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Prevalence of plasmid-borne benzalkonium chloride resistance cassette *bcrABC* and cadmium resistance *cadA* genes in nonpathogenic *Listeria* spp. isolated from food and food-processing environments

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ABSTRACT

The sixty-seven nonpathogenic *Listeria* spp. strains isolated from food and food processing environments in Poland were examined for the presence of benzalkonium chloride (BC) resistance cassette (*bcrABC*) and four different variants of cadmium resistance determinants (*cadA1-cadA4*). All the strains were phenotypically resistant to cadmium and 22 among them were also resistant to BC. PCR-based analysis revealed that *bcrABC* cassette was harbored by 95.5% of the strains phenotypically resistant to BC. All of them harbored also either *cadA1* or *cadA2* genes (none carried *cadA3* or *cadA4*), which corresponded to the presence of plasmids with two restriction patterns. The strains resistant to cadmium but susceptible to BC harbored only the *cadA1* gene variant. DNA-DNA hybridization analysis showed that all the identified *bcrABC*, *cadA*1 and *cadA2* genes were located within plasmids, classified into 11 groups of RFLP profiles. Only one of the plasmids – pLIS1 of *Listeria welshimeri* (carrying *bcrABC* and *cadA2*) – was capable of efficient conjugal transfer from nonpathogenic *Listeria* isolates to a pathogenic *Listeria monocytogenes* strain. Analysis of the complete nucleotide sequence of pLIS1 (the first sequenced plasmid of *L. welshimeri* species) revealed the presence of genes involved in plasmid replication, stabilization and transfer as well as genes conferring resistance phenotypes. Comparative analysis showed that pLIS1 genome is highly similar to a group of plasmids originating from *L. monocytogenes* strains. A common feature of pLIS1 and its relatives, besides the presence of the resistance genes, is the presence of numerous transposable elements (TEs). The analysis revealed the important role of TEs in both promoting genetic rearrangements within *Listeria* spp. plasmids and the acquisition of resistance determinants.

1. Introduction

The genus *Listeria* (rod-shaped gram-positive bacteria) currently includes 17 recognized species. Several of them – *Listeria monocytogenes*, *Listeria ivanovii* (both considered pathogens) as well as *Listeria seeligeri* and *Listeria welshimeri* – are widely distributed and commonly found in different food processing environments (Orsi and Wiedmann, 2016). The growing number of *Listeria* spp. strains resistant to quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC), which are widely applied in food processing and health care environments and household, is becoming an increasing problem. BC resistance has been detected both in *L. monocytogenes* strains of different serotypes and diverse sources of isolation (Dutta et al., 2013; Elhanafi et al., 2010; Jiang et al., 2016; Mereghetti et al., 2000; Møretrø et al., 2017; Mullapudi et al., 2008; Ratani et al., 2012; Xu et al., 2014) and in other *Listeria* spp. (Korsak and Szuplewska, 2016).

It has been demonstrated that the genetic background of QAC resistance may vary. The resistance has been correlated with the presence of several efflux pump genes, such as: (i) *qacH* of chromosomally located transposon Tn*6188* (Müller et al., 2013; Müller et al., 2014), (ii) *qacA* and *qacEΔ1-sul* (Xu et al., 2014), (iii) *emrE* of genomic island LGI1 of *L. monocytogenes* strains responsible for the deadliest listeriosis outbreak in Canada in 2008 (Kovacevic et al., 2016), (iv) *mdrL* located in the chromosome (Mereghetti et al., 2000; Romanova et al., 2002; Romanova et al., 2006) or (v) *bcrABC*, originally identified in plasmid pLM80 as a part of a putative composite transposon (Dutta et al., 2013; Elhanafi et al., 2010). Moreover, the *bcrABC* cassette was found in the chromosomes of several *L. monocytogenes* strains (Dutta et al., 2013).

Many *L. monocytogenes* strains show tolerance to cadmium (Cd) as well. The persistence of Cd and other heavy metals in various environments can exert long-term selective pressure on bacteria (Alam et al., 2011). The co-occurrence of heavy metal resistance genes with

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other resistance determinants (e.g. within a single transposon or plasmid) may result in the co-selection of different resistance phenotypes.

Four distinct energy-dependent efflux systems have been associated with Cd resistance in *L. monocytogenes*: (i) *cadA1*, found e.g. in a plasmid-borne transposon Tn*5422* (Lebrun et al., 1994a; Lebrun et al., 1994b), (ii) *cadA2*, harbored by several large plasmids, e.g. the aforementioned pLM80 (Kuenne et al., 2010; Mullapudi et al., 2008; Nelson et al., 2004), (iii) *cadA3*, associated with an integrative and conjugative element (ICE) of *L. monocytogenes* EGDe (Glaser et al., 2001) and (v) *cadA4* identified in the chromosome of *L. monocytogenes* Scott A strain (Briers et al., 2011).

Many studies were conducted on the distribution of the *bcrABC* resistance cassette and *cad*A genes among foodborne *L. monocytogenes*. However, not much information is currently available on the prevalence of these determinants among other *Listeria* spp. originating from food and food processing plant environments. Likewise, not much is known about the horizontal transmission of these elements, e.g. within conjugative plasmids.

Bacteria of the genus *Listeria* are characterized by high level of conservation of their genomes, which is a unique feature among the entire *Firmicutes* type (Shintani et al., 2015). A relatively small amount of exogenous DNA in their chromosomes suggests that plasmids might be the major factor determining the variability of these microorganisms (den Bakker et al., 2010).

So far, little is known about the extrachromosomal replicons of *Listeria* spp. The NCBI database contains 43 complete plasmid sequences – 40 of *L. monocytogenes*, 2 of *L. innocua* and 1 of *L. grayi* (Bertsch et al., 2013; Canchaya et al., 2010; den Bakker et al., 2012; Gilmour et al., 2010; Kuenne et al., 2010; Kuenne et al., 2013; Nelson et al., 2004; Portmann et al., 2018). The plasmids are mostly in the 50 kb to 80 kb size range, with the smallest being pDB2011 (7.6 kb) and the largest pCFSAN021445 (152 kb).

Most of the identified *Listeria* plasmids increase tolerance of their hosts to environmental stress conditions (Schmitz-Esser et al., 2015). Within the plasmids numerous *loci* were identified, encoding (i) multidrug efflux systems, (ii) ABC transporters involved in protection against osmotic stress, (iii) heat shock proteins and (iv) transcriptional regulators to cold adaptation and cold growth (Kuenne et al., 2010). About 50% of the plasmid genes encode proteins of unknown function that may potentially contribute to the unusual plasticity of their hosts metabolism.

A characteristic feature of the *Listeria* plasmids is the presence of numerous mobile genetic elements (MGE). Nearly each plasmid carries genes encoding transposases (characteristic for insertion sequences, ISs, and transposons) and other recombinases (integrases, resolvases) typical for MGEs (Kuenne et al., 2010), which is an unprecedented number among bacterial plasmids. Many of these elements carry genes that confer resistance to heavy metals and disinfectants.

Among the plasmids only a few confer antibiotic resistance. These are: (i) pDB2011 of *Listeria innocua* TTS-2011 determining resistance to trimethoprim, spectomycin and macrolides (Bertsch et al., 2013), (ii) conjugative plasmid pIP811 of *L monocytogenes* conferring resistance to chloramphenicol, erythromycin, streptomycin and tetracycline (Poyart-Salmeron et al., 1990) and (iii) pWDB100 of *L. monocytogenes* carrying genes responsible for resistance to chloramphenicol, macrolides and tetracycline (Hadorn et al., 1993). The three aforementioned plasmids are broad host range replicons and therefore they successfully replicate in other gram-positive bacteria as well as in representatives of gram-negative bacteria (pDB2011).

In our previous work, a collection of 127 nonpathogenic *Listeria* spp. strains (*L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*) isolated from food and various food processing environments in Poland was examined in terms of susceptibility to different antimicrobials, disinfectants and heavy metals (Korsak and Szuplewska, 2016). As a result, 67 of phenotypically cadmium resistant strains, including 22 resistant to BC were identified. Our objective here was to identify the genetic basis of the resistance, genomic localization of the resistance genes and possibility of their transfer into *L. monocytogenes* cells.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The sixty-seven nonpathogenic strains of *Listeria* spp. used in this study were isolated from large retail outlets, smaller retail stores and food-producing factories between 2001 and 2010 in Poland (Korsak and Szuplewska, 2016). All the strains (listed in Supplementary Material, Table S1) are included in the collection of microorganisms of the Institute of Microbiology at the University of Warsaw (Poland). Bacteria were grown in brain-heart infusion broth (BHI) (Oxoid, UK) or on BHI agar (BHI with 1.5% agar) at 37 °C for 24 or 36 h, depending on the strain.

2.2. DNA isolation and DNA manipulations

Genomic DNA was extracted from *Listeria* spp. cells using a Chelex-100 (Bio-Rad, Hercules, USA) resin-based technique. Three to five colonies from a BHI plate were suspended in 50 µl of 5% Chelex-100. The suspensions were mixed and after incubation for 20 min at 95 °C the samples were cooled on ice for 5 min and centrifuged at 2.400 ×*g* for 3 min (Eppendorf MiniSpin Plus Centrifuge). The resulting supernatants were used as DNA templates in the PCR mixture.

Plasmid DNA was isolated from 3 ml of overnight *Listeria* spp. cultures using alkaline lysis procedure as described by Sambrook and Russell (2001). Prior to isolation bacterial cells were centrifuged, suspended in 500 µl of SET buffer [25% sucrose (w/v), 50 mM EDTA, 50 mM Tris, pH 8.0, 5 mg/ml lysozyme] and incubated at 37 °C for 30 min.

Common DNA manipulation techniques (transformation of plasmid DNA into bacterial cells, restriction enzymes digestion, agarose gel electrophoresis for the separation of DNA fragments) were performed according to Sambrook and Russell (2001).

2.3. PCR amplifications

PCR amplification was used for (i) detection of *bcrABC* cassette and *cadA* genes, (ii) preparation of specific DNA-DNA hybridization probes, and (iii) verification of transconjugants. To detect *bcrABC* cassette and *cadA* genes the following oligonucleotide primer pairs were used: bcr1/bcr2 (Elhanafi et al., 2010), cadA1-F/cadA1-R, cadA2-F/cadA2-R, cadA3-F/cadA3-R, cadA4-F/cadA4-R (Mullapudi et al., 2010; Lee et al., 2013) (Table 1). Genomic DNA from control strains (i) *L. innocua* 62/06 – for *bcrABC* and *cadA1* genes, (ii) *L. welshimeri* 49/06 – for *cadA2* gene, (iii) *L. monocytogenes* EGDe – for *cadA3*, and (iv) *L. monocytogenes* 28 – for *cadA4* gene were included in all PCR reactions. The *bcrABC* and *cadA* PCR products from selected control strains were sequenced using the same primers as were used in PCR. *L. monocytogenes* transconjugants were verified by species specific primers: Lmo-F and Lmo-R, described by Huang et al. (2007) (Table 1).

2.4. Plasmid profile analysis, restriction fragment length polymorphism (RFLP)

Plasmid DNAs were digested with *Hin*dIII and/or *Nco*I restriction endonucleases (in conditions recommended by the supplier – Thermo Scientific) and separated by electrophoresis in 0.8% agarose gel.

2.5. DNA-DNA hybridization

DNA-DNA hybridization (Southern blot) was performed to analyze the genomic localization of the *bcrABC*, *cadA1* and *cadA2* genes in

Table 1
Primers used in this study.

Gene	Primer	Sequence (5′ → 3′)	Size (bp)	Reference
bcrABC	bcr1	CATTAGAAGCAGTCGCAAAGCA	1130	Elhanafi et al. (2010)
	bcr2	GTTTTCGTGTCAGCAGATCTTTGA		
cadA1	cadA1-F	CAGAGCACTTTACTGACCATCAATCGTT	594	Mullapudi et al. (2010)
	cadA1-R	CTTCTTCATTTAACGTTCCAGCAAAAA		
cadA2	cadA2-F	ACAAGTTAGATCAAAAGAGTCTTTTATT	590	Mullapudi et al. (2010)
	cadA2-R	ATCTTCTTCATTTAGTGTTCCTGCAAAT		
cadA3	cadA3-F	TGGTAATTTCTTTAAGTCATCTCCCATT	468	Mullapudi et al. (2010)
	cadA3-R	GCGATGATTGATAATGTCGATTACAAAT		
cadA4	cadA4-F	GCATACGTACGAACCAGAAG	1135	Lee et al. (2013)
	cadA4-R	CAGTGTTTCTGCTTTTGCTCC		
0733	Lmo-F	CGCAAGAAGAAATTGCCATC	453	Huang et al. (2007)
	Lmo-R	TCCGCGTTAGAAAAATTCCA		

Listeria spp. isolates. Plasmid DNAs were isolated from *Listeria* strains and separated by electrophoresis in 0.8% agarose gels. DNA was blotted onto nylon membranes and hybridized under high-stringency conditions at 68 °C overnight. DNA fragments for hybridization probes (specific for *bcrABC*, *cadA1* and *cadA2*) were amplified using appropriate primer pairs (Table 1) and DNA templates, which were genomic DNA samples of (i) *L. innocua* 62/06 – for *bcrABC* and *cadA1* genes, (ii) *L. welshimeri* 49/06 – for *cadA2* gene. The amplicons were gel-purified and labeled with digoxigenin (Roche). DNA-DNA hybridization and visualization of bound digoxigenin-labeled probes procedures were performed as recommended by the supplier (Roche).

2.6. Bacterial mating procedure

The biparental mating between *Listeria* spp. was performed on solid medium using *Listeria* spp. isolates carrying resistance plasmids (as donor strains) and streptomycin-resistant *L. monocytogenes* 10403S (as a recipient). The mating experiments were performed with different variants of temperature, concentration of streptomycin and cadmium chloride.

Overnight cultures of the donor strain and the recipient strain (spun down and washed to remove antibiotics) were mixed at a ratio of 1:9. A 100 µl aliquot of such mixture was spread on a plate of solidified BHI medium. After 24 h of incubation at 30 or 37 °C, bacteria were washed off the plates with BHI medium and suitable dilutions were plated on selective media containing streptomycin (50 or 100 µg/ml; selective marker of the recipient strain) and cadmium chloride (20 or 25 µg/ml) and incubated at 30 or 37 °C for up to 96 h to select the transconjugants. Spontaneous resistance to cadmium in the recipient strains was not detectable under these experimental conditions. Transconjugants were verified by PCR using *L. monocytogenes*-specific primers and by their growth on ALOA medium, on which the colonies of *L. monocytogenes* produce a characteristic halo. The presence of transferred plasmid in transconjugants was confirmed by restriction analysis of isolated plasmid DNA.

The conjugation frequency was determined as a ratio of the number of transconjugants over CFU of the recipient strain on BHI plates supplemented with streptomycin (100 µg/ml).

2.7. DNA sequencing

DNA sequencing of *bcrABC*, *cadA1*, *cadA2*, *cadA3*, *cadA4* genes and pLIS1 plasmid of *L. welshimeri* strain 40/07 was performed in the DNA Sequencing and Oligonucleotide Synthesis Laboratory (oligo.pl) at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The pLIS1 plasmid was sequenced on Illumina MiSeq instrument in a paired-end (2 × 300 bp) mode using 600 cycle (v3) chemistry kit. The obtained sequence reads were filtered for quality using FastX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and residual Illumina adapters were removed using Cutadapt (https://github.com/marcelm/cutadapt). Filtered reads were assembled using Newbler v3.0 software (Roche). Final gap closure was performed by primer walking.

The nucleotide sequence of pLIS1 was deposited in GenBank (NCBI) with accession number MH382833.

2.8. Bioinformatic analysis

Automatic annotation of the plasmid nucleotide sequence was performed using RAST on the PATRIC platform (Wattam et al., 2017). It was followed by manual refinement in Artemis (Rutheford et al., 2000), based on homology searches (BLASTp, BLASTn) through the National Centre for Biotechnology (NCBI) website (http://www.ncbi.nlm.nih.gov/) using default settings (Altschul et al., 1990). EasyFig (Sullivan et al., 2011) was used to perform comparative genomic analyses and visualize the results. Transposable elements were identified using ISfinder website (Siguier et al., 2006). Novel elements were appropriately designated, assigned into appropriate IS families and their sequences were deposited in the ISfinder database.

3. Results

3.1. The prevalence of gene cassette bcrABC, cadA1 and cadA2 determinants among Listeria spp. strains

The sixty-seven nonpathogenic *Listeria* spp. strains isolated from food and food processing environments (Korsak and Szuplewska, 2016) was analyzed for the presence of different variants of *cadA* gene (*cadA1-cadA4*) (corresponding to cadmium resistance) and *bcrABC* genes (corresponding to BC resistance). All the strains were previously shown to be resistant to cadmium and 22 among them were also resistant to BC (Table 2).

The PCR-based analysis revealed that *cadA* genes were present in total of 52 strains – 38 of them carried *cadA1* and 14 *cadA2* gene variants (Table 2). None of the strains harbored *cadA3* or *cadA4* resistance determinants. Among the strains resistant to BC, 21 harbored *bcrABC* resistance cassette – 14 of them carried also *cadA2* and in 7 *cadA1* gene was identified (Table 2).

The phenotypic resistance of the tested strains generally correlates with the presence of *bcrABC*, *cadA1* or *cadA2* genes and plasmids. However, there are some exceptions. Among 67 cadmium-resistant strains, 15 *L. innocua* isolates (12 plasmid-less and 3 with plasmids with the same RFLP profile) lacked any of the four known cadmium resistance genes. Moreover, one BC and cadmium-resistant strain (*L. innocua* 27/06; Table S1) was found to be negative for *bcrABC* and positive for *cadA1*. The results suggest that phenotypic resistance to BC and cadmium chloride of these strains may have a different genetic background. Phenotypic description of individual strains has been presented in Supplementary Material, Table S1.

Table 2
Presence of *bcrABC, cadA* genes and conjugative plasmids in the analyzed pool of *Listeria* spp. strains resistant to benzalkonium chloride and cadmium.

Phenotype (no. of strains)	Plasmid RFLP group	Species (no. of strains)	Identified resistance genes				Conjugative plasmid
			cadA1	*cadA2*	*cadA3/4*	*bcrABC*	
Cd^R BC^R (22)	III	*L.welshimeri* (14)	−	+	−	+	+
	I	*L. innouca* (6)	+	−	−	+	−
		L. welshimeri (1)					
	IV	*L. innocua*[a] (1)	+	−	−	−	−
Cd^R BC^S (45)	IV	*L. innocua* (8)	+	−	−	−	−
	II	*L. innocua* (1)	+	−	−	−	−
	V	*L. innocua* (5)	+	−	−	−	−
		L. welshimeri (1)					
	VI	*L. innocua* (4)	+	−	−	−	−
	VII	*L. innocua* (4)	+	−	−	−	+[b]
	IX	*L. welshimeri* (1)	+	−	−	−	−
	X	*L. innocua* (1)	+	−	−	−	−
	XIV	*L. innocua* (5)	+	−	−	−	−
	XIII	*L. innocua*[c] (3)	−	−	−	−	−
	−	*L. innocua*[c] (12)	−	−	−	−	−

Cd^R – resistance to cadmium chloride;
BC^R – resistance to benzalkonium chloride;
BC^S – sensitivity to benzalkonium chloride;
[a] *L. innocua* strain resistant to BC but not harboring *bcrABC* cassette – undetermined mechanism of resistance;
[b] Only one transconjugant colony obtained;
[c] Undetermined mechanism of resistance to Cd.

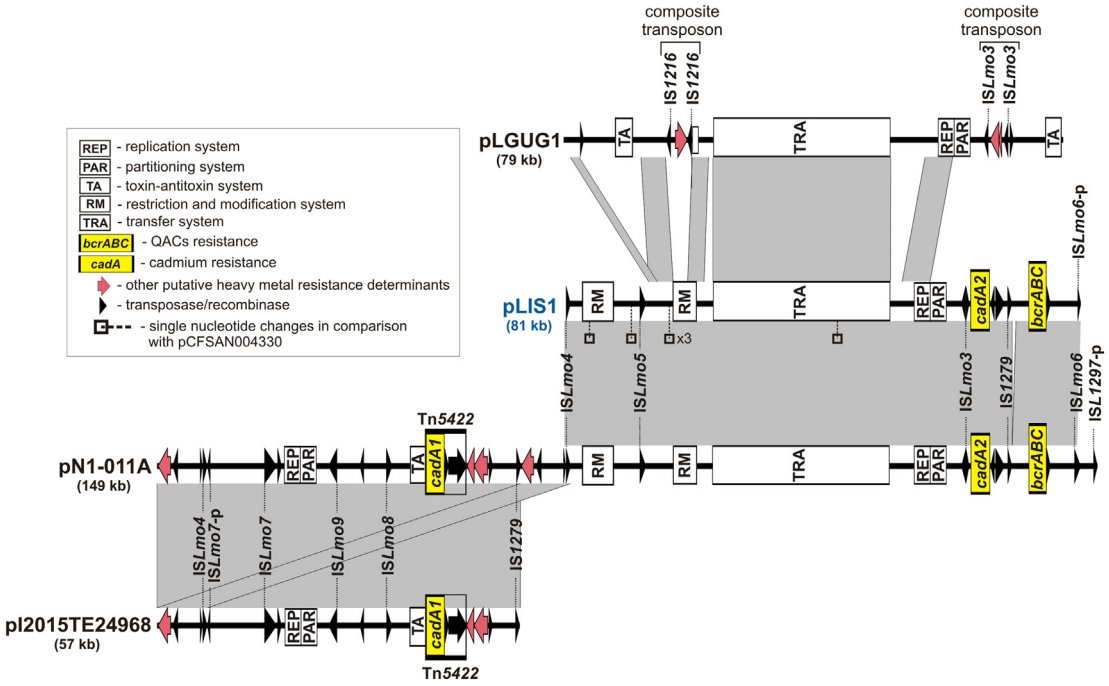

Fig. 1. Comparative analysis of pLIS1 of *L. welshimeri* and three plasmids – pLGUG1, pN1-011A and pI2015TE24968 (accession nos. FR667693, CP006611 and CP015985, respectively) originating from *L. grayi* (pLGUG1) and foodborne pathogenic strains of *L. monocytogenes* (pN1-011A and pI2015TE24968). Plasmid pCFSAN004330 of *L. monocytogenes* CFSAN004330, nearly identical with pLIS1, was not included in the analysis, however location of 6 single nucleotide changes between these replicons has been indicated. A simplified structure of the plasmids is presented, showing only the resistance and transposase genes as well as REP, PAR, RM, and TRA genetic modules. Plasmid sequences were aligned and visualized using EasyFig. The gray-shaded areas connect DNA regions with at least 95% nucleotide sequence identity and at least 1000 bp in size. IS*1297*-p, IS*Lmo6*-p, IS*Lmo7*-p – partial elements.

3.2. Genomic localization of the resistance genes

As shown in Table 2, all the *Listeria* spp. strains, in which the *bcrABC* and *cadA* genes were identified, contained plasmids. In our previous study, in which a larger set of strains was analyzed, plasmids were classified into 12 groups (I-XII) based on their RFLP patterns (Korsak and Szuplewska, 2016). To differentiate clearly the replicons present in BC- and Cd-resistant strains, the analysis was repeated with the application of two additional restriction enzymes (*Hin*dIII and *Nco*I). This allowed distinguishing between two additional RFLP patterns – XIII and XIV (Table 2).

To test whether the *bcrABC* and *cadA* determinants are located within extrachromosomal replicons, Southern blotting was performed using plasmids representing all the RFLP profiles. The analysis revealed that all the identified resistance genes are indeed located within plasmids (Table 2).

The *bcrABC* cassette was present in plasmids of two groups of RFLP profiles – associated with *cadA1* (profile I – plasmids of 6 strains of *L.*

innocua and 1 strain of *L. welshimeri*) or *cadA2* (profile III – plasmids of 14 strains of *L. welshimeri*) (Table 2). The *cadA1* resistance determinants were present in a majority of the plasmids, representing 10 RFLP groups. Only one group of plasmids (3 replicons of *L. innocua* – profile XIII; Table 2) did not contain any of the tested resistance genes.

3.3. Inter-species conjugal transfer of Listeria spp. plasmids

The identified resistance plasmids were tested for their ability to transfer to streptomycin-resistant *L. monocytogenes* 10403S cells. To this end bi-parental mating was performed with *L. innocua* and *L. welshimeri* as donor strains, carrying plasmids representing each RFLP group.

The analysis showed that only one plasmid (pLIS1 of *L. welshimeri* strain 40/07, carrying *bcrABC* and *cadA2* genes) could be transferred with high frequency (between 10^{-6} and 10^{-8}) into *L. monocytogenes* recipient strain (Table 2). In the case of another plasmid (pLIS2 of *L. innocua* 24/04, carrying *cadA1* gene) only one transconjugant colony was obtained (with mating procedure repeated eight times), which indicates extremely low transfer frequency of this replicon. Other donor strains (carrying plasmids with *cadA1* or both *bcrABC* and *cadA1*) yielded no transconjugants (Table 2).

3.4. Genomic analysis of conjugative plasmid pLIS1

The complete nucleotide sequence of pLIS1 was determined. The plasmid is 81,588 bp in length, with an average G + C content of 37%, and contains 89 predicted protein-coding sequences (CDSs) (Fig. 1). Comparative analysis with the NCBI GenBank database revealed that pLIS1 sequence is nearly identical (99% identity, 100% sequence coverage) with plasmid pCFSAN004330 of the foodborne pathogen *L. monocytogenes* CFSAN004330 (accession no. CP020834) – these plasmids differ in only 6 single nucleotide positions. It should be highlighted that these related plasmids were identified in strains of different species, *L. welshimeri* and *L. monocytogenes*, and isolated in different geographical locations – in Poland and the USA. pLIS1 also shows a high degree of similarity to many incomplete plasmid sequences obtained from whole genome sequencing of *L. monocytogenes* strains, such as the previously mentioned plasmid pLM80 (2 contigs: NZ_AADR01000010, NZ_AADR01000058) (data not shown).

Additionally, pLIS1 shares 99% identity (98% sequence coverage) with the nucleotide sequences of two much larger, highly related with each other *L. monocytogenes* plasmids: pN1-011A, which is 149 kb in length (accession no. CP006611) and 152 kb in length pCFSAN021445 (accession no. CP022021). Both plasmids have the same genetic backbones. As shown in Fig. 1, pN1-011A (as well as pCFSAN021445) encodes two separate replication systems. Therefore, these are to be considered composite plasmids, which had most probably been formed by recombination events between two smaller replicons – resembling pLIS1 and pl2015TE24968 of *L. monocytogenes* strain 2015TE24968 (accession no. CP015985) (Fig. 1).

The analysis of pLIS1 sequence revealed that the plasmid contains three predicted genetic modules, which determine replication (REP), stabilization (PAR) and transfer (TRA) functions. The REP module encodes a putative replication initiation protein RepA, characteristic for group 2 of *Listeria* replication systems (Kuenne et al., 2010). The PAR module contains two overlapping genes, *parA* and *parB*, encoding proteins whose homologues ensure active segregation of plasmid copies into daughter cells during cell division. The predicted TRA module is approximately 20 kb in size and includes genes encoding three putative type IV secretion system proteins, peptidase, endonuclease and 23 hypothetical proteins of unknown function. One of the genes encodes also a putative relaxase – a key protein involved in initiation of plasmid conjugal transfer – as judged from the weak sequence similarity with predicted relaxase of pLM5578 plasmid of *L. monocytogenes* (Gilmour et al., 2010). As shown in Fig. 1, the predicted TRA region of pLIS1 is shared with a distantly related plasmid pLGUG1 of *L. grayi* DSM 20601 (accession no. FR667693). Additionally, the plasmid contains two putative restriction and modifications systems (RM) (Fig. 1).

The *bcrABC* and *cadA2 loci* are located in the pLIS1 genome in the close neighborhood of transposable elements (TEs). pLIS1 carries 10 CDSs annotated as putative transposases/recombinases, which indicates that TEs are important components of the plasmid. Within this replicon four complete insertion sequences (ISs) (IS*Lmo3*, IS*Lmo4* and an isoform of IS*1297* – all of the IS*6* family, and IS*Lmo5* of the IS*3* family) and one partial IS (terminal part of IS*Lmo6* – IS*30* family) were identified.

Transposable elements of the aforementioned *L. monocytogenes* plasmids used for the comparative analysis with pLIS1 (Fig. 1) were also analyzed. The plasmids contain numerous TEs, including a few known elements, e.g. cadmium resistance non-composite transposon Tn*5422* (Tn*3* family) residing in plasmids pN1-011A and pI2015TE24968 (Fig. 1), and several novel ISs (IS*Lmo3*-IS*Lmo9*), as well as two predicted resistance composite transposons (located in pLGUG1) (Fig. 1). One of them (4.1 kb) is a putative cadmium resistance transposon carrying two flanking copies of IS*1216* (Fig. 1). This element contains on both sides 8-bp-long directly repeated sequences (DRs), which may correspond to a target site duplicated upon transposition.

4. Discussion

Molecular mechanisms of resistance to heavy metals and quaternary ammonium compounds have been extensively studied in *L. monocytogenes*. However, the prevalence of resistance determinants to these compounds in other *Listeria* species and possible correlations between disinfectant resistance and heavy-metal resistance have been poorly recognized. The *bcrABC* cassette was first identified in *L. monocytogenes* strain H7550 from the 1998–1999 hot dog outbreak (Elhanafi et al., 2010). A previous study demonstrated that in *L. monocytogenes* the *bcrABC* cassette conferred high-level resistance to BC and other QACs, and that this module is harbored by the vast majority of BC-resistant strains, regardless of serotype or source of isolation (Dutta et al., 2013).

Our results showed that the *bcrABC* cassette was harbored by 95.5% *Listeria* spp. strains phenotypically resistant to BC. All the strains carrying *bcrABC* encoded also cadmium resistance determinants – either *cadA1* or *cadA2* – which corresponds with results reported previously by Mullapudi et al. (2010). The most strains harbored *bcrABC* and *cadA2* gene (66.7%) (plasmid RFLP profile III). This is in agreement with the observation made by Dutta et al. (2013) in their study of BC-resistant isolates of *L. monocytogenes*, which concluded that the majority of the isolates carried a pLM80-like *bcrABC* region and *cadA2*. The second group of strains was that with *bcrABC* and *cadA1* (plasmid RFLP profile I). As previously shown, *cadA1* may be harbored by a plasmid-borne transposon Tn*5422* (Lebrun et al., 1994a; Lebrun et al., 1994b), and thus it may be located within the same plasmid that carries *bcrABC* (Dutta et al., 2013). In our study all the identified *bcrABC*, *cadA1* and *cadA2* genes were located in plasmids.

Analysis of the cadmium-resistant but BC-susceptible strains revealed that *cadA1* was present in 68.6% strains and none carried a *cadA2* gene. The high frequency of *cadA1* in the nonpathogenic *Listeria* spp. is consistent with the high frequency of occurrence of this gene in *L. monocytogenes* (Mullapudi et al., 2010; Ratani et al., 2012; Xu et al., 2014). The reasons for the relatively high prevalence of *cadA1* among all *Listeria* spp. are still to be identified.

Among the cadmium-resistant strains, fifteen did not harbor any known *cadA* determinants, and among the BC-resistant strains one did not contain the *bcrABC* cassette. This suggests novel gene variants of the resistance determinants or the presence of other adaptation mechanisms that may confer resistance. Further studies are needed to characterize the molecular mechanisms mediating cadmium and BC tolerance in these strains.

The possibility of the conjugal transfer of plasmids harboring *cadA1*, *cadA2* and *bcrABC* was tested by inter-species bi-parental mating. Only one plasmid – pLIS1 of *L. welshimeri* strain 40/70 (RFLP profile III),

carrying *cadA2* and *bcrABC* genes, was capable of efficient conjugal transfer to *L. monocytogenes* 10403S. Katharios-Lanwermeyer et al. (2012) conducted a similar experiment. They studied the conjugal transfer of plasmids from nonpathogenic *Listeria* spp. to other listeriae, including *L. monocytogenes*. In their study the donor strains harboring *cadA2* produced strikingly more transconjugants, which is consistent with our findings. Efficient conjugal transfer of plasmids containing *cadA2* may reflect the relatedness of these replicons. However, the studies by Katharios-Lanwermeyer et al. (2012) were not concluded by sequencing of the conjugative plasmids, which makes it impossible to perform any comparative analysis. To our best knowledge none of the sequenced *Listeria* spp. plasmids was experimentally tested in terms of conjugal transfer. In this study we showed that pLIS1 is a self-transmissible replicon and it contains a predicted TRA module, conserved also in other *Listeria* spp. plasmids (Fig. 1).

Interestingly, none of the plasmids carrying *cadA1* analyzed in this study was capable of conjugal transfer. They may be not self-transmissible, however it cannot be excluded that these replicons may require as yet not identified specific factors or conditions for efficient transfer.

The nucleotide sequence of pLIS1 showed similarities to many pLM80-like plasmids isolated from *L. monocytogenes* strains. The high level of conservation of these replicons occurring in numerous strains isolated from different environments suggests that their maintenance is possible due to a strong selective pressure.

Listeria spp. plasmids encode multiple recombinases. As shown in Fig. 1, the DNA regions of high nucleotide sequence similarity between e.g. pLIS1 and pN1-011A or pN1-011A and pl2015TE24968 are bordered by ISs, suggesting that these elements play an important role in promoting genetic rearrangements. Additionally, some ISs (e.g. isoform of IS*1297* in pLIS1) are bordered by partial ORFs, encoding different incomplete proteins, which indicates that also homologous recombination between identical IS copies might have occurred.

Likewise, transposons play a role in the dissemination of foreign DNA of adaptive value, such as heavy-metal or disinfectants resistance determinants. Known examples include Tn*6188* containing *qacH* (Müller et al., 2013) or Tn*5422* – a transposon harboring *cadA1* (Lebrun et al., 1994b) present in many *Listeria* spp. plasmids, including pN1-011A and pl2015TE24968 (Fig. 1). It has been suggested that the *bcrABC* and *cadA2* resistance genes identified in numerous isolates of L. *monocytogenes* (Dutta et al., 2013) are part of a composite transposon (Elhanafi et al., 2010). These *loci* are accompanied by complete or partial transposase genes and it cannot be excluded that the resistance determinants may have been acquired independently by different transposition events.

The presence of numerous TEs and heavy metal resistance genes in *Listeria* spp. plasmids points to the important role of transposition in the shaping of plasmid structure and in the dissemination of resistance determinants among them.

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