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SigB-Dependent In Vitro Transcription of *prfA* and Some Newly Identified Genes of *Listeria monocytogenes* Whose Expression Is Affected by PrfA In Vivo

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Recent studies have identified several new genes in *Listeria monocytogenes* which are positively or negatively affected by PrfA and grouped into three classes (E. Milohanic et al., Mol. Microbiol. 47:1613–1625, 2003). In vitro transcription performed with promoters of some class III genes showed strict SigB-dependent but PrfA-independent transcription initiation. Transcription starting at the *prfA* promoter P*prfA2* was also optimal with SigB-loaded RNA polymerase, suggesting a direct link between SigB- and PrfA-dependent gene expression.

Virulence of *Listeria monocytogenes* is determined by a number of well-characterized genes (for a recent review, see reference 26), most of which are regulated by the transcriptional activator PrfA (14, 17), which recognizes a 14-bp sequence of dyad symmetry located 40 to 41 bp in front of the transcriptional start sites of the PrfA-regulated virulence genes (9, 25). PrfA belongs to the Crp/Fnr family of transcriptional activators (13, 16). There is evidence suggesting that PrfA activity is modulated in vivo by an as yet essentially unknown mechanism(s) (2–5, 7, 14).

Recently, we established an in vitro transcription system allowing PrfA-mediated transcription initiation at all known PrfA-dependent promoters in a dose-dependent fashion (2, 15, 18, 22). These studies revealed some features essential for PrfA-dependent promoters, including the requirement for a SigA-recognized −10 box, a fixed distance between the PrfA box and the −10 box of 22 or 23 bp, a purine start nucleotide 5 to 8 bp apart from the 3′ end of the −10 box, and high GTP concentration for transcription initiation (18).

A recent comparative transcriptome analysis (21) revealed listerial genes (divided into three groups) that were also affected in their expression by PrfA. Some of these genes contained putative PrfA binding sites (with one to two mismatches) and putative SigB-dependent promoters (group III genes) in their regulatory 5′ upstream regions (UTRs). Kazmierczak et al. also showed with microarrays using RNA from a *sigB* mutant and the wild-type strain that transcription of most of these genes, including lmo2067, is SigB dependent (12).

Here, we used the in vitro transcription system for studying the possible involvement of PrfA and SigB in transcription initiation at some of these newly identified PrfA-affected genes, concentrating in particular on the group III genes *rsbV*, lmo0596, and lmo2067 (*bsh*).

Sigma A and B proteins of *L. monocytogenes* were purified, and SigB-dependent in vitro transcription was established. For the isolation of sigma A and B proteins, His_6 tags were placed at the N termini of both proteins. To generate the SigA and SigB constructs, the *sigA* and *sigB* gene sequences were amplified by PCR (Table 1) and cloned into the vector pQE-30 (QIAGEN). Purification of His_6-tagged PrfA proteins (PrfA and PrfA* [24, 27]) and RNA polymerase (RNAP) of *L. monocytogenes* A42 (Δ*prfA*) grown in brain heart infusion at 37°C was performed as described previously (2, 3). To construct template plasmids for the in vitro transcription assays, the promoter region and adjacent sequences were cloned into pUC18 by using the oligonucleotides listed in Table 1. In vitro transcription and primer extension were carried out with various amounts of RNAP, PrfA protein, and/or additional SigA or SigB protein (2, 18).

SigA- and SigB-dependent in vitro transcription of P*rsbV*. In vitro transcription was first performed with the promoter of *rsbV*, which is the first gene of an operon encoding the anti-anti-sigma (RsbV), the anti-sigma (RsbW), and the sigma B factor (RsbB) itself and was shown to be transcribed by SigB-loaded RNA polymerase in *Bacillus subtilis* (10, 11). The UTR of *rsbV* in *L. monocytogenes* contains the sequence 5′-ATGTTT-N15-GGGTAA-3′, which is closely related to the *B. subtilis* SigB recognized consensus sequence AGGTTT-N16-GGG TAT (1). Using RNAP loaded with SigA (15) and [^{32}P]CTP as the labeled nucleoside triphosphate (NTP) (0.08 μM; the other three NTPs, 200 μM), we obtained a major transcript (t2), while labeling with [^{32}P]GTP led to the synthesis of a weaker transcript (t1) (Fig. 1A). The absence of t2 in the presence of labeled [^{32}P]GTP suggested that this transcript contains a G within the first 4 nucleotides (15, 18). Addition of increasing amounts of purified sigma B protein to the assay resulted in a dose-dependent increase of t1 and a corresponding decrease of t2, confirming that t1 starts at the anticipated SigB-dependent promoter, whereas t2 probably starts at a SigA-dependent promoter. The start sites of t1 and t2 were determined by primer extension with RNA transcripts as templates. These transcripts were obtained with SigA- and SigB-loaded RNAP in the presence of the 4 nucleotides in equally high concentrations (200 μM each) (Fig. 1B). With SigA-loaded RNAP, we obtained a major cDNA (reverse transcript 2 [rt2]) (Fig. 1B), suggesting nucleotide T as the start site, 7 bp downstream from the an-

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TABLE 1. PCR primers used[a]

Sequence (5′→3′)	Application
GAGGAATACAGGGTACCATGAGTGATAAAACAAAAAACACAAAAC; GGTTTGTCC TCCTGCAGGGTTGTGCATTATTCCAGGAAG	Construct for His_6-tagged SigA
AAAAGCAGGTGGGGTACCATGCCAAAAGTATCTCAACC; TTTGATTCAACTGCAGT GTTCATTTACTCC	Construct for His_6-tagged SigB
AAGTGGGGAATTCTGTATTTATTATGTC; TTTGGCGCTGCAGAAGCATCGATCTC	pUC18*P*rsbV*, template for in vitro transcription
GTATTGTGGATCCAACTAAAGTAACACG; CCATCACTGCAGCAATACCAATAAGTG	pUC18*P*bsh*, template for in vitro transcription
AAGGATTGGTACCTTTTCCGACTTATC; ATAATCACTGCAGTCCCCGCTAATAA	pUC18*P*lmo0596*, template for in vitro transcription
AATCCGTTTCTAGATATGTATGC; ATACAATAAGCTTGTGGATCCCA	pUC18*P*prfA*, template for in vitro transcription
CAACAAATATGTCTATGTGGTC	Primer extension of P*rsbV*
CGGCGTAACAACCACAACTTC	Primer extension of P*bsh*
CCGCTAATAAAACAAGAATTCGTG	Primer extension of P*lmo0596*
GGTTTTATCCCGTTAG	Primer extension of P*prfA*

[a] Primers were used for His_6-tagged SigA and SigB constructs and cloning of in vitro transcription templates. Oligonucleotides were used for primer extension. Restriction sites that were used are underlined.

ticipated SigA-dependent −10 box (TATACT). With SigB-loaded RNAP, the reverse transcript rt1 becomes the major cDNA, while rt2 decreased. The size of rt1 suggests A as the start site, 8 bp downstream of the anticipated SigB-dependent −10 box (GGGTAA).

These data clearly indicate that the two purified sigma proteins function as expected.

SigB-dependent but PrfA-independent in vitro transcription of *bsh* and lmo0596. To test whether SigB-loaded RNAP also drives transcription of lmo2067 (*bsh*) and lmo0596 (possibly in a PrfA-dependent fashion), we carried out in vitro transcription with UTR-DNA fragments of these genes (containing the putative SigB-dependent promoters and PrfA boxes) and SigB-loaded RNAP in the presence or absence of PrfA. As shown in Fig. 2A, efficient in vitro transcription was obtained with the SigB-loaded RNAP from the *bsh* promoter, but this transcription was not further enhanced by increasing amounts of PrfA. Primer extension with SigB-loaded RNAP (Fig. 2B) confirmed that transcription of *bsh* is indeed initiated at the anticipated SigB-dependent promoter (Fig. 2C), which is apart from the previously proposed PrfA box (6).

In vitro transcription with the UTR-DNA fragment of lmo0596 also resulted in PrfA-independent transcription, which was again highly activated with SigB-loaded RNAP. The SigB-dependent transcription starts at A, 9 bp downstream of the −10 box of the anticipated SigB-dependent promoter (Fig.

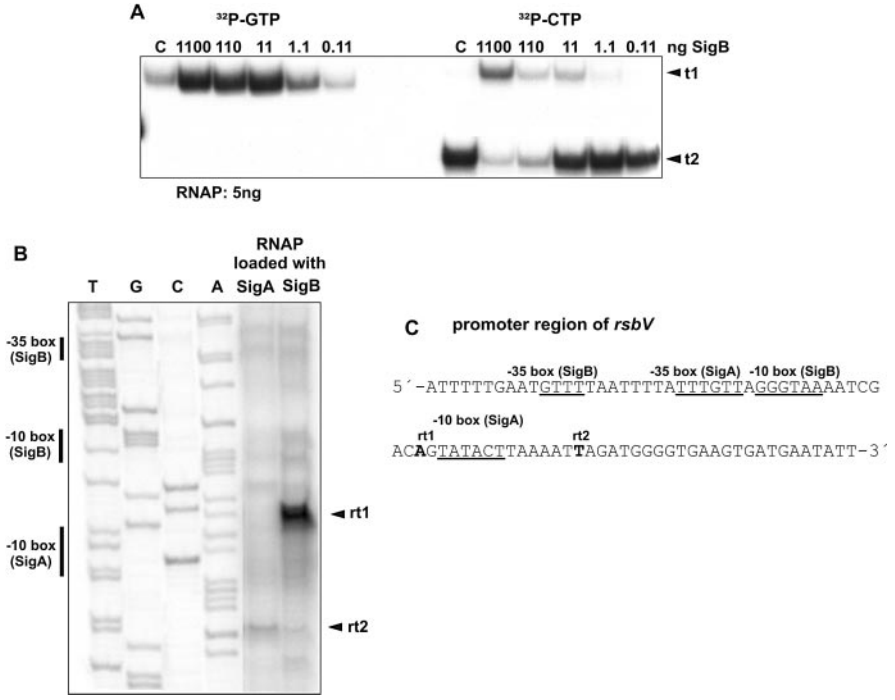

FIG. 1. (A) In vitro transcription with the promoter region of *rsbV*. RNAP was loaded with different amounts of purified SigB protein, as indicated. The obtained transcripts are indicated as t1 and t2. C indicates control without addition of SigB protein. (B) Analysis of in vitro derived transcripts by primer extension. (C) The promoter sequence of *rsbV*. RNAP loaded either with SigA or with SigB was used for in vitro transcription. The main cDNA fragments obtained after reverse transcription are labeled as rt1 and rt2. Putative −35 and −10 boxes as well as transcription start sites are indicated. A sequence ladder was used for determining the transcription start site.

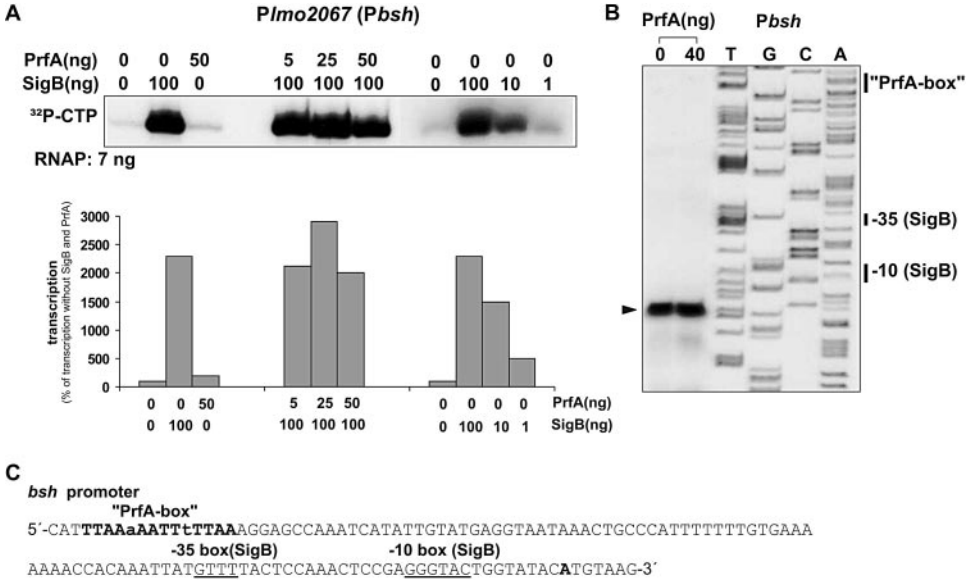

FIG. 2. (A) In vitro transcription assay with the promoter region of lmo2067 (*bsh* gene). RNAP of *L. monocytogenes* was loaded with different amounts of purified SigB protein, as indicated. (B) The corresponding in vitro transcripts were taken for primer extension to determine the transcriptional start sites. The putative −35 and −10 boxes of the SigB-recognized promoters are indicated. (C) The transcription start site is shown in bold letters.

3). There is a faint but reproducible band 24 bp upstream from the transcription start site of the SigB-dependent promoter, the intensity of which also seems to increase in the presence of SigB. Whether this band represents a transcriptional start site of another weak SigB-dependent promoter or simply a primer extension artifact cannot be determined (Fig. 3B).

SigB-dependent in vitro transcription starting at P*prfA2*. One possible link between PrfA- and SigB-dependent gene expression is the recently suggested SigB-dependent transcription of the *prfA* gene itself (23). The *prfA* gene is transcribed together with *plcA* in a bicistronic mRNA from an autoregulated promoter located in front of *plcA* (9, 20) and in addition from two promoters (P*prfA1* and P*prfA2*) located in the UTR of *prfA*; the latter ones seem to be essential for basal-level transcription of *prfA* in the absence of PrfA.

SigB-dependence of P*prfA2* was directly demonstrated by in vitro transcription with RNAP in the presence and absence of SigB by using as template a DNA fragment containing P*prfA1* and P*prfA2*. With [^{32}P]UTP, we obtained, in accord with previously reported data (15), mainly a *prfA* transcript

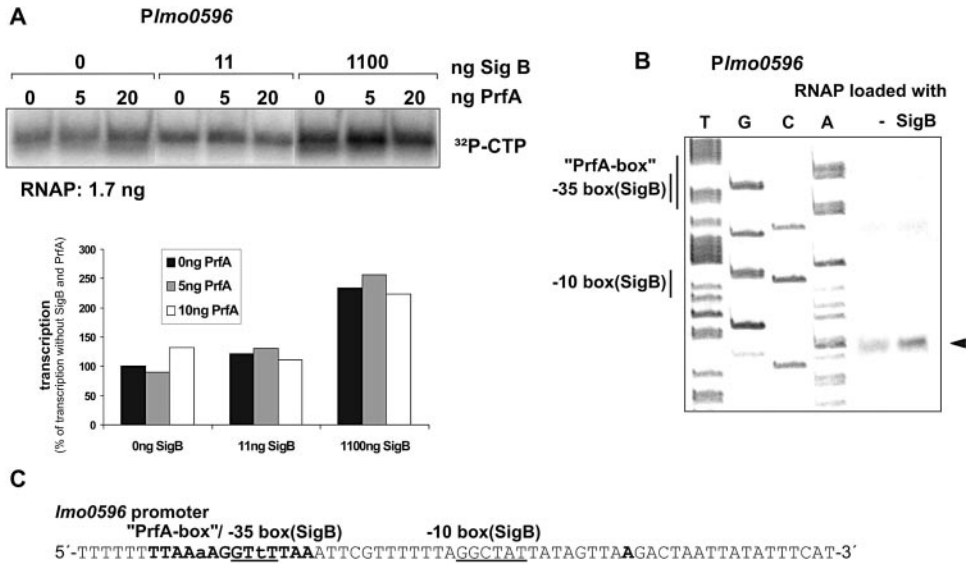

FIG. 3. (A) In vitro transcription assay with the promoter region of lmo0596. RNAP of *L. monocytogenes* was loaded with different amounts of purified SigB protein, as indicated. (B) The corresponding in vitro transcripts were taken for primer extension to determine the transcriptional start sites. The putative −35 and −10 boxes of the SigB-recognized promoters are indicated. (C) The transcription start site is shown in bold letters.

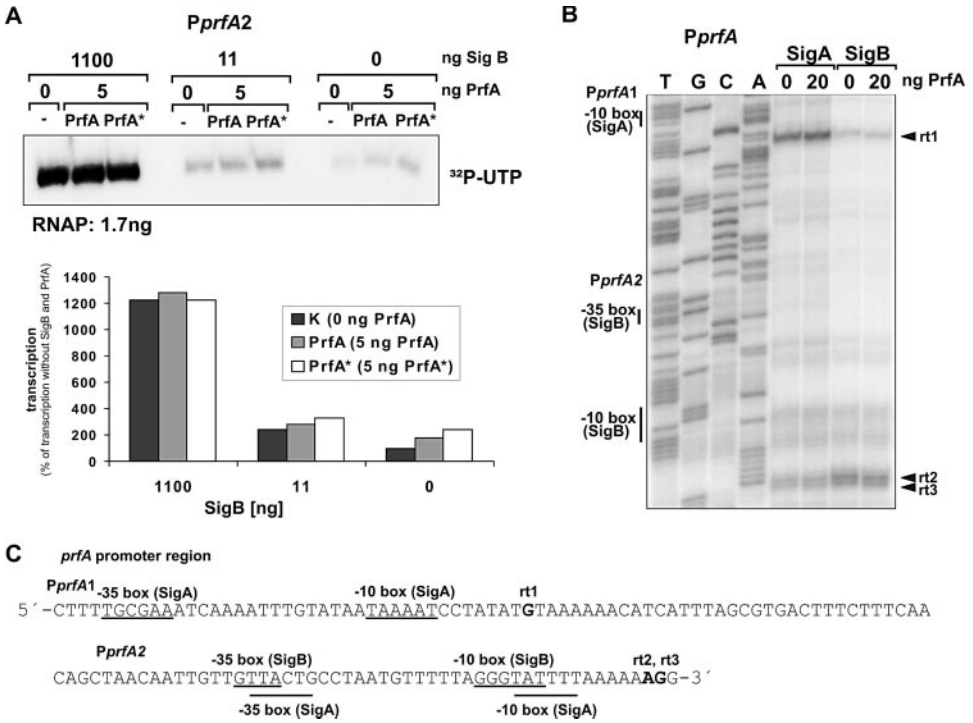

FIG. 4. In vitro transcription with the 5′ upstream region of *prfA* containing the two previously mapped promoters P*prfA1* (P1) and P*prfA2* (P2) (8). (A) Wild-type PrfA and hyperactive PrfA* (24, 27) proteins and RNAP loaded with different amounts of purified SigB protein were used as indicated. (B) The primer extensions were carried out to determine the transcriptional start sites. (C) The putative −35 and −10 boxes of SigB- and SigA-recognized promoters and the transcription start sites (rt1, rt2, and rt3) are indicated.

starting at P*prfA2*, the amount of which was significantly enhanced by increasing the SigB concentration (Fig. 4). No difference in the size of this transcript was observed, however, in the presence of SigA (and the absence of SigB), suggesting that P*prfA2* may actually consist of two overlapping promoters, one being SigA dependent and the other being SigB dependent (Fig. 4C).

To prove this assumption, primer extension was performed with transcripts produced in the presence of the four unlabeled NTPs and RNAP loaded with either SigA or SigB. With SigA-RNAP, we obtained as major reverse transcript mainly rt1 (Fig. 4B), suggesting a transcriptional start at G, 8 bp downstream of the −10 box (TAAAAT) of P*prfA1*, and at a lower concentration rt2 and rt3, two reverse transcripts which suggested transcription starts from P*prfA2*, presumably 6 and 7 bp downstream of a possible SigA-recognized −10 box (TAT TTT) (Fig. 4C). Transcription with RNAP loaded with additional SigB resulted mainly in two reverse transcripts apparently identical to rt2 and rt3, suggesting that SigB-dependent transcription is initiated 9 and 10 bp downstream of the SigB-specific −10 box of P*prfA2* (GGGTAT) (Fig. 4B). These data suggested that P*prfA1* is a typical SigA-dependent promoter, whereas P*prfA2* seems to consist of two overlapping promoters, one being SigB dependent and the other being SigA dependent. Transcription from neither of these *prfA* promoters was affected by PrfA (Fig. 4B).

In conclusion, our in vitro transcription data indicate that the tested group III genes (21) are controlled by SigB-dependent promoters but that PrfA has no direct influence on their transcription. There appears to be, however, a link between PrfA and SigB through the partial transcription of *prfA* by SigB-RNAP. In vitro transcription (data not shown) with several other newly identified genes (lmo0788, lmo2219, lmo0178, and lmo0278), all belonging to class I or II, which are positively or negatively affected by PrfA in vivo (21), indicated transcription by SigA-loaded RNAP which was also not directly activated or repressed by PrfA, suggesting that transcription of (at least most of) these newly identified PrfA-affected genes is not directly regulated by PrfA, as in the case of the PrfA-dependent virulence genes, but possibly by activators or repressors whose expression or activity might be influenced by PrfA (19).

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