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The virulence regulator protein of *Listeria ivanovii* is highly homologous to PrfA from *Listeria monocytogenes* and both belong to the Crp–Fnr family of transcription regulators

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Summary

The two pathogenic *Listeria* species, *L. ivanovii* and *L. monocytogenes*, can be differentiated biochemically and show different host ranges. Virulence of *L. monocytogenes* is dependent on the integrity of *prfA* which positively and co-ordinately regulates transcription of several virulence genes. Until now, a *prfA* homologue had not been identified in *L. ivanovii*. We have now cloned a chromosomal region from *L. ivanovii* comprising two genes with high homology to the *plcA* and *prfA* genes from *L. monocytogenes*. Distal from *prfA*, an open reading frame highly homologous to a phosphoribosyl pyrophosphate synthetase gene (*prs*) was newly identified, defining the border of the virulence gene cluster. Transcription of the gene for ivanolysin O and expression of other genes of the virulence gene cluster in *L. ivanovii* were dependent on PrfA. The pattern of PrfA-dependent proteins (PdPs) expressed in *L. ivanovii* was similar, but not identical to that of *L. monocytogenes*. The PrfA proteins, as predicted from nucleotide sequences of both pathogenic *Listeria* species, are very similar and show significant homology to the Crp–Fnr family of global transcription regulators.

Introduction

The two pathogenic *Listeria* species (*L. ivanovii* and *L. monocytogenes*) are facultative intracellular bacteria, a group that also includes the causative agents of tuberculosis, leprosy, shigellosis, typhoid fever and Legionnaires' disease. *L. ivanovii* (Ivanov, 1962; Seeliger *et al.*, 1984) very rarely infects humans, rather this species is primarily an animal pathogen of sheep and cattle that causes abortion, neonatal sepsis, etc. (Ivanov, 1962; Cooper and Dennis, 1978). It is different from *L. monocytogenes* using the criteria of biochemical and serological tests (Ivanov, 1975; Rocourt *et al.*, 1983; Seeliger *et al.*, 1984). The most virulent listerial species, *L. monocytogenes*, can cause severe illness (listeriosis) in animals and man (Gellin and Broome, 1989), predominantly meningitis and septicaemia in immuncompromised or pregnant individuals.

A cholesterol-binding (SH-activated) cytolysin (Alouf and Geoffroy, 1991), termed listeriolysin O, was the first virulence factor of *L. monocytogenes* for which an essential role in pathogenesis could be unequivocally demonstrated and the gene (*hly*) sequenced (Mengaud *et al.*, 1988; Cossart *et al.*, 1989). Other genes which are involved in various steps of the complex intracellular life cycle of *L. monocytogenes* include genes for lecithinase (*plcB*), metalloprotease (*mpl*), actin polymerization (*actA*) and phosphatidylinositol-specific phospholipase C (PI-PLC, *plcA*) (reviewed in Portnoy *et al.*, 1992). These four genes, together with *hly*, constitute a chromosomal virulence gene cluster co-ordinately and positively regulated at the transcriptional level by *prfA*, the most proximal gene of the cluster located next to *plcA*. The precise mechanism of transcriptional activation by PrfA is not known, and it has been claimed that it has no extensive homology to any known protein (Leimeister-Wächter *et al.*, 1990; Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992). A 14 bp palindromic DNA sequence, which is present upstream of *hly* (Mengaud *et al.*, 1989) and in modified form in front of the other transcription units of the virulence cluster, is required for efficient listeriolysin synthesis (Freitag *et al.*, 1992). This so-called 'PrfA box' is commonly believed to be the target site for PrfA-dependent regulation. Recently we have shown that under specific culture conditions PrfA-dependent proteins (PdPs) are the main surface proteins synthesized *de novo* in *L. monocytogenes* (Sokolovic *et al.*, 1993).

It has been reported earlier that a gene probe specific for *prfA* of *L. monocytogenes* did not hybridize to *L. ivanovii*

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chromosomal DNA; from this it was concluded that virulence gene regulation in the latter species is different from *L. monocytogenes* (Wernars *et al.*, 1992). However, lecithinase and PI-PLC, in addition to Ivanolysin O, can also readily be detected in *L. ivanovii*, suggesting that at least some of the virulence genes in this listerial species might be similar to those of *L. monocytogenes*. Here we show that indeed *L. ivanovii* contains genes that are highly homologous to both *prfA* and *plcA* from *L. monocytogenes*.

Results

*Characterization of the Tn*1545 *mutant 8/6*

In many instances, insertional mutagenesis of Gram-positive bacteria by conjugal transfer of enterococcal transposons, e.g. Tn*916* or Tn*1545*, has proven to be a powerful genetic tool (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987). In our laboratory, several Tn*1545*-derived mutants of *L. ivanovii* have previously been constructed and described (Schlesinger, 1988; Kreft *et al.*, 1990). One of these, mutant 8/6, exhibits a pleiotropic phenotype with a concomitant reduction in haemolytic activity (Fig. 1A) and a loss of expression of lecithinase (Fig. 1B) as well as of PI-PLC (Fig. 1C). In *L. monocytogenes*, the genes for lecithinase and PI-PLC (Leimeister-Wächter *et al.*, 1991) are under the control of *prfA*. Culture supernatants from the mutant *L. ivanovii* strain had about 3% (4 HU) of the wild type (128 HU) haemolytic activity when measured in a liquid assay. On blood agar plates, haemolysis by this mutant was only moderately reduced (Fig. 1A). However, no measurable amounts of Ivanolysin O were detectable (see Fig. 6 below) and the residual haemolytic activity was attributed to the sphingomyelinase secreted by *L. ivanovii* (Kreft *et al.*, 1989; Vazquez-Boland *et al.*, 1989). Later, it was shown that mutant 8/6 was unable to multiply in Caco-2 colon carcinoma cells *in vitro* (Karunasagar *et al.*, 1993), and that it is completely avirulent in mice and chicken embryos (J. Kreft *et al.*, unpublished). Therefore, it had properties very similar to the known *prfA*-negative mutants of *L. monocytogenes* (Mengaud *et al.*, 1991; Chakraborty *et al.*, 1992), suggesting that this mutant had Tn*1545* inserted in a gene at least functionally homologous to *prfA* from *L. monocytogenes*.

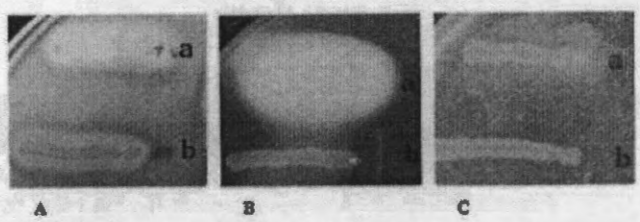

Fig. 1. Phenotype of *L. ivanovii* wild-type (a) and its *prfA*-mutant 8/6 (b).
A. Haemolysis on blood agar after 24 h at 37°C.
B. Lecithinase production, visualized by precipitate on egg yolk agar.
C. Production of PI-PLC, visualized by halo formation on BHI agar with an overlay containing 0.2% phosphatidylinositol. In (B) and (C) incubation was for 48 h at 37°C.

Isolation, cloning, and sequencing of the prfA *and* plcA *genes*

Based on the assumption that the chromsomal organization of *L. ivanovii* was similar to *L. monocytogenes*, we performed a polymerase chain reaction (PCR) amplification of mutant 8/6 chromosomal DNA with oligonucleotide primers derived from the known ends of the transposon Tn*1545* (Caillaud and Courvalin, 1987) and from a previously determined *ilo*-upstream sequence (J. Kreft and M. Weber, unpublished). The reaction with primers M395 (Tn*1545* right end) and M397 (*ilo*-upstream) resulted in the amplification of a 1.3 kb fragment. Sequence determination revealed that this fragment contained two continuous, yet incomplete, open reading frames with significant homology to the *C*-terminal part of *plcA* and the *N*-terminal part of *prfA* from *L. monocytogenes*. The complete *plcA* gene was PCR amplified using primers derived from the intergenic sequence upstream of *ilo* (J. Kreft, unpublished) and the *N*-terminus of *prfA*. Inverse PCR on *Dde*I-digested and self-ligated DNA from *L. ivanovii* wild type yielded a 0.8 kb fragment containing the *C*-terminus of *prfA*. Using the sequence information obtained so far, the complete *prfA* wild-type gene was PCR amplified. The entire double-stranded sequence of *prfA* as well as of *plcA* was verified using different sequencing primers and has been deposited in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X72685. Figure 2A is a schematic representation of the chromosomal region from *L. ivanovii* described here. A computer homology search (TFASTA) in the EMBL database initially detected only a rather low homology of the PrfA protein, as deduced from the nucleotide sequence, to NtcA from *Synechococcus*. The latter protein is a member of the Crp–Fnr family of global transcription regulators (Vega-Palas *et al.*, 1992). A manual alignment of the PrfA proteins from both *L. ivanovii* and *L. monocytogenes* with NtcA and with Crp–Fnr from *Escherichia coli*, optimized with respect to known functional residues and domains, then revealed an extended and significant homology among these proteins (Fig. 3). Compared to NtcA, the two PrfA proteins contained identical or similar amino acids at 79 positions; the homology to Crp was 75 amino acids (*L. ivanovii*) and 73 amino acids (*L. monocytogenes*), respectively.

Figure 3 includes a comparison of the deduced amino acid sequences for the PrfA proteins from both *L. ivanovii* and *L. monocytogenes* strain LO28. The latter strain was chosen because its PrfA is identical in length to the

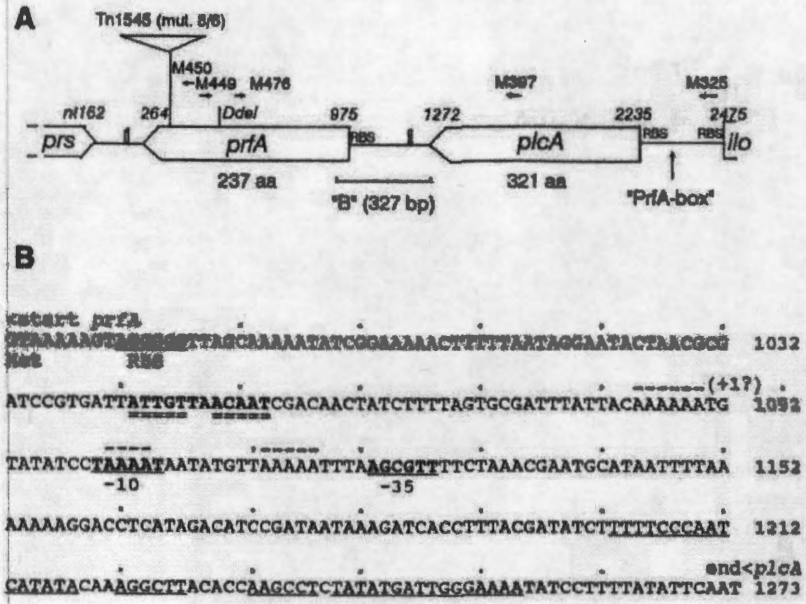

Fig. 2. A. Schematic representation of the chromosomal region from *L. ivanovii* analysed here. The complete nucleotide sequence appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession no. X72685. The orientation is according to the common scheme for the *Listeria* virulence gene cluster. RBS: putative ribosome-binding site. The vertical bars in the intergenic regions indicate the position of putative transcription terminators. The horizontal bar ('B') indicates the position of the DNA fragment analysed in Fig. 5. The position and orientation of the PCR primers used here is indicated (arrows with M-numbers) as well as the insertion site of Tn*1545* in the mutant 8/6.
B. Nucleotide sequence of the intergenic region between *prfA* and *plcA* from *L. ivanovii*. Putative −10/−35 regions, the putative ribosome-binding site (RBS) and the stem-loop (putative transcription terminator) distal from *plcA* are underlined. The presumptive transcription start site (+1?) has been located by analogy with *L. monocytogenes* (Mengaud *et al.*, 1991). Regularly spaced $(dA)_{4-5}$ tracts are indicated by a broken line above the sequence, and the palindrome downstream from the *prfA* promoter is indicated by a broken double line below the sequence.
C. Comparison of 'PrfA boxes'. The sequences for *L. monocytogenes hly/plcA* and *mpl* are from Mengaud *et al.* (1989) and Domann *et al.* (1991); the sequence for *L. ivanovii mpl* is from our own unpublished results. Non-symmetrical nucleotides are given in lower case.

L. ivanovii protein whereas PrfA from strain EGD is less similar at its *C*-terminus; in particular it is two amino acids shorter. Other relevant features of *plcA* and *prfA* and of the deduced amino acid sequences are summarized in Table 1. The homology between the PI-PLCs from the two *Listeria* species was evenly distributed over the entire length of the protein.

It has been shown that integration of Tn*1545* occurs via homologous recombination between the transposon ends and the target sequence (Trieu-Cuot *et al.*, 1993). Our sequence analysis supported this notion. In the mutant 8/6, insertion of Tn*1545* occurred in-frame at nucleotide 623 of *prfA* (--TACAAA--), thus creating an altered *C*-terminus (206-FYVQTKYKFLIIFLYFLKCS* 225) in

Table 1. Comparison of the *prfA/plcA* chromosomal region from *L. ivanovii* and *L. monocytogenes* and of the respective gene products (as deduced from the nucleotide sequences).

(a) Comparison at the nucleotide sequence level

	prfA	*prfA–plcA*	*plcA*	*plcA–hly/lllo*
L. monocytogenes (bp)	711[a]	272	951[b]	242
L. ivanovii (bp)	711	297	963	240
Per cent identity	79.1	78.2	72.8	77.9

(b) Comparison at the amino acid sequence level

	PrfA	PI-PLC
L. monocytogenes, No. aa (kDa)	237 (27.31)[a]	317 (36.29)[b]
L. ivanovii, No. aa (kDa)	237 (26.88)	321 (36.65)
Per cent identity	77.2	68.4
Per cent similarity[c]	89.9	80.1

aa: amino acids.
a. Strain LO28 (Mengaud *et al.*, 1991).
b. Strain EGD (Leimeister-Wächter *et al.*, 1991).
c. Calculated by the program BESTFIT of the UWGCG program package (Devereux *et al.*, 1984).

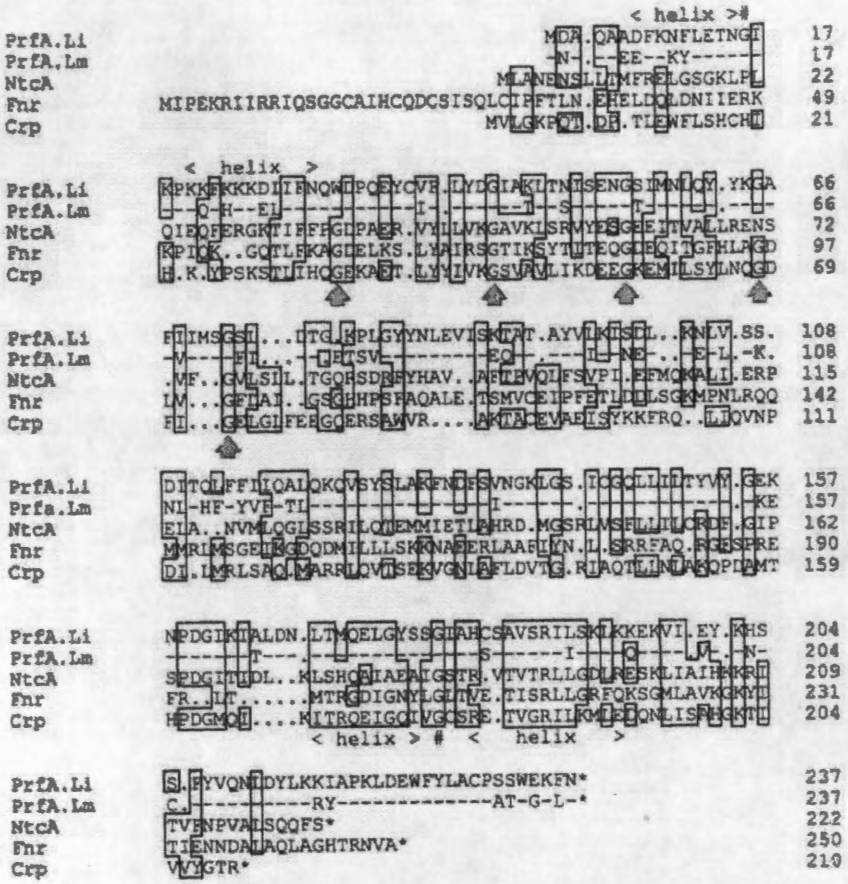

Fig. 3. Multiple amino acid sequence alignment of PrfA from *L. ivanovii* (PrfA.Li; this work) and *L. monocytogenes* LO28 (PrfA.Lm; Mengaud *et al.*, 1991), NtcA from *Synechococcus* (Vega-Palas *et al.*, 1992), Fnr and Crp from *E. coli* (Spiro and Guest, 1990; Cossart and Gicquel-Sanzey, 1982). The putative helix-turn-helix at the *N*-terminus of PrfA and the DNA-binding helix-turn-helix of Crp are indicated above or below the sequence, respectively. The glycine in the turn region is marked by #, and highly conserved glycines in the *N*-terminal part are indicated by arrows. Amino acids which are similar in PrfA and in at least two of the other proteins are boxed. Groups of similar amino acids were as follows: i) A, S, T, P, G; ii) N, D, E, Q; iii) H, R, K; iv) M, L, I, V; v) F, Y, W.

which amino acids 210–225 were changed and which was 12 amino acids shorter than the wild-type protein (compare to Fig. 3).

In the intergenic region between *ilo* and *plcA*, a 20 bp palindromic sequence (Fig. 2C) could be detected which was centred between the putative −10 regions of the two genes. It contained the 14 bp palindromic sequence ('PrfA box', Mengaud *et al.*, 1989) identified in front of *prfA*-regulated genes in *L. monocytogenes*. A similar palindrome, 12 bp long, was found in front of *prfA*, in this case, however, between the putative promoter and the ribosome-binding site in the transcribed part of the *prfA* gene (Fig. 2B).

The *C*-terminus of a third open reading frame (53 amino acids, see EMBL accession number X72685) was detected distal of *prfA*; sequencing of a DNA region further upstream identified another part (75 amino acids, data not shown) of the same open reading frame. A computer homology search in the EMBL database revealed a significant 66% identity/85% similarity of these polypeptides to the respective regions at the *C*-terminus of the enzyme phosphoribosyl pyrophosphate synthetase (PRS, ATP:D-ribose-5-phosphate pyrophosphotransferase, E.C. 2.7.6.1) from *Bacillus subtilis* (Nilsson *et al.*, 1989).

Transcription analysis of the ivanolysin gene (ilo)

In order to test if in *L. ivanovii* the efficient transcription of the ivanolysin O gene (*ilo*) is dependent on the integrity of *prfA* as has repeatedly been demonstrated for *hly* from *L. monocytogenes* (Leimeister-Wächter *et al.*, 1990; Mengaud *et al.*, 1991), primer-extension experiments were performed on total RNA isolated from *L. ivanovii* wild type and its mutant 8/6, respectively. For comparison, total RNA from *L. monocytogenes* NCTC7973 was investigated in parallel. Figure 4 shows that in the *L. ivanovii* wild type (lane b) transcription of *ilo* started at position +131 from the initiation codon and a second weak start site was located at position +121. In the *prfA* mutant 8/6, efficient transcription of *ilo* was abrogated (lane c). However, weak secondary transcription start sites further downstream (positions +87, +83) could be detected).

Electrophoretic mobility of the DNA fragment between plcA *and* prfA

The intergenic region between *plcA* and *prfA* (Fig. 2C) contains several oligo-dA and oligo-dT tracts. In particular, three $(dA)_{4-6}$ tracts (indicated by a broken line above the sequence) spaced 9–10 bp apart are found in the

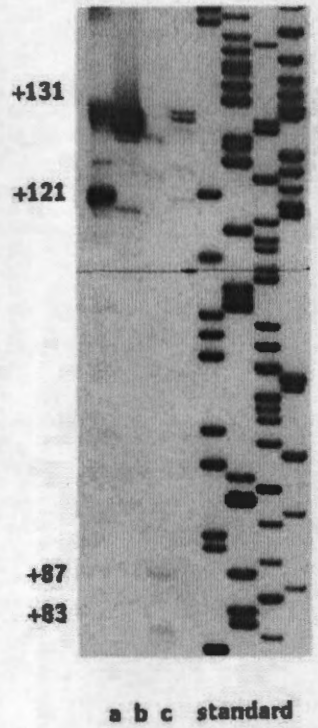

Fig. 4. Primer-extension analysis of listeriolysin O transcription. Lane a: *L. monocytogenes* NCTC 7973 (*hly*-specific primer); lane b: *L. ivanovii* wild type and lane c: mutant 8/6 (both with an *ilo*-specific primer). Transcript lengths were determined from standard sequencing reactions (right panel).

putative promoter region of *prfA*. Such a pattern is typical for DNA with an intrinsic curvature (Wu and Crothers, 1984). A recombinant plasmid containing the intergenic region described above was digested with *AvaII*/*KpnI* and electrophoresed on 6% polyacrylamide gels at 4°C and in parallel at 60°C (Mizuno, 1987). Figure 5 shows that the 327 bp fragment ('B' in Fig. 2A), which contained the three $(dA)_{4-5}$ tracts in its central part, had an unusual slow mobility at 4°C but migrated close to normal at 60°C. This behaviour was clearly indicative of a bent DNA structure.

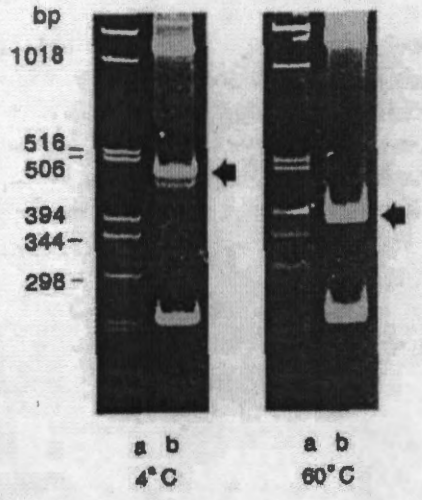

Fig. 5. Electrophoretic mobility of the intergenic DNA fragment between *prfA* and *plcA* from *L. ivanovii*. From a recombinant plasmid, a 327 bp DNA fragment ('B' in Fig. 2A), comprising the entire intergenic sequence, was cut out by *AvaII*/*KpnI* and electrophoresed on 6% polyacrylamide gels at 4°C or 60°C, respectively (lanes b). Lanes a: length standard (1 kb ladder, Bethesda Research Laboratories); fragment sizes are indicated on the left. Staining was with ethidium bromide.

PrfA-dependent proteins (PdPs) of L. ivanovii

Efficient expression from the virulence gene cluster in *L. monocytogenes*, and presumably also in *L. ivanovii*, is strictly dependent on an intact *prfA* gene. Recently, we have demonstrated that under specific nutritional conditions, i.e. during cultivation in a minimal cell culture medium (MEM) and a 5% CO_2 atmosphere, primarily PdPs were synthesized *de novo* in *L. monocytogenes*. Many of these [^{35}S]-methionine-labelled PdPs were localized on the outer cell surface and thus could be released by a mild treatment with detergent. Protein analyses on SDS–PAGE could detect all the known gene products from the virulence cluster among the PdPs plus several additional proteins (Sokolovic *et al.*, 1993). Figure 6 shows the results of a similar experiment with wild-type *L. ivanovii*, its *prfA* mutant 8/6 and a previously characterized isogenic mutant deficient for lecithinase. *L. monocytogenes* NCTC 7973 and its *prfA*-defective mutant SLCC53 were included for comparison (lanes a, b). In the mutant 8/6 of *L. ivanovii* (lane d), at least 14 proteins could no longer be detected which were labelled in the wild-type strain (lane c), identifying them as PdPs. By comparison with the isogenic transposon mutant 34/26 (lane e), lecithinase was identified as one of the PdPs. As was shown above, transcription of the ivanolysin O gene (*ilo*) was also *prfA*-dependent. Immunoblotting with a specific polyclonal rabbit antiserum confirmed the transcriptional results when ivanolysin O could not be detected in the *prfA* mutant 8/6 (Fig. 6, lane g). No appropriate mutants were available for PI-PLC (*plcA*) or Mpl. However, the 34 kDa and 57 kDa proteins that were missing in mutant 8/6 were tentatively identified as PI-PLC (34 kDa) and Mpl (57 kDa), based on the known molecular weight of PI-PLC from *L. ivanovii* (Table 1) and of Mpl from *L. monocytogenes*, respectively (Domann *et al.*, 1991). Interestingly, no PdP of 92 kDa, which in *L. monocytogenes* has been identified as the *actA* gene product (Kocks *et al.*, 1992; Domann *et al.*, 1992), was detectable in *L. ivanovii*. One protein of about 70 kDa and of unknown function was detected in mutant 8/6 at a greater level than in the wild-type *L. ivanovii*. Figure 6 also shows that the pattern of PdPs is similar for both *Listeria* species but also clearly exhibits a number of differences with regard to PdPs of yet unknown function,

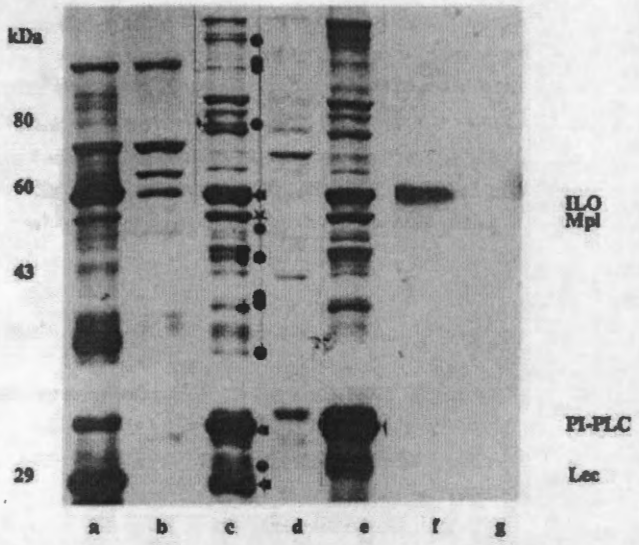

Fig. 6. PrfA-dependent proteins (PdPs). Surface proteins specifically labelled with [^{35}S]-methionine in MEM and solubilized with 1% SDS (see the *Experimental procedures*) were separated by SDS–PAGE (13% polyacrylamide/1% SDS) and autoradiographed (lanes a–e). Lane a: *L. monocytogenes* wild type (NCTC 7973); lane b: *L. monocytogenes* PrfA-deficient mutant (SLCC53); lane c: *L. ivanovii* wild type; lane d; mutant 8/6 (PrfA-deficient); lane e: mutant 34/26 (deficient for lecithinase). Ivanolysin O (ILO) was detected in non-labelled samples by immunoblotting with anti-ivanolysin antiserum. Lane f: *L. ivanovii* wild type, lane g: mutant 8/6. Positions of molecular weight markers are indicated on the left, and identified gene products (arrows) on the right (PI-PLC: *plcA* gene product; Lec: lecithinase). The putative Mpl protein is marked by an asterisk, and PdPs of unknown function by black dots.

particularly in the molecular mass range between 40–50 kDa and above 80 kDa.

Discussion

The chromosomal organization of the virulence gene cluster in *L. monocytogenes* is known in detail (e.g. Portnoy *et al.*, 1992). In the case of *L. ivanovii*, the gene (*ilo*) for the species-specific listeriolysin O, ivanolysin O (ILO), has previously been cloned and sequenced (Haas *et al.*, 1992). However, nothing was known about the genetic organization and the regulation of virulence genes in *L. ivanovii*. In this study, we have characterized a 2477 bp chromosomal region from *L. ivanovii* upstream of the *ilo* gene.

The *C*-terminal part of an open reading frame which showed 66% identity/85% similarity to the *C*-terminus of the enzyme PRS from *B. subtilis* (Nilsson *et al.*, 1989) was identified at the most distal end. An almost identical open reading frame can be found distal from *prfA* in the sequences published for *L. monocytogenes* (Leimeister-Wächter *et al.*, 1990; Mengaud *et al.*, 1991) but was not identified as part of a presumptive *prs* gene. PRS catalyses the biosynthesis of 5-phospho-α-ribosyl-1-pyrophosphate (PRPP), a key intermediate in the biosynthesis of pyrimidines, purines, histidine, tryptophan and NAD^+. From this, it is clear that PRS is indispensable for normal bacterial growth. Although the sequence is incomplete and the protein functionally untested, the significant homology of the open reading frame from pathogenic *Listeriae* to PRS must be taken as strong evidence that this region defines the previously unknown 'left' border of the virulence gene cluster. A gene encoding lactate dehydrogenase has already been identified as comprising the 'right' border (Vazquez-Boland *et al.*, 1992). In the region between *prs* and *ilo*, we identified two genes with significant homology to the *L. monocytogenes prfA* and *plcA* genes. Differences in the DNA sequence of the *prfA* genes from the two *Listeria* species were mainly found in GC base pairs; this could explain why a *prfA* gene probe from *L. monocytogenes* did not hybridize under stringent conditions to *L. ivanovii* chromosomal DNA (Wernars *et al.*, 1992). The PrfA protein from *L. ivanovii*, as deduced from the coding sequence, is very similar to PrfA from *L. monocytogenes* (Fig. 3 and Table 1), only the region between amino acids 100–120 being rather heterologous (35% identity/85% similarity).

Our primer-extension experiments clearly have demonstrated that in *L. ivanovii* an efficient transcription of the listeriolysin gene (*ilo*) is dependent on an intact *prfA* gene. For wild-type *L. ivanovii*, we could show that *ilo* transcription starts from two closely spaced sites, primarily at position +131 from the *ilo* initiation codon. Upstream from there, no extensive homology to consensus −10/−35 promoter boxes could be found; however, a 20 bp palindromic sequence ('PrfA box') centred around position −42 was detectable. In the *prfA*-negative mutant 8/6, a weak transcription of *ilo*, starting at position +87/+83, was seen. These results are similar to those recently described for *hly* from *L. monocytogenes* (Domann *et al.*, 1993). No measurable amounts of ivanolysin O were detectable in the culture supernatant of this mutant, and, in contrast to *prfA* mutants of *L. monocytogenes*, no evidence for the escape of the *L. ivanovii* mutant from the phagosome of infected Caco-2 cells has been found (Karunasagar *et al.*, 1993).

In *L. monocytogenes* PdPs can specifically be labelled and isolated (Sokolovic *et al.*, 1993). The pattern of PdPs from *L. ivanovii*, when assayed by the same method, was similar to that from *L. monocytogenes*. Ivanolysin O, PI-PLC and lecithinase clearly could be identified as PdPs. The presence of a functional Mpl could not be directly proven because of the lack of mutants and specific antisera. However, our preliminary and unpublished data revealed the presence in *L. ivanovii* of a sequence with high homology to *mpl* from *L. monocytogenes*. In addition, a protein of a size appropriate for Mpl (57 kDa, Domann *et al.*, 1991) was missing in the *prfA* mutant 8/6. No PdP of a size comparable to ActA could be found in

L. ivanovii. Recently it has been shown that in infected eukaryotic cells, *L. ivanovii* also induces actin-tail formation, although it is less efficient than *L. monocytogenes* (Karunasagar *et al.*, 1993). Taken together, we conclude that all the constituents of the virulence gene cluster from *L. monocytogenes*, with the possible exception of *actA*, are also present in *L. ivanovii* and are co-ordinately regulated by *prfA*. The chromosomal organization of the *prfA*, *plcA* and *ilo* genes was found to be identical to that of *L. monocytogenes*. It remains to be elucidated whether this is also true for *plcB* and *mpl* genes. Interestingly, a number of yet unidentified PdPs from *L. ivanovii* showed differences compared to *L. monocytogenes*. We cannot rule out the possibility that a few of the low molecular mass proteins constitute degradation products of larger proteins. Among the differing proteins was one of M_r 70 that was obviously enhanced in the absence of a functional *prfA* gene, similarly to the effect described for a 64 kDa protein of *L. monocytogenes* (Sokolovic *et al.*, 1993). It is not known if the expression of these proteins is directly repressed by PrfA or is under the control of another PrfA-activated gene.

There is good evidence that PrfA specifically interacts with the palindrome ('PrfA box') found upstream from PrfA-dependent genes (Freitag *et al.*, 1992; 1993). Figure 2C shows a comparison of the two 'PrfA boxes' for which a sequence is available for both *L. ivanovii* and *L. monocytogenes*. In this figure we have expanded the *hly/plcA* box (*L. monocytogenes*) up to the GC pair, indicating one mismatch. The sequence for *mpl* of *L. ivanovii* is from our own unpublished work. *ilo/plcA* and *hly/plcA*, respectively, are transcribed in opposite directions but use the same 'PrfA box', which is 4 bp longer than the respective *mpl* box. The palindromes from *L. ivanovii* are 2 bp longer than in *L. monocytogenes*. In both listerial species the *mpl* palindrome is imperfect. This may influence the binding of PrfA and therefore might be related to the observation by others (Freitag *et al.*, 1993) that apparently *mpl* needs higher amounts of PrfA for its activation than *hly/plcA*.

The analysis of the intergenic regions between *ilo* or *hly* and *plcA* on the one hand and between *plcA* and *prfA* on the other hand revealed several interesting features of these DNA sequences. First, both intergenic regions are remarkably conserved among the two *Listeria* species (see Table 1). Figure 2B shows that upstream from *prfA* rather well conserved −35/−10 boxes can be found, and it has been demonstrated (Mengaud *et al.*, 1991; Freitag *et al.*, 1993) that in *L. monocytogenes* a monocistronic transcript of *prfA* can be initiated from this promoter, in addition to the bicistronic *plcA/prfA* transcript. In this case, no 'PrfA box' was found overlapping with the −35 region. However, we have now detected a shorter, but otherwise almost identical, palindrome (TAACAATTGTTA in *L. ivanovii*, TAACAATTGTTg in *L. monocytogenes*) downstream from the transcription start site in the non-translated region of *prfA*. Furthermore, in the case of *L. ivanovii* we could demonstrate experimentally that this DNA region is intrinsically bent, presumably through the presence of regularly spaced $(dA)_{4-5}$ tracts. This intergenic region is 25 bp shorter in *L. monocytogenes* but contains similar $(dA)_n$ tracts at comparable positions. No such sequence peculiarities can be found upstream of other PrfA-regulated genes. It has been shown by others (Freitag *et al.*, 1993) that in *L. monocytogenes* the monocistronic transcript from this promoter is greatly enhanced in the absence of a functional PrfA protein. These results as well as our own suggest that in this case PrfA negatively controls transcription of its own gene, most probably by binding to the palindrome mentioned above. In the absence of PrfA, transcription may be stimulated by the intrinsic curvature of this DNA region.

The symmetry of the binding site on the DNA suggests that PrfA, like other DNA-binding proteins, may bind as a dimer. Our computer secondary structure predictions, using the algorithms of Chou and Fasman (1978) and Rose (1978), for the PrfA proteins from the two *Listeria* species identified several regions as potential candidates for DNA-binding or dimerization domains. At the *N*-terminus, centred around G-16, a helix-turn-helix (HTH) motif was predicted which fulfilled almost all of the stereochemical constraints established for true DNA-binding HTHs (Dodd and Egan, 1990). At the very *C*-terminus a rather long α-helical domain was predicted. Within this region, leucine and some similar amino acids were found in heptad intervals (L-193, V/E-200, Y-207, L-214, L-221). Such a heptad array is characteristic of the so-called leucine zipper (Landschulz *et al.*, 1988), a dimerization domain found in both eukaryotes and prokaryotes. Whether these predicted structures constitute functionally important domains is not known at present.

More importantly, however, a multiple amino acid sequence alignment revealed a striking homology of the entire PrfA sequence with the Crp–Fnr family of global transcription regulators, which includes NtcA from *Synechococcus* (Vega-Palas *et al.*, 1992) Crp and Fnr from *E. coli* (Cossart and Gicquel-Sanzey, 1982; Spiro and Guest, 1990) (Fig. 3). This finding fits well into the proven role of PrfA as a pleiotropic regulatory protein.

The three-dimensional structure of Crp is known at a 0.25 nm resolution (Weber and Steitz, 1987); the properties of this regulator have been reviewed recently (Kolb *et al.*, 1993). The DNA-binding domain of Crp has been characterized as a HTH, spanning amino acids 168–191 and centred around G-178. PrfA as well as the other members of the protein family show a particularly high degree of similarity to this domain at a comparable position (in the case of PrfA, 17 amino acids out 24).

From this we conclude that this region constitutes the DNA-binding domain of PrfA. In the region between amino acids 133–152 of PrfA, we could not detect any particular homology to Crp nor was a HTH predicted here, which is in contrast to previous claims (Freitag *et al.*, 1992). No homology could be found for the amino acids involved in cAMP binding by Crp (E-72, R-82, S-83, R-123, T-127 and S-128) and for the critical cysteine residues (C-16, C-20, C-23 and C-29) in Fnr (Spiro and Guest, 1990). In this context it is interesting that cellobiose but not glucose was shown to influence PrfA-regulated listeriolysin expression in *L. monocytogenes* (Park and Kroll, 1993; Datta and Kothary, 1993). Four of the glycines structurally important in Crp, which are highly conserved among Crp-like proteins, were found at appropriate positions in PrfA (indicated by arrows in Fig. 3). Furthermore, a high probability for short β-strands was predicted in this region. The predicted α-helicity for the domain between amino acids 109–130 of PrfA was rather low. This is in some contrast to Crp where an extended α-helix at this position has been identified experimentally as the dimer contact area (Weber and Steitz, 1987). Other differences from Crp include a potential second *N*-terminal HTH and a leucine-containing α-helix at the *C*-terminus, although possible functions remain unclear at present. It seems premature to conclude from these data that the three-dimensional structure of PrfA is similar to Crp, including the equivalent of a nucleotide-binding domain with β-roll structure near the *N*-terminus.

To our knowledge PrfA is the second example of a Crp/Fnr-like regulatory protein in Gram-positive bacteria, the first one being Flp from *Lactobacillus casei* (Irvine and Guest, 1993).

Transcription of *prfA* as well as PrfA-regulated gene expression are affected by sugars (see above) and a number of other conditions, e.g. temperature, culture medium and pH (Leimeister-Wächter *et al.*, 1992; Sokolovic *et al.*, 1993; Datta and Kothary, 1993). Future investigations will be designed to further elucidate the mechanisms by which transcription of *prfA* is regulated, how PrfA itself is activated and how it regulates virulence gene expression in *Listeria*.

Experimental procedures

Bacterial strains and plasmids

L. ivanovii ATCC 19119 (SLCC 2379) and *L. monocytogenes* NCTC 7973 (SLCC 2371) and SLCC53 were obtained from the *Listeria* strain collection of the Institute of Hygiene and Microbiology of the University of Würzburg. The mutants of *L. ivanovii* ATCC 19119 used in this study have been obtained by conjugal transfer of the streptococcal shuttle transposon Tn*1545* from *L. monocytogenes* BM4140, kindly provided by P. Courvalin (Pasteur Institute, Paris) and have been described previously (R. Schlesinger, 1988; Kreft *et al.*, 1990). The *E. coli* strain DH5-alpha was used for transformation and cloning. The *E. coli* vector plasmid pTZ18R (Mead *et al.*, 1986) was purchased from Pharmacia.

Media and antibiotics

L. ivanovii and *L. monocytogenes* were grown in brain–heart infusion broth (BHI, Difco), whereas *E. coli* strains were passaged in Luria–Bertani (LB) broth at 37°C. For Tn*1545* mutants of *L. ivanovii*, tetracycline was added at 4 μg ml^{-1}. *E. coli* transformants were grown with 100 μg ml^{-1} ampicillin. Blood agar consisted of blood agar base (Oxoid No. 2) supplemented with 5% (v/v) sheep blood. Egg yolk agar was prepared by adding 0.5% (v/v) of fresh egg yolk to a basal medium (1% bactopeptone, 0.2% beef extract, 0.5% NaCl, 0.03% cysteine-HCl, 2% agar, pH 7.4).

Restriction enzymes and ligase were purchased from Pharmacia or Boehringer and used as recommended by the manufacturer.

Haemolysin and phospholipase C assays

Haemolytic titres of culture supernatants were determined in microtitre plates as described (Dominguez-Rodriguez *et al.*, 1986). PI-PLC activity of *L. ivanovii* strains was estimated after incubation at 37°C for 48 h by halo formation around bacterial colonies on LB agar plates with an 0.7% (w/v) agar overlay containing 1% (w/v) L-*d*-phosphatidylinositol (Sigma Chemicals). Lecithinase production was visualized by the formation of a precipitation zone around bacterial streaks on egg yolk agar (see above) after 48 h incubation at 37°C.

Polymerase chain reaction

Chromosomal DNA fragments of *L. ivanovii* wild type and its mutant 8/6 were amplified by PCR according to published procedures (Saiki *et al.*, 1988; Bubert *et al.*, 1992), using *Taq* DNA polymerase (Pharmacia). Thermal cycling (30 cycles) consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 52°C, and primer extension for 2.5 min at 72°C. The oligonucleotide primers, synthesized on a A373 DNA synthesizer (Applied Biological Instruments) were M325: 5′-GTATGTTCCAGGCCTCTCCTTC-3′ (*ilo*-proximal); M395: 5′-CATAGAATAAGGCTTTACGAGC-3′ (Tn*1545* right end); M396: 5′-GATAAAGTGTGATAAGTCCAG-3′ (Tn*1545* left end); M397: 5′-CCTACACTAAAAGGCCTCCGCGG-3′ (*ilo*-upstream); M449: 5′-TCCGCAAATAGAACCTAGC-3′ and M450: 5′-TGTTAGCAGAATTCTTTC-3′ (for inverse PCR); M476: 5′-TTTTCCTGTATCAAGGGAAC-3′ (*prfA*-internal).

Nucleotide sequence and primer-extension analyses

Nucleotide sequences were determined from both strands of recombinant plasmids containing different segments of the respective *L. ivanovii* genes. Sequencing reactions were performed with a commercial kit (Pharmacia) using supercoiled templates and synthetic oligonucleotide primers (15–18 bases). Computer analyses and homology searches in the EMBL database were performed with the University of

Wisconsin Genetics Computer Group program package (Devereux *et al.*, 1984) run on a VAX/VMS computer or with the PROSIS program package (Hitachi) on a MS-DOS personal computer.

Total RNA was isolated from log-phase listerial cells, kept on ice for 15 min in 1 M Tris-HCl, 0.1 M EDTA, pH 8.0 and 1 mg ml^{-1} lysozyme. Triton X-100 was added to a final concentration of 0.1%, followed by 15 min incubation on ice. The lysates were extracted twice with 1 volume of phenol/chloroform (1:1 v/v) at 65°C. Nucleic acids were precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate pH 4.8 at −70°C, then centrifuged through a CsCl density gradient (rotor TLA100.2, 80 000 × *g*, 15°C, 16 h). The RNA pellets were dissolved in water, reprecipitated with ethanol as above, then treated with RNAse-free DNAse (0.25 U µl^{-1} in 20 mM Tris-HCl, 1.5 mM $MgSO_4$, pH 7.5) for 40 min at 25°C. These preparations were then extracted twice with phenol/chloroform. Primer-extension reactions were carried out according to a previously described protocol (Leimeister-Wächter *et al.*, 1990), using synthetic oligonucleotides spanning 20 nucleotides immediately upstream of the initiation codon of *ilo* or *hly* as primers.

Electrophoresis of DNA fragments at 4°C and 60°C was performed in 6% (w/v) polyacrylamide gel with 40 mM Tris-acetate, 5 mM sodium acetate, 1 mM EDTA, pH 7.5 as buffer.

Labelling, isolation and gel electrophoresis of PrfA-dependent proteins from Listeria

PdPs from *L. ivanovii* and *L. monocytogenes* were labelled with [^{35}S]-methionine in MEM (minimum essential medium with Earle's salts, Gibco) and analysed as published (Bokolovic *et al.*, 1993). In brief, bacteria were grown at 37°C to OD_{600} 1.0, washed and resuspended in MEM without L-glutamine and L-methionine. After preincubation without shaking for 30 min at 37°C in a 5% CO_2 atmosphere, 25 µCi of [^{35}S]-methionine were added and the bacteria were incubated for an additional 60 min. For the isolation of non-radiolabelled PdPs, radioactive methionine was omitted. Cells were harvested by centrifugation and washed three times with phosphate-buffered saline (PBS). Surface proteins were released from the bacteria by shaking for 15 min in the presence of 1% SDS at room temperature. All released proteins were precipitated with trichloroacetic acid (7% final concentration) at 4°C. Gel electrophoresis was performed on SDS slab gels (13% acrylamide; 1% SDS).

Other techniques

Transformation of *E. coli* DH5-alpha, all DNA manipulations and immunoblotting of non-labelled PdPs were performed according to standard procedures (Sambrook *et al.*, 1989). Polyclonal rabbit anti-Ivanolysin O antiserum was obtained by repeated subcutaneous injection of toxin purified as previously described (Kreft *et al.*, 1989).

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