Complete genome of the barotolerant Listeria monocytogenes RO15 strain and comparison with other strains isolated from food and food processing environments

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Abstract

Background Consumption of Listeria monocytogenes contaminated food can cause infection with a high mortality rate in humans and animals. High pressure processing (HPP) is a non-thermal preservation technique adopted by the food industry to inactivate food pathogens, including L. monocytogenes. Strains of L. monocytogenes show different level of resistance to the high pressure. Some strains resist up to 500 MPa pressure. Here, we tested the pressure resistance of 10 different L. monocytogenes strains, including field isolates and widely used type strains, to 400 and 600 MPa pressure treatments. Genome sequencing, and genome comparison of the tested L. monocytogenes strains were performed to investigate the relation between genomic profile and pressure resistance. Results In this study, we showed that none of the tested strains were resistant to 600 MPa, more than 5 log 10 reduction observed for all strains after 1 minute 600 MPa pressure treatment. However, L. monocytogenes strain RO15 showed no significant reduction in viable cell counts after 400 MPa for 1 minute and it was defined as barotolerant. Genome sequencing of so far unsequenced L. monocytogenes strain RO15, 2HF33, MB5, AB199, AB120, C7, and RO4 allowed us to compare their gene content. Genome comparison of 10 tested strains showed that the three most pressure tolerant strains had more than one CRISPR system with self-targeting spacers. Further, several anti-CRISPR genes were detected in these strains. Pan-genome wide analysis showed that 10 prophage genes were significantly associated with the three most barotolerant strains. Conclusions L. monocytogenes strain RO15 was the most pressure tolerant among the selected strains. Genome comparison suggests that there might be a relationship with prophages, CRISPR systems and pressure resistance in L. monocytogenes .

Background

Listeria monocytogenes is a well-known foodborne pathogen that may cause listeriosis, which can be fatal in animals and humans [1]. Although being a relatively rare food-borne disease, increasing numbers of listeriosis cases have been reported in the EU/EEA countries since 2008 [2]. L. monocytogenes is generally found in food processing, agricultural, and aquacultural environments [3]. Listeriosis outbreaks have been associated with consumption of contaminated food, such as meat, milk, cheese, fish [4]. Therefore, it is critical for the food industry to process food effectively for health and economic reasons. Processes related to L. monocytogenes are challenging because it has a high ability to adapt to different environments, and to survive under various stress conditions [5]. In addition, during food processing, it is important to preserve food quality and nutritional value. High pressure processing (HPP) is an alternative to the thermal processes to inactivate pathogens, including L. monocytogenes, and maintain a high quality of the product. Depending on the products, between 100 to 800 MPa pressure is used in the food industry. HPP has been tested and reported as an effective processing method for several products including fish [6], vegetable [7], meat [8] and cheese [9].

It has been shown that HPP has different effects on different target organisms. For example, a pressure of 300 MPa is enough to inactivate most Gram-negative bacteria, while more than 400 MPa is needed for an inactivation of Gram-positive bacteria [10, 11]. It is also known that pressure resistance can differ among different strains of the same species. Differences in pressure resistance have been reported for strains of several species including Cronobacter sakazakii [12], Salmonella enterica [13], and L. monocytogenes [14, 15]. In L. monocytogenes strains, pressure resistance varies between 300–500 MPa [14]. To our knowledge, genomic profiling and comparison has not been performed for barotolerant and barotosensitive L.

monocytogenes strains. In this study, we selected 10 L. monocytogenes strains to test their resistance against 400 and 600 MPa, compared their genomes, and investigated whether genetic traits may be associated with pressure resistance.

Results

Pressure treatment and reduction of viable cell counts

Reduction of viable cell counts (colony forming units, cfu) after pressure treatment at 400 and 600 MPa for 1 minute showed that pressure resistance differs between strains. The variance in cfu of the treated samples was significantly larger compared to control, indicating large variance in the level of resistance of L. monocytogenes. At 400 MPa for 1 minute, the 10 strains exhibited an average $\log_{10}$ reduction of 0.57 cfu/ml, ranging from 0.05 $\log_{10}$ cfu/ml for the most barotolerant strain (RO15) to 2.07 $\log_{10}$ cfu/ml for the most pressure sensitive strain (EGD-e). Similar variance was observed at 600 MPa. Here, the 10 strains exhibited an average $\log_{10}$ cell number reduction of 7.06 cfu/ml, with a range of 5.42 to 8.27 $\log_{10}$ cfu/ml (Table 1). Strain RO15 was also the most barotolerant strain based on an initial screening including several other L. monocytogenes strains (Supplementary text 1, Table S1, Figure S1).

Table 1
Reduction in viable cell counts after 1 minute 400 and 600 MPa pressure treatment. Table shows the average reduction in $\log_{10}$(cfu/ml) of selected L. monocytogenes strains after 1 minute at 400 or 600 MPa pressure compared to untreated control samples (n = 6 to 9 treated/untreated samples of per strain).

	Log_{10} reduction compared to control -$\Delta\log_{10}$ (cfu/ml)	
Strain	400 MPa	600 MPa
RO15	0.05	5.42
2HF33	0.15	6.97
MB5	0.22	8.27
AB199	0.26	6.95
AB120	0.35	7.30
C7	0.37	7.52
F2365	0.47	7.10
ScottA	0.68	5.86
RO4	1.00	7.70
EGD-e	2.07	7.49

One-way analysis of variance (ANOVA), comparing the pressure (400 MPa) treated samples of each strain to the control samples, showed that the mean cfu/ml for treated samples was significantly lower than the control for all strains ($p < 0.02$), except for strain RO15 ($p = 0.15$) (Fig. 1a). The same statistical analysis for HPP at 600 MPa indicated that all strains including strain RO15 had significantly lower cfu/ml in treated samples compared to controls (Fig. 1b).

Genome sequencing, general features and RNA-Seq

We sequenced and assembled seven L. monocytogenes strains (RO15, 2HF33, MB5, AB199, AB120, C7, and RO4). Sequenced seven strains and three additional strains (ScottA, F2365 and EGD-e, genome sequences of which are available in public databases) were used for comparative genome analysis. All genomes had a similar size, GC content and number of coding sequence (CDS) (Table 2). Strain 2HF33 had the largest genome with the highest number of CDS (3.11 Mbps; 3092 CDS) whereas strain RO4 had the smallest genome size with the least number of CDS (2.88 Mbps; 2806 CDS). As RO15 was defined as barotolerant strain, we sequenced the genome of L. monocytogenes strain RO15 using PacBio RSII long read technology. Obtained data was assembled using HGAP3 resulting in one continuous 3,042,507 bp sized chromosome (Fig. 2) at average 308X

sequencing coverage. In addition to the chromosome, a contig with a complete, circular prophage sequence of 38,811 bp was obtained, which was also found as part of the chromosome (2729417–2770759 bp).

Table 2
General genome summary of selected L. monocytogenes strains. The table shows the genome size, number of predicted CDS and serotypes of selected strains.

Strain	Size, Mbp	#CDS	Serotype	MLST	Lineage	Genome Source	Source	Reference
RO15	3.04	3022	1/2a	155	II	This study (Complete)	Herring with spices	[16]
2HF33	3.11	3092	1/2a	121	II	This study (Contigs)	Salmon filleting section	[20, 21]
MB5	2.97	2975	1/2a	7	II	This study (Contigs)	Salmon gutting machine	[20]
AB199	2.99	2914	1/2a	204	II	This study (Contigs)	Drain/processing room	[16]
AB120	3.00	2922	1/2a	204	II	This study (Contigs)	Sausage filler machine	[16]
C7	3.09	3050	1/2a	8	II	This study (Contigs)	Salmon gutting machine	[20]
F2365	2.91	2808	4b	1	I	GenBank: AE017262.2	Mexican-style cheese	[17]
ScottA	3.02	2966	4b	290	I	GenBank: CM001159.1	Clinical isolate	[18]
RO4	2.88	2806	1/2a	20	II	This study (Contigs)	Dry cured salami	[16]
EGD-e	2.94	2875	1/2a	35	II	GenBank: AL591824.1	Rabbit tissue	[19, 22]

The serotype of the strains was reported in previous studies [16, 17, 18, 19, 20] (Table 2). Although RO15 was reported as a serovar 4b strain [16], our genome-based prediction suggests that it belongs to serovar 1/2a. Sequences for ORF2110 and ORF2819 primers used for identification of serovar 4b strains were not found in the genome of strain RO15, whereas sequences for primers targeting lmo0737 and prs, which are indicators of serovar 1/2a strains were present. Multilocus sequence typing (MLST) of the strains were also assigned based on the genome sequences (Table 2). Lineage assignment showed that only ScottA and F2365 belong to lineage I and all other strains were members of lineage II (Table 2, Figure S2).

In addition to genome sequencing, transcriptome analysis using RNA-seq for strain RO15 and ScottA provided a basic view of transcriptional activity of the genomes (Table S3).

Methylated DNA Motifs

High PacBio sequencing coverage allowed us to analyze DNA methylation modifications motifs in strain RO15 in addition to assembling the genome. Using the long read modification data eleven methylated sequence motifs were detected in the genome (Table 3). None of the detected motifs had a partner motif, i.e. a reverse-complementary sequence, but all detected motifs were only partially modified in the genome with less than 50% methylated motifs. While most of the motifs have already been deposited in the REBASE database [23] DADGYATYA, WNNTVVGCNTWNH, AHNBAACA, AGNNARNWW were novel, i.e. they have not been described as potential methylation sites previously. None of the detected motifs have been reported as recognition sequence motif for a restriction enzyme in the REBASE database.

Table 3
A summary of motifs. The table shows the detected methylated motifs and total number of motifs in the genome.

Motifs	Type	% Motifs Detected	# of Motifs Detected	# of Motifs in Genome	mean Coverage
ADGYACYTV	m6A	44.03%	420	954	149.9
ADDTGGCA	m6A	30.97%	455	1469	148.5
TVVARARG	unknown	22.20%	1835	8265	148.8
ANNYASYA	m6A	22.10%	3289	14879	149.0
DADGYATYA	m6A	21.02%	326	1551	147.7
WNNTVVGCNTWNH	unknown	18.14%	582	3208	149.2
AHNBAACA	m6A	13.36%	839	6282	150.2
AGNNARNWW	m6A	9.85%	2225	22596	148.6
TNNNDNNH	unknown	9.22%	114413	1240313	148.9
TNNNCRVHNH	unknown	7.59%	6652	87598	149.0
TVNNNNNG	unknown	3.26%	7834	240556	149.6

We predicted one type II cytosine-5 DNA methyltransferase gene (OCPFDLNE_00657), and three type II N4-cytosine or N6-adenine DNA methyltransferase genes (OCPFDLNE_02168, OCPFDLNE_02626, OCPFDLNE_02808) in strain RO15 genome. In addition, genes for a type IV methyl-directed restriction enzyme (OCPFDLNE_00324) and type II restriction enzymes (OCPFDLNE_00658, OCPFDLNE_02625, OCPFDLNE_02807) were predicted. These predicted methyltransferases and restriction enzymes had high e-value (> 1E-50) BLASTP hits in the REBASE protein sequences database [23]. However, their recognition sequences are unknown. OCPFDLNE_02625 and OCPFDLNE_02807 type II restriction enzyme genes in prophage regions were expressed based on RNA-seq data.

Genomic comparison of the selected strains.

In the genome assembly we also identified a circular phage as an independent contig. While looking after phage originating genome parts prophage prediction revealed five prophage regions (10.7 kb to 47.9 kb sized)

in the RO15 genome (Fig. 2). Prophage (region) 5 had the same sequence as separate circular phage. RNA-Seq count data suggested that most of the genes of prophage 1,3 and 5 in RO15 were transcribed. Following, the prophage prediction was performed for all strains, which showed that all strains had at least one or more prophage region in the genome with a maximum of six prophage regions in strain C7 (Table 4). One of the predicted prophage regions in ScottA was also transcribed based on the RNA-seq data.

Table 4

Comparison of CRISPR/Cas systems, anti-CRISPR genes, and prophage regions amongst the selected L. monocytogenes strains.

Strain	Average reduction of log_{10}(cfu/ml) at 400 MPa	CRISPR/Cas systems	Number of spacers	Self-targeting spacer predicted	anti-CRISPR gene predicted	Number of prophage predicted
RO15	0.05	RliB-CRISPR, CRISPR I	64	yes	AcrIIA1, A2	5
2HF33	0.16	RliB-CRISPR, CRISPR I, CRISPR II	62	yes	AcrIIA1, A2, A3, A4	5
MB5	0.22	RliB-CRISPR, CRISPR II	39	yes	AcrIIA1, A2, A3	5
AB199	0.26	RliB-CRISPR	3	no	no	1
AB120	0.34	RliB-CRISPR	3	no	no	1
C7	0.37	RliB-CRISPR	7	no	no	6
F2365	0.48	RliB-CRISPR	3	no	no	1
ScottA	0.68	RliB-CRISPR	3	no	no	3
RO4	1.00	RliB-CRISPR	5	no	AcrIIA1, A2, A3	1
EGD-e	2.07	RLIB-CRISPR	4	no	AcrIIA1, A2, A3	2

Additional files

Additional File 1: Supplementary Text 1. L. monocytogenes strain selection, including Table S1, Figure S1.

Additional File 2: Figure S2. Maximum-likelihood tree shows lineages of strains. Concatenated sequences of the seven MLST gene fragments were used to create approximately maximum-likelihood tree to assign lineage of strains. Bold written strains were used in this study. The lineage information of other strains (not bold written) in this figure were shown in a previous study [64]. L. innocua strain Clip11262 was used as outgroup.

Additional File 3: Table S2. Table shows Average Nucleotide Identity (ANI) (based on BLAST) calculation results of each strain. For strain ScottA and F2365 the ANI score with other strains were lower than 95. ANI score between ScottA and F2365 were higher than 95. For the other strains ANI scores between each other were more than 98.

Additional File 4: Table S3. Gene count data for both strain RO15 and ScottA based on RNA-Seq.

Additional File 5: Table S4 and Figure S3. Table S4 shows identified antibiotic resistance genes and percentage Identity of matching region in all strains using CARD database. Figure S3 shows average distance trees from alignments of protein sequences of identified antibiotic resistance genes.

Strain	Average reduction of log_{10}(cfu/ml) at 400 MPa	CRISPR/Cas systems	Number of spacers	Self-targeting spacer predicted	anti-CRISPR gene predicted	Number of prophage predicted
Additional File 6: Table S5. T-test against RO15 log_{10} reduction.						
Additional File 7: Table S6. Barotolerant strains specific genes based on pan-genome wide association analysis. Table shows genes and its annotation that have $p < 0.01$ based on pan-genome wide association analysis for barotolerant strains. Gene ID of strain RO15 was used for this table. Orthologs of these genes with 95% identity was also seen in strain MB5 and 2HF33.						

Genome alignment of the selected nine strains against the strain RO15 (Fig. 3) showed large sequence gaps between strain RO15 and the other strains. The gaps were mainly related to prophage regions with prophage region 3 being specific for strain RO15. Prophage region 2 was only seen in the strain 2HF33. Prophage region 4 was partially seen in strains 2HF33, EGD-e, MB5 and ScottA. Similarly, prophage region 5 was partially seen in strains 2HF33, C7, and RO4. In addition, strains ScottA and F2365 had less aligned regions compared to the other strains. Average nucleotide identity (ANI) results showed that ANI scores of strain ScottA and F2365 with other strains were lower than 95 (Table S2).

Annotation of CRISPR systems revealed that RliB-CRISPR system was seen in all the strains, which is in line with previous RliB-CRISPR system studies [24, 25]. Interestingly, CRISPR I or CRISPR II system genes were present only in strain RO15, 2HF33, and MB5 (Fig. 4), which exhibited the lowest reduction in log_{10} cfu/ml with the 400 MPa pressure treatment (Table 1). The alignment of the spacer sequences of the CRISPR systems back to the genome itself revealed that only strain RO15, 2HF33, and MB5 contained self-targeting spacers with 100% identity. As expected, spacer sequences were aligned to the prophage regions, except one spacer sequence in RO15. It aligned to the gene ATP-dependent helicase addB (OCPFDLNE_02427) located on the chromosome.

We predicted that strain RO15, 2HF33, MB5, RO4, and EGD-e had anti-CRISPR genes in the prophage regions (Table 4). Homologues of all four previously annotated Listeria anti-CRISPR genes, i.e. acrIIA1, acrIIA2, acrIIA3, and acrIIA4 [26], were seen in strain 2HF33. Gene acrIIA4 was not seen in strains MB5, RO4, and EGD-e, but the rest of the anti-CRISPR genes were present. RO15 contains two copies of acrIIA1 (OCPFDLNE_02770, OCPFDLNE_02583) and acrIIA2 (OCPFDLNE_02582) (Fig. 2) and we observed expression of the anti-CRISPR genes in strain RO15.

As antibiotic resistance and pressure resistance were linked in a previous study [14], we also searched antibiotic resistance genes in all strains to identify relation of antibiotic resistance genes and pressure resistance. Four same antibiotic resistance gene families were detected in all the 10 strains (Table S4). Multiple sequence alignment of detected antibiotic resistance genes and following average distance tree generation showed that amino acid sequence of norB gene (encoding for a quinolone resistance protein) (OCPFDLNE_03068) was slightly different in barotolerant strain RO15 compared to other strains (Figure S3). Similarly, for lin gene (encoding for a lincomycin resistance protein) (OCPFDLNE_00980) amino acid difference was seen for strain 2HF33 and RO15 (Figure S3).

Roary pan-genome pipeline suggested that the 10 L. monocytogenes strains contain a total of 4825 orthologous gene clusters. Of these genes, 2250 were core genes found in all strains and 2575 genes were accessory genes found in at least one strain. The core genome was used for phylogenetic tree construction. This indicates that the two serovar 4b strains (ScottA and F2365) are closely related to each other and located closer to the serovar 1/2a strains in the phylogenetic tree. In addition, there was also a clear difference in accessory genome for serovar 4b strains compared to serovar 1/2a strains (Fig. 5).

To test any significant association between pressure resistance and genes (clusters) in the accessory genome, we performed pan-genome wide association analysis using Scoary. Based on pairwise comparison of reduction in $\log_{10}$ (cfu/ml) against strain RO15 using Student's t-test (Table S5), statistically difference compared to RO15 was seen in all strains except MB5 and H2F33. Therefore, strain RO15, 2HF33, MB5 were used as barolerant strains for pan-genome association analysis. Scoary results showed that 13 gene clusters (Table S6) had p-values < 0.01 for the association with barotelerant strains (genes were only seen in barotolerant strains). Of these, 10 gene clusters were located in prophage regions but most of the genes were annotated as hypothetical proteins (Table S6).

Discussion

HPP is commonly used in the food industry to inactivate food pathogens and spoilage organisms. Depending on the type of food product, up to ~ 800 MPa pressure is applied [27]. The pressure resistance level varies between different species, strains, and even isolates [11, 14, 28, 29]. Here, we tested pressure resistance of 10 L. monocytogenes strains, compared their genomes and predicted genome features related to pressure resistance.

Pressure treatment using 600 MPa for 1 minute caused more than 5 $\log_{10}$ reduction in all selected strains, which is generally considered sufficient for food safety regulations. According to the Food Safety and Inspection Service (United States Department of Agriculture), 5 $\log_{10}$ reduction is considered a full lethality treatment for L. monocytogenes [30]. This suggests that the strains used in this study were relatively sensitive to 600 MPa pressure treatment. Similarly, an earlier study reported that pressure treatment at 500 MPa for 10 minutes provided sufficient reduction in viable counts to reach desired level of safety for all except one L. monocytogenes strains tested [14]. However, the lower pressure levels, such as 400 MPa, are more relevant for industrial applications, therefore, we focused more on the results obtained with the 400 MPa treatment. Among the strains tested, RO15 was the most barotolerant strain when processed at both 400 and 600 MPa, which is in line with the initial selection process of strains (Supplementary text 1). Based on ANOVA, RO15 was the only strain for which no statistically significant reduction was observed with the 400 MPa treatment (Fig. 1a). Therefore, we defined the strain RO15 as barotolerant. A difference in viable count (cfu) reduction between experiment 1 and 2 was observed for all strains. Distribution of the $\log_{10}$ cfu concentrations also indicated a large variance in the resistance of L. monocytogenes.

Serotypes of the selected strains were shown in the earlier studies based on agglutination method, multiplex-PCR and genome sequencing [16, 17, 18, 19, 20] (Table 2). Here, we predicted the serotype of all strains based on their genome sequences. This confirmed the previous serotyping of all strains except RO15, which was reported to be a serotype 4b strain [16] that should contain serotype 4 marker genes (ORF2110 and ORF2819)

and lack lmo0737 marker gene sequence as described by Doumith et al. [19]. However, according to the genome sequence, serotype 4 marker genes were absent, and a lmo0737 homologue was present suggesting that the serotype of strain RO15 is indeed 1/2a. The phylogenetic tree that we created based on the core genome showed that there is considerable genetic variation between serovars. Serovar 4b strains (ScottA and F2365) clustered separately from the other strains (Fig. 4). Strain RO15 was not clustered together with other serovar 4b strains, hence these results also supported our gene based prediction of serotype (1/2a) of strain RO15.

PacBio sequencing provides not only the genome sequence but also methylation data [31], which gives an opportunity to analyze restriction-modification systems and their recognition motifs. In this study, we have not observed a genuine methylated motif based on PacBio sequencing data of strain RO15. Similarly, PacBio methylation data of nine other L. monocytogenes strains without genuine methylated motifs were also seen in REBASE database in REBASE PacBio list [23], which might show that it is not uncommon to have only partially methylated motifs in L. monocytogenes.

Previous studies based on L. monocytogenes strain ScottA and LO28 barotolerant isolates showed that there is a phenotypic and genotypic variation between barotolerant isolates [28, 29, 32], which may indicate that there are different factors which cause pressure resistance. Therefore, it is a challenge to link a genomic profile with pressure resistance in different strains. Nevertheless, our annotation results suggest there was a genotypic difference between barotolerant and barosensitive strains. Two or more CRISPR-Cas systems, self-targeting spacers, and anti-CRISPR genes were seen together only in three most barotolerant strains (RO15, MB5, and 2HF33). Since the existence of self-targeting spacer indicates that the activity of the CRISPR/Cas systems is inhibited [33], it can be predicted that anti-CRISPR proteins detected in barotolerant strains inactivate CRISPR/Cas system. Previous studies have also shown that anti-CRISPR proteins are able to inhibit several types of CRISPR systems [34, 35]. Observed active transcription of anti-CRISPR genes in RO15 prophages based on RNA-seq may also support this prediction.

Both restriction-modification and CRISPR-Cas systems are defense systems against foreign DNA in bacteria [36]. Since no genuine methylated motifs have been observed and CRISPR-Cas systems could be inhibited in barotolerant strains, we speculate that potential of acquiring new genes by horizontal gene transfer and harboring prophages is higher in barotolerant strains. This might provide extra genes which gives selective advantage to bacteria [37]. Interestingly, most of the barotolerant associated genes (Table S6) were located in the prophage regions in the studied genome(s) .The genome comparisons also showed that barotolerant strains harbored a slightly higher number of prophages compared to barosensitive strains implying that phages and inactivated CRISPR-Cas system could give an advantage for pressure resistance in L. monocytogenes.

A previous study concluded that antibiotic resistant L. monocytogenes strains are more resistant to pressure at 400 MPa [14]. Here, annotation results did not show any strain specific antibiotic resistance gene. However, multiple sequence alignment of predicted antibiotic resistant genes showed that there were slight differences in amino acid sequences across the strains (Figure S3). However, it is not known that these amino acid differences cause antibiotic resistance advantage, further studies are required to link the amino acid sequence variations of antibiotic resistance genes and pressure resistance.

Conclusions

In this study we reported that none of the 10 selected L. monocytogenes strains was resistant to 600 MPa 1 minute pressure treatment, all showed more than 5 $\log_{10}$ reduction. Strain RO15 was identified as the most barotolerant strain for 400 MPa 1 minute pressure treatment. Genome sequence of seven new strains and genome comparison of 10 strains revealed that the three most barotolerant strains have CRISPR-Cas genes and anti-CRISPR genes in their genome, and they have slightly more prophage regions compared to barosensitive strains. Furthermore, we have predicted 10 phage genes that might be related to pressure resistance based on the pan-genome wide association test. Therefore, we conclude that prophages and prophage defense systems might have a relation with pressure resistance.

Methods

Strains, Growth conditions and Pressure Treatment

Strains Scott A (CIP103575) and EGD-e (CIP107776) were obtained from Centre de Ressources Biologiques de l'Institut Pasteur, Paris, France, and strain F2365 (LMG23356) from Laboratorium voor Microbiologie, UGent, Gent, Belgium. Strains RO4, RO15, AB120 and AB199 are from Dunarea de Jos University of Galati, Romania, and have been isolated in the Promise FP7 project either from food illegally introduced to Romania or from meat processing environments. All other strains were isolated from the environment as indicated in Table 2. The strains were stored on Microbank beads with cryopreservatives (Pro-Lab Diagnostics) at − 80 °C prior to use. The bacteria were passaged twice in tryptic soy broth (TSB) supplemented with 0.6% (w/v) yeast extract (TSBYE; Oxoid). Prior to pressure treatments, bacteria were grown overnight in 50 mL TSBYE at 37 °C with rotary agitation (150 rpm), resulting in cells in early stationary phase and with a target concentration of approx. 10^9 cfu/mL. Aliquots (10 mL) of the cultures were packaged in sous-vide plastic pouches and sealed without using vacuum, and 10 mL was transferred to 15 mL falcon tubes to serve as untreated controls.

HPP was carried out using the QFP 2L-700 (Avure Technologies Inc., Columbus, USA). The cylindrical pressure vessel had 10 × 25.4 cm dimensions, 2 L capacity and 690 MPa upper pressure limit. Temperature of the pressure medium (water) was tracked with a K-type thermocouple located on the external surface of the samples. A holding time of 1 minute was used, pressures of 400 and 600 MPa, and ambient vessel water temperature (20–22 °C). Due to adiabatic heating, water temperatures in the middle of the vessel at the end of pressure treatment had risen to 31–33 °C after the 400 MPa treatment, and 36–38 °C after the 600 MPa treatment. Straight after pressure treatment, pressurized and untreated samples were serial diluted and plated in triplicate on tryptic soy agar with 0.6% yeast extract (TSAYE; Oxoid) by using a spiral plater (Eddy Jet; IUL Instruments, Spain). TSAYE plates were incubated at 37 °C for 48 hours prior to counting the colonies and estimating bacterial inactivation. Two consecutive trials of the methodology were performed (Exp. 1 and 2).

DNA extraction

DNA of each strain was extracted from 5 ml of a culture grown overnight in BHI broth at 37 °C with aeration on a rotary shaker. Wizard Genomic DNA purification kit (Promega, Madison, USA) was used according to the manufacturer's instructions.

Library preparation, genome sequencing, de novo assembly, base modification detection and motif analysis

L. monocytogenes strain RO15 was sequenced using PacBio RSII (Pacific Bioscience, Menlo Park, CA, USA) and Illumina Miseq (Illumina, San Diego, CA, USA). Pacbio reads were assembled using HGAP3 protocol [38] in SMRTPortal 2.3.0. Obtained assembly of chromosomal and phage sequences were checked and circularized using Gap4 program [39] and finally the chromosomal DNA sequence was set to start from dnaA gene. cutadapt v1.8.1 [40] was used with -m 200 and -q 25 options to trim Nextera adapter sequences and quality filtering. Trimmed reads were mapped against circularized chromosomal and phage sequences using bwa-mem [41]. Short indels within homopolymeric regions were corrected using pilon v1.16 [42]. Average sequencing coverages at the whole genome level were 308X in Pacbio data and 157X in Illumina data, respectively. DNA base modifications and motifs were analyzed using Modification and Motif analysis protocol implemented in SMRTPortal 2.3.0 with default parameters.

For L. monocytogenes strain RO4, AB199, and AB120, Nextera DNA Library preparation and paired-end sequencing were performed using Illumina MisEq. Adaptor removal and quality filtering was done using Trimmomatic v0.36 [43]. Spades v3.13.0 [44] with default options was used for assembling the reads and creating the contigs.

For strain 2HF33, MB5, and C7, Nextera XT DNA Library preparation and paired-end (2 × 300) sequencing were performed on the Illumina MiSeq platform aiming for > 50 x coverage. RTA v1.18.54 and bcl2fastq v2.17.1.14 were used for base calling, demultiplexing and converting data to fastq format. Prior to downstream analysis, adapters used for sequencing and low quality reads were trimmed using Trimmomatic v0.33 [43] following the recommendations from the developer. Reads aligning to PhiX genome that was used as spike-in during Illumina sequencing were removed by using bbmap v34.56 [45]. Sequence reads were trimmed until an average base quality of 30 was reached in a window of 20 bases. de novo assembly was performed using Velvet 1.1.04 [46] with default settings.

RNA extraction

Cells previously stored in RNA Protect (Qiagen) were pelleted by centrifugation for 10 minutes at 5000 g. RNA extraction was performed with NucleoSpin RNA kit (Macherey-Nagel) according to manufacturer's instructions with some modifications in the cell disruption phase. The cell pellets were suspended with 700 µl RA1 buffer and 7 µl β-mercaptoethanol (Sigma-Aldrich). Cells were then mechanically disrupted using Lysing Matrix B tubes (MP Biomedicals) and FastPrep 24 tissue homogenizer (MP Biomedicals) at 6 m/s for 3 × 30 sec. Cells were rested on ice for five minutes between cycles. After spinning the cells briefly and transferring the supernatant to NucleoSpin filter column, manufacturer's protocol was followed. Quantity and quality of RNA extractions were analyzed using the Agilent 2100 Bioanalyzer and RNA 6000 Nano kit.

RNA-seq library preparation

Ribosomal RNAs were removed from the total RNAs (ScottA 9.4 µl; RO15 14 µl) with Ribo-Zero rRNA Removal kit for bacteria (Illumina) according to manufacturer's instructions with 1/3 (ScottA) or 1/2 (RO15) volumes of kit solutions. rRNA-depleted RNA was purified with RNeasy MinElute Cleanup Kit (Qiagen) according to modified protocol of the Ribo-Zero kit manual and eluted in 12 µl of RNA-free water. Eight µl of rRNA-depleted RNA was used to prepare RNA sequencing libraries with SENSE Total RNA-seq Library Prep Kit for Illumina (Lexogen) according to manufacturer's instructions. In reverse transcription and ligation phase, incubation time was extended to two hours. After second-strand synthesis, purification and size-selection of the libraries was

performed with 13 µl of Bead Diluent and 27 µl of Purification Solution. PCR amplification program was slightly modified by increasing the denaturation times: from 30 to 60 sec in the beginning, and from 10 to 30 sec during the cycles; cycle number was increased to 40. Concentration of amplified libraries was measured with Qubit fluorometer and dsDNA HS assay kit (Invitrogen), and size distribution visualized with Fragment Analyzer and High Sensitivity NGS Fragment Analysis kit (Advanced Analytical). Aliquots of amplified libraries were pooled twice for both strains. After pooling, libraries were concentrated using Amicon Ultra 100K columns (Millipore). To remove fragments under 200 bp, size selection of ScottA pools was performed using BluePippin and 2% agarose gel cassette (Sage Science). In size selection of RO15 library pools, bead purification with 0.9 x AMPure XP beads (Beckman Coulter) and PEG/NaCl precipitation on MyOne™ carboxylic acid beads (Invitrogen) were additionally used. Concentration of the pooled libraries was measured with Qubit fluorometer, and libraries were sequenced with NextSeq 500 (Illumina).

RNA-seq

During stationary stage Strain RO15 and ScottA were treated with 200 MPa and 400 MPa pressure for two, eight and sixty minutes at 20 °C. RNA was extracted from triplicate treated and control samples (total of 36 samples per strain). After RNA extraction and RNA-seq library preparation, RNA-seq was performed using Illumina NextSeq500. Quality filtering and adapter trimming for RNA-seq reads was done using Trimmomatic v0.36 [43]. SortMeRNA [47] was used to filter rRNA reads. Reads were mapped to the genomes using Bowtie2. Aligned reads were sorted using Samtools [48]. HTSeq [49] with union mode was used for read counts.

Genome annotation, prophage prediction and genome alignment

Assembled genomes were annotated using Prokka v1.13 [50] with default options. To improve functional annotation PANNZER2 annotation webserver [51] was used. CRISPR repeat regions were detected using CRISPRCasFinder version 4.2.19 [52]. Anti-CRISPR genes were annotated using BLAST alignment against known anti-Crispr genes [26]. The prophage prediction for all genomes was done using PHASTER web tool [53]. Serotype of the selected strains were predicted by checking the marker primers [19] in the genome using EMBOSS primersearch v 6.6.0 [54]. Multilocus sequence typing of strains was determined using Center for Genomic Epidemiology MLST 1.8 [55] with L. monocytogenes as the MLST configuration. Lineages of strains were assigned based on the MLST gene fragments using BIGSdb-Lm database [56]. Antibiotic resistance genes were detected using Resistance Gene Identifier (RGI) tool with CARD database [57]. Heuristic neighbor-joining phylogeny tree based on concatenated sequences of the MLST gene fragments was created using FastTree v2.1 [58]. Whole genome alignment and ring figure was created using CGView Comparison Tool [59]. The ANI scores were calculated using JSpeciesWS webtool [60].

Pan-genome and Pan-GWAS analysis

The pan-genome analysis was done using Roary Pan-genome Pipeline [61] with default settings, to create alignment of core genes using PRANK [62] the “-e” setting was used. The core genes alignment was used for phylogenetic tree construction using FastTree [58]. Pan-genome-wide association analysis was done using Scoary [63] with default settings.

List Of Abbreviations

Analysis of variance (ANOVA), average nucleotide identity (ANI), coding sequence (CDS), colony forming units (cfu), high pressure processing (HPP), megapascals (MPa), multilocus sequence typing (MLST)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All sequencing data and assembled genomes have been deposited in the European Nucleotide Archive (ENA) under accession code PRJEB35939.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PA, AIN, TL, CUR, and NB conceived and designed the study. TMR, TL and FIB collected the samples and performed the pressure treatment and viable cell count. ICD, MA, and PL performed the bioinformatics analyses. AY performed RNA extraction and sequencing preparation. PC extracted DNA. NB performed ANOVA statistical analysis. ICD drafted the manuscript. All authors have read, commented, and approved the final manuscript.

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Additional File Legends

Additional File 1: Supplementary Text 1. *L. monocytogenes* strain selection, including Table S1, Figure S1.

Additional File 2: Figure S2. Maximum-likelihood tree shows lineages of strains. Concatenated sequences of the seven MLST gene fragments were used to create approximately maximum-likelihood tree to assign lineage of strains. Bold written strains were used in this study. The lineage information of other strains (not bold written) in this figure were shown in a previous study [64]. *L. innocua* strain Clip11262 was used as outgroup.

Additional File 3: Table S2. Table shows Average Nucleotide Identity (ANI) (based on BLAST) calculation results of each strain. For strain ScottA and F2365 the ANI score with other strains were lower than 95. ANI score between ScottA and F2365 were higher than 95. For the other strains ANI scores between each other were more than 98.

Additional File 4: Table S3. Gene count data for both strain RO15 and ScottA based on RNA-Seq.

Additional File 5: Table S4 and Figure S3. Table S4 shows identified antibiotic resistance genes and percentage Identity of matching region in all strains using CARD database. Figure S3 shows average distance trees from alignments of protein sequences of identified antibiotic resistance genes.

Additional File 6: Table S5. T-test against RO15 $\log_{10}$ reduction.

Additional File 7: Table S6. Barotolerant strains specific genes based on pan-genome wide association analysis. Table shows genes and its annotation that have $p < 0.01$ based on pan-genome wide association analysis for barotolerant strains. Gene ID of strain RO15 was used for this table. Orthologs of these genes with 95% identity was also seen in strain MB5 and 2HF33.

Figures

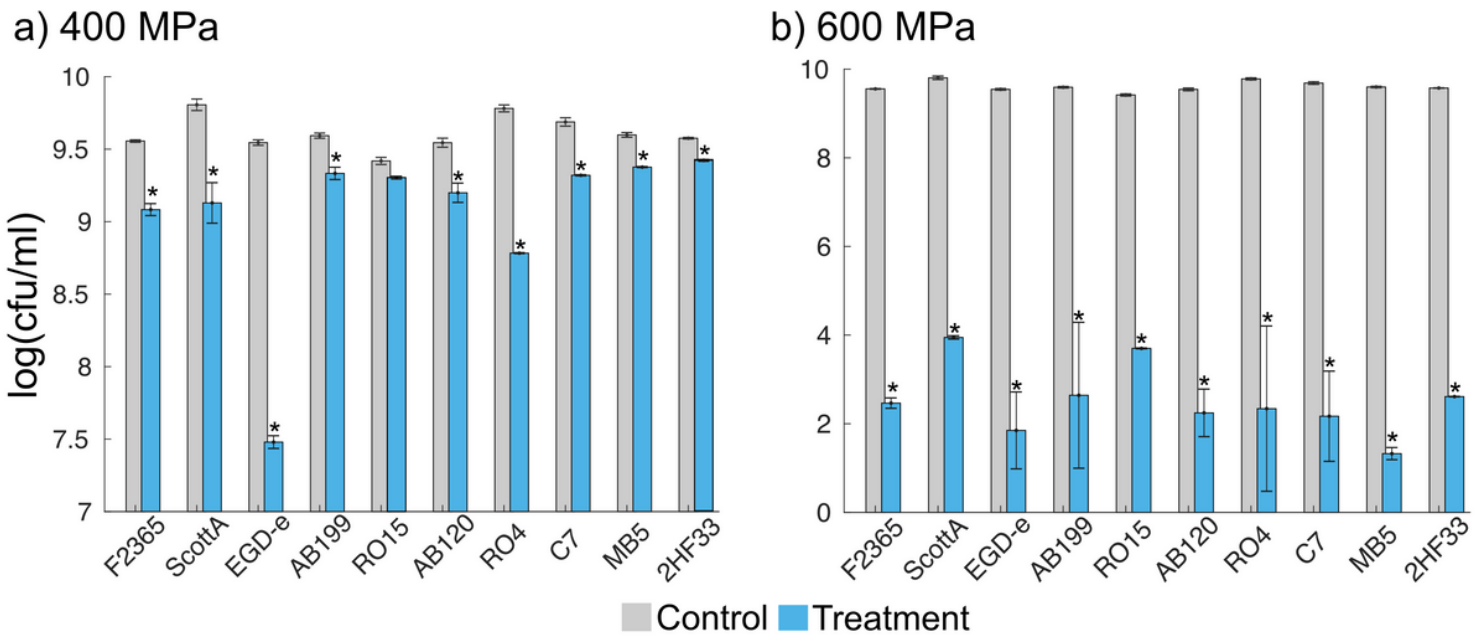

Figure 1

Viable cell counts bar chart. Viable cell counts log10(cfu/ml) of untreated controls and samples treated for 1 minute at 400 MPa (a) or 600 MPa (b). Gray bar represents control samples, blue bar represents treated sample. Error bars represent standard deviation (ANOVA; *, $p < 0.02$).

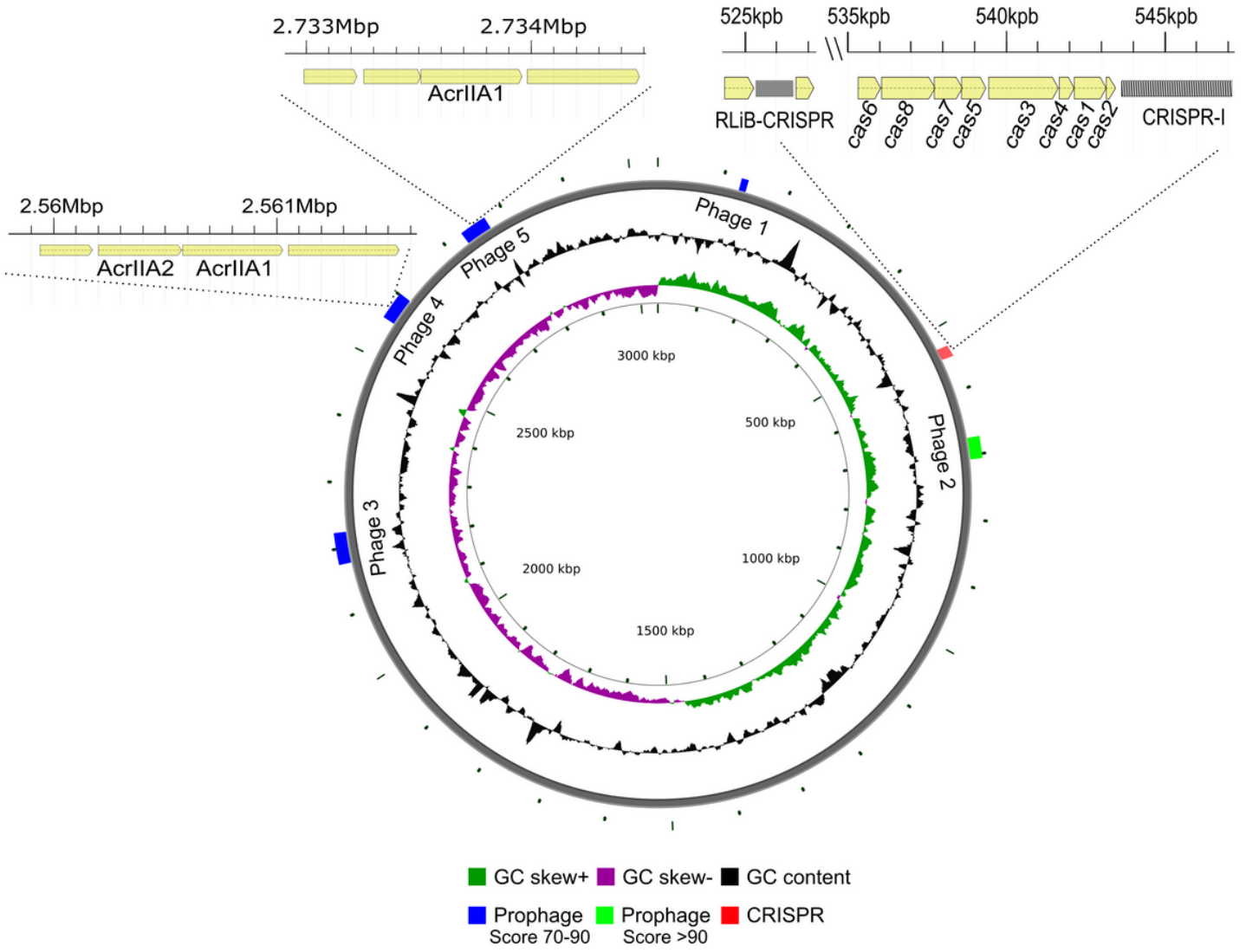

Figure 2

Circular map of the L. monocytogenes strain RO15 chromosome with predicted prophage regions, CRISPR region and anti-CRISPR genes. Five prophage regions were predicted in the chromosome of L. monocytogenes strain RO15 using PHASTER tool, shown in figure with blue and green color. Two anti-CRISPR gene regions were annotated within the prophage region 4 and 5. RliB-CRISPR/Cas system was located at position 525-527 kbp, following CRISPR-I system located the position 535-547 kbp with cas1-8 genes and a CRISPR array with 54 spacers.

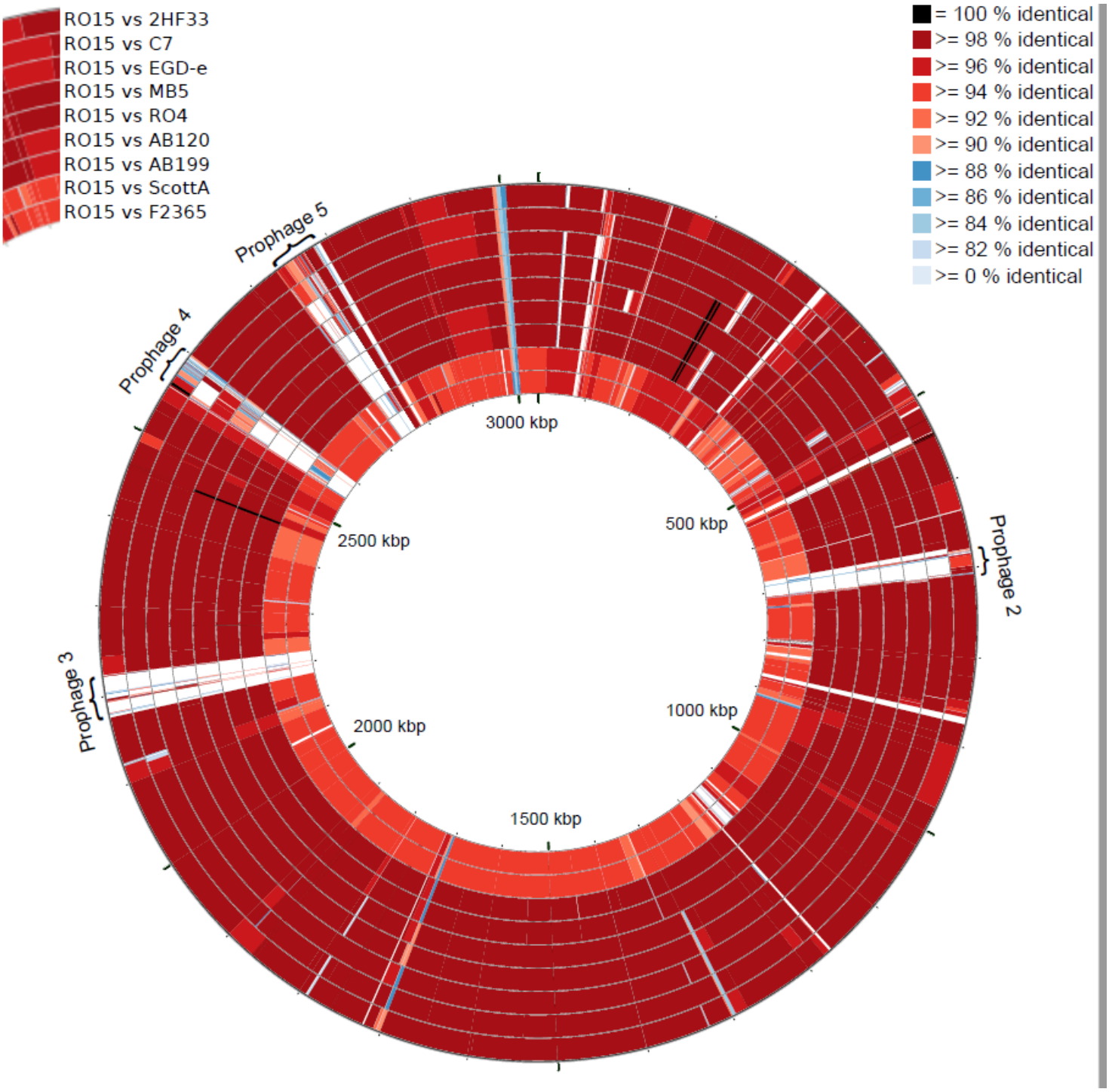

Figure 3

Genome comparison of the selected nine strains mapped against strain RO15. The genomes were aligned using BLAST and visualized as ring figures. Each ring represents the genome alignment to strain RO15. From inside to outside, strains are located according to increasing hits of high percent identity to strain RO15 (Order from inside to outside: strain F2365, ScottA, AB199, AB120, RO4, MB5, EGD-e, C7, and 2HF33. Color represents BLAST hit identity percentage).

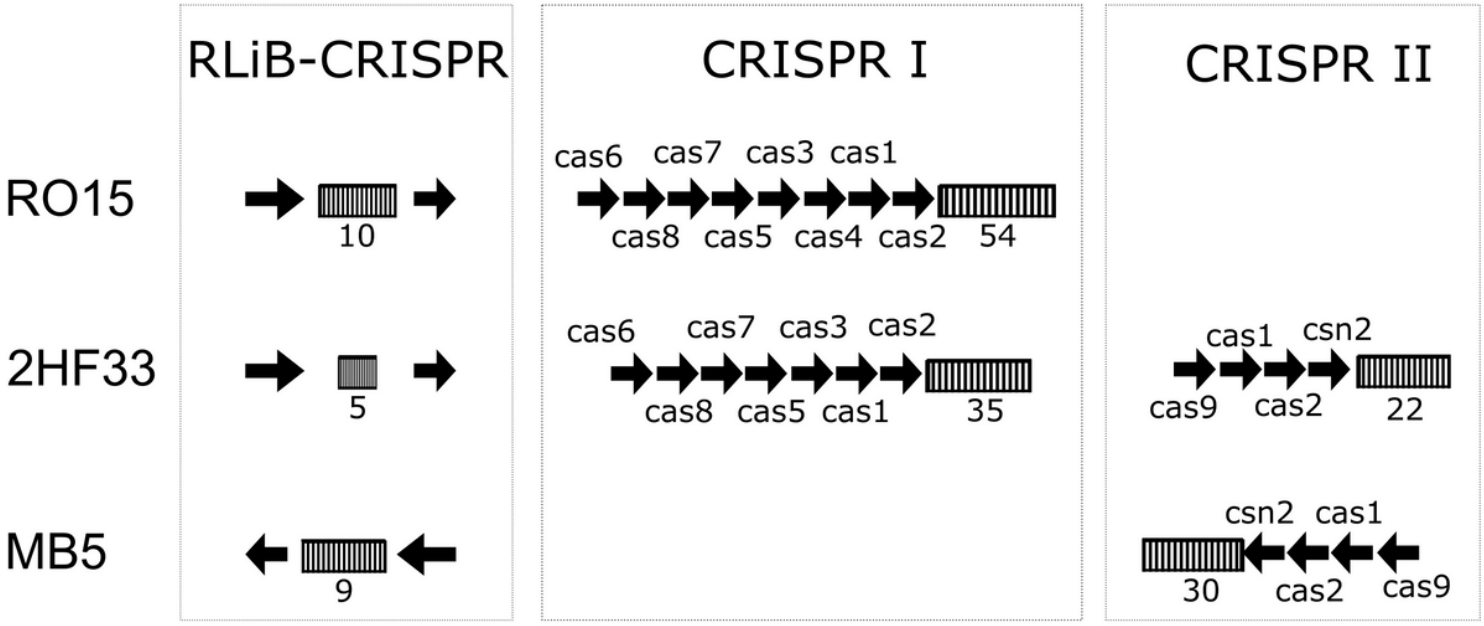

Figure 4

Visualization of CRISPR systems in strain RO15, 2HF33, and MB5. The figure shows a representation of CRISPR systems in strain RO15, 2HF33, and MB5. These three strains contained more than one CRISPR system, while the rest of the selected strains in the study contained only the RIiB-CRISPR system. Arrows represent genes, rectangles represent repeat/spacer arrays, the numbers below the rectangles indicate number of spacers in the array. For simplification, sizes of the arrows do not correspond to the actual size of the genes.

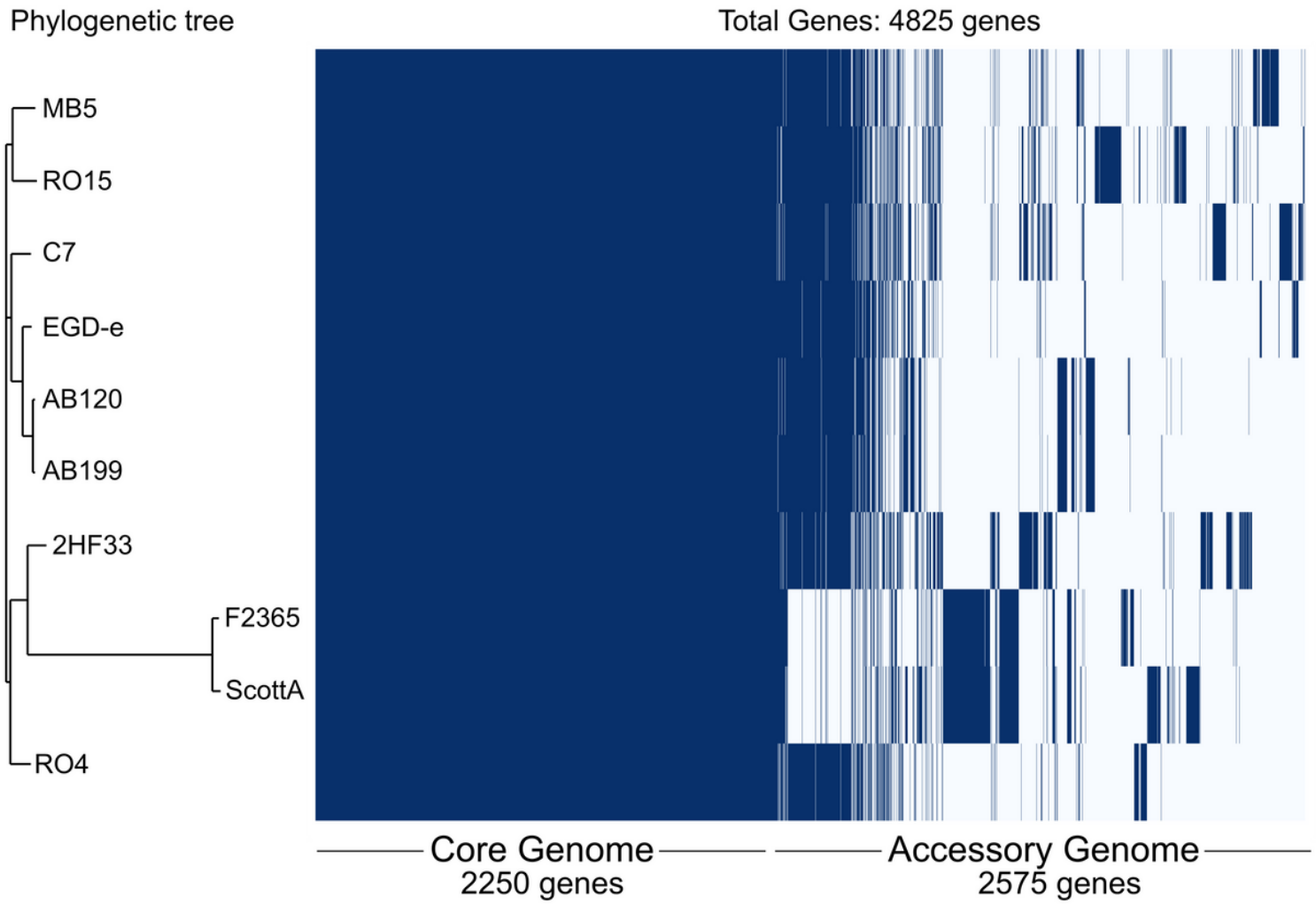

Figure 5

Pan-genome comparison of selected strains. A phylogenetic tree was created based on core genome alignments of the selected strains. The matrix shows presence and absence of core and accessory genes (blue: gene is present; white: gene is absent). Core genes are found in all strains, accessory genes are found in at least one strain.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile3TableS2.xlsx
- AdditionalFile1.docx
- AdditionalFile7TableS6.xlsx
- AdditionalFile6TableS5.xlsx
- AdditionalFile4TableS3.xlsx
- AdditionalFile2FigureS2lineage.png

- AdditionalFile5TableS4FigureS3.pdf