Genetic Carriers and Genomic Distribution of cadA6—A Novel Variant of a Cadmium Resistance Determinant Identified in Listeria spp.

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Abstract: Listeria monocytogenes is a pathogen responsible for severe cases of food poisoning. Listeria spp. strains occurring in soil and water environments may serve as a reservoir of resistance determinants for pathogenic L. monocytogenes strains. A large collection of Listeria spp. strains (155) isolated from natural, agricultural, and urban areas was screened for resistance to heavy metals and metalloids, and the presence of resistance determinants and extrachromosomal replicons. Of the tested strains, 35% were resistant to cadmium and 17% to arsenic. Sequence analysis of resistance plasmids isolated from strains of Listeria seeligeri and Listeria ivanovii, and the chromosome of L. seeligeri strain Sr73, identified a novel variant of the cadAC cadmium resistance efflux system, cadA6, that was functional in L. monocytogenes cells. The cadA6 cassette was detected in four Listeria species, including strains of L. monocytogenes, isolated from various countries and sources—environmental, food-associated, and clinical samples. This resistance cassette is harbored by four novel composite or non-composite transposons, which increases its potential for horizontal transmission. Since some cadAC cassettes may influence virulence and biofilm formation, it is important to monitor their presence in Listeria spp. strains inhabiting different environments.

Keywords: Listeria; Listeria ivanovii; Listeria seeligeri; Listeria monocytogenes; heavy-metal resistance; cadmium; arsenic; plasmid; transposon; cadA

1. Introduction

The genus Listeria currently comprises 20 species [1]. Listeria spp. are distributed ubiquitously in diverse environments including soil, surface waters, and vegetation. The presence of Listeria spp. in agricultural environments represents a potential source of contamination for food products [2]. L. monocytogenes and L. innocua are the species identified most frequently in foodstuffs and sites of food processing [3–10]. L. monocytogenes is a human and animal pathogen, so its incidence in food industries is closely monitored. The frequency with which different Listeria species are identified in soil and water samples varies depending on the source of isolation and geographical factors, although L. seeligeri was the most common isolate in a number of studies [11–13].

Most studies on resistance to heavy metals and metalloids in Listeria spp. have focused on L. monocytogenes strains [14–17], although a few have examined the resistance to cadmium and arsenic...
The National Centre of Biotechnology Information (NCBI) database (GenBank) contains 57 complete
encoded proteins CadA1–CadA3 show approximately 70% amino acid (aa) sequence identity
processing environments may be the main source of selection pressure that leads to the acquisition
pressure if they result in impaired bacterial growth [30]. However, Parsons et al. [35] suggests that food
estimated worldwide mean cadmium concentration in soils is 0.36 \( \mu g/g \) [31], but local
concentrations vary depending on the regions, soil acidity, and local pollution levels [32–34]. Even low
concentrations of metals can induce transcription of resistance genes and potentially exert selective
pressure if they result in impaired bacterial growth [30]. However, Parsons et al. [35] suggests that food
processing environments may be the main source of selection pressure that leads to the acquisition
of cadmium resistance determinants by Listeria spp. strains. Interestingly, Parsons et al. [30] and
Pombinho et al. [36] observed that the presence of some cadAC resistance cassettes in Listeria can
influence other phenotypic traits, such as virulence and biofilm formation, which suggests that these
heavy-metal resistance determinants may have additional functions.

Arsenic resistance in Listeria spp. is usually chromosomally determined, either by a Tn554-like
transposon (arsCBADR) or genomic islands LGI2 and LGI2-1 (arsR1D2R2A2B1B2 and upstream arsA1D1
cassette) [15,28]. However, the occurrence of arsenic resistance genes within Listeria spp. plasmids has
also been observed [23,37].

The mechanisms regulating intracellular levels of copper in L. monocytogenes remain poorly
understood. The first copper transporter identified in this species was the plasmid-encoded
efflux pump CtpA from L. monocytogenes strain DRDC8, which, however, appears to be present
in very few strains [38,39]. CtpA plays a role not only in copper uptake but also in virulence [40].
Other putative copper-translocating P-type ATPases and multi-copper oxidases have been identified
via sequence analysis of L. monocytogenes plasmids [23], but their function has not been tested
experimentally. A conserved chromosomal copper resistance operon, csor-copA-copZ, was described
in L. monocytogenes EGD [38]. This encodes the copper-sensing transcriptional regulator CsoR,
a copper-exporting P1B-type ATPase CopA and the copper metallochaperone CopZ. It was also shown
that the penicillin-binding protein PBP4, encoded by chromosomal gene lmo2229, is involved in copper
tolerance in L. monocytogenes [41].

So far, little is known about the extrachromosomal replicons of Listeria spp. other than L. monocytogenes.
The National Centre of Biotechnology Information (NCBI) database (GenBank) contains 57 complete
Listeria spp. plasmid sequences: 52 from L. monocytogenes, 3 from L. innocua, 1 from L. grayi, and 1
from L. welshimeri (as of 30 August 2020). Listeria spp. plasmids can harbor genes influencing
bacterial tolerance to various environmental stresses, including (i) heavy metals [23,25,27,37],
(ii) sanitizers [18,20,27,42], (iii) antibiotics [43–45], (iv) high temperatures [46], and (v) osmotic and
oxidative stress [47]. In addition, many of the plasmid-borne genes encode proteins of unknown
function that may potentially contribute to bacterial survival under stressful conditions.

Listeria spp. strains from soil environments can potentially serve as a reservoir of resistance
determinants, which, if encoded by mobile elements, may be transferred to pathogenic L. monocytogenes
strains. The aim of this study is to characterize a collection of 155 Listeria spp. strains isolated from soil
and water samples collected from Poland and the United States in terms of (i) their susceptibility to heavy metals and metalloids, (ii) the presence of resistance determinants, and (iii) the occurrence and distribution of plasmids commonly harboring resistance genes.

2. Results and Discussion

2.1. Isolation and Taxonomic Position of Listeria spp. Isolates

In this study, 143 Listeria spp. strains were obtained from 555 soil and 15 water samples were collected from diverse locations representing natural, urban, and agricultural environments. PCR analysis showed that L. seeligeri was the predominant species (67 strains), followed by L. innocua (29 strains), L. monocytogenes (24 strains), L. ivanovii (16 strains), and L. welshimeri (7 strains). In soil and water samples, L. seeligeri strains are usually identified more often than other Listeria species, e.g., [12,13]. A further 12 strains provided by the Food Safety Laboratory at Cornell University in the USA (3 L. seeligeri, 1 L. innocua, 4 L. welshimeri, 4 L. marthii), also originating from soil and water samples, were added to the collection. In total, 155 Listeria strains representing six species were examined in this study (listed in Supplementary Table S1).

2.2. Susceptibility of Listeria spp. Strains to Heavy Metals and Metalloids

All strains were tested for their susceptibility to heavy metals and metalloids (cadmium, arsenic, and copper) using an agar dilution assay. The presence of resistance determinants to cadmium (cadA1–cadA5 genes), arsenic (arsA1 and arsA2 genes associated with LGI2; arsA gene associated with the Tn554-like transposon), and copper (ctpA gene) was determined by PCR amplification (Figure 1) using primers listed in Table 1.

Figure 1. Resistance profiles, resistance determinants, and plasmids identified in Listeria spp. strains isolated from soil samples. Identified determinants conferring resistance to cadmium (cadA1, cadA4, cadA6) and arsenic (Tn554-associated arsA, LGI2-associated arsA); ND—undetermined resistance gene. The number of strains carrying identified resistance determinants and plasmids (representing 19 RFLP groups: p1–p19) is given in parentheses. The total number of strains within a species is indicated by the extent of the gray bar (n). Strains harboring Tn554-associated arsA genes, but not classified as resistant to arsenic, are indicated in gray.

The frequency of resistant strains varied depending on the species (Figure 1). Overall, 35% of the strains were determined to be resistant to cadmium and 17% to arsenic (minimum inhibitory concentrations (MICs), or lower bounds of MIC values if growth was still observed at highest tested concentrations, are presented in Supplementary Table S1). There are several reports on the prevalence of cadmium and arsenic resistance among L. monocytogenes strains [17,22,48] and a few studies that include other Listeria species [19–21], which indicate that resistance to cadmium is widespread, especially among food-associated isolates, while arsenic resistance is usually found at lower frequencies.

However, in contrast to Listeria spp. isolated from food products [16–18,20,21], few of the resistant strains identified in this study harbored previously known cadmium resistance determinants (Figure 1, Supplementary Table S1). We identified cadA1 in only 16 strains (4 L. monocytogenes, 5 L. seeligeri, 4 L. ivanovii and 3 L. innocua strains) and cadA4 in 1 strain (L. monocytogenes). None of the strains carried
cadA2, cadA3, or cadA5 resistance determinants. In total, only 31% of the cadmium-resistant strains harbored one of the known gene variants cadA1–cadA5, compared to 95% [16], 78% [18], and 61% [21] reported among food industry isolates in previous studies.

The Tn554-associated arsenic resistance cassette was present in most of the arsenic-resistant strains (62%). Interestingly, the Tn554-associated arsenic resistance cassette was also detected in 14 strains (4 of L. seeligeri, 10 of L. ivanovii) that were capable of growth at 250 µg/mL of sodium(meta) arsenite, but not at 500 µg/mL. According to the applied criteria, these strains were not considered resistant. It has yet to be determined why the presence of the resistance cassette confers tolerance only to lower concentrations of arsenic in these strains.

Cadmium- and arsenic-resistant strains that were PCR negative for the tested genes may either harbor yet to be identified resistance determinants or sequence divergence may have occurred in the DNA regions where the primers anneal.

In the case of copper compounds, there are no determined breakpoint concentrations for Listeria spp. Among the tested strains, only 1 L. monocytogenes strain (Sr118) yielded confluent growth at 2400 µg/mL copper(II) sulfate pentahydrate, while 78 strains were capable of growth at up to 1200 µg/mL. Some Listeria spp. plasmids harbor genes potentially involved in copper detoxification [23,39]; however, only one of them, ctpA, has had its role in copper tolerance experimentally confirmed [39]. The ctpA gene was not detected in any of the strains tested in this study.

Available data from locations close to some of the sampling areas (Warsaw city parks and Kampinoski National Park) showed relatively low median concentrations of cadmium (<1 µg/g), arsenic (<5 µg/g), and copper (<20 µg/g) in soil samples [49–51]. However, even low concentrations of toxic metals and metalloids can potentially exert selective pressure on bacteria.

Table 1. Primers used in PCRs to amplify resistance determinants.

| Primer | Sequence (5′→3′) | Annealing [Tm, ◦C] | Amplified DNA Fragment | Reference |
|--------|------------------|-------------------|------------------------|-----------|
| cadA1-F | CAGAGCACTTTACTGACCATCAATCGTT | 53 | cadA1 (Tn5422) 594 | [16] |
| cadA1-R | CTTCITCATTAAACGTTCCAGCAAAAA | | | |
| cadA2-F | ACAAGTTAGATCAAAAGAGTCTTTTATT | 53 | cadA2 (plM80) 590 | [16] |
| cadA2-R | ATCTTCTCTTATGTTCTGCAAAT | | | |
| cadA3-F | GCGATGATGATGTAATGTCGATTACAAAT | 52 | cadA3 (ICEEtn1 of EGD-e strain) 468 | [16] |
| cadA3-R | TGGTAAATTTCTTTAAGTCATCTCCCATT | | | |
| cadA4-F | GCATACGTCAGAAGCAGATITCTCCCTTTC | 51 | cadA4 (genomic island LGI2) 1135 | [14] |
| cadA4-R | CATGTGTTTCTGCTTTTGCTCC | | | |
| cadA5-F | GTTGATGTAATGACGTGCGGAAG | 47 | cadA5 (genomic island LGI2-1) 1178 | [32] |
| cadA5-R | GTGTTTCTGCTTTTGCTCC | | | |
| cadA6-F | ACTGGTACAAGCTGTGCATATC | 51 | cadA6 (plLB4, plL56) 2027 | This study |
| cadA6-R | AGTAATGTTGCTCCCATATCG | | | |
| arsA1-F | CAACCTTGACCCACATCGGAG | 50 | arsA1 (genomic island LGI2) 1466 | [14] |
| arsA1-R | CTTCCTCAATCACTACGGTG | | | |
| arsA2-F | CACCCAGATGACCTTACAATCAAAC | 50 | arsA2 (genomic island LGI2) 1710 | [14] |
| arsA2-R | TATCCCTCAGATTTCTCCTC | | | |
| arsATn54F | TAACAAATAAGCCAACACCG | 64 | arsA (Tn545-like transposon) 1219 | [15] |
| arsATn54R | CTTCCTTCACCAGATTTCTCCTC | | | |
| FB001 | CACACTGTAATGTTAACTGG | 47 | ctpA (plasmid-associated) 396 | [39] |
| LM2004 | TCCACCTGGCTGAGAATAAC | | | |

Cloning of cadA6 Genes

| Primer | Sequence (5′→3′) | Annealing [Tm, ◦C] | Amplified DNA Fragment | Reference |
|--------|------------------|-------------------|------------------------|-----------|
| cadA6a-F | CCCGACATTGCCCGCTCTAAATGGAAAC | 49 | cadA6a and cadC6a cassette (plLB4, strain Sr12) 2843 | This study |
| cadA6a-R | ATGGCGCCCGCGTCTCTGGCTTCTTCCTC | | | |
| cadA6b-F | CCCGACTGATGCTCCAGGCTCTTCTTCTGTC | 49 | cadA6b and cadC6b cassette (plLB6, strain Sr11) 3072 | This study |
| cadA6b-R | AAGCGCGCGCCGCAACAAAAAGGCTGCTTCTGTC | | | |
2.3. Plasmid Occurrence and Distribution in Listeria spp. Strains

Among the Listeria spp. strains tested in this study, 31 (20%) harbored large plasmids. The reported incidence of plasmids in other studies usually lies in the range 15–50% and is higher among food and environmental isolates than in clinical isolates [19,25,48]. The identified plasmids were classified into 19 groups (named p1–p19) based on their RFLP patterns (Supplementary Table S1). None of the tested L. weisshimeri and L. marthii strains harbored plasmids. No plasmids sharing the same restriction pattern were identified in strains of different species. Interestingly, plasmids of the p1 group showed an identical RFLP pattern to those identified in 92 L. monocytogenes strains (isolated from food products in Poland) analyzed in our previous study [52].

2.4. Nucleotide Sequences of L. seeligeri and L. ivanovii Replicons Containing Resistance Determinants

Listeria spp. plasmids often carry cadmium resistance determinants and can contribute to the horizontal transfer of this resistance phenotype [18,20,25]. All strains harboring plasmids of the p6 (L. seeligeri, 4 strains) and p8 (L. seeligeri, 2 strains) RFLP profiles were resistant to cadmium, while not containing any of the known cadA1–cadA5 cadmium-resistance determinants. Only one of the two cadmium-resistant L. ivanovii strains harboring a p16 profile plasmid encoded a known resistance determinant (cadA1). Therefore, the complete nucleotide sequences of the plasmids from the following strains were determined: L. seeligeri Sr12 (named pLIS4; plasmid profile p6), L. seeligeri Sr73 (pLIS5; p8), and L. ivanovii Sr11 (pLIS6; p16). Two of these plasmids, pLIS4 and pLIS6, contained a new variant of the cadAC efflux system, named cadA6 (Figure 2). Plasmid pLIS5 did not encode any cadmium resistance determinant, however the cadA6 resistance cassette was identified in the complete genome sequence of its host strain (L. seeligeri Sr73), determined in this study. Comparative analysis of the chromosomes of Sr73 (2,867,282 bp) and L. seeligeri SLCC3954 (the only available complete genomic sequence of this species; NC_013891.1) is presented in Supplementary Figure S2.

Figure 2. Comparative analysis of plasmids pLIS4 (L. seeligeri Sr12), pLIS5 (L. seeligeri Sr73) and pLIS6 (L. ivanovii Sr11). Plasmid sequences were aligned and visualized using EasyFig. Gray shading indicates conserved DNA regions with at least 75% nucleotide sequence identity of at least 1 kb in length. A simplified structure of the plasmids is presented, showing only the putative REP, PAR, TA, TRA modules, partial or complete transposase/recombinase genes and heavy-metal resistance determinants.

These are the first published complete sequences of plasmids from L. seeligeri and L. ivanovii species. The largest of these plasmids, pLIS4, is 101,192 bp in length and contains 102 predicted protein-coding sequences (CDSs), including 14 pseudogenes. pLIS5 is 60,659 bp long and encodes 77 CDSs (15 pseudogenes), while pLIS6 is 59,142 bp long with 68 CDSs (3 pseudogenes) (Figure 2). The plasmids share few regions of nucleotide sequence similarity with other complete plasmid sequences available in the NCBI GenBank database. The shared regions are mostly limited to transposable elements (TEs). In contrast, many L. monocytogenes plasmids from food-associated isolates share similar genetic backbones and show a high level of sequence conservation [23,37,53,54]. The CDSs identified in these plasmids are described in Supplementary Table S2.

The positions of the predicted genetic modules responsible for replication (REP), stabilization (PAR) and conjugative transfer (TRA) are shown in Figure 2. Large Listeria spp. plasmids are usually
categorized into two major groups based on replication initiation protein (RepA) phylogeny [23]. However, the RepA proteins of plasmids pLIS4–6 are placed in two additional and distinct phylogenetic groups (Supplementary Figure S3). The replication proteins of plasmids pLIS5 and pLIS6 cluster together with the RepA of plasmid pLMIV from *L. monocytogenes* strain FSL J1-208 (GenBank acc. no. NZ_CM001470.1) [55], while the pLIS4 RepA is located on a separate branch. The putative PAR modules encode two proteins probably involved in active partitioning of plasmid molecules during cell division and are present adjacent to the repA gene in all three analyzed plasmids (Figure 2).

In addition, we identified a 15-kb-long sequence representing a possible conjugative transfer module (TRA) that is shared by pLIS4 and pLIS6 (85% nucleotide sequence identity), but absent from pLIS5 (Figure 2). This module is also present in 2 *L. seeligeri* sequence contigs (probable plasmid sequences) present in the NCBI database (NZ_JAASVW010000014.1; NZ_JAASVC010000008.1). This TRA module includes genes encoding two putative type IV secretion system proteins, a relaxase of the MobL family (protein required for initiation of plasmid conjugal transfer), a lysozyme family protein, and nine hypothetical proteins of unknown function. However, no transconjugants containing pLIS4 or pLIS6 were obtained in a bi-parental mating with a streptomycin-resistant *L. monocytogenes* strain 10403S. The strain *L. welshimeri* 40/70 [18] harboring a conjugative plasmid pLIS1 was used in a control mating, and this plasmid was transferred with high frequency. Therefore, plasmids pLIS4 and pLIS6 are either non-self-transmissible or require specific conditions for efficient transfer.

The three sequenced plasmids also contain numerous predicted TEs and genes encoding recombinases and integrases, both complete and partial, which represent traces of previous insertion events. All of the identified insertion sequences (ISs), including three novel ones, ISLse1, ISLse2, ISLse3 (the first defined ISs of *L. seeligeri*; ISFinder database), were classified as members of the IS3 family. In addition, plasmids pLIS4 and pLIS5 harbor a Tn554-like arsenic resistance transposon. This transposon is also present in some *L. monocytogenes* plasmids [37], although it is usually chromosomally encoded [15,28]. Furthermore, two novel putative transposons carrying the cadA6 cadmium-resistance cassette were identified in plasmids pLIS4 and pLIS6 (Figure 2).

### 2.5. Identification of cadA6—a Novel Cadmium Resistance Cassette Variant

Pairwise comparisons revealed that the novel CadA6 protein shares approximately 65% aa sequence identity with CadA1–CadA3, and 36% with CadA4–CadA5 (Figure 3 and Supplementary Figure S4). The cadA6 sequences identified in plasmid pLIS4 and the *L. seeligeri* Sr73 chromosome are identical (named cadA6a), but the determinant present in pLIS6 is slightly divergent (91% aa and 86% nucleotide sequence identity), so was named cadA6b. A primer pair, cadA6-F/cadA6-R, was designed to enable detection of both cadA6a and cadA6b in *Listeria* spp. strains (Table 1, Supplementary Figure S5). These primers yielded strong PCR products for positive control strains (*L. seeligeri* Sr12, *L. ivanovii* Sr11), while no products of the expected size were produced with control strains containing cadA1, cadA2, cadA3, or cadA4 genes. PCR analyses revealed that 65% of the cadmium-resistant strains tested in this study harbored a cadA6 efflux system (Supplementary Table S1). Therefore, cadA6 was the most prevalent cadmium resistance determinant identified in this study, being present in four species: *L. monocytogenes*, *L. seeligeri*, *L. innocua*, and *L. ivanovii*. The PCR products from one representative strain of each species (*L. monocytogenes* Sr119, *L. seeligeri* Sr12, *L. innocua* Sr103, *L. ivanovii* Sr11) were sequenced to confirm the specificity of the amplification reaction. Comparative analysis with the GenBank database showed that CDSs homologous to CadA6 (91–100% aa sequence identity, 100% query coverage) are present in species of various Gram-positive genera, including *Listeria*, *Bacillus*, *Solibacillus*, and *Enterococcus*.
2.6. Transposons Harboring the cadA6 Efflux System

The cadA6 cadmium resistance determinants in plasmids pLIS4, pLIS6 and the chromosome of L. seeligeri Sr73 are localized within three novel putative TEs (Figure 4). The cadA6a cassette present in pLIS4 is located within a 27-kb-long composite transposon, named Tn6869, formed by two flanking copies of ISLse2 (IS3 family). This putative mobile element disrupts a hypothetical gene and is flanked by 3-bp-long direct repeats (DRs), which are probably the result of target site duplication upon transposition. Other copies of ISLse2 found in Listeria sequences present in GenBank also feature 3-bp-long DRs.

In pLIS6, the cadA6b cassette was probably introduced on a 6-kb-long non-composite transposon. Apart from the cadA6b efflux system, this novel transposon Tn6870 encodes a transposase, a serine recombinase, and an ATPase, which shows aa sequence similarity to the CDSs of transposon Tn552 (6.5 kb) from Staphylococcus aureus (GenBank acc. no. X52734.1). No DRs flanking Tn6870 were identified in pLIS6. However, we discovered the same putative transposon in the genome sequences of several L. monocytogenes strains in the GenBank database (e.g., NZ_NYDG01000002), where it is flanked by 6-bp-long DRs. These L. monocytogenes strains originated from Italy and are food-associated or clinical isolates.

Another non-composite cadA6a-harboring transposon, Tn6871 (7 kb), was identified in the L. seeligeri Sr73 chromosome sequence (nucleotide positions of TEs identified in the chromosome are presented in Supplementary Table S2). This putative transposon has a similar structure to Tn6870, but harbors an additional methyltransferase domain-containing protein. Identification of an empty insertion site in the chromosome of a related L. seeligeri strain RR4 (CP034772) enabled
definition of the transposon borders with flanking 6-bp-long DRs. Tn6871 is present in the genomic sequences (99–100% nucleotide sequence identity) of several strains of L. monocytogenes (e.g., NZ_FFER01000001), L. ivanovii (e.g., KR780025), L. seeligeri (e.g., NZ_JAATNZ010000010.1), and L. innocua (e.g., NZ_JRYX01000002). In many of these cases the transposon is flanked by 7- or 8-bp-long DRs of the target site. This transposon was found in both food-associated and clinical isolates originating from various locations, including (i) China (NZ_VOXH01000052; source—delicatessen), (ii) Switzerland (NZ_QYIR01000006; raw sausage), (iii) Ireland (NZ_LABG01000002; milk), (iv) Italy (NZ_NXUN01000005; smoked salmon), (v) Netherlands (NZ_FFGL01000001, NZ_FFER01000001; blood, cerebrospinal fluid), and (vi) Canada (NZ_QADA01000002; food).

In addition, a Tn3 family transposon, Tn6947 (7 kb), harboring a cadA6 cassette was identified in a few L. monocytogenes strains originating from the United States (e.g., strain CDPHFDLB-FM17-00092, NZ_PQHI01000019), for which draft sequences are available in the GenBank database. The putative transposon disrupts a DNA polymerase gene and is flanked by 5-bp-long DRs. It encodes a Tn3-family transposase, a resolvase and a slightly divergent cadA6 cassette (named cadA6c). CadA6c shows around 91% aa sequence identity to CadA6a (86% nucleotide sequence identity). The transposase shows around 43% aa sequence identity to that of Tn5422.

In all the analyzed cases, cadA6 is harbored by putative transposons, which increases the potential for its dissemination. Two other cadmium resistance determinants frequently encountered in Listeria spp., cadA1 and cadA2, are also carried by TEs–Tn5422 a Tn3-family transposon [24] and a putative composite transposon formed by flanking IS3-family ISs [27], respectively.

2.7. Functional Analysis of the cadA6 Resistance Cassette

To test whether cadA6 encodes a functional efflux system conferring cadmium tolerance, plasmid pLIS4 was removed from L. seeligeri Sr12 cells by repeated subculturing in BHI broth supplemented with subinhibitory concentrations of novobiocin. Plasmid-cured strains (Sr12-C1, Sr12-C2) were indeed incapable of growth on BHI agar plates supplemented with cadmium chloride (35 µg/mL). Despite repeated attempts, we did not succeed in obtaining a derivative of L. ivanovii Sr11 that had been cured of plasmid pLIS6. Interestingly, plasmid pLIS4 encodes a putative toxin-antitoxin (TA) stabilization system, while pLIS6 from L. ivanovii Sr11 does not contain such addiction modules.

Next, the putative cadmium resistance cassettes cadA6a (pLIS4, L. seeligeri Sr12) and cadA6b (pLIS6, L. ivanovii Sr11) were amplified by PCR and cloned into the mobilizable E. coli–Listeria spp. shuttle vector pDKEL. The constructed plasmids (pDKEL_cadA6a and pDKEL_cadA6b) were transferred from E. coli into a cadmium-susceptible L. monocytogenes strain 10403S. The obtained transconjugants acquired cadmium tolerance and were able to grow at a cadmium chloride concentration of 75 µg/mL. In contrast, both the recipient strain L. monocytogenes 10403S and a 10403S derivative carrying “empty” vector pDKEL were incapable of growth at cadmium chloride concentrations of ≥20 µg/mL. The cloned cadA6a and cadA6b efflux systems did not seem to influence the susceptibility of L. monocytogenes 10403S to copper(II) sulfate pentahydrate or zinc chloride, which is consistent with previous reports [24,30], that the cadA1–cadA4 cassettes did not confer resistance to zinc. Our results strongly support the predicted cadmium detoxification function of the cadA6 efflux system.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

A total of 143 Listeria spp. strains (listed in Supplementary Table S1) were isolated from soil and water samples collected in this study (see Section 3.2), while another 12 were obtained from the Food Safety Laboratory collection at Cornell University (USA) (L. seeligeri: FSL S4-0003, FSL S4-0057, FSL S4-0073; L. innocua: FSL H4-0088; L. welshimeri: FSL H4-0277, FSL S4-0070, FSL S4-0101, FSL S4-0105; L. marthii: FSL S4-0120, FSL S4-0696, FSL S4-0710, and FSL S4-0965). Escherichia coli DH5α pir [56] and streptomycin-resistant L. monocytogenes strain 10403S (serotype 1/2a) [57] were used to clone...
and analyze the cadA6 cadmium-resistance determinant. Unless otherwise specified, bacteria were grown in brain–heart infusion (BHI) broth (BioMaxima, Lublin, Poland) or on BHI agar plates (1.5% w/v agar) at 37 °C. When necessary, the media were supplemented with kanamycin (Km; BioShop, Burlington, Canada)—50 µg/mL, streptomycin (Str; BioShop)—100 µg/mL for L. monocytogenes 10403S, erythromycin (Erm; Sigma-Aldrich, Saint-Louis, MO, USA)—2 µg/mL for Listeria spp., and 250 µg/mL for E. coli. For long-term storage, the isolates were suspended in BHI containing 20% glycerol and frozen at −80 °C.

3.2. Soil and Water Sample Collection and Isolation of Listeria spp.

For this study, 555 soil and 15 water samples were collected from 53 different locations in Poland in the years 2016 and 2017. Samples were collected from natural environments (328 samples), and urban (171) and agricultural (71) areas. Listeria spp. were isolated from these samples using a modified PN-EN ISO 11290-1 procedure. Briefly, 10 g of a soil sample or 10 mL of a water sample was added to 90 mL of selective enrichment half-Fraser broth (BioMaxima) and incubated at 30 °C for 24 h. A sample of 100 µL of the enriched culture solution was then added to 10 mL of Fraser Broth (BioMaxima) and incubated at 37 °C for 48 h. After incubation, 10 µL of the culture solution was streaked onto Chromogenic Listeria Lab-Agar plates (BioMaxima) and incubated at 37 °C for 48 h. Blue-green colonies were re-streaked onto Chromogenic Listeria Lab-Agar and BHI plates. If a single sample gave colonies displaying a distinctly different morphology on Chromogenic Listeria Lab-Agar (e.g., a different shade of color, with or without an opaque halo), representatives of each colony type were picked for further analysis.

3.3. Identification of Listeria Species

Listeria species were differentiated based on the multiplex PCR analysis, as described previously [58]. The following control strains were used: L. monocytogenes ATCC 13932, L. grayi ATCC 25401, L. welshimeri ATCC 35987, L. seeligeri ATCC 35967, L. innocua PZH 5/04, and L. ivanovii PZH 7/04 from the collection of the Department of Molecular Microbiology, University of Warsaw (Poland). In the case of presumptive Listeria spp. strains for which the species could not be determined by multiplex PCR, 16S rDNA amplicons (primers 27f/1492R; [59]) were sequenced.

3.4. Bacterial Mating between Listeria spp. Strains

Bi-parental matings between Listeria spp. strains were performed on solid BHI medium using L. seeligeri Sr12, L. ivanovii Sr 11, and L. welshimeri 40/07 [18] as donor strains, and streptomycin-resistant L. monocytogenes 10403S as the recipient. The mating experiments were performed in four independent repetitions at two different temperatures. Overnight cultures of the donor and recipient strains were mixed at a ratio of 1.2 and 100 µL samples of these mixtures were spread on solidified BHI medium and incubated at 30 or 37 °C for 24 h. Bacteria were then washed off the plates with BHI medium and suitable dilutions were plated on selective media containing streptomycin (100 µg/mL; selective marker of the recipient strain) and cadmium chloride (25 µg/mL), and incubated at 30 or 37 °C for up to 96 h. Transconjugants were verified by PCR using L. monocytogenes-specific primers and by plasmid DNA isolation.

3.5. Heavy-Metal and Metalloid Susceptibility

The susceptibility of Listeria spp. to heavy metals was determined using an agar dilution method as described previously [19], with some modifications. Briefly, several colonies picked from BHI plates were suspended in saline solution to a turbidity of 0.5 McFarland units. Spots of 3 µL of each bacterial suspension were applied to cation-adjusted Mueller Hinton 1.2% agar plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), supplemented with 2.5% defibrinated horse blood and 0, 35, 75, 150 µg/mL (which corresponds to: 0, 0.19, 0.41, 0.82 mM) cadmium chloride (Sigma-Aldrich) or 0, 250, 500, 1000 µg/mL (0, 1.92, 3.85, 7.70 mM) sodium (meta)arsenite (Sigma-Aldrich), or to BHI plates supplemented with 0, 600, 1200, 2400 µg/mL (0, 2.40, 4.81, 9.61 mM) copper(II) sulfate.
pentahydrate. The plates were then incubated at 37 °C for 48 h (cadmium chloride, sodium (meta) arsenite) or 72 h (copper(II) sulfate pentahydrate). Strains were classified as resistant to heavy metals if they produced confluent growth on plates containing ≥75 µg/mL (0.41 mM) cadmium chloride or ≥500 µg/mL (3.85 mM) sodium arsenite. In the case of copper compounds, there are no determined breakpoint values for Listeria spp.

3.6. Isolation of Genomic DNA

Genomic DNA was extracted from bacterial cells using a Chelex-100 resin-based technique. Colonies were suspended in 50 µL of 5% Chelex-100 (Bio-Rad, Hercules, CA, USA), incubated at 95 °C for 20 min, then cooled on ice for 5 min and centrifuged at 2400×g for 3 min. The DNA-containing supernatants were then used as the templates for PCR amplifications.

3.7. Plasmid DNA Isolation and Restriction Fragment Length Polymorphism (RFLP) Profile Analysis

Each strain was cultured overnight in BHI broth at 37 °C. Samples of 3 mL were centrifuged and the cell pellets resuspended in 500 µL of diluted SSC buffer (15 mM sodium chloride, 1.5 mM trisodium citrate, pH 7.0). The cells were collected by centrifugation and resuspended in 500 µL of SET buffer (25% sucrose (w/v), 50 mM EDTA, 50 mM Tris, pH 8.0, 5 mg/mL lysozyme). After incubation at 37 °C for 30 min, plasmid DNA was isolated according to the alkaline lysis procedure [60]. Purified plasmid DNA was digested with EcoRI according to the manufacturer’s protocol (Thermo Scientific in the case of all restriction enzymes used in this study). The resulting DNA fragments were separated by electrophoresis in 0.8% agarose gels. Plasmid DNA for sequencing was isolated using a larger scale preparation method [61]. Sequence assembly was verified by RFLP analysis (enzymes: EcoRI, BamHI and NcoI).

3.8. Plasmid Curing

Plasmid pLIS4 was removed from cells of L. seeligeri Sr12 as described previously [62], with some modifications. The strain was grown overnight at 37 °C in 2 mL of BHI broth supplemented with a subinhibitory concentration (0.15 µg/mL) of novobiocin (Sigma). The resulting culture was subcultured (1:100) in the same medium and again grown overnight at 37 °C. This procedure was repeated each day for 15 days. Dilutions of the final culture were then plated on Chromogenic Listeria Lab-Agar plates, and incubated at 37 °C overnight. Single colonies were screened for the presence of plasmids by PCR using primers targeting the cadA6 gene. Negative and positive controls were used in all PCR experiments. Plasmid loss was confirmed by plasmid DNA extraction and by testing the ability of strains to grow on BHI plates supplemented with 35 and 75 µg/mL cadmium chloride.

3.9. Detection of Resistance Determinants by PCR

The presence of resistance determinants was detected by PCR performed with DreamTaq polymerase (Thermo Scientific, Waltham, MA, USA) using primers listed in Table 1. A primer pair for the novel cadA6 gene was designed based on the sequences of plasmids pLIS4 (L. seeligeri Sr12) and pLIS6 (L. ivanovii Sr11). The following thermocycle conditions were applied: initial denaturation for 5 min at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s at the temperatures listed in Table 1, and elongation at 72 °C for 45–150 s, depending on the expected product size; final elongation at 72 °C for 5 min. Positive control strains were included for all tested genes, apart from cadA5 and ctpA for which no such controls were available. The following control strains were used: (i) L. innocua 62/06 [18]—cadA1 gene, (ii) L. welshimeri 49/06 [18]—cadA2 gene, (iii) L. monocytogenes EGDe [63]—cadA3 gene, (iv) L. monocytogenes Lmo28 [52]—cadA4, arsA1, and arsA2 genes associated with LGI2, and (v) L. monocytogenes 2270/03 [52]—arsA gene associated with the Tn554-like transposon. The PCR products amplified from positive control strains were sequenced.
3.10. Functional Analysis of the cadA6 Resistance Cassette

3.10.1. Construction of a Mobilizable Shuttle Vector pDKEL

Plasmid pDKEL was constructed based on mobilizable E. coli vector pDS132 [64]. The construction of pDKEL and its derivatives is described in detail in Supplementary Figure S1. Briefly, a kanamycin resistance cassette, obtained from pDIY-Km [65] was cloned in plasmid pDS132. Then, a 3707-bp restriction fragment from this construct was ligated with an erythromycin resistance cassette obtained from plasmid pHPI3 [66]. Next, the lacZ multiple cloning site (MCS) from plasmid pBBR1MCS was added to the construct [67]. Finally, a replication system, functional in Gram-positive bacteria, obtained from plasmid pHPI3, was cloned into the MCS. The resulting plasmid pDKEL is a mobilizable E. coli—Listeria spp. shuttle vector containing kanamycin (selection marker for E.coli strains) and erythromycin (selection marker for L. monocytogenes strains) resistance cassettes.

3.10.2. Cloning of the Cadmium Resistance Cassette into pDKEL

Novel cadmium resistance cassettes (genes cadA6 and cadC6) were amplified from plasmids pLIS4 (cadA6a; L. seeligeri strain Sr12; nucleotide position: 18,685–21,508) and pLIS6 (cadA6b; L. ivanovii strain Sr11 nucleotide position: 12,664–15,715) using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and the primer pairs cadA6aF/cadA6aR and cadA6bF/cadA6bR (Table 1). The amplified DNA fragments (2843- and 3072-bp-long, respectively) and pDKEL were digested using enzymes SpeI and NotI, ligated using T4 DNA ligase, and the mixtures used to transform chemically competent E. coli DH5α with selection on LA plates containing kanamycin. Plasmid DNA was isolated from transformants and restriction analysis was used to confirm their identity as recombinants.

3.10.3. Introduction of Constructed Plasmids into L. monocytogenes 10403S

The plasmids pDKEL_cadA6a, pDKEL_cadA6b, and empty pDKEL were introduced into the cadmium-sensitive and streptomycin-resistant L. monocytogenes strain 10403S by conjugation. Overnight cultures of the donor (E. coli DH5α pir strains harboring pDKEL_cadA6a, pDKEL_cadA6b or pDKEL), recipient (L. monocytogenes 10403S), and helper (E.coli DH5α harboring helper plasmid pKR2013; [68]) strains were harvested by centrifugation and the cells were washed to remove the antibiotics. The cell suspensions were mixed in a 1:1:1 ratio and 100 µL of the mixtures were spread on BHI agar plates. After incubation at 37 °C for 24 h, bacterial cells were washed off the plates with BHI medium and dilutions were plated on BHI agar plates supplemented with streptomycin, and depending on the introduced vector, erythromycin (in the case of pDKEL) or 20 µg/mL of cadmium chloride (pDKEL_cadA6a and pDKEL_cadA6b). These plates were then incubated at 37 °C for up to 48 h to allow transconjugant colonies to form. The presence of transferred plasmids in these strains was confirmed by restriction analysis of isolated plasmid DNA.

3.10.4. Heavy-Metal Susceptibility of L. monocytogenes 10403S Strains Harboring the Constructed Plasmids

The susceptibility of the following strains to cadmium was tested using the agar dilution method: (i) L. monocytogenes 10403S carrying pDKEL_cadA6a, (ii) L. monocytogenes 10403S carrying pDKEL_cadA6b, (iii) L. monocytogenes 10403S carrying empty pDKEL, (iv) L. monocytogenes 10403S without any plasmids (negative control), (v) L. seeligeri Sr12 harboring the cadA6a cassette, and (vi) L. ivanovii Sr11 harboring the cadA6b cassette (positive controls). Assay plates were supplemented with cadmium chloride at 0, 35, 75, 150, and 300 µg/mL. The experiment was performed in two independent replications. The same strains were used in agar dilution assays to test their susceptibility to copper(II) sulfate pentahydrate (0, 600, 1200, 2400 µg/mL) and zinc chloride (0, 500, 1000, 2000, 3000 µg/mL), in order to determine whether the cadA6 efflux system influences the susceptibility to these metals.
3.11. DNA Sequencing

Sequencing of plasmids pLIS4 (L. seeligeri Sr12), pLIS5 (L. seeligeri Sr73), and pLIS6 (L. ivanovii Sr11), as well as the L. seeligeri Sr73 chromosome was performed in the DNA Sequencing and Oligonucleotide Synthesis Laboratory (oligo.pl) at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The sequences were obtained using a combination of MiSeq (Illumina, San Diego, CA, USA) and GridION (Oxford Nanopore Technologies, Oxford, UK) sequencing and assembled with Unicycler [69]. The nucleotide sequences of the plasmids have been deposited in GenBank (NCBI, Bethesda, MD, USA) under the accession numbers MW124301 (pLIS4), CP063072 (pLIS5), MW124302 (pLIS6), and CP063071 (chromosome of L. seeligeri Sr73).

3.12. Bioinformatic Analysis

Automatic annotation of the nucleotide sequences was performed using RAST on the PATRIC platform [70], followed by manual refinement in Artemis [71], based on the homology searches (BLAST) of the NCBI database (http://www.ncbi.nlm.nih.gov). Sequences were aligned and visualized using EasyFig [72]. Insertion sequences were identified using the ISfinder website and the nucleotide sequences of novel elements were deposited in the ISfinder database [73]. Transposon numbers were registered at the Transposon Registry [74]. The phylogenetic tree of the RepA proteins of complete Listeria spp. plasmid sequences available in the GenBank database and plasmid pOX2 of Bacillus anthracis was constructed in MEGAX [75] using the maximum-likelihood algorithm (Le and Gascuel model). Statistical support for the internal nodes was determined by 1000 bootstrap replicates. Multiple sequence alignments were performed using MUSCLE [76] and visualized using Jalview [77].

4. Conclusions

Analysis of the first complete sequences of plasmids from L. seeligeri and L. ivanovii as well as the chromosome of L. seeligeri Sr73 identified a novel variant of the cadAC efflux system. The presence of cadA6 in four different novel transposons increases the potential for its dissemination. The cadA6 resistance determinant has been detected in strains representing four Listeria species, including L. monocytogenes, isolated from various countries and sources—environmental, food-associated, and clinical samples. We have experimentally confirmed the involvement of the cadA6 determinant in the increased cadmium tolerance of Listeria spp. strains and designed molecular probes to verify the presence of this novel variant in other strains. Previous studies have suggested that some cadAC cassettes may additionally influence the phenotypic traits not related to heavy-metal resistance [30,36]. Therefore, the prevalence of various cadAC efflux systems and the means of their dissemination should be monitored in Listeria spp. strains isolated from different environments.

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