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NOTES

Identification and Purification of Novel Internalin-Related Proteins in *Listeria monocytogenes* and *Listeria ivanovii*

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Monoclonal antibodies were generated against a 30-kDa protein fraction derived from culture supernatants of a *Listeria monocytogenes* strain complemented with additional copies of the *prfA* regulator gene. Several of the antibodies reacted specifically with a hitherto unidentified, secreted 30-kDa polypeptide. By immunoblot analysis, the expression of this 30-kDa polypeptide was found to be dependent on the presence of the PrfA regulator protein. Microsequencing of peptides derived from the partially purified 30-kDa protein revealed homologies to the InlA and InlB polypeptides of *L. monocytogenes*, which are required for the internalization of the bacteria into nonphagocytic cell lines. This prompted us to term the 30-kDa polypeptide internalin-related protein (Irp). Irp-specific monoclonal antibodies cross-reacted with a 24-kDa polypeptide present in culture supernatants of *Listeria ivanovii*, indicating the existence of an Irp-related protein in this pathogenic *Listeria* species.

Listeria monocytogenes is a gram-positive bacterium that causes listeriosis, a rare but severe form of meningitis in humans (7, 9). Analysis of the genes involved in the infection has led to the identification of a chromosomal gene cluster harboring six genes (*prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*) which are essential for the pathogenicity of *L. monocytogenes*. These genes are involved in the escape of bacteria from the phagolysosomal compartment as well as in the process of intracellular and intercellular spreading (reviewed in references 20 and 23).

With the exception of *hly* (3), the virulence genes are strictly regulated by the PrfA regulator protein (1, 14, 25). Mutants lacking the *prfA* gene do not express the respective polypeptides, and as a consequence such mutants are avirulent when tested in an animal infection model (1; also references 20 and 23 and references therein). Conversely, mutants complemented with additional copies of the *prfA* gene produce larger quantities of the virulence factors (20, 23).

In addition to the genes located in the virulence gene cluster, an independent operon harboring two genes, *inlA* and *inlB*, has been detected in *L. monocytogenes* by transposon-induced mutagenesis and isolation of noninvasive mutants (8). Generation of isogenic mutants with a chromosomal in-frame deletion in either *inlA* or *inlB* showed that both the InlA and InlB polypeptides are required for invasion of *L. monocytogenes* into nonphagocytic tissue culture cells (4, 15). Although the *inlAB* genes are not part of the virulence gene cluster, their expression was shown to be regulated by temperature as well as by *prfA* (6, 15). The regulation mechanism, however, differs from that of the genes of the virulence gene cluster in that InlA and InlB are both detectable in isogenic *prfA* mutants and are also expressed at 20°C, albeit in small amounts (15). In a manner similar to that of the other virulence factors, the *inlAB* genes are positively regulated by PrfA (15).

Since the expression of all the listerial virulence factors so far characterized is controlled by the PrfA regulator, the search for novel *prfA*-regulated proteins represents a promising approach to identify additional virulence factors in *L. monocytogenes*. In order to identify potential novel *prfA*-regulated virulence factors, we have analyzed culture supernatants derived from the EGDpERL3 50-1 strain, which is complemented with additional copies of the *prfA* gene. We noted that, compared with the EGD*prf*A1 strain lacking the PrfA polypeptide, the expression of a number of additional polypeptides, particularly those with molecular masses of about 90 and 30 kDa (compare lanes 1 and 2 in Fig. 1a), was enhanced. Recently, analysis of such culture supernatants also enabled us to identify InlA (15).

The only known virulence factors of *L. monocytogenes* with molecular masses of about 30 kDa are the two phospholipases PlcA (34 kDa [13, 16]) and PlcB (29 and 33 kDa [17, 24]) and presumably also the mature form of the *mpl* product (2). We were therefore especially interested in analyzing the proteins of culture supernatants with molecular masses of approximately 30 kDa (Fig. 1a, lanes 1 and 2). To this end, concentrated culture supernatants of EGDpERL3 50-1 were separated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands of interest were excised, electroeluted, and injected into mice for generation of monoclonal antibodies (MAbs) by the procedure recently described for obtaining InlAB MAbs (15). Hybridoma supernatants were analyzed by enzyme-linked immunosorbent assay using microtiter plates coated with concentrated culture supernatants of EGDpERL3 50-1 (data not shown). Positive supernatants were further tested by comparative immunoblotting of concentrated supernatants derived from the PrfA-negative EGD*prf*A1 and the PrfA-overproducing EGDpERL3 50-1 strains as described recently (15). We identified several

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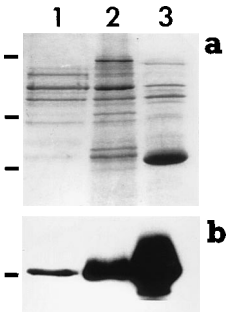

FIG. 1. Identification of the Irp polypeptide in culture supernatants of *L. monocytogenes* strains EGD*prf*A1 (lanes 1) and EGDpERL3 50-1 (lanes 2) and in a Mono-Q fraction derived from EGDpERL3 50-1 culture supernatants (lanes 3). (a) The Coomassie blue-stained SDS gel (15%); (b) the corresponding immunoblot processed with MAb O301 revealing an apparent molecular mass of 32 kDa for Irp. Molecular mass markers in panel a are as follows from top to bottom: 97, 45, and 31 kDa; that in panel b is 31 kDa.

MAbs specific for a 30-kDa polypeptide of *L. monocytogenes* which was highly enriched in the supernatants of EGDpERL3 50-1 (Fig. 1b, lanes 1 and 2). For further studies we concentrated on MAb O301 (immunoglobulin G1), which detected the 30-kDa polypeptide in culture supernatants derived from a *plcA1* insertional mutant (13), an *mpl2* deletion mutant as well as in supernatants from a *plcB2* deletion mutant (10). The observation that the 30-kDa polypeptide was also detectable in EGD*prf*A1 supernatant ruled out the possibility that MAb O301 reacts with *plcA*, *plcB*, and *mpl* products, whose expression is strictly *prfA* regulated (20). Interestingly, we have observed that the InlA and InlB polypeptides are also expressed in the absence of PrfA (15), suggesting that the expression of the 30-kDa polypeptide is similarly regulated.

To obtain N-terminal amino acid sequences of the 30-kDa protein, we partially purified this polypeptide by separating concentrated supernatants of EGDpERL3 50-1 by Mono-Q ion-exchange chromatography (HR 5/5; Pharmacia). The MAb O301 enabled us to identify those fractions that were highly enriched with the 30-kDa protein (Fig. 1, lanes 3). For N-terminal amino acid sequencing, the 30-kDa polypeptide was separated by SDS-PAGE, blotted onto Problot membranes, and processed as described recently (15). The sequence that was obtained, ESIQRPTINQVF, was unique and unrelated to any polypeptide of *L. monocytogenes* previously described.

In order to obtain more protein sequence information from the new 30-kDa polypeptide by microsequencing, we used the agarose-based gel concentration system recently described by Rider and coworkers (21). This method enabled us to concentrate the partially purified 30-kDa protein. After protease digestion with endolysin C and high-pressure liquid chromatographic (HPLC) purification, microsequencing of the resulting peptides yielded six different peptide sequences (see Table 1). The peptides, especially Irp-1, -2, -5, and -6, exhibited significant homologies to the InlA and InlB polypeptides (Table 1). Furthermore, there was a striking similarity between the Irp-5 and Irp-6 peptides and the central regions of InlA and InlB (Table 2). Since our protein sequence data unambiguously show that this novel 30-kDa polypeptide is a member of the internalin multigene family recently suggested to exist in *Listeria* (8, 23), we have termed it internalin-related protein (Irp).

For further analysis Irp was purified from concentrated culture supernatants of the PrfA-overproducing strain EGDpERL3 50-1 grown overnight. Growth media were prepared and concentrated by ultrafiltration as described previously (15). Preparative gel filtration using a Sephadex G-50-course column (XK; 5 by 50 cm; Pharmacia), which removed medium components of lower molecular weight, turned out to be essential for further purification steps. For the gel filtration, 20 mM Tris-Cl buffer, pH 7.0, containing ammonium sulfate

TABLE 1. Peptides identified after digestion of Irp with endolysin C and sequence alignments with A and InlB[a]

Peptide[b]	Sequence[c]
InlA(36–67)	ATITQDTPINQIFTDAALAEKMKTVLGKTNVT
	.* . *****.* *..**. .* *** . *
Irp-1[d]	ESIQRPTPINQVFPDPGLANAVKQNLGKQSXT
	. .***.*.*** ..*...*.** *.* *
InlB(36–67)	ETITVSTPIKQIFPDDAFAETIKDNLKKKSVT
InlA(74–97)	DLDQVTTLQADRLGIKSIDGLEYL
	.* ** ...* * *. * ..
Irp-2	ELSGVXNFNGDNSNIXSLAGXXFF
	... .* *. * ..
InlB(74–97)	ELNSIDQIIANNSDIKSVQGIQYL
InlA(147–156)	LTLFNNQITD
	* * * .. *
Irp-3	LFLDNXELXD
	* *.. . *
InlB(147–156)	LSLEHNGISD
InlA(209–218)	LERLDISSNK
	....*....*
Irp-4	VNWIDLTGQK
	.. . *. .
InlB(210–219)	LQNLYLSKNH
InlA(425–435)	KANVSIPNTVK
	.... * * **
Irp-5	QPELYIXNXVK
	.. . * **
InlB(250–260)	QSNLVVPNTVK
InlA(473–495)	FSQPVTIGKGTTTFSGTVTQPLK
	. . .* . * ****.*
Irp-6	FSEYINVGETEAIFDGTVTQPIK
	* . . .* . * *.* ****.*
InlB(298–320)	FYQPVTIGKAKARFHGRVTQPLK

[a] Homologies were analyzed with Clustal V (11).
[b] Numbers in parentheses are amino acid residues.
[c] X indicates a residue not yet identified by sequence analysis. InlA and InlB sequences are from references 5 and 8. Asterisks indicate amino acid identity, and dots indicate isofunctional amino acids.
[d] N terminus.

TABLE 2. Alignment of Irp-5 and Irp-6 peptides with the central regions of InlA and InlB[a]

Peptide	Sequence[b]
InlA	$_{415}$**QA**W**T**N**A**P**V**N**V**K**A**N**V**S**IPNTVKN**V**T**G**A**L**I**A**P**A**T**ISD**G**G**S**Y**A-
InlB	$_{240}$**Q**E**C**L**N**K**P**I**N**H**Q**S**N**L**V**V**P**NTVKN**T**D**G**S**L**V**T**P**E**I**ISD**D**G**D**Y**E-
Irp-5	**Q**P**E**L**Y**I**X**N**X**V**K**
InlA	**E**P**D**I**T**W**N**L**P**S**Y**T**NEVS**Y**T**F**SQPVIIGK**G**T**T**I**F**S**G**T**V**T**Q**P**L**K**$_{495}$
InlB	**K**P**N**V**K**W**H**L**P**E**F**T**NEVS**F**I**F**Y**Q**PVIIGK**A**K**A**R**F**H**G**R**V**T**Q**P**L**K**$_{320}$
Irp-6	**FS**E**Y**I**N**V**G**E**T**E**A**I**F**D**G**T**V**T**Q**P**I**K**

[a] According to references 5 and 8.
[b] Subscript numbers are amino acid residues. Identical amino acids are in boldface.

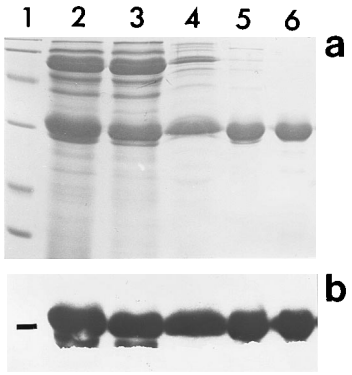

FIG. 2. Purification of Irp by ion-exchange and gel filtration chromatography. (a) The SDS gel (15%); (b) the corresponding immunoblot, processed with Irp MAb O301. Lanes 2, concentrated supernatant of *L. monocytogenes* EGD-pERL3 50-1. Lanes 3 to 6, pooled fractions after Sephadex G-50-course gel filtration (lanes 3), fractionated ammonium sulfate precipitation (lanes 4), Mono-Q ion-exchange chromatography (lanes 5), and Sephacryl S-100 gel filtration (lanes 6). Molecular mass markers in panel a (lane 1) from top to bottom are as follows: 97, 66, 45, 31, 21, and 14 kDa; that in panel b is 31 kDa.

(20% saturation), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol was used. Fractions enriched with Irp were identified by dot blot analysis using MAb O301 and further concentrated by fractionated ammonium sulfate precipitation (50 to 70% saturation). Desalted protein samples were fractionated by Mono-Q ion-exchange chromatography (Fig. 2, lanes 5). Irp was further purified to homogeneity by gel filtration in the presence of 1 M NaCl using a Sephacryl S-100 column (XK; 1.6 by 90 cm; Pharmacia). Although the purified Irp appeared to be a single 30-kDa polypeptide as judged by SDS-PAGE with Coomassie blue (Fig. 2, lanes 6) and silver staining (date not shown), we confirmed its purity on the basis of the following analytical method. Extremely sensitive mass spectrometric analysis using MALDI mass spectrometry not only revealed a molecular mass of 29.763 kDa for the native Irp but also demonstrated that the Irp fraction contained no additional polypeptides (Fig. 3). Measurements of Irp samples were performed on a Bruker Reflex MALDI/TOF mass spectrometer as described previously (18, 22). Furthermore, isoelectric focusing analysis (19) showed that the pI of Irp was approximately 5.6, and gel filtration analysis indicated that Irp exists as a monomer in bacterial supernatants (data not shown).

The purification procedure yielded approximately 2 mg of pure Irp from 3 liters of bacterial supernatant.

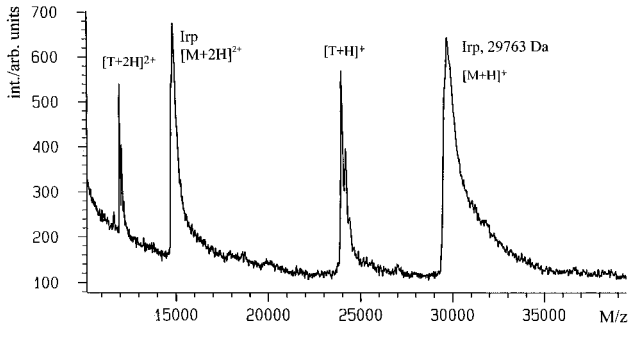

FIG. 3. Analysis of Irp by MALDI-TOF mass spectrometry with trypsinogen (T) as the internal calibration standard. $[M+H]^+$ and $[M+2H]^{2+}$ represent the singly and doubly charged molecular ions. The molecular mass of Irp was determined to be 29.763 kDa.

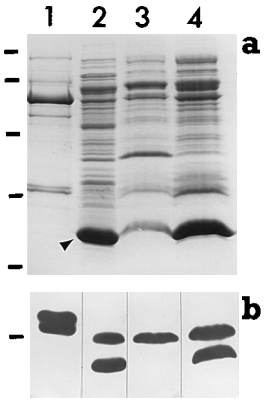

FIG. 4. Cross-reactivity of Irp-specific MAbs with internalin-related polypeptides in culture supernatants derived from different *L. ivanovii* strains analyzed by SDS-PAGE and immunoblotting. Lanes 1, *L. monocytogenes* EGD; lanes 2, *L. ivanovii* SLCC 2379 (ATCC 19119); lanes 3, SLCC 4770; lanes 4, SLCC 5378. (a) The Coomassie blue-stained SDS gel; (b) the low-molecular-weight region of the corresponding immunoblot processed with Irp-specific MAb O301. The arrowhead in panel a indicates the iIrp1 polypeptide. Molecular weight markers in panel a are as follows from top to bottom: 97, 66, 45, 31, and 21 kDa; that in panel b is 31 kDa.

TABLE 3. Peptides identified after digestion of iIrp1 with trypsin and sequence alignments with InlA and InlB[a]

Peptide[b]	Sequence[c]
InlA(36–58)	ATITQDTPINQIFTDAALAEKMK
	* * . ***** *. **
iIrp1-1[d]	ANILHPMPINQIPFDPDLAKVKV
	* . ***** * .*
InlB(36–58)	ETITVSTPINQIFPDDAFAETIK
InlA(417–432)	WTNAPVNYKANVSIPN
	..** * ... **
iIrp1-2	IVETPVTYEPDLVIPV
	.. *. ...**.*
InlB(242–257)	CLNKPINHQSNLVVPN
InlA(77–86)	QVTTLQADRL
	. *
iIrp1-3	IYDDIIAXNQ
	*.*** *
InlB(77–86)	SIDQIIANNS
InlA(558–563)	NLYAQF
	** *
iIrp1-4	NLPAYK
	* *
InlB(383–388)	TLYAVF
InlA(156–176)	DIDPLKNLTNLNRLELSSNTI
	.. *... .*. * *.** *
iIrp1-5	SLEGLQHFNKLEVLFLASNQI
	..** *. .** *.*..*.*
InlB(156–176)	DINGLVHLPQLESLYLGNNKI
InlA(70–82)	VSQTDLDQVTTLQ
	... . .
iIrp1-6	ITDNGAYIYDDII
	.*.* *.**
InlB(70–82)	VTQNELNSIDQII

[a] Homologies were analyzed with Clustal V (11).
[b] Numbers in parentheses are amino acid residues.
[c] X indicates a residue not yet identified by sequence analysis. InlA and InlB sequences are from references 5 and 8. Asterisks indicate amino acid identity, and dots indicate isofunctional amino acids.
[d] N terminus.

TABLE 4. Comparison of the N-terminal sequences of InlA, InlB, Irp, and iIrp1

Protein	Species	Strain(s)	N-terminal sequence[a]
InlA	*L. monocytogenes*	EGD, EGD-SmR	AT**I**TQD**TPINQIF**T**D**AA**L**A**L**AEKM**K**[b]
InlB	*L. monocytogenes*	EGD, EGD-SmR	ET**I**TVS**TPI**K**QIFPD**DAFA**E**T**I**K**[b]
Irp	*L. monocytogenes*	EGD	ES**I**QRP**TPINQ**V**FPD**PG**L**ANAV**K**
iIrp1	*L. ivanovii*	SLCC 2379	AN**I**LHPM**PINQIFPD**PD**L**AKVKV

[a] Identical amino acids are in boldface.
[b] According to references 5, 8, and 15.

When culture supernatants of *Listeria ivanovii* strains SLCC 2379 and SLCC 5378 were analyzed by immunoblotting, two cross-reacting polypeptides of 24 and 31 kDa were identified with the Irp MAbs (Fig. 4, lanes 2 and 4). In *L. ivanovii* SLCC 4779 only the 31-kDa protein reacted with the antibodies (Fig. 4, lanes 3). The 24-kDa polypeptide of *L. ivanovii* is the prominent protein band in Coomassie blue-stained gels following ammonium sulfate concentration (70% saturation) of the supernatants of strains SLCC 2379 and SLCC 5378. Since cross-reactivity with the 24-kDa polypeptide was also observed with specific InlA and InlB MAbs (14a), we decided to characterize the 24-kDa protein in more detail. In order to generate N-terminal end sequences and additional internal sequences of the *L. ivanovii* 24-kDa polypeptide, we used supernatants of strain SLCC 2379 and concentrated the protein in the agarose-based gel concentration system (21). After digestion with trypsin, peptides were purified by HPLC. Microsequencing identified six peptides (Table 3). When the peptide sequences were compared with the InlA and InlB amino acid sequences, most were partially homologous to InlA as well as to InlB (Table 3). The N terminus of the *L. ivanovii* 24-kDa polypeptide was highly homologous to the N termini of *L. monocytogenes* InlA, InlB, and Irp (Table 4). Interestingly, a 24-kDa polypeptide with an identical N-terminal sequence was recently copurified with listeriolysin of *L. ivanovii* SLCC 2379 (12). In addition, one peptide (iIrp1-5) harbors a leucine-rich sequence which is closely related to similar repeats in InlA and InlB (Table 5).

Cross-reactions with MAbs specific for InlA, InlB, and Irp; the amino acid sequence homology of the 24-kDa polypeptide to InlA, InlB, and Irp; and the presence of a leucine-rich repeat consensus sequence typical of InlA and InlB all strongly indicate that this polypeptide of *L. ivanovii* is a further member of the *Listeria* internalin family. We therefore suggested that it be termed iIrp1.

The presence of further *inlA*-related genes in *L. monocytogenes* and *L. ivanovii* had been postulated on the basis of DNA hybridization studies using an *inlA* gene probe (8). Peptide sequences obtained from Irp suggest that it may be a further bona fide member of the internalin family. Indeed, homologies between the InlA, InlB, and Irp polypeptides are not restricted only to the respective leucine-rich repeat regions of InlA and InlB (15) but also include sequences flanking this region in either protein (Table 1). Although Irp is a secreted polypeptide and is not associated with the bacterial surface, it is tempting to speculate that it is also involved in mediating internalization of bacteria. Cloning of the *irp* and *iirp1* genes is currently in progress and will enable detailed analysis of the roles of these genes in the invasion process. Furthermore, the availability of purified Irp and specific Irp antibodies will facilitate the development of biochemical assays and provide useful tools for the localization of Irp during listerial infection.

TABLE 5. Alignment of iIrp1-5 and Irp-3 peptides with the leucine-rich repeat consensus sequence of InlA and InlB[a]

Peptide	Sequence[b]
iIrp1-5	S **L** E G **L** Q H F N K **L** E V **L** F **L** A S N Q **I**
InlA	- D **I** - P **L** - - **L** T N **L** - - **L** - **L** - - N Q **I** -
	I
InlB	- D **L** - - **L** - - **L** - - **L** - - **L** - **L** - - N - **I** -
	I
Irp-3	**L** F **L** D N X E **L**

[a] According to reference 8.
[b] Leucine and isoleucine residues are in boldface, hyphens represent miscellaneous residues in the consensus sequence, and X indicates a residue not yet identified by sequence analysis.

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