Essential Features of a PrfA-dependent Promoter of *Listeria monocytogenes*

DISSERTATION

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vorgelegt von

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Die vorliegende Arbeit wurde von mir selbständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt.

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Contents

1. Abstract

The gram-positive, facultative intracellular pathogen *Listeria monocytogenes* is the causal agent of listeriosis, severe food-borne, opportunistic infection of humans and animals symptomatized by meningoencephalitis, abortion, and septicaemia. Most of well-known virulence genes are controlled by PrfA that belongs to the Crp-Fnr family of transcriptional activators.

A PrfA-mediated transcription initiating at a virulence gene promoter, *inlC* promoter (P*inlC*) that regulates the expression of the small, secreted internalin C, was in-depth characterized by an *in vitro* transcription system to unravel the essential features of a PrfA-dependent promoter in this study. The obtained results indicate a dual promoter for *inlC* that leads to PrfA-dependent and -independent transcription *in vitro* and *in vivo*. The PrfA-dependent transcription requires, as expected, the PrfA-box, a conserved 14 bp sequence of dyad symmetry located about 40 bp upstream of the transcriptional start site of each PrfA-regulated gene. Another important structural feature for this PrfA-dependent promoter is the distance between the 3´-end of the PrfA-box and the 5´-end of the SigA-recognized –10 box fixed to 22 or 23 bp, which is observed in the interspace regions of the other known PrfA-dependent promoters, e.g. P*actA*, P*plcA*, P*hly* and P*mpl*. The –35 box of P*inlC* is not necessary for PrfA-dependent transcription. The –10 box of P*inlC* and also that of the other PrfA-dependent promoters of *L. monocytogenes* closely resemble SigA-recognized –10 promoter sequences of the well-characterized gram-positive bacterium *B. subtilis*. Even the extended –10 motif (5´-TRTG-3´) considered to be a basic element for many SigA-recognized promoters in *B. subtilis* is present in P*inlC*. Primer extension studies reveal that both the PrfA-dependent and the independent promoter share the same –10 box. The PrfA-independent transcription of *inlC* depends on a –35 box located directly downstream of the PrfA-box, and the close proximity of the two sites inhibits strongly the transcription activity of the PrfA-independent promoter when the PrfA-RNA polymerase complex binds to the PrfA-box. Deletion of the PrfA-box results in PrfA-independent transcription from P*inlC,* which is no longer inhibited by PrfA. High concentration of GTP appears to be necessary for PrfA-dependent transcription initiated at the *inlC* promoter and at other PrfA-dependent promoters.

Recent studies have shown that in addition to the known virulence genes the expression of a rather large number of *L. monocytogenes* genes seems to be positively or negatively affected by PrfA. Based on transcriptome analysis, Milohanic and his co-workers identified three groups of genes that were regulated differently by PrfA. Some of these genes containing putative PrfA-boxes in their 5´-upstream regulatory regions were selected for analysis of their transcriptional dependency on PrfA using again the *in vitro* transcription system. The data show that among these “PrfA-regulated” promoters tested, only the promoter of the *hpt* gene belonging to group I is clearly activated by PrfA. This promoter is also the only one that exhibited all essential features of a typical PrfA-dependent promoter as described above. *In vitro* transcription starting at most of the other promoters was neither positively nor negatively affected by PrfA. Transcription initiated at some of the promoters of group III genes (*lmo0596* and *lmo2067*) is rather inefficient with SigA-loaded RNA polymerase, but is highly activated with RNA polymerase loaded with purified SigB. Addition of purified PrfA protein has no

effect on the SigB-dependent transcription. These *in vitro* transcription results indicate that the *in vivo* observed PrfA effect on the expression of most of the new genes is either indirect or PrfA-mediated transcription of these genes requires - in contrast to the PrfA-dependent transcription of the known virulence genes (including *hpt*) - additional factors not present in the *in vitro* transcription assay. In addition to these new genes described by Milohanic, the promoters of two genes (*lmo2420* and *lmo2840*) that contain putative PrfA-boxes with only a single mismatch in their upstream regulatory regions were analyzed in this study. However, transcription of none of these genes is regulated by PrfA, suggesting that these genes are either not truly regulated by PrfA or regulated by other global transcription activators that interact with PrfA by yet unknown mechanisms.

There are some promoters of *L. monocytogenes* genes containing putative PrfA-boxes and appropriate SigA-recognized –10 boxes, but *in vitro* transcription from them is not affected by PrfA. By exchanging corresponding sequences between a functionally inactive promoter P*aroA*P2 and a typical PrfA-dependent promoter P*plcA,* it is found that PrfA-dependent *in vitro* transcription can be initiated from the hybrid promoter containing the putative PrfA-box and the SigA-recognized –10 box (TTTAAT) from the putative PrfA-dependent *aroA*P2 promoter, but it is inhibited strongly by the interspace sequence between these two sites apparently due to an additional RNA polymerase binding site [the –10 box (TAATAT) for the PrfA-independent transcription of P*aroA*P1)] within this region. Furthermore, a symmetric sequence downstream of the –10 box (TTTAAT) is also shown to be a strongly inhibitory for PrfA-dependent transcription from the putative PrfA-dependent *aroA*P2 promoter.

Zusammenfassung

Listeria monocytogenes, ein gram-positives, fakultativ intrazelluläres Bakterium, ist der Krankheitserreger der Listeriose und kann bei Mensch und Tier schwere Infektionen auslösen, die zu Meningoenzephalitis, Totgeburten und Sepsis führen können. Die meisten bekannten listeriellen Virulenzgene werden durch den positiven Regulationsfaktor PrfA, der zur Crp-Fnr-Familie von Transkriptionsfaktoren zählt, reguliert. Zu den durch PrfA regulierten Genen zählt auch *inlC*, das das kleine sekretierte Internalin C kodiert.

Mit Hilfe des *in vitro* Transkriptionssystems wurde in dieser Arbeit die PrfA-abhängige Transkription des *inlC*-Promotors (P*inlC*) untersucht, um die essentiellen Eigenschaften eines PrfA-abhängigen Promotors zu charakterisieren. Die hier erhaltenen Ergebnisse deuten auf einen dualen Promotor für *inlC* hin, der für eine PrfA-abhängige und -unabhängige Transkription *in vitro* und *in vivo* verantwortlich ist. Die PrfA-abhängige Transkription von P*inlC* benötigt wie erwartet die PrfA-Box, eine konservierte, 14 bp lange Sequenz, die 40 bp upstream des Transkriptionsstarts PrfA-regulierter Gene liegt. Ein weiteres wichtiges Merkmal für diesen PrfA-abhängigen Promotor ist der Abstand zwischen dem 3'-Ende der PrfA-Box und dem 5'-Ende der SigA-abhängigen –10 Box, der 22 oder 23 bp beträgt und auch bei anderen bekannten PrfA-abhängigen Promotoren wie P*actA*, P*plcA*, P*hly* und P*mpl* zu finden ist. Untersuchungen zeigten, dass die –35 Box von P*inlC* nicht notwendig für eine PrfA-abhängige Transkription ist. Die –10 Box von P*inlC* und anderen PrfA-abhängigen Promotoren von *L. monocytogenes* ähnelt stark den SigA-abhängigen –10 Promotorsequenzen des gut untersuchten gram-positiven Bakteriums *B. subtilis*. Sogar das erweiterte –10 Motiv (5'-TRTG-3'), das in *B. subtilis* als Hauptbestandteil vieler SigA-abhängiger Promotoren betrachtet wird, ist auch in P*inlC* zu finden. Untersuchungen mit Primer Extension zeigten, dass der PrfA-abhängige und PrfA-unabhängige *inlC*-Promotor die gleiche –10 Box verwenden. Die PrfA-unabhängige Transkription von *inlC* ist abhängig von einer –35 Box, die direkt downstream der PrfA-Box liegt. Durch die enge Nachbarschaft dieser beiden Sequenzen wird die Transkriptionsaktivität des PrfA-unabhängigen Promotors stark inhibiert, wenn der PrfA-RNA-Polymerase-Komplex an die PrfA-Box bindet. Deletion der PrfA-Box führt zu einer PrfA-unabhängigen Transkription von P*inlC*, die nicht länger durch PrfA inhibiert wird. Hohe Konzentration an GTP scheint zudem für die PrfA-abhängige Transkripitonsinitiation am *inlC*-Promotor und anderen PrfA-abhängigen Promotoren notwendig zu sein.

Jüngste Untersuchungen haben gezeigt, dass neben den bereits bekannten Virulenzgenen die Expression einer Vielzahl weiterer *L. monocytogenes*-Gene positiv oder negativ durch PrfA beeinflusst zu sein scheint. Basierend auf Transkriptomanalysen identifizierte Milohanic *et al*. drei Gruppen von Genen, die differentiell durch PrfA reguliert werden. Einige dieser Gene besitzen putative PrfA-Boxen in ihren Promotorbereichen und wurden in dieser Arbeit mit Hilfe des *in vitro* Transkriptionssystems auf ihre PrfA-Abhängigkeit untersucht. Die hier erhaltenen Ergebnisse zeigen, dass unter allen untersuchten "PrfA-regulierten" Promotoren nur der Promotor des *hpt*-Gens - einem Mitglied der Gruppe I - deutlich durch PrfA aktiviert wird. Dieser Promotor ist auch der Einzige, der alle essentiellen Eigenschaften eines typischen PrfA-abhängigen Promotors wie oben beschrieben aufwies. Die *in vitro*

Transkription ausgehend von den meisten anderen Promotoren wurde weder positiv noch negativ durch PrfA beeinflusst. Die Transkription von einigen Promotoren der Gruppe III Gene (*lmo0596* und *lmo2067*) ist relativ ineffizient mit SigA-beladener RNA-Polymerase, wird aber stark aktiviert, wenn RNA-Polymerase mit gereinigtem SigB beladen wird. Zugabe von gereinigtem PrfA-Protein hat keinen Einfluss auf die SigB-abhängige Transkription. Diese *in vitro*-Transkriptionsergebnisse deuten darauf hin, dass der *in vivo* beobachtete PrfA-Effekt auf die Expression der meisten neu identifizierten Gene entweder indirekt ist oder die PrfA-vermittelte Transkription dieser Gene im Gegensatz zur PrfA-abhängigen Transkription der bekannten Virulenzgene (einschließlich *hpt*) zusätzliche Faktoren benötigt, die im *in vitro* Transkriptionsansatz nicht vorhanden sind. Neben diesen neuen von Milohanic *et al*. beschriebenen Genen wurden die Promotoren von zwei weiteren Genen (*lmo2420* und *lmo2840*) untersucht, die putative PrfA-Boxen mit nur einem Mismatch aufwiesen. Die Transkription dieser beiden Gene zeigte jedoch keine Abhängigkeit von PrfA. Dies lässt vermuten, dass diese Gene entweder nicht von PrfA reguliert werden oder ihre Regulation über andere globale Transkriptionsaktivatoren, die auf bisher unbekannte Weise mit PrfA interagieren, erfolgt.

Es gibt einige Promotoren in Genen von *L. monocytogenes*, die putative PrfA-Boxen und dazu passende SigA-abhängige –10 Boxen besitzen, deren *in vitro* Transkription jedoch nicht von PrfA beeinflusst wird. Durch Austausch entsprechender Sequenzen zwischen einem funktionell inaktiven Promotor P*aroA*P2 und einem typischen PrfA-abhängigen Promotor P*plcA* konnte gezeigt werden, dass PrfA-abhängige *in vitro* Transkription von einem Hybridpromotor initiiert werden kann, der die putative PrfA-Box und SigA-abhängige –10 Box (TTTAAT) des möglicherweise PrfA-abhängigen *aroA*P2-Promotors besitzt. *In vitro* Transkription wird allerdings durch die zwischen PrfA und –10 Box liegende Sequenz des *aroA*P2-Promotors stark inhibiert, da offensichtlich eine zusätzliche RNA-Polymerase-Bindungsstelle [die –10 Box (TAATAT) für die PrfA-unabhängige Transkription von P*aroA*P1] in dieser Region vorliegt. Außerdem konnte gezeigt werden, dass eine symmetrische Sequenz downstream der –10 Box (TTTAAT) die PrfA-abhängige Transkription vom *aroA*P2-Promotor stark inhibiert.

2. Introduction

2.1. Pathogenicity of *Listeria* species

The genus *Listeria* consists of a group of gram-positive, rod-shaped of 0.4 by 1 to 1.5 μm, nonsporulating, and facultative anaerobic bacteria. They belong to the group of low G+C content bacteria closely related to *Bacillus*, *Staphylococcus*, *Streptococcus, Clostridium* and *Enterococcus* (Sallen *et al*., 1996; Rocourt, 1999). *Listeria* spp. are widespread in nature and have been isolated from water, soil, a large variety of foods, plant, and the feces of humans and animals (Gray and Killinger, 1966; Weis and Seeliger, 1975; Watkins and Sleath, 1981; Schlech *et al*., 1983; Fleming *et al*., 1985; Linnan *et al*., 1988; Farber and Peterkin, 1991). Furthermore, they have capacity to adapt and survive in extreme environments such as high salt concentration (10% NaCl), a broad pH range (from 4.5 to 9.0) and a wide temperature range (between –1°C to 45°C), even long periods of drying and freezing with subsequent thawing (Junttila *et al*., 1988; Peel *et al*., 1988; Schuchat *et al*., 1991; Lou and Yousef, 1997).

Currently, the genus *Listeria* includes six species: *L. monocytogenes*, *L. ivanovii*, *L, seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* (Rocourt, 1999). Two of these species, *L. monocytogenes* and *L. ivanovii* are considered to be pathogens that can cause the infectious disease known as listeriosis. A third species, *L. seeligeri* is generally regarded as nonpathogenic, although it has been implicated in at least one case of human listeriosis (Rocourt *et al*., 1986). The other three are harmless saprophytes. *L. monocytogenes* is the major food-borne pathogen potentially lethal in humans and animals, while *L. ivanovii* often infects ungulates such as sheep and cattle. Human cases of *L. ivanovii* infection are rare (Sergeant *et al*., 1991; Alexander *et al*., 1992; Chand and Sadana, 1999; Ramage *et al*., 1999; Wesley, 1999).

In 1921, the first recorded culture of *L. monocytogenes* was isolated in France from a patient with meningitis (Dumont and Cotoni, 1921; Seeliger, 1988), whereas the first official discovery of *L. monocytogenes* was in 1924 in England (Murray *et al*., 1926). Infection with *L. monocytogenes* can cause a rare (normally 2-8 cases annually per million population in Europe and the United States) but very severe disease, listeriosis, with a mortality rate in humans of 20 to 30% or higher, despite early antibiotic treatment (Rocourt and Brosch, 1992; Tappero *et al*., 1995). Due to the ubiquitous occurrence of *L. monocytogenes* and its ability to grow at refrigeration temperature, it can cause a number of large outbreaks involving several hundreds of individuals (Bille, 1990; Schlech *et al*., 1983; Linnan *et al*., 1988; Schwartz *et al*., 1989; Dalton *et al*., 1997). The major source of infection is contaminated food, such as corn, chocolate, milk, shrimp, and rice salad (Schlech, 2000). The gastrointestinal tract is thought to be the primary site of infection (Dalton *et al*., 1997). The high-risk groups of listeriosis occurred in humans are pregnant women, newborns, the elderly (55 to 60 years and older) immunocompromised or debilitated adults with underlying diseases. The clinical symptoms range from flu-like illness, septicaemia, abortion, febrile gastroenteritis, granulomatous disease and meningoencephalitis (Vazquez-Boland *et al*., 2001).

The reasons that a majority research of the molecular pathogenesis of *L. monocytogenes* in the

past years are not only that *L. monocytogenes* is an important paradigm for immunological investigation, i.e. it has the potential as a live vaccine for the specific delivery of antigens to the cell-mediated immune system (Pan *et al*., 1995), but also it is an important model system for the study of intracellular pathogens. Although a number of intracellular pathogens are significant agents of serious human diseases, such as *Mycobacterium leprae* in Hanson`s disease and *Chlamydia trachomatis* for the sexually transmitted infection, they are not experimentally tractable. However, the infection of *L. monocytogenes* is easily reproducible in laboratory by a number of simple tissue culture systems as well as murine models have allowed rigorous analysis of different aspects of the pathogenesis (Shen *et al*., 1998).

2.2. Virulence of *L. monocytogenes*

2.2.1. The infection process of host cells by *L. monocytogenes*

The pathogenesis of *L. monocytogenes* can be traced by its ability to invade and grow intracellularlly in the host cells. The intracellular life cycle involves the following steps (Goebel *et al*., 2000; Kreft and Vazquez-Boland, 2001; Vazquez-Boland *et al*., 2001):

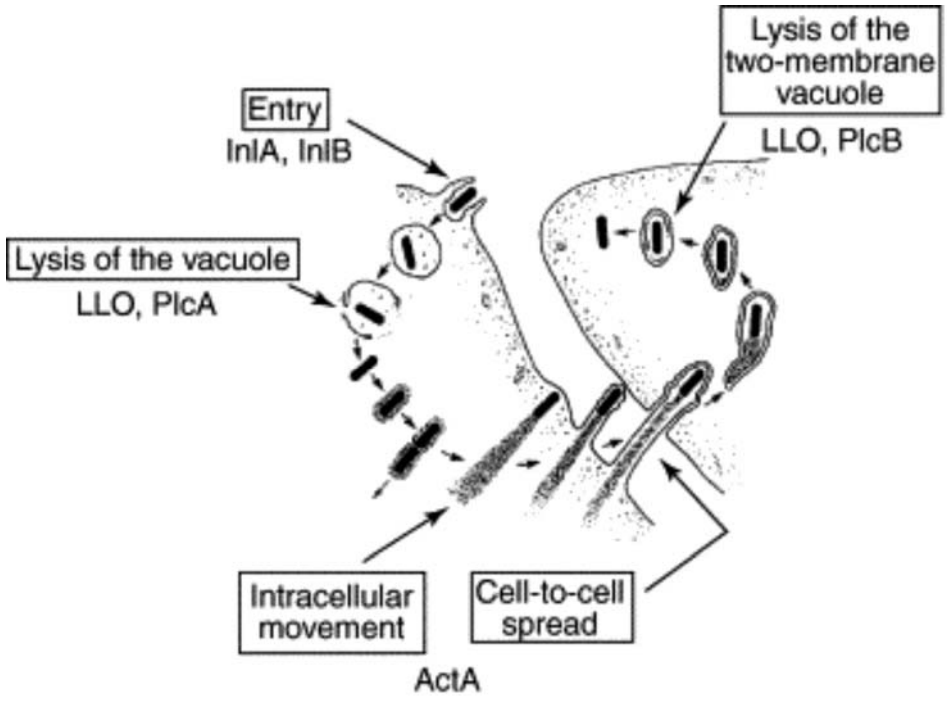


Fig. 1. The infection process of host cells by *Listeria monocytogenes* (from Tilney and Portnoy, 1989 and Cossart *et al*., 2003)

(i) Uptake of the bacterium, either by normal phagocytosis or the bacterium induces its own phagocytosis (e.g. in an epithelial or endothelial cell).

L. monocytogenes gets into host cells by the process of phagocytosis. Professional phagocytes, such as macrophages engulf bacteria, whereas epithelial and endothelial cells cannot normally phagocytize bacteria, rather they should be induced to do so. *Listeria* induced phagocytosis is triggered mainly by two surface proteins: Internalin A (InlA) and Internalin B

(InlB). InlA is the first well characterized protein that is required for uptake of *L. monocytogenes* into epithelial cell cultures, it recognizes and binds E-cadherin, a kind of host cellular receptor; whereas InlB activates the tyrosine kinase receptor Met, the receptor gC1qR and proteoglycans and plays a role in invasion of hepatocytes in the liver (Gaillard *et al*., 1991; Dramsi *et al*., 1995; Braun *et al*., 1997; Greiffenberg *et al*., 1998; Parida *et al*., 1998; Cossart *et al*., 2003). By binding host cell receptor, the host cellular signalling pathways and cytoskeletal reorganization mechanisms are subverted by *L. monocytogenes*, leading to bacterial entry into host cells (Schubert and Heinz, 2003). Additional bacterial factors were reported as being involved in this process, e.g. InlA-dependent internalization (in the absence of InlB) into non-phagocytic mammalian cells requires the support of other internalins, InlC and InlGHE (Bergmann *et al*., 2002).

(ii) Escape from the primary phagosome.

During invasion, *Listeria* is engulfed within a phagocytic vacuole (Gaillard *et al*., 1987). After 30 minutes, the bacteria begin to lyse the membrane of this vacuole and escape from it with the help of a pore-form bacterial toxin, listeriolysin-O (LLO) and two secreted phospholipases C, PlcA and PlcB. How LLO and these two phospholipases disrupt vacuolar membranes remains unknown.

(iii) Replication inside the cytosol, actin-based intracellular movement and cell to cell spread.

Once in the cytosol, bacteria multiply without upregulation of known listerial stress proteins (Hanawa, *et al*., 1995). Recent experimental evidence indicates that *L. monocytogenes* exploits hexose phosphates (HP) from the host cell as a source of carbon and energy for efficient intracellular growth. HP uptake is mediated by Hpt, a bacterial homolog of the microsomal glucose-6-phosphate translocase. Moreover, loss of Hpt results in impaired listerial intracytosolic proliferation and attenuated virulence in mice, it is thus the first virulence factor identified as being involved in the replication phase of a facultative intracellular pathogen (Chico-Calero *et al*., 2002).

The intracytoplasmic movements and cell-to-cell spread of bacteria are mediated by actin polymerization with the help of the listerial surface protein ActA (reviewed in Cossart, 2000; Vazquez-Boland *et al*., 2001), the two bacterial phospholipases (PlcA and PlcB) and metalloprotease (Mpl) play also a role in the cell-to-cell spread by lysing the double membraned vacuole formed after uptake by the neighbouring cells (Smith *et al*., 1995; Sokolovic *et al*., 1996; Marquis *et al*., 1997), which allows the bacteria to avoid both the humoral and cellular immune response.

2.2.2.Virulence gene organization and virulence factors

2.2.2.1. Central virulence gene cluster

Six of the virulence factors (PrfA, PlcA, Hly, Mpl, ActA and PlcB) playing essential roles in *Listeria* infection are encoded by a 9-kb virulence gene cluster localized on the chromosome of *L. monocytogenes* between the two house keeping genes *ldh* and *prs*, and referred now to

as LIPI-1 (Fig. 2; *Listeria* Pathogenicity Island I) (Portnoy *et al*., 1992; Kreft and Vazquez-Boland, 2001). This region is absent from the non-pathogenic species (Gouin *et al*., 1994) including:

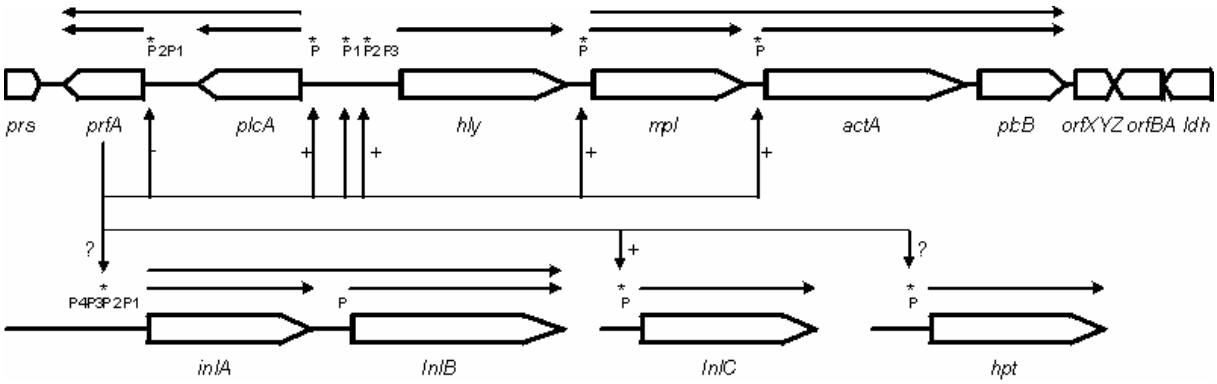


Fig. 2. The central gene cluster (LIPI-1) and other PrfA-regulated genes (from Kreft and Vazquez-Boland, 2001; Vazquez-Boland *et al*., 2001). P: promoter, an asterisk above means the presence of a PrfA-box within the promoter. Thin arrows above the gene symbols indicate the different transcripts. Arrows below with a (+) or (–) sign indicate transcriptional induction or repression by PrfA.

(i) *hly* encodes a cholesterol-binding, pore-forming hemolysin, or listeriolysin (LLO) responsible for the bacteria escape from the primary phagosomes of host cells into the host cytosol before they are killed in the phagolysosomes.

(ii) *plcA* encodes a phosphatidylinositol-specific phospholipase C (PlcA) and *plcB* encodes phosphatidylcholine phospholipase C with a broad substrate range (PlcB). Loss of either one of these genes causes very slight reductions in virulence. However, the virulence of *plcA-plcB* double mutant is reduced obviously in an intravenous mouse model.

(iii) *mpl* encodes a metalloprotease required for the proteolytic processing of PlcB into the mature form (Raveneau *et al*., 1992).

(iv) *actA* encodes the ActA protein that is one of key elements involved in the bacterial intracellular movement.

(v) *prfA* encodes the PrfA protein (Positive Regulatory Factor A), 27 kDa, known as the master regulator of virulence, which is discussed in detail in the following text.

The physical and transcriptional organization of this gene cluster is comprised of three units:

(i) the *hly* monocistron;

(ii) the lecithinase operon, containing the *mpl*, *actA*, and *plcB* genes; These genes transcribed either as *mpl-actA-plcB* transcript under the control of the *mpl* promoter or as shorter transcripts, *mpl*-transcript and *actA-plcB* transcript.

(iii) the *plcA-prfA* bicistron.

2.2.2.2. Internalin islands

Besides the central virulence gene cluster, some genes such as the *inlAB* operon and *inlC* encoding the protein products belonging to the internalin family are located outside this region (Fig. 2) and are also contributed to the virulence of *Listeria*. These internalin genes are clustered in *Listeria* chromosomal islands (Fig. 3). Except for *inlA* and *inlB* located on the *inlAB* operon, and *inlC* itself, the other internalin locus are found in different arrangements in two isolates of *L. monocytogenes* (EGD and LO28), i.e. three genes in the order *inlG*, *inlH* and *inlE* (operon *inlGHE*) in one strain and *inlC2*, *inlD* and *inlE* clustered in the other strain at the same position on the listerial chromosome as the *inlGHE*. Comparison of these two clusters reveals that *inlH* represents a recombination product of *inlC2* and *inlD*, (Dramsi *et al*., 1997), while *inlF* has been identified as separate gene in both strains (Raffelsbauer *et al.*, 1998).

The role of *inlA* is demonstrated by expression in the non-invasive bacterium *L. innocua*, which becomes able to invade Caco-2 cells and suggested that InlA protein may be sufficient for entry (Gaillard *et al*., 1991). The role of *inlB* is elucidated by deleting each of these two genes and testing the corresponding mutants in various cell lines (Dramsi *et al*., 1995, 1997; Ireton *et al*., 1996). These experiments show that InlB is also an invasion protein involved in entry into some hepatocyte-like cell lines, Hela cells, Vero cells, CHO cells and fibroblasts. The role of *inlC* in virulence remains unknown. Internalization and intracellular growth of *inlC* deletion mutant are found to be similar to that of wild-type when tests in several cell types *in vitro*. The *inlC* deletion mutant, however, results in significantly lower virulence in mice after infection by intravenous or oral route (Engelbrecht *et al*., 1996).

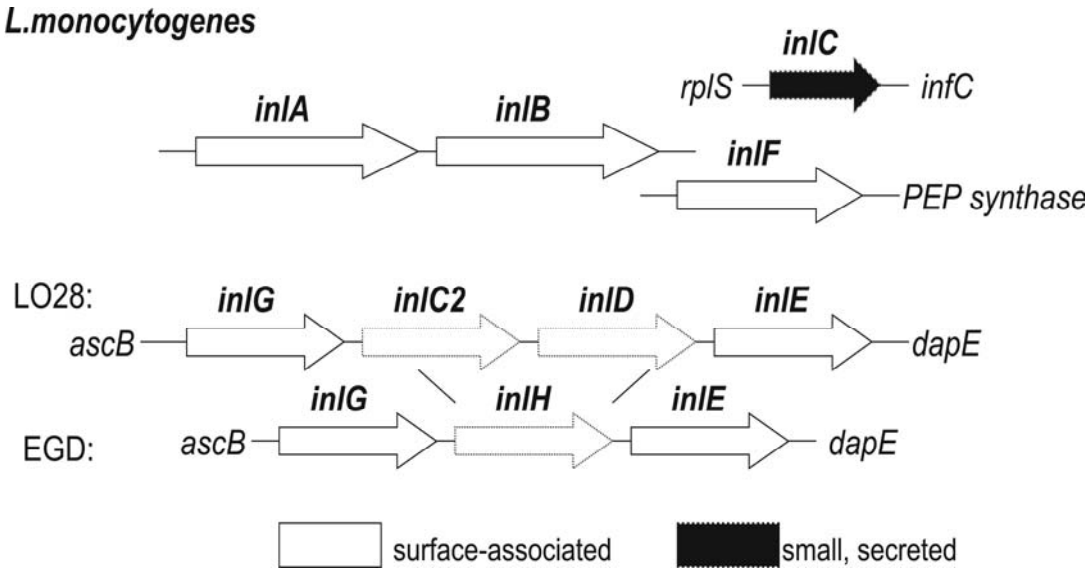


Fig. 3. The internalin islands (from Kreft and Vazquez-Boland, 2001; Vazquez-Boland *et al*., 2001)

All proteins of the internalin family share a common sequence motif, a leucine-rich repeat (LRR) domain, consisting of a tandem repeat arrangement of mostly 22 amino acids with leucine or isoleucine residues at positions 3, 6, 9, 11, 16, and 22 (xxLxxLxxLxLxxNxIxxI/LxxL). The LRR domain is assumed to be essential for specific protein-protein interaction (Marino *et al*., 2000). Except InlB and InlC, the C-termini of the

internalins (InlA, InlC2, InlD, InlE, InlF, InlG, and InlH) carry a LPXTG (Leu-Pro-X-Thr-Gly) motif, another conserved region, which anchors these proteins to the peptidoglycan of the cell wall (Gaillard *et al*., 1991; Dramsi *et al*., 1997; Raffelsbauer *et al*., 1998). InlB has a so-called GW (Gly-Trp) motif, which mediates its loose attachment to the lipoteichoic acid of the bacteria cell wall (Braun *et al*., 1997; Jonquieres *et al*., 1999). Only InlC has been identified to date in *L. monocytogenes* as a small secreted protein without any anchor sequences (Engelbrecht *et al*., 1996).

InlA and InlB, the two internalins best characterized in terms of structure and function, are encoded by an operon *inlAB*, the transcription of which is controlled by a complex regulatory region located upstream of *inlA* containing one PrfA-regulated promoter and several other promoters (Fig. 2; Dramsi *et al*., 1993; Lingnau *et al*., 1995; Sheehan *et al*., 1995; Bohne *et al*., 1996). The host cell receptor for InlA is E-cadherin (Mengaud *et al*., 1996). E-cadherin is a transmembrane glycoprotein containing five extracellular cadherin domains (EC1-EC5) and an intracytoplasmic domain. It regulates calcium-dependent cell-cell adhesion through homophilic interactions mediated by the EC1 and EC2 domains (Yap *et al*., 1998). InlA interacts through the LRR region and the first extracellular domain of E-cadherin (Lecuit *et al*., 1999), but entry is mediated by the intracytoplasmic domain of E-cadherin, which presumably leads to actin cytoskeleton rearrangement (Lecuit *et al*., 2000). InlB, in contrast to InlA, is attached loosely to the cell wall and released partially into the environment (Jonquieres *et al*., 1999). There are three receptors for InlB identified: a) gC1q-R, the cellular ligand of the globular part of the C1q complement fraction. The GW motif of InlB mediates specific binding to this receptor (Marino *et al*., 2002); b) Met, the tyrosine kinase receptor that physiologically serves as ligand for hepatocyte growth factor (HGF) (Shen, *et a*l., 2000). InlB interacts with the extracellular domain of Met through its LRR domain; c) glycosaminoglycans (GAGs), binds to InlB through LRR domain as with HGF and increases InlB-dependent activiation of Met (Jonquieres *et al*., 2001).

The experiment demonstrated recently that deletion of *inlGHE* enhanced the expression of InlA and InlB, and InlA by itself triggered invasion poorly and needs the support of other internalins for efficient internalization of *L. monocytogenes* by non-phagocytic mammalian cells (Bergmann *et al*., 2002). Although some molecular functions of internalins have been analysed using various combinations of in-frame deletions in the internalin genes, the true role of them in *Listeria* biology and pathogenesis is still not well understood.

2.2.2.3. Other virulence factors

In addition to the products of the major *Listeria* pathogenicity island (LIPI-1) and the internalin islands which participate directly in the pathogenicity of *Listeria*, other listerial proteins have been required in saprophytic life of bacteria.

(i) Protein p60, encoded by the *iap* (invasion associated protein) gene, is a murein hydrolase enzyme that catalyzes a reaction during the final stage of cell division of *L. monocytogenes*. This 60-kDa extracellular protein is present both on the cell surface and in the culture supernatant (Kuhn and Goebel, 1989; Ruhland *et al*., 1993). Some experimental evidences

indicate that it is important for phagocytosis of *L. monocytogenes* by some cell types (Kuhn and Goebel, 2000). The expression of *iap* is independent on PrfA and *iap* is normally used as an reference gene for the study of PrfA-dependent regulation of *L. monocytogenes* (Bubert *et al*., 1997 and 1999).

(ii) Clp proteases (caseinolytic protein), belong to a group of recently identified virulence-associated stress response proteins in *L. monocytogenes*, may be chaperones that assist in the proper refolding of proteins or assembly of proteins that can not be altered conformationally, ensuring the essential function of bacteria correctly under adverse environmental conditions (such as high or low pH, temperature, osmotic conditions). ClpC, a member of Clp family of HSP-100 stress proteins, encoded by *clpC*, is a general stress protein that aids in lysis of the phagosomal membrane and intracellular survival of *L. monocytogenes* (Rouquette *et al*., 1998). ClpC also modulates *inlA*, *inlB* and *actA* gene transcription (Nair *et al*., 1999) and is almost not found in a *L. monocytogenes prfA** mutant, in which the transcriptional regulator PrfA is overexpressed (Ripio *et al*., 1998), which indicates a direct expression crosstalk between virulence determinant and stress protein. ClpB, another member of the Clp-HSP100 family reported recently, is involved in the pathogenicity of *L. monocytogenes* since the *clpB* deletion mutant is significantly affected by virulence in a murine model of infection (Chastanet *et al*., 2004). ClpE acting like ClpC, is upregulated in a *clpC* mutant (Nair *et al*., 1999). ClpP serine protease is required for growth under stress conditions and has been shown to affect the activity of listeriolysin O (LLO) (Caillot *et al*., 2000).

There are still some other virulence factors identified to be involved in the listerial infection process in various specific ways, such as Hpt, a hexose phosphate transporter encoded by the *hpt* gene, which are required for efficient replication of *L. monocytogenes* in the cytosol of infected host cells (Chico-Calero *et al*., 2002).

2.3. Regulation of virulence gene expression

Regulation of virulence genes occurs in many pathogenic bacteria, in a fashion that allows the coordinate and differential expression of the virulence factors at the right time during the infection.

2.3.1. PrfA, the master regulator of virulence genes

PrfA is the only regulatory factor molecularly characterized up to now that is crucial for the virulence gene expression. All LIPI-1 genes (central gene cluster) and *inlC* (encodes a small secreted internalin) are strongly regulated by PrfA, whereas the *inlAB* operon is partially regulated by PrfA. Furthermore, the *hpt* gene encoding a hexose phosphate, which is required for efficient interacellular replication of *L. monocytogenes,* is identified recently to be a PrfA-dependent virulence gene (Chico-Calero *et al*., 2002).

All of these PrfA-regulated promoters possess 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. Binding affinity to PrfA-boxes is considered to be dependent on the number of nucleotide mismatches of the PrfA-boxes of PrfA-regulated

promoters, becoming more active at promoters that possess a perfectly symmetrical PrfA-box (*hly*/*plcA*) than at promoters with substitutions in the PrfA-box (*mpl* and *actA*) (Sheehan *et al*., 1995), which is so-called "PrfA-box hierarchy" model (Freitag *et al*., 1993; Freitag and Portnoy, 1994; Sheehan *et al*., 1995; Bubert *et al*., 1997: Ripio *et al*., 1997; Williams *et al*., 2000). The temporal and spatial expression of *Listeria* virulence genes seems to partially account for it: at first, when *L. monocytogenes* just adheres to and penetrates into the surface of host cell, PrfA is present at low to moderate levels and binds and activates transcription of promoters with high qualified PrfA-boxes (such as *hly*/*plcA* without a mismatch in their PrfA-boxes). Upon entry into the cytosol, PrfA protein synthesis increases to provide sufficient PrfA to occupy the promoters with low-affinity PrfA-boxes (such as *mpl*, *actA* and *inlC* with one or two mismatches in their PrfA-boxes). However, evidence has shown that sequences outside of this palindrome play also a role in the regulation of virulence gene expression, since the replacement of the PrfA-box of *actA* by *hly* did not improve transcription of *actA* (Williams *et al*., 2000).

On the basis of structural functional features, PrfA is clearly related to the Crp/Fnr family of transcriptional regulators (Lampidis *et al*., 1994). Crp/Fnr-like proteins play a key role in virulence gene regulation in bacterial, mammalian and plant pathogens (Goebel *et al*., 2000; Kreft *et al*., 1995; West *et al*., 1994). Crp (catabolite activator protein, named also CAP) is the major regulator in catabolite repression and Fnr regulates the cellular response to anaerobic growth conditions in *E. coli* (Spiro and Guest, 1990; Kolb *et al*., 1993). Comparison of Crp and PrfA (Herler *et al*., 2001), several functionally important features are found to be shared by each other, e.g. the N-terminal β-roll structures, the C-terminal helix-turn-helix (HTH) motif, and the activating regions AR (Fig. 4). The β-roll structure (amino acids 19 to 99) consists of series of short antiparallel β-sheets delimited by Gly residues. In Crp, this structure is required for binding of the cofactor cAMP. The Crp-cAMP complex recognizes and binds to 22 bp twofold-symmetric consensus sequence, 5´-AAATGTGATCTAGATCACATTT-3´, which is located at the position between -40 and -200 bp from the transcriptional start sites of Crp-regulated genes (Ebright, 1993; Busby and Ebright, 1994 and 1997). However, the addition of exogenous cAMP does not activate the PrfA-regulated transcription of *L. monocytogenes* (Vega *et al*., 1998) and accordingly, most of amino acids crucial for cAMP binding in Crp are not conserved in PrfA. Other two similarities in PrfA and Crp are alpha-helix D (amino acids 138 to 155) involved in transmission of the allosteric effect from cAMP-binding N-terminal domain to the DNA-binding C-terminal domain in Crp, and activation region 1 (amino acids 156 to 164) involved in Crp-RNA polymerase interaction. The C-terminal domain of Crp contains a helix-turn-helix (HTH) motif (amino acids 171 to 191), which mediates specific interaction of proteins with target DNA sequences. This domain has 70% similarity between PrfA and Crp. Moreover, direct experimental evidence by site-direct mutagenesis indicates that this region is essential for binding PrfA to DNA (Sheehan *et al*., 1996). There are two domains present only in PrfA, an additional putative HTH motif at its N-terminus (amino acids 8 to 27) and a putative leucine zipper motif at its extended C-terminus (amino acids 193 to 237), the loss of latter abolishes the activity of PrfA (Lampidis *et al*., 1994). However, the exact role of these two regions is not yet known.

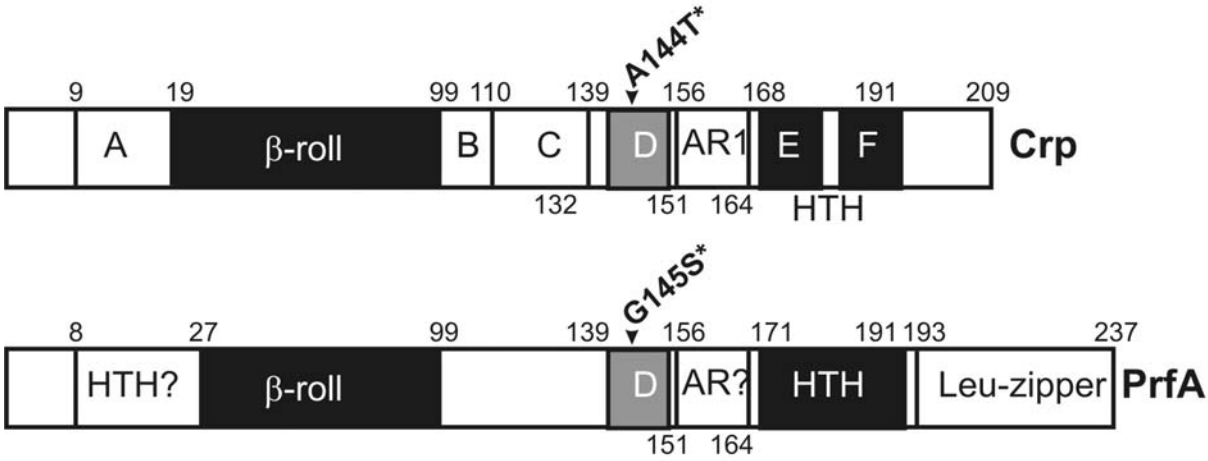


Fig. 4. Schematic comparison of Crp from *E. coli* and PrfA from *L. monocytogenes* (from Vazquez-Boland *et al*., 2001). Numbers below indicate the amino acids where the domain starts or ends. HTH: the helix-turn-helix motif; A-D: alpha helices in Crp and PrfA; β-roll structure: antiparallel β–sheets, cAMP-binding region in Crp from *E. coli*, similar structure in PrfA; AR1: activation region in Crp; AR?: domain with similarity to the activation region 1 of Crp from *E. coli*.

Convincing evidence placing PrfA into the Crp/Fnr family came from the characterization of the PrfA* mutant of *L. monocytogenes* (Ripio *et al*., 1996). This mutant protein carries a Gly145Ser substitution, which aligns well with Ala144Thr in Crp, such substitution resulting in a constitutive expression of Crp protein at the absence of cofactor cAMP (Kolb *et al*., 1993). Like Crp*, the Gly145Ser mutant (PrfA*) also increases the binding affinity of PrfA to the specific target DNA sequence and the expression of all PrfA-dependent virulence genes is induced to a high level (Bohne *et al*., 1996; Ripio *et al*., 1997), which suggests that PrfA may function via a cofactor-mediated allosteric transition mechanism similar to that of Crp.

The arrangement of the PrfA-box, a conserved 14 bp sequence of dyad symmetry (TTAACANNTGTTAA) in the PrfA-dependent promoters of *L. monocytogenes* located at position around –40 from the transcriptional start site, is similar to that of the Crp-dependent class II promoters. The Crp-dependent class I promoters of *E. coli* carry the Crp-binding site at variable positions from the transcriptional start site, while the location of Crp-binding site of the class II promoters, e.g., *gal*P1, is centred at position –41.5, thus overlapping the –35 promoter region (Ebright, 1993; Busby and Ebright, 1994 and 1997). The transcription activation at class I promoters requires a direct protein-protein contact between a surface-exposed β-turn in the downstream subunit of Crp (amino acids 154 to 164, AR1; Fig. 4) and a target in the RNA polymerase α subunit C-terminal domain (αCTD). Transcription activation at class II promoters is more complex than that of class I, involving two mechanistic components. The first one is protein-protein interaction between AR1 of the upstream subunit of the Crp dimer and αCTD of RNA polymerase to overcome an inhibitory of αCTD. The second component is "direct activation", mediated by protein-protein interaction between AR2 of the downstream subunit of Crp and RNA polymerase α subunit N-terminal domain (αNTD), which catalyzes the transition of the closed RNA polymerase-promoter complex into the transcription-competent open complex (Niu *et al*., 1996; Rhodius *et al*., 1997). The PrfA-dependent promoters resemble more closely to the class II Crp-dependent promoters not only due to the position of transcriptional activator binding site, but also based on the following

experimental evidences: DNase I footprinting studies have identified an approximately 26 bp region, beginning 10 bp upstream of the 14 bp palindrome (PrfA-box) and ending 2 bp drownstream, that is protected from DNase I digestion in the present of PrfA protein (Dickneite *et al*., 1998), Furthermore, electrophoretic mobility shift assays (EMSA) have shown that purified PrfA protein binding to its target sequence PrfA-box forms a faster migrating complex CIII. This complex can be shifted to the slower migrating complex CI after the addition of partially purified RNA polymerase from *L. monocytogenes*. The intermediately migrating complex CII consists of RNA polymerase bound to the promoter region, this complex can also be transformed into CI in the presence of purified PrfA. Competition experiments further showed that in EMSA, RNA polymerase less stably bound in the CII complex than in CI, indicating that PrfA is really necessary to mediate a strong and specific binding of RNA polymerase to the PrfA-regulated promoters. Binding of PrfA together with RNA polymerase in DNA footprint enlarged the protected region by PrfA at both upstream and downstream areas (Böckmann *et al*., 2000), which resembles also the situation known for the class II Crp-dependent promoters (Busby and Ebright, 1997). However, it is still not clear whether PrfA requires a cofactor for its activity, such as cAMP for transcriptional activation of Crp. It has been reported that an unknown component from PrfA-free extracts (obtained from a *prfA* deletion mutant of *L. monocytogenes* EGD) enhances the formation of a stable CI complex (Böckmann *et al*., 1996 and 2000; Dickneite *et al*., 1998).

2.3.2. Regulatory mechanism of PrfA

While it is clear that PrfA is a key regulatory element required for the control of virulence gene expression in *L. monocytogenes*, it is not clear how PrfA regulates gene expression. The transcription of the *prfA* gene and hence the cellular level of PrfA is shown to be a complex regulation circuit. *prfA* is transcribed in two ways: a) Transcription from two promoters of the *prfA* gene, *prfA*P1 and *prfA*P2 leads to monocistronic transcripts of 0.9 and 0.8 kb, respectively. This transcription seems to be negatively regulated by PrfA, as the amount of transcripts is significantly increased in the absence of functional PrfA (Freitag *et al*., 1993); b) Transcription from a promoter located in front of *plcA* generates a bicistronic *plcA-prfA* transcript, which is activated by PrfA (Camilli *et al*., 1993). PrfA positively regulates its own expression through the activation of *plcA* transcription, and the increase in PrfA synthesis resulting from the generation of the *prfA-plcA* transcript is essential for full virulence (Camilli *et al*., 1993; Freitag *et al*., 1993; Freitag and Portnoy, 1994). As recently shown (Johansson *et al*., 2002) the non-transcribed 5´-region of the mRNA starting at *prfA*P1 can fold into a secondary structure which strongly influences translation of this *prfA* transcript. At temperatures below 30°C no translation of this transcript occurs, whereas at 37°C PrfA is translated.

Fundamental functional similarity between PrfA and Crp protein leads to a proposal that PrfA has two functional forms, inactive and active, and can shift from one to the other when binds to a cofactor, in similar way as cAMP to Crp protein (Ripio *et al*., 1997; Vega *et al*., 1998). Evidence for the existence of this cofactor is still under investigation. Furthermore, there is evidence for the interaction of PrfA with other listerial factors which seem to modulate the

activity of this regulatory factor (Böckmann *et al*., 1996, 2000; Dickneite *et al*., 1998; Vazquez-Boland *et al*., 2001). Mutations within specific regions of PrfA render this transcriptional regulator permanently active and external parameters do no longer modulate its activity (Vega *et al*., 1998) suggesting that these mutations may alter the conformation of PrfA in a way that is similar to alterations in structure resulting from co-factor binding, as has been described for the analogous substitutions in CRP (Kolb *et al*., 1993).

2.3.3. Environmental parameters affecting virulence gene expression

A number of studies have shown that the expression of the *Listeria* virulence genes is significantly influenced by temperature, pH, carbon source and various stress conditions.

Listeria can survive and grow at low temperature, but below 20°C, the expression of the PrfA-regulated virulence genes is strongly inhibited, however, it will be recovered at 37°C such as in the warm-blooded host and the expression of *hly* and *actA* are even induced at heat shock temperature (Leimeister-Wächter *et al*., 1992; Sokolovic *et al*., 1993). As already mentioned the non-transcribed 5′-region of the mRNA starting at *prfA*P1 can fold into a secondary structure which strongly influences translation of *prfA* transcripts. At temperature below 30°C no translation of this transcript occurs whereas at 37°C PrfA is translated (Johansson *et al*., 2002).

High concentration of iron is probably required for invasiveness of *L. monocytogenes* by Caco-2 cells (Conte *et al*., 1996), while low iron seems to induct the expression of *actA* (Conte *et al*., 2000).

Starvation condition, e.g., incubation of *L. monocytogenes* in minimal essential medium (MEM), induces most of PrfA-regulated genes (Sokolovic *et al*., 1993 and Milenbachs *et al*., 1997), moreover, the transcription of the *prfA* gene is also enhanced in the brain heart infusion medium (BHI) containing activated charcoal (Ripio *et al*., 1996).

Different carbon sources have different influences on the virulence of *L. monocytogenes*. Cellobiose and the beta-glucoside arbutin have been described to repress the expression of *hly* and *plcA* (Park and Kroll, 1993; Park, 1994; Brehm *et al*., 2001), whereas hexose phosphate utilization stimulates the growth of *L. monocytogenes* without causing virulence gene repression and a PrfA-dependent gene *hpt* encoding a sugar phosphate transporter is responsible for this phenotype (Ripio *et al*., 1997; Chico-Calero *et al*., 2002). When *L. monocytogenes* is grown in the presence of utilizable sugars, expression of its virulence genes is downregulated (Milenbachs *et al*., 1997; Renzoni *et al*., 1997). However, sugars do not affect the level of the PrfA protein, the positive regulator of virulence determinants in *L. monocytogenes*. Based on these results, Milenbachs *et al*. proposed that regulation of virulence genes by sugars may represent an aspect of global catabolite control and could occur by modifying the activity of PrfA. This is consistent with the observation that a mutation in PrfA (PrfA*) results in the deregulated expression of *hly* in the presence of utilizable sugars and other environmental factors (Behari and Youngman, 1998; Ripio *et al*., 1997). Furthermore, analysis of the molecular basis of the observed strong growth inhibition in glucose-containing minimal media of *L. monocytogenes* over-expressing PrfA and

especially PrfA* by comparing the gene expression patterns with the help of whole genome microarrays suggest interference of PrfA(*) with the catabolite repression system (A.K. Marr *et al*., manuscript submitted).

2.4. Aims

In the past years, several *L. monocytogenes* genes involved in the various steps of the intracellular infection cycle have been identified. The regulation of most of the virulence genes is intimately connected with the regulator protein PrfA. By the complete genomic sequence of *L. monocytogenes* EGDe and transcriptome analyses recently published, a large number of additional genes were identified which could be possibly regulated by PrfA. The primary goal of this doctoral work was to reveal the essential features of the typical PrfA-dependent gene promoter, thus contributing to a better understanding of the fine regulation of PrfA-dependent transcription of virulence genes. By *in vitro* transcription analysis of several newly identified putatively PrfA-regulated genes of *L. monocytogenes* suggests a more indirect interaction of PrfA with other global regulatory circuits, such as catabolite repression system and sigma B regulon was demonstrated. Finally, a series of hybrid promoters were constructed in which corresponding elements of a PrfA-dependent promoter (P*plcA*) and a PrfA-independent promoter (P*aroA*) with sequence similarities to a PrfA-dependent promoter were excnanged. The obtained data suggest that primary but also secondary structures in the promoter region are essential for transcription initiation from a PrfA-dependent promoter.

3. Materials and Methods

3.1. Bacteria strains and culture conditions

L. monocytogenes EGDe came from S.H.E. Kaufmann. P14 is an *L. monocytogenes* wild-type strain of serovar 4b and P14a is *prfA** mutant of P14 (Gly145Ser). *L. monocytogene* EGD is wild-type of serovar 1/2a and Δ*prfA*EGD is its deletion mutant of *prfA*. The various mutants of *L. monocytogenes* such as Δ*aroA*EGDe, Δ*aroA*Δ*prfA*EGDe were constructed by J. Stritzker (PhD work). All *L. monocytogenes* stains were grown in brain heart infusion (BHI) broth (Difco) or minimal medium (MEM) for overnight at 37°C with vigorous shaking (190 rpm/min).

E. coli DH5α used for cloning was purchased from Gibco BRL and cultured with single clone in Luria-Bertani (LB) medium or YT medium for about 16 hours at 37°C with vigorous shaking (190 rpm/min).

3.2. Plasmids

Plasmid pUC18 used for cloning contains a multiple cloning site, Amp^r and *ori*E1 for replication in *E. coli*. Shuttle plasmid pUNK1 with Em^r was obtained from S. Pilgrim (PhD work). Other plasmids used for *in vitro* transcription and β-galactosidase activity assay are listed as follows:

(i) Plasmids used for *in vitro* transcription of various gene promoters of *L. monocytogenes*:

Name	Character	Sources
P*hly*	pUC18 x Promoter of *hly*	M. Lalic-Mülthaler
P*plcA*	pUC18 x Promoter of *plcA*	J. Bohne
P*actA*	pUC18 x Promoter of *actA*	J. Bohne
P*hpt*	pUC18 x Promoter of *hpt*	F. Engelbrecht
P*inlC*	pUC18 x Promoter of *inlC*	This work
P*aroA*	pUC18 x Promoter of *aroA*	A.K. Marr
P*lmo2420*	pUC18 x Promoter of *lmo2420*	F. Engelbrecht
P*lmo0178*	pUC18 x Promoter of *lmo0178*	S. Müller-Altrock
P*lmo0788*	pUC18 x Promoter of *lmo0788*	This work
P*lmo0596*	pUC18 x Promoter of *lmo0596*	M. Rauch
P*lmo2219*	pUC18 x Promoter of *lmo2219*	This work
P*lmo0278*	pUC18 x Promoter of *lmo0278*	This work
P*lmo2067*	pUC18 x Promoter of *lmo2067*	This work
P*lmo2840*	pUC18 x Promoter of *lmo2840*	This work
P*plcA*-IS20	pUC18 x P*plcA* interspace mutant	This work
P*plcA*-IS21	pUC18 x P*plcA* interspace mutant	This work
P*plcA*-IS23	pUC18 x P*plcA* interspace mutant	This work
P*plcA*-IS24	pUC18 x P*plcA* interspace mutant	This work

P*lmo2420*+AG	pUC18 x P*lmo2420* interspace mutant	This work
P*lmo2840*-IS22	pUC18 x P*lmo2840* interspace mutant	This work
P*lmo2840*-pa	pUC18 x P*lmo2840* mutant of exchange T, 6 bp downstream from the putative –10 box by A	This work
P*lmo2840*-IS22pa	pUC18 x P*lmo2840* mutant with interspace 22 bp and in addition replacement of T 6 by A	This work

(ii) Plasmids used for *in vitro* transcription of altered *inlC* promoter mutants:

Name	**Character**	**Sources**
P*inlC*-m1	Change of the putative –35 box TTTAAA to TTGACA	This work
P*inlC*-m2	Change of the putative –35 box TTTAAA to GGGAAA	This work
P*inlC*-m3	Change of the putative –35 box TTTAAA to CCCGGG	This work
P*inlC*-m4	Deletion of the original start nucleotide G (ΔG)	This work
P*inlC*-m5	Deletion of the original –10 box (ΔTAACA**)** and in addition ΔG	This work
P*inlC*-m6	Change of the putative –35 box TTTAAA to TTGACA and in addition ΔTAACA (–10 box) and ΔG	This work
P*inlC*-m7	Deletion of 5 bp in the interspace region between the PrfA-box and the –10 box **(**ΔCTTAT) and in addition ΔG	This work
P*inlC*-m8	Deletion of 10 bp in the PrfA-box (ΔTTAACGCTTG**)**	This work
P*inlC*-m9	ΔTTAACGCTTG and ΔG	This work
P*inlC*-m10	ΔTTAACGCTTG and ΔCTTAT	This work
P*inlC*-m11	ΔTTAACGCTTG and ΔCTTAT and ΔG	This work
P*inlC*-m12	Deletion of 8 bp (ΔTTAACGCT**)** in the PrfA-box and ΔCTTAT	This work
P*inlC*-m13	ΔTTAACGCT and ΔCTTAT and ΔG	This work
P*inlC*-m14	Deletion of 1 bp in the interspace region (ΔC) to 21 bp	This work
P*inlC*-m15	Deletion of 2 bp in the interspace region (ΔTC) to 20 bp	This work
P*inlC*-m16	Insertion of 1 bp (G) into the interspace region to 23 bp	This work
P*inlC*-m17	Insertion of 2 bp (TC) into the interspace region to 24 bp	This work
P*inlC*-m18	Change of G5 to A5 and in addition change of the original –10 box TAACAT to TATAAT	This work

P*inlC*-m19	Deletion of 9 bp in the pseudo PrfA-box (ΔCAGCGTTTG)	This work
P*inlC*-m20	Deletion of 3 bp in the pseudo PrfA-box (ΔGCG) converting the pseudo PrfA-box into a new perfect PrfA-box	This work
P*inlC*-m21	ΔTTAACGCTTG (PrfA-box) and in addition ΔGCG (converting pseudo PrfA-box to perfect one)	This work
P*inlC*-m22	ΔTTAACGCTTG (PrfA-box) and ΔGCG (converting pseudo PrfA-box to the perfect one) and change TTTTGT to TATAAT, 23bp downstream from the modified PrfA-box	This work
P*inlC*-m23	ΔTTAACGCTTG (PrfA-box) and ΔTAACAT (–10 box) and ΔGCG (converting pseudo PrfA-box to the perfect one) and change TTTTGT to TATAAT, 23 bp downstream from the modified PrfA-box	This work
P*inlC*-m24	ΔTTAACGCTTG (PrfA-box) and ΔTAACAT (–10 box) and ΔGCG (converting pseudo PrfA-box to the perfect one) and change TTTTGT to TATAAT, 23 bp downstream from the modified PrfA-box and in addition change C (5 bp downstream from TATAAT) to G	This work
P*inlC*-m25	On the basis of P*inlC*-m24, change CT to TG (construction of an extented –10 box for the perfect PrfA-box)	This work
P*inlC*-m26	ΔTTAACGCTTG (PrfA-box) and ΔTAACAT (–10 box) and ΔGCG (converting pseudo PrfA-box to the perfect one) and change TTTTGT to TATAAT, 23 bp downstream from the modified PrfA-box and in addition change G (1 bp downstream from TATAAT) to A	This work
P*inlC*-m27	ΔTTAACGCTTG (PrfA-box) and ΔTAACAT(–10 box) and ΔGCG (converting pseudo PrfA-box to the perfect) and change CT to TG (construction of an extented –10 box for the perfect PrfA-box) and change TTTTGT to TATAAT, 23 bp downstream from the modified PrfA-box and in addition change C (5 bp downstream from TATAAT) to G	This work

(iii) Plasmids used for *in vitro* transcription of P*plcA*-P*aroA* hybrid mutants:

Name	Character	Sources
P*plcA*-P*aroA*-m1	Exchange of the PrfA-box of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m2	Exchange of the –10 box of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m3	Exchange of the PrfA-box and the –10 box of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m4	Exchange of the PrfA-box, interspace sequences and the –10 box of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m5	Exchange of the PrfA-box, interspace sequences and the –10 box of P*plcA* by that of P*aroA*P2 and in addition change of the –10 box of P*aroA*P1 TAATAT to TAATGC	This work
P*plcA*-P*aroA*-m6	Exchange of the upstream and downstream fragments of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m7	Exchange of the interspace region of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m8	Deletion of the –35 box of P*aroA*P1 (ΔTTGTAA)	This work
P*plcA*-P*aroA*-m9	Exchange of the upstream region of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m10	Exchange of the downstream region of P*plcA* by that of P*aroA*P2	This work
P*plcA*-P*aroA*-m11	Exchange of the upstream region of P*aroA*P2 by that of P*plcA* and in addition change of –10 box of P*aroA*P1 TAATAT to TAATGC	This work
P*plcA*-P*aroA*-m12	Change of –10 box of P*aroA*P1 TAATAT to TAATGC and insertion of G into interspace region of P*aroA*P2 to 22 bp	This work
P*plcA*-P*aroA*-m14	Exchange of the downstream region of mutant P*plcA*-P*aroA*-m12 by that of P*plcA*	This work
P*aroA*-ΔGCG	Deletion of GCG from the downstream region of P*aroA*P2	A.K. Marr
P*aroA*+G	Insertion of G into the interspace region of P*aroA*P2 to 22 bp	This work
P*aroA*+A	Insertion of G into the interspace region of P*aroA*P2 to 22 bp	This wok

(iv) Plasmids used for β-galactosidase activity assays:

Name	Character	Sources
P*inlC*-*lacZ*	pUNK1 x *inlC* promoter- *lacZ* gene fusion	This work
P*inlC*-m8-*lacZ*	pUNK1 x P*inlC*-m8- *lacZ* gene fusion	This work

P*inlC*-m14-*lacZ*	pUNK1 x P*inlC*-m14-*lacZ* gene fusion	This work
P*inlC*-m15-*lacZ*	pUNK1 x P*inlC*-m15-*lacZ* gene fusion	This work
P*inlC*-m16-*lacZ*	pUNK1 x P*inlC*-m16-*lacZ* gene fusion	This work
P*inlC*-m17-*lacZ*	pUNK1 x P*inlC*-m17-*lacZ* gene fusion	This work

3.3. Oligonucleotides and primers

All primers were procured from Sigma-ARK GmbH and dissolved in dH_2O to a final concentration of 50 pmol/µl. For the sequencing reaction, the primer was diluted in dH_2O to a final concentration of 5 pmol/µl. Primers were stored at –20°C.

1) Primers used for construction of plasmids (i) indicated in 3.2. in this work:

Plasmid Name	**The Sequence of Primer (5`→3`)**	**Templates**
P*inlC*	CATTGTTGCGGCGGTACCTTACTTCTTATAC CATTGCTATTACTGCAGTTTGTAACCAATT	EGDe chr. DNA
P*lmo0788*	CAGTATGGTACCTGATTTTAGGTATAG CTGCATGAAGCTTTTTCTCATCCCTTTC	EGDe chr. DNA
P*lmo2219*	CCGGTAGGTACCTATTATTACTATCTG CACCGCAAGCTTCTAGACTGAACAATG	EGDe chr. DNA
P*lmo0278*	GGATTTGGTACCAAAGAATTAAGC GGCGCAAGCTTGTTCATTACTTTACC	EGDe chr. DNA
P*lmo2067*	CTAAAGTAACACGTTCCGCTCTAC CCATCACTGCAGCAATACCAATAAGTG	EGDe chr. DNA
P*lmo2840*	GCACGGGTACCGCAAACGCAACAACG GCTCCTTCTTCTAGATAGAAC	EGDe chr. DNA
P*plcA*-IS20	CAAATGTTAATGCCTCAATAAAAGTCACTTTA AG CTTAAAGTGACTTTTATTGAGGCATTAACATT TG	P*plcA* plasmid
P*plcA*-IS21	CAAATGTTAATGCCTCAAATAAAAGTCACTTT AAG CTTAAAGTGACTTTTATTTGAGGCATTAACAT TTG	P*plcA* plasmid
P*plcA*-IS23	CAAATGTTAATGCCTCAACTATAAAAGTCACT TTAAG CTTAAAGTGACTTTTATAGTTGAGGCATTAAC ATTTG	P*plcA* plasmid
P*plcA*-IS24	CAAATGTTAATGCCTCAACTGATAAAAGTCAC TTTAAG CTTAAAGTGACTTTTATCAGTTGAGGCATTAA CATTTG	P*plcA* plasmid
P*lmo2420*+AG	CAATTCTTAAAAGTTTCTAGTTCCGCGTGATT	P*lmo2420* plasmid

	TATG CATAAATCACGCGGAATAGAAACTTTTAAGAA TTG	
P*lmo2840*-IS22	CTTTTAAAAAAATGTARGACTCAGTTATTTAA AATCGGC GCCGATTTTAAATAACTGAGTCATACATTTTT TTAAAAG	P*lmo2840* plasmid
P*lmo2840*-pa	GTTATTTAAAATCGGCTATTGAGATAGTGC GCACTATCTCAATAGCCGATTTTAAATAAC	P*lmo2840* plasmid
P*lmo2840*-IS22pa	GTTATTTAAAATCGGCTATTGAGATAGTGC GCACTATCTCAATAGCCGATTTTAAATAAC	P*lmo2840*-IS22 plasmid

2) Primers used for construction of plasmids (ii) indicated in 3.2. in this work:

Plasmid Name	**The Sequence of Primer (5`→3`)**	**Templates**
P*inlC*-m1	CGCTTGTTAATTGACACATCTCTTATTTTTGC GCAAAAATAAGAGATGTGTCAATTAACAAGCG	P*inlC*
P*inlC*-m2	CGCTTGTTAAGGGAAACATCTCTTATTTTTGC GCAAAAATAAGAGATGTTTCCCTTAACAAGCG	P*inlC*
P*inlC*-m3	TTAACGCTTGTTAACCCGGGCATCTCTTATTTTTGC GCAAAAATAAGAGATGCCCGGGTTAACAAGCGTTAA	P*inlC*
P*inlC*-m4	GCTAACATATAATATACAAAGGGAC GTCCCTTTGTATATTATATGTTAGC	P*inlC*
P*inlC*-m5	CATCTCTTATTTTTGCTATAATATACAAAGGGAC GTCCCTTTGTATATTATAGCAAAAATAAGAGATG	P*inlC*-m4
P*inlC*-m6	CGCTTGTTAATTGACACATCTCTTATTTTTGC GCAAAAATAAGAGATGTGTCAATTAACAAGCG	P*inlC*-m5
P*inlC*-m7	GTTAATTTAAACATCTTTTTGCTAACATATAATATAC GTATATTATATGTTAGCAAAAAGATGTTTAAATTAAC	P*inlC*-m4
P*inlC*-m8	CTGATTTTCGATTATTATTAATTTAAACATCTC GAGATGTTTAAATTAATAATAATCGAAAATCAG	P*inlC*
P*inlC*-m9	CTGATTTTCGATTATTATTAATTTAAACATCTC GAGATGTTTAAATTAATAATAATCGAAAATCAG	P*inlC*-m4
P*inlC*-m10	GTTAATTTAAACATCTTTTTGCTAACATATAAG CTTATATGTTAGCAAAAAGATGTTTAAATTAAC	P*inlC*-m8
P*inlC*-m11	GTTAATTTAAACATCTTTTTGCTAACATATAATATAC GTATATTATATGTTAGCAAAAAGATGTTTAAATTAAC	P*inlC*-m9
P*inlC* -m12	CTGATTTTCGATTATTATGTTAATTTAAACATC GATGTTTAAATTAACATAATAATCGAAAATCAG	P*inlC*-m10
P*inlC*-m13	CTGATTTTCGATTATTATGTTAATTTAAACATC GATGTTTAAATTAACATAATAATCGAAAATCAG	P*inlC*-m11
P*inlC*-m14	CTTGTTAATTTAAACATCTTTATTTTTGCTAAC GTTAGCAAAAATAAAGATGTTTAAATTAACAAG	P*inlC*

P*inlC*-m15	CTTGTTAATTTAAACATCTTATTTTTGCTAAC GTTAGCAAAAATAAGATGTTTAAATTAACAAG	P*inlC*
P*inlC*-m16	GTTAATTTAAACATCTCTCTATTTTTGCTAACATATAAG CTTATATGTTAGCAAAAATAGAGAGATGTTTAAATTAAC	P*inlC*
P*inlC*-m17	GTTAATTTAAACATCTCTCTTATTTTTGCTAACATATAAG CTTATATGTTAGCAAAAAZAAGAGAGATGTTTAAATTAAC	P*inlC*
P*inlC*-m18	CTCTTATTTTTGCTATAATATAAATATACAAAGGGAC GTCCCTTTGTATATTTATATTATAGCAAAAATAAGAG	P*inlC*-m4
P*inlC*-m19	GGGACATAAAAAGGTTAATTAAATAGGAAGTATATG CATATACTTCCTATTTAATTAACCTTTTTATGTCCC	P*inlC*
P*inlC*-m20	GATAAAAAGGTTAACATTTGTTAAATAGGAAG CTTCCTATTTAACAAATGTTAACCTTTTTATG	P*inlC*
P*inlC*-m21	GATAAAAAGGTTAACATTTGTTAAATAGGAAG CTTCCTATTTAACAAATGTTAACCTTTTTATG	P*inlC*-m8
P*inlC*-m22	GAAAATCCTCTATAATGTTTCTAAATT ATTTTAGAAACATTATAGAGGATTTTC	P*inlC*-m21
P*inlC*-m23	CTCTTATTTTTGCATAAGTATACAAAG CTTTGTATACTTATGCAAAAATAAGAG	P*inlC*-m22
P*inlC*-m24	CCTCTATAATGTTTGTAAATTTATTTTTAAG CTTAAAAATAAATTTACAAACATTATAGAGG	P*inlC*-m23
P*inlC*-m25	GGAAGTATATGAAAATCTGCTATAATGTTTG CAAACATTATAGCAGATTTTCATATACTTCC	P*inlC*-m24
P*inlC*-m26	GAAAATCCTCTATAATATTTCTAAATTTATTTTTAAGG CCTTAAAAATAAATTTAGAAATATTATAGAGGATTTTC	P*inlC*-m23
P*inlC*-m27	GAAAATCTGCTATAATATTTGTAAATTTATTTTTAAGG CCTTAAAAATAAATTTACAAATATTATAGCAGATTTTC	P*inlC*-m25

3) Primers used for construction of plasmids (iii) indicated in 3.2 in this work:

Plasmid Name	**The Sequence of Primer (5`→3`)**	**Templates**
P*plcA*-P*aroA*-m1	CTTTATCGTCGTTAAAACATGTTAATGCCTC AAC GTTGAGGCATTAACATGTTTTAACGACGATA AAG	P*plcA*
P*plcA*-P*aroA*-m2	CATAAAAGTCACTTTTTAATAGGAATATACT AATC GATTAGTATATTCCTATTAAAAAGTGACTTT TATG	P*plcA*
P*plcA*-P*aroA*-m3	CTTTATCGTCGTTAAAACATGTTAATGCCTC AAC GTTGAGGCATTAACATGTTTTAACGACGATA AAG	P*plcA*-P*aroA*-m2
P*plcA*-P*aroA*-m4	ATTAGTGTTTACATTATTCACTTTAATAGGA AT	P*plcA* and P*aroA*+G

P*plcA*-P*aroA*-m5	CGTTAAAACATGTTAATGCTAGTGTTTACAT TATTC GAATAATGTAAACACTAGCATTAACATGTTT TAACG	P*plcA*-P*aroA*-m4
P*plcA*-P*aroA*-m6	TTGAGGCATTAACATTTGTTAATCAAATTAC AAGC CAAATGTTAATGCCTCAACATAAAAGTCACT TTAAG CTTAAAGTGACTTTTATGTTGAGGCATTAAC ATTTG CATAAAAGTCACTTTAAGATGCTTAAAAATT AAGTATAAAAG	P*plcA* and P*aroA*-ΔGCG
P*plcA*-P*aroA*-m7	CTTTATCGTCGTTAACAAATGTTAATATTAG TG CACTAATATTAACATTTGTTAACGACGATAA AG GTTTACATTATTCACTAAGATAGGAATATAC TAATC GATTAGTATATTCCTATCTTAGTGAATAATG TAAAC	P*plcA*-P*aroA*-m4
P*plcA*-P*aroA*-m8	GAAAAACACATTATCTGCTTTGATTTAAAAC ATG CATGTTTTAAATCAAAGCAGATAATGTGTTT TTC	P*aroA*
P*plcA*-P*aroA*-m9	TTGAGGCATTAACATTTGTTAATCAAATTAC AAGC CAAATGTTAATGCCTCAACATAAAAGTCACT TTAAG	P*plcA* and P*aroA*
P*plcA*-P*aroA*-m10	CATAAAAGTCACTTTAAGATGCTTAAAAATT AAGTATAAA CTTAAAGTGACTTTTATGTTGAGGCATTAAC ATTTG	P*plcA* and P*aroA*-ΔGCG
P*plcA*-P*aroA*-m11	GAATAATGTAAACACTAGCATTAACATGTTT TAACG CGTTAAAACATGTTAATGCTAGTGTTTACAT TATTC	P*plcA*-P*aroA*-m5 and P*plcA*
P*plcA*-P*aroA*-m12	GATTTAAAACATGTTAATGCTAGTGTTTACA TTATTC GAATAATGTAAACACTAGCATTAACATGTTT TAAATC	P*aroA*+G
P*plcA*-P*aroA*-m14	CATTATTCACTTTAATAGGAATATACTAATC GATTAGTATATTCCTATTAAAGTGAATAATG	P*plcA*-P*aroA*-m12 and P*plcA*
P*aroA*+G	CATGTTAATATTAGTGTTTACATTATTCAC GTGAATAATGTAAACACTAATATTAACATG	P*aroA*

P*aroA*+A	CATGTTAATATTAGTATTTACATTATTCAC GTGAATAATGTAAATACTAATATTAACATG	P*aroA*

4) Primers used for construction of plasmids (iv) indicated in 3.2. in this work:

The promoterless *lacZ* was amplified from *E. coli* W3110 genomic DNA with the primer A: 5′-GTGGAGAATGTTGAAAATGACCATGATTACGG-3′ and the primer B 5′-AAAAAACCCGGGTTATTTTTGACACCAGACC-3′ for construction of P*inlC* or P*inlC* mutants fusion plasmids. The DNA fragment containing the *inlC* promoter region (involving the rbs and the translation start site of the *inlC*) or its altered mutants was amplified from the correspondent plasmids with the primer C: 5′-AGCAGACAACCCCGGGAGGTAGAACATGTTTTG-3′ and the primer D: 5′-CCGTAATCATGGTCATTTTCAACATTCTCCAC-3′ (the single underlined regions correspond to the *inlC* promoter sequences, the double underlined regions to the *lacZ* sequences and the dotted underlined regions to the *Cfr*9I site)

5) Primers used for primer extension experiments:

Primer Name	**The Sequence of Primer (5`→3`)**
PE-P*inlC*	TTTCAACATTCTCCACTCC
PE-P*plcA*	CTAATGGGAAAGTAAAAAAG
PE-P*hly*	GCCAAATACCGTTTGCCACCCCTC
PE-P*actA*	GAACAAGGAAAATTCGGCCTTC
PE-P*hpt*	CGATGAGTATTGTTACGC
PE-P*aroA*	ACCATTTAACTTCCACCCTTC
PE-P*lmo0788*	GTTACGCACTACTTAATATGTATATG
PE-P*lmo2219*	CTGAACAATGCCATCATCATGACAAG
PE-P*lmo2067*	CGGCGTAACAACCACAACTTC
PE-P*lmo2840*	GACTTTTCCATTTCCTTAATATC
PE-P*lmo0178*	GGGTTTATGTCTTTAATTAAATC
PE-P*lmo2420*	CTGCAGGTCGACTCTAGAGGATCCC
PE-P*lmo0596*	CCGCTAATAAAACAAGAATTCGTG

3.4. Media

3.4.1. Liquid medium and agar plates for *E. coli* culture

2xYT Medium	bacto trypton	16 g
	yeast extract	10 g
	NaCl	5 g

These were dissolved in 1000ml dH_2O and autoclaved at 121°C, 15psi for 20 minutes.

1xYT Agar Plate	bacto trypton	8 g
	yeast extract	5 g
	NaCl	5 g
	agar	15 g

These were dissolved in 1000 ml dH_2O and autoclaved at 121°C, 15psi for 20 minutes. After autoclaving, the medium was cooled down to 60°C to add respective antibiotic, immediately distributed in bacteria dishes under the laminar hood and allowed to solidify at RT. The plates were stored at 4°C.

LB Medium	bacto trypton	10 g
	yeast extract	5 g
	NaCl	10 g

The pH of the medium was adjusted to 7.0 with 10 N NaOH. The total volume was added up to 1000 ml with dH_2O and autoclaved at 121°C, 15psi for 20 minutes.

3.4.2. Liquid medium and agar plates for *L. monocytogenes*

BHI medium	BHI	37 g

Powder was dissolved in 1000 ml dH_2O and autoclaved at 121°C, 15psi for 20 minutes.

BHI Agar Plate	BHI	37 g
	agar	15 g

These were dissolved in 1000 ml dH_2O and autoclaved at 121°C, 15psi for 20 minutes. After autoclaving, the medium was cooled down to 60°C to add respective antibiotic, immediately distributed in bacteria dishes under the laminar hood and allowed to solidify at RT. The plates were stored at 4°C.

Listeria Minimal Medium (MEM)	KH_2PO_4	6.56 g/l
	$Na_2HPO_4 * 7H_2O$	30.96 g/l
	$MgSO_4 * 7H_2O$	0.41 g/l
	glucose	10 g/l
	ferric citrate	0.088 g/l
	L-leucine, L-isoleucine, L-valine, L-methionine,	
	L-arginine, L-cysteine	0.1 g/l
	L-glutamine	0.6 g/l
	riboflavin	0.5 mg/l
	thiamine	1.0 mg/l
	biotin	0.5 mg/l
	thioctic acid	0.005 mg/l

Listeria Minimal Essential Medium (MEM) was prepared as follows to prevent precipitation or denaturation of essential nutrients. The buffer component salt A (Na_2HPO_4 * $7H_2O$ and KH_2PO_4) was dissolved in dH_2O, and the volume was brought up to 500 ml; salt B. $MgSO_4$ * $7H_2O$, was dissolved in dH_2O, and the volume was brought up to 100 ml. Both salt A and salt B solutions were autoclaved for 15 min. Glucose was prepared as a 20% stock solution and filter sterilized. Amino acids required for biosynthesis (leucine, isoleucine, arginine, methionine and valine) were mixed in 100-fold excess (10 g/liter) steamed to dissolve in dH_2O and filter sterilized after cooling. Cysteine and glutamine were dissolved in 50-fold excess and filter sterilized. Cysteine and glutamine must be added fresh to the medium. A 100-fold stock solution of Ferric citrate was prepared in hot dH_2O and bringing the volume up to 100 ml. Vitamins were also prepared as 100-fold solutions: riboflavin was dissolved in 1 N formic acid; biotin was dissolved in hot dH_2O and cooled to room temperature; thiamine was dissolved in a small volume of 70% ethanol and diluted with dH_2O. The vitamin solutions were filter sterilized and kept refrigerated (Premaratne *et al*., 1991).

3.4.3. X-Gal plate

100 mM IPTG	0.5 ml
X-Gal 2% in dimethylformamid	1.2 ml
liquid medium	1000 ml

Liquid medium was autoclaved and cooled down to 60°C to add IPTG, 2% X-Gal and respective antibiotic.

3.5. Chemicals, antibiotics and instruments

All chemicals of molecular biology research grade were procured from respective manufactures (Merck, Roth, Serva and Sigma) and all solutions were prepared using pure distilled water. Wherever necessary, solutions were sterile filtered or autoclaved.

DEPC H_2O used in RNA analysis (such as *in vitro* transcription and primer extension) was treated previously as follows: add 1 ml of DEPC (diethyl pyrocarbonate) into 1 liter dH_2O, shake thoroughly, take this solution at 37°C overnight, then autoclave it.

Radioactive labelled nucleotides [α-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]GTP and [α-^{32}P]UTP (3000 Ci/mmol) used for *in vitro* transcription, [γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{33}P]dATP (3000 Ci/mmol) used for primer extension and sequencing, were purchased from Amersham Biosciences.

The general used instruments are listed as follows:

Autoclave	Webeco
Cold centrifuge	Eppendorf
DNA sequencer	Beckman Coulter
Gel dryer	BioRad 1125 B

Heating blocks	Hartenstein
Ice machine	Scotsman
Microliter pipettes	Eppendorf, Gilson
Microcentrifuge	Eppendorf
PCR machine	Bio-med, Thermocycler 60
PH meter	WTW pH523, Metrohm-Herisau
DNA/RNA Calculator	Gene Quant II, Amersham
Phosphorimager	Taifun, Molecular Dynamics
Refrigerators (-20°C; -70°C)	Privileg
Shaking incubator	Hartenstein
Electrophoresis apparatus	Bio-Rad, Hartenstein
Spectrophotometer	Amersham
Vortexter	Hartenstein
Incubator	Heraeus
Magnetic stirrer	BIOSAN
Photoelectric colorimeter	Klett MEG
Autoradiography machine	Kodak M 35 X-OMAT Processor
Laminar flow	NUAIR
Speedvac-concentrator	Univapo 150 H, Savant

Antibiotics were purchased from Sigma, prepared as stock solutions and stored at -20°C.

Antibiotic	**Stock Solution**	**Working Concentration in *E. coli***	**Working Concentration in *Listeria***	**Incubation time after Transformation**
Ampicillin	100 mg/ml in H_2O	100 µg/ml	–	1 h (*E. coli*)
Chloramphenicol	30 mg/ml in 100% ethanol	30 µg/ml	5-10 µg/ml	1 h (*E. coli*) 3 h (*L. m.*)
Erythromycin	100 mg/ml in 100% ethanol	300-600 µg/ml	5 µg/ml	3 h (*E. coli*) 3-6 h (*L. m.*)
Kanamycin	25 mg/ml in H_2O	25 µg/ml	15 µg/ml	1 h (*E. coli*) 3-6 h (*L. m.*)
Streptomycin	30 mg/ml in H_2O	30 µg/ml	–	1 h (*E. coli*)
Tetracyclin*	20 mg/ml in 50% ethanol	20 µg/ml	7.5 µg/ml	1 h (*E. coli*) 3-6 h (*L. m.*)
Penicillin G	5 mg/ml	–	5 µg/ml	–

*Store solution in light-tight containers

3.6. General cloning techniques

3.6.1. DNA extraction methods

3.6.1.1. Plasmid DNA isolation

Mini preparation of plasmid DNA (analytical): One colony from the bacterial transformed plate was inoculated in 3 ml LB medium supplemented with selective antibiotic and cultured overnight at 37°C, 190 rpm/min in a shaking incubator. Mini preparation of plasmid DNA was done according to the instructions of GFX^{TM} Micro Plasmid Prep kit (Amersham Biosciences).

Maxi preparation of plasmid DNA (Preparative): One colony or 1ml of overnight fresh grown culture was inoculated into 50 ml of LB or 2xYT medium supplemented with antibiotics and cultured overnight at 37°C, 190 rpm/min in a shaking incubator. Plasmid DNA was isolated using Nucleoband kit (Macherey-Nagel), usually 50-200 µg of DNA was obtained from 50ml culture. Plasmid DNA was dissolved in dH_2O and stored at –20°C.

3.6.1.2. Isolation of chromosomal DNA from *L. monocytogenes*

1 ml of overnight culture was briefly centrifuged and the pellet was suspended in 500 µl TE (20 mM Tris, 10mM EDTA, pH 8.2) containing freshly added 10 µl of 120 mg/ml lysozyme. This suspension was incubated at 37°C for 10 to 15 minutes, spun briefly in a microfuge and the supernatant was removed. The pellet was gently resuspended in 1 ml of DNA-Zol (BRL) with a wide bore pipette tip (cutting 2-3 mm from the ends of plastic pipette tips) 500 µl of 100% ethanol was added and mixed by inversion. The supernatant was removed after a quick spin, the pellet was washed twice with 1 ml of 95% ethanol, and dried briefly in air before being resuspended in 100 µl 8 mM NaOH solution. After DNA was solubilized, 0.1 M HEPES [*N*-(2-hydroxyethyl) piperazine-*N*`-(2-ethanesulfonic acid)] was added to adjust the DNA solution to a desired pH. The isolated DNA was stored at 4°C.

3.6.2. DNA digestion, ligation and dephosphorylation

Restriction endonucleases and corresponding buffers were procured from Pharmacia and Stratagene respectively, and used as recommended by the manufacturers.

DNA ligation (enzyme and buffer from New England Biolab) was performed as follows:

	Blunt end	**Sticky end**
Volume	20 µl	20 µl
Insert:Vector (molar ratio)	5:1	5:1
Insert (fmol)	45-180	9-90
Vector (fmol)	15-60	3-30
DNA total (I+V in µg)	0.1-1	0.01-0.1
T4 ligase	1 U	0.1 U
Incubation time and temperature	16°C overnight	16°C overnight or 1 h at 24°C-28°C

A general formula for calculating the concentration of vector and DNA fragment which is to be inserted is: ng of insert = (ng of vector x kb size of insert / kb size of vector) x molar ratio of (insert/vector)

Dephosphorylation of DNA fragments with alkaline phosphatase:

If the ends of the prepared vector are identical (e.g., following a single digestion), it is advantageous to treat the vector with Calf Intestinal Alkaline Phosphatase (CIAP, Promega) to remove the phosphate groups from the 5′-ends to prevent self-ligation of the vector.

DNA solution (1-20 pmol DNA)	10-40 µl
reaction buffer (10x)	5 µl
add H_2O up to	49 µl
alkaline phosphatase	1 µl

Incubate the reaction mix at 37°C for 45 min, then stop it by heating at 85°C for 15 min or extract DNA with phenol-chloroform.

3.6.3. Transformation of *E. coli* and *L. monocytogenes*

3.6.3.1. Transformation of *E. coli*

1) Preparation of competent *E .coli* cells

Method I (calcium chloride method; according to Cosloy and Oishi 1973):

1 ml of overnight culture was inoculated into 49 ml LB (or 2xYT) (1:50) in side arm flasks and grown at 37°C with vigorous agitation (about 3.5 h) till to 120 Klett (midlog). Transfer the bacterial cells to sterile 50 ml polypropylene tubes and centrifuge at 6000 rpm for 10 min at 4°C. The pellet was washed with 5 ml ice-cold 50 mM $CaCl_2$ and then resuspended in 10 ml ice-cold 50 mM $CaCl_2$. After incubation of cells on ice for about 30 minutes, the cells were centrifuged again at 6000 rpm for 10 min at 4°C, The pellet is resuspended gently in 1.5 ml 50 ml ice-cold $CaCl_2$-20% glycerin. These competent cells could be used directly for each transformation. Unused cells were frozen at –70°C.

Method II:

Solution 1 (fresh preparation)	1 M $MgSO_4$	1% (v/v)
	20% (w/v) glucose	1% (v/v)
	add LB medium to 10 ml and filter sterilize.	
Solution 2	glycerine	36% (v/v)
	1 M $MgSO_4$	1.2% (v/v)
	PEG (7500)	12% (v/v)
	Add LB medium to 100 ml and filter sterilize.	

Overnight culture (0.5 ml) was inoculated into 49 ml LB (1:100) in side arm flasks and grown at 37°C with vigorous agitation (about 3 h) till to OD 0.5 (80-90 Klett). After incubation for 10 min at 4°C, transfer the bacterial cells to sterile 50 ml polypropylene tubes and centrifugate

at 3000 rpm for 10 min at 4°C The pellet was washed with 0.5 ml solution 1 and then in 2.5 ml solution 2 resuspended. This competent cell could be used directly for each transformation. Unused cells were frozen at –70°C.

To transform competent *E. coli* cells, 1-20 µl of DNA (5-100 ng) were added into 100 µl of competent cells and mix briefly. Kept the mix on ice for 45 minutes and then heat it at 43°C for exactly 3 minutes. Rapidly transfer the tubes on ice again for 5 minutes, add 800 µl of pre-warmed LB (or 2xYT) to each tube, incubate the cultures for 1 h or more hours (dependent on selection antibiotic) to allow the bacteria to recover and to express the antibiotic resistance marker encoded in the DNA. The cells are plated on selective plates and incubated 12-16 hours at 37°C.

2) Transformation of *Listeria monocytogenes* (summarized from Park and Stewart, 1990)

a) Preparation of electrocompetent *Listeria* cells

3.5x SMHEM	sucrose	952 mM
	$MgCl_2$	3.5 mM
	HEPES	7 mM

1 ml of *Listeria* overnight culture was inoculated into 49 ml BHI (1:50) in side arm flasks and grown at 37°C with vigorous shaking till early to midlog (80-90 Klett units). Penicillin G was added to a final concentration of 5 µg/ml (5µl of 50mg/ml Penicillin G stock), and the cells were grown till 120 Klett units.The cells harvested by centrifugation at 6000 rpm for 10 minutes at 4°C. The pellet was washed twice with 5 ml ice-cold 3.5x SMHEM. The final pellet was resuspended in 0.5 ml 3.5x SMHEM, 200 µl were used for each electroporation. Unused cells were frozen at –70°C:

b) Electroporation

Electroporation was used to transform *Listeria* in this study. It was carried out with fresh or frozen competent cells prepared as described above. 5-20 µl of desalted DNA (500 ng) was added to the 200 µl of competent cells on ice. After briefly votexing, the mix was gently transferred to pre-chilled electroporation cuvettes (0.2 cm gap length, Equibio), and electroporated at 2.25 KV (Micropulser, Bio-Rad). 1 ml of BHI medium was immediately added to the electroporated cells and gently transferred into sterile, 10 ml tubes. The cells were incubated with shaking (190 rpm) at 37°C for 1 hour for Ampicillin selection, or up to 4 hours for Erythromycin and other antibiotic selection. The cells were plated on selective plates and incubated one to two days at 37°C

3.7. Electrophoresis techniques

General used electrophoresis buffer

10x TBE	Tris	108 g
	boric acid	55 g

	Na_2EDTA	9.3 g

Add dH_2O up to 1 Liter and pH should be adjusted to pH 8.3. 1xTBE is usually used as the working solution.

3.7.1. DNA agarose gel electrophoresis

6xDNA-loading buffer	bromophenol blue	0.01 g
	xylene cyanol FF	0.02 g
	sucrose	4.00 g

Add dH_2O up to 10 ml and store the solution at 4°C.

Agarose gel electrophoresis was employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA isolation or PCR reaction, and to size fractionate DNA molecules, which then could be eluted from the gel. An agarose gel was prepared according to the protocol described by Sambrook and Russell (2001). DNA samples were electrophoresed in 1xTBE buffer and ethidium bromide was added to a final concentration of 0.5 µg/ml.

3.7.2. Denaturing polyacrylamide gel

In 1978, Fred Sanger and Alan Coulson devised a method to pour and run thin polyacrylamide gels, which are now used ubiquitously to resolve the products of DNA sequencing reactions. In this study it was also used to separate the RNA transcripts from *in vitro* transcription. 6% gel was normally used in the experiment as follows:

urea	24 g
10 x TBE	5 ml
30% polyacrylamid	10 ml
dH_2O	10 ml

Heat this solution to dissolve the urea and filter though a filter paper into a probet. Fill the volume to 50 ml with dH_2O. Add 200 µl of freshly prepared 10% ammonium persulfate (APS) and 36 µl of TEMED and mix gently. Pour carefully the gel solution into the clean glass plate that has been clamped together with clips. Place the sharkstooth comb into the open end of the gel to form the wells for loading sample later. Before running the gel, pour the 1xTBE buffer into the electrophoresis tank and remove the comb. Put buffer with a syringe into the wells to clean them from urea and put the aluminium plate in contact with the glassplates to allow heat dispersion. Run the gel without sample for 15-30 min at 30 mAmp, 40 Watt and 1500 volt, then load sample on the well under same condition. Run the gel until the loading buffer is in the middle or end of the gel. It takes about 2-3 hours.

The following table is polyacrylamide (Roth) solutions for denaturing gels (total volume: 50 ml).

	6% Gel	8% Gel	10% Gel	12% Gel
30% polyacrylamide	10 ml	13.4 ml	16.7 ml	20 ml
10xTBE buffer	5 ml	5 ml	5 ml	5 ml
H_2O	10 ml	10 ml	5 ml	5 ml
urea	24 g	24 g	24 g	24 g

Percentage of denaturing polyacrylamide/urea gel for separation of various DNA fragments in primer extension experiment is shown as follows.

Percentage gel (%)	Size of band (nt)
4	>250
6	60-250
8	40-120
10	20-60
12	10-50

3.8. Polymerase chain reaction (PCR) methods

3.8.1. General PCR method

PCR is an enzymatic method for *in vitro* synthesis of multiple copies of specific sequences of DNA. The reaction mixture for general PCR contained the following components:

PCR reaction mixture	10 x PCR buffer	5 µl
	25 mM $MgCl_2$	3.4 µl
	20 mM dNTPs	0.5 µl
	primer 1 (0,5-1 µg/µl)	0.5 µl
	primer 2 (0,5-1 µg/µl)	0.5 µl
	template-DNA (0.1-0.25 µg)	2 µl
	Taq polymerase (5 U/µl)	0.2 µl
	ddH_2O	up to 50 µl

The PCR mixture was immediately incubated in PCR machine for amplification using the cycling program as follows:

Program	"first delay"	5 min	94°C
30 cycles (step of 1-3)	1. denaturing	30 sec	95°C
	2. annealing	30 sec	55°C
	3. elongation	90 sec	72°C
	"last delay"	5 min	72°C

The annealing temperature is dependent on primers, normally 5-10°C below the Tm of the

primer. The time of elongation is determined by polymerase and the length of desired DNA fragments. *Taq* polymerase can synthesize 2 kb of DNA in 1 min, and *Pfu* polymerase requires 2 min to synthesize 1 kb DNA.

3.8.2. Site-directed mutagenesis

PCR mediated site-directed mutagenesis using double-stranded DNA template was modified from "Molecular Cloning" (Sambrook and Russell, 2001).

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationship, identifying intramolecular regions or amino acids, which may mediate these functions, gene expression, and vector modification.

The basic procedure (see Fig. 5) utilizes a supercoiled, double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of *Pfu* DNA polymerase (Promega), which replicates both plasmids strands with high fidelity and without displacing the mutant oligonuleotide primers, and a thermal cycler. On incorporation of the oligonuleotide primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with *Dpn*I (Biolab): The *Dpn*I endonuclease (target sequence: 5`-$G^{m6}$ATC-3`) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *Escherichia coli* strains is *dam* methylated and therefore susceptible to *Dpn*I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into *E. coli* (DH5α).

a) The design of primer

Mutagenic primers introduce specific experimental mutations. The mutagenic oligonuleotide primers for use in this protocol must be designed individually according to the desired mutation. The following considerations should be made for designing mutagenic site and selection primers:

1: Both mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid.

2: Primers should be between 25 and 45 bases in length, and the melting temperature (Tm) of the primers should be 10°C above the extension temperature of 68°C.

3: The desired mutation (deletion or insertion) should be in the middle of the primer with 10-15 bases of correct sequence on both sides.

4: The primers optimally should have a minimum GC content of 40% and should terminate in one or more C or G base.

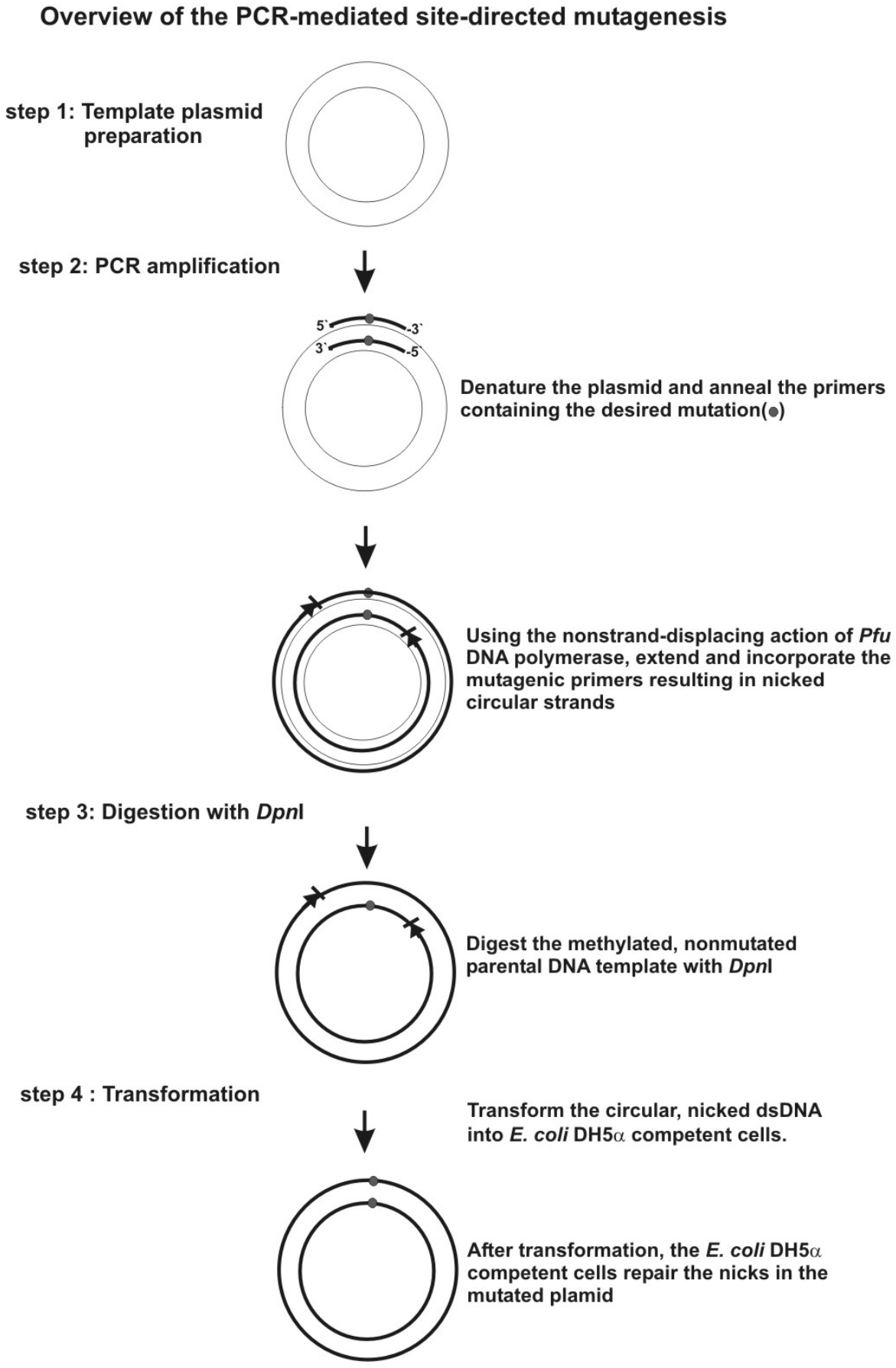


Fig. 5. Schematic of the site-directed mutagenesis method

5: Primers need not be 5`phosphorylated but must be purified either by fast polynucleotide liquid chromatography (FPLC) or by polyacrylamide gel electrophoresis (PAGE).

b) Temperature cycling

10x*Pfu* polymerase Buffer	5 µl
dsDNA template	5 - 50 ng
primer forward (100 pmol)	1µl
primer reward (100 pmol)	1µl
dNTPs(20mM)	1µl
Pfu polymerase (3 U/µl)	1µl
add ddH2O (Milli-Q degree) up to	50µl

PCR program: 95°C/30s [95°C/30s; 50-55°C/1min; 68°C/7min]$^{12\text{-}18}$ 72°C/10min

Adjust the number of thermal cycles in accordance with the type of mutation desired (see the following table):

Type of mutation desired	Number of cycles
Point mutations	12
Single amino acid changes	16
Multiple amino acid deletions or insertions	18

c) Digestion with *Dpn*I and transformation into *E. coli*

Digesting the products: Add 1µl of the *Dpn*I restriction enzyme (20,000U/ml, Biolab) into PCR products, incubate 1 hour at 37°C to digest the parental supercoiled dsDNA, then transfer 10-20 µl of the *Dpn*I-treated DNA into *E. coli* (DH5α).

3.8.3. PCR-mediated recombination

This method is based on the idea that a PCR product can be engineered by adding or changing sequences at its ends so that the product can itself be used to prime DNA synthesis in a subsequent overlap-extension reaction which creates mutant or recombinant molecules. The protocol is modified from Higuchi (1990) and S. Pilgrim (PhD work).

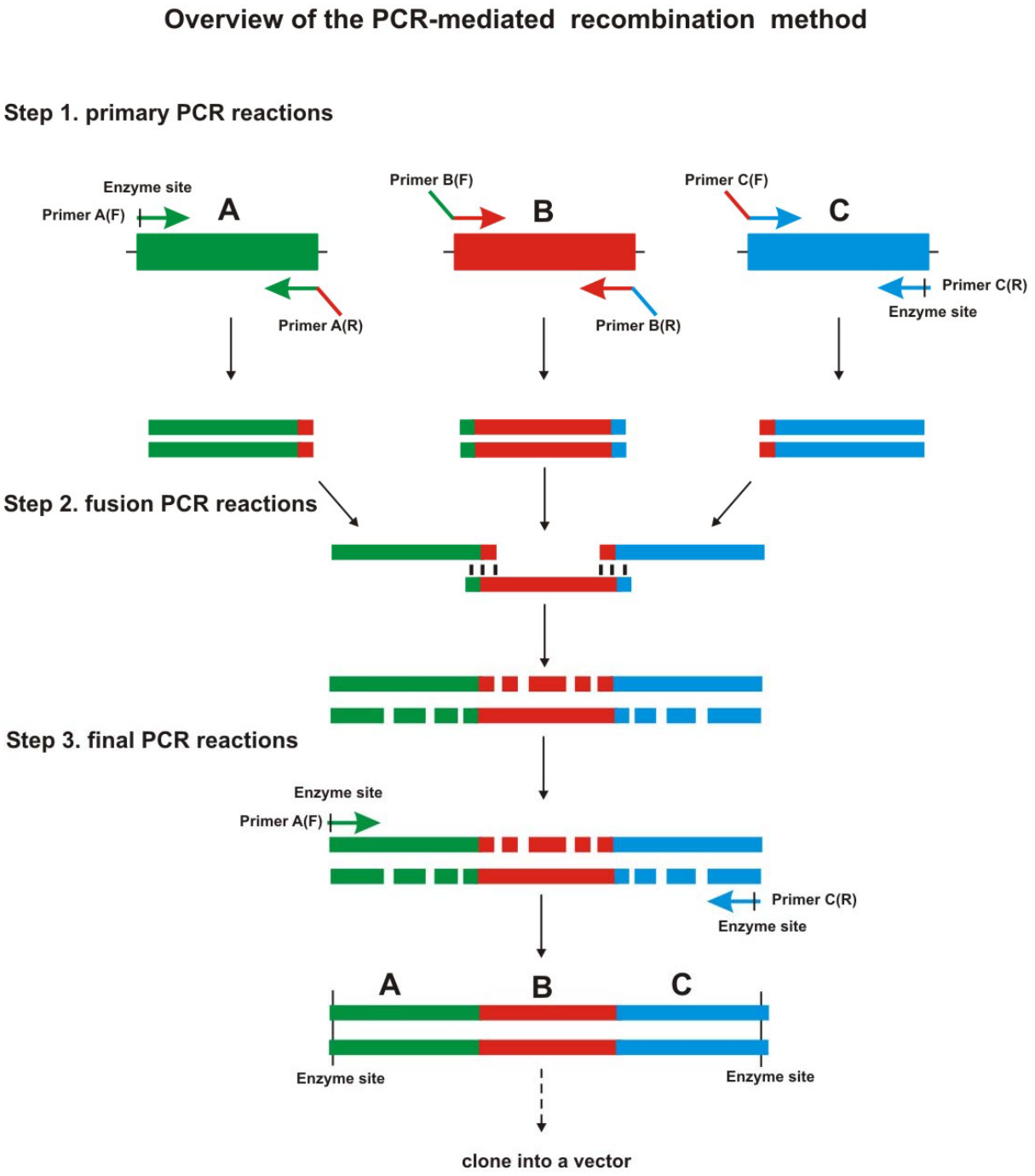


Fig. 6. Schematic of the PCR-mediated recombination method. The DNA fragments to be assembled are shown as colour rectangles with the capital letters A-C on the top. The 5´-end of the primer either carries a 10-20 bp of flanking region complementary to similar colour fragments or a restriction endonuclease site. PCR amplified products are shown as two paired strands and are colour coded according to the corresponding fragments.

In the first PCR, the fragments were amplified respectively by high fidelity PCR employing *Pfu* polymerase. Then, the products from each individual PCR were mixed in an equal molar ratio for second PCR. The condition for this PCR was quite special: only 6-8 cycles of PCR were carried out at 95°C for 30 sec, 45°C for 1 min, and 72°C for 2-10 min (2 min per kilobase DNA fragment to be amplified), and PCR reaction was mixed without addition of primers. So that the excess strand of the first PCR products could pair with each other. The

strands then acted as megaprimers to make the intermediate PCR products. In the final overlap extension step primers created the full length recombinant DNA fragments, which later could be digested with corresponding enzymes and introducted into a plasmid vector (Fig. 6).

3.9. " Run-off" *in vitro* transcription assay

In vitro transcription is a biochemical assay designed to measure transcription. It can be used to investigate the mechanism of action of general transcription factors or transcriptional activators and repressors. *In vitro* transcription requires a DNA template (usually it is a linearized plasmid containing a promoter), 5´-ribonucleotide triphosphates (ATP, GTP, UTP, and CTP), magnesium cation (as in magnesium chloride) and a DNA-directed RNA polymerase. These transcription reaction components are incubated with the desired transcription factor (s) or transcriptional activator(s) and repressor(s), and the resulting amount of transcription is quantified. The RNA polymerase for functional transcription is extremely promoter-specific (i.e., there is almost no transcriptional cross-talk). In this study, the RNA polymerase of *L. monocytogenes* A42 (Δ*prfA*) grown in BHI at 37°C was purified by ammonium sulphate precipitation and heparin sepharose affinity chromatography as described by Böckmann *et al*. (2000). This procedure yields a partially purified RNA polymerase preparation (about 60% RNAP subunit proteins) that is free of DNase, RNase and GTPase activities). In *in vitro* transcription reaction, the RNA polymerase binds firstly to the specific base sequences of the desired promoter. Transcription is then initiated by the coupling of the first two NTP´s and RNA chain elongation occurs in the 5´ to 3´direction. Transcription is terminated when the RNA polymerase reaches the end of the DNA template. Because the plasmid served as the template here is linearized at an appropriate restriction site prior to the transcription reaction, only discrete "run-off" transcripts are obtained. The reagents and protocol for *in vitro* transcription are described as follows:

2xMix	Tris-HCl (pH 8.0)	1 M
	EDTA	0.1 M
	$MgCl_2$	1 M
	K_3PO_4* $3H_2O$	0.1 M
	BSA (globulin free)	0.125%
PrfA Buffer	Tris HCl (pH 8.0)	50 mM
	NaCl	150 mM
	EDTA	1 mM
	DTT	1 mM
	$CaCl_2$	2.5 mM
	glycerin	20%

The stock solutions of Tris-HCl, NaCl and EDTA (nuclease free) purchased from Ambion were diluted in DEPC H_2O, the other solutions were also dissolved in DEPC H_2O. PrfA buffer is aliquoted and stored at –20°C.

RNAP Buffer	Tris-HCl (pH 8.0)	10 mM
	$MgCl_2$	10 mM
	$CaCl_2$	5 mM
	KCl	50 mM
	glycerin	10%
	EDTA	1 mM
	DTT	0.2 mM

The stock solutions of Tris-HCl, EDTA (nuclease free) were diluted in DEPC H_2O. The other solutions were also dissolved in DEPC H_2O. RNAP buffer is aliquoted and stored at –20°C.

a) DNA template preparation

To prepare a plasmid for the production of "run-off" transcripts, linearize the vector with a suitable restriction endonuclease (*Hind*III used in this study). After the restriction digestion, extract the linearized plasmid with phenol / chloroform, then ethanol precipitate and suspend in TE or dH_2O before using the DNA for *in vitro* transcription reactions.

b) Synthesis of radiolabeled RNA probes

Reaction mix	2 x Mix	5.7 µl
	50 % glycerin	4 µl
	0.1 M DTT	0.088 µl
	01 M EDTA (pH 8.0)	0.088 µl
	RNase inhibitor	0.5 µl
	linearized DNA	750 ng
Stop mix	urea	10M
	bromophenol blue	0.02%
	xylene cyanol FF	0.02%
	store in aliquots at –20°C	

According to the protocol of Böckmann *et al*. (2000) and Lalic-Mülthaler *et al*. (2001), each premix contained a single DNA template (750 ng), three out of the four NTPs buffered in the reaction system (containing RNase inhibitor, Pharmacia), an equal amount of RNA polymerase and a given amount of PrfA protein. After 2-10 minutes incubation at room temperature the fourth [α-^{32}P]-labelled NTP (3000 Ci/mmol; Amersham Biosciences) was added at low concentration (0.08 µM; 0.5 µl) to initiate the synthesis of the transcripts. The assay was stopped by the addition of 40 µl of stop mix. Each sample (14 µl) was subjected to electrophoresis on a 6% (w/v) acrylamide/7M urea gel and the transcripts were visualized by autoradiography or by phosphorimage.

If the transcripts were used as RNA templates for primer extension studies to determine the respective transcriptional start sites, the fourth NTP was added as unlabelled NTP either at

low concentration (0.08 µM) or at the same concentration as the other three NTPs (200 µM).

c) RNA polymerase of *L. monocytogenes* loaded with SigB factor

SigB Buffer	Tris HCl pH 8.0	50 mM
	EDTA pH 8.0	0.1mM
	DTT	1 mM
	$MgCl_2$	10 mM
	KCl	200 mM
	glycerin	20 %

In order to check the transcriptional activity of the SigB-dependent promoter, the purified SigB factor (provided by M. Rauch; Luo *et al*., submitted) should be loaded on RNA polymerase of *L. monocytogenes* for 20 min at 30°C in front of the step of synthesis of *in vitro* transcripts described above.

3.10. Primer extension

Primer extension is used chiefly to map the 5´-termini of mRNAs. In this study it was used to measure the exact site of 5´-terminus of the RNA obtained from "run-off" *in vitro* transcription assay.

a) Preparation of the oligonucleotide probe

The primer extension assay is carried out using synthetic oligonucleotide primers, 20-30 nucleotides in length. The best results are obtained when primers are used that hybridize to target sequences located within 150 nucleotides of 5´-terminus of the RNA. Primers that hybridize to more distant sites can give rise to heterogeneous extension products because reverse transcriptase may stop or pause in regions of high secondary structure in the template RNA. Wherever possible, primers should have a G+C content of about 50% and should have a G or C residue at the 3´-terminus. The primer should be present in about ten fold molar excess over the target RNA in the hybridization reaction. Before hybridization and extension of primer to the target RNA, it should be phosphorylated by [γ-^{32}P]-ATP (6000 Ci/mmol; Amersham Biosciences) in a reaction containing:

oligonucleotide primer (5-10 pmol)	1 µl
distilled deionized H_2O	15 µl
polynucleotide kinase (10 U/µl, Fermentas)	1 µl
kinase buffer (10x)	1 µl
[γ-^{32}P]-ATP 2 µl	

The reaction was incubated for 30 min at 37°C. Then unincorporated primers were removed by the QIAquick Nucleotide Removal Kit (QIAGEN). 2 µl of radiolabeled primer were counted in 3 ml of scintillation fluid in a liquid scintillation counter.

b) Hybridization of radiolabeled primer to the RNA

Labelled primer (2000000 c.p.m) was added to 10-30 µg of mRNA, which was generated previously in an in vitro transcription reaction. Primer/RNA mixtures were precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 4.8, RNase free) and 2.5 volumes of ethanol for overnight at –20°C.

c) Extension of primer

Stop buffer (formamid)	EDTA (pH 7.5)	10 mM
	deionized formamid	97.5%
	bromphenolblau	0.3%
	xylencyanol	0.3%

The overnight Primer/RNA mixtures were recovered by centrifugation two times at 14000 rpm for 15 min at 4°C, using 70% ethanol to wash. The pellet was carefully dried in the Speedvac and resuspended in 9 µl of reverse transcriptase mix [containing 2 µl of 2 mM dNTP, 5 µl of DEPC H_2O, and 2 µl of AMV reverse transcriptase buffer (Roche)]. After 2 min at 100°C, 1µl of AMV reverse transcriptase (Roche) was added to each sample, and the samples were incubated for 45 min at 45°C to allow reverse transcription. To digest the remaining mRNA, 1µl of RNase (10 mg/ml; Merck) was added and incubated for 10 min at room temperature. The reaction was stopped by adding 4 µl of stop solution. Before loading onto the 6% polyacrylamide-urea gel for electrophoresis, the samples were heated to 75-80°C for 2 min.

DNA sequencing of the cloned promoters performed by the dideoxynucleotide chain termination method (T7 Sequencing Kit; Pharmacia) were run in parallel on a 6% polyacrylamide-urea gel to determine the transcriptional start nucleotide with the same primers used in the primer extension experiments.

3.11. DNA sequencing

a) DNA sequencing using automatic sequencer

DNA sequencing was done using automatic sequencer (Beckman Coulter) based on a method which is a variant of dideoxynucleotide method. PCR for sequencing included 15 µl of DNA template (200-1000 ng), 1 µl of 5 pmol primer and 4 µl of sequencing mix (provided by Beckman Coulter in the CEQ Dye terminator Cycle Sequencing Kit). The thermal cycling is: 96°C/20s; 50°C/20s; 60°C/4 min, for 30 cycles followed by holding at 4°C. The reactions were cleaned by sodium acetate (pH 5.2) and ethanol precipitation as recommended by the manufacturer.

b) DNA sequencing using ^{T7}Sequencing^{TM} Kit (Amersham Pharmacia Biotech)

This Dideoxy sequencing depends on base-specific termination of enzyme-catalyzed primer

extension reaction. Therefore, in this study it was almost performed parallel with primer extension to determine the transcriptional start site of a tested promoter. The protocol is following the instructions of kit and [α-^{33}P]-dATP (3000 Ci/mmol) was used for labelling reaction.

3.12. β-galactosidase assay

Z-Buffer	$Na_2HPO_4.7H_2O$	60 mM
	NaH_2PO_4	40 mM
	KCl	10 mM
	$MgSO_4.7H_2O$	1 mM
	β-mercaptoethanol	l50 mM
	add β-mercaptoethanol on day of use	

Overnight cultures were vortexed and subcultured 1:50 into the fresh brain heart infusion (BHI) medium or MEM medium (Premaratne *et al*., 1991) containing 5 μg/ml erythromycin. After 3 h of growth at 37°C, an OD_{595} reading was taken and β-galactosidase activity was measured as described by Miller but with the following modifications. 1 ml of cultures were pelleted and resuspended in 0.64 ml of Z buffer. The samples were lysed by mechanical shaking for 45 s, 3 times using Fastprep instrument (QBiogene). The bacteria lysates were then centrifuged and the supernatants were incubated shortly at 37°C to warm the reaction mixture. 200 μl of Z-buffer with ONPG (o-nitrophenyl-β-D-galactopyranoside; 4.0 mg/ml) were added and incubated at 37°C. The reaction was timed until a yellow colour had developed and stopped by the addition of 400 μl of 1M Na_2CO_3 (approximately at 0.2-0.8 OD_{420} to get the linear range of assay). 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 should be repeated at least three times.

4. Results

4.1. Activity of the *inlC* promoter and its altered mutants determined by *in vitro* transcription and β-galactosidase assays

In this study the PrfA-dependent transcriptional activity of the *inlC* promoter and its various site-directed mutagenesis mutants were analysed, using the recently established "run-off" *in vitro* transcription assay (Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001) in the presence of low concentration (0.08 μM) of different radiolabelled NTP ([α-^{32}P]ATP, [α-^{32}P]CTP, [α-^{32}P]GTP and [α-^{32}P]UTP) or of high concentration (200 μM) of four nonlabelled NTPs. Furthermore, β-galactosidase assays were also used in this study to test under *in vivo* condition whether PrfA-independent transcription starts at P*inlC*, the effect of altered spacing between 3′-end of the PrfA-box and 5′-end of the –10 box in the *inlC* promoter region can occur, and the 17 bp of PrfA-box-like sequence (also termed "pseudo-PrfA-box") downstream of the –10 box of P*inlC* participates in transcription of the *inlC* gene.

4.1.1. *In vitro* transcription of the *inlC* promoter

The *inlC* gene encodes a small secreted internalin, the function of which is still unknown. An *inlC* deletion mutant shows significant reduction in virulence (Engelbrecht *et al*., 1996) and recent studies indicate that it may play a supportive role in InlA-mediated internalization of *L. monocytogenes* by non-phagocytic cells (Bergmann *et al*., 2002). Transcription of *inlC* is strongly PrfA regulated *in vivo* (Engelbrecht *et al*., 1996). Moreover, the *in vivo* observed PrfA-dependent transcription initiating from most of the well-known virulence gene promoters, such as P*hly*, P*plcA* and P*actA* can be reproduced in the *in vitro* transcription system, which works with linearized DNA and purified RNA polymerase (probably loaded mainly with SigA) and PrfA protein from *L. monocytogenes* (Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001), thus excluding effects of specific DNA topology and all additional factors which may positively or negatively affect PrfA binding to its target DNA sequence.

The promoter region of the *inlC* gene contains a PrfA-box with a single mismatch (Fig. 7) and primer extension studies using the total mRNA from the wild-type EGD strain as template identified a guanine (G) 40 bp downstream of the centre of this PrfA-box as the transcription start site (Engelbrecht *et al*., 1996), suggesting 5′-TAACAT-3′ located 5 bp upstream of this G as the –10 box of the *inlC* promoter (P*inlC*).

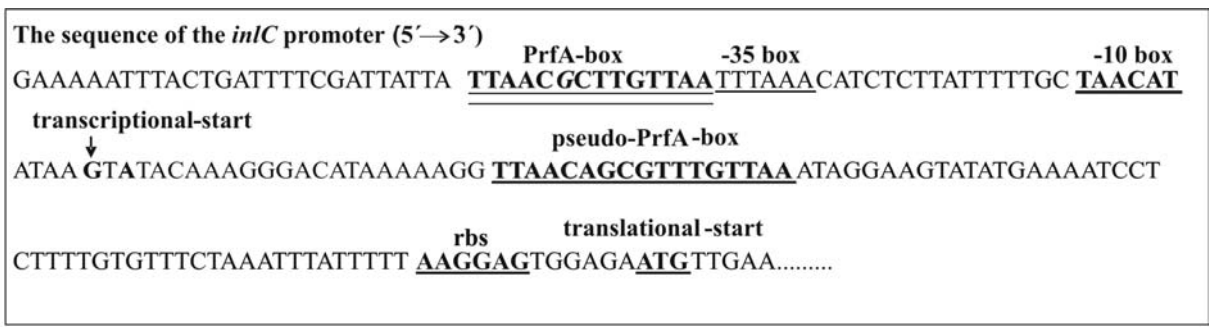


Fig. 7. Sequence of the *inlC* promoter (according to the primer extension results using *in vivo* obtained

total mRNA as template; Engelbrecht *et al*., 1996). The putative PrfA-binding site (PrfA-box) is double underlined and the deviation from the consensus sequence of the PrfA binding site (TTAACANNTGTTAA) is shown by the italic letter. The putative –35 box, –10 box, the palindrome of the PrfA-box-like sequence (pseudo-PrfA-box), the putative ribosome-binding site and the translational start codon ATG are underlined. The transcriptional start site identified under *in vivo* condition is marked by an arrow. Dots in the nucleotide sequence represent that the *inlC* structural gene continues.

Using the recently established *in vitro* transcription assay (Lalic-Mülthaler *et al*., 2001) with ^{32}P-CTP as radioactively labelled nucleotide, the PrfA-dependent transcription resulting in a major transcript starting at the *in vivo* mapped G, 5 bp downstream of the –10 box (G5) (henceforth, start sites +1 will be indicated by the start nucleotide followed by a number giving the distance in bp between the 3´ -end of the –10 box and the start nucleotide) and a minor transcript starting at adenine (A7) 2 bp further downstream were observed as expected (Fig. 8A). The start sites were determined by primer extension (Fig. 8B). However, it was not expected that the PrfA-independent transcription could be initiated in the presence of ^{32}P-GTP, because it has been shown previously, for listerial RNA polymerase, *in vitro* transcription is only initiated if nucleotides present in low concentrations (as is the case for the labelled NTP) do not belong to the first 4 nucleotides of the transcript (Lalic-Mülthaler *et al*., 2001). Indeed there was no transcription observed with ^{32}P-ATP and ^{32}P-UTP. Moreover, primer extension using low concentration of nonlabeled GTP determined A7, 7 bp downstream of the –10 box, was the transcriptional start site for this PrfA-independent transcription (Fig. 8B). As this PrfA-independent transcription uses apparently the same –10 box as the PrfA-dependent one, it must be a –35 box in an appropriate position upstream of this –10 box that is negatively influenced by PrfA binding, suggesting an overlap or close proximity of the –35 box with the PrfA-box.

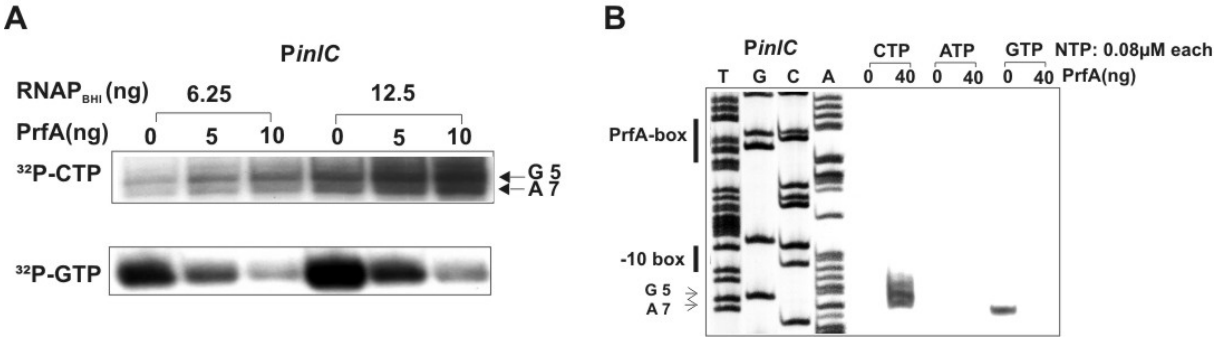


Fig. 8. *In vitro* transcription starting at the *inlC* promoter (P*inlC*). The ^{32}P- labelled rNTP (corresponding to the rNTP present in lowest concentration, 0.08 µM, in the assay) and the increasing amounts of PrfA and RNA polymerase isolated from BHI grown *L. monocytogenes* Δ*prfA* cells (RNAP$_{BHI}$) are indicated. (A) transcription with ^{32}P-CTP and ^{32}P-GTP in the presence of wild-type PrfA, (B) primer extension with the corresponding transcripts (serving as RNA template for the reverse transcriptase).

Similar results were also obtained when the wild-type PrfA protein was replaced by the *in vivo* constitutively hyperactive mutant PrfA* protein (Ripio *et al*., 1997). In this case (Fig. 9) transcription was about 2 fold enhanced (relative to the same concentration of PrfA) which is in accordance with *in vitro* transcription data using other PrfA-dependent promoters (Vega *et al*., 2004). The PrfA-independent transcription was more strongly inhibited by PrfA* (Fig. 9), which obviously reflects the stronger affinity of PrfA* to its binding sites (Vega *et al*., 1998).

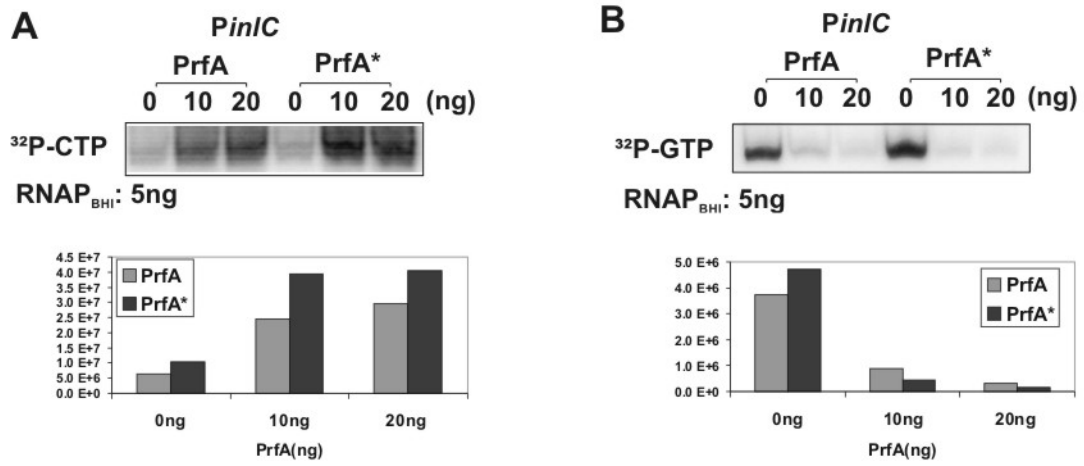


Fig. 9. ***In vitro*** **transcription with ^{32}P-CTP (A) and ^{32}P-GTP (B) in the presence of hyperactive PrfA*. Quantification of the transcripts was performed by phosphorimaging and is shown in the lower panels.**

As it has previously been shown that *in vitro* transcription of some PrfA regulated gene promoters, such as P*actA*, P*hly* and the second promoter of *prfA* (P*prfA*P2), functioned differently with three kind of RNA polymerases purified from *L. monocytogenes* that was cultured under either in rich culture medium ($RNAP_{BHI}$), exposed to heat shock conditions ($RNAP_{48}$) or conditioned in minimal essential medium ($RNAP_{MEM}$) (Lalic-Mülthaler *et al*., 2001), the similar test was also performed on the *in vitro* transcription activity starting at the *inlC* promoter with $RNAP_{BHI,}$ $RNAP_{48}$ and $RNAP_{MEM.}$ Fig. 10 showed that the highest transcription efficiency initiating from P*inlC* was obtained with $RNAP_{BHI}$ and the lowest with $RNAP_{48.}$ This activity profile showed no significant difference between PrfA -dependent and -independent transcription (Fig. 10). Although it is presently unknown what causes the observed change in *in vitro* transcription with the three RNA polymerase preparations, it has been reported that RNA polymerase of *Bacillus subtilis* can be loaded with different sigma factors (Tatti and Moran, 1996). Moreover, recently research in our lab showed that $RNAP_{MEM}$ contains a higher percentage of SigB and thus processes *in vitro* transcription more efficiently on SigB-dependent P*prfA*P2 than other two RNA polymerases (Q. Luo *et al*., manuscript submitted). Therefore, it is probable that $RNAP_{BHI}$ is loaded mainly with SigA and $RNAP_{48}$ may contain other sigma factor(s).

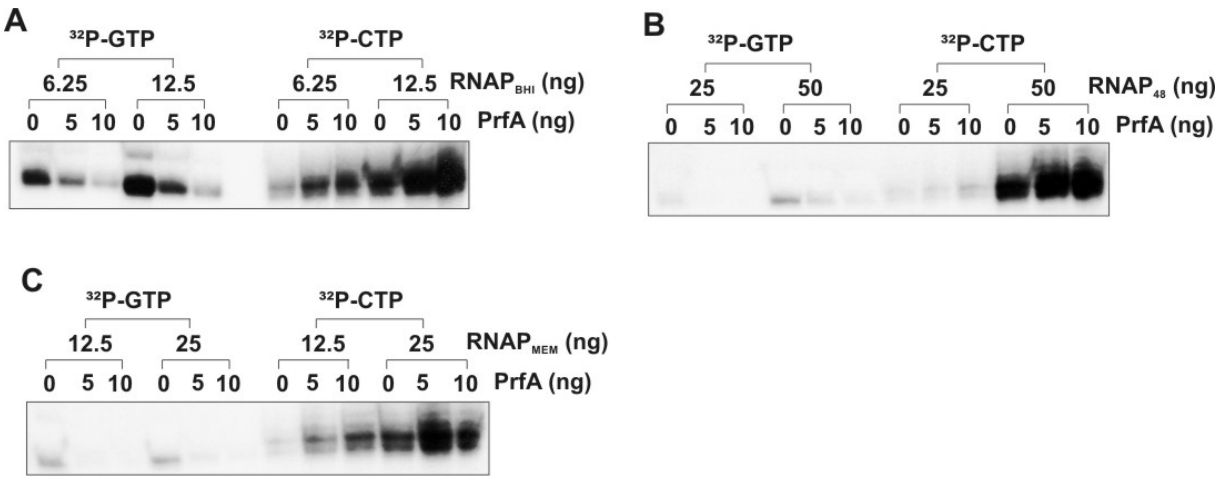


Fig. 10. ***In vitro*** **transcription of P*inlC* with RNA polymerases of *L. monocytogenes* from different**

preparations: fraction 16 of $RNAP_{BHI}$, fraction 19 of $RNAP_{MEM}$ and fraction 22 of $RNAP_{48}$. The concentration and activity profiles of fractions of three RNA polymerases are indicated in Fig. 3. in Lalic-Mülthaler *et al* (2001).

4.1.2. *In vitro* transcription with P*inlC* mutants altered in the putative –35 box

In a distance of 16 bp from the –10 box (a suitable distance for SigA recognized promoters in *B. subtilis*; for reviews see Haldenwang, 1995; Helmann, 1995; Wösten, 1998) there is a TTTAAA sequence directly 3´ adjacent to the PrfA-box (underlined in Fig. 7), which represents a possible –35 box (SWISS-PROT, SubtiList). Conversion of this sequence to either GGGAAA or CCCGGG [such G(C)-rich sequences are not recognized by SigA in *B. subtilis*, SWISS-PROT, SubtiList; mutants P*inlC*-m2 and -m3 in Fig. 11] completely abolished the PrfA-independent transcription and resulted in an efficient, strictly PrfA-dependent transcription starting at G5 (with ^{32}P-CTP as labelled nucleotide) (Fig. 11A and B) and at low efficiency at A7 (with ^{32}P-GTP as labelled nucleotide). Surprisingly, even conversion of TTTAAA into TTGACA (the consensus –35 box for SigA-loaded RNA polymerase in *B. subtilis*) abolished PrfA-independent transcription (mutant P*inlC*-m1; Fig. 11C), suggesting that this –35 box does not function in combination with TAACAT as –10 box as PrfA-independent promoter in the given context of P*inlC*.

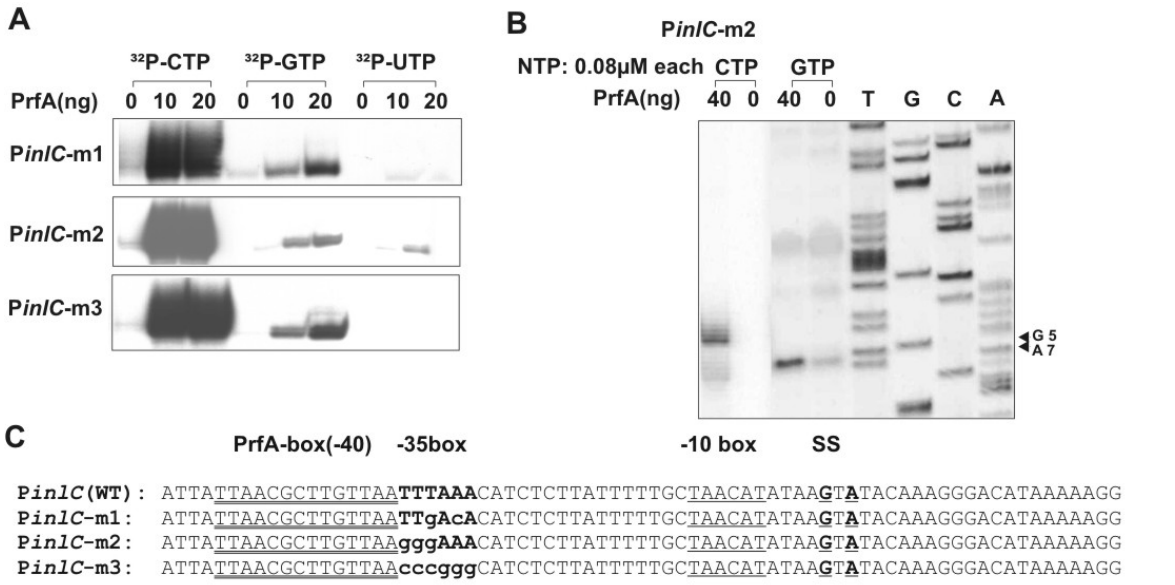
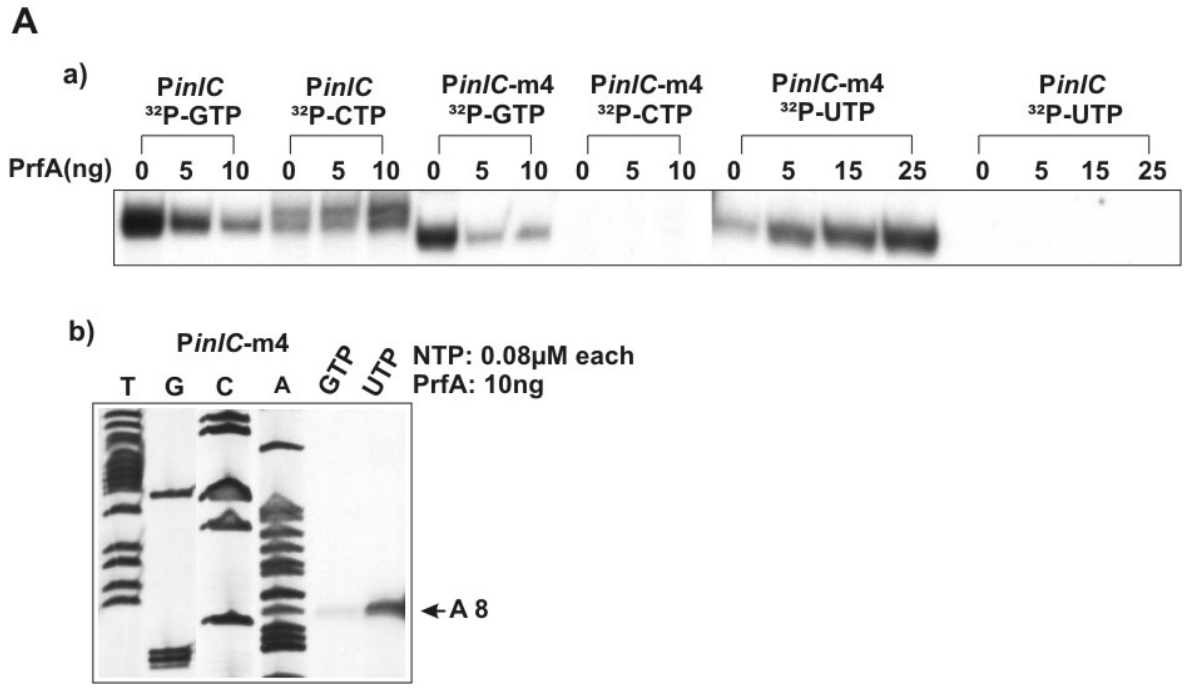


Fig. 11. *In vitro* transcription with P*inlC* mutants altered in the putative –35 box (P*inlC*-m1, -m2 and -m3) using the indicated ^{32}P-rNTP, a constant amount of $RNAP_{BHI}$ (about 20 ng) and increasing amounts of PrfA (A). Primer extensions with the *in vitro* transcripts of P*inlC*-m2 [using unlabelled CTP or GTP at the same low concentration (0.08 µM) as above] to determine the transcriptional start site (B), a smaller amount of the reverse CTP transcript than of the GTP transcript was used in order to obtain similar intensities of the bands. The sequence ladder serves for the identification of the transcriptional start sites indicated by arrowheads. (C) Alterations in the sequence of –35 box of P*inlC* are shown. Underlined letters designate the PrfA-box and –10 box of the promoter. Bold letters indicate –35 box and the small bold letters indicate base change. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.

4.1.3. *In vitro* transcription with P*inlC* mutants altered in the combination of the –10 and –35 box

Deletion of the G5 start site (for the PrfA-dependent transcription) created a new –10 box (TATAAT, the consensus –10 box for SigA-dependent promoters in *B. subtilis*), which has, however, a longer distance to the PrfA-box and the –35 box of the PrfA-independent promoter than the original –10 box (TAACAT). As shown in Fig. 12A (mutant P*inlC*-m4), PrfA-independent transcription started still in the presence of ^{32}P-GTP at A (now 8 bp downstream of the –10 box) and was inhibited by PrfA. There was no transcription in the presence of ^{32}P-CTP (C would be within the first 4 nucleotides of the expected transcript), but PrfA-dependent transcription occured in the presence of ^{32}P-UTP starting at A8, i.e. the newly created TATAAT –10 box was not used for PrfA-dependent (as expected) but also not for PrfA-independent transcription, suggesting the lack of a suitable –35 box at an appropriate distance from this new –10 box.

However, when TAACA from the primary –10 box was deleted (mutant P*inlC*-m5), the new –10 box (TATAAT) could be used for PrfA-dependent transcription in the presence of ^{32}P-CTP and ^{32}P-UTP starting at adenine 7 bp downstream of the new –10 box (previous position 13, see Fig. 12D), and no transcription occured in the presence of ^{32}P-GTP (Fig. 12B). Little PrfA-independent transcription took place with the new promoter combination consisting of TATAAT as –10 box and TTTAAA as –35 box. However, when the –35 sequence TTTAAA was replaced by the consensus –35 sequence TTGACA (mutant P*inlC*-m6), efficient PrfA-independent transcription occured that was less affected by PrfA than in the combination TTTAAA (–35 box) and TATAAT (–10 box) (Fig. 12), suggesting that the PrfA-independent promoter with the combination TTGACA (–35 box) and TATAAT (–10 box) is dominant over the PrfA-dependent one.



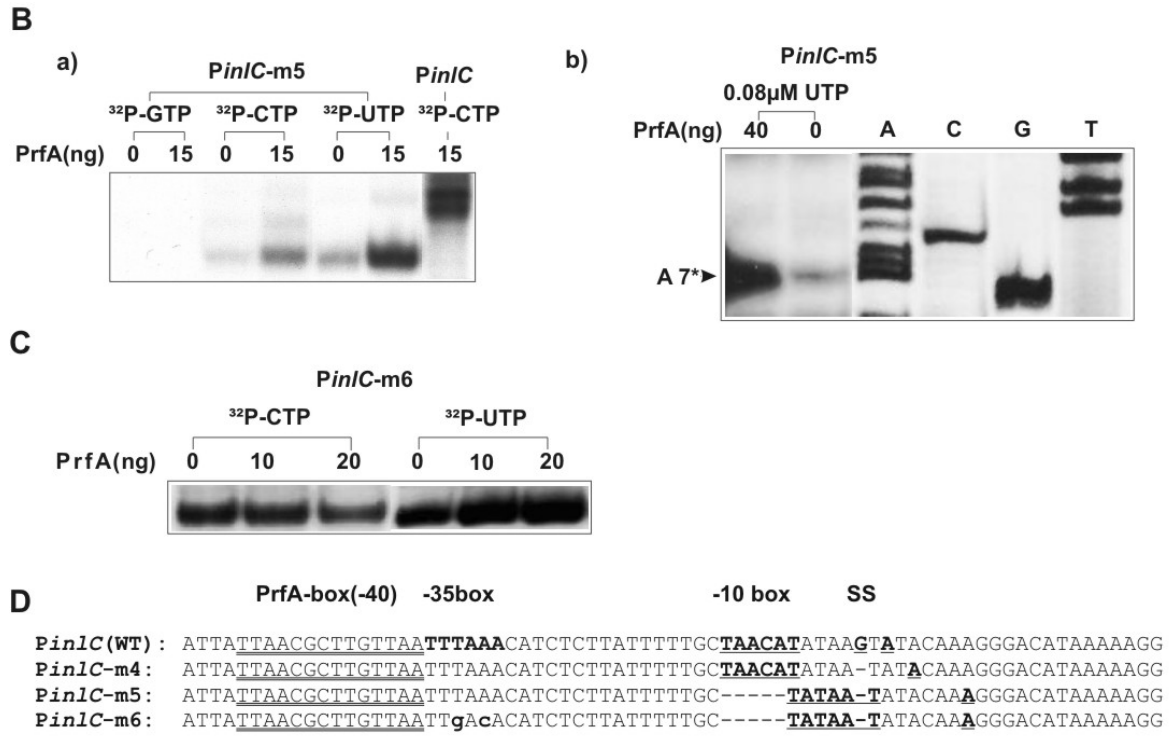


Fig. 12. (A) *In vitro* transcription of the G deletion mutant (P*inlC*-m4) (G is the dominant start site for *inlC* transcription starting from wild-type P*inlC*) and P*inlC* as control (a); (b) primer extension to determine the start site of transcription. (B) Transcription starting at P*inlC*-m5 (deletion of the –10 box and the original start site G of P*inl*C) with primer extension (b) and (C) at P*inlC*-m6 (additional change of the putative –35 box to the consensus sequence TTGACA) always compared to that at P*inlC* (WT). *In vitro* transcription was carried out with $RNAP_{BHI}$ (20 ng) and ^{32}P-labelled CTP, GTP or UTP (0.08 µM) to mark the transcripts. The corresponding transcriptional start sites are indicated by arrows. A7* indicates that the transcriptional start site is 7 bp downstream of the new –10 box (TATAAT). (D) Underlined start sites and alterations in the sequence of P*inlC* are shown. Dashes without letters indicate the position where bases have been removed. Small bold letters indicate base changes. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.

Deletion of 5 bp (CTTAT, mutant P*inlC*-m7) from the interspace region between the PrfA-box and the original –10 box (TAACAT), in addition to the G deletion led to strict PrfA-dependent transcription starting again at A7*, i.e. 7 bp downstream from the newly generated –10 box (TATAAT) in the presence of ^{32}P-CTP (Fig. 13A and B), but PrfA-independent transcription (strongly inhibited by PrfA) now started at A 8 bp downstream from the original –10 box (TAACAT) with ^{32}P-GTP as labelled nucleotide (Fig. 13A and B). Again, RNA polymerase did not use TATAAT as –10 box in combination with the original –35 box (TTTAAA) but instead used the original –10 box (TAACAT) in combination with a new –35 box that must be located within the PrfA-box at an appropriate distance from this –10 box (possibly TGTTAA located completely in the PrfA-box). This PrfA-independent transcription was again strongly inhibited by PrfA, further suggesting that this –35 sequence is overlapping with the PrfA binding site. With ^{32}P-UTP as labelled nucleotide, two transcripts were obtained (Fig. 13A). One initiated at the PrfA-dependent promoter (using TATAAT as –10 box) and the other at the PrfA-independent promoter [TGTTAA (–35 box) and TAACAT (–10 box)]. The latter transcription was only weakly inhibited by PrfA, suggesting that transcription from each of these two overlapping promoters can occur with equal efficiency.

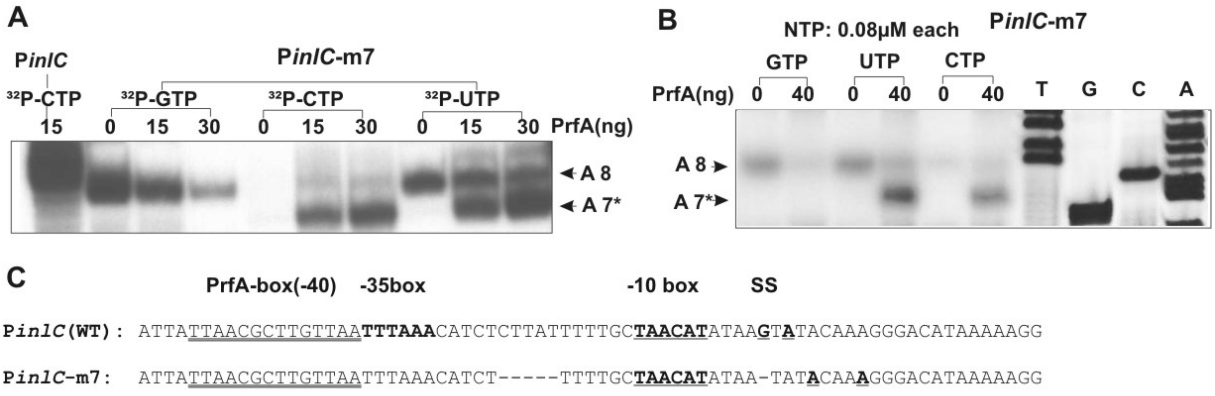


Fig. 13. (A) *In vitro* transcription starting at P*inlC*-m7 (carrying the G deletion and an additional 5 bp deletion in the interspace region between –10 and –35 boxes). Reaction was carried out with $RNAP_{BHI}$ (20 ng) and transcripts were marked with either ^{32}P-labelled CTP, GTP or UTP (0.08 µM). The corresponding transcriptional start sites were again determined by primer extension (B), A8 indicates the transcriptional start of the PrfA-independent transcription which is 8 bp downstream of the original –10 box of P*inl*C (TAACAT) and A7* indicates the transcriptional start of the PrfA-dependent promoter which is 7 bp downstream of the new –10 box (TATAAT). (C) Underlined start sites and alterations in the sequence of P*inlC* are shown. Dashes without letters indicate the position where bases have been removed. Small bold letters indicate base changes. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.

To characterise further the –35 box of this newly generated promoter for the PrfA-independent transcription, two deletions of different lengths were introduced into the PrfA box (mutants P*inlC*-m8 to P*inlC*-m13; Fig. 14). In all these mutants, transcription proceeds as expected, solely in a PrfA-independent manner. The data shown in Fig. 14A suggest that, in mutant P*inlC*-m8 (carrying the 10 bp deletion TTAACGCTTG in the PrfA binding sequence) TTTAAA was again used as –35 box in combination with TAACAT as –10 box. The transcription start site was A7 and this PrfA-independent transcription was no more inhibited by PrfA, further supporting the assumption that the inhibition of transcription by PrfA shown above results from an overlap of PrfA binding to a putative –35 box. The most efficient transcription was observed with ^{32}P-GTP, slightly less efficient transcription with ^{32}P-CTP; no transcription was seen with ^{32}P-UTP (Fig. 14A). Additional deletion of G5 (mutant P*inlC*-m9) led to transcription still initiated at the same promoter, but the start site moved to A (now 8 bp downstream of the –10 box), and transcription was optimal with ^{32}P-GTP, less efficient with ^{32}P-UTP and below detection with ^{32}P-CTP (Fig. 14A).

Even in the absence of the functional PrfA-box, no PrfA-independent transcription was observed in the combination TATAAT as –10 box and TTTAAA as –35 box (see mutant P*inlC*-m11; Fig. 14B), whereas weak transcription occurred with the combination TAACAT (–10 box) and possibly TATTAA as –35 box (P*inlC*-m10; Fig. 14B).

However, when only TTAACGCT were removed from the PrfA-box, leaving behind TGTTAA of the 3´ part of the PrfA-box, efficient PrfA-independent transcription (with ^{32}P-GTP) was obtained. In this case, the combination TAACAT (as –10 box) and TGTTAA (as –35 box) is used as promoter (mutants P*inlC*-m12 and -m13); again the combination TATAAT and TTTAAA was not used (mutant P*inlC*-13).

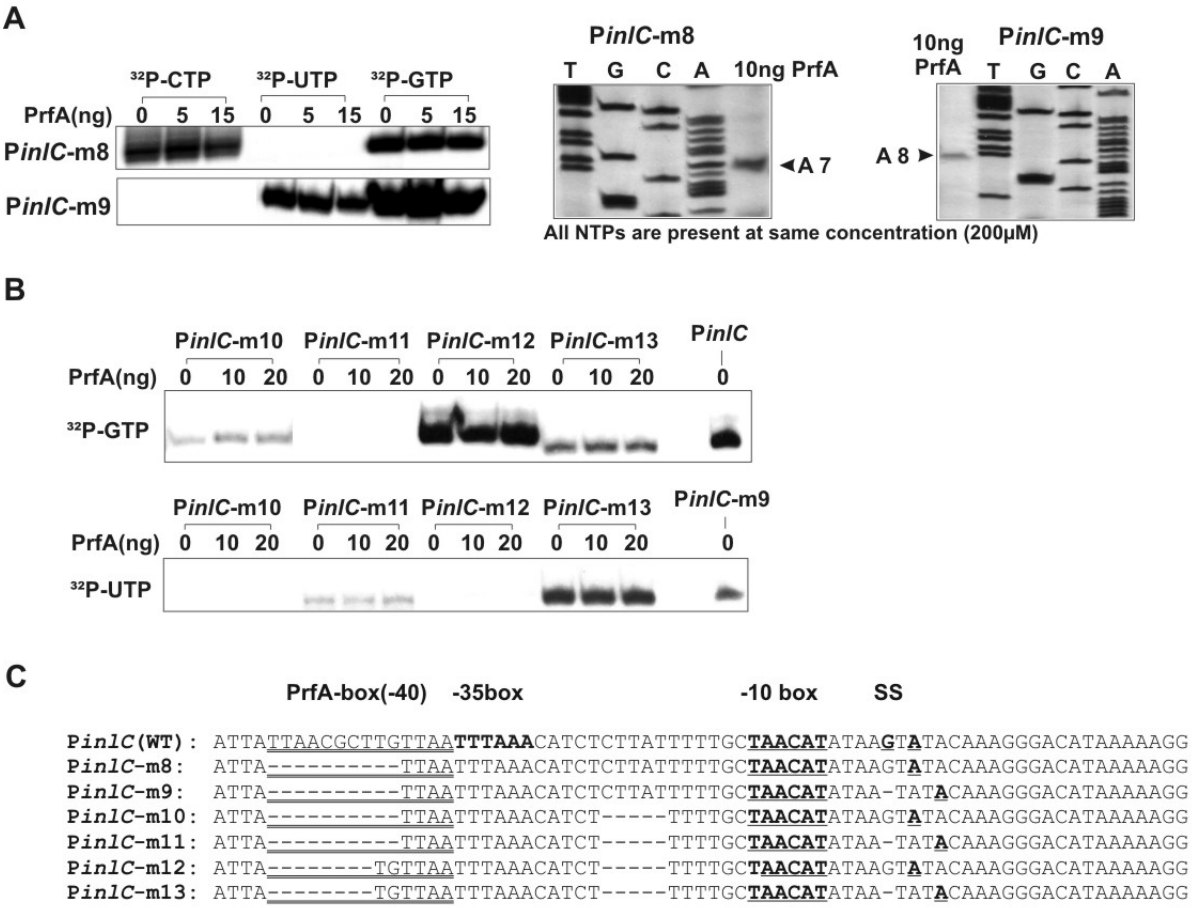
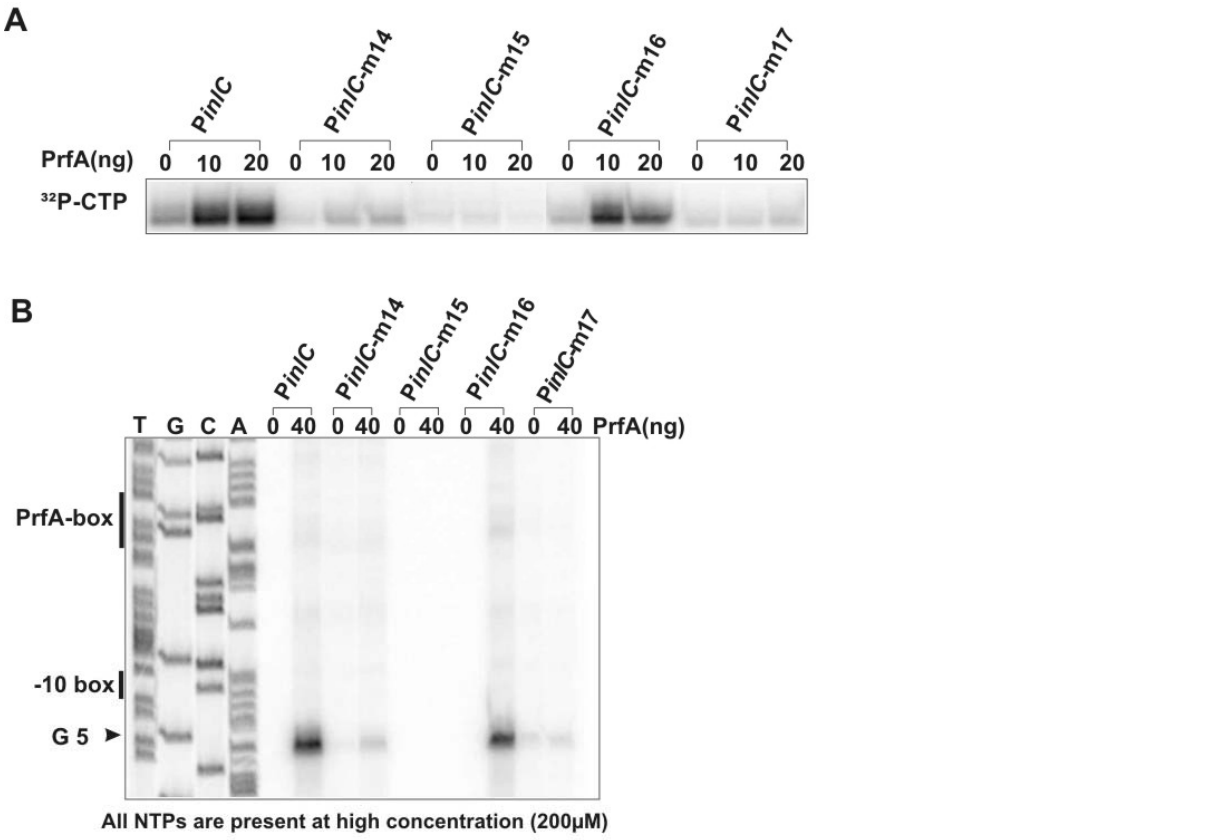


Fig. 14. ***In vitro*** **transcription starting at the promoter mutants carrying deletions in the PrfA-box of P*inlC*. Mutants P*inlC*-m8 to -m11 carry a 10 bp deletion (TTAACGCTTG), while mutants P*inlC*-m12 and P*inlC*-m13 contain a deletion of 8 bp (TTAACGCT) in the PrfA-box (Fig. 14C). P*inlC*-m9, -m11, -m13 contain in addition the G deletion while P*inlC*-m10 to -m13 carry an additional 5 bp deletion (CTTAT) in the interspace region between the –10 box and the PrfA-box. All *in vitro* transcription assays (A and B) were carried out with $RNAP_{BHI}$ (20 ng) and the indicated labelled rNTPs. Start sites of transcription from P*inlC*-m8 and -m9 were determined by primer extension carried out as described above. (C) Underlined start sites and alterations in the sequence of P*inlC* are shown. Dashes without letters indicate the position where bases have been removed. Small bold letters indicate base changes. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.**

4.1.4. *In vitro* transcription and β-galactosidase assay with P*inlC*- and P*plcA*- mutants altered in the interspace region

The above data indicate that, in PrfA-dependent transcription the distance between the –10 box and the transcriptional start site can vary between 5 and 8 bp and two different –10 boxes (TAACAT and TATAAT) can be used in combination with the PrfA-box of P*inlC*. In all the analysed combinations, the distance between the –10 box and the PrfA-box is fixed to 22 bp. In order to test the flexibility of this interspace region, deletions and insertions of 1 and 2 bp (mutants P*inlC*-m14 to -m17; Fig. 15) were introduced into this region and their PrfA-dependent transcription was tested. Whereas insertion of a single base pair (mutant P*inlC*-m16) reduced the efficiency of PrfA-dependent transcription (in the presence of ^{32}P-CTP) only slightly, deletion of one base pair (mutant P*inlC*-m14) led to a 10-fold reduction in transcription efficiency. Insertion or deletion of 2 bp (mutant P*inlC*-m15 and -m17) resulted in transcription at a very low level which was no longer activated by PrfA. These data suggest that the optimal distance for the interspace region of P*inlC* is 22 or 23 bp.



C

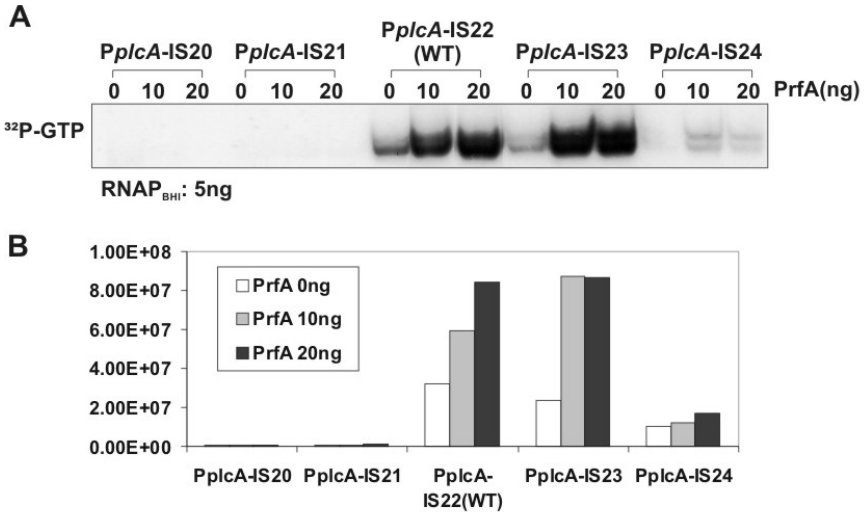
PrfA-box(-40) -35box -10 box SS

```
PinlC(WT): ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGG

PinlC-m14: ATTATTAACGCTTGTTAATTTAAACATCT-TTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGG
PinlC-m15: ATTATTAACGCTTGTTAATTTAAACATC--TTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGG
PinlC-m16: ATTATTAACGCTTGTTAATTTAAACATCTCTgTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGG
PinlC-m17: ATTATTAACGCTTGTTAATTTAAACATCTCtcTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGG
```

Fig. 15. ***In vitro*** **transcription starting at the promoter mutants P*inlC*-m14 to -m17 carrying either 1 or 2 bp insertions or deletions in the interspace region between the PrfA-box and the –10 box. A constant amount of $RNAP_{BHI}$ (20 ng) and the ^{32}P-labelled CTP was used as indicated (A). The corresponding *in vitro* transcripts were taken for primer extension to determine the transcriptional start sites (B). (C) Underlined start sites and alterations in the sequence of P*inlC* are shown. Dashes without letters indicate the position where bases have been removed. Small bold letters indicate base changes. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.**

To determine whether this would also be the case with the *plcA* promoter that contains a "high quality" PrfA-box, a reasonable SigA-recognized –10 box and a 22 bp-optimal interspacer, similar variants with deletions and insertions of 1 and 2 bp in the interspace region of P*plcA* were constructed and the *in vitro* transcription activity was tested (Fig. 16).



C

PrfA-box -10 box P

PplcA (WT) 5`-ATCGTCG**TTAACAAATGTTAA**TGCCTCAACATAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC-3`

PplcA-IS20 5`-ATCGTCG**TTAACAAATGTTAA**TGCCTCAA--TAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC-3`
PplcA-IS21 5`-ATCGTCG**TTAACAAATGTTAA**TGCCTCAA-ATAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC-3`
PplcA-IS23 5`-ATCGTCG**TTAACAAATGTTAA**TGCCTCAAC**t**ATAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC-3`
PplcA-IS24 5`-ATCGTCG**TTAACAAATGTTAA**TGCCTCAAC**tg**ATAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC-3`

Fig. 16. *In vitro* transcription starting at the promoter mutants P*plcA*-IS20, -IS21, -IS23 and -IS24 carrying either 1 or 2 bp insertions or deletions in the interspace region between the PrfA-box and the –10 box. (A) A constant amount of $RNAP_{BHI}$ (20 ng) and the ^{32}P-labelled CTP was used as indicated; (B) The *in vitro* transcription efficiency is analyzed by phosphorimage. Alterations in the sequence of P*plcA* are shown in (C). Dashes without letters indicate base deletions and small bold letters indicate base insertions. The transcriptional start sites are shown as P and underlined.

Apparently, 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 and 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* and the interspace regions observed previously for the other known PrfA-dependent promoters, i.e. P*hly*, P*mpl*, and P*actA* (promoter sequences see Table. 2 in Discussion).

To compare interspace altered *inlC* promoter strengths *in vivo*, the activities of a promoter-driven reporter gene, β-galactosidase, were determined in EGDe and its isogenic *prfA* deletion mutant (EGDeΔ*prfA*) transformed with the shuttle plasmid pUNK1 bearing the cloned promoter variants. The results shown in Fig. 17 are in agreement with that observed previously *in vitro* (Fig. 15). In the case of the low concentration of PrfA protein (e.g. in EGDeΔ*prfA*), all promoters showed very weak and similar activities, while in the presence of relatively more PrfA protein (e.g. in EGDe), the promoters with 22 bp or 23 bp of interspacer were remarkable stronger than other promoter variants. All these *in vitro* and *in vivo* data reveal that PrfA-regulated transcription is dependent on the length of interspacer region, which optimal distance is fixed to 22bp or 23 bp.

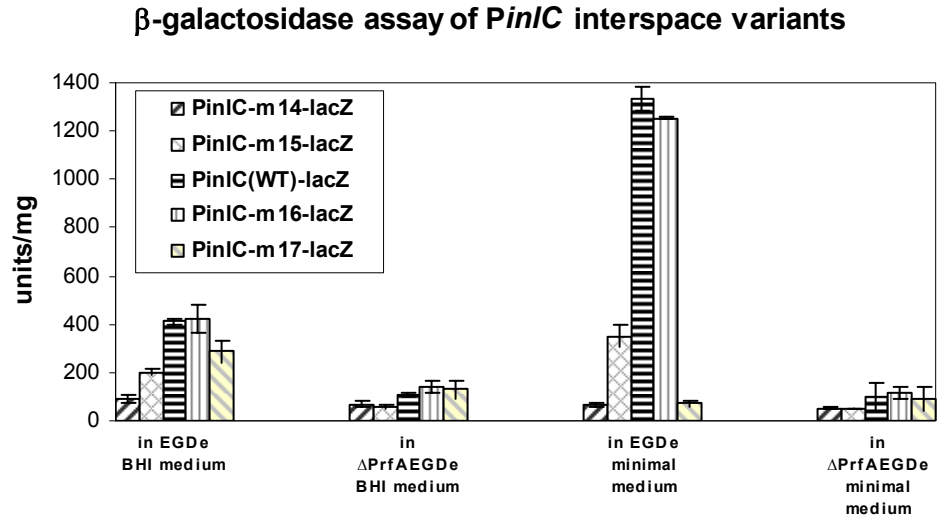


Fig. 17. β-galactosidase assay of *inlC* promoter interspacer variants. The values shown are averages of at least 5 experimental results. A background value of β-galactosidase activity, measured on EGDe and EGDeΔ*prfA* transformed with a pUNK-promoterless-lacZ plasmid, has been subtracted from all values.

4.1.5. Influence of high concentration of GTP on *in vitro* transcription from the *inlC* promoter and other PrfA-dependent promoters

The data described above showed that PrfA-dependent transcription of P*inlC* did not (or only to a very low level) occur at low GTP concentration even under conditions in which this nucleotide is not present in the first four nucleotides of the transcript, e.g. replacement of G5 by A5 (mutant P*inlC*-m18) still led to low efficient PrfA-independent transcription (Fig. 18A) in the presence of ^{32}P-GTP (GTP concentration 0.08 µM), although there is now no G within the first eight nucleotides of the expected transcript. Therefore *in vitro* transcription from the *inlC* promoter was tested in the presence of equally high concentrations (200 µM) of all four nucleotide triphosphates. The start sites of the transcripts synthesized were determined by primer extension with [γ-^{32}P]-ATP. With the wild-type *inlC* promoter (P*inlC*) a single transcript starting exclusively at G5 was obtained and this transcription was PrfA-dependent (Fig. 18B). Deletion of this G (mutant P*inlC*-m4) still led to a single transcript also synthesized in a PrfA-dependent manner (Fig. 18C) that now started at A8. Note that this transcript does also not contain G within the first four nucleotides. As already shown in Fig. 8, lowering the GTP concentration to 0.08 µM resulted in a PrfA-independent transcript starting at the same A (now 7 bp downstream of the –10 box), whereas the decrease in ATP to 0.08 µM stopped transcription completely, and the decreased concentration of CTP still resulted in PrfA-dependent transcription starting at G5. To determine the critical GTP concentration for optimal PrfA-dependent transcription starting at P*inlC*, the GTP concentration over a range from 0.1 to 200 µM (Fig. 18D) was varied while fixing the concentration of the other NTPs to 200 µM. The results show that the optimal GTP concentration for PrfA-dependent transcription from P*inlC* is around 200 µM.

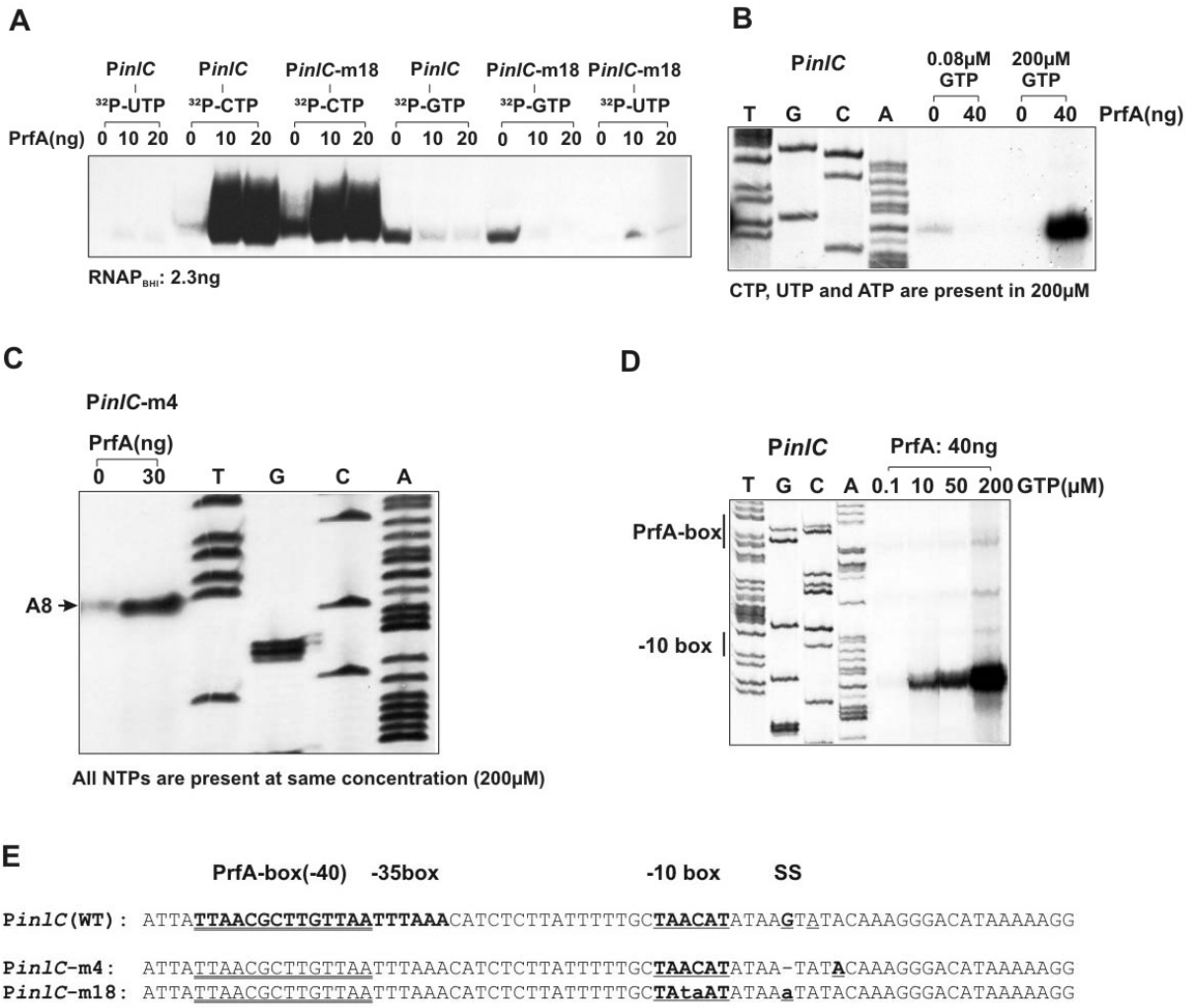


Fig. 18. (A) Comparison of transcription starting at P*inlC* (start site G5) and at P*inlC*-m18 (G5 to A5 exchange). (B) Primer extensions to determine the transcriptional start sites at P*inlC* in the presence of low (0.08 µM) or high (200 µM) GTP with or without PrfA. (C) Primer extensions to determine the transcriptional start sites at P*inlC*-m4 with *in vitro* transcripts synthesised in the presence of all four rNTPs in equal concentrations of 200 µM each. (D) Transcription in the presence of increasing GTP concentrations and fixed concentrations (200 µM) of the other three NTPs, transcripts were determined by primer extension. (E) Underlined start sites and alterations in the sequence of P*inlC* are shown. Dashes without letters indicate the position where bases have been removed. Small bold letters indicate base changes. The underlined transcriptional start sites are indicated as "SS" above the sequences and marked by bold letters.

These data suggest that a PrfA-dependent transcription initiation complex at the P*inlC* promoter is only formed in the presence of high GTP (and possibly ATP) concentrations. In contrast the initiation complex at the PrfA-independent promoter can be formed at low GTP, and PrfA-independent transcription from P*inlC* is then dominant.

To test whether the PrfA-independent transcription starting at P*inlC in vitro* can also occur *in vivo*, a plasmid which carries *lacZ* as reporter gene fused to P*inlC* or P*inlC*-m8 was constructed (Fig. 19A). This multicopy plasmid was introduced into the wild-type EGDe strain and an isogenic *prfA* deletion mutant. The strains were grown in brain-heart infusion (BHI) medium in which *L. monocytogenes* shows only low PrfA activity (Ripio *et al*., 1997). The high plasmid-borne P*inlC-lacZ* copies were expected to amplify PrfA-independent *lacZ*

transcript and to titrate out the cellular PrfA, which as shown *in vitro* (Fig. 8), inhibits PrfA-independent transcription from P*inlC*. As shown in Fig. 19B, β-galactosidase activity was observed in the wild-type strain and at a fourfold reduced level also in the *prfA* mutant. When the two strains were grown in a minimal medium (Premaratne *et al*., 1991) that strongly induces expression of active PrfA (A. K. Marr *et al*., manuscript in preparation), strongly enhanced β-galactosidase activity was observed in the wild-type strain. In the *prfA* mutant β-galactosidase activity was at a similar level to that in the BHI-grown *prfA* mutant. To confirm that the observed expression of β-galactosidase in the *prfA* mutant is indeed due to transcription of *lacZ* from the PrfA-independent P*inlC*, a similar experiment was performed with *lacZ* fused to the promoter of mutant P*inlC*-m8 (deletion in the PrfA-box). This time, similar β-galactosidase activity was obtained in the wild-type and the *prfA* mutant strain carrying P*inlC*-m8-*lacZ* regardless of whether the strains were grown in BHI or minimal medium (Fig. 19B, a). These data indicate that PrfA-independent transcription from P*inlC* can also occur *in vivo*. To simulate a low cellular GTP level, the EGDe wild type and *prfA* mutant strains were cultured under microaerophilic conditions in BHI (Fig. 19B, b). Under these conditions, expression of β-galactosidase is PrfA-independent, and β-galactosidase activity in both strains reaches a level similar to that in the wild-type strain grown in BHI under aerobic conditions. These data indicate that PrfA-independent transcription starting at P*inlC* can also take place *in vivo* and seems to occur preferentially when the cellular energy level is reduced.

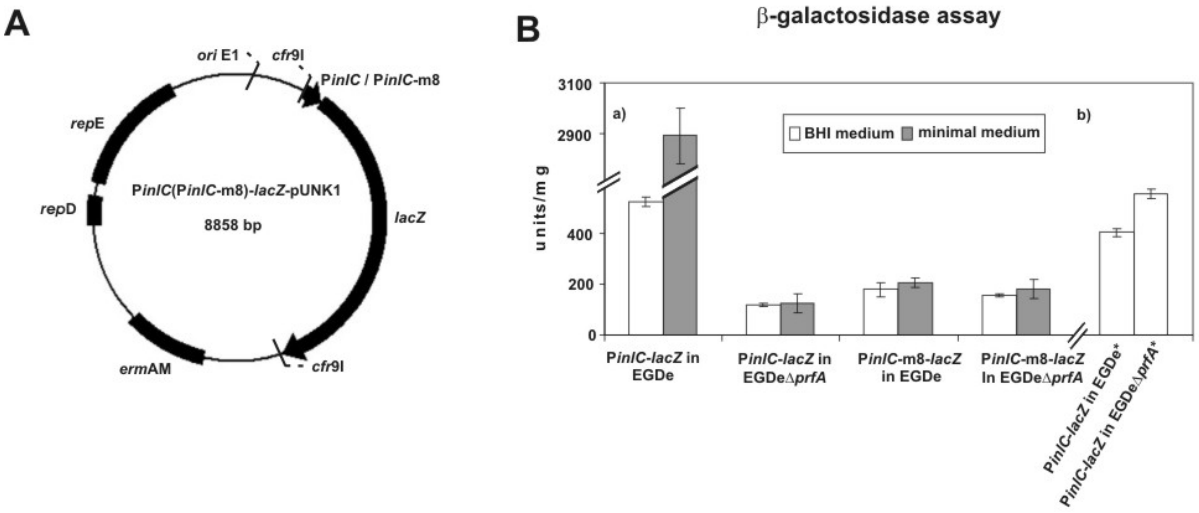


Fig. 19. (A) Construction of a multicopy plasmid carrying *lacZ* under the control of P*inlC* or P*inlC*-m8. (B) Beta-galactosidase activity of *L. monocytogenes* EGDe and an isogenic *prfA* deletion mutant (EGDe*ΔprfA*) carrying either one of the two plasmids after growth of the strains in BHI or minimal medium under aerobic conditions (a) and under microaerophilic conditions (b).

The observation that PrfA-dependent and -independent transcription can occur at the *inlC* promoter led to re-examine the *in vitro* transcription initiated at the other PrfA-dependent promoters, such as P*hly* and P*actA*. No such dual promoter activities were observed in previous *in vitro* transcription studies with the PrfA-dependent promoters of the other known virulence genes (*hly, plcA*, *actA*, Lalic-Mülthaler *et al*., 2001). Indeed PrfA-dependent *in vitro* transcription was obtained with ^{32}P-GTP starting at P*hly* and P*actA* albeit with low efficiency. This is not unexpected as there is no G within the first four nucleotides in the *hly* and the *actA*

transcript respectively (Fig. 20 A and B). However, when transcription is carried out with unlabelled NTPs, there is again a very significant increase in transcription efficiency at both promoters in the presence of 200 µM GTP compared to 0.08 µM GTP (Fig. 20 A and B). In contrast, there was no difference in the amount of transcripts synthesized from the PrfA-independent promoter P*inlC*-m8 in the presence of low or high GTP (Fig. 20C)

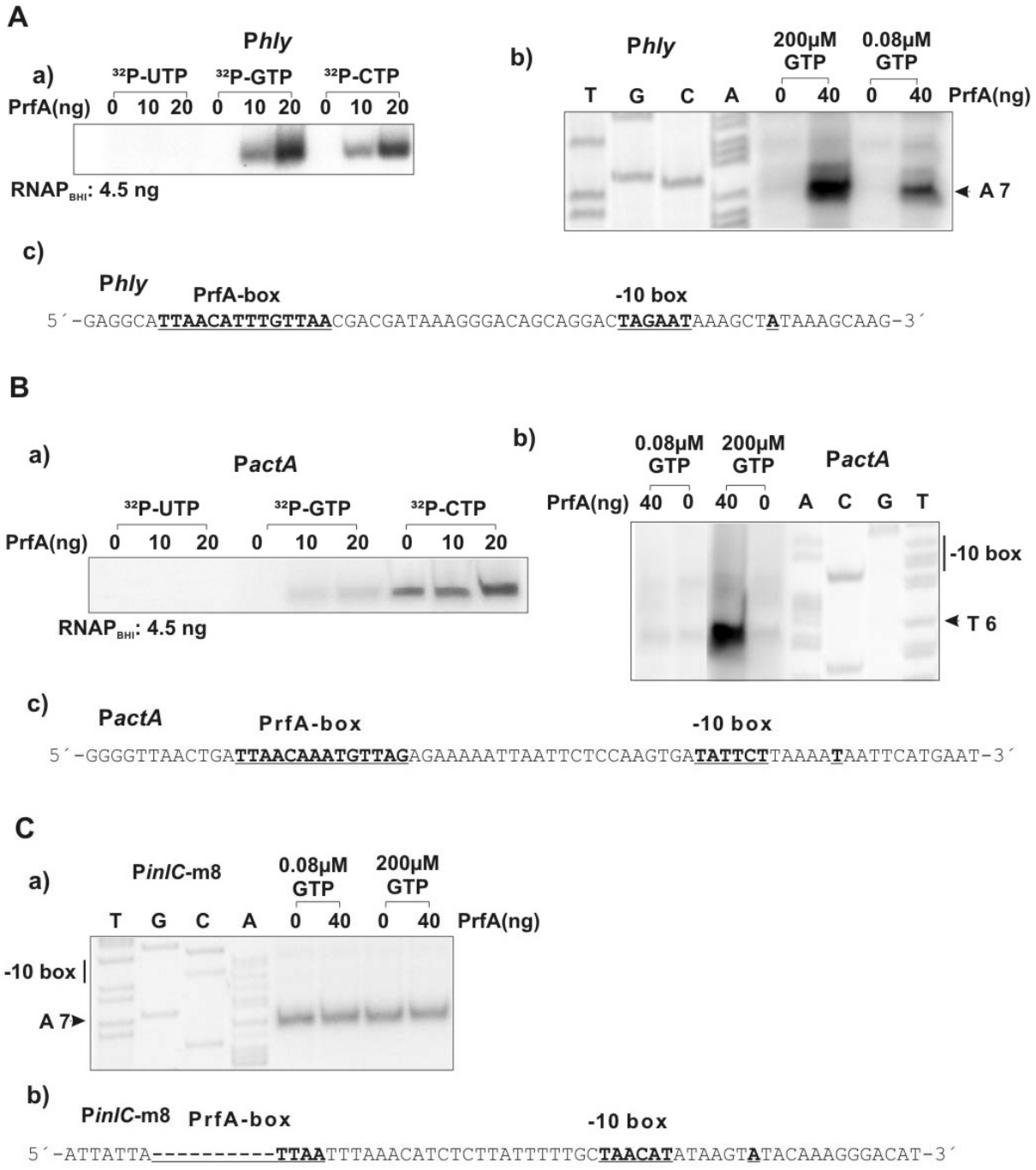


Fig. 20. ***In vitro*** **transcription starting at PrfA-dependent (P*hly* and P*actA*) and PrfA-independent (P*inlC*-m8) promoters in the presence of low (0.08 µM) or high (200 µM) GTP concentrations. (A) Transcription from P*hly* in the presence of ^{32}P-labelled GTP (0.08 µM) with and without PrfA (a). Primer extension of** ***in vitro*** **synthesised transcripts initiated at P*hly* in the presence of low (0.08 µM) or high (200 µM) GTP with and without PrfA (b). (B) and (C) Similar assays with P*actA* and P*inlC*-m8. The PrfA- and the –10 boxes of the corresponding promoters are underlined and the transcriptional start sites are indicated by bold letters. Dashes without letters indicate the position where bases have been removed. The sequence ladders**

(A, C, G and T) are always used for determining the transcription start nucleotides.

4.1.6. *In vitro* transcription and β-galactosidase assay with P*inlC* mutants altered in the pseudo-PrfA-box region

As previously described (Engelbrecht *et al*., 1996), there is a PrfA-box-like sequence 27 bp downstream of the –10 sequence of P*inlC* (Fig. 21), which was termed "pseudo-PrfA-box". This 17 bp sequence exhibits the dyad symmetry typical for PrfA-boxes in the flanking 6 bp parts but has an inner loop-forming part, which comprises 5 bp instead of the normal 2 bp. According to the above described transcription analyses this sequence is also positioned in the *inlC* transcript.

```
                PrfA-box(-40) -35box              -10 box   SS                  pseudo-PrfA-box

PinlC(WT): ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACAGCGTTTGTTAAATAGGAAGTATATGAAAATCCTCTTTTGTGTTTCTAAATTTATTT

PinlC-m19: ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAA---------TTAAATAGGAAGTATATGAAAATCCTCTTTTGTGTTTCTAAATTTATTT
PinlC-m20: ATTATTAACGCTTGTTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTTTTGTGTTTCTAAATTTATTT
PinlC-m21: ATTA----------TTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTTTTGTGTTTCTAAATTTATTT
PinlC-m22: ATTA----------TTAATTTAAACATCTCTTATTTTTGCTAACATATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTaTaaTGTTTCTAAATTTATTT
PinlC-m23: ATTA----------TTAATTTAAACATCTCTTATTTTTGC-----TATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTaTaaTGTTTCTAAATTTATTT
PinlC-m24: ATTA----------TTAATTTAAACATCTCTTATTTTTGC-----TATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTaTaaTGTTTgTAAATTTATTT
PinlC-m25: ATTA----------TTAATTTAAACATCTCTTATTTTTGC-----TATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCtgCTaTaaTGTTTgTAAATTTATTT
PinlC-m26: ATTA----------TTAATTTAAACATCTCTTATTTTTGC-----TATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCCTCTaTaaTaTTTgTAAATTTATTT
PinlC-m27: ATTA----------TTAATTTAAACATCTCTTATTTTTGC-----TATAAGTATACAAAGGGACATAAAAAGGTTAACA---TTTGTTAAATAGGAAGTATATGAAAATCtgCTaTaaTaTTTgTAAATTTATTT
```

Fig. 21. The sequences of P*inlC* mutants altered in the pseudo PrfA-box region and the original PrfA-box region.

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 (P*inlC*-m19), while the other converts the pseudo-PrfA box into a consensus PrfA-box (P*inlC*-m20). None of these mutations had any influence on the transcription from either of the two overlapping *inlC* promoters (Fig. 22). There were only very faint bands observed at the putative position of that modified PrfA-box region, which is now indicated as P2 in relation to the major transcripts (P1) from the original PrfA-box region.

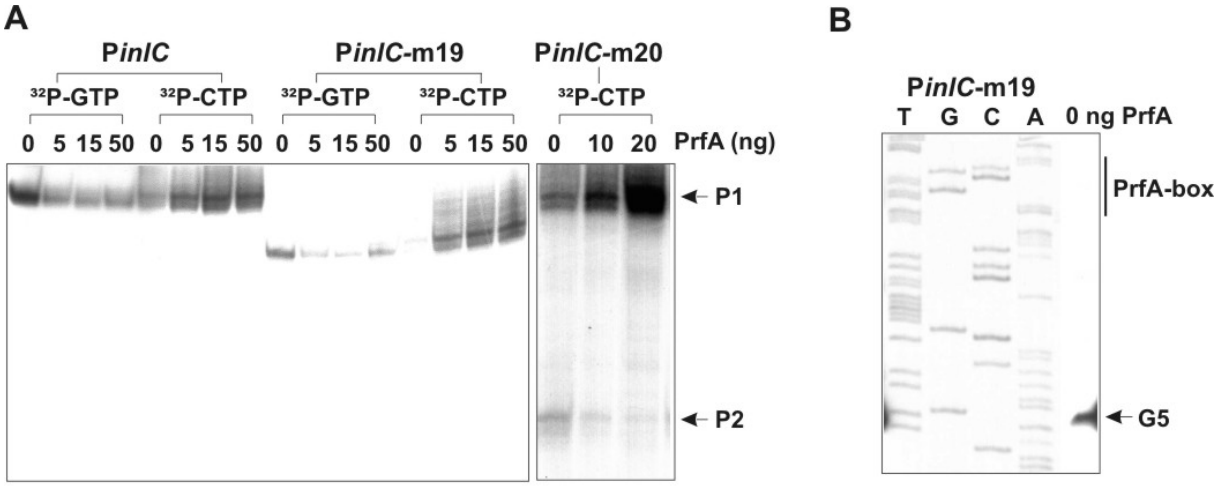


Fig. 22. (A) *In vitro* transcription of P*inlC*-m19 and P*inlC*-m20; reaction was carried out with $RNAP_{BHI}$ (20 ng) and transcripts were marked with either ^{32}P-labelled CTP or GTP (0.08 µM). (B) The transcriptional

start site of P*inlC*-m19 was determined by primer extension.

Even *in vivo* β-galactosidase assays showed no difference between wild-type (P*inlC-lacZ*) and pseudo-PrfA-box mutants (P*inlC*-m19) tested in *L. monocytogenes* wild-type strain (P14), *prfA** mutant (P14a) and *prfA* deletion mutant (A42), in which the expression level of PrfA will change in the cells (Fig. 23).

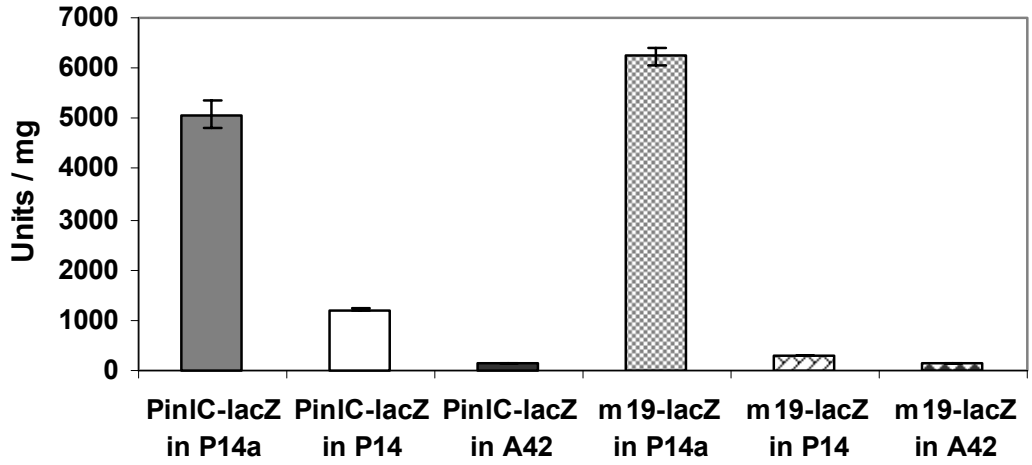


Fig. 23. Beta-galactosidase activity of *L. monocytogenes* P14, a *prfA mutant P14a, and an isogenic *prfA* deletion mutant A42 carrying either one of the two plasmids P*inlC*-lacZ and P*inlC*-m19-lacZ. The values shown are averages of at least 5 times of repeats.**

To test whether the binding of RNA polymerase and (or) PrfA protein to the original promoter sequence led to the inhibition of transcription from the modified PrfA-box region, mutant P*inlC*-m21 carrying a deletion of the original PrfA-box and a modified one was constructed. However, the transcript P2 was still very weak (Fig. 24). A comparison of both promoter sequences bearing either original or modified PrfA-box region shows that the downstream region of the latter is far away from the basic requirement of a PrfA-dependent promoter, which has been described in above experiments. Therefore, the sequence of TTTTGT 23 bp from the modified PrfA-box of mutant P*inlC*-m22 to -m27 was converted to TATAAT a consensus –10 box of SigA-recognized promoters. In addition to that, in P*inlC*-m23 to -m27 original PrfA-boxes and –10 boxes were deleted; P*inlC*-m25 and P*inlC*-m27 were changed TCCT in the interspace region of P2 to TCTG, so-called "extended –10 box"; P*inlC*-m24, -m25, -m26 and -m27 were changed C5, 5 bp downstream from TATAAT to G5; and P*inlC*-m26 and P*inlC*-m27 were converted G1, one bp downstream from TATAAT to A1 (see Fig. 21). All those effects led the primary sequence of modified PrfA-box region P2 to be similar to the original one. However, no transcription was shown from modified promoter of these variants (Fig. 24).

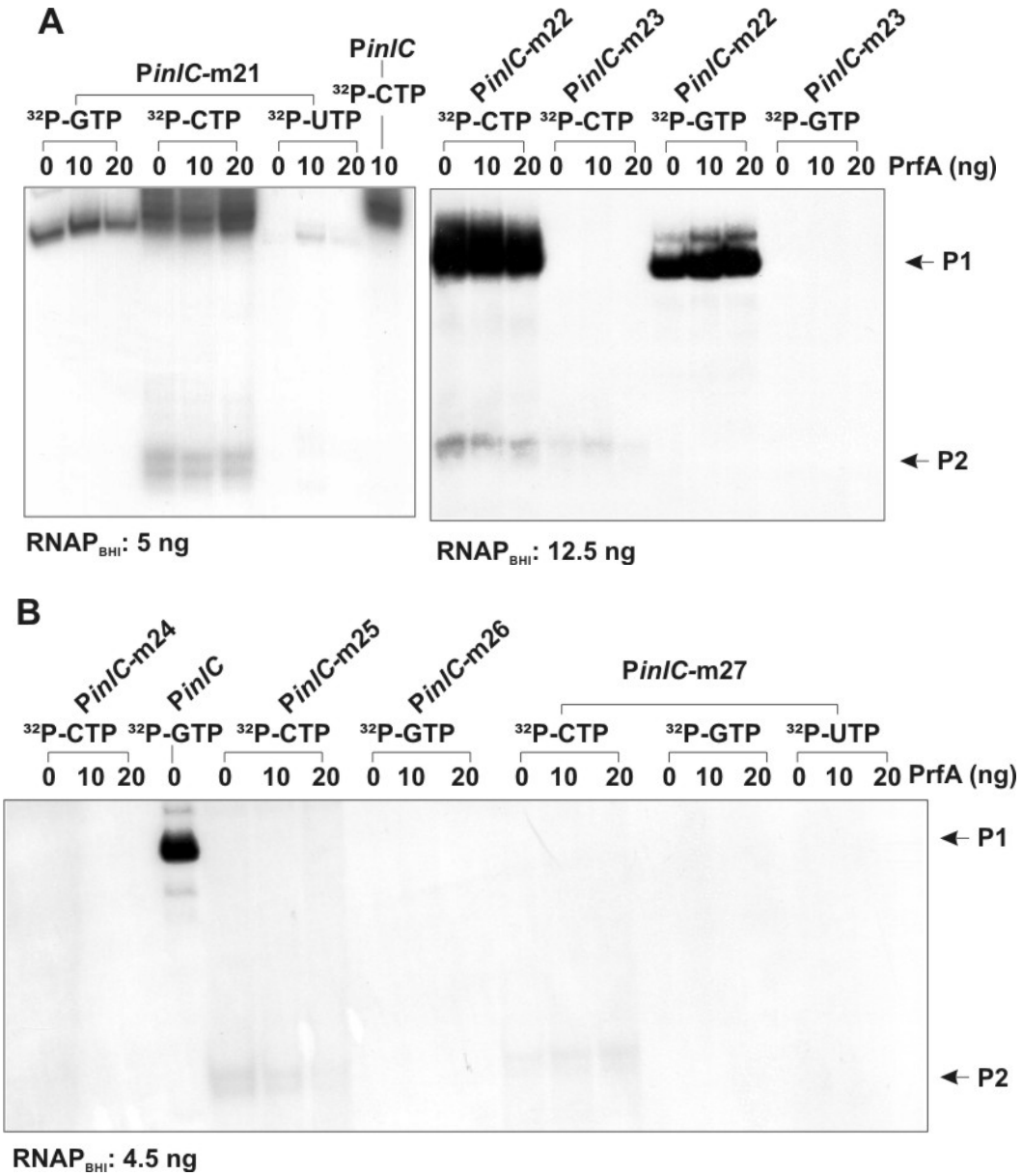


Fig. 24. (A and B) *In vitro* transcription starting at the pseudo-PrfA-box mutants P*inlC*-m21 to -m27. A constant amount of $RNAP_{BHI}$ and the ^{32}P-labelled NTPs were used as indicated. P1 is the transcription start site of the *inlC* promoter containing the original PrfA-box and P2 is the putative transcription start site of the promoter involving the pseudo-PrfA-box.

4.2. *In vitro* transcription of newly identified, putatively PrfA-regulated genes of *L. monocytogenes*

Recently, extensive comparative transcriptome analyses were performed (Milohanic *et al*., 2003) using transcripts from wild-type *L. monocytogenes*, an isogenic *prfA* deletion mutant and a *L. monocytogenes* wild-type strain expressing a permanently activated PrfA mutant protein (Ripio *et al*., 1997), that were hybridized to whole genome macroarrays. This study revealed also a larger number of new genes (in addition to the experimentally proven PrfA-regulated genes), which were affected in their expression by PrfA. Based on their different response to conditions known to activate or repress PrfA-dependent gene expression (Park and Kroll, 1993; Brehm *et al.*, 1996; Ripio *et al.*, 1996; Renzoni *et al.*, 1997; Kreft and Vazquez-Boland, 2001), these genes were divided into three groups (Table. 1). Group I

comprises 12 genes: 10 already known genes (*hly*, *plcA*, *actA*, *mpl*, *inlC et al.*) and two new genes, *lmo2219* and *lmo0788*, both positively regulated and containing a putative PrfA-box; Group II comprises 8 negatively regulated genes: 7 genes (*lmo0178*-*lmo0184*) are organized in an operon. *lmo0178* and the remaining gene *lmo0278* contain a putative PrfA-box; Group III comprises 53 genes, of which only two (*lmo0596* and *lmo2067*) contain a putative PrfA-box and a putative sigma B (SigB) recognized –10 box.

In addition to these three groups of genes, using whole genome microarray, A. K. Marr and B. Joseph in our lab have also identified three groups of genes that differ in expression in the presence of over-expressing PrfA when bacteria grow in minimal essential medium (MEM) with glucose (A. K. Marr *et al*., manuscript submitted). One of these, gene *lmo2840,* encoding a putative sucrose phosphorylase carries a single mismatch PrfA-box in the upstream regulatory region. This gene is found up-regulated under all conditions which also cause up-regulation of PrfA-dependent virulence genes.

	gene	function	upstream region (5´→3´)
Group I	*hpt*	Hexose phosphate transporter	TGCATG**aTAACAAGTGTTAA**TGACGGAAAGAGAGTATCTGGTT**TATATT**TTTATC**A**GCGCAAAA
	lmo0788	unknown	TATAGC**TTAtCAGATtTTAA**AAGCTTCCAATCGAAAGACAAAAATTCGACATAATTAACAATCA AGCAAGCATTCGCTAAATGAAGCTATTTTAGGGAATGCTTGCTTTTTTTTACTGTCAAAATGCTC CGCGAAATTCTTTACAAATGAAAAATATCGTG**TAGAAT**GATAGG**T**GTTTAGAGCATTTTGTCGT ATTTACGCATTTCGAGTGAGTACTCACTCATTTT**TaAACAACTaTTtA**TGTGAAGCGAAGTACA
	lmo2219	similar to PrsA of *B. subtilis*	TCTGAT**TTtACACATaTTAA**AAAATTATGGAAAATGCCGAAGAATTGCTTTATCGAAAGTGCAG AACGTGGATTTTCTGTTTTAATTTCTGACTAAACCATAAGAATATCATTAAATTTCTTTCATCT TTTCTTTTTACCTTTAATTTATGT**TATGAT**AGTTAT**T**GTGTTAAGCGGGAATTATTCAACAAC
Group II	*lmo0178*	similar to xylose repressor	TCTAAC**TTAAaACCTGTgAA**ACTTCGCGGACTAATGAGCGAAGGAATGATTCTCTCAGGCGAAA AAGATGGCAAGCTAAGCGTAATTGAAGCAAGTAGCGCACTTCCAAATGGTGCGAAAGTAAAATA GTAAAAACGAGTCCTAATTCACTTGAATTAGGGCTCATTTTTTTCGTTTAAAAAAACTTTAAAA AAAAGATTGCATATTGcAAACGATTACAA**TATAAT**GTAAC**T**ATACTTACTTAATTCATTGAAC
	lmo0278	similar to sugar ABC transporter, ATP-binding protein	TTTTTA**TTAcCATTTGTTcA**TTAAGTTGTTCCACGTACATGATAATCCCCCACTAACTATATAT TGTGTCTGCCTTAATAATATAACACAAGATGAAGTTTTTTTGTAATGATATCTGAGGAATTTTC GGATTTGGGACTAAAGAATTAAGCGCTTTGAAAAATCTTTCACATAAAAGGTCTTTTGTCCCTT TACTTCTTTTTACTTTTTTGCTACTTATGCAAGTTGCCCAACAGATGATTTTTTTCTTTGTGCA AATTGCCATAGTTTTTGTAAAGCGTTTTCATATACAA**TgAACACAaGTTAA**AAAATAGAAAGAG GCGAAGAAAGTTGGCACAATTATCACTAGAACATATTTATAAAATTTATGATA
Group III	*lmo0596*	unknown	TTTTTT**TTAAaAGGTtTTAA**ATTCGTTTTTTA**GGCTAT**TATAGTTA**A**GACTAATTATATTTCAT
	lmo2067	bile salt hydrolase	GTACAT**TTAAaAATTtTTAA**AGGAGCCAAATCATATTGTATGAGGTAATAAACTGCCCATTTTT TTGTGAAAAAAACCACAAATTATGTTTTACTCCAAACTCCGA**GGGTAC**TGGTATAC**A**TGTAAGG
not classified	*Lmo2840*	Similar to sucrose phosphorylase	**TTAACAACTtTTAA**AAAAATGTATGATAGTTATTTAAAATCGGCTTTTGAGATAGTGCATTTCC
	lmo2420	unknown	TAATCT**TTAACAATTcTTAA**AAGTTTCTTTCCGCGTGATTTATGTTCTTTCTG**TAGAAT**ATAAA TC**A**CGGAGGAGAG

Table. 1. Promoter regions and functions of putatively PrfA-regulated genes of *L. monocytogenes* EGDe. The classification of the genes is based on the recently published transcriptome analysis of Milohanic *et al.* (2003). *Lmo2420* and *lmo2840* described in the text are classified into a group. The putative PrfA boxes are in bold print and double underlined; The used –10 sites and start points determined by primer extension are in bold print. Deviations from the consensus sequence of the PrfA binding site are shown by small letters.

In silico search for PrfA-boxes in the genome sequence of *L. monocytogenes* has identified several additional putative PrfA-binding sites belonging possibly to PrfA-dependent promoters (Glaser *et al*., 2001). Among them, *lmo2420* contains a putative PrfA-box with

only one single mismatch and a SigA-recognized –10 box in its upstream regulatory region (Table. 1).

As the *in vitro* transcription system proved to yield highly reliable PrfA-dependent transcription initiation at all known PrfA-dependent promoters tested under appropriate conditions [including P*plcA*, P*hly*, P*mpl*, P*actA* and P*inlC* (Lalic-Mülthaler *et al*., 2001; Luo *et al*., 2004)], this system was applied to analyse transcriptional dependency on PrfA of the new "PrfA-affected" genes mentioned above, except *lmo0596* and the *hpt* gene that has been partially done by my colleagues M. Rauch and S. Müller-Altrock (Luo and M. Rauch *et al*., manuscript submitted). The nine analysed upstream regions of these genes showing the putative PrfA-boxes are listed in Table 1.

4.2.1. *In vitro* transcription of group I genes *hpt*, *lmo0788* and *lmo2219*

The *hpt* gene encodes a transporter for phosphorylated hexoses enabling *L. monocytogenes* to grow on glucose-6-phosphate and glucose-1-phosphate (Chico-Calero *et al*., 2002), the gene product of *lmo0788* is of unknown function and *lmo2219* (*prsA*) encodes a putative chaperone for secreted proteins (Kontinen *et al*., 1991, Kontinen and Sarvas, 1993; Milohanic *et al*., 2003).

Previous studies have shown that the *hpt* gene is hardly expressed in a *prfA* mutant and the upstream regulatory region of *hpt* contains a PrfA-box with a single mismatch (Chico-Calero *et al*., 2002; see also Table 1). Recombinant *L. monocytogenes* carrying GFP cDNA linked to a DNA fragment containing the putative *hpt* promoter shows poor fluorescence upon growth in rich brain-heart infusion (BHI) medium, which is strongly induced upon shift to minimal essential medium (MEM) (M. Beck PhD work); this procedure is known to strongly induce PrfA-regulated genes (Sokolovic *et al*., 1993). In addition, microinjection (Götz *et al*., 2001) of *prfA*+ and *prfA*- versions of this recombinant strain into the cytosol of epithelial Caco-2 cells shows also strong PrfA-dependent expression of GFP 4-6 h post injection. Using the previously described "run-off" assay (Lalic-Mülthaler *et al*., 2001), little transcription in the absence of PrfA was obtained with ^{32}P-UTP as labelled nucleotide triphosphate (0.08 µM) and the other unlabeled rNTPs (200 µM) and addition of purified PrfA activated transcription in a dose-dependent fashion (Luo and M. Rauch *et al*., manuscript submitted). There is a 2-3 fold increase in the amount of this transcript when the wild-type PrfA protein is replaced by the *in vivo* hyperactive PrfA* protein (Vega *et al*., 1998; Luo and M. Rauch *et al*., manuscript submitted). This enhancement becomes particularly apparent when comparing transcription activation from P*hpt* at low PrfA (PrfA*) concentration which is in accord with similar data obtained before with other PrfA-regulated promoters (Vega, *et al*., 2004). These data clearly indicated that the chosen DNA fragment contains all elements for PrfA-dependent transcription of *hpt.* Primer extension revealed that the transcription start site (+1) is A, 7 bp downstream of the anticipated –10 box (Fig. 25). The distance between the –10 box and the PrfA-box is 23 bp (Table 1) in perfect agreement with previous findings.

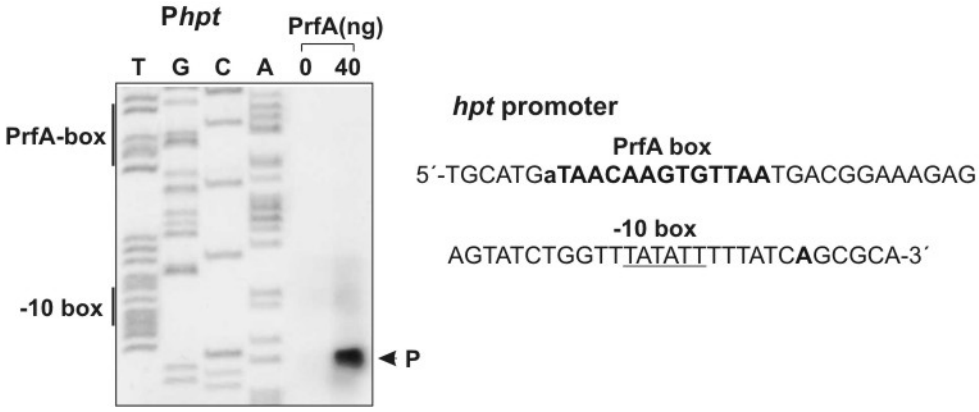
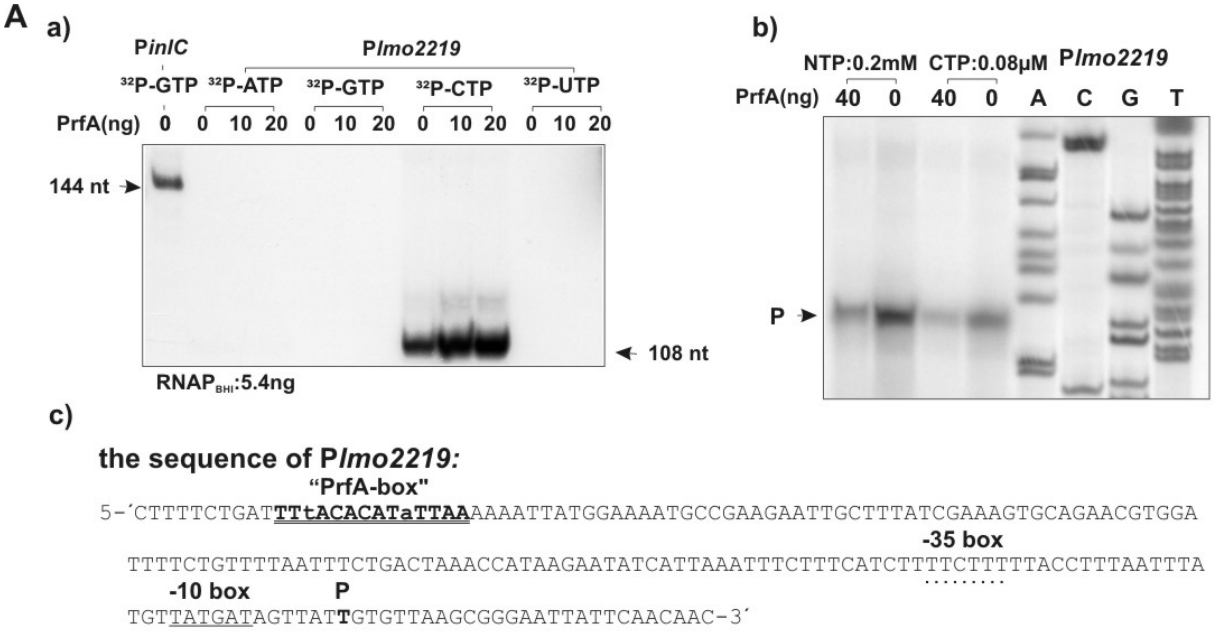


Fig. 25. Determination of the promoter sequence of *hpt* gene by primer extension. The putative PrfA-box is shown in bold letters and the small letter indicates the deviation from the consensus sequence of the PrfA binding site. The –10 box is underlined and transcriptional start site is in bold print. The sequence ladders (A, C, G and T) were used for determining the transcription start site.

A putative PrfA-box was proposed by Milohanic *et al*. 2003 (see also Table 1) which may be involved in PrfA-dependent transcription of *lmo2219*. However, *in vitro* transcription starts in the presence of ^{32}P-CTP as labelled NTP (0.08 µM) or with all 4 unlabelled NTPs in equally high concentration (Luo *et al*., 2004) at a T position 7 bp downstream of a –10 box (TATGAT) which is located 132 bp downstream of the proposed PrfA-box (Fig. 26A, a and b). Transcription efficiency is already high in the absence of PrfA and not significantly enhanced by increasing amounts of PrfA and PrfA* (S. Müller-Altrocker, personal communication) suggesting a PrfA-independent promoter for transcription of *lmo2219*. A suitable –35 box (TTCTTT) is indeed found in an appropriate distance (17 bp) to the identified –10 box. From these data it seems to be more likely that the *in vivo* observed PrfA-mediated enhancement of *lmo2219* transcription is caused by an indirect PrfA effect.



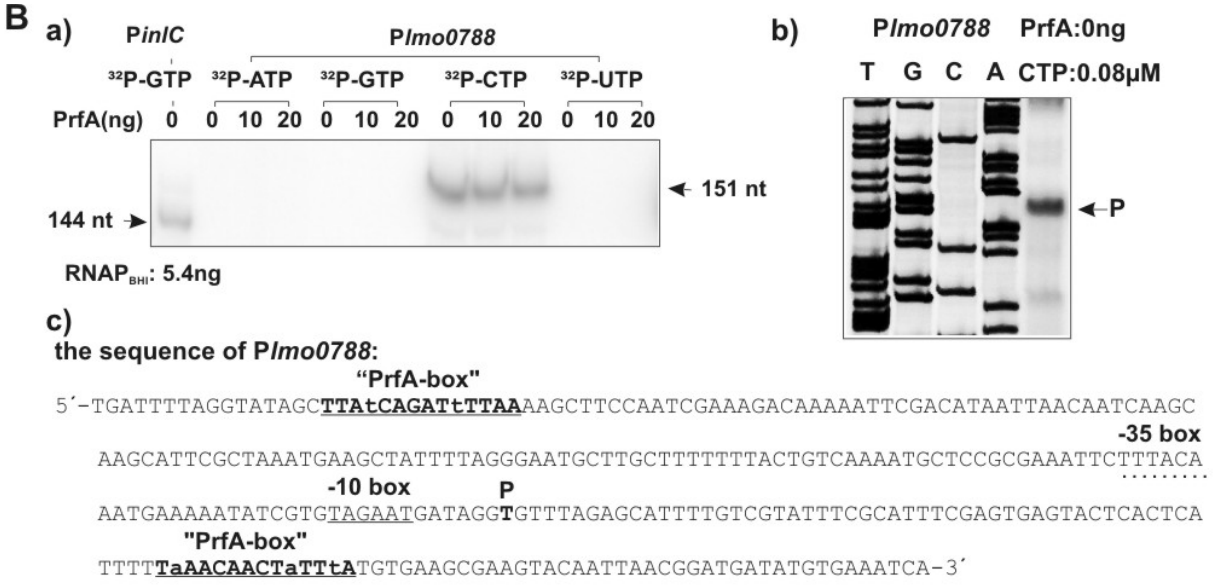


Fig. 26. (A) and (B) *In vitro* transcription with the promoter regions of the genes *lmo2219* and *lmo0788* and analysis of the *in vitro* derived reverse transcripts by primer extension (A, b for *lmo2219*; B, b for *lmo0788*). The mapped promoter sequences of the corresponding genes are shown. For initiation of *in vitro* transcription, RNAP of *L. monocytogenes*, increasing amounts of wild type PrfA and the indicated labelled [α-^{32}P]-NTP were added to the reaction mix. Putative PrfA-boxes (bold letters and underlined), –10 boxes (underlined), putative –35 boxes (dotted underlining) and the used transcriptional start sites (bold nucleotide) are indicated. The sequence ladders (A, C, G and T) were used for determining the transcription start nucleotides.

Two putative PrfA-boxes were recognized in the 5´-upstream region of *lmo0788* (Glaser *et al*., 2001; Milohanic *et al*., 2003; see Table 1), one of which contain however three mismatches and is hence unlikely to be functional. No PrfA-dependent *in vitro* transcription was observed from a promoter of gene *lmo0788* which would include any of these proposed PrfA-boxes. This was not unexpected as no suitable –10 boxes were identified in appropriate distances to any of the two "PrfA-boxes". Instead, PrfA-independent transcription was obtained at rather low efficiency in the presence of ^{32}P-CTP (Fig. 26B, a) and primer extension suggests TAGAAT as –10 box and TTTACA as –35 box (Fig. 26B, b). Hence, *lmo0788* seems to be also transcribed from a typical SigA-recognized promoter which may be also influenced *in vivo* by PrfA in an indirect way.

4.2.2. *In vitro* transcription of group II genes *lmo0178* and *lmo0278*

The genes *lmo0178* and *lmo0278* belong to those listerial genes which seem to be negatively affected by PrfA, according to the transcriptome analysis described by Milohanic *et al*., 2003. The gene *lmo0178* encodes a hypothetical xylose repressor (Glaser *et al*., 2001) and *lmo0278* the ATP-binding protein of a sugar ABC transporter. Putative PrfA-boxes with two mismatches are recognized in the regulatory up-stream sequence of the *lmo0178* gene –286 bp, of the *lmo0278* gene –324 and –37 bp, respectively from the start codons (Table 1). However, there are no suitable –10 boxes in an appropriate distance to these putative PrfA binding sites. Hence it is not surprising that *in vitro* transcription from these two putative promoters is not affected by PrfA. Instead strong PrfA-independent *in vitro* transcription starting at the *lmo0178* promoter is observed with ^{32}P-GTP and primer extension analysis determines the start site at T 6 bp downstream of a perfect –10 box (TATAAT) for SigA-

recognised promoters (Fig. 27). No *in vitro* transcription was observed with a fragment containing 340 bp upstream (including the putative PrfA-box) from the start codon of *lmo0278*.

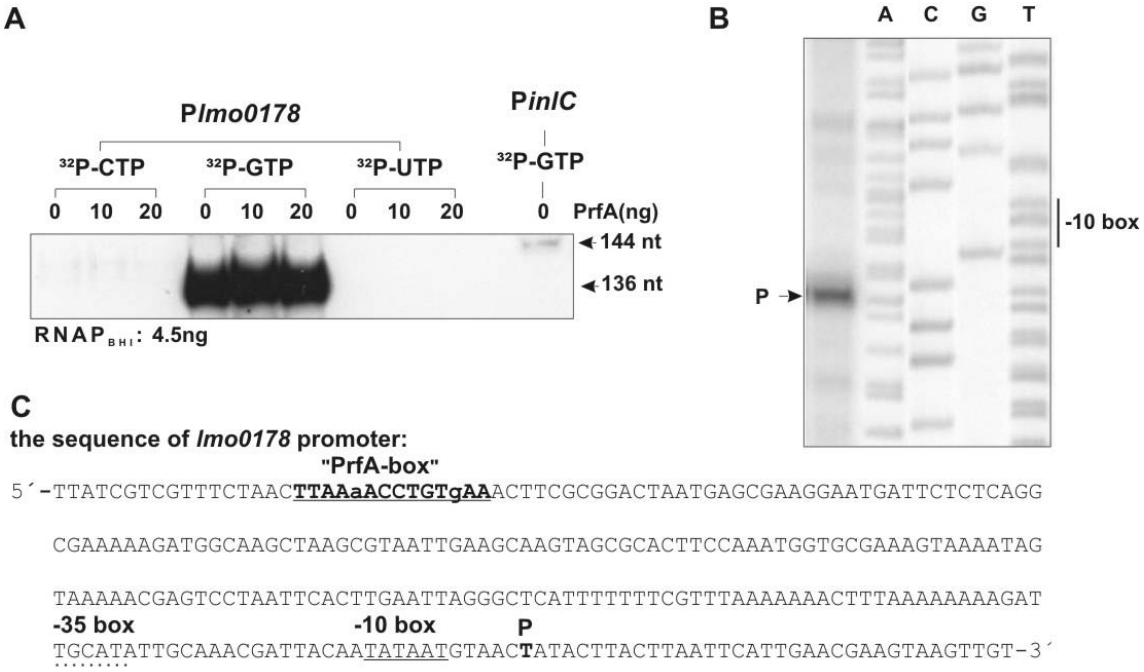


Fig. 27. ***In vitro*** **transcription starting at the promoter of** ***lmo0178*** **(A) and primer extension to determine the transcriptional start site (B); Putative PrfA-boxes (bold letters and underlined), –10 boxes (underlined), putative –35 boxes (dotted underlining) and the used transcriptional start sites (bold nucleotide) are indicated in (C). The sequence ladders (A, C, G and T) were used for determining the transcription start nucleotides.**

4.2.3. *In vitro* transcription of group III genes *lmo0596* and *lmo2067*

The gene product of *lmo0596* is of unknown function while *lmo2067* encodes a bile acid hydrolase (Bsh). Both genes carry in their upstream regulatory regions putative PrfA-boxes and at least for the *bsh* gene this PrfA-box has been claimed to be active *in vivo* (Dussurget *et al*., 2002). In addition putative sigma B (SigB) recognised –10 boxes have been observed (Milohanic *et al*., 2003) which might eventually function in combination with these PrfA-binding sites (see Table 1). However, none of these two "PrfA-boxes" showed suitable –10 boxes for SigA-dependent promoters in an appropriate distance to these putative PrfA-boxes in the regulatory regions of these two genes (Table 1). DNA fragments from the upstream regions of *lmo2067* (*bsh*) and *lmo0596* including the hypothetical PrfA-boxes and the SigB-recognized –10 boxes were used for *in vitro* transcription. No PrfA-dependent transcription was obtained from any of the two "promoter" fragments and only weak PrfA-independent transcription when SigA-loaded RNAP was used (Fig. 28 and M. Rauch PhD work). As also shown in Fig. 28, efficient *in vitro* transcription occurred with SigB-loaded RNAP in the absence of PrfA, but this transcription was not affected by increasing amounts of PrfA, suggesting that *bsh* is indeed transcribed by SigB-loaded RNA polymerase but this transcription does not involve the proposed PrfA-box.

Primer extension of *in vitro* transcripts of *lmo0596* revealed also a SigB-dependent –10 box overlapping the putative PrfA-box, and this SigB-dependent transcription is starting at A, 9 bp downstream of the anticipated SigB-recognized –10 box (Fig. 28).

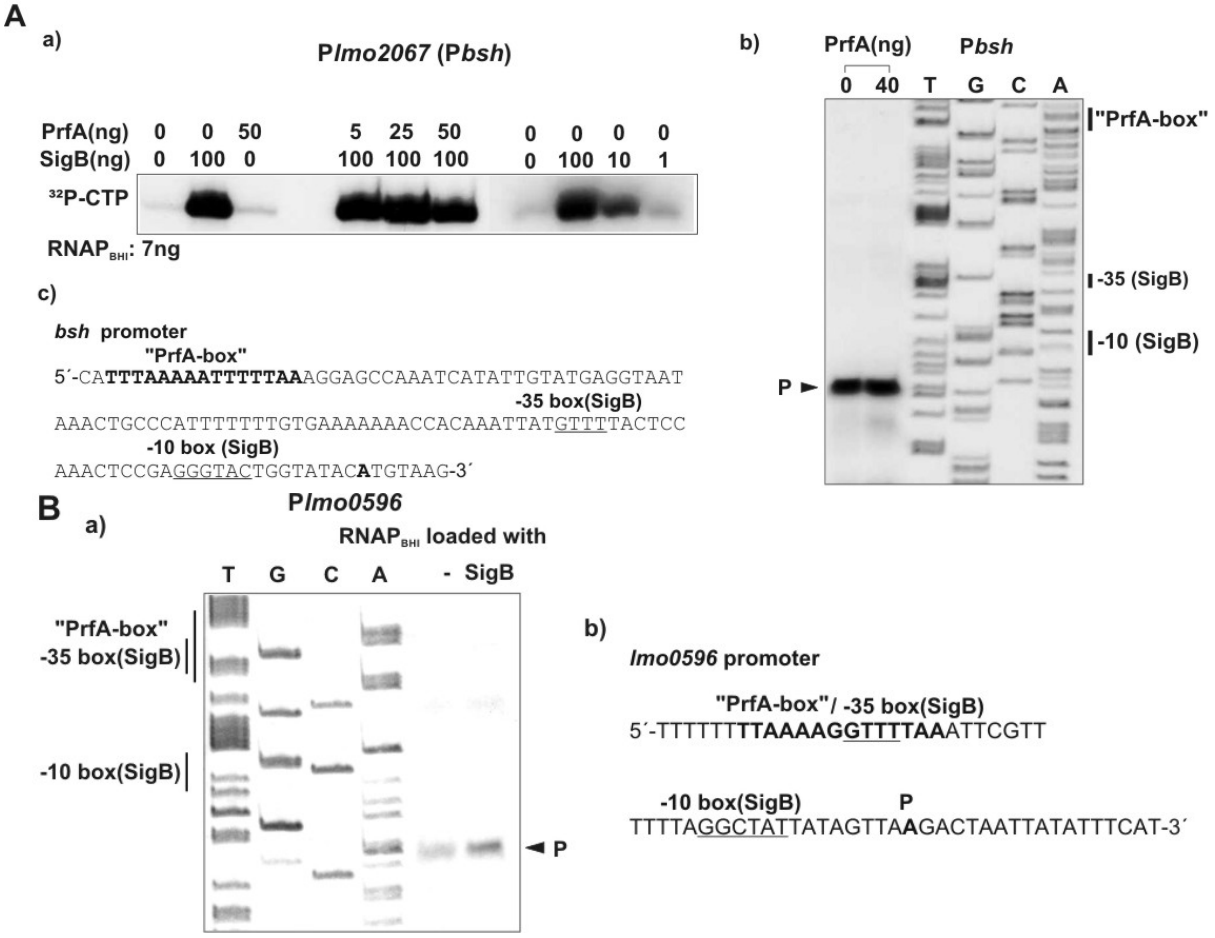


Fig. 28. (A, a) *In vitro* transcription assay with the promoter regions of the *lmo2067* (*bsh* gene). RNAP of *L. monocytogenes* was loaded with different amounts of purified SigB protein as indicated. (A. b and B. a). The corresponding *in vitro* transcripts were taken for primer extension to determine the transcriptional start sites of *lmo2067* and *lmo0596*. The putative PrfA-boxes, –35 and –10 boxes of the SigB-recognised promoters as well as the transcription start sites are indicated in A. c and B. b.

4.2.4. *In vitro* transcription of *lmo2420* and *lmo2840*

The *lmo2420* gene was included in this study since a putative PrfA-box with only a single bp deviation from the consensus sequence of the PrfA binding site was identified in the upstream regulatory region of this gene (Table 1). However, transcription of this gene did not show up as PrfA regulated in the transcriptome study of Milohanic *et al*., 2003. Furthermore, in the anticipated promoter region of gene *lmo2420* a reasonable –10 box (TATGTT) is located 20 bp apart from the putative PrfA-box. In order to fulfil all the elements of a PrfA-dependent promoter described previously, a mutant *(lmo2420+AG*) with two bp (A and G) of insertion into interspace region between the 3´-end of PrfA-box and 5´-end of the –10 box was constructed. Transcription from the wild-type P*lmo2420* and its mutant is, however, initiated in the absence of PrfA at a promoter which includes a different –10 box (TAGAAT) starting with an A 8 bp downstream of this –10 box when ^{32}P-UTP was used (Fig. 29). This transcription was not enhanced in the presence of PrfA but, unexpectedly, rather strongly

inhibited in the presence of higher concentrations of PrfA (Fig. 29, b), suggesting that PrfA interferes with RNAP binding to the *lmo2420* promoter in a yet unknown way. Such inhibitory effects of PrfA have been already demonstrated in the firset part of this work when a functional –35 box is in close proximity or even overlaps with a PrfA-binding.

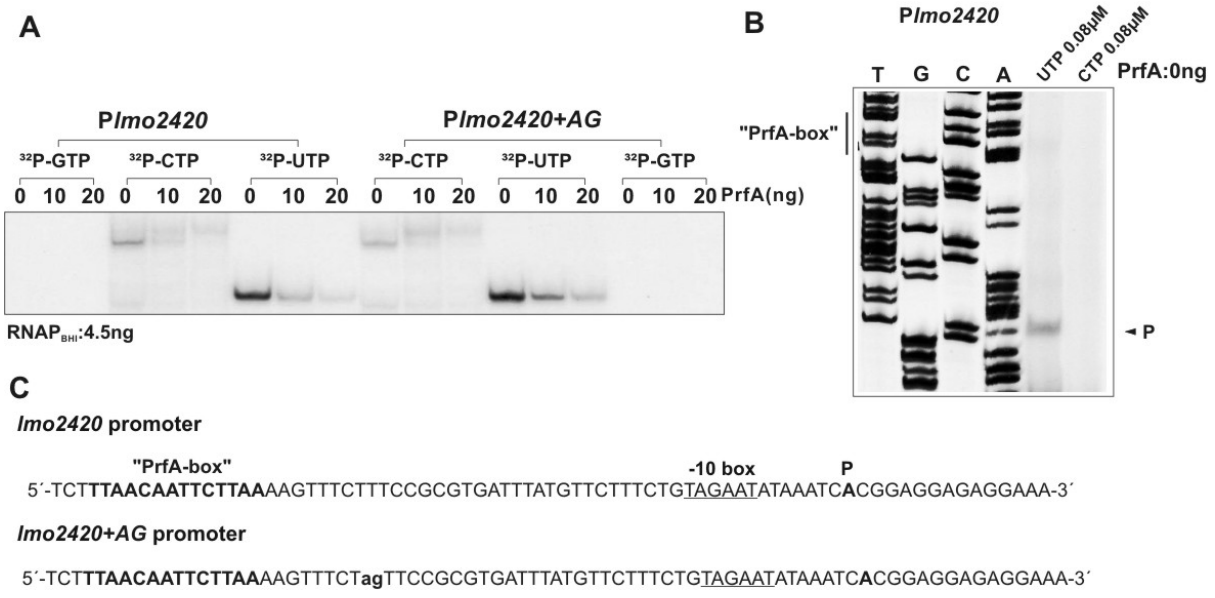
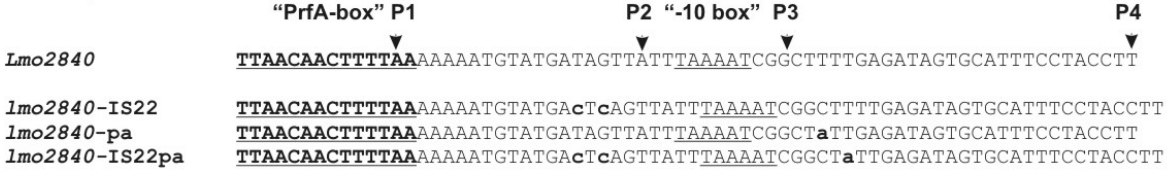


Fig. 29. ***In vitro*** **transcription starting at the promoter of** ***lmo2420*** **and its interspace mutant** ***lmo2420+AG*** **(A); the transcriptional start site of** ***lmo2420*** **was determined by primer extension (B); (C) Insertion of two base pairs in the interspace region are indicated in small bold letter in the sequence of promoters; the –10 box is underlined and the transciptional start site is indicated as P and printed in bold letter.**

lmo2840 encoding a putative sucrose phosphorylase carries also a putative PrfA-box with one single base pair deviation in the upstream regulatory region (Table 1). This gene is not included in the groups of PrfA-regulated genes found by Milohanic and his co-workers (2003), but the transcription of this gene is up-regulated in the presence of over-expressing PrfA when bacteria grow in minimal medium (MM) with glucose (A. K. Marr *et al*., unpublished results).

However, no *in vitro* transcription with four kinds of labelled ^{32}P-NTPs was observed from this gene promoter. It occured also initiation from the altered mutants P*lmo2840*-IS22, P*lmo2840*-pa, and P*lmo2840*-IS22pa that contains almost all elements of a PrfA-dependent promoter including a putative SigA-recognized –10 box (TAAAAT), a fixed distance between the PrfA-box and the –10 box of 22 bp, a putative purine start nucleotide 6 bp apart from the 3´-end of the –10 box (Fig. 30). The transcripts obtained with high concentration of four non-labelled NTP (200 µM) were analysed by primer extension, suggesting no reasonable transcriptional start site related to the putative PrfA-box and this transcription was not increased in the presence of higher concentration of PrfA (Fig. 30).

The sequences of *lmo2840* promoter and its altered mutants:



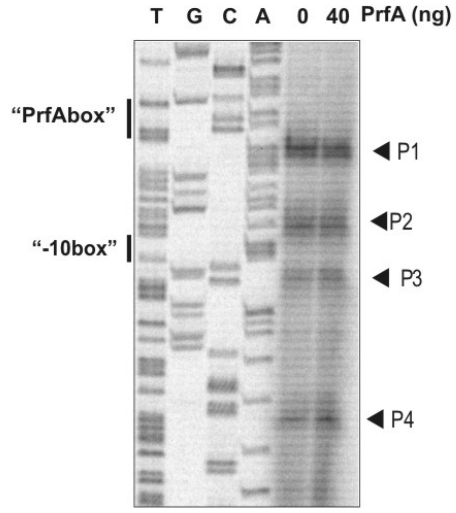


Fig. 30. The sequences of *lmo2840* and its mutants. The putative PrfA-box and –10 box are underlined; the transcription start sites are indicted by arrows and marked by P1 to P4. Insertions and base change are shown as small bold letters.

4.3. Exchange of corresponding elements of P*aroA* and P*plcA* and efficiencies of *in vitro* transcription starting at these hybrid promoters

This study attempts to understand why sequences which apparently contain the all known elements of PrfA-dependent promoters, i.e. a PrfA-box and a –10 box recognized by SigA-loaded RNA polymerase in an appropriate distance (Luo, *et al*., 2004), do not function as PrfA-dependent promoters. For this goal, the *plcA* promoter, which is functional as PrfA-dependent promoter *in vivo* and *in vitro* was compared with an upstream regulatory sequence of the *aroA* gene (termed P*aroA*P2) which contains similar PrfA- and –10 boxes (in the same distance) as P*plcA* but does not function as PrfA-dependent promoter.

4.3.1. Comparison of *in vitro* transcription starting at P*plcA* and P*aroA*

plcA encodes a phosphatidylinositol-specific phospholipase C (PlcA). Transcription starting at the promoter of *plcA* (P*plcA*) has been shown to be dependent on PrfA *in vivo* (Leimeister-Wächter *et al*., 1991; Mengaud *et al*., 1991) and *in vitro* (Fig. 31 and Lalic-Mülthaler *et al*., 2001). The sequence of the PrfA binding site of P*plcA* which serves also as PrfA binding site for the *hly* promoter (P*hly*) exhibits a perfect dyad symmetry (Kreft and Vazquez-Boland, 2001).

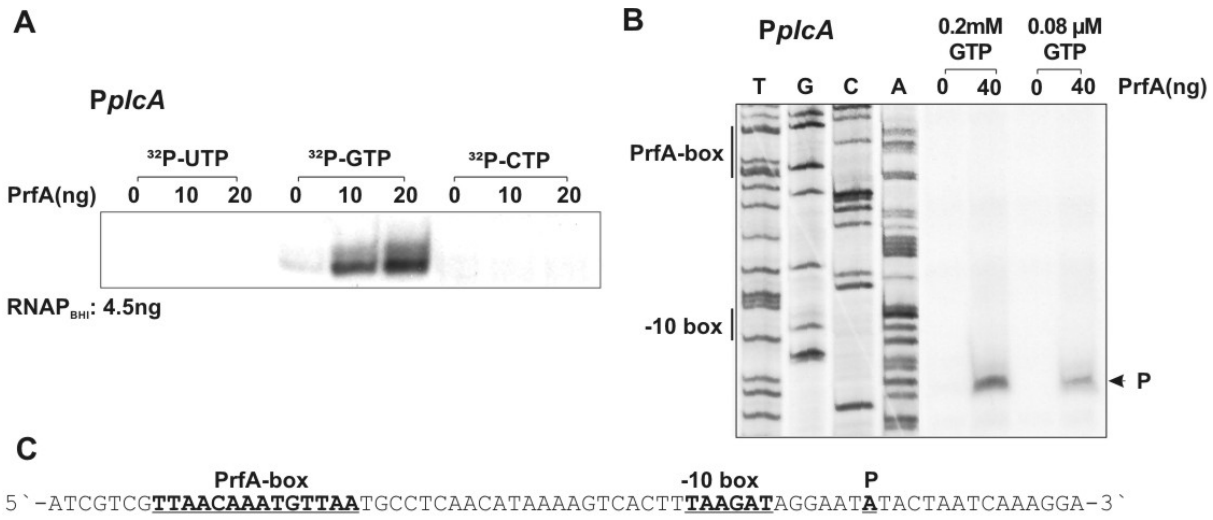


Fig. 31. (A) *In vitro* transcription starting at the *plcA* promoter; (B) Primer extension of *in vitro* synthesized transcripts initiated at P*plcA* in the presence of low (0.08 µM) or high (200 µM) GTP with and without PrfA. The PrfA-box, –10 box, and transcription start site are indicated in (C). The sequence ladders (A, C, G and T) were always used for determining the transcription start nucleotides.

aroA is the first gene of the operon encoding the enzymes for the common pathway of aromatic amino acids biosynthesis (Glaser *et al*., 2001). A putative PrfA binding site with only a single mismatch compared to the PrfA-binding site of P*plcA* and an appropriate –10 box (TTTAAT) 21 bp downstream of this sequence are identified in the regulatory upstream region of the *aroA* gene (Fig. 32). However *in vitro* transcription assays did not result in PrfA-dependent transcription but rather showed that *in vitro* transcription started from a PrfA-independent promoter (in the following termed P*aroA*P1) with a –10 box (TAATAT) partially overlapping the putative PrfA-box and a –35 box (TTGTAA) upstream of this PrfA-box (Fig. 32). The PrfA-independent transcription observed from P*aroA*P1 is considerably more efficient than that from P*plcA* even in the presence of PrfA. The presence of G in the region downstream of the –10 box of the putative PrfA-dependent *aroA* promoter (which is termed in the following P*aroA*P2) is not the reason for the failure of PrfA-dependent transcription from this promoter since the use of ^{32}P-UTP (U would not be within the first 4 nucleotides of a possible RNA transcribed from this promoter) or the removal of the GCG from this downstream sequence did not result in PrfA-dependent transcription from P*aroA*P2 either (A.K. Marr PhD work). Previous results have also shown that the optimal length of the interspace region between the PrfA-box and the –10 box is 22 or 23 bp while that of the putative *aroA* promoter is only 21 bp (Fig. 32). Therefore one additional bp (G or A) was inserted into the interspace sequence of P*aroA*P2 (the promoter carrying the putative PrfA-box and TTTAAT as –10 box) but none of these changes resulted in PrfA-dependent transcription. The insertions rather resulted in a decreased efficiency of the PrfA-independent transcription which still started at P*aroA*P1 but the start site moved now from A in position 8 to T in position 6 downstream of the –10 box (Fig. 32).

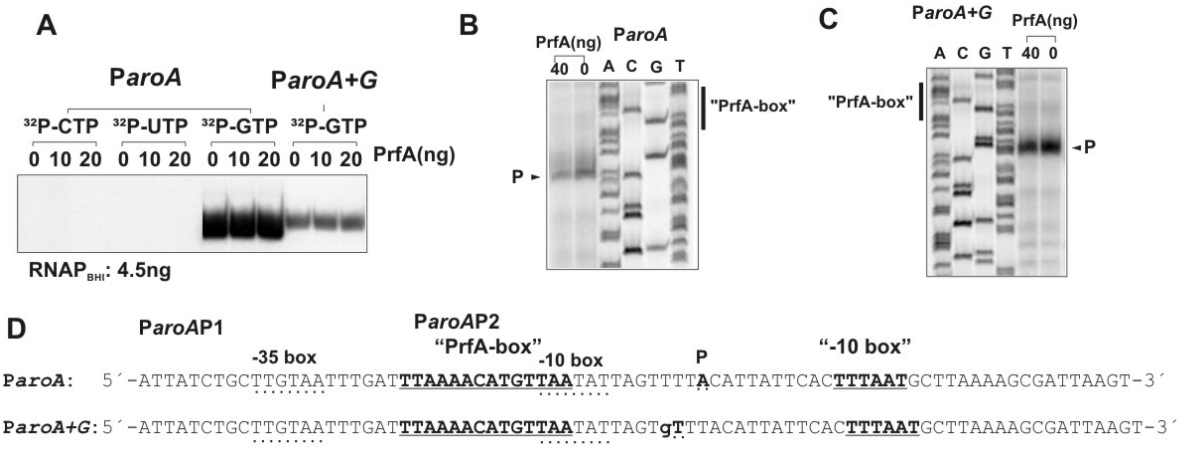


Fig. 32. (A) *In vitro* transcription of P*aroA* and its interspace mutant P*aroA+G*. (B and C) The transcriptional start site of P*aroA* and mutant P*aroA+G* were determined by primer extension. (D) The sequence of P*aroA* and mutant P*aroA+G.* Putative PrfA-boxes and putative –10 boxes of P*aroA*P2 are indicated by bold letters and underlined; the used –35 boxes and –10 boxes and the used transcriptional start sites of P*aroA*P1 are dotted underlined. The insertion nucleotide is shown by the small bold letter. The sequence ladders (A, C, G and T) were used for determining the transcription) start nucleotides.

4.3.2. *In vitro* transcription of P*plcA*-P*aroA* hybrid promoters

Since none of the above described changes in P*aroA*P2 led to PrfA-dependent transcription, longer sequence elements between the sequences of P*plcA* and P*aroA*P2 were exchanged using the P*aroA*P2 sequence with the 22 bp interspace region (P*aroA*+G; see Fig. 33).

Exchange of the PrfA-box of P*plcA* by that of P*aroA*P2 (mutant P*plcA*-P*aroA*-m1; Fig. 34A) led to PrfA-dependent transcription which was only slightly reduced compared to that of the original P*plcA,* suggesting that the PrfA-box of P*aroA*P2 is functional. Exchange of the –10 box in P*plcA* by that of P*aroA*P2 (mutant P*plcA*-P*aroA*-m2) also resulted in PrfA-dependent transcription with further reduced transcription efficiency compared to P*plcA* (Fig. 34A). Exchange of both elements, the PrfA-box and the –10 box of P*plcA* by those of P*aroA*P2 (mutant P*plcA*-P*aroA*-m3), resulted in further reduction of transcription efficiency of this hybrid promoter but transcription remained PrfA-dependent. In contrast, exchange of the P*plcA* sequence by the two elements of P*aroA*P2 including the interspace region (mutant P*plcA*-P*aroA*-m4) led to PrfA -independent transcription starting now again at the original PrfA-independent P*aroA*P1. Although, however, the transcription efficiency of this promoter is reduced compared to the original P*aroA*P1 promoter (Fig. 34A), probably due to the replacement of the original –35 box of P*aroA*P1 by a less efficient new –35 box in the corresponding P*plcA* sequence. The importance of the –35 sequence of P*aroA*P1 in the PrfA-independent transcription was demonstrated by deletion of this sequence (mutant P*plcA*-P*aroA*-m8) (Fig. 34C). The obtained results suggest that the –10 box of P*aroA*P1 now present in the interspace region of the new hybrid promoter may inhibit PrfA-dependent transcription. Therefore, this –10 site was inactivated by converting the TAATAT sequence into TAATGC (mutant P*plcA*-P*aroA*-m5). This alteration indeed resulted in efficient PrfA-dependent transcription starting at this hybrid promoter (Fig. 34B), showing that the –10 box

in the interspace region of P*aroA*P2 is indeed an inhibitory element for PrfA-dependent transcription from P*aroA*P2. This assumption is further supported by the exchange of the interspace region of P*plcA* by that of P*aroA*+G (mutant P*plcA*-P*aroA*-m7). This hybrid promoter led to transcription from the –10 site of P*plcA* which was only weakly activated by PrfA but also to weak PrfA-independent transcription starting from the –10 box of P*aroA*P1 (Fig. 34 B).

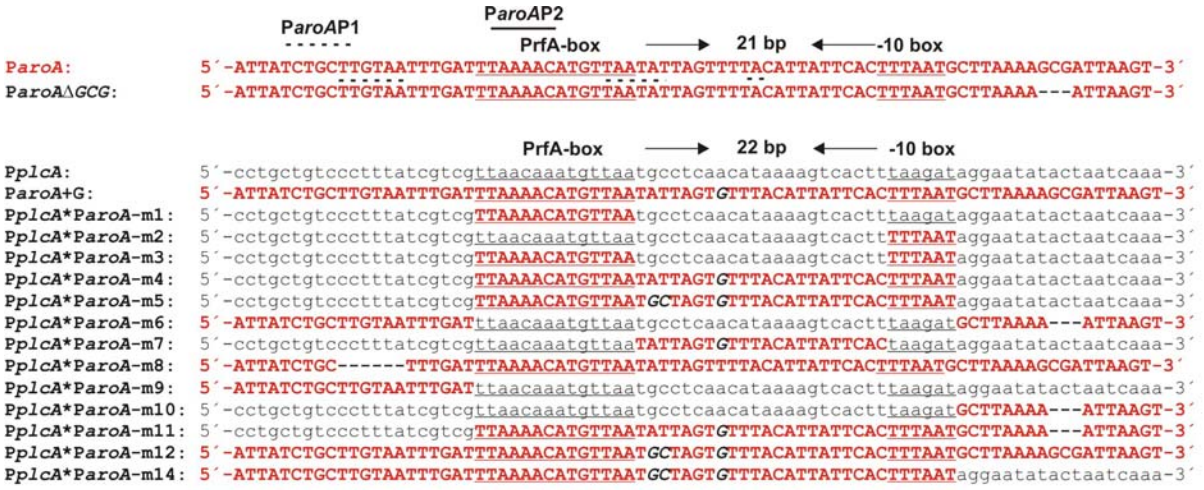


Fig. 33. The sequences of *aroA* promoter, *plcA* promoter (indicated by small letters) and the hybrid mutants. The corresponding fragments of *aroA* promoter are indicated by red colour and capital letters. The deletion sequences are replaced by dashed lines. The insertion and exchange are shown by capital bold dark letters. Putative PrfA-boxes and putative –10 boxes of P*aroA*P2 are underlined; the used –35 boxes and –10 boxes and the used transcriptional start site of P*aroA*P1 are dotted underlined.

Interestingly, exchange of the sequence downstream of the –10 site of P*plcA* by the corresponding sequence of P*aroA*P2 (mutant P*plcA*-P*aroA*-m10) completely blocked transcription of this hybrid promoter, whereas the exchange of the sequence upstream of the PrfA site of P*plcA* by the corresponding sequence of P*aroA*P2 (mutant P*plcA*-P*aroA*-m9) did not alter PrfA-dependent transcription starting at the –10 site of P*plcA* and transcription efficiency of this hybrid promoter seems to be even slightly higher compared to P*plcA* (Fig. 34). The opposite exchange, i.e. exchange of this PrfA-box upstream sequence of P*aroA*P2 by the corresponding P*plcA* sequence (mutant P*plcA*-P*aroA*-m11) abolished transcription from this hybrid promoter entirely, suggesting that the P*plcA* upstream sequence does not contain a –35 box necessary for PrfA-independent transcription from P*aroA*P1 (Fig. 34C).

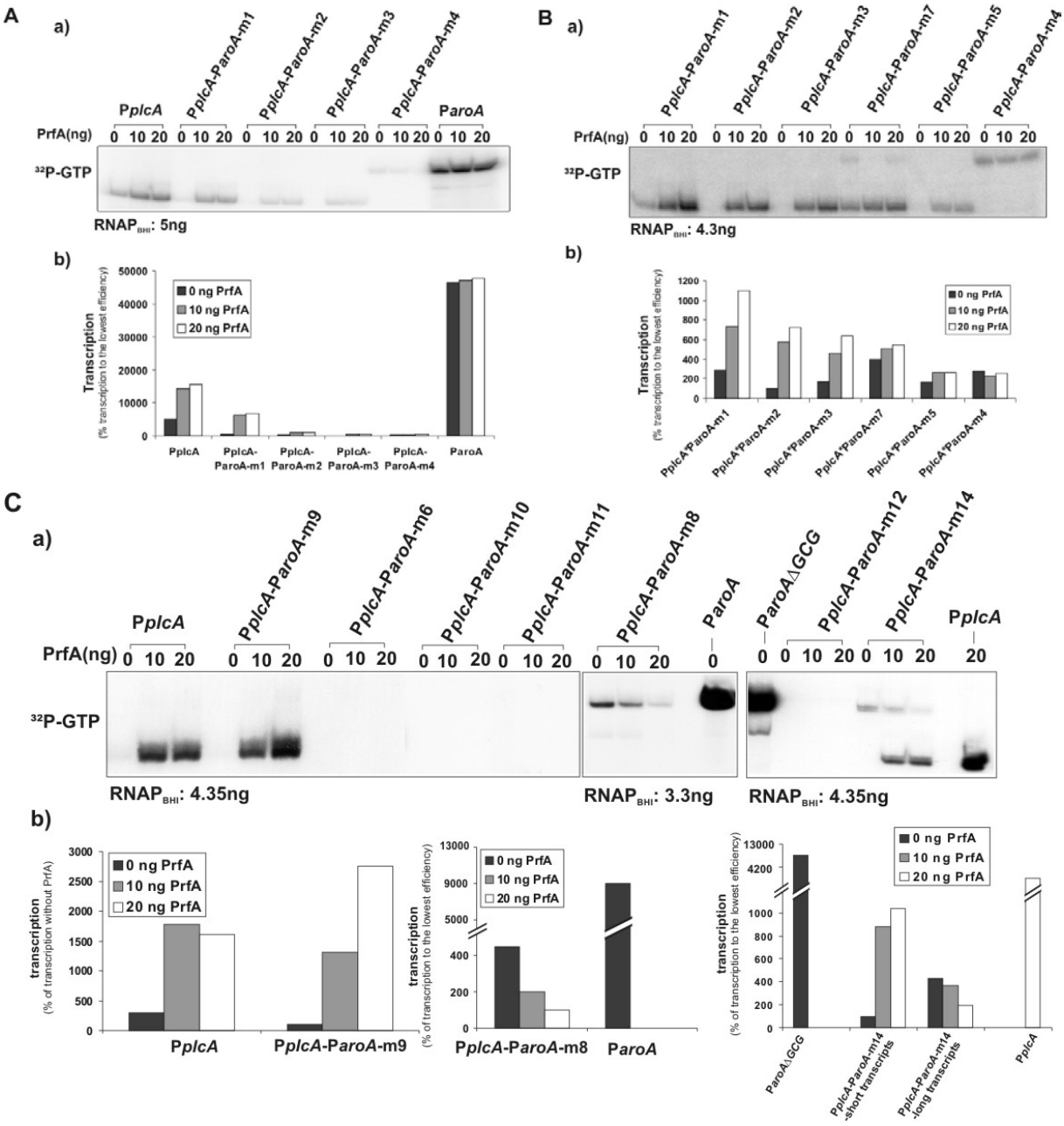


Fig. 34. *In vitro* transcription of hybrid mutants P*plcA*-P*aroA*-m1 to -m14 with indicated radiolabeled ^{32}P-NTP, the amount of RNA polymerase and PrfA protein. The relative efficiency of *in vitro* transcription of hybrid promoters was analysed by phosphorimaging.

These data indicate that the sequence downstream of the –10 box but not the sequence upstream of the PrfA-box of P*aroA*P2 is inhibitory for transcription. Using the MFOLD program developed by Zuker and Turner (Zuker *et al*., 1991; Zuker, 2003; http://www.bioinfo.rpi.edu/applications/mfold/) a stable hairpin loop structure is predicted to appear in the downstream region of P*aroA*P2, which may inhibit the transcription from the PrfA-dependent P*aroA*P2 promoter (Fig. 35).

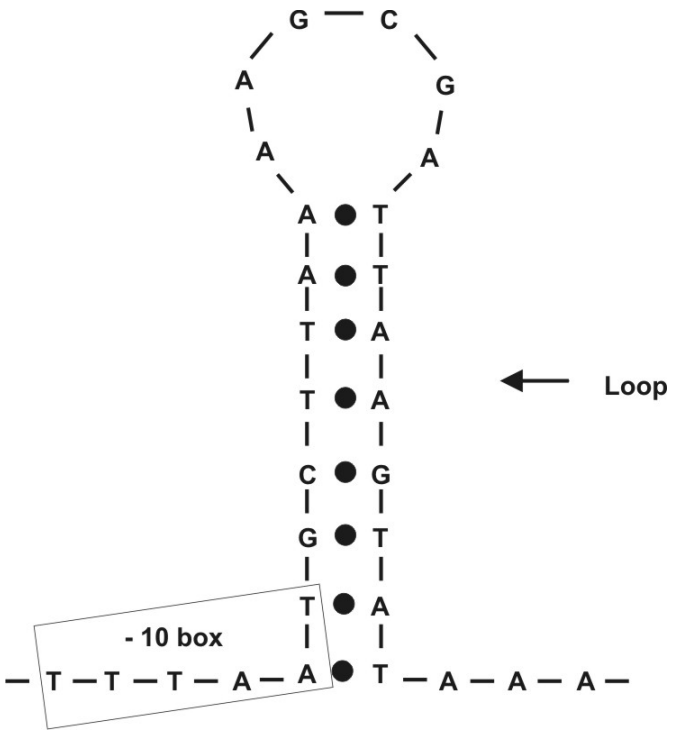


Fig. 35. Predicted secondary structure of the downstream region of the –10 box of P*aroA*P2. The hairpin loop is indicated by an arrow and the –10 box of P*aroA*P2 is boxed.

5. Discussion

5.1. Essential features of a PrfA-dependent promoter of *L. monocytogenes*

5.1.1. Interaction of PrfA, RNA polymerase and PrfA-dependent promoters of *L. monocytogenes*

Promoters are specific DNA sequences to which RNA polymerase(s) bind(s) and initiate(s) transcription. In prokaryotes, the interaction of RNA polymerase with promoters is best characterized in the gram-negative bacterium *Escherichia coli* and the gram-positive bacterium *Bacillus subtilis*. Four important promoter elements are distinguished: two hexamers centered at or near positions –10 and –35 upstream from the transcription start site (designated by their locations as the –10 and –35 region); the spacer DNA with a consensus length of 17 bp between the –10 and –35 region; a very A + T -rich region between positions –40 and –60 (termed UP element) identified recently in some promoters as an additional important determinant of promoter activity and an "extended –10" region with a TGN motif in *E. coli* and TRTG motif in *B. subtilis* located just upstream of the –10 region, which enhances promoter activity in the absence of –35 region (Voskuil and Chambliss, 1998; Keilty and Rosenberg, 1987; Belyaeva *et al*., 1993; Camacho and Salas, 1999). The RNA polymerase (RNAP) holoenzyme contains five subunits with the stoichiometry $\alpha_2\beta\beta'\sigma$. Sigma factor is responsible for specific promoter recognition. The primary sigma factor in *E. coli* is encoded by the *rpo*D gene and is known as σ^{70}. In *B. subtilis* and other gram-positive bacteria, this sigma factor is known as SigA (σ^A) (Wösten, 1998; Haldenwang, 1995), therefore, the major form of *E. coli* RNA polymerase is holozyme $E\sigma^{70}$ and in *B. subtilis* $E\sigma^A$. The binding of RNAP holoenzyme to the promoter results in a so-called "closed complex", which is converted into an "open complex" by melting of the –10 promoter region. In this open form the RNAP holoenzyme starts to produce small RNA molecules 2 to 12 bp. A ternary complex is then formed. After dissociation of the sigma subunit from the holoenzyme, the RNAP core enzyme moves along the DNA, synthesizing the nascent RNA molecule. A locally unwound region of DNA moves with the enzyme. The RNAP core enzyme and RNA are released when a terminator structure or factor is encountered and the DNA is then fully restored to the duplex form (Record *et al*., 1996)

L. monocytogenes belongs to gram-positive facultative intracellular bacterial pathogens that can cause serious infections in human (Gray and Killinger, 1966; Vazquez-Boland *et al*., 2001). 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* (see recent reference: Kreft and Vazquez-Boland, 2001; Vazquez-Boland *et al*., 2001). On the basis of structural and functional similarities, PrfA belongs to the Crp/Fnr family of transcription regulators (Lampidis *et al*., 1994). PrfA binds to a palindromic PrfA regulation sequence termed PrfA-box that is located at around position –40 from the transcription start site in PrfA-regulated promoters (Mengaud *et al*., 1989; Sheehan *et al*., 1996). Electrophoretic mobility shift assays (EMSA) show that purified PrfA protein can bind to a 28 bp *hly* probe containing only the entire PrfA-box and form the CIII complex. A slower migrating PrfA-RNAP-DNA complex

corresponding to "CI" can not be generated, whereas a 109 bp fragment carrying the entire *hly* promoter in addition to this PrfA-box can generate both CI and CIII complexes, suggesting that although PrfA protein alone is able to bind to the target DNA sequence containing the anticipated PrfA-box, a more extended target sequence than just the PrfA-box is apparently necessary for formation of CI complex and hence for PrfA-dependent transcriptional initiation (Dickneite *et a*l., 1998). The complex "CII" that represents the binding of RNAP alone to the promoter is unstable and can be converted into CI by addition of purified PrfA (Böckmann *et al*., 2000). This result is in line with the data obtained from the DNase I footprint assays that purified PrfA alone protects the PrfA-box, 8-10 nucleotides upstream and 2 nucleotides downstream of it (Vega *et al*., 1998; Dickneite *et al*., 1998). Upon addition of partially purified RNA polymerase of *L. monocytogenes*, the region protected from DNase digestion extends down to the –10 box of the promoter and also enlarges the 5' -end upstream protected region of the palindromic PrfA binding site, suggesting that bound RNAP probably overlaps the PrfA protein (Böckmann *et al*., 2000). However, RNAP of *L. monocytogenes* is not capable of protecting the promoter regions of PrfA-regulated genes on its own, indicating that PrfA is absolutely necessary to mediate a strong and specific binding of RNAP to the promoter region of the PrfA-regulated genes (Böckmann *et al*., 2000). The PrfA-dependent promoters resemble more closely class II CRP-dependent promoters of *E. coli* (Busby and Ebright, 1997), not only in both cases the binding site of the transcriptional activator is centred at position –41 and overlaps the –35 region but also a PrfA/RNAP complex contacting regions upstream and downstream of the PrfA-box is similar to the situation known for class II CRP-dependent promoters, i.e. CRP contacts the α-subunit of the RNAP at its N- as well at its C-terminal domain and RNAP binds upstream as well as downstream of CRP thus spanning the region where CRP is bound (Belyaeva *et al*., 1996; Busby and Ebright, 1997).

In this study, the characterization of a PrfA-dependent virulence gene promoter, *inlC* promoter, has been described in detail, using the recently established "run-off*" in vitro* transcription assay (Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001). The *in vivo* observed PrfA-dependent *inlC* transcription (Engelbrecht *et al*., 1996) could be reproduced in the *in vitro* transcription system using purified RNA polymerase (isolated from logarithmically grown bacteria cultivated in rich BHI medium and hence probably loaded mainly with SigA (Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001) and PrfA protein from *L. monocytogenes* when a high concentration of GTP (200 μM) is present in the reaction mix, whereas the *in vitro* transcription starting from a PrfA-box deletion mutant (mutant P*inlC*-m8, Fig. 14) is absolutely independent of PrfA, suggesting that the PrfA binding site (PrfA-box) is indeed necessary for the PrfA-dependent transcription, which is in agreement with previously obtained *in vivo* and *in vitro* results (Freitag *et al*., 1993; Sheehan *et al*., 1996; Vega *et al*., 1998; Dickneite *et al*., 1998; Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001).

The –10 box of P*inlC* closely resembles the SigA-recognized –10 promoter sequences of *B. subtilis*, which is also observed in the other known PrfA-dependent promoters of *L. monocytogenes*. Compilation and analysis of six of the strongly PrfA-dependent promoters (P*hly*, P*plcA*, P*mpl*, P*actA*, P*inlC* and P*hpt*) identified both *in vivo* and *in vitro* to date as well

as one partially PrfA-dependent promoter (P*inlA*P3) of *L. monocytogenes* reveal a highly conserved Pribnow box-like sequence (TATAAT) around the –10 position (Table 2). The conserved –10 sequences are 100% T, 100% A, 57.1% T, 42.9% A, 71.4% A and 100% T. The first, second, and sixth base pairs in this hexameric sequence (T, A, and T, respectively), the positions of which are important for the function of SigA-dependent promoters in *B. subtilis* (Helmann, 1995) are also strongly conserved in these PrfA-dependent promoters. Interestingly, the *mpl* promoter, the only one carrying a perfect –10 consensus sequence TATAAT related to SigA-dependent promoters in *B. subtilis*, requires a high concentration of GTP for the PrfA-dependent transcription under *in vitro* condition (Luo, *et al*., 2004), suggesting a complex interaction between the PrfA-RNAP and the PrfA-dependent promoter.

	PrfA-box (– 40) ⟶ TTAACANNTGTTAA	interspace	⟵ –10 box	+1	Description
hly:	AGGCA**TTAACATTTGTTAA**CGACGATAAAGGGACAGCAGGAC**TAGAAT**AAAGCT**A**TAAAGCA	23bp			Listeriolysin O
plcA:	CGTCG**TTAACAAATGTTAA**TGCCTCAACATAAAAGTCACTT**TAAGAT**AGGAAT**A**TACTAATC	22bp			Lecithinase
mpl*:	AAGAA**TTAACAAATGTaAA**AGAATATCTGACTGTTTATCCA**TATAAT**ATAAGC**A**TATCCCAA	22bp			Metalloprotease
actA:	ACTGA**TTAACAAATGTTAg**AGAAAAATTAATTCTCCAAGTGA**TATTCT**TAAAA**T**AATTCATG	23bp			Actin-assembly inducing protein
inlC:	TATTA**TTAACgCTTGTTAA**TTTAAACATCTCTTATTTTTGC**TAACAT**ATAA**G**TATACAAAGG	23bp			Internalin C
hpt:	GCATG**aTAACAAGTGTTAA**TGACGGAAAGAGAGTATCTGGTT**TATATT**TTTAT**C**AGCGCAAA	23bp			Hexose phosphate transport protein
***InlA*P3:**	AGAGG**aTAACATAaGTTAA**TTCTTTTTTTTGGAAAAATAGT**TATTAT**TATTTAATGGGCTTT	22bp			Internalin A

Table 2. DNA sequences of PrfA-dependent promoters from *L. monocytogenes* (Domann *et al*., 1993; Lingnau *et al*., 1995; Engelbrecht *et al*., 1996; Brehm *et al*., 1996; Lalic-Mülthaler *et al*., 2001; Luo *et al*., 2004; Luo *et al*., manuscript submitted). *mpl: The PrfA-dependent *in vitro* transcription of *mpl* gene requires high concentration of GTP (Luo *et al*.,2004). Minor case letters indicate deviation from the consensus sequence of the PrfA-box.**

Another feature of PrfA-dependent promoters different from that of *B. subtilis,* is that none of the –35 regions of these seven PrfA-regulated promoters shows any homology with TTGACA, a consensus sequence present in the –35 region of most SigA-dependent promoters in *B. subtilis*. An explanation may be that the PrfA-box is centered proximal to this region and thus overlaps it. In the case of the *inlC* promoter, it is found that this –35 region can tolerate a greater variety of sequences without effect of PrfA-dependent activity (Fig. 11). Moreover, the extended –10 motif (5´-TRTG-3´) frequently found in SigA-recognised promoters (contributing to the maintenance of DNA-strand separation; Voskuil and Chambliss, 1998) is observed in P*inlC.* The absence of this typical prokaryotic –35 region consensus sequence TTGACA appears to be a distinctive feature of *mycobacterial* and *streptomycete* promoters, owing presumably to the presence of multiple sigma factors with different or overlapping specificities for –35 regions (Strohl, 1992; Westpheling *et al*., 1985; Bashyam *et al*., 1996). *L. monocytogenes* encodes five sigma factors: SigA, SigB, SigL, SigH and SigECF (Glaser *et*

al., 2001). Moreover, it was previously shown that *in vitro* transcription from some PrfA regulated gene promoters, such as P*actA*, P*hly,* functioned differently with RNA polymerase purified from *L. monocytogenes* which was cultured either in rich culture medium ($RNAP_{BHI}$), or exposed to heat shock conditions ($RNAP_{48}$) or conditioned in minimal essential medium ($RNAP_{MEM}$) (Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001). It is therefore presumed that this RNA polymerase is loaded with different sigma factor(s). Similar results were also observed in the *in vitro* transcription starting at the *inlC* promoter (Fig. 10). Furthermore, there is growing evidence that in addition to the sigma factor, the α subunit of RNA polymerase (especially αCTD) also plays a direct role in promoter recognition via binding to the A + T -rich sequence located immediately upstream of the –35 element (Ross *et al*., 1993; Estrem *et al*., 1999). This α recognition element is named the "upstream element" (UP), consisting of a region of about 20 bp from –59 to –38 (5´-NNAAAWWWTWTTTTNNNAAANNN-3´; W= A or T, N= any base) in the *E. coli rrnB* P1 promoter (Ross *et al*., 1993; Rao *et al*., 1994; Estrem *et al*., 1999). UP elements are more prevalent in gram-positive bacteria promoters than in *E. coli* (Helmann, 1995). Interactions between αCTD bound to the proximal UP element and σ bound to the –35 hexamer could potentially stabilize initiation complexes and thus contribute to the efficient transcription. There is no additional consensus region found in the upstream region of PrfA-dependent promoters, except for the PrfA-box, it would therefore be reasonable to assume that PrfA may also interact with sigma A and αCTD together to form a transcription complex. The same situation is found in the λ cI protein activating the λP_{RM} promoter by binding to a site centered at position –42 and overlapping the –35 element (Hochschild *et al*., 1983; Busby and Ebright, 1994), and genetic evidence shows that λ cI and σ^{70} are in direct physical proximity (and possibly in direct contact) in the ternary complex of λ cI, RNAP and promoter P_{RM} (Li *et al*., 1994; Estrem *et al*., 1999; Gourse *et al*., 2000).

The distance between the PrfA-box and the –10 box of the *inlC* promoter is critical and functions optimally when the two sites are 22 bp apart (a distance of 23 bp is tolerated with a slightly reduced transcription efficiency) while a distance of 21 or 24 bp already leads to a drastic reduction in PrfA-dependent transcription (Fig. 15 and Fig. 16). The importance of the length of this interspace region for PrfA-dependent transcription is also confirmed *in vivo* by β-galactosidase analysis of different interspace mutants in the *inlC* promoter (Fig. 17). 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 similar to the spacer DNA between –35 and –10 region in the SigA-dependent promoters of *B. subtilis* and σ^{70} -dependent promoters of *E. coli* (von Hippel *et al*., 1984; McClure, 1985). 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 (Stefano and Gralla, 1982; Auble and deHaseth, 1988; Ayers *et al*., 1989) 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 (Siebenlist *et al*., 1980): the spacer DNA is untwisted by RNAP to align the –35 and –10 regions on the same face of the double helix and to interact with RNAP. The

untwisting of the spacer DNA places stress on the DNA, resulting in generation of free energy thus stored in the DNA, which would drive the nucleation of the strand separation process (Mulligan *et al*., 1985; Stefano and Gralla; 1980, 1982; Ayers and deHaseth, 1988; deHaseth and Helmann, 1995).

The position of the start sites for PrfA-dependent transcription initiation at P*inlC* or the generated P*inlC* mutants (either G or A) seems to be rather flexible (5 bp to 8 bp downstream of the –10 box). The preferred start positions of the PrfA-independent transcription are A7 or A8, similar to those observed for promoters recognized by SigA-loaded RNA polymerase of *B. subtilis* (Helmann, 1995). The distance between the –10 box and the transcription start site of *B. subtilis* varies from four to ten bases and the average value is seven bases. Furthermore, *L. monocytogenes* RNAP appears to initiate transcription preferentially with a purine, as also noted previously in *B. subtilis* and *E. coli* (Hawley and McClure, 1983; Helmann, 1995).

5.1.2. Transcription from the PrfA-independent promoter and the second PrfA-box-like region (modified from a pseudo PrfA-box) of *inlC*

PrfA-independent transcription occurs in the absence of the PrfA-box, suggesting that P*inlC* can be considered as consisting of two overlapping promoters, one being PrfA-dependent and the other PrfA-independent. Moreover, inactivation of the –35 box yields exclusively PrfA-dependent transcription, indicating that the –35 box is not needed for PrfA-dependent transcription from the *inlC* promoter but necessary for PrfA independent transcription. The PrfA-independent promoter of *inlC* obviously shares the –10 box (TAACAT) with the PrfA-dependent promoter and uses TTTAAA as –35 box; the two sites essential for binding of RNA polymerase are 16 bp apart, which is in line with the promoter pattern in *B. subtilis*. Most of these SigA-dependent promoters have distances of 16 or 17 bp between the –35 and the –10 boxes (Helmann, 1995; Jarmer *et al*., 2001; SWISS-PROT, SubtiList). The same holds true for the different –10 and –35 sites of the various P*inlC* mutants constructed. However, unexpectedly it is found that only certain combinations of –10 and –35 sites (with a fixed distance of 16 bp adapted to the fixed 22 bp interspace region between the –10 box and the PrfA-box of the PrfA-dependent promoter) function as overlapping PrfA-independent *inlC* promoter. The original –10 box of the *inlC* promoter (TAACAT) combines with TTTAAA (and even better with TGTTAA) as –35 box functions efficiently, whereas the replacement of this –10 box by TATAAT does not show efficient PrfA-independent transcription with these –35 boxes. Likewise, the combination of the original –10 box of P*inlC* (TAACAT) with the consensus –35 box (TTGACA) is also inactive, whereas the combination TATAAT (–10 box) and TTGACA (–35 box) is again highly active as PrfA-independent promoter. This complex combination for PrfA-independent transcription appears to correlate well with the observation that gram-positive bacterial RNAPs commonly utilize different combinations of several elements in addition to the –35 and –10 regions to form functional promoters (Voskuil and Chambliss, 1998).

When the latter promoter (and also the TAACAT and TGTTAA combination) overlaps with the PrfA-dependent *inlC* promoter, PrfA-independent transcription is no longer suppressed in the presence of high GTP and PrfA-dependent and -independent transcription from both

promoters occurs with similar efficiency, suggesting that binding of RNA polymerase to both overlapping promoters and the formation of transcriptional active complexes can occur with similar efficiency in these combinations.

There is no or only a very weak transcription starting from the second modified promoter of *inlC* in which three bp are deleted from a 17 bp -symmetric region (pseudo-PrfA-box) in order to match the consensus sequence of the PrfA-box, even when the original PrfA-box and –10 box are deleted to eliminate the putative competitive binding of PrfA and RNAP to them. Together with data from the *in vivo* β-galactosidase assay one could conclude that (Fig. 19) that this PrfA-box-like sequence plays no role in the *inlC* transcription; or perhaps *L. monocytogenes* RNAP requires the level of RNAP associated proteins or some other factor(s) which is not sufficient in our RNAP preparation, or it could be that the PrfA-RNAP complex binds too tightly to this modified promoter to form an active transcription open complex.

5.1.3. High concentration of GTP is required for the PrfA-dependent transcription

PrfA-dependent transcription starting at P*inlC* requires high concentrations of GTP and ATP but not of CTP and UTP. In the presence of PrfA and high GTP (ATP) concentration, there is little PrfA-independent transcription, indicating that transcription starting at the PrfA-independent promoter is suppressed. Even without PrfA, transcription starting at the PrfA-independent promoter seems to be less efficient at high GTP than at low GTP concentration. Lowering the GTP concentration below 1 μM activates PrfA-independent transcription from the *inlC* promoter (starting at A7) and suppresses PrfA-dependent transcription (Fig. 20). Apparently because of the close proximity of the now utilized –35 box (TTTAAA) of P*inlC* to the PrfA-box, this PrfA-independent transcription is inhibited with increasing amounts of PrfA. The dependency of the PrfA-dependent transcription on high GTP concentration is not only a consequence of the previously established rule (Lalic-Mülthaler *et al*., 2001) which postulates that the nucleoside triphosphate used for labelling the *in vitro* synthesized transcript (and hence present in low concentration; 0.08 μM) in the reaction (mix) must not be present in the first four nucleotides of the transcript, as removal or G to A replacement of the start nucleotide (G5) only leads to PrfA-independent transcription in the presence of low GTP, although there is no G in the first eight nucleotides of the expected transcript (Fig. 18).

The requirement of high GTP for transcription initiation seems to be a general requirement for PrfA-dependent promoters, as the same dependency on high GTP concentration for the efficient transcription initiation at the PrfA-dependent promoters is found for the *actA* and *hly* genes. It is unlikely that high GTP is necessary for the interaction of PrfA with RNA polymerase or for the formation of stable complexes between PrfA, RNA polymerase and the PrfA-dependent promoter as such complexes are readily formed with these PrfA-dependent promoters in the absence of GTP or ATP at least *in vitro* (Dickneite *et al*., 1998). It suggests rather that high GTP is needed for the conversion of a transcriptionally inactive (closed complex) into a transcriptionally active (open) complex when RNA polymerase is bound to the PrfA-dependent promoter (in the presence of PrfA) but not when RNA polymerase is bound to the PrfA-independent promoter. Dependency on high GTP has been shown for the

formation of active open complexes with T7 RNA polymerase (Nierman and Chamberlin, 1979; Villemain *et al*., 1997) and the presence of high ATP is required for the open complex formation with *Escherichia coli* RNA polymerase loaded with Sig54 and appropriate promoters (Qureshi *et al*., 1997; North and Kustu, 1997). Activators of the σ^{54}-holoenzyme catalyze the isomerization of closed complexes between this polymerase and a promoter to open complexes in a reaction that depends upon hydrolysis of a nucleoside triphosphate. The *gln* promoter (P*glnA*) in *Salmonella* is one such example: P*glnA* is recognized by a form of RNAP in which Sig70 is replaced by an alternative sigma factor (Sig54). In the absence of the activator NTRC, this Sig54-dependent promoter binds RNAP in an instable and transcriptionally inactive, closed complex. NTRC binds to enhancer-like sequences and catalyzes the isomerization of this closed complex to a transcriptionally active open complex, in an ATP-dependent reaction (North and Kustu, 1997).

Whether high ATP concentration has the same function for PrfA-dependent transcription as high GTP concentration or is required for the stabilization of the first DNA-RNA hybrid formed during transcription initiation can not be decided from obtained results, as A other than G is present within the first four nucleotides of all formed transcripts starting at P*inlC* and the constructed P*inlC* variants.

Alternate PrfA-dependent and -independent *in vitro* transcription depending on the GTP concentration was also observed at P*mpl* (the promoter of the gene *mpl* coding for the metalloprotease Mpl), which is also composed of two overlapping PrfA-dependent and -independent promoters (Luo *et al*., 2004).

The described experimental results lead one to propose a model (Fig. 36) for this type of promoters (possessing apparently overlapping PrfA-dependent and -independent promoter activities) in which transcription is modulated by the cellular concentration of GTP. At low GTP, RNA polymerase, loaded with SigA, binds to the PrfA-independent promoter, forms an open complex and initiates PrfA-independent transcription. Under these conditions PrfA is either not produced or present in an inactive form. At higher GTP concentration and in the presence of PrfA, a ternary open complex (with PrfA, RNA polymerase and the PrfA-dependent promoter) is formed and PrfA-dependent transcription can be initiated while PrfA-independent transcription will be suppressed. However, suppression of the PrfA-independent promoter activity at high GTP was observed only in certain –10/–35 combinations whereas in others, both PrfA-dependent and -independent transcription can occur at the same time, i.e. in order to allow alternate PrfA-dependent or -independent transcription in response to high/low GTP from such dual promoters, the sites of the two promoters essential for RNA polymerase binding (PrfA-box, –35 box and the same –10 box) and the distances between these sites have to be balanced precisely, which is apparently the case for P*inlC* and P*mpl*.

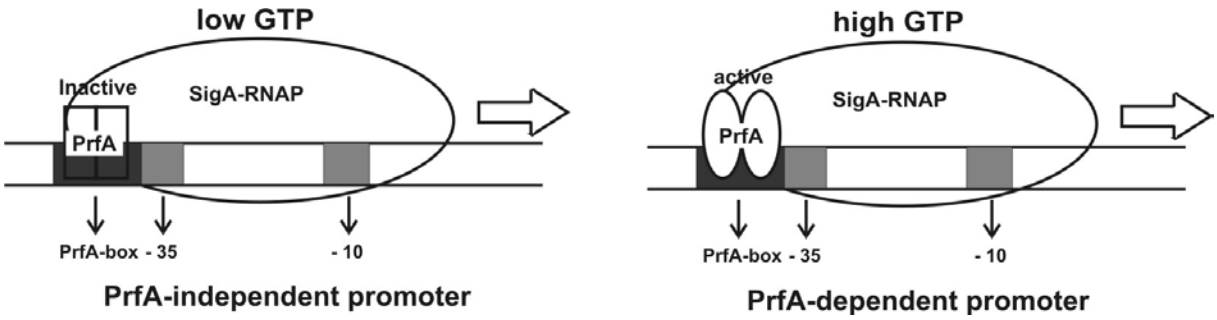


Fig. 36. Model for the type of promoters (possessing apparently overlapping PrfA-dependent and -independent promoter activities) in which transcription is modulated by the cellular concentration of GTP. At low GTP, RNA polymerase, loaded with SigA, binds to the PrfA-independent promoter, forms an open complex and initiates PrfA-independent transcription. Under these conditions PrfA is either not produced or present in an inactive form. At higher GTP concentration and in the presence of PrfA, a ternary open complex (with PrfA, RNA polymerase and the PrfA-dependent promoter) is formed and PrfA-dependent transcription can be initiated while PrfA-independent transcription will be suppressed.

The *in vitro* observed alternate PrfA-dependent and -independent transcription (in response to GTP) also seems to play a role for the regulation of the *in vivo* expression at least of *inlC*. Significant PrfA-independent expression of β-galactosidase is observed under conditions in which PrfA is either relatively inactive (growth in rich BHI medium) or highly active (growth in a minimal medium). Furthermore, the data indicate that expression of β-galactosidase is strongly PrfA-dependent when *L. monocytogenes* wild-type strain is cultured under aerobic conditions (presumably high GTP) but becomes basically PrfA-independent when the same strain is cultured under microaerophilic conditions (presumably low GTP). *L. monocytogenes* may encounter similar conditions causing low GTP levels during an infection in certain extracellular niches (Traut, 1994) and increased expression of *inlA, B* (also regulated by PrfA-dependent and independent promoters; Lingnau *et al*., 1995) together with *inlC* (Bergmann *et al*., 2002) by PrfA-independent transcription of these genes may allow invasion of the bacteria into neighbouring host cells where the bacteria will find more favourable growth conditions in the cytosolic compartment of the host cells.

In conclusion, a PrfA-dependent promoter contains the following essential features (Fig. 37): a) a PrfA-box; b) a –10 box for SigA-loaded RNAP; c) a –35 box not necessary; d) a fixed distance of 22 or 23 bp between 3´ -end of PrfA-box and 5´ -end of –10 box; e) a purine (A or G) at position 5~8 downstream of the –10 box as the preferred transcription start site and f) high concentration of ATP and GTP for efficient transcription initiation.

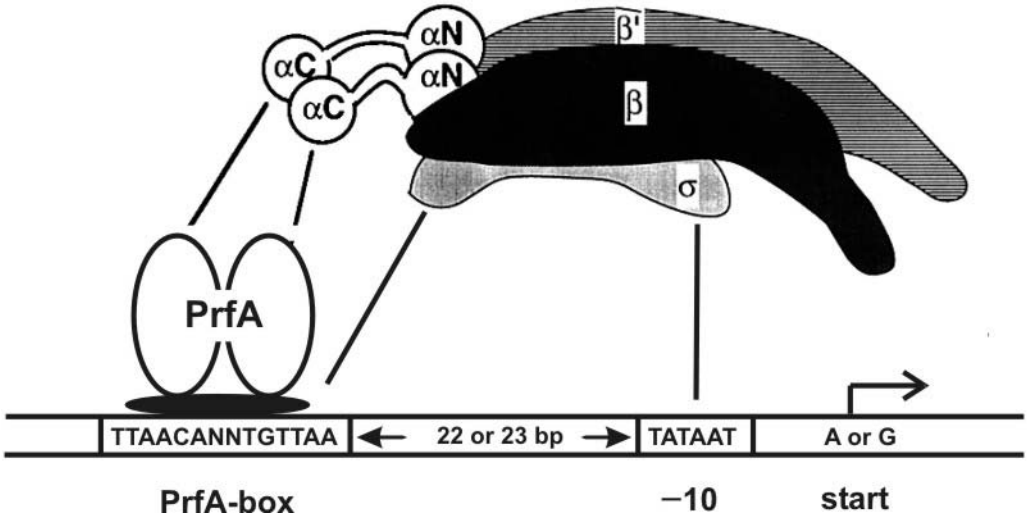


Fig. 37. Schematic representation of a PrfA-dependent promoter and its interaction with the SigA-recognized RNA polymerase of *L. monocytogenes*. The consensus –10 box (TATAAT) and the PrfA-box (TTAACANNTGTTAA), the length of the distance between these two sites and the preferred transcription start nucleotide(s) are indicated. Putative contacts between RNA polymerase and promoter are shown by the solid lines.

5.2. The new putative PrfA-regulated promoters of *L. monocytogenes*

PrfA-dependent promoters have been identified in front of virtually all virulence-specific genes or operons of *Listeria monocytogenes*, suggesting that this transcriptional activator is a central regulator of virulence in *L. monocytogenes* (for recent reviews see Kreft and Vazquez-Boland, 2001; Vazquez-Boland *et al*., 2001). The binding site for PrfA is a conserved 14 bp sequence of dyad symmetry (TTAACANNTGTTAA) that is rather rich in A+T. It is therefore not surprising that this sequence has been found in many copies within the genome sequence of this microorganism belonging to the group of A+T -rich gram-positive bacteria. [There are 286 putative PrfA-boxes found on the *L. monocytogenes* EGDe chromosome, if one allows two mismatches in the PrfA-binding consensus sequence - sometimes also present in functional PrfA-boxes (Glaser *et al*., 2001)] However, it is rather unlikely that so many listerial genes (most of them apparently unrelated to virulence and also present in the non-pathogenic *L. innocua* species) should be under the control of PrfA. On the other hand, a recent transcriptome study demonstrated that, in addition to the known virulence genes, surprisingly many additional genes are affected by PrfA *in vivo* (Milohanic *et al*., 2003). Some of them showed putative PrfA binding sites (with 1-2 mismatches) in their upstream regulatory regions, whereas others, positively or negatively affected in their expression by PrfA, do not contain readily identifiable conserved PrfA-boxes. Based on their different response to PrfA regulation under different conditions, these genes were divided into three groups1) Group I comprises 12 genes: 10 already known genes (*hly*, *plcA*, *actA*, *mpl*, *inlC*, *hpt et al*) and two new genes, *lmo2219* and *lmo0788*, both are positively regulated and contain a putative PrfA-box. 2) Group II comprises 8 negatively regulated genes: 7 genes (*lmo0178-lmo0184*) are organized in an operon; *lmo0178* and the remaining gene *lmo0278* contain a putative PrfA-box. 3) Group III comprises 53 genes, of which only two (*lmo0596* and *lmo2067*) contain a putative PrfA-box and a putative SigB-recognized –10 box.

In addition to these three groups of genes, our colleagues (A. K. Marr *et al*., unpublished results) using whole genome microarrays also found three groups of genes differently regulated in the presence of highly expressed PrfA i.e. bacteria grown in minimal medium with glucose. Among them, the gene *lmo2840* encoding a putative sucrose phosphorylase is quite remarkable. It carries a single mismatch PrfA-box in the upstream regulatory region and is found upregulated under all conditions which also cause upregulation of PrfA-dependent virulence genes.

However, the results reported here cast doubts whether PrfA is directly involved in the transcription of most of these new genes that comprise putative PrfA-boxes and are up- or down-regulated by PrfA. Here, the *in vitro* transcription system using purified RNA polymerase and PrfA protein is carried out to test the interaction between PrfA and the promoters of these new genes (Table 1).

5.2.1. *In vitro* transcription activity of the newly identified, putative PrfA-regulated promoters of *L. monocytogenes*

It is found that with the exception of the *hpt* promoter, none of the other eight analysed promoters is directly activated or repressed by PrfA. Moreover, only the promoter of the *hpt* gene which belongs to group I (Milohanic *et al*., 2003) fully meets all features described in the first part of this work: the transcription start site is A 7 bp downstream of the anticipated –10 as shown by primer extension (Fig. 25); containing a typical SigA-recognized –10 box; and the distance between the –10 box and the PrfA-box is 23 bp (Table 2). Other group I gene promoters tested in this study, *lmo0788* and *lmo2219*, contain reasonable PrfA-boxes as pointed out in table 1, but there is no appropriate –10 box located at 22 (23) bp downstream of this box and primer extension results show that transcription starting from both promoters does not involve the putative PrfA-boxes, suggesting that these two genes are not directly up-regulated by PrfA.

In vitro transcription results do not allow any conclusions concerning the negative influence of PrfA on group II genes (Milohanic *et al*., 2003) as none of the putative promoter fragments of the two selected genes, *lmo0178* and *lmo0278* that include putative PrfA-boxes (Table 1) yielded transcription activity in the presence or absence of PrfA. Also the promoter region of these two genes does not contain the characteristic features of a PrfA-dependent. Therefore, it is likely that transcription of these genes may require transcription factors the expression of which may be somehow negatively influenced by PrfA.

In addition to these new genes described by Milohanic (Milohanic *et al*., 2003), the promoters of two genes, *lmo2420* and *lmo2840,* contain putative PrfA-boxes with only a single mismatch in their upstream regulatory regions. However, transcription of none of these genes is regulated by PrfA, and both of them do not possess a suitable –10 box in an appropriate distance (22 bp or 23 bp) to the putative PrfA-box. It is suggested that these genes are either not truly regulated by PrfA or regulated by other global transcription activators that interact with PrfA by yet unknown mechanisms.

5.2.2. SigB-dependent *in vitro* transcription starting at *lmo2067* and

lmo0596 promoters

A large number of *in vivo* PrfA-upregulated genes belongs to group III (Milohanic *et al*., 2003). Two genes among them, *lmo0596* and *lmo2067,* with putative PrfA-boxes in their upstream regulatory regions were analyzed in respect to their dependency on PrfA using *in vitro* transcription assays (Table 1). None of these two genes yield PrfA-dependent transcription *in vitro* with SigA-loaded RNA polymerase. This is not surprising since none of the two promoters shows the characteristic features of a PrfA-dependent promoter although PrfA-dependent transcription of *lmo2067* (*bsh*) is recently claimed from *in vivo* studies (Dussurget *et al*., 2002).

As already pointed out by Milohanic *et al*., 2003, most group III genes are preceded by putative SigB-dependent promoter sequences. A recent comparative transcriptome analysis of the SigB deletion mutant and the wild-type strain also shows that transcription of most of these genes including *lmo2067* is SigB-dependent (Kazmierczak *et al*. 2003). Transcription efficiency of *lmo0596* and *lmo2067* is strongly activated in a dose-dependent manner by RNA polymerase loaded with additional purified *listerial* SigB and this SigB-promoted transcription could not be enhanced by addition of PrfA protein. These results suggest that the *in vivo* demonstrated PrfA-dependent transcription starting from *lmo2067* (*bsh*) promoter could not be reproduced in the *in vitro* transcription assays either with SigA- or SigB-loaded RNA polymerase. The reason for this apparent discrepancy between the *in vitro* and *in vivo* data is currently unknown, but since the claimed P*lmo2067* does not show the characteristic features of a PrfA-dependent promoter, *in vitro* transcription initiated at this promoter is certainly not expected. Of course, the results in this study do not exclude the possibility that factors not present in the *in vitro* transcription assay may be necessary to render the *lmo2067* promoter dependent on PrfA *in vivo*. Alternatively PrfA-mediated transcriptional activation of the group III genes might be indirect as also suggested by Milohanic *et al*., 2003, and may be part of overlapping PrfA and SigB regulons.

In conclusion, the data indicate that most of the newly identified genes found positively or negatively affected by PrfA *in vivo* (Milohanic *et al*., 2003) are not transcribed by promoters which are directly activated or repressed by PrfA. These genes seem to be rather under the control of alternative sigma factors (such as SigB) or may be regulated *in vivo* by other global transcription activators or repressors that interact with PrfA by yet unknown mechanisms.

5.3. Supportive and inhibitory elements of PrfA-dependent promoters in *L. monocytogenes*

The results reported above revealed that the the PrfA-box, the SigA-dependent –10 box, 22 bp or 23 bp interspace distance between them and a purine as the prefered transcription start site are essential elements for a PrfA-dependent promoter. However, it was also observed that *in vitro* transcription of some mutants, such as the modified pseudo-PrfA-box of *inlC* promoter mutants and *lmo2840* and *lmo2420* mutants, which met all requirements indicated previously, did not show PrfA-dependent transcription activity. In order to better understand the basic elements necessary for a PrfA-dependent promoter, a series of hybrid mutants which

contained various combinations of the elements of a PrfA-independent promoter (P*aroA*) and a typical PrfA-dependent promoter (P*plcA*) were constructed and then analyzed in respect to their dependency on PrfA using the *in vitro* transcription assay.

5.3.1. The influence of the PrfA-box, the –10 box and the interspace region of P*aroA*P2 on PrfA-dependent *in vitro* transcription

aroA is the first gene of the operon encoding the enzymes for the common pathway of aromatic amino acids biosynthesis. In the regulatory upstream region of *aroA* a putative PrfA binding site with only a single mismatch gene is identified compared to the PrfA-binding site of P*plcA* (Fig. 32) and an appropriate SigA-recognized –10 box (TTTAAT) located at 21 bp downstream from this putative PrfA is also observed. However, no transcription is initiated from this putative PrfA-dependent promoter (termed P*aroA*P2; Fig. 32), while PrfA-independent transcription is strongly expressed using TAATAT as the –10 box located in the interspace region of P*aroA*P1 and TTGTAA as the –35 box (this PrfA-independent promoter is named ParoAP1; Fig. 32). Insertion of one bp into the interspace region of P*aroA*P2 and the deletion of GCG 8 bp downstream from the putative –10 box (TTTAAT) to match the requirements for a PrfA-dependent promoter as described earlier did not yield any transcript starting from P*aroA*P2 (mutants P*aroA*+G and P*aroA*ΔGCG; Fig. 33 and 34), suggesting that there are some other important unknown features or factor(s) for PrfA-dependent transcription.

plcA encoding a phosphatidylinositol-specific phospholipase C (PlcA), is a typical PrfA-dependent virulence gene with so called a "perfect PrfA-box" and fulfils all the characters of a PrfA-dependent promoter. *In vitro* transcription starting from *plcA* promoter is absolutely dependent on PrfA.

The PrfA-independent transcription starting from P*aroA*P1 is more efficient than that from P*plcA* even in the presence of saturating amounts of PrfA (Fig. 34 A, a). This is not surprising as ^{32}P-labelled GTP [which is present in low concentration (0.08 μM) in the reaction mix compared to 200 μM of the other three unlabelled NTPs] was used for the *in vitro* transcription due to the presence of the other three NTPs in the first four nucleotides of the RNA transcribed from P*plcA* (Lalic-Mülthaler *et al*., 2001). As shown in the first part of this work high GTP concentration is, however, required for efficient transcription initiation at PrfA-dependent promoters but not at PrfA-independent ones (Luo *et al*. 2004).

The *in vitro* transcription efficiency of *PplcA* is about two times stronger than that of the hybrid mutant P*plcA*-P*aroA*-m1, in which the PrfA-box of P*plcA* is replaced by that of P*aroA*P2 (Fig. 33 and Fig. 34), suggesting that PrfA binds with higher affinity to the ideal PrfA-box of P*plcA* than to the PrfA-box of P*aroA* with one mismatch. Difference in binding efficiency of PrfA to the PrfA-boxes with different number of nucleotide mismatches is considered to determine the transcription efficiency of PrfA-regulated genes and thereby causes the differential expression of PrfA-regulated genes *in vivo* (Freitag *et al*., 1993; Sheehan *et al*., 1995; Brehm *et al*., 1996; Williams *et al*., 2000). Electrophoretic mobility shift experiments (EMSA) also show that the imperfect PrfA-boxes of P*actA* and P*mpl* bind PrfA

less efficiently than the ideal PrfA-boxes of P*hly* and P*plc*A (Dickneite *et al*., 1998; Böckmann *et al*., 2000). The data in this study confirm these previously obtained results.

In addition to the "quality" of PrfA-boxes, the consensus sequences of the –10 boxes play a significant role in the PrfA-dependent transcription efficiency, because the *in vitro* transcription activity of the hybrid mutant P*plcA*-P*aroA*-m2, in which only the –10 box of P*plcA* is exchanged by that of P*aroA*P2, is obviously reduced (Fig. 33). The sequences TAGAAT of the –10 box of the P*plcA* more closely resemble the SigA-recognized –10 box (TATAAT) of *B. subtilis* than the –10 box TTTAAT of P*aroA*P2, since three positions [the first (T), second (A) and sixth base (T) pairs] important for the function of SigA-dependent promoters are all conserved in the P*plcA* but not in the P*aroA*P2. This could be responsible for the weaker binding of RNA polymerase to the promoter of the hybrid mutant P*plcA*-P*aroA*-m2 and the lower transcription observed at this promoter.

The functionality of the individual PrfA- and –10 boxes of P*aroA*P2 was demonstrated by separately exchanging the PrfA-box (P*plcA*-P*aroA*-m1) or the –10 site (P*plcA*-P*aroA*-m2) of P*plcA* by the corresponding P*aroA*P2 sequence, i. e. both hybrid promoters led to PrfA-dependent *in vitro* transcription initiation, albeit at lower efficiency as P*plcA*. However the simultaneous exchange of both, the PrfA- and the –10 boxes of P*plcA* by the corresponding P*aroA*P2 sites resulted in very low transcription efficiency at this hybrid promoter (P*plcA*-P*aroA*-m3) suggesting a close interaction of these two sites in binding of PrfA and/or RNAP. This assumption was confirmed by direct PrfA-, RNAP- and PrfA+RNAP- binding assays (Böckmann *et al*., 2000) which showed indeed very low PrfA and PrfA/RNAP binding to P*aroA*P2 and to P*plcA*-P*aroA*-m3, but rather efficient binding to P*plcA*-P*aroA*-m1 and P*plcA*-P*aroA*-m2 (M. Herler PhD work). Thus the strength of PrfA binding and of the ternary PrfA/RNAP/promoter complex formation parallels the *in vitro* transcription efficiency. The results also indicate that the strength of PrfA binding is not only determined by the PrfA-box but also by the –10 box and the fitting of the two sites leads to optimal binding, a conclusion which is also supported by previous findings (Williams *et al*., 2000; Luo *et al*., 2004)

The replacement of both sites of P*plcA* including the interspace region by those of P*aroA2* (P*plcA*-P*aroA*-m4) completely abolished PrfA binding and ternary complex formation. The "poisonous" element in the P*aroA2* interspace sequence was pinned down to the –10 box (TAATAT) of the functional (PrfA-independent) promoter of *aroA* (P*aroA*P1) located in this interspace region. Inactivation of this –10 box restored ternary complex formation and PrfA-dependent transcription (P*plcA*-P*aroA*-m5; Fig. 34), suggesting that the combination of three basic elements in the P*aroA*P2, i. e. the PrfA-box, the –10 box and the 22 bp interspace region between these two sites can be utilized for the PrfA-dependent transcription starting from the *aroA* promoter. Absence of a PrfA-dependent transcript starting from the intact P*aroA*P2 region, e.g. from mutant P*plcA*-P*aroA*-m12 (Fig. 34), which contains a similar disruption of the –10 box (TAATAT) of the PrfA-independent promoter (P*aroA*P1), indicates that the promoter regions of upstream and downstream play a role in the active transcription.

5.3.2. The influence of the upstream and downstream fragments of *plcA* and *aroA* promoters on PrfA-dependent *in vitro* transcription

In recent years it has been clearly elucidated that the A+T -rich sequences upstream of the –35 element are needed for efficient transcription from some promoters (Gourse *et al*., 2000; Rao *et al*., 1994; Estrem *et al*., 1999). However, there is no *in vitro* transcription observed from the P*aroA*P2, when its upstream region is replaced by that of P*plcA* (mutants P*plcA*-P*aroA*-m10 and -m11), whereas the opposite exchange, i.e. the upstream region of the P*plcA* replaced by that P*aroA*P2 (mutant P*plcA*-P*aroA*-m9), yields a slightly stronger PrfA-dependent transcription than that of P*plcA*. These data show that no additional UP elements are required for the efficient PrfA-dependent transcription besides the 14 bp consensus PrfA-box. It is the downstream region of P*aroA*P2 that inhibits strongly the PrfA-dependent transcription. Furthermore, electrophoretic mobility shift assays (EMSA) showed that the binding of PrfA (CIII) and the formation of the ternary CI complex to the hybrid mutant P*plcA*-P*aroA*-m10 was not blocked (M. Herler PhD work), suggesting that this sequence inhibits transcription initiation rather than formation of the closed transcription complex.

Structural modelling by the MFOLD program (Zuker *et al*., 1991; Zuker, 2003; http://www.bioinfo.rpi.edu/applications/mfold/) suggests that the immediate downstream sequences of P*aroA*P2 could form a secondary stem-loop structure including part of the –10 box of the P*aroA*P2 promoter (Fig. 35) when the DNA double helix is melted by the RNA polymerase to form the single-stranded transcription initiation bubble. Indeed replacement of this inhibitory sequence by the downstream fragment of P*plcA* (mutant P*plcA*-P*aroA*-m14), which is unable to form such a secondary structure with the –10 box, did no longer block *in vitro* transcription from this hybrid promoter (Fig. 34 C). This putative secondary structure does not lead to transcription termination since *in vitro* transcription starting at P*aroA*P1 about 15 bp upstream of the anticipated stem-loop structure is not affected by this sequence. It rather suggests that due to the formation of this secondary structure the initiation nucleotide at position +1 may be inaccessible for the RNA polymerase.

However, inhibition of transcription initiation by such secondary structures has not been reported for other promoters and it therefore remains to be shown whether this is a general effect in transcription initiation from any promoter or specific for initiation from PrfA-dependent promoters. The requirement of high GTP concentration for transcription initiation at PrfA-dependent promoters in contrast to PrfA-independent ones (Luo *et al*., 2004) may be taken as a further indication that this early phase of abortive initiation is a particularly sensitive step in transcription starting from PrfA-dependent promoters.

In conclusion, this study shows that putative PrfA-dependent promoters even if they fulfil all basic features, i. e. a well-balanced combination of PrfA-box and –10 box (for SigA-loaded RNA polymerase) in the appropriate distance (22-23 bp) and a suitable purine start nucleotide, may not lead to PrfA-dependent transcription initiation (even in the presence of high GTP concentrations) if other binding sites for RNA polymerase (and possibly other DNA binding proteins) are present in the promoter sequence or secondary structures may interfere with the formation of the open complex.

5.4. Perspectives

Transcriptome and proteome analyses comparing the expression patterns between prfA-positive and negative *L. monocytogenes* strains and *in silico* search for PrfA-box sequences in the whole genome sequence of *Listeria monocytogenes* were applied recently to unravel the entire PrfA regulon of *L. monocytogenes* (Glaser, *et al*., 2001; Milohanic, *et al*., 2003). However, neither method has delivered reliable results concerning *bona fide* new PrfA-regulated genes beyond those which were already known from previous conventional genetic and biochemical studies (Freitag *et al*., 1992; 1993; Bohne *et al*., 1994; Dickneite *et al*., 1998; Böckmann *et al*., 2000; Lalic-Mülthaler *et al*., 2001). The former methods identified a large number of genes whose expression is apparently positively or negatively affected by PrfA due to yet unknown interaction(s) of PrfA with other global regulators (Milohanic, *et al*., 2003; A. K. Marr *et al*., manuscript submitted), while the latter one identified a number of additional genes which contain in their upstream noncoding (and hence probably regulatory) regions sequences typical of PrfA binding sites (PrfA-boxes) as identified in the established PrfA-dependent promoters of virulence genes of *L. monocytogenes* (Glaser, *et al*., 2001). However, neither *in vivo* nor *in vitro* transcription studies could demonstrate direct involvement of PrfA in the transcriptional activations or repression of these newly identified genes (Milohanic, *et al*., 2003; Luo, *et al*., manuscript submitted), suggesting that PrfA may interact with other global regulatory circuits, such as catabolite repression system (CR). Indeed, the interaction of PrfA regulon and sigma B regulon has been reproduced in this study with partially purified *L. monocytogenes* RNAP loaded with additional SigB protein. This work has opened up avenues for the elucidation of the complex regulatory mechanism of PrfA using *in vitro* system with purified *L. monocytogenes* RNA core polymerase loaded with purified alternative sigma factors, and other putative regulatory factor (s), which will be a rational approach to get an insight into the mechanism(s) of pathogenesis of *L. monocytogenes* at the molecular level. On the other hand, it has been shown in this study that PrfA-dependent transcription initiating from P*aroA*P2 was inhibited by a secondary structure. However, it is unknown whether this is a reasonable explanation for *in vitro* transcription starting unsuccessfully at promoters which apparently contain all known elements of PrfA-dependent promoters such as mutants of the pseudo PrfA-box in P*inlC*, P*lmo2840* mutant and P*lmo2420* mutant described in the first and second parts of this work, Therefore, the influence of the putative secondary structures on PrfA-dependent *in vitro* transcription will be investigated in detail in the future.

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7. Appendix

Commonly used abbreviations and chemical symbols

A	adenine or adenosine
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine or cytidine
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
CTP	cytosine triphosphate
C-Terminus	carboxy-terminus
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EDTA	ethylene-diamine-tetraacetate
e.g.	for example
et al.	and others
EtOH	ethanol
G	guanine or guanosine
GTP	guanosine triphosphate
IPTG	isopropyl-ß-D-thiogalactopyranoside
HCl	hydrochloric acid
Kb	kilobase (s) ; kilobase pairs
KCl	potassium chloride
KH_2PO_4	potassium phosphate, monobasic; potassium dihydrogenphophate
K_2HPO_4	potassium phosphate, dibasic; potassium hydrogenphosphate
L.	liter
LB	Luria-Bertani
LRR	Leucine-rich repeat
M	molar
MEM	Minimum Essential Medium
$MgCl_2$	magnesium chloride
mg	milli gram
min	minutes
ml	milli liter
mM	milli Molar
mRNA	messenger RNA
MW	molecular weight
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
ng	nano gram
nmol	nano mole
N-Terminus	amino-terminus
ORF	Open reading frame
ONPG	o-nitrophenyl-β-D-galactosidase
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
pmol	pico mole
rbs	ribosomal binding site
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature (20-25 degrees)
spp.	species
TEMED	N,N,N´,N´-tetramethylethylenediamine
TTP	Thymidine triphosphate
u	unit
U	uracil or uridine
UTP	uridine triphosphate
µl	micro liter
µg	micro gram
µM	micro Molar
vol.	volume
X-Gal	5-bromo-4-chlor-Indolyl-ß-D-galactopyranoside
WT	wildtype

Common conversions of nucleic acids and proteins

1. Standards

1 kb of double-stranded DNA (sodium salt) = 6.6 x 10^5 Daltons
1 kb of single-stranded DNA (sodium salt) = 3.3 x 10^5 Daltons
1 kb of single-stranded RNA (sodium salt) = 3.4 x 10^5 Daltons
The average MW of a nucleotide = 330 Daltons

2. Spectrophotometric conversions

1 A_{260} unit of double-stranded DNA = 50 µg/ml
1 A_{260} unit of single-stranded DNA = 33 µg/ml
1 A_{260} unit of single-stranded RNA = 40 µg/ml

3. DNA molar conversions

1 µg of 1000 bp DNA = 1.52 pmol (3.03 pmol of ends)
1 pmol of 1000 bp DNA = 0.66 µg

4. Formulas for DNA molar conversions

For dsDNA:
To convert pmol to µg:
pmol x N x 660 pg / pmol x 1 µg / 10^6 pg = µg

To convert µg to pmol:
µg x 10^6 pg / 1 µg x pmol / 660 pg x 1 / N = pmol

Where N is the number of nucleotide pairs and 660 pmol / pg is the average MW of a nucleotide pair.

For ssDNA:
To convert pmol to µg:
pmol x N x 330 pg / pmol x 1 µg / 10^6 pg = µg

To convert µg to pmol:
µg x 10^6 pg / 1 µg x pmol / 330 pg x 1 / N = pmol

Where N is the number of nucleotide pairs and 660 pmol / pg is the average MW of a nucleotide pair.

5. Protein molar conversions

100 pmol of 1kDa protein = 100 ng

6. Protein/DNA conversions

1 kb of DNA = 333 amino acids of coding capacity = 37 kDa protein
10 kDa protein = 270 bp DNA

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Publications

Qin Luo, Marcus Rauch, Alexandra K. Marr, Stefanie Müller-Altrock and Werner Goebel. 2004. *In vitro* transcription of the *Listeria monocytogenes* virulence genes *inlC* and *mpl* reveals overlapping PrfA-dependent and -independent promoters which are differentially activated by GTP. ***Mol Microbiol* 52:** 39-52

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Posterpresentation

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