Novel Internalin P homologs in *Listeria*

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Impact Statement

The intracellular bacterial pathogen *Listeria monocytogenes* can breach protective barriers in the pregnant host, allowing for the colonization of the placenta in pregnant women and resulting in numerous adverse pregnancy outcomes. Previous studies aimed at delineating the mechanisms behind placental colonization of *L. monocytogenes* identified a key virulence factor, internalin P (InIP). The internalin family of proteins has been studied extensively due to their conservation in the *Listeria* genus and their contribution to virulence and pathogenicity in *L. monocytogenes*. Still, many questions remain regarding the evolution of internalins and their potential roles in non-pathogenic *Listeria*. Our work addresses this gap in knowledge by 1) identifying additional InIP homologs in *Listeria*, including *L. ivanovii*, *L. seeligeri*, *L. innocua*, and *L. costaricensis*, and 2) characterizing these homologs using computational evolutionary methods to compare their primary sequences, domain architectures, and structural models. Together, our findings contribute to the field by providing insights into the evolution of one key member of the internalin family as well as serving as a catalyst for future studies of InIP and its role in *Listeria* pathogenesis.

Abstract

Listeria monocytogenes (*Lm*) is a bacterial pathogen that causes listeriosis in immunocompromised individuals, particularly pregnant women. Several virulence factors support the intracellular lifecycle of *Lm* and facilitate cell-to-cell spread, allowing it to occupy multiple niches within the host and cross protective barriers, including the placenta. One family of virulence factors, internalins, contributes to *Lm* pathogenicity by inducing specific uptake and conferring tissue tropism. Over 25 internalins have been identified thus far, but only a few have been extensively studied. Internalins contain leucine-rich repeat (LRR) domains which enable protein-protein interactions, allowing *Lm* to bind host proteins. Notably, other *Listeria* species express internalins but cannot colonize human hosts, prompting questions regarding the evolution of internalins within the genus *Listeria*. Internalin P (InIP) promotes placental colonization through interaction with the host protein afadin. Though prior studies of InIP have begun to elucidate its role in *Lm* pathogenesis, there remains a lack of information regarding homologs in other *Listeria* species. Here, we have used a computational evolutionary approach to identify InIP homologs in additional *Listeria* species. We found that *L. ivanovii londoniensis* (*Liv*) and *L. seeligeri* (*Ls*) encode InIP homologs. We also found InIP-like homologs in *L. innocua* and the recently identified species *L. costaricensis*. All newly identified homologs lack the full-length LRR6 and LRR7 domains found in *Lm*'s InIP. These findings inform on the evolution of one key *Lm* virulence factor, InIP, and serve as a springboard for future evolutionary studies of *Lm* pathogenesis as well as mechanistic studies of *Listeria* internalins.

Introduction

Prenatal infection remains a major public health concern. Annually, nearly 13 million infants are born prematurely worldwide, and an estimated 30% of these preterm births can be attributed to prenatal infection, though the actual number may be higher due to the subclinical nature of many prenatal infections¹. To better detect and treat these infections to prevent adverse pregnancy outcomes, we must better understand the pathogens that cause them. *Listeria monocytogenes* is widely used in prenatal infection research due to its well-characterized lifecycle and ease of use in laboratory experiments^{2,3}.

The *Listeria* genus comprises 17 species, including the human pathogens *L. ivanovii* (*Liv*) and *L. monocytogenes* (*Lm*)⁴. These Gram-positive facultative intracellular bacterial pathogens are the most typical causative agent of listeriosis in humans^{4,5}. While relatively rare, listeriosis can result in severe morbidity and mortality in immunocompromised individuals^{5,6}. Pregnant women are particularly at risk for listeriosis, as *Lm* can colonize the placenta and cause adverse pregnancy outcomes such as preterm birth, neonatal meningitis, miscarriage, and stillbirth⁵. *Lm* employs several virulence factors that aid in its invasion of various host niches and the breach of protective host barriers^{5,6}. Previous studies have addressed the roles of various *Lm* virulence factors, such as ActA, Internalin A (InlA), and Internalin B (InlB) in the context of pregnancy^{7,8}. Faralla *et al.* followed up with two studies focusing on Internalin P (InlP), a key virulence factor for the invasion of the placenta^{9,10}.

Typically, listeriosis begins with the consumption of contaminated food items. Once in the digestive system, *Lm* uses several virulence factors, including the internalins, to colonize gut epithelial cells and spread throughout the host^{5,6,11}. Internalins contribute to this spread by conferring tissue tropism; for example, InlA binds E-cadherin on gut epithelial cells while InlB binds C-Met expressed by hepatocytes¹¹. These interactions are enabled by Leucine-Rich Repeat (LRR) domains found in all internalins^{11,12}. LRR domains are found in an array of functionally diverse proteins across the domains of life, such as ribonuclease inhibitors in eukaryotes (humans, pigs) and virulence factors in prokaryotes (*Yersinia pestis*, *L. monocytogenes*)¹². Internalins may have as few as four (InlG) or as many as fourteen (InlA) LRR domains^{5,11}. Notably, internalins and other virulence factors

are relatively well-conserved across the genus, including species that are considered non-pathogenic to humans, such as *L. seeligeri* and *L. innocua*^{4,13,14}. While the details remain unclear, differences in pathogenicity have been attributed to minor genetic variations and differences in the expression of virulence factor genes¹⁵. The precise roles of the various internalin genes in *Listeria* and their evolutionary relationships remain critical open questions in *Listeria* biology.

Internalin P (InIP) is an *Lm* virulence factor known to enhance placental colonization in the pregnant host. This is likely accomplished by enabling *Lm* to transcytose through the basal membrane underlying the syncytiotrophoblast, the protective outer layer of placental cells that serves as a barrier between maternal and fetal blood⁹. Further characterization revealed that InIP encompasses nine LRR domains and binds the human protein afadin, which is a nectin-like protein found in cell-cell junctions and thought to play a significant role in cellular adhesion¹⁰.

Initial InIP studies identified a structural homolog of InIP, Lmo2027, in *Lm*, but information regarding InIP homologs in other *Listeria* species has been incomplete⁹. In this study, we used comparative genomics and protein sequence-structure-function analyses to identify InIP homologs in the genomes of *L. seeligeri* (*Ls*), *L. ivanovii londoniensis* (*Liv*), *L. innocua* (*Lin*), and *L. costaricensis* (*Lc*) (**Fig. 3, Table S1**). The bioinformatic analysis presented here serves as a springboard for future studies of *Listeria* evolution and pathogenesis pertaining to the internalin protein family, including its ability to colonize the human placenta.

Materials and Methods

Identification of InIP Homologs. To identify InIP_{*Lm*} homologs across evolutionary lineages, we submitted the InIP_{*Lm*} amino acid sequence (accession: WP_014601135.1) to MolEvolVR (<http://www.jravailab.org/molevolvr>)¹⁶. The query returned hits for homologous proteins across bacterial phyla. While many species carried homologous proteins (*e.g.*, *Nostoc spp.* and *Beggiatoa leptomitiformis*), we chose to filter out hits with low similarity and divergent domain architectures and genomic contexts for our detailed study; we thus focused on the *Listeria* genus (including 45,530 *L. monocytogenes*, 740 *L. innocua*, 169 *L. seeligeri*, 44 *L. ivanovii*, and 1 *L. costaricensis* genomes). Within this

dataset, we selected the hits with the highest percent similarity and unique domain architectures as representative homologs for further analysis (**Figure 1; Table S1**). Accession numbers provided by MolEvolVR were used to query the NCBI RefSeq Protein Database for corresponding nucleotide sequences and locus tags for homologous genes¹⁷. BioCyc¹⁸ (<https://biocyc.org>) and NCBI RefSeq¹⁷ protein databases were used to identify genomic contexts (neighboring genes).

Calculation of Percent Identity and Percent Similarity. Percent identity and percent similarity values for predicted homologs were provided by MolEvolVR¹⁶ (**Table S1**; https://github.com/jrabilab/inlp_listeria). Nucleotide sequences for *inlP* in *L. monocytogenes*, *L. ivanovii londoniensis*, *L. seeligeri*, and *L. costaricensis* were aligned using Clustal Omega¹⁹ (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), and resulting alignments were submitted in FASTA format to the Sequence Manipulation Suite²⁰ (SMS; <http://www.bioinformatics.org/sms2>) to calculate percent nucleotide identity. The homolog similarity and identity matrix was generated using MatGAT2.01 with the BLOSUM 62 matrix and default options²¹.

Multiple Sequence Alignment, Phylogenetic Trees, and Protein Models. Multiple sequence alignments for homologous amino acid sequences were generated using Kalign²⁴ and visualized using JalView (Version 2.11.1.4)²⁵. Phylogenetic trees were constructed using Kalign²⁴, FastTree, and FigTree. Three-dimensional protein models were produced using SWISS-MODEL with the *L. monocytogenes* Internalin P crystal structure (PDB: 5hl3) as a template^{26,27}. All data, analyses, and visualizations for InlP *Listeria* homologs are available here: https://github.com/jrabilab/inlp_listeria.

Results

Listeria ivanovii londoniensis and *Listeria seeligeri* encode Internalin P Homologs.

To begin investigating evolutionary conservation of the *L. monocytogenes* Internalin P (InIP_{Lm}), we started with an extensive homology search and protein characterization of InIP-like proteins in diverse lineages across the tree of life using MolEvolvR¹⁶ (<http://iravilab.org/molevolvr>). Most homologs were present only within the genus *Listeria*. To further ensure that all homologs are being identified, we picked other representative InIP homologs from *L. ivanovii* and *L. seeligeri* as new starting points for our homology search and characterization (using MolEvolvR¹⁶; see *Methods*). We found several hits in our multi-start search including proteins that resembled other Internalins (e.g., InIB), and others that carried neither the signature Internalin_N or LRR domains characteristic of internalins (**Fig. 2 and S1**). Therefore, we restricted our full set of homologs to only InIP-like proteins resulting in 64 representative proteins with distinct domain architectures from each *Listeria* species including *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. innocua*, and *L. costaricensis* (**Fig. 2 and S1; Table S1**). Homologs from *L. seeligeri* (Ls) and *L. ivanovii* (Liv) showed >65% amino acid similarity compared to InIP_{Lm}, while homologs from *L. innocua* (Lin) and *L. costaricensis* (Lc) showed <35% and 55% similarity, respectively (**Fig. 3**). We found that several homologs lacked predicted signal peptide domains suggesting they are not secreted like InIP; this was further corroborated by the presence of predicted transmembrane LPXTG motifs, which indicate that these homologs are more likely to be membrane-anchored InIB-like proteins rather than secreted InIP homologs (**Fig. 2 and S1**). Investigation of the InIP-like proteins in *Lc* and *Lin* showed that it is unlikely that they are functional InIP homologs (discussed below). Further investigation of domain architectures and genomic contexts of putative homologs ultimately revealed one InIP homolog encoded within the *L. ivanovii londoniensis* genome and three InIP homologs encoded by *L. seeligeri* (discussed below). Here, we refer to these homologs as InIP_{Lm}, InIP_{Li}, InIP_{Ls1}, InIP_{Ls2}, and InIP_{Ls3}, respectively, to indicate species and gene order.

We determined the similarity of InIP homologs in *Liv* and *Ls* at the nucleotide and amino acid levels towards functional characterization. The *inIP* gene in *L. ivanovii londoniensis* (*inIP_{Liv}*), as well as the three *L. seeligeri* paralogs (*inIP_{Ls1}*, *inIP_{Ls2}*, and

inIP_{LS3}), shared ~70% identity with the *inIP_{Lm}* gene and ~52–65% identity at the amino acid level when compared to *InIP_{Lm}* (**Fig. S2**). However, the newly identified homologs in *L. ivanovii londoniensis* and *L. seeligeri* shared much higher percent amino acid similarity with *InIP_{Lm}* — *InIP_{Liv}*, *InIP_{LS1}*, and *InIP_{LS3}* showed ~70% similarity to *InIP_{Lm}* (**Fig. 3**). *InIP_{LS2}* showed a striking 94% similarity to the *L. monocytogenes* *InIP* (**Fig. S2**). Notably, the flanking *L. seeligeri* paralogs, *InIP_{LS1}* and *InIP_{LS3}*, were more similar to each other than to the third paralog or to *InIP_{Lm}* (**Fig. 3**). To further investigate these new *Listeria* *InIP* proteins, we next explored their genomic neighborhoods.

Because the *Listeria* genus maintains a high degree of synteny across species, we investigated the genomic contexts of identified homologs compared to the *InIP_{Lm}* gene, which is flanked upstream by an amino acid permease gene and downstream by an NADPH dehydrogenase gene (**Fig. 4A**). We hypothesized that functional homologs of *InIP_{Lm}* would be flanked by these same genes in other *Listeria* species. We, therefore, determined the genomic neighborhoods of *inIP* homologs in *L. ivanovii londoniensis*, *L. ivanovii ivanovii*, *L. seeligeri*, *L. costaricensis*, and *L. innocua* (see *Methods*). We found that the *inIP* genes in *L. ivanovii londoniensis* and *L. seeligeri* were flanked upstream by an amino acid permease gene and downstream by an NADPH dehydrogenase gene mirroring the *L. monocytogenes* genomic context, suggesting the identified homologs are likely true homologs of the *inIP* gene (**Fig. 4**). Interestingly, we found that the gene encoding the *InIP*-like protein in *Lc* was flanked upstream by an amino acid permease and downstream by a gene encoding a LapB repeat-containing protein, inconsistent with genomic neighborhoods seen in other *Listeria* species (**Fig. 4E**). Also inconsistent with other genomic neighborhoods, the *InIP*-like protein in *Lin* was flanked upstream by a DUF5110-containing protein-encoding gene and downstream by the *ssrA* gene (**Fig. 4F**). To quantify the similarity of the flanking genes in *L. ivanovii londoniensis*, *L. seeligeri*, and *L. monocytogenes*, we calculated their pairwise similarity. At the amino acid level, the products of these flanking genes had >95% similarity to *L. monocytogenes* (**Fig. S3**). Notably, while *L. ivanovii londoniensis* encoded an *inIP* homolog in this region, *L. ivanovii ivanovii* did not (**Fig. 4**). Additionally, while *L. ivanovii londoniensis* encoded only one copy of *inIP* (EL212_RS12905; *inIP_L*), *L. seeligeri* encoded three copies (LSE_RS12040, LSE_RS12045, and LSE_RS12050; *inIP_{LS1}*, *inIP_{LS2}*, and *inIP_{LS3}*, respectively) (**Fig. 4**).

Internalin P Homologs in *L. ivanovii* and *L. seeligeri* lack the full-length LRR6 and LRR7 domains found in *L. monocytogenes* InIP.

To delineate the evolution of InIP within *Listeria*, we generated a multiple sequence alignment (**Fig. 5**) and constructed a phylogenetic tree of the homologs (**Fig. 1**). While we observed several amino acid substitutions throughout the length of the proteins, the most striking difference between InIP_{Lm} and its homologs was in the Leucine-Rich Repeat (LRR) regions — a partial lack of LRR6 and complete lack of LRR7 — in the *L. ivanovii* and *L. seeligeri* homologs (**Fig. 1**). Additionally, we noted a lack of conservation in a previously described calcium-binding loop¹⁰ present in InIP_{Lm} (amino acid residues 132–135; **Fig. 1**). This observation was of particular interest since this calcium-binding loop might play a role in protein signaling or stabilization of protein-protein interactions between InIP_{Lm} and host afadin. To better visualize the structural differences in these homologs, we generated models of InIP_{Li}, InIP_{Ls1}, InIP_{Ls2}, and InIP_{Ls3} based on the previously resolved crystal structure of InIP_{Lm} (**Fig. 1**; see *Methods*). These models illustrate the similarity in the overall structure of the five homologous proteins, and the lack of LRR7 and full-length LRR6 are visible in *Li* and *Ls* homologs (**Fig. 1**; green/yellow regions). Additionally, the calcium-binding loop region is discernible in all five homologous proteins but appears structurally diverse in *L. ivanovii* and *L. seeligeri* homologs.

In summary, we have discovered novel Internalin P homologs in *Listeria*, traced their evolution, and uncovered potential functional implications pertaining to heterogeneity in key InIP domains. All InIP homolog data (along with characterizations in terms of domain architectures and modeling) are available at https://github.com/jravidlab/inlp_listeria.

Discussion

While previous studies have addressed many of the physical and mechanistic properties of InIP, the conservation of the *inIP* gene within or outside of the *Listeria* genus remains incompletely characterized. Here, we have provided insight into Internalin P in other *Listeria* species aside from *L. monocytogenes* that will drive future mechanistic studies of InIP as well as evolutionary studies of *Listeria* pathogenesis pertaining to the internalins.

First, we analyzed the InIP amino acid sequence with MolEvolvR¹⁶ to retrieve homologous proteins across evolutionary lineages. MolEvolvR¹⁶ is a powerful new bioinformatic web application to characterize protein families using molecular evolution and phylogeny (<http://jrvilab.org/molevolvr>). The MolEvolvR¹⁶ InIP search returned a list of potential homologs including those found in *Listeria* species. In this article, we focus on homologs in *Listeria* since these species carried the classic Internalin and LRR domains. Using MolEvolvR¹⁶, we identified InIP-like proteins in *L. innocua*, *L. seeligeri*, *L. ivanovii*, and *L. costaricensis*; only homologs in *L. seeligeri* and *L. ivanovii londoniensis* expressed an amino acid percent similarity value >65%. We found that the lower identity proteins are more likely to be Internalin B homologs based on their sequence, domain architecture, and structure.

To determine if the newly identified proteins were true homologs of InIP_{Lm}, we explored their domain architectures and genomic context. Consistent with the synteny observed in *Listeria* genomes, we found that the *inIP* domain architectures and genomic neighborhoods were highly conserved in *L. ivanovii londoniensis* and *L. seeligeri*, but not in *L. ivanovii ivanovii*, *L. innocua*, or *L. costaricensis*. While it is possible that these species could encode *inIP* homologs elsewhere in their genomes, it seems unlikely considering their domain architectures and lower conservation in sequence compared to other homologs. It is more likely that the homologous proteins identified in *Lc* and *Lin* are independent of InIP, but in the same class of small, secreted internalins that encompasses InIP, InIC, and InIH, among others¹¹.

Notably, we found that *L. ivanovii londoniensis* encoded a homolog for *inIP* while *L. ivanovii ivanovii* did not. Historically, *L. ivanovii londoniensis* and *L. ivanovii ivanovii* have been distinguished biochemically²⁸. Recently, Hupfeld *et al.*, noted that the two

subspecies could also be distinguished based on bacteriophage susceptibility: *L. ivanovii* strains are sensitive to bacteriophages, while *L. ivanovii londoniensis* strains encode a type II-A CRISPR-Cas system rendering them resistant to many phages²⁹. Our finding that only *L. ivanovii londoniensis*, and not *L. ivanovii ivanovii*, encodes the *inIP* gene provides another avenue for distinguishing between these two subspecies and could be beneficial to public health laboratories seeking to differentiate between them among clinical and food isolates. Additionally, because the evolution of virulence factors in *Listeria* remains mysterious, the specific presence of *inIP* in subspecies such as *ivanovii londoniensis* and its absence in *ivanovii ivanovii* may provide clues as to how *Listeria* evolves the ability to infect different cells and tissues.

Since *L. ivanovii* has been implicated in human and animal placental infection, it was not entirely surprising to find that it encoded the gene for InIP, an internalin known to enhance placental colonization. It was surprising, however, to find three copies of the *inIP* gene in *L. seeligeri* since it has not been significantly indicated in human or animal pathogenesis³⁰. Our analyses suggested that InIP_{LS2} was the most similar to InIP_{Lm} and InIP_{Liv}. It is possible that this copy (InIP_{LS2}) is the ancestral one, and InIP_{LS1} and InIP_{LS3} resulted from subsequent duplication events. The presence of multiple homologs suggests that InIP could have alternative functions apart from enhancing placental colonization. *Listeria* species are frequently found in environmental isolates, as they readily reside in soil. It is possible that InIP provides a fitness advantage in this environment.

One of the main questions resulting from the discovery of InIP homologs centers on the evolutionary timeline of the *Listeria* genus: which InIP came first? Our discovery that InIP_{Liv}, InIP_{LS1}, InIP_{LS2}, and InIP_{LS3} do not contain the full-length LRR6 and LRR7 domains found in InIP_{Lm} begins to offer potential answers to this question. It is plausible that *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii londoniensis* shared a common ancestor that passed down the *inIP* gene, and a subsequent insertion event in *L. monocytogenes* led to the full-length InIP containing LRR6 and LRR7. Conversely, it is likely that *L. monocytogenes* carries the ancestral copy of InIP (InIP_{Lm}); the full-length *inIP* gene could have undergone a deletion resulting in the loss of LRR6 and LRR7 in InIP_{Liv} and InIP_{LS}, although it is less likely to observe several deletion events as against a single

insertion event. Future studies on the evolution of the *Listeria* genus and the larger family of internalin proteins will be required to answer this question more rigorously and determine their possible links to pathogenicity.

An additional structural difference noted between newly identified InIP homologs resides in the Ca²⁺-binding loop of LRR3. Previously, this loop has been hypothesized to play a role in InIP signaling, activation, or stabilization in complex with its binding partner afadin¹⁰. Structural heterogeneity is visible in the Ca²⁺ regions of InIP homolog models; InIP homologs in *Liv* and *Ls* appear to have more open loops compared to InIP_{Lm}. The ability of these loops to bind calcium, and their relative binding affinities will be an important avenue for future investigation, especially as more details regarding the function and regulation of InIP_{Lm} come to light.

Recent studies made several fundamental discoveries regarding the physical and mechanistic properties of InIP_{Lm} and its activity in the placenta, but many questions remain unanswered^{9,10}. The discovery of InIP homologs in *L. ivanovii londoniensis* and *L. seeligeri*, two species that have not been substantially implicated in cases of placental infection, is compelling. Future studies will investigate the activity of these homologs to determine if they bind afadin and if they are able to enhance placental colonization of *L. monocytogenes* as well as endogenous InIP. Further, structural differences between these homologs suggest potential binding sites for the InIP-afadin interaction, which has not been resolved to date.

In summary, we report that *L. ivanovii londoniensis* and *L. seeligeri* encode homologs for the *L. monocytogenes* virulence factor InIP. Identified homologs in all three species are housed within similar genomic neighborhoods, flanked by the same housekeeping genes upstream and downstream; further, *L. seeligeri* encodes three copies of the *inIP* gene in this region. All four homologs are similar (>70%) to InIP in *L. monocytogenes*, the main structural difference resulting from the lack of full-length LRR6 and LRR7 regions in InIP_{Li}, InIP_{Ls1}, InIP_{Ls2}, and InIP_{Ls3}. Our findings will serve as a springboard for future evolutionary studies of internalins in the *Listeria* genus and will bolster future *in vitro* and *in vivo* studies of InIP in the context of virulence and pathogenicity.

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Author contributions

Conceptualization: KNC, JTB, JR, JWH. *Methodology:* KNC, JTB, JR. *Software:* JTB, JR. *Validation:* KNC, JTB, JR, JWH. *Formal Analysis:* KNC, JTB, JR. *Investigation:* KNC, JTB, JR. *Resources:* JR, JWH. *Data Curation:* KNC, JTB, JR. *Writing:* KNC, JR, JWH. *Visualization:* KNC, JTB, JR. *Supervision:* JR, JWH. *Project Administration:* JR, JWH. *Funding:* JR, JWH.

Data availability and reuse

All the data, analyses, and visualizations are available in our GitHub repository, https://github.com/JRaviLab/inlp_listeria. Text, figures, and data are licensed under Creative Commons Attribution CC BY 4.0.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Figures

Figure 1: Phylogeny and Structure Models of InIP and Representative Homologs. A phylogenetic tree was generated using the amino acid sequences of InIP homologs in *L. monocytogenes* (blue), *L. ivanovii londoniensis* (Purple), and *L. seeligeri* (green). Three-dimensional models were generated using SWISS-MODEL with the crystal structure for InIP_{Lm} (PDB: 5hl3) as a template. Multiple sequence alignments were generated using MolEvolvR and illustrate the complete LRR6 and LRR7 insertion present in InIP_{Lm}. The legend shows the colors of the amino acid residues indicated in the multiple sequence alignment

Figure 2: Phylogeny and Domain Architectures of Putative Internalin P Homologs.

A multiple sequence alignment and phylogenetic tree were generated using the amino acid sequences of putative InIP homologs identified using MolEvolVR and five *Listeria* InIP starting points (See *Methods*). The phylogeny of InIP-like proteins has been overlaid with their domain architectures (generated using Pfam database, MolEvolVR). The legend shows the colors of the amino acids indicated in the multiple sequence alignment and Pfam domain annotations.

Figure 3: Percent Similarity and Identity of Internalin P Homologs in representative *Listeria* species. Percent similarity and percent identity were calculated for the amino acid sequences for representative Internalin P homologs from five *Listeria* species, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. costaricensis*, and *L. innocua*. Matrix showing similarity and identity values was generated using MatGAT2.01 with the BLOSUM 62 matrix and default options selected.

Representative InIP homologs	<i>Liv</i>	<i>Lm</i>	<i>Ls1</i>	<i>Ls2</i>	<i>Ls3</i>	<i>Lin</i>	<i>Lc</i>	Percentage identity
BFirmic_Livanovii_WP_126300113.1		58.1	74.6	78.6	75.2	37.8	32.1	
BFirmic_Lmonocytogenes_WP_014601135.1	70.9		57.9	57.5	57.2	32.4	38.2	
BFirmic_Lseeligeri_WP_012986389.1	85.7	72.7		77.3	90.5	36.9	33.1	
BFirmic_Lseeligeri_WP_012986390.1	89.7	70.9	87.0		76.7	36.5	30.8	
BFirmic_Lseeligeri_WP_012986391.1	84.9	72.2	94.8	85.3		36.6	32.5	
BFirmic_Linnocua_WP_003769168.1	61.7	52.6	58.9	60.3	58.6		28.1	
BFirmic_Lcostaricensis_WP_099224870.1	45.7	53.0	46.1	43.4	45.9	43.0		

Percentage similarity

Figure 4: Genomic Context of Newly Identified Internalin P Gene Homologs. Genes homologous to the *L. monocytogenes inIP* (A) were identified in various other *Listeria* species. Gene order was maintained in *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii londoniensis*. Notably, *L. seeligeri* encodes three copies of the *inIP* gene. All homologs categorized as “true” homologs were flanked upstream by an amino acid permease gene (blue) and downstream by an NADPH dehydrogenase gene (orange). *L. ivanovii ivanovii* contains a pseudogene (purple) and an uncharacterized gene encoding a hypothetical protein (red) in this region. Putative homologous genes in *L. costaricensis* and *L. innocua* did not mirror genomic neighborhoods seen in the other *Listeria* species. All *inIP* homologs are represented in green. Genomic context was determined using RefSeq genomic records and the BioCyc genome browsers for each species (see *Methods*).

A. *Listeria monocytogenes* Genomic Context

B. *Listeria ivanovii londoniensis* Genomic Context

C. *Listeria seeligeri* Genomic Context

D. *Listeria ivanovii ivanovii* Genomic Context

E. *Listeria costaricensis* Genomic Context

F. *Listeria innocua* Genomic Context

Supplementary Figures

Figure S1: Phobius and Gene3D Domain Architectures of Identified Internalin P Homologs. Cellular localizations (Phobius) and domain architectures (Gene3D) were determined and visualized using MolEvolvR. Figure legends correspond to different domain predictions.

Figure S2: Percent Nucleotide Identity of InIP Homolog Genes in *L. monocytogenes*, *L. ivanovii londoniensis*, and *L. seeligeri*. Heatmap representing pairwise percent identities of the nucleotide sequences of Internalin P homologs in *L. monocytogenes*, *L. ivanovii londoniensis*, and *L. seeligeri*. Darker and lighter shades of blue represent higher and lower percent identities, respectively.

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