studied in detail (33–42). One study concerns strain 10403S (36), and all the other studies concern the EGD-e strain. We compiled a list of small noncoding RNAs (sRNAs) from these various publications. Altogether, 305 noncoding RNA elements have now been reported in *L. monocytogenes*, with 155 sRNAs, 46 *cis* regulatory elements (cisRegs), and 104 antisense RNAs (asRNAs).

A comparison of EGD-e RNAs by a BLASTN search (−e 0.001 –W 4) in EGD and 10403S showed a very high conservation (Data Set S1) with regard to protein-coding genes. Regarding sRNAs, 142 out of the 155 (92%) are common to the three strains (Fig. 2B); 100% of cisRegs are conserved, as are 97% of the asRNAs. Only 9 sRNAs are found only in EGD-e (Fig. S2A and B). The particular case of Rli38 is interesting, as it seems that the whole region from *lmo1097* (which encodes an integrase) up to the 5′ end of the Rli38 gene, has been integrated in EGD-e upstream from *lmo1116* (Fig. S2C).

Conservation of internalin genes. *L. monocytogenes* encodes a large family of proteins known as internalins, which possess a leucine-rich repeat (LRR)-containing domain. Twenty-five members of this family, including several virulence factors, that have been classified into three types (Fig. S3A) were described for strain EGD-e (43). InlA, the prototype internalin, and InlB promote *L. monocytogenes* internalization into mammalian cells and were initially identified in EGD (44–46). We found that InlA and InlB show, respectively, 8 and 6 nonsynonymous amino acid differences in EGD and 10403S compared to their counterparts in EGD-e. With a BLASTP search of the different internalins already described in EGD-e, we found 27 internalins present in the different genomes of strains EGD-e, EGD, and 10403S (Fig. 2C and Table 2).

Our new analysis of the EGD-e genome revealed the presence of a type IV internalin represented by Lmo0460, a predicted lipoprotein (47) containing an atypical LRR domain (Fig. 2D). Whether Lmo0460 is a bona fide lipoprotein remains to be confirmed. The lipobox of Lmo0460 is located at the expected distance from the N terminus and differs only slightly from the consensus lipobox, L − 3-S/A − 2-A/G − 1-C + 1. Lmo0460 displays a novel type of LRR domain, with 10 repeats diverging from the internalin-LRR prototype motif by a longer length (26 instead of 22 amino acids [43]) and the presence of an MFXXCX sequence at the end of most repeats (Fig. 2D). The unusual Lmo0460 LRR repeats with the M-F motif are found in various predicted surface proteins (often lipoproteins) from other species, such as *Listeria innocua*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Mycoplasma mycoides*, and *Helicobacter hepaticus*. The functions of these proteins are still unknown. A BLASTP search did not reveal any homolog of Lmo0460 in EGD and 10403S (Table 2). However, the gene is conserved in many *L. monocytogenes* strains of different serovars.

As previously reported, *inlH* from EGD-e comprises the 5′ end of *inlC2* and the 3′ end of *inlD*, both found in EGD and 10403S, and likely results from a recombination event (Fig. S3B). InlH and InlC2 proteins are highly homologous; they have the same LRR domain and C-terminal regions that differ by only 13 amino acids (Fig. S3C).

Presence of a PrfA* mutation in EGD. Expression of virulence genes at the right time and place during infection is critical for the outcome of the disease and is thus highly regulated. PrfA is a regulator of the major virulence genes (48–50). It belongs to the cyclic AMP (cAMP) receptor protein (Crp)/fumarate nitrate reductase regulator (Fnr) family of bacterial transcription factors. PrfA is itself regulated by an RNA thermosensor allowing PrfA-regulated genes to be expressed at 37°C (41), the temperature of infected hosts. PrfA is also regulated by nutrient availability via a short noncoding RNA generated by a riboswitch (40).

Among the SNPs detected between the different genomes, a remarkable one is present in the *prfA* gene of strain EGD. We found 2 amino acid changes in PrfA of strain EGD compared to PrfA in EGD-e and 10403S; one glycine is changed into a serine at position 145, and one cysteine is changed into a tyrosine at position 229 (Fig. 3A). While the impact of the latter change, located in the G α-helix, on PrfA function is not known, the former, located in the D α-helix, is well known. It is a PrfA* mutation (51). This Gly145Ser mutation is believed to induce a conformational change in the PrfA protein, leading to a constitutively active protein and overexpression of the virulence locus and of the whole PrfA regulon (52). Strikingly, PrfA is the only protein in the whole virulence locus with an amino acid sequence in EGD that is different from that of 10403S. All the other proteins are similar in the two strains but show some differences in strain EGD-e (Fig. 3B and Data Set S1). This result predicted that EGD might express the PrfA regulon in a way very different from that of EGD-e and 10403S (see below).

It is noteworthy that our analysis of NrdD, a class III anaerobic ribonucleotide reductase (RNR), in EGD showed that a KITPFE

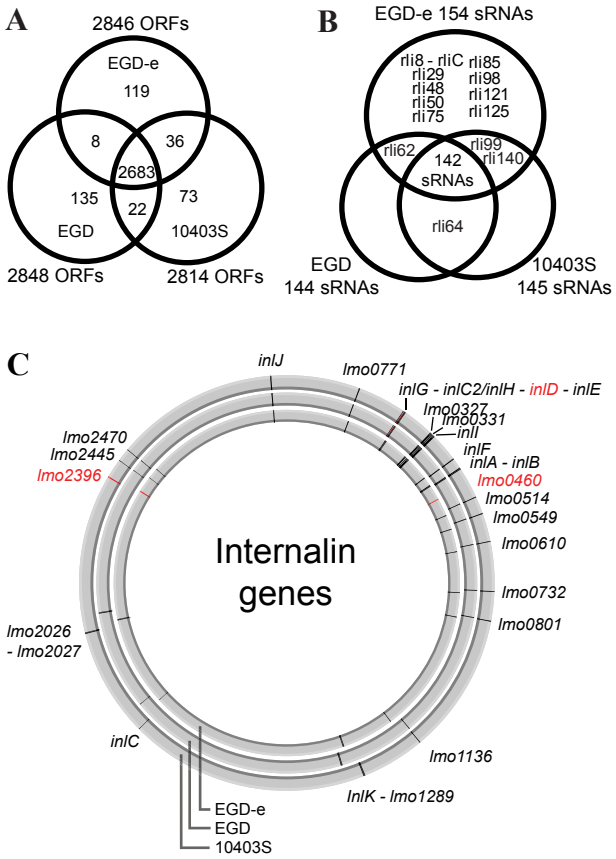

FIG 2 Conservation of ORFs, small RNAs, and internalins in EGD, EGD-e, and 10403S. (A) Venn diagram showing the numbers of ORFs common to the different strains. A bidirectional best-hit search with an E value score lower than 1e−4 was used to determine homologies. (B) Venn diagram of the small RNAs found in the three strains. The percentage of similarity was calculated

(Continued)

TABLE 2 List of 27 internalins found in EGD-e, EGD, and 10403S[c]

Internalin (no. of SNPs) for:			
EGD-e	EGD	10403S	*inl* gene name[a]
lmo0171	LMON_0168 (6)	LMRG_02416 (6)	
lmo0262	LMON_0259 (0)	LMRG_02647 (0)	*inlG*
lmo0263	LMON_0260 (7)	LMRG_02646 (3)	*inlH*, *inlC2*[b]
	LMON_0261	**LMRG_02851**	*inlD*
lmo0264	LMON_0262 (6)	LMRG_02612 (6)	*inlE*
lmo0327	LMON_0334 (77)	LMRG_00021 (172)	
lmo0331	LMON_0336 (20)	LMRG_00023 (25)	
lmo0333	LMON_0338 (14)	LMRG_00025 (14)	*inlI*
lmo0409	LMON_0418 (6)	LMRG_00102 (6)	*inlF*
lmo0433	LMON_0441 (24)	LMRG_00126 (24)	*inlA*
lmo0434	LMON_0442 (13)	LMRG_00127 (15)	*inlB*
lmo0460			
lmo0514	LMON_0514 (19)	LMRG_00195 (19)	
lmo0549	LMON_0549 (17)	LMRG_00231 (27)	
lmo0610	LMON_0611 (7)	LMRG_00293 (7)	
lmo0732	LMON_0737 (6)	LMRG_00420 (5)	
lmo0801	LMON_0805 (8)	LMRG_02867 (6)	
lmo1136	LMON_1129 (12)	LMRG_00579 (9)	
lmo1289	LMON_1350 (0)	LMRG_00739 (0)	
lmo1290	LMON_1352 (13)	LMRG_00740 (13)	*inlK*
lmo1786	LMON_1853 (2)	LMRG_02825 (2)	*inlC*
lmo2026	LMON_2097 (16)	LMRG_01175 (16)	
lmo2027	LMON_2098 (2)	LMRG_01176 (1)	
lmo2396		**LMRG_01852** (8)	
lmo2445	LMON_2456 (0)	LMRG_01803 (0)	
lmo2470	LMON_2481 (5)	LMRG_01778 (0)	
lmo2821	LMON_2840 (5)	LMRG_01877 (5)	*inlJ*

[a] Internalin genes extensively studied.
[b] *inlH* is in EGD-e; *inlC2* is in EGD and 10403S.
[c] Boldface indicates internalin genes present in one or two strains.

motif present in strain EGD (Fig. S1C), as well as in 10403S, is absent in EGD-e (53), revealing a higher capacity for the first two strains to live under anaerobic conditions, including the gastrointestinal tract.

The PrfA core regulon in EGD is overexpressed. To assess the impact of the PrfA* mutation in EGD, we constructed a PrfA* mutant in EGD-e by generating a Gly145Ser mutation and compared the phenotypes of the two strains (EGD and EGD-e PrfA*) to the EGD-e strain in exponential phase, after growth in brain heart infusion (BHI) at 37°C. We first performed a whole-genome transcriptomic analysis of the resulting EGD-e PrfA* strain using our Affymetrix tiling array (35). As EGD-e and EGD share more than 95% of their ORFs and sRNAs, our tiling arrays could also be used for EGD transcriptomic analysis. We found that in both EGD and EGD-e PrfA*, the core PrfA regulon (54), which contains the whole virulence locus, the *inlA-inlB* operon, *inlC* (*lmo1786*), and *hpt* (*lmo0838*), is overexpressed compared to its expression in the reference strain EGD-e (Fig. 3C and Data Set S3), confirming the effect of the Gly145Ser PrfA* mutation on the core PrfA regulon.

Figure Legend Continued

from BLASTN results. Small RNAs with a percentage lower than 10% were not considered conserved. (C) Genomic locations of 27 internalins in the EGD-e, EGD, and 10403S genomes (using CGView [81]). In red are indicated internalins present only in one or two strains. (D) Lmo0460 amino acid sequence. Lmo0460 is a predicted lipoprotein present only in EGD-e which contains an atypical leucine-rich repeat (LRR) domain.

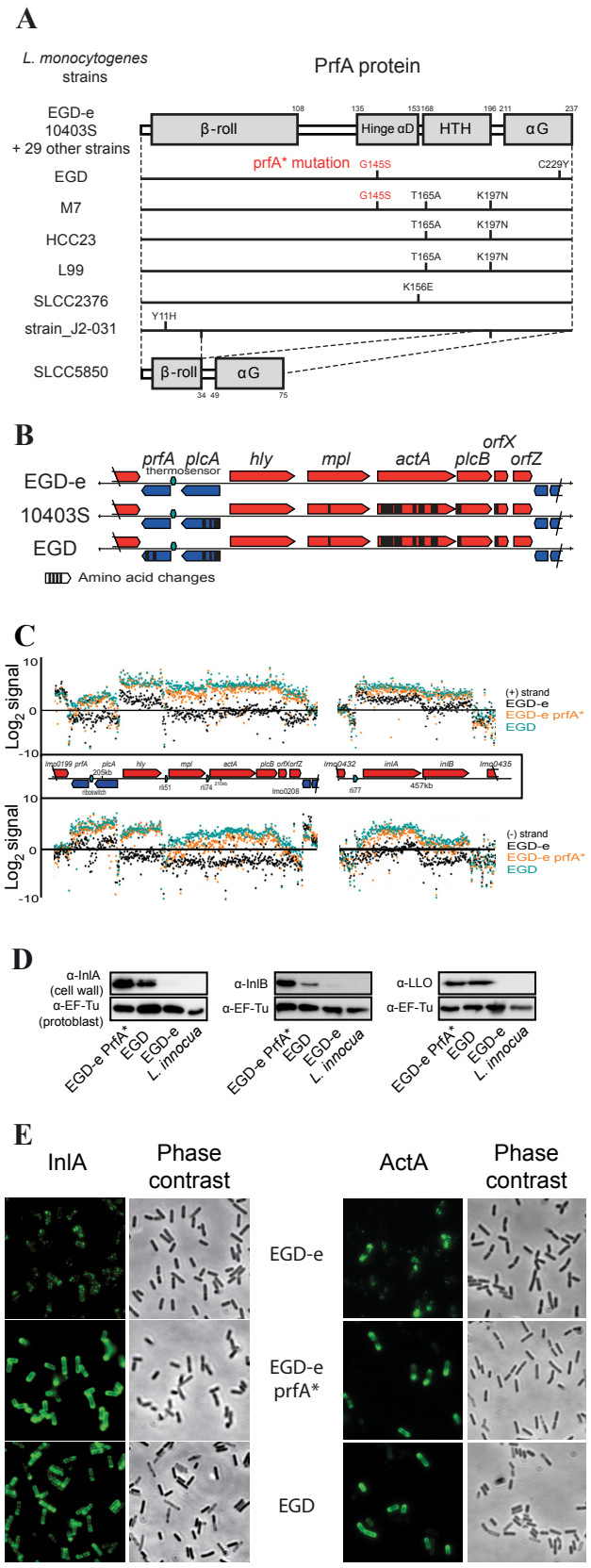

FIG 3 PrfA* mutation and the overexpression of the PrfA core regulon in EGD. (A) Protein sequence alignment of PrfA in 43 *L. monocytogenes* strains. The well-known PrfA* mutation G145S (51, 82) is highlighted in red and

(Continued)

All these genes have a canonical PrfA box upstream from their start codon, which allows the direct binding of PrfA.

Notably, we found that only 15 genes in the EGD-e PrfA* strain (Fig. S4A) are expressed differently from those in EGD-e when bacteria are grown to an optical density at 600 nm (OD_{600}) of 1.0 at 37°C. This list includes the 11 genes of the PrfA core regulon. *Lmo2269* was found to be differently expressed in *L. monocytogenes* P14prfA* versus P14ΔprfA after growth in BHI (85). The three remaining genes, *argC* (*lmo1591*), *argG* (*lmo2090*), and *lmo0640*, have to our knowledge never been described in PrfA regulation studies. In EGD, the number of genes expressed differently (128 genes) compared to EGD-e under reference conditions is much larger (Fig. S4A) than for EGD-e PrfA*, but also includes the core PrfA regulon (54). Overexpression of *inlA*, *inlB*, *inlC*, *hly*, and *lmo0042* (which is similar to the gene for the *Escherichia coli* DedA protein, an inner membrane protein) was confirmed by quantitative reverse transcription-PCR (qRT-PCR) in the three strains (Fig. S5). The overproduction of the InlA, LLO, and InlB proteins was also confirmed by Western blotting (Fig. 3D). Examination of InlA at the bacterial surface (Fig. 3E) by immunofluorescence assay (55) showed that InlA decorates the bacterial body and accumulates at poles in EGD-e PrfA* and EGD. In contrast, in EGD-e, InlA is detected at the surface as helical dots, in agreement with the results of our previous studies (56). ActA was more highly expressed at the bacterial surface in EGD-e PrfA* and EGD than in EGD-e. Of interest, exposure of ActA on the surface seems to be a bistable process, as only half of the cells express it (Fig. 3E).

We also looked at differently expressed RNAs. Our statistical analysis revealed, in total, 27 sRNAs that were expressed in EGD and EGD-e PrfA* differently from in EGD-e (Fig. S4B and S5 and Data Set S3).

Phenotypic effect of the PrfA* mutation. The two PrfA* strains EGD-e PrfA* and EGD grow more slowly in broth than strains EGD-e and 10403S. This was confirmed by a colony size analysis showing larger colonies for EGD-e than for EGD-e PrfA* and for 10403S than for EGD on BHI agar plates after 24 h of growth (Fig. 4A). This is in accordance with the defect already observed in 10403S PrfA* strains (57).

We performed classical gentamicin invasion assays (58) using strains EGD, 10403S, EGD-e PrfA*, and EGD-e in three different cell lines: HeLa (in which entry is InlB dependent), JEG3 (in which entry is InlA and InlB dependent), and Raw264 (macrophages). In HeLa and JEG3 cells, strains EGD and EGD-e PrfA* were more invasive than EGD-e and 10403S (Fig. 4B). There were also higher bacterial counts in mouse Raw264 macrophages for EGD-e PrfA* than for EGD-e and for EGD than for 10403S.

Figure Legend Continued

appears only in the EGD and M7 strains. All other amino acid changes found are drawn showing their positions in the different domains of PrfA (52). HTH, helix turn helix. (B) Schematic representation of the *v*irulence locus synteny in EGD-e, EGD, and 10403S. Amino acid differences from EGD-e's sequence are displayed. (C) Genome browser view showing tiling array whole-transcriptome coverage of the virulence locus and the *inlA-inlB* operon in EGD-e, EGD-e PrfA*, and EGD. Each tiled probe indicating expression from the two genomic strands (top for plus strand, bottom for minus strand) is represented as a black dot for EGD-e, an orange dot for EGD-e PrfA*, and a green dot for EGD. (D) Comparison of expression levels of InlA, InlB, and LLO in EGD-e, EGD-e PrfA*, EGD, and *L. innocua* Clip11262 (used as a nonpathogenic reference bacterium) in whole bacterial lysates or in the cell wall fraction (InlA). (E) Immunofluorescence of InlA and ActA in EGD and EGD-e PrfA* in BHI medium.

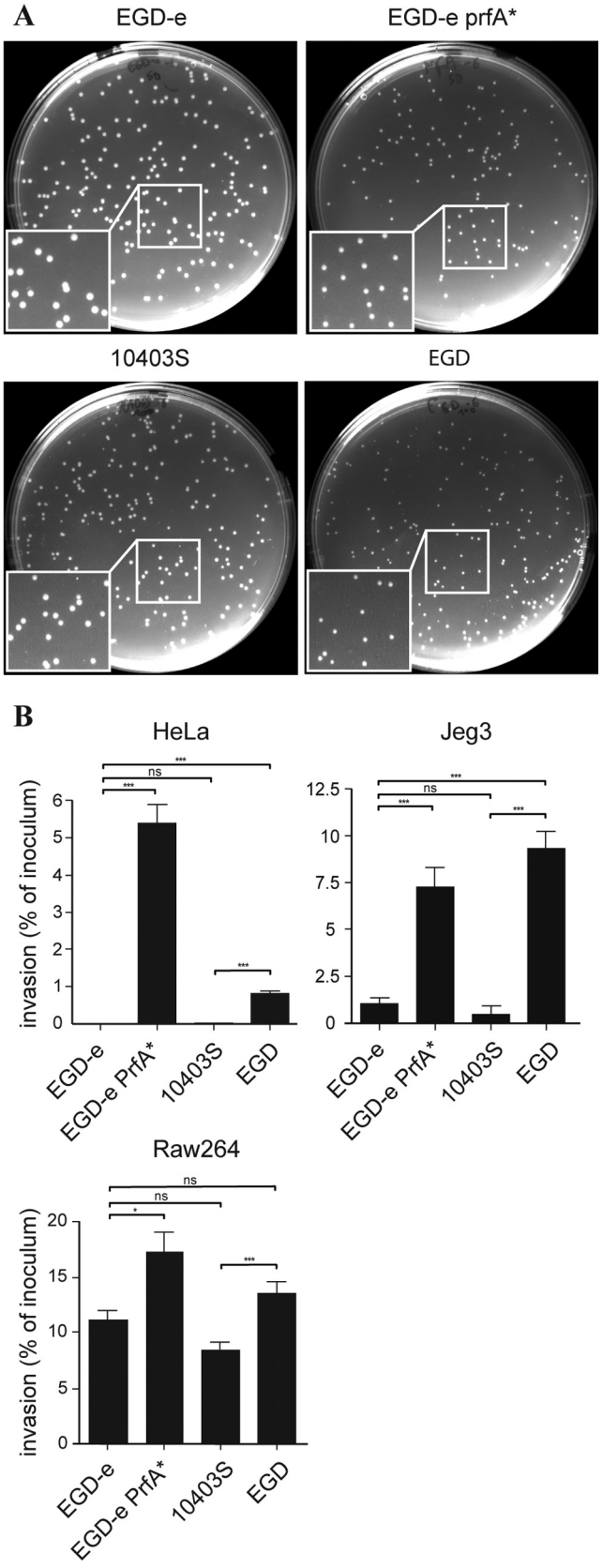

FIG 4 Differential bacterial invasion phenotypes *in vitro*. (A) Colony sizes of the three strains after 24 h of growth on solid BHI agar plates reveal that EGD-e

(Continued)

We performed plaque assays in L2 fibroblast cells. We observed a larger number of plaques for each PrfA* strain (Fig. 5A and B). Strikingly, plaque size was larger for the 10403S strain than for the EGD-e, EGD-e PrfA*, and EGD strains (Fig. 5C).

We then evaluated the virulence of the different strains in mice. Clearly, strains EGD and 10403S are less virulent than EGD-e, as revealed by lower bacterial counts in blood, liver, and spleen (Fig. 5D), showing that many factors control virulence. In addition, the number of EGD-e PrfA* bacteria was higher than the number of wild-type EGD-e bacteria in blood, liver, and spleen, in agreement with the observed overexpression of the PrfA regulon (Fig. 5D). An increased virulence was also observed for 10403S PrfA* (57). Strikingly, despite a clear phenotypic difference in tissue culture cells, bacterial counting in mice spleen and liver 72 h after intravenous infection did not demonstrate clear differences between EGD and 10403S strains.

DISCUSSION

Here we report the genome sequence of *L. monocytogenes* strain EGD and compare it to the genomes of strains EGD-e and 10403S, the two other *Listeria* strains widely used by immunologists and listeriologists. Despite the fact that more than 95% of the ORFs are conserved in EGD, 10403S, and EGD-e, we found many critical differences between these strains. Altogether, our study revealed that the EGD strain is closer to 10403S than to EGD-e and that EGD-e is quite different from EGD. We detected a PrfA* mutation in EGD.

We confirmed the effect of the PrfA* mutation on the invasion of cells both by invasion assays and by plaque assays, but we did not observe an increased virulence of EGD in mice. Finally, the plaque size comparison revealed no difference between the EGD-e, EGD-e PrfA*, and EGD strains. However, we detected larger plaques for 10403S. These many discrepancies, which cannot be explained only by the overexpression of PrfA-regulated virulence factors, need more investigation. A first element to decipher is the complete role of ActA in these phenotypes. ActA is known to trigger intra- and intercellular movements (59) and to mediate escape from autophagy (60), and it is also implicated in interbacterial adhesion during intestinal colonization (61). Here were found 27 amino acid changes between EGD-e ActA and the ActA proteins of strains EGD and 10403S (Fig. 5C); it is the most variable protein within the whole virulence locus (Fig. 3B). The highest proportion of amino acid variation is found in the actin nucleation motif of ActA (Fig. 5E). A thorough comparative analysis of the actin tail lengths and intracellular speeds of the different strains may provide insight into the implication of these ActA amino acid changes for plaque size differences.

Altogether, our analysis indicates that PrfA* mutation does not confer an advantage during the whole process of *Listeria* infection of cells. We performed a comparison of all PrfA protein sequences in 39 published *L. monocytogenes* genomes. The PrfA* mutation appears only in the EGD and M7 strains (Fig. 3A). (M7 is a nonpathogenic serovar 4a strain isolated from cow's milk [62].) We

Figure Legend Continued

PrfA* has smaller colonies than EGD-e and that EGD has smaller colonies than 10403S. (B) Gentamicin assays at 2 h postinfection of HeLa, JEG3, and Raw264 cells by the four different strains, EGD, EGD-e PrfA*, 10403S, and EGD. ns, not significantly different; *, *P* value of <0.05; **, *P* value of <0.005; ***, *P* value of <0.0005.

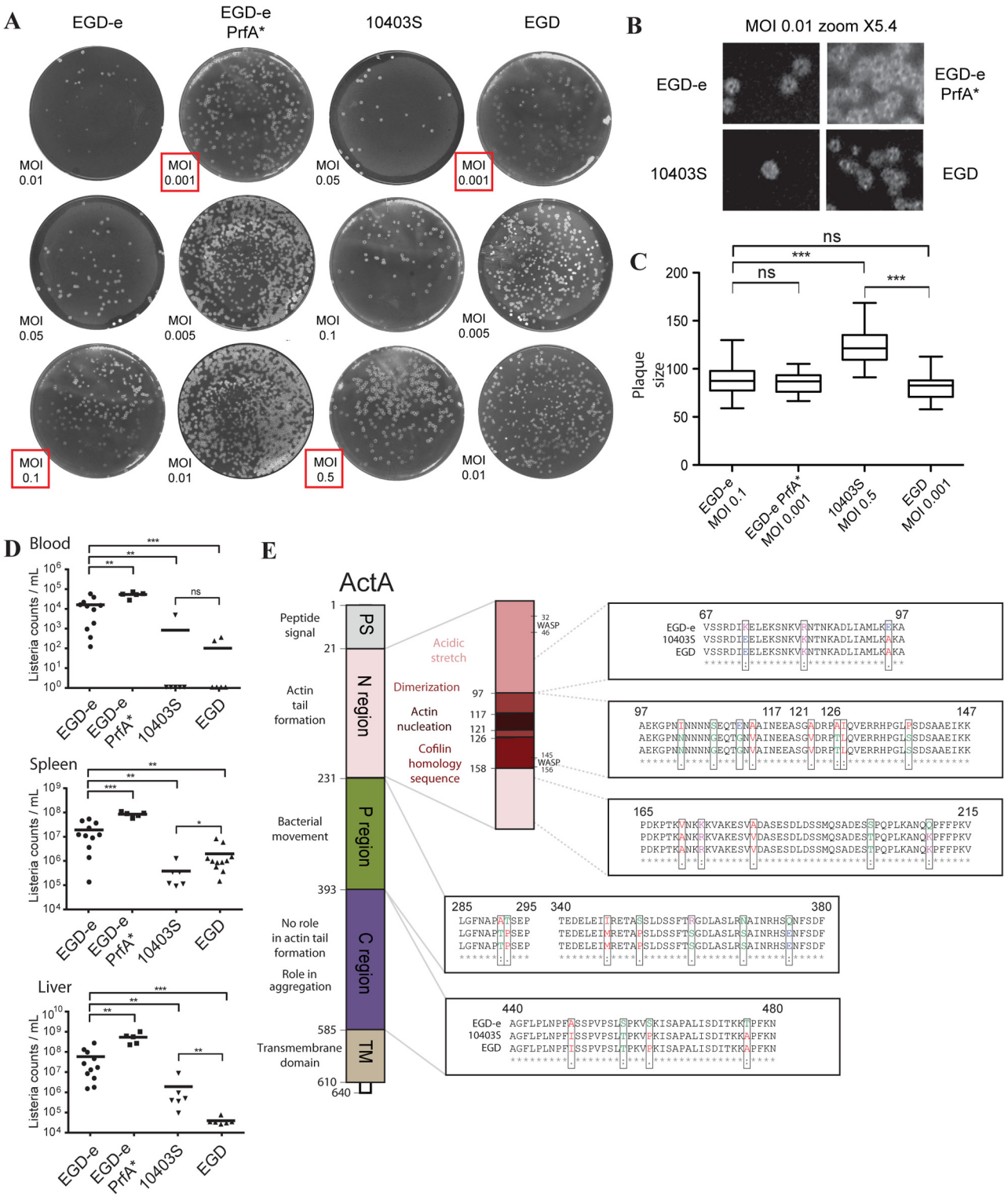

FIG 5 Plaque assays, virulence in mice, and ActA amino acid changes. (A) Plaque assays of EGD-e, EGD-e PrfA*, 10403S, and EGD at different MOIs show different sizes depending on the strain. Highlighted in red are the MOIs used for plaque size measurement. (B) Magnifications (×5.4) of the plaques for EGD-e, EGD-e PrfA*, 10403S, and EGD at an MOI of 0.01. (C) Measurement of plaque size (in square pixels) using Icy image analysis software (80). Different MOIs were used for each strain in order to have the same number of plaques in each well. Differences between strains were assessed by unpaired *t* test. Plaques from 10403S are bigger than the ones from EGD-e and the two PrfA* strains (EGD-e PrfA*, EGD). (D) CFU counts measured 72 h after intravenous infection with EGD-e, EGD-e PrfA*, 10403S, and EGD. Each dot represents the value for one mouse, and asterisks indicate Mann-Whitney statistical test results; results are from two independent experiments. (E) Motifs of the ActA protein (adapted from reference 61) and the different amino acid changes between EGD-e and EGD are shown. ActA has the same amino acid sequence in EGD and 10403S. The two WASP-like sequences of ActA present no differences between EGD-e, EGD, and 10403S.

conclude that the PrfA* mutation does not provide an advantage. It would otherwise have been found in many more strains. In the specific case of the EGD strain, the PrfA* mutation might have been acquired through years of passage in mice, followed by plating on blood agar.

To understand whether the differences between EGD and EGD-e come from a mislabeling or an accumulation of mutations during evolution, we searched for the phylogenetic strains closest to EGD and EGD-e. According to the NCBI genome database, the strain phylogenetically closest to EGD is strain SLCC5850. It is a serotype 4b strain isolated in 1983 from a man with meningitis, according to the Seeliger collection database (63). Its main feature is the loss of important motifs in its PrfA protein (Fig. 3A). The loss of these motifs was also found in the nonhemolytic SLCC53 strain when PrfA was sequenced in 1991 (49). SLCC53 is the type strain of *Listeria* (64) and thus originates from the rabbit strain isolated by E. G. D. Murray in 1924 (see reference 13). The strains phylogenetically closest to EGD-e are SLCC2372, a serotype 1/2c strain isolated from human in 1935, and SLCC2479, a serotype 3c strain which is of unknown origin, isolated in 1966 (12). By comparing the close phylogenetic neighbors of each strain, it seems more likely that EGD is closer to the original type strain and that EGD-e has been mislabeled and exchanged for another strain. However, a complete answer cannot be given until a phylogenomics analysis of all *Listeria* strains available is performed. It would be the only solution to characterize the relationship between strains. However, we face almost a century of *Listeria* strain isolation and cultures, and it clearly seems impossible to decipher completely the many events which might have occurred to create what seems to be a mislabeling of strains.

Since the pioneer work of Mackaness, *L. monocytogenes* has been used and is still widely used as a tool to study the induction of a T-cell response as well as to analyze the response to infection in knockout mice (65–68). In these studies, infections are performed with a variety of *L. monocytogenes* strains, including the three strains EGD, EGD-e, and 10403S. However, strain-specific differences are not taken into account except when using mutants, such as the nonhemolytic mutant or the ActA mutant strains. We consider that many factors in addition to LLO and ActA can affect survival in the host. It is thus of the utmost importance in any report to precisely indicate which strain has been used. It is to be noticed that *Listeria* has recently been engineered as a promising live-vaccine strain against viral infection and cancer (69–74). In most cases, strain 10403S is the original strain used. Given the results reported here, it will be important to use the same original strain in future constructions and vaccine trials. In conclusion, our results highlight strain-specific genomic differences with important consequences for the interpretation of results in both infection biology and immunology. We hope the genomic comparisons that we provided here will help listeriologists to go further in their investigations and strongly recommend that authors always indicate the names and origins of the *Listeria* strains used in their studies.

MATERIALS AND METHODS

For more information on materials and methods, see Text S4 in the supplemental material.

***Listeria monocytogenes* EGD and EGD-e sequencing and annotation.** Briefly, genomic DNA was prepared as described in reference 75. Library preparation was achieved using NEBNext DNA sample prep master mix set 1 with the multiplexing sample preparation oligonucleotide according to the manufacturer's recommendations. Libraries were then sequenced on a HiSeq 2000 sequencer in 100-base single-end reads. Sequence files were generated using Illumina Analysis Pipeline version 1.7 (CASAVA). After quality filtering, 25,827,948 reads were aligned with the *Listeria monocytogenes* 10403S genome sequence (GenBank accession number CP002002) using CLC Genomics Workbench (version 3.20), and more than 98.4% of reads mapped successfully. The remaining 407,195 reads were then used to sequentially fill gaps in the final sequence. The overall final coverage was 875×, with only 47,125 unmapped reads. EGD-e was sequenced using the same protocol, with a total of 13,414,584 reads.

The consensus sequence of EGD has been exported and annotated using RAST annotation software (76). Automatic annotation provided by RAST was curated using homology to proteins in *Listeria monocytogenes* EGD-e (7) and *Listeria monocytogenes* 10403S, and the sequence was submitted to the ENA database (accession number HG421741). Interactive visualization of the syntenic organization of *Listeria* genomes is available with the Flash-based SynTView (77) software available at http://genopole.pasteur.fr/SynTView/flash/Listeria_monocytogenes/SynWebEGD_final.html.

Transcriptomic analysis. Bacterial overnight cultures were diluted in BHI, and bacteria were grown to an OD of 1. RNA was extracted and samples for each chip were prepared as previously described (35). The tiling chip works with two types of arrays: the gene expression array (link E-MTAB-1676; https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1676/) and tiling array (link E-MTAB-1677; https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1677/). Genes having an average false-discovery rate (Benjamini and Yekutieli method [84]) (FDRBY) under 0.05 and an absolute log fold change (|logFC|) value of >1.5 were selected as potential differentially expressed genes. For small RNAs, we applied the cutoff *t* test *P* value of <0.05 and an |logFC| value of >1.5; after manual curation, we obtained a potential list of differentially expressed sRNAs. Genes and sRNAs of interest were then studied using the real-time PCR system ABI PRISM 7900HT (Applied Biosystems), normalized to expression of the gyrase (*lmo0007*) gene, and values were compared by an unpaired *t* test.

***Listeria* strains used.** For every experiment in this paper, we used the following strains: EGD (BUG600), 10403S (BUG1361), EGD-e (BUG1600), EGD-e PrfA* (BUG3057), and *L. innocua* Clip11262 (BUG499). BUG numbers are identification numbers of the Unité des Interactions Bactéries Cellules laboratory's *Listeria* strain collection.

Bacterial lysis, cell wall extraction, and protein detection. For preparation of whole bacterial lysates, 1×10^9 bacteria of overnight cultures were washed 3 times in phosphate-buffered saline (PBS), lysed in 200 μl Laemmli buffer containing 10% dithiothreitol (DTT), boiled for 10 min, and sonicated. Cell wall extraction and Western blotting of InlA, InlB, LLO, and EF-Tu were performed as previously described (78).

Gentamicin invasion assay and *in vivo* studies. We performed classical gentamicin invasion assays as described in reference 58. Cells were plated in 24-well plates the day before infection with the indicated strains at a multiplicity of infection (MOI) between 1 and 25 depending on the host cell type. Bacteria on BHI agar plates for the inoculum and output after gentamicin treatment were counted. Invasion was quantified as a percentage of the inoculum.

All experiments involving mice were handled in accordance with the Pasteur Institute's guidelines for animal welfare. Eight-week-old BALB/c mice (Charles River) were injected intravenously with 10^4 CFU of *Listeria monocytogenes* per mouse. Liver, spleen, and blood samples were recovered 72 h after infection. The organs were disrupted in 2 ml of PBS. Serial dilutions of organ homogenates and of the mouse blood were plated on BHI agar plates and numbers of CFU determined.

Plaque assay. The plaque assay procedure was adapted from the work of Kuhn et al. (79). L2 from cells were grown in Ham's F-12K medium (GIBCO, Life Technologies). Before the infection, monolayers were in-

fected at different MOIs. Infected cells were subsequently incubated at 37°C for 1 h and then washed several times with medium. Following a 48-h incubation at 37°C, cells were fixed with paraformaldehyde (4% in PBS for 20 min) and stained with crystal violet.

In order to measure the plaque size, we needed to have the same number of plaques on each well for each bacterium. We thus selected the following MOIs: 0.1 for EGD-e, 0.001 for EGD-e PrfA*, 0.5 for 10403S, and 0.001 for EGD. Directly on a picture of the plaques, we measured plaque size (in square pixels) using the interior value of the region of interest (ROI) manually defined by Icy software (80). Almost 30 plaque sizes were measured for each bacterium. An unpaired *t* test was used to assess plaque size differences between strains.

Nucleotide sequence accession number. The sequence of EGD was submitted to the ENA database under accession number HG421741.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00969-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Data Set S1, XLS file, 1.1 MB.
Data Set S2, XLS file, 1.5 MB.
Data Set S3, XLS file, 0.1 MB.
Figure S1, PDF file, 1.1 MB.
Figure S2, PDF file, 1.5 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 1.1 MB.
Figure S5, PDF file, 0.5 MB.

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P.C. conceived and designed the experiments. C. Bécavin, C. Bouchier, C.A., Z.W., A.K., M.G.P., J.P.-C., and H.B. performed the experiments. C. Bécavin, C. Bouchier, P.L., C.A., S.C., F.G.-D.P., I.M., and H.B. analyzed the data. S.B. and I.M. provided analysis tools. C. Bécavin, T.H., D.A.P., T.C., M.L., H.B., and P.C. wrote the manuscript.

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