Some essential elements on the inlC promoter for PrfA-dependent regulation in Listeria monocytogenes

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Abstract: To study some essential elements of a PrfA-dependent promoter of Listeria monocytogenes, a series of promoter mutants incorporated into upstream of a promoterless lacZ gene were constructed from a known listerial PrfA-dependent promoter inlC promoter by PCR-mediated site-directed mutagenesis and recombinant PCR technique and then electroporated into L. monocytogenes wild-type strain PL4 with prfA* mutant Pl4A and prfA deletion mutant A42. The corresponding transcription activities of altered promoters were measured by the β-galactosidase assay. The results showed that a PrfA-box-like sequence “pseudo-PrfA-box” TTAACACGGTGTAA 22bp downstream of the transcriptional start site of PinlC had no ability to enhance or inhibit the PrfA-dependent transcription of inlC promoter even it was modified to the “ideal PrfA-box” TTAACATGGTAA. However, there was almost no PrfA-dependent transcriptional activity from the mutants deletion of the inlC original PrfA-box. Moreover, altered spacing between 3′-end of the PrfA-box and 5′-end of the -10 box in the inlC promoter region affected transcription efficiency dramatically which also happened in another promoter-dependent promoter plcA promoter. Those above suggested that besides the “PrfA-box” additional unknown PrfA-dependent promoter structure or sequence might be required for the PrfA binding to the promoter and initiation of transcription. Furthermore, the distance between the PrfA-box and the -10 box should be fixed to 22 or 23bp for the PrfA-dependent transcription.

Keywords: Listeria monocytogenes; inlC promoter; PinlC; PrfA; PrfA-box; transcriptional regulation

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Listeria monocytogenes is a gram-positive facultative intracellular bacterial pathogens that can cause serious food-borne illness in pregnant women and newborns, the elderly and immunocompromised or debilitated adults with underlying diseases. The processes which are of major importance for the pathogenesis of L. monocytogenes infection require several well-characterized virulence factors including Internalin A InlA and Internalin B InlB which are required for uptake of L. monocytogenes into non-professional phagocytes such as epithelial and endothelial cells, a pore-form bacterial toxin listeriolysin-O LLO or Hly and two secreted phospholipases C PlcA and PlcB which play an important role in disruption vacuolar membranes and escape from it into host cytosol ActA which mediates the intracytoplasmic movements and cell-to-cell spread of bacteria. Mpl metalloprotease which helps lysis of the double membrane vacuole formed after uptake by the neighbouring cells. Hpt hexose phosphates translocate a recent identified virulence factor involving in listerial replication inside the host cytosol. These virulence factors are encoded by inla, inlb, hly, plcA, plcB, mpl and hpt genes respectively which are regulated strongly or partly by a transcription factor PrfA encoded by prfA gene. PrfA is the only regulatory protein identified to date to be necessary for the regulation of the expression of most of the virulence genes in L. monocytogenes. On the basis of structural and functional similarities PrfA belongs to the Cep/Fnr family of transcription regulators. It recognizes a conserved symmetric sequence of 14 bp TTAACANTGGTAA commonly referred to as the “PrfA-box” at around position-40 from the transcriptional start site.

The inlC gene encodes a small secreted internalin, the function of which is still unknown. An inlC deletion mutant shows significant reduction in virulence and recent studies indicate that it may play a supportive role in InlA-mediated internalization of L. monocytogenes by non-phagocytic cells. Transcription of inlC is PrfA regulated in vivo.

Although the number of genes for which PrfA-dependent transcription has been clearly established is relatively low and contains mainly the known virulence genes recent transcriptome analysis using whole genome microarrays revealed a much larger number of genes being positively and negatively affected by PrfA. These results suggest that PrfA may function in a more complex way.

In order to investigate some essential features of PrfA-dependent promoters we have undertaken an in-
depth study at the PrfA-dependent inIC promoter\[ Pmi-
C\] using the site-directed mutagenesis and PCR-mediated recombination techniques. The data revealed that the conserved 14bp\[ TTAACGCTTGTAA\] sequences of dyad symmetry located at about 40bp upstream of the transcriptional start site of PmiC played a significant role in the PrfA-dependent transcription. While the 17bp of PrfA-box-like sequences\[ also termed “pseudo-PrfA-
box”\] downstream of the transcriptional start site of PmiC had no influence on the PrfA-dependent transcription of the inIC gene. Furthermore, altered spacing between 3’-end of the PrfA-box and 5’-end of the 10 box in the inIC promoter region affected transcription efficiency. The optimal length of it is 22 or 23bp.

1 MATERIALS AND METHODS

1.1 Bacteria strains and culture conditions

P14 is a L. monocytogenes wild-type strain of sero-
avar 4b and P14a is prfA+ mutant of P14\[ Gly145Ser\] with high expression of PrfA protein\[11\]. A42 is P14 de-
letion mutant of prfA. All L. monocytogenes strains were grown in brain heart infusion\[ BH\] broth\[ Difco\] overnight at 37°C with vigorous shaking\[ 190r/min\].

1.2 Construction of template plasmids for site directed 
mutagenesis

The concerning promoter regions of PmiC and PpLA 
were amplified from chromosomal DNA of L. monocyo-
togenes P14 using the oligonucleotides 5’-CATTTGTTGGC-GCGGTACCTTTAATTTAAC3’ and 5’-CATTTGCTATTATC TGCAGTTGTAACCAATT-3’ for the inIC promoter and 
5’-CTTTTATTGACCTGTCGTTCC TTATCG-3’ and 5’-GCCTAATGCTTTC GTCCCTAATG-3’ for the pLC promoter. Both amplified fragments were cloned into pUC18 
using the underlined restriction site\[ Kpn I \] and Pst I \] for 
inIC promoter and Xba I \] and Hind III \] for pLC promoter\[. The generated plasmids were used as templates to 
 obtain various promoter mutants in PCR mediated site-directed 
mutagenesis.

1.3 Site-directed mutagenesis

PCR mediated site-directed mutagenesis using double-
stranded DNA template was modified from “Molecular 
Cloning”[12]. The primers and templates are listed in 
the Table 1. The oligonucleotide primers each complement-
ary to opposite strands of the vector extended during 
temperature cycling by means of Pfu DNA poly-
merase\[ Promega\] which replicates both plasmids 
strands with high fidelity. PCR program\[ 95°C 30s for 
the first delay\[ 95°C 30s 50 – 55°C 1min\] 68°C 7min\] 72°C 
10min for the last delay\[ 12 – 18 cycles. The PCR 
products were treated with Dpn I \[ BioLa\] to digest the 
parental DNA template. The nicked vector DNA incorpo-
rating the desired mutations was then transformed into E. 
coli\[ DH5a\]. The constructs were confirmed by se-
quence analysis.

| Table 1 | PCR primers used in the site-directed mutagenesis for construction of the inIC or pLC promoter mutants |
|---|---|---|
| Plasmid name | The sequence of primer \[ 5’ – 3’ \] | Templates |
| PmiC-m1 | 1-GGGACATAAAAGGTTAATTTAATAGGAAGTATG
2-CATATACTTCTTATTTAATTCCTTTATGTTC
1-GATAAAAAGGTTACATTTGTAAATAGGAAG
2-CITCCTATTTAAACATTTGACCTTTTATGT |
| PmiC-m2 | 1-CTGATTTCTAGATTATATATTAAATTTAAACATYT
2-GAGAAGTTTTAAATTTAAATATTTGACCTTT |
| PmiC-m3 | 1-GATAAAAAGGTTACATTTGTAAATAGGAAG
2-CITCCTATTTAAACATTTGACCTTTTATGT |
| PmiC-m4 | 1-CTGATTTCTAGATTATATATTAAATTTAAACATYT
2-GAGAAGTTTTAAATTTAAATATTTGACCTTT |
| PmiC-m5 | 1-CTGATTTCTAGATTATATATTAAATTTAAACATYT
2-GAGAAGTTTTAAATTTAAATATTTGACCTTT |
| PmiC-IS20 | 1-CTTGAATTTAAACATTTGACCTTTTATGT
2-TTAGACAAAAAAATTAGATTTAATTTAAC |
| PmiC-IS21 | 1-GTAAATTTAAACATTTGACCTTTTATGT
2-TTAGACAAAAAAATTAGATTTAATTTAAC |
| PmiC-IS22 | 1-GTAAATTTAAACATTTGACCTTTTATGT
2-TTAGACAAAAAAATTAGATTTAATTTAAC |
| PpLC-IS20 | 1-CAAGTATATGCTCCTAATAAAGATGCATTCAAA
2-CTAAAGGAGGACCTTTTATTGAGGCAATTGAC |
| PpLC-IS21 | 1-CAAGTATATGCTCCTAATAAAGATGCATTCAAA
2-CTAAAGGAGGACCTTTTATTGAGGCAATTGAC |
| PpLC-IS22 | 1-CAAGTATATGCTCCTAATAAAGATGCATTCAAA
2-CTAAAGGAGGACCTTTTATTGAGGCAATTGAC |
1.4 Construction of shuttle plasmid carrying promoterless lacZ reporter gene fused to the promoters of inIC and plcA as well as their mutants

The plasmids containing promoterless lacZ were constructed by the PCR-mediated recombination method Fig. 1[10]. This method depends on DNA fragment fusion by the PCR technique and requires three steps of PCR to obtain a sufficient amount of the gene fusion fragment. In the first step the promoterless lacZ gene was amplified from Escherichia coli W3110 genomic DNA with the primer A 5'-GTGGAGAATGT-TGAAAATGACCATGATTACGG-3' and the primer B 5'-AAAAAACCCGGTTATTTTGACACCAGACC-3'. The DNA fragment containing the inIC promoter region involving the rhs site and the translation start site of the inIC with or without mutagenesis was amplified from the plasmids listed in Table 1 with the primer C 5'-AGCAGACAACCGGGGATTGAAACATGTGGT-3' and the primer D 5'-TCGTAATCATGCTACATCCATTTCC-3'. The single underlined regions correspond to inIC promoter sequences the double underlined regions to the lacZ sequences and the dotted underlined regions to the Cfr 91 site. For construction of recombinant PplcA-lacZ shuttle plasmids with or without mutagenesis in the plcA promoter region the primer A is 5'-GGGGGCTATTTGTATATGCCATGATTACGG-3' the primer B 5'-AAAAAACCCGGTTATTTTGACACCAGACC-3' the primer C is 5'-AGCAGACAACCGGGGATTGAAACATGTGGT-3' and the primer D is 5'-TCGTAATCATGCTATATCACCAATGGGCC-3'. After amplification by standard PCR procedure with Pfu DNA polymerase Promega both PCR products were purified by QIAquick PCR purification kit Qiagen respectively and the equal molar amounts of each were mixed for 6 amplification cycles without primers thereby the inIC or plcA promoter fragments annealed to the promoterless lacZ fragments at the overlapping regions. Therefore in the final step the full length of the fusion gene fragment of Promoter-lacZ or Promoter-mutant-lacZ was amplified with primers B and C. After digestion with Cfr 91 the fusion gene fragment was cloned into the unique Cfr 91 site of the shuttle plasmid pUNK S. Pilgrim personal communication and transformed into E. coli DH5α. The blue clones containing the plasmids with the fusions were selected on erythromycin 600 μg/mL containing X-Gal plates and the constructs were confirmed by sequence analysis.

1.5 Transformation of L. monocytogenes with the lacZ fusion constructs

Recombinant plasmids were used to transform L. monocytogenes Pl4 Pl4a and A42 by electroporation[10] with erythromycin 5 μg/mL selection.

1.6 β-galactosidase assay

β-galactosidase assay was carried out as described by Miller[10]. The β-galactosidase activity Miller units was determined by the equation OD420 ×1000 re-action time × volume of culture mL used in the assay × OD95. For accurate measurements of β-galactosidase activity the amount of total protein in the cell lysate was determined using Bio-Rad protein assay kit BIO-RAD for protein concentration determination. Therefore β-galactosidase activity in this study was expressed in units/mg of lysate. The experiments had repeated at least five times.

2 RESULTS

2.1 Construction of multicopy plasmids carrying lacZ under the control of P inIC or P inIC mutants

As previously described[10] the promoter region of the inIC gene contains a Prfa-box with one mismatch compared to the “ideal” Prfa-box TTAACANTGT-TAA and a Prfa-box-like sequence 22bp downstream of the transcription start site of P inIC Fig. 2 P inIC WT which was termed “pseudo-Prfa-box”. This 17bp sequence exhibits the dyad symmetry typical for Prfa-boxes in the flanking 6bp parts but has an inner loop-forming part which comprises 5bp instead of the normal 2bp. According to the above described transcription analyses this sequence is also positioned in the inIC transcript.

In order to analyse whether this pseudo Prfa-box participates in the transcription of inIC two deletions
were introduced into this sequence one of which should inactivate a possible function of this pseudo-PrfA-box by deletion of 9bp in i) PinIC-m1[3] while the other converts the pseudo-PrfA box into a consensus PrfA-box with 3bp deletion[4] PinIC-m2[6]. Moreover the –10bp of deletion in the original PrfA-box PinIC-m3[2] combined with altered pseudo-PrfA-box were also constructed as shown in Fig. 2 PinIC-m4 and PinIC-m5[2]. In addition the altered interspace mutants of PinIC and PpCAs were constructed as in Fig. 2 to investigate the optimal distance between 3-end of the PrfA-box and 5-end of the -10 box in a PrfA-regulated promoter. All these inIC promoter mutants were fused to the upstream of a non-promoter lacZ reporter gene and inserted to a plasmid pUNK[10] Fig. 1. After electroporation[10] the recombinant plasmids were introduced into L. monocytogenes wild-type strain P14[5] prfA[9] mutant P14a[10] and prfA deletion mutant A42[9] in which the expression level of PrfA is the highest in the P14a and the lowest in A42.

![PrfA-box (-10 box) SS pseudo-PrfA-box](image)

**Fig. 2** The sequences of PinIC mutants altered in the pseudo PrfA-box region[10] the original PrfA-box region[10] A and the interspace region of PinIC as well as of PpCAs[10]. The deletion sequences are replaced by dashed lines. The insertion[10] i) [10] are marked by the small letter. Pseudo-PrfA-box putative PrfA-boxes[-10 boxes] transcription start site) SS of PinIC and PpCAs are underlined and the positions are indicated.

### 2.2 β-galactosidase assay with PinIC mutants altered in the PrfA-box and pseudo-PrfA-box region

As shown in Fig. 3 compared to the wild type PinIC[10] the β-galactosidase activities of deletions in the pseudo-PrfA-box[10] no matter which may inactivate its possible function PinIC-m1[10] or convert it into a consensus PrfA-box PinIC-m2[10] there were no significant influence on the PrfA-dependent transcription from the original PrfA-box in three listerial strains野生-type strain P14[5] prfA[9] mutant P14a[10] and prfA deletion mutant A42[9] which express PrfA protein in different level. On the contrary in the case of deletions in the original PrfA-box PinIC-m3[10] the transcription activities tested were very weak and similar to each other in PinIC-m4 and PinIC-m5[10] as well as cultured in P14a[9] expression of high amount of PrfA[10]. These results indicated that PrfA recognized the 14bp of dyad symmetry located termed "PrfA-box" at about 40bp upstream of the transcriptional start site of PinIC[10] and initiation of the PrfA-dependent transcription together with listerial RNA polymerase did not be affected by the downstream dyad symmetry in pseudo-PrfA-box. The more PrfA a cell contains the more PrfA-dependent activity a virulence promoter has.


### 2.3 β-galactosidase assay with PinIC-and PpCAs-mutants altered in the interspace region between 3'-end of the PrfA-box and 5'-end of the -10 box

In order to test the flexibility of the interspace region between 3'-end of the PrfA-box and 5'-end of the -10 box in the inIC promoter[10] deletions and insertions of 1 and 2bp[9] mutants PinIC-IS20 to IS24[10] Fig. 2 were introduced into this region using the site-directed mutagenesis method.
genesis. To compare interspace altered inlC promoter strengths in vivo the activities of a promoter-driven reporter gene $\beta$-galactosidase were determined in L. monocytogenes wild-type strain P14 $\beta$-galactosidase $\beta$-galactosidase mutant P14a and prfA deletion mutant A42 transformed with the shuttle plasmid pUNK bearing the cloned promoter variants. The results show in Fig. 4 in the case of the low concentration of PrfA protein e.g. in prfA deletion mutant A42 all promoters showed very weak and similar activities while in the presence of relatively more PrfA protein e.g. in prfA mutant P14a the promoters with 22bp or 23bp of interspace were quite stronger than other promoter variants even were about 2 to 3 folds of transcriptional activities than in the normal concentration of PrfA e.g. in wild-type strain P14. Whereas insertion of a single base pair mutant PinlC-IS23 reduced the efficiency of PrfA-dependent transcription in the presence of high PrfA protein only slightly deletion of one base pair mutant PinlC-IS21 led to a remarkable reduction in transcription efficiency. Insertion or deletion of 2bp mutant PinlC-IS24 and IS20 resulted in transcription at a very low level that was no longer activated by PrfA. These data suggest that the optimal distance for the interspace region of PinlC is 22 or 23bp.

Fig. 4 $\beta$-galactosidase assay of inlC promoter interspace variants. The values shown are averages of at least 5 experimental results. A background value of $\beta$-galactosidase activity measured on wild-type strain P14 $\beta$-galactosidase mutant P14a and prfA deletion mutant A42 transformed with a pUNK-promoterless-lacZ plasmid has been subtracted from all values.

To determine whether the importance of such interspace region in the inlC promoter would also occur in other well-known PrfA-dependent promoters the plcA promoter was chose which contains a“ high quality” PrfA-box TTAACAAATGTAA compared to the consensus sequence of the PrfA-box TTAACANTTGTAAA a reasonable SigA-recognized 10 box and a 22bp-optimal interspace. Similar variants with deletions and insertions of 1 and 2bp in the interspace region of Ppica were constructed and the $\beta$-galactosidase of the recombinant plcA promoter mutants was tested Fig. 5. Apparently under conditions of presence of high or normal level of PrfA protein in P14a and P14b in the case of the deletions even with one base pair the effects of changes in the interspace region between 5’-end of the -10 box and 3’-end of the PrfA-box of Ppica resulted in a greater reduction in promoter transcription activity while this is not the case if only one base pair of insertion was introduced. Insertion of two base pairs reduced also transcription to a very low level. This result is very well in accordance with that of PinlC. All these data reveal that PrfA-regulated transcription is dependent on the length of interspace region which optimal distance is fixed to 22bp or 23bp.

Fig. 5 $\beta$-galactosidase assay of plcA promoter interspace variants. The values shown are averages of at least 5 experimental results. A background value of $\beta$-galactosidase activity measured on wild-type strain P14 $\beta$-galactosidase mutant P14a and prfA deletion mutant A42 transformed with a pUNK-promoterless-lacZ plasmid has been subtracted from all values.

3 DISCUSSION

In this study some important characteristics of a PrfA-dependent virulence gene promoter inlC promoter has been studied in detail by testing $\beta$-galactosidase activities of a series of recombinant mutants altered in the inlC promoter region under the presence of different lever of PrfA protein in the strains P14 P14a and A42.

As previously mentioned PrfA was absolutely necessary to mediate a strong and specific binding of RNA polymerase RNAP to the promoter region of the PrfA-regulated genes and Electrophoretic mobility shift assays EMSA indicated that PrfA protein alone was able to bind to the target DNA sequence containing only an entire PrfA-box which is in line with our study that PrfA-dependent transcription activities were only present in PinlC and mutants with entire PrfA-box PinlC-m1 and -m2 no or weak in PrfA-box deletion mutants PinlC-m3-m4 and m5. However our in vivo data also showed that although the mutant PinlC-m4 contained a conserved PrfA-box by deletion of 3bp from a 17bp-symmetric region pseudo-PrfA-box almost no PrfA-dependent transcription activities were observed from it even when the original PrfA-box was deleted to eliminate the putative competitive binding of PrfA and RNAP to hemi-mutant PinlC-m4 Fig. 3 suggesting that be.

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sides the PrfA binding site, PrfA-box more unknown essential elements for a PrfA-dependent promoter might be required to convert the region containing the pseudo-PrfA-box to the PrfA-dependent one.

The distance between the PrfA-box and the -10 box of the inlC promoter or of the plcA promoter is critical and functions optimally when the two sites are 22bp apart. A distance of 23bp is tolerated with a slightly reduced transcription efficiency while a distance of 21 or 24bp already leads to a drastic reduction in PrfA-dependent transcription [Fig. 4 and Fig. 5]. These results are agreement with our previous in vitro data [18]. Furthermore, the requirement of the interspace distance fixed to 22 or 23bp can also be observed in the other known PrfA-dependent promoters i.e. Phly, PmpI, PxtA, Phpt and PinA as analysis of their published transcription data [40].

Since the centre of a PrfA-box is located at around 40bp from the transcription start site and no consensus sequence present in the 35 region of all well-known PrfA-dependent promoters suggesting that the PrfA-box is centered proximal to this region and thus overlaps it. Hereby, an explanation of the function of the conserved length between the 3'-end of PrfA-box and the 5'-end of the -10 box may involve the requirement of this distance to form an open complex in the transcription initiation steps which is similar to the spacer DNA between 35 and 10 region with requirement of 16 or 17bp in the SigA-dependent promoters of B. subtilis and sigma70-dependent promoters of E. coli [19, 20]. The main role of the spacer is thought to be maintaining the 10 and 35 regions in the proper orientation for initial binding of RNA polymerase and subsequent formation of a complex that is competent to initiate RNA synthesis. This has been explicitly formulated in the "untwist and melt" model for formation of a functional RNA polymerase-promoter open complex where strand separation has taken place in the region around the start site of transcription. The deletion or insertion in this spacer would affect the formation of the functional open complex and in turn inhibit transcription initiation.

REFERENCES


PrfA

*%

\textit{inlC}

L. monocytogenes