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 P14, *prfA** mutant P14a 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"), TTAACAGCGTTTGTTAA, 22bp downstream of the transcriptional start site of P*inlC* had no ability to enhance or inhibit the PrfA-dependent transcription of *inlC* promoter, even it was modified to the "ideal PrfA-box" TTAACATTTGTTAA. 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(s) or sequence(s) 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 (P*inlC*); 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, newborns, the elderly, immunocompromised or debilitated adults with underlying diseases[1, 2]. The processes, which are of major importance for the pathogenesis of *L. monocytogenes* infection, require several, well-characterized virulence factors including[2]: 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 a important role in disruption vacuolar membranes and escapement 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 membraned vacuole formed after uptake by the neighbouring cells; Hpt (hexose phosphates translocase), a recent identified virulence factor involving in listerial replication inside the host cytosol[3]. 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*[2, 4]. On the basis of structural and functional similarities, PrfA belongs to the Crp/Fnr family of transcription regulators[5]. It recognizes a conserved symmetric sequence of 14 bp (TTAACANNTGTTAA), commonly referred to be as the "PrfA-box", at around position-40 from the transcriptional start site[6, 7].

The *inlC* gene encodes a small secreted internalin, the function of which is still unknown. An *inlC* deletion mutant shows significant reduction in virulence[8] and recent studies indicate that it may play a supportive role in InlA-mediated internalization of *L. monocytogenes* by non-phagocytic cells[9]. Transcription of *inlC* is PrfA regulated *in vivo*[8].

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[10]. 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-

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depth study at the PrfA-dependent *inlC* promoter (P*inlC*) using the site-directed mutagenesis and PCR-mediated recombination techniques. The data revealed that the conserved 14bp (TTAACGCTTGTTAA) sequences of dyad symmetry located at about 40bp upstream of the transcriptional start site of P*inlC* 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 transcription start site of P*inlC* had no influence on the PrfA-dependent transcription of the *inlC* gene. Furthermore, altered spacing between 3′-end of the PrfA-box and 5′-end of the 10 box in the *inlC* 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 serovar 4b and P14a is *prfA** mutant of P14 (Gly145Ser) with high expression of PrfA protein[11]. A42 is P14 deletion mutant of *prfA*. All *L. monocytogenes* stains were grown in brain heart infusion (BHI) broth (Difco) overnight at 37℃ with vigorous shaking (190r/min).

1.2 Construction of template plasmids for site directed mutagenesis

The concerning promoter regions of P*inlC* and P*plcA* were amplified from chromosomal DNA of *L. monocytogenes* P14 using the oligonucleotides 5′-CATTGTTGCGGCGGTACCTTACTTCTTATAC-3′and 5′-CATTGCTATTACTGCAGTTTGTAACCAATT-3′ for the *inlC* promoter and 5′-CTTTATTCTAGACCTGCTGTCCC TTTATCG-3′ and 5′-GCGAATAAGCTTTTC CGCCTAATG-3′ for the *plcA* promoter. Both amplified fragments were cloned into pUC18 using the underlined restriction sites (*Kpn* Ⅰ and *Pst* Ⅰ for *inlC* promoter and *Xba* Ⅰ and *Hin*d Ⅲ for *plcA* 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 complementary to opposite strands of the vector, extended during temperature cycling by means of *Pfu* DNA polymerase (Promega), which replicates both plasmids strands with high fidelity. PCR program: 95℃ 30s for the first delay; 95℃ 30s, 50 ~ 55℃ 1min, 68℃ 7min; 72℃ 10min for the last delay; 12 ~ 18 cycles. The PCR products were treated with *Dpn* Ⅰ (Biolab) to digest the parental DNA template. The nicked vector DNA incorporating the desired mutations was then transformed into *E. coli* (DH5α). The constructs were confirmed by sequence analysis.

Table 1 PCR primers used in the site-directed mutagenesis for construction of the *inlC* or *plcA* promoter mutants

Plasmid name	The sequence of primer (5′ ~ 3′)	Templates
P*inlC*-m1	1-GGGACATAAAAAGGTTAATTAAATAGGAAGTATATG 2-CATATACTTCCTATTTAATTAACCTTTTTATGTCCC	P*inlC*
P*inlC*-m2	1-GATAAAAAGGTTAACATTTGTTAAATAGGAAG 2-CTTCCTATTTAACAAATGTTAACCTTTTTATG	P*inlC*
P*inlC*-m3	1-CTGATTTTCGATTATTATTAATTTAAACATCTC 2-GAGATGTTTAAATTAATAATAATCGAAAATCAG	P*inlC*
P*inlC*-m4	1-GATAAAAAGGTTAACATTTGTTAAATAGGAAG 2-CTTCCTATTTAACAAATGTTAACCTTTTTATG	P*inlC*-m3
P*inlC*-m5	1-CTGATTTTCGATTATTATTAATTTAAACATCTC 2-GAGATGTTTAAATTAATAATAATCGAAAATCAG	P*inlC*-m1
P*inlC*-IS20	1-CTTGTTAATTTAAACATCTTATTTTTGCTAAC 2-GTTAGCAAAAATAAGATGTTTAAATTAACAAG	P*inlC*
P*inlC*-IS21	1-CTTGTTAATTTAAACATCTTTATTTTTGCTAAC 2-GTTAGCAAAAATAAAGATGTTTAAATTAACAAG	P*inlC*
P*inlC*-IS23	1-GTTAATTTAAACATCTCTCTATTTTTGCTAACATATAAG 2-CTTATATGTTAGCAAAAATAGAGAGATGTTTAAATTAAC	P*inlC*
P*inlC*-IS24	1-GTTAATTTAAACATCTCTCTTATTTTTGCTAACATATAAG 2-CTTATATGTTAGCAAAAAZAAGAGAGATGTTTAAATTAAC	P*inlC*
P*plcA*-IS20	1-CAAATGTTAATGCCTCAATAAAAGTCACTTTAAG 2-CTTAAAGTGACTTTTATTGAGGCATTAACATTTG	P*plcA*
P*plcA*-IS21	1-CAAATGTTAATGCCTCAAATAAAAGTCACTTTAAG 2-CTTAAAGTGACTTTTATTTGAGGCATTAACATTTG	P*plcA*
P*plcA*-IS23	1-CAAATGTTAATGCCTCAACTATAAAAGTCACTTTAAG 2-CTTAAAGTGACTTTTATAGTTGAGGCATTAACATTTG	P*plcA*
P*plcA*-IS24	1-CAAATGTTAATGCCTCAACTGATAAAAGTCACTTTAAG 2-CTTAAAGTGACTTTTATCAGTTGAGGCATTAACATTTG	P*plcA*

1.4 Construction of shuttle plasmid carrying promoterless *lacZ* reporter gene fused to the promoters of *inlC* and *plcA* as well as their mutants

The plasmids containing promoterless *lacZ* were constructed by the PCR-mediated recombination method (Fig.1)[13]. 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′-GTGGAGAATGTTGAAAATGACCATGATTACGG-3′ and the primer B: 5′-AAAAAACCCGGGTTATTTTTGACACCAGACC-3′. The DNA fragment containing the *inlC* promoter region (involving the rbs site and the translation start site of the *inlC*) with or without mutagenesis was amplified from the plasmids listed in Table 1 with the primer C: 5′-AGCAGACAACCCGGGAGGTAGAACATGTTTTG-3′ and the primer D: 5′-CCGTAATCATGGTCATTTTCAACATTCTCCAC-3′ (The single underlined regions correspond to *inlC* promoter sequences, the double underlined regions to the *lacZ* sequences and the dotted underlined regions to the *Cfr* 9I site). For construction of recombinant P*plcA*-*lacZ* shuttle plasmids with or without mutagenesis in the *plcA* promoter region, the primer A is 5′-GGGGGCCATTTTGTATATGACC ATGATTACGG-3′; the primer B: 5′-AAAAAAC-CCGGGTTATTTTTGACACCAGACC-3′; the primer C is 5′-AGCAGACAACCCGGGGCTGTCCCTTTATCG-3′ and the primer D is 5′-CCGTAATCATGGTCATATACAAAATGGCCCCC-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 *inlC* 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* 9I, the fusion gene fragment was cloned into the unique *Cfr* 9I 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 (600μg/mL) containing X-Gal plates and the constructs were confirmed by sequence analysis.

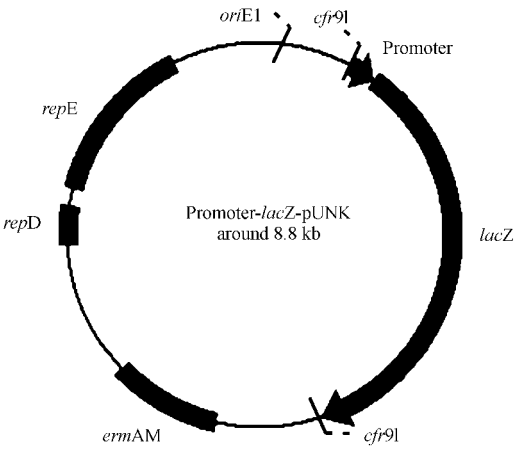

Fig.1 The scheme of construction of a multicopy plasmid carrying *lacZ* under the control of P*inlC* or P*plcA* mutants.

1.5 Transformation of *L. monocytogenes* with the *lacZ* fusion constructs

Recombinant plasmids were used to transform *L. monocytogenes* P14, P14a and A42 by electroporation[14] with erythromycin (5μg/mL) selection.

1.6 β-galactosidase assay

β-galactosidase assay was carried out as described by Miller[15]. The β-galactosidase activity (Miller units) was determined by the equation [OD_{420} × 1000] / [reaction time (min) × volume of cultures (mL) used in the assay × OD_{595}]. 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*inlC* or P*inlC* mutants.

As previously described[8], the promoter region of the *inlC* gene contains a PrfA-box with one mismatch compared to the "ideal" PrfA-box (TTAACANNTGTTAA) and a PrfA-box-like sequence 22bp downstream of the transcription start site of P*inlC* [Fig. 2, P*inlC* (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 *inlC* transcript.

In order to analyse whether this pseudo PrfA-box participates in the transcription of *inlC*, 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 it (P*inlC*-m1), while the other converts the pseudo-PrfA box into a consensus PrfA-box with 3bp deletion (P*inlC*-m2). Moreover, the −10bp of deletion in the original PrfA-box (P*inlC*-m3) combined with altered pseudo-PrfA-box were also constructed as shown in Fig. 2 (P*inlC*-m4 and P*inlC*-m5). In addition, the altered interspacer mutants of P*inlC* and P*plcA* 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 *inlC* promoter mutants were fused to the upstream of a non-promoter *lacZ* reporter gene and inserted to a plasmid pUNK (Fig. 1). After electroporation, the recombinant plasmids were introduced into in *L. monocytogenes* wild-type strain (P14), *prfA** mutant (P14a)[11] and *prfA* deletion mutant (A42), in which the expression level of PrfA is the highest in the P14a and the lowest in A42.

(A)

PrfA-box(-40) -10 box SS pseudo-PrfA-box

P*inlC*(WT): 5`-ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`

P*inlC*-m1: 5`-ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAA---------TTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-m2: 5` ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAAT 3`
P*inlC*-m3: 5`-ATTA----------TTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-m4: 5`-ATTA----------TTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-m5: 5`-ATTA----------TTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAA---------TTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-IS20: 5`-ATTATTAACGCTTGTTAATTTAAACATC--TTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-IS21: 5`-ATTATTAACGCTTGTTAATTTAAACATCT-TTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-IS23: 5`-ATTATTAACGCTTGTTAATTTAAACATCTCTgTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`
P*inlC*-IS24: 5`-ATTATTAACGCTTGTTAATTTAAACATCTCtcTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAAT-3`

(B)

PrfA-box -10 box SS

P*plcA* (WT) 5`-ATCGTCGTTAACAAATGTTAATGCCTCAACATAAAAGTCACTTTAAGATAGGAATATACTAATC-3`

P*plcA*-IS20 5`-ATCGTCGTTAACAAATGTTAATGCCTCAA--TAAAAGTCACTTTAAGATAGGAATATACTAATC-3`
P*plcA*-IS21 5`-ATCGTCGTTAACAAATGTTAATGCCTCAA-ATAAAAGTCACTTTAAGATAGGAATATACTAATC-3`
P*plcA*-IS23 5`-ATCGTCGTTAACAAATGTTAATGCCTCAACtATAAAAGTCACTTTAAGATAGGAATATACTAATC-3`
P*plcA*-IS24 5`-ATCGTCGTTAACAAATGTTAATGCCTCAACtgATAAAAGTCACTTTAAGATAGGAATATACTAATC-3`

Fig. 2 The sequences of P*inlC* mutants altered in the pseudo PrfA-box region, the original PrfA-box region (A) and the interspace region of P*inlC* as well as of P*plcA* (B). The deletion sequences are replaced by dashed lines. The insertion (s) is (are) marked by the small letter. Pseudo-PrfA-box, putative PrfA-boxes, -10 boxes, transcription start site (SS) of P*inlC* and P*plcA* are underlined and the positions are indicated;

2.2 β-galactosidase assay with P*inlC* mutants altered in the PrfA-box and pseudo-PrfA-box region

As shown in Fig. 3, compared to the wild type P*inlC*, the β-galactosidase activities of deletions in the pseudo-PrfA-box, no matter which may inactivate its possible function (P*inlC*-m1) or convert it into a consensus PrfA-box (P*inlC*-m2), there were no significant influence on the PrfA-dependent transcription from the original PrfA-box in three listerial strains, wild-type strain (P14), *prfA** mutant (P14a) and *prfA* deletion mutant (A42), which express PrfA protein in different level. On the contrary, in the case of deletions in the original PrfA-box (P*inlC*-m3), the transcription activities tested were very weak and similar to each other in P*inlC*-m4 and P*inlC*-m5, as well as cultured in P14a (expression of high amount of PrfA). 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 P*inlC*, 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.

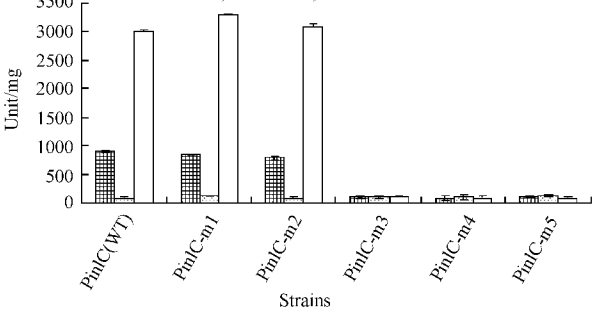

Fig. 3 β-galactosidase activity of *L. monocytogenes* P14, a *prfA** mutant P14a, and an isogenic *prfA* deletion mutant A42 carrying the recombinant *inlC* promoter mutants altered in the PrfA-box and pseudo-PrfA-box region. The values shown are averages of at least 5 times of repeats. A background value of β-galactosidase activity, measured on wild-type strain P14, *prfA** mutant P14a and *prfA* deletion mutant A42 transformed with a pUNK-promoterless-*lacZ* plasmid, has been subtracted from all values.

2.3 β-galactosidase assay with P*inlC*-and P*plcA*-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 *inlC* promoter, deletions and insertions of 1 and 2bp (mutants P*inlC*-IS20 to IS24; Fig. 2) were introduced into this region using the site-directed muta-

genesis. To compare interspace altered *inlC* promoter strengths *in vivo*, the activities of a promoter-driven reporter gene, β-galactosidase, were determined in *L. monocytogenes* wild-type strain P14, *prfA** 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 interspacer 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 P*inlC*-IS23) reduced the efficiency of PrfA-dependent transcription (in the presence of high PrfA protein) only slightly, deletion of one base pair (mutant P*inlC*-IS21) led to a remarkable reduction in transcription efficiency. Insertion or deletion of 2bp (mutant P*inlC*-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 P*inlC* is 22 or 23bp.

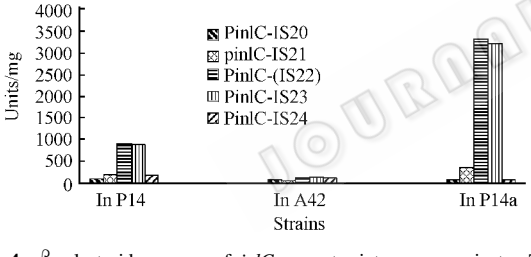

Fig.4 β-galactosidase assay of *inlC* promoter interspacer variants. The values shown are averages of at least 5 experimental results. A background value of β-galactosidase activity, measured on wild-type strain P14, *prfA** mutant P14a and *prfA* deletion mutant A42 transformed with a pUNK-promoterless-*lacZ* 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 TTAACAAATGTTAA compared to the consensus sequence of the PrfA-box TTAACANNTGTTAA[4], a reasonable SigA-recognized 10 box and a 22bp-optimal interspacer. Similar variants with deletions and insertions of 1 and 2bp in the interspace region of P*plcA* were constructed and the β-galactosidase of the recombinant *plcA* promoter mutants was tested (Fig.5).

Apparently, under conditions of presence of high or normal level of PrfA proteins (in P14a and P14), 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 P*plcA* 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 P*inlC*. All these data reveal that PrfA-regulated transcription is dependent on the length of interspacer region, which optimal distance is fixed to 22bp or 23bp.

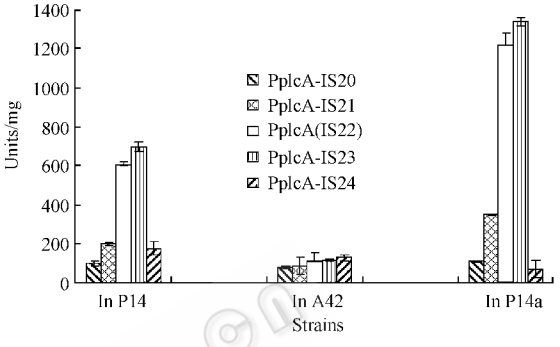

Fig.5 β-galactosidase assay of *plcA* promoter interspacer variants. The values shown are averages of at least 5 experimental results. A background value of β-galactosidase activity, measured on wild-type strain P14, *prfA** mutant P14a and *prfA* deletion mutant A42 transformed with a pUNK-promoterless-*lacZ*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

3 DISCUSSION

In this study, some important of characteristics of a PrfA-dependent virulence gene promoter, *inlC* promoter, has been studied in detail, by testing β-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[16] 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[17], which is in line with our study that PrfA-dependent transcription activities were only present in P*inlC* and mutants with entire PrfA-box (P*inlC*-m1 and -m2), no or weak in PrfA-box deletion mutants (P*inlC*-m3,-m4 and m5). However, our *in vivo* data also showed that although the mutant P*inlC*-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 hem (mutant P*inlC*-m4, Fig.3), suggesting that be-

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. P*hly*, P*mpl*, P*actA*, P*hpt* and P*inlA* as analysis of their published transcription data[4]. Since the centre of a PrfA-box is located at around of 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 sigma^{70}-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[21, 22] 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.

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依赖 PrfA 转录调控的单核细胞增生李斯特菌毒力基因 *inlC* 启动子结构特点的初步研究

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摘 要 为了研究单核细胞增生李斯特菌毒力基因启动子的结构特点与转录调控因子 PrfA 蛋白之间的关系,应用 PCR 定点突变和重组 PCR 技术缺失了该菌毒力基因 *inlC* 启动子上可能与 PrfA 蛋白结合以及诱发转录起始相关的碱基序列,构建了一系列突变启动子与 *lacZ* 报告基因融合表达质粒,使 *lacZ* 基因的表达置于 *inlC* 突变启动子下,并分别电转化单核细胞增生李斯特菌野生株 P14、PrfA 蛋白高表达突变株 P14a 和 *prfA* 基因等位缺失突变株 A42 中,检测相应的 β-半乳糖苷酶活性。结果表明:位于 *inlC* 启动子转录起始点下游 22bp 处的一段 17bp 的类似 PrfA 蛋白结合序列 TTAACAGCGTTTGTTAA 并没有增强和抑制 PrfA 转录调控活性的功能;甚至将其改造成"完美的"PrfA 蛋白结合序列 TTAACATTTGTTAA 后,也不影响 *inlC* 依赖于 PrfA 的转录活性地表达;但是,如果缺失 *inlC* 启动子上原始的 PrfA 蛋白结合序列,则使 *inlC* 依赖于 PrfA 的转录活性完全丧失;另外,单核细胞增生李斯特菌毒力基因 *inlC* 和 *plcA* 依赖于 PrfA 的转录活性的表达也与启动子上 PrfA 蛋白结合区(PrfA-box)距离-10 区的碱基个数有关:最适为 22 或 23bp,长于 23bp 或短于 22bp 的突变启动子的依赖 PrfA 的转录活性大大降低,甚至没有活性。说明除 PrfA 蛋白结合序列外,受 PrfA 调控的毒力基因启动子上还可能存在其它尚未阐明的结构和序列影响 PrfA 蛋白的结合以及启动转录表达。

关键词 单核细胞增生李斯特菌 (*Listeria monocytogenes*); *inlC* 启动子; PrfA; PrfA 蛋白结合区(PrfA-box); 转录调控

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