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Negative control of *Listeria monocytogenes* virulence genes by a diffusible autorepressor

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Summary

Virulence genes from the facultative intracellular pathogen *Listeria monocytogenes* are controlled by the transcriptional regulator PrfA. Although PrfA synthesis is activated at 37°C, PrfA-dependent expression remains low in rich medium. However, a strong induction of the PrfA regulon is observed when *L. monocytogenes* is cultured in the presence of activated charcoal. Here, we show that the 'charcoal effect' results from the adsorption of a diffusible autorepressor substance released by *L. monocytogenes* during exponential growth. Analyses using an *L. monocytogenes* strain in which the *prfA* gene is expressed constitutively at 37°C from a plasmid indicate that the autoregulatory substance represses PrfA-dependent expression by inhibiting PrfA activity. PrfA presumably functions via an allosteric activation mechanism. The inhibitory effect is bypassed by a PrfA* mutation that locks PrfA in fully active conformation, suggesting that the autorepressor interferes with the allosteric shift of PrfA. Our data indicate that the listerial autorepressor is a low-molecular-weight hydrophobic substance. We suggest that this diffusible substance mediates a quorum-sensing mechanism by which *L. monocytogenes* restricts the expression of its PrfA virulence regulon. This autoregulatory pathway could serve *L. monocytogenes* to ensure the silencing of virulence genes during extracellular growth at 37°C. It may also play a role during intracellular infection, by limiting the damage to the host cell caused by an excess production of cytotoxic PrfA-dependent virulence factors in the PrfA-activating cytosolic compartment.

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Introduction

The virulence genes that enable the soil bacterium *Listeria monocytogenes* to live as an intracellular parasite are co-ordinately regulated by PrfA, a member of the Crp/Fnr family of bacterial transcriptional activators. The known PrfA-regulated products include surface proteins involved in host cell invasion and cell-to-cell spread (InlA, InlB and ActA), secreted membrane-damaging factors mediating escape from the phagocytic vacuole (a pore-forming toxin, listeriolysin O, and two phospholipases, PlcA and PlcB) and a transporter by which *Listeria* steal sugar phosphates, mediating rapid growth in the host cytosol (Hpt) (Vázquez-Boland *et al*., 2001; Chico-Calero *et al*., 2002; Portnoy *et al*., 2002; Cossart *et al*., 2003). There is evidence that PrfA integrates a variety of environmental signals to modulate virulence gene expression according to the specific needs determined by the saprophytic and parasitic lifestyles of *L. monocytogenes* (Kreft and Vázquez-Boland, 2001).

The levels of listerial virulence gene expression depend on the amounts of the PrfA protein. These are controlled via two mechanisms. One involves the deinhibition of translation of the *prfA* mRNA in response to an elevation of the ambient temperature to 37°C, the temperature of the warm-blooded animal host (Johansson *et al*., 2002). The other involves a PrfA-dependent bicistronic *plcA–prfA* transcript that creates a positive feedback loop by which PrfA induces its own synthesis. The insertional disruption of this autoregulatory circuit leads to total loss of virulence, even if the *prfA* gene and its promoters remain intact (Mengaud *et al*., 1991; Camilli *et al*., 1993; Freitag *et al*., 1993), showing that it is essential for the normal function of the PrfA regulon. However, in wild-type *L. monocytogenes*, PrfA-dependent genes are only very weakly expressed *in vitro* at 37°C during growth in bacteriological culture media (Ripio *et al*., 1996), indicating that additional mechanisms are involved in the induction of the PrfA regulon.

Critical insight into the mechanism of PrfA regulation was provided by the identification of PrfA* mutations that cause the constitutive overexpression of *L. monocytogenes* viru-

lence genes (Ripio *et al*., 1997; Vega *et al*., 1998; 2004). Interestingly, the PrfA* mutant proteins exhibit structure–function characteristics similar to those of the Crp* (cAMP-independent) mutations characterized in *Escherichia coli*, thought to mimic the conformational change induced by the Crp-activating cofactor, cAMP. This suggests that the listerial regulator functions via a cofactor-mediated allosteric shift mechanism similar to that of Crp. Like the Crp* mutant proteins, the PrfA* mutant forms show enhanced DNA-binding and transcriptional activities, consistent with the notion that they are locked in an active conformation. Based on these findings, we proposed a model in which, on entry into an appropriate host compartment, PrfA, normally weakly active, becomes fully active and boosts its own synthesis via the *plcA–prfA* transcript, triggering an abrupt and sustained activation of the PrfA regulon (Ripio *et al*., 1997; Vega *et al*., 1998; 2004). There is evidence that the PrfA regulon is strongly induced, well above the levels seen in broth culture at 37°C, when *L. monocytogenes* grows intracellularly after escape from the phagocytic vacuole (Freitag and Jacobs, 1999; Moors *et al*., 1999; Shetron-Rama *et al*., 2002). It is therefore very tempting to suggest that the chemical composition of the host cell cytosol provides activating signals for PrfA.

That chemical signalling plays a major role in the control of PrfA-dependent gene expression is most clearly suggested by the finding that listerial virulence factor expression is significantly enhanced at 37°C if the culture medium is supplemented with activated charcoal (Ripio *et al*., 1996). A recent genome-wide transcriptomic analysis has confirmed that growth in the presence of this adsorbent specifically activates *L. monocytogenes* genes that are preceded by a PrfA-box (the target DNA sequence to which PrfA binds) (Milohanic *et al*., 2003). In this study, we investigated the mechanism of the charcoal-mediated induction of the PrfA regulon. We present evidence that the 'charcoal effect' results from the sequestration of a diffusible autorepressor substance that is released by *L. monocytogenes* during exponential growth and which affects, directy or indirectly, the activity of PrfA.

Results

Characterization of the 'charcoal effect'

Figure 1A illustrates the effect of the presence of charcoal in the culture medium [brain-heart infusion (BHI)] on the transcription of *prfA* and PrfA-dependent virulence genes. Charcoal-mediated induction of these genes correlates with a clear increase in the production of the corresponding PrfA-regulated virulence factors (Fig. 1B). The activity of one of these factors, PlcB, a wide-substrate-range phospholipase C (PC-PLC) or leci-

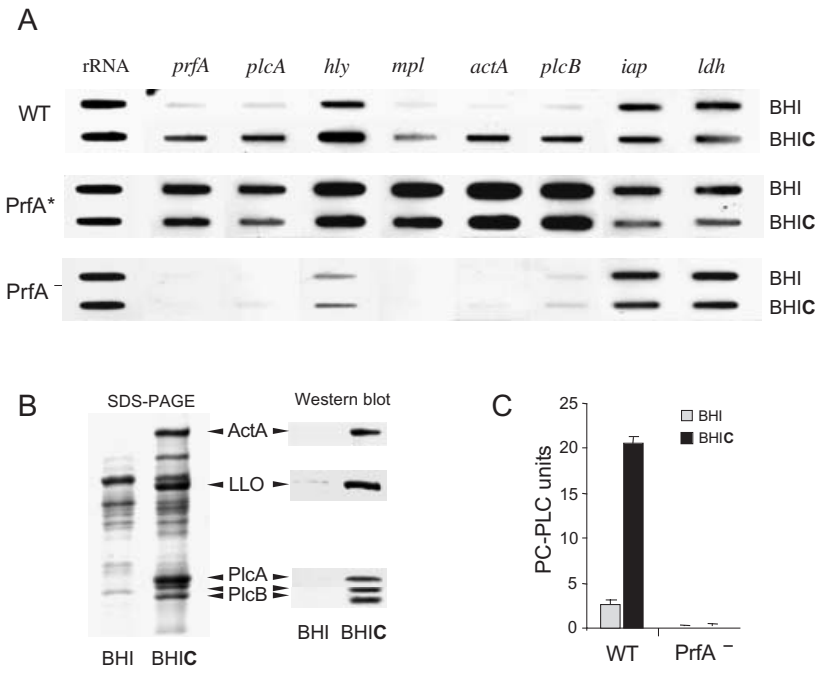

Fig. 1. Induction of PrfA-dependent expression by activated charcoal.
A. Slot-blot transcription analysis of *prfA* and PrfA-dependent genes in *L. monocytogenes* wild type (WT) and PrfA* and PrfA$^-$ mutants grown in BHI and BHI supplemented with 0.2% charcoal (BHIC). Each slot was loaded with 5 μg of total listerial RNA extracted in the mid-exponential phase (OD_{600} = 1.5). The PrfA-independent, constitutively expressed gene *iap*, encoding the p60 protein (Bubert *et al*., 1999), and the housekeeping gene *ldh*, encoding lactate dehydrogenase (Vázquez-Boland *et al*., 1992), were used as controls; the plasmid pKK3535 carrying the *rrnB* rRNA operon of *E. coli* was used to monitor the amount of RNA present in the blots (Ripio *et al*., 1996; 1997).
B. Detection of PrfA-dependent virulence factors in the culture supernatant of BHI- and BHIC-grown wild-type *L. monocytogenes* by Western immunoblotting. Proteins from 1.5 ml of culture supernatant were loaded in each lane. The double band detected by the anti-PlcB monoclonal antibody corresponds to the pro-PlcB and mature PlcB forms (Vázquez-Boland *et al*., 1992; Niebuhr *et al*., 1993). ActA polypeptides are released from the bacterial surface and are detectable in the culture supernatant (Suárez *et al*., 2001).
C. PrfA-dependent expression in wild-type (WT) and Δ*prfA* mutant (PrfA$^-$) *L. monocytogenes* in BHI and BHIC as determined using the *plcB* gene reporter system. Mean values ± standard error (SE) from many experiments with similar results.

thinase, can be quantified easily in the culture supernatant and was used in this study to monitor the effect of charcoal on PrfA-dependent expression (Fig. 1C). In our hands, the *plcB* gene, which is controlled by a strictly PrfA-dependent promoter (P*actA*) with no substantial background expression, provides an easy, sensitive and clean 'natural' reporter system for monitoring PrfA-dependent expression in *L. monocytogenes* (Vázquez-Boland *et al*., 1992; Ripio *et al*., 1997; Brehm *et al*., 1999; Vega *et al*., 2004). The induction of the P*actA* promoter by charcoal during mid-exponential growth (OD_{600} = 1.0–1.5) as determined via the PC-PLC activity (between 4.8 and 13.4 times depending on the strain) is similar to that determined for *plcB* and *actA* by slot-blot transcriptional analysis (between 4.3 and 17.6) (Fig. 1A). Activation of virulence genes by charcoal does not occur in the absence of a functional *prfA* gene (Fig. 1A and C), indicating that the mechanism involves PrfA.

The charcoal effect is concentration dependent, with maximum induction of PrfA-dependent expression at 0.2% (w/v) in broth medium and 0.5% in solid medium (see Fig. 3A). The efficacy of charcoal diminishes as it becomes saturated or as its particle size increases, indicating that its effect is related to its adsorptive properties (Ermolaeva *et al*., 1999; other unpublished data). The underlying mechanism does not involve a global physiological response resulting from the sequestration of essential nutrients or pH changes because the supplementation of BHI with 0.2% charcoal (BHIC medium) affects neither the bacterial growth (Ripio *et al*., 1996) nor the pH of the culture (not shown). A possibility was that charcoal adsorbs a component of the nutrient medium with PrfA-repressing activity. To test this, BHI was treated with 0.2% charcoal at 37°C for a minimum of 24 h, and then the charcoal particles were completely removed by centrifugation and filtration through a 0.2 μm pore size membrane. Significant induction of PrfA-dependent expression was not observed in this charcoal-pretreated medium unless subsequently supplemented with 0.2% charcoal (Fig. 2).

A recent report claimed that the heating of the BHI medium gives rise to Maillard reaction products that repress virulence gene expression in *L. monocytogenes*. Its authors suggested that the charcoal effect could result from the sequestration of these repressor products (Sheikh-Zeinodin *et al*., 2000). We did not find any difference in the levels of PrfA-dependent expression between the heat-sterilized and filter-sterilized BHI, and supplementation of the latter with charcoal caused the same degree of *plcB* induction as in heat-sterilized BHI (Fig. 2).

Thus, (i) charcoal does not seem to act by sequestering any component of the BHI medium that could act as a PrfA-repressing signal; and (ii) to exert its effect, charcoal needs to be present in the culture medium while bacteria are growing.

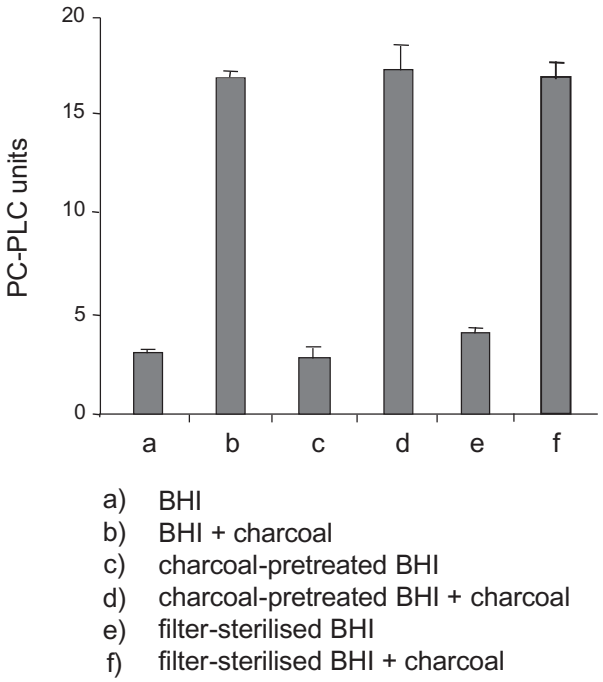

Fig. 2. The effect of charcoal does not involve the removal of PrfA-repressing signals derived from the BHI medium composition or generated by heat sterilization of BHI. The same batch of BHI medium was: (a) heat sterilized in the autoclave; (b) heat sterilized and supplemented with 0.2% charcoal; (c) treated with 0.2% charcoal for 24 h at 37°C, followed by removal of the adsorbent by centrifugation and filtration through 0.2 μm pore size membrane; (d) treated as in (c) and supplemented with 0.2% charcoal; (e) filter sterilized; (f) filter sterilized and supplemented with 0.2% charcoal. These media were inoculated with *L. monocytogenes* and the PC-PLC activity was measured in the culture supernatant as indicated in *Experimental procedures*. Means ± SE from at least three independent experiments done in duplicate.

The 'charcoal effect' is mediated by a diffusible substance from L. monocytogenes

Given the above observations, two possibilities were considered to explain the charcoal effect. One was that the mechanical contacts between bacteria and charcoal particles trigger a PrfA-activating pathway. This explanation, however, was not supported by the fact that charcoal also activates PrfA-dependent expression on agar plates (Fig. 3A), in which direct interactions between bacteria and the adsorbent particles are minimal. The other option was that charcoal adsorbs a listerial product with virulence gene repressor activity. To test this, we cultured *L. monocytogenes* in BHI using Transwell permeable chambers fitted with a microporous membrane that excludes bacteria. The same strong *plcB* reporter induction as in BHIC was observed when bacteria grew

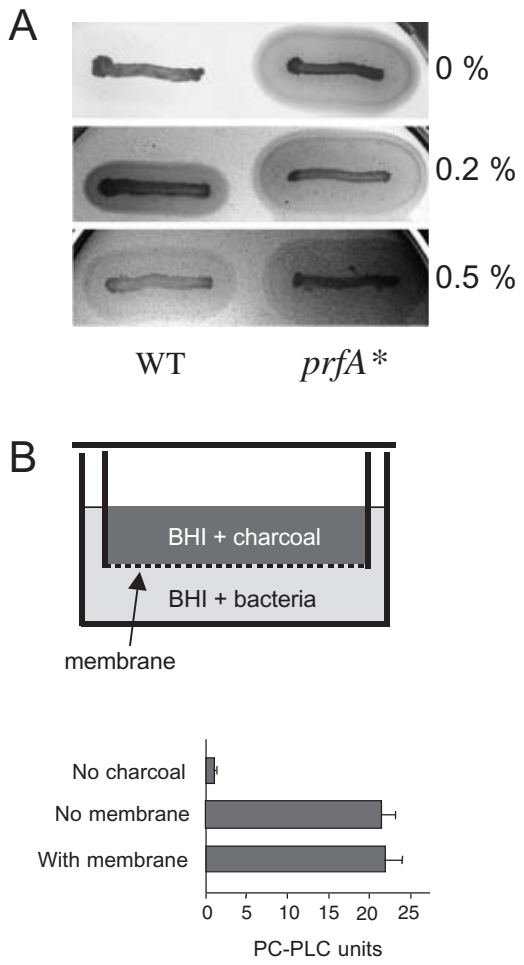

Fig. 3. Demonstration that *L. monocytogenes* produces a diffusible, charcoal-sequestrable virulence gene autorepressor substance.
A. PrfA-dependent virulence gene induction by charcoal as shown in agar plates. Wild-type *L. monocytogenes* (WT) and an isogenic *prfA** derivative were streaked out on egg yolk BHI agar without and with activated charcoal (0.2% and 0.5% w/v) supplementation. When egg yolk phospholipids are split by the product of the *plcB* PrfA-dependent reporter gene, a halo of diacylglycerol/fatty acid precipitation develops around the bacterial colonies, the width of which is directly proportional to the degree of PlcB production. Plates were incubated at 37°C for 48 h. Note that, in wild-type *L. monocytogenes*, charcoal induces *plcB* expression in a concentration-dependent manner. Also, that the maximal diameter of the precipitate is smaller than that reached by the *prfA** mutant, indicating that the PrfA-dependent gene activation induced by charcoal, although strong, does not reach the levels expressed from bacteria producing a constitutively active PrfA* mutant form. In solid medium, a greater concentration of charcoal than in broth (0.5% versus 0.2%) is required to achieve full *plcB* induction.
B. Charcoal activates PrfA-dependent expression when separated by a microporous membrane from *L. monocytogenes* (pore sizes tested: 0.4 and 0.1 μm). Top, schematic representation of the experimental set-up in a Transwell vessel. As controls, *L. monocytogenes* was grown in BHI without charcoal or in physical contact with the adsorbent (membrane removed). Bottom, mean ± SE of the results obtained in at least three independent duplicate experiments. Similar results were obtained regardless of whether the charcoal and bacteria were reciprocally placed in the upper or lower chamber.

in BHI physically separated from charcoal, demonstrating that the effect is mediated by a diffusible listerial autorepressor (Fig. 3B).

Listeria monocytogenes *releases a charcoal-sequestrable autorepressor during exponential growth.*

To assess better the impact of the putative charcoal-sequestrable substance on *L. monocytogenes* virulence gene regulation, we carried out a time-course analysis of *plcB* expression in BHI and BHIC. PC-PLC reporter activity levels remained constantly low in BHI whereas they increased progressively with bacterial growth in BHIC (Fig. 4A). Induction was already detectable at an OD_{600} as

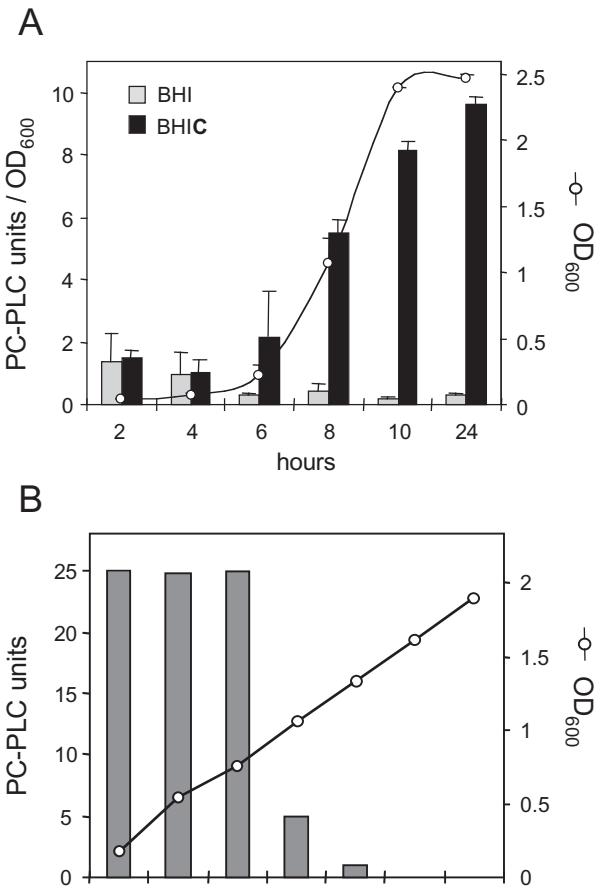

Fig. 4. The charcoal-sequestrable autorepressor is released by *L. monocytogenes* from the early exponential growth phase.
A. Kinetics of *plcB* reporter gene induction in BHI and BHIC. Media were inoculated 1:200 with a stationary culture of *L. monocytogenes*, and PC-PLC activity was measured at the indicated time points. PC-PLC units were normalized for the optical density at 600 nm (OD_{600}) of the BHI culture (mean values ± SE of two representative experiments).
B. Effect of the *L. monocytogenes* growth phase at which charcoal is added to the culture medium. At the indicated OD_{600} (from 0.1 to 2.0), 0.2% charcoal was added to the exponentially growing *L. monocytogenes* BHI culture, and *plcB* gene reporter activity was measured after a total cultivation time of 24 h. A representative experiment from several replicates with similar results is shown.

low as 0.25, indicating that the repressor substance is released by *L. monocytogenes* early during exponential growth. To confirm this, charcoal was added to an *L. monocytogenes* BHI culture at different points of the exponential growth phase (Fig. 4B). PrfA-dependent gene expression was only fully induced when charcoal was added before mid-exponential phase ($OD_{600} < 1.0$). No induction was detected if charcoal was added once the culture had reached the mid-exponential phase ($OD_{600} > 1.5$) (Fig. 4B). These results indicate that the repressor substance is produced during the early exponential phase and has to be removed by charcoal at early stages of listerial growth for maximal PrfA-dependent gene induction to be detected.

To demonstrate that the charcoal effect is mediated by a soluble *L. monocytogenes*-released substance, we assessed PrfA-dependent gene induction in BHIC conditioned with spent growth medium. BHI culture supernatants were collected at mid-exponential ($OD_{600} = 1.0$) and post-exponential (12 h culture, $OD_{600} \geq 2.5$) growth phase, lyophilized and incorporated into the BHIC medium at a ratio 2:1 (original v/v) of spent to fresh medium as described in *Experimental procedures*. PrfA-dependent expression was clearly repressed in BHIC conditioned with mid-exponential BHI culture supernatant (Fig. 5A). This effect was not attributable to differences in bacterial growth (Fig. 5B). Treatment of the mid-exponential BHI culture supernatant with 0.2% charcoal totally removed the repressor activity (Fig. 5A). These results show that a charcoal-sequestrable repressor substance is released into the *L. monocytogenes* culture supernatant during the exponential growth phase. Interestingly, the activity of that substance was no longer detectable in the stationary phase BHI culture supernatant, suggesting that its production ceases at high population density and that it is degraded and/or incorporated/adsorbed by growing bacteria.

The autorepressor substance affects PrfA activity

The amount of PrfA is significantly greater in wild-type *L. monocytogenes* grown in BHIC than in BHI (see Fig. 7C). The existence of the autoregulatory loop mediated by the PrfA-dependent *plcA–prfA* bicistronic message complicates the interpretation of this finding as an initial increase in either the amount of PrfA or the activity of the protein would immediately be amplified by positive feedback (Ripio *et al*., 1997; Vega *et al*., 1998). To determine whether the increased PrfA synthesis results primarily from the activation of monocistronic *prfA* expression or is secondary to an increase in PrfA activity, we used PAM 374, a Δ*prfA* mutant *trans*complemented with a plasmid carrying a monocistronic *prfA* construct (Fig. 6). In PAM 374, the autoregulatory loop is not operational and *prfA*

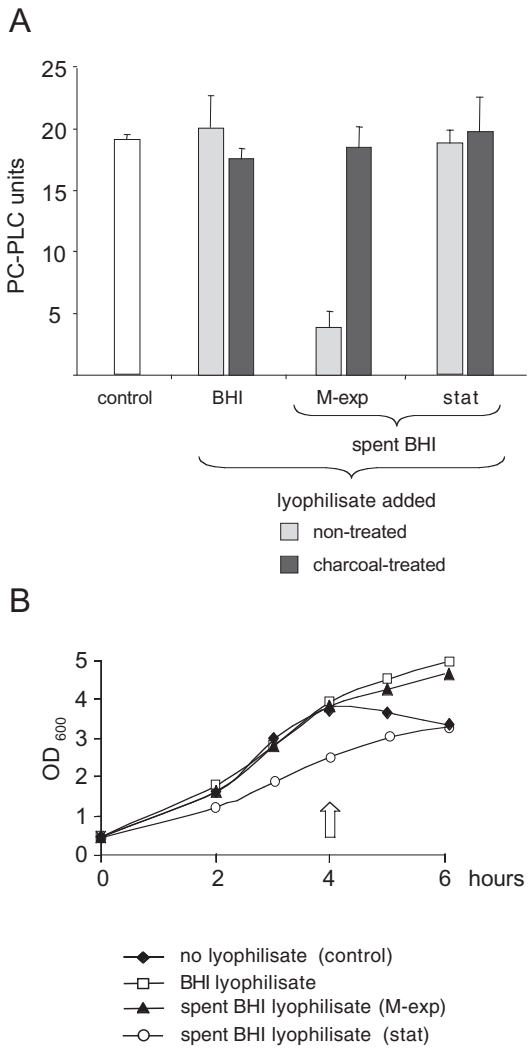

Fig. 5. PrfA-dependent expression in BHIC conditioned with *L. monocytogenes* spent medium.
A. A charcoal-sequestrable PrfA-dependent gene repressor is released into the culture medium by *L. monocytogenes* during exponential growth. Sterile BHI medium was conditioned with lyophilisates obtained from mid-exponential (M-exp) or post-exponential (stat) phase BHI culture supernatants (see *Experimental procedures*) of two types: one was treated for 8 h at 37°C with 0.2% charcoal, with the adsorbent being subsequently removed by centrifugation and filtration (dark grey bars); the other was incubated in the same conditions but without charcoal (light grey bars). The conditioned BHI media (50 ml) were supplemented with 0.2% charcoal, inoculated with a suspension of BHI-washed bacteria from an equivalent volume (50 ml) of an exponentially growing ($OD_{600} = 0.5$) *L. monocytogenes* BHI culture and incubated for 4 h at 37°C (indicated by arrow in B), after which the PC-PLC activity was measured in the culture supernatant. Controls: non-conditioned BHIC (white bar) and medium conditioned with an equivalent amount of non-inoculated BHI lyophilisate. The mean values ± SE from three experiments are represented.
B. The effect of BHI medium conditioning on *L. monocytogenes* growth was assessed in parallel using the same spent BHI media as above. In BHI medium conditioned with non-inoculated BHI or mid-exponential spent BHI lyophilisates, growth was identical to that in normal BHI (except that the exponential growth was extended possibly because of the additional nutrients provided by the medium supplement). In medium conditioned with stationary phase spent BHI lyophilisate, a reduction in the growth rate was observed.

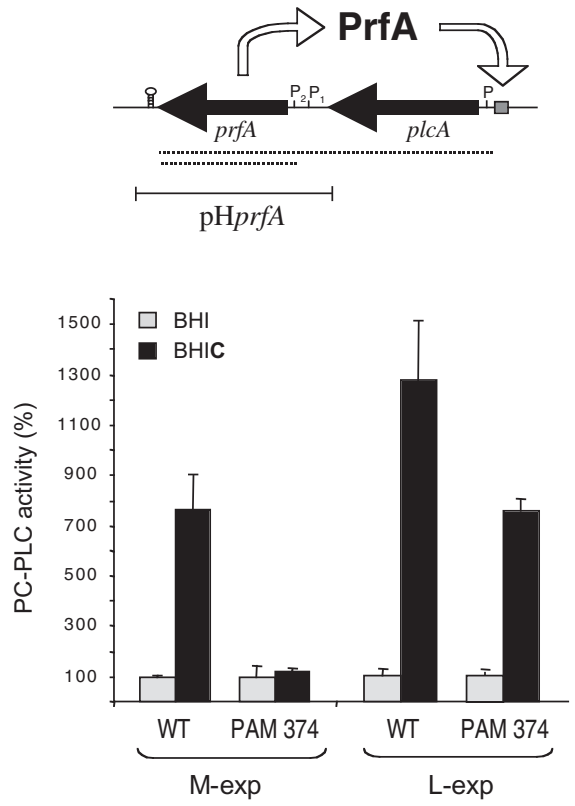

Fig. 6. Impact of the PrfA positive autoregulatory loop in the charcoal-mediated induction of PrfA-dependent genes. *plcB* gene reporter activity was measured at mid- (OD_{600} ≈1.5) and late exponential (OD_{600} ≈2.3–2.5) growth phases in wild-type *L. monocytogenes* (WT, functional autoregulatory loop) and in a Δ*prfA* isogenic mutant complemented with pH*prfA* (PAM 374, autoregulatory loop missing) grown in BHI and BHIC. Results are represented as a percentage of the mean value in BHI, which was arbitrarily set to 100. Mean ± SE of at least three independent experiments. *plcB* expression was not activated by charcoal in Δ*prfA* transformed with a vector without insert (not shown). Top, a schematic representation of the PrfA positive feedback loop mediated by the PrfA-dependent *plcA–prfA* operon. The relevant transcripts (dotted lines below the genes) and promoters (P), the PrfA-box in front of the P*plcA* promoter (grey box) and the DNA fragment inserted into the pH*prfA* plasmid are indicated.

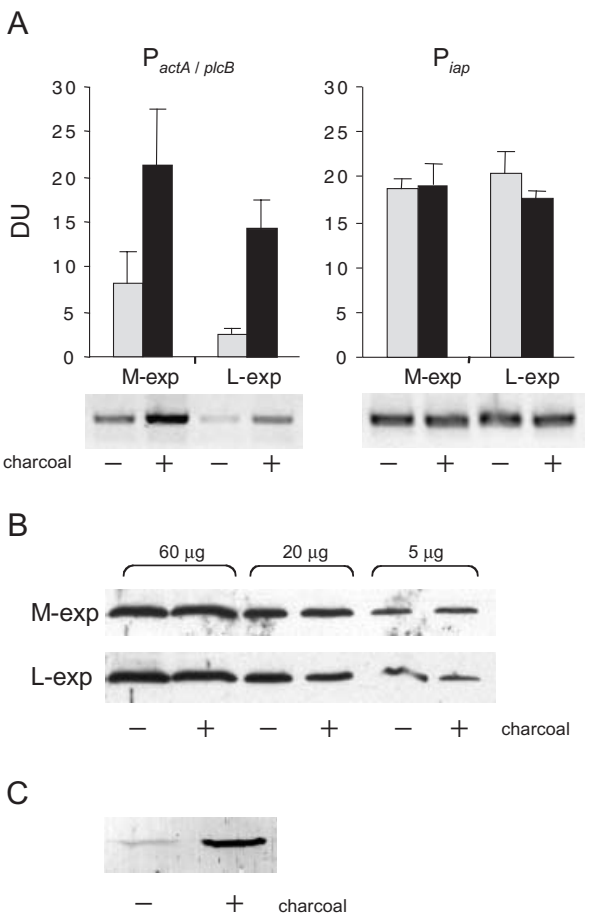

Fig. 7. The charcoal-adsorbable virulence gene autorepressor interferes with PrfA activity. The experiments were carried out using PAM 374 to avoid the effect of the PrfA autoregulatory loop in identical conditions to those described in the legend to Fig. 6. BHI and BHIC media are indicated by grey and black bars and the signs '–' and '+', and mid- and late exponential growth phases by 'M-exp' and 'L-exp' respectively.
A. RT-PCR analysis of the effect of charcoal on the activity of the strictly PrfA-dependent P*actA* promoter from which the reporter gene *plcB* is expressed. As a control, transcription from the constitutive *iap* gene promoter was analysed on the same samples. RNA was extracted during the mid- (OD_{600} ≈1.5) and late exponential (OD_{600} ≈2.3–2.5) growth phases, and RT-PCR products from the RNA dilution giving the best discrimination were subjected to densitometric analysis. Results are expressed in integrated density units with background subtracted (DU) (mean ± SE of three experiments). The products of a representative RT-PCR are shown below the graphs.
B. Western immunoblot analysis of PrfA in cell extracts of parallel samples of PAM 374 grown to mid- and late exponential phases in BHI and BHIC. No differences in the amounts of PrfA are observed in the absence or presence of charcoal. The amount of soluble listerial intracellular protein extract loaded in each lane is indicated in µg. Representative experiment of three replicates with identical results.
C. Same analysis as in (B) but with wild-type *L. monocytogenes*. Bacteria were grown in BHI and BHIC until mid-exponential phase, and PrfA was detected using 30 µg of cytosolic *L. monocytogenes* extract.

is expressed constitutively from its own promoters at 37°C in rich medium.

Growth in BHIC also induced *plcB* expression in PAM 374, albeit later during growth and with a lower intensity than in wild-type bacteria (Fig. 6). The increase in PC-PLC activity in PAM 374 was associated with a clear induction of the P*actA* promoter from which the *plcB* gene is expressed (Vázquez-Boland *et al*., 1992; Shetron-Rama *et al*., 2003) (Fig. 7A) but was not accompanied by a change in the amounts of PrfA protein (Fig. 7B) (unlike in wild-type bacteria with a functional autoregulatory loop; Fig. 7C). These results suggest that (i) the charcoal-sequestrable autorepressor affects PrfA activity; and (ii) the autoregulatory loop enhances the sensitivity of the PrfA system to changes in the extracellular concentration of the autorepressor.

Nature of the autorepressor substance

Activated charcoal is often substituted by the polymeric non-polar adsorbent Amberlite™ XAD-4 for the removal of small organic substances from aqueous solutions. Owing to its small pore size, high exchange area and aromatic surface, this synthetic resin is especially effective against low-molecular-weight hydrophobic substances (Chriswell *et al*., 1977; Held *et al*., 1999). Amberlite does not adsorb highly polar compounds (M. Held, personal communication). Supplementation of the BHI medium with 1% (w/v) Amberlite™ XAD-4 was almost as effective as activated charcoal at inducing PrfA-dependent gene expression. Diaion HP-20, a polystyrene resin with similar physical and polyaromatic characteristics to Amberlite™ XAD-4 but with ion exchange capability, also had a clear, albeit weaker, PrfA-dependent gene-inducing effect (Fig. 8A). In contrast, macroreticular Amberlite™ resins that preferentially adsorb high-molecular-weight organic compounds via an ion-exchange mechanism, such as the basic anion exchanger, IRA-904, or the acidic cation exchanger, IRC-50, did not affect PrfA-dependent gene expression when added to the BHI culture (Fig. 8A). Treatment of a reconstituted mid-exponential phase BHI culture supernatant lyophilisate at 100°C for 10 min did not affect its repressor properties, but treatment with proteinase K reduced this activity (Fig. 8B). These data suggest that the *L. monocytogenes* soluble autorepressor is a low-molecular-weight hydrophobic/aromatic moiety-containing, thermostable molecule, possibly of proteinaceous nature.

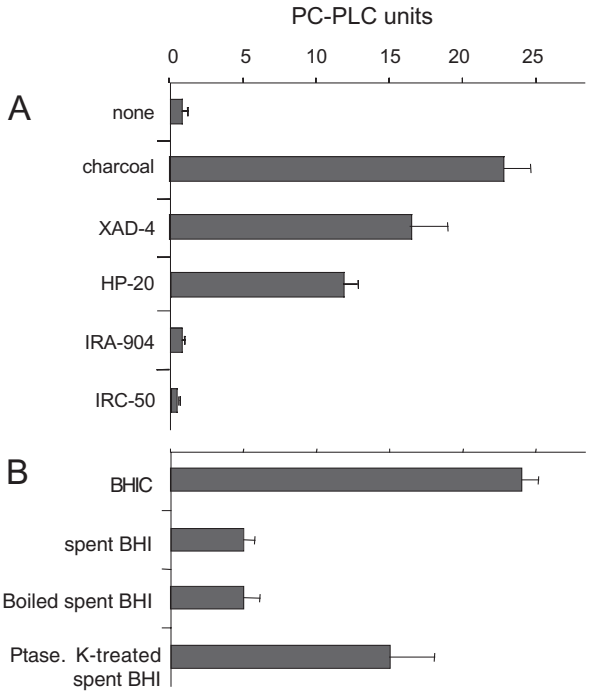

Fig. 8. Preliminary characterization of the virulence gene autorepressor substance.
A. Effect of different synthetic resins on PrfA-dependent expression. Mean ± SE of at least three independent experiments.
B. The autorepressor substance is heat stable and Proteinase K sensitive. Spent BHI lyophilisate from mid-exponential culture supernatant (20 ml) was reconstituted with sterile saline (2 ml) and incubated at 100°C for 5 min or with 100 μg ml^{-1} Proteinase K (Sigma) at 37°C for 2 h, followed by heat inactivation by boiling for 10 min. After these treatments, the reconstituted BHI lyophilisates were added to 8 ml of 1.25× sterile BHI to give a final medium volume of 10 ml, and 0.2% charcoal was added. As controls, 2 ml of distilled water (BHIC) and 2 ml of reconstituted untreated spent BHI lyophilisate were present in the BHIC medium. Media were inoculated, and the PC-PLC activity was determined as described in the legend to Fig. 5A.

Discussion

We present here evidence that *L. monocytogenes* produces a diffusible substance during exponential growth that inhibits PrfA activity, almost completely abolishing PrfA-dependent virulence factor production. This repression is over-ridden by a *prfA** mutation (Figs 1A and 3A), in which a single amino acid substitution locks PrfA in a highly active conformation, suggesting that the listerial autorepressor interferes with the allosteric mechanism thought to be involved in the activation of PrfA transcriptional function (Vega *et al*., 1998; 2004; Vázquez-Boland *et al*., 2001). The crystal structure of PrfA shows that the N-terminal domain of PrfA, which is structurally very similar to the cAMP-binding domain of Crp, can potentially accommodate a small molecule (Thirumuruhan *et al*., 2003; Vega *et al*., 2004). The putative autorepressor might compete directly for this site with the hypothetical PrfA-activating cofactor, blocking the protein in its low-activity conformation. It is also conceivable that the autorepressor molecule is the only PrfA cofactor and that the PrfA* mutant proteins represent a form that is no longer sensitive to inhibition. However, this 'only repression' mechanism does not explain why the PrfA* mutant forms are more active than wild-type PrfA in *in vitro* assays involving only purified proteins (Vega *et al*., 1998; 2003). According to our slot-blot transcription analyses (Fig. 1A), the average increase in PrfA-dependent gene expression operated by charcoal during mid-exponential growth (≈ 5.6 times) is lower than that caused by a *prfA** mutation (≈ 31.9 times). Interestingly, the latter value is similar to the fold induction reported for P*actA* during intracellular infection respect to BHI levels (46 times) (Shetron-Rama *et al*., 2002). These observations suggest that mechanisms other than just derepression are required for PrfA to reach its maximum transcriptional activity. The autorepressor substance might also prevent PrfA function indirectly, even from the exterior of the bacterial cell via a receptor, by regulating the activity of an accessory transcription factor or the levels of the hypothetical PrfA-activating cofactor.

The diffusible autorepressor can obviously play a prin-

cipal role in preventing the production of virulence factors when *L. monocytogenes* is growing in microcolonies outside an animal host, especially at elevated ambient temperatures. However, a role during infection can also be envisaged. There is evidence that *L. monocytogenes* induces PrfA-dependent virulence genes upon entry into host cells (Bubert *et al*., 1999; Freitag and Jacobs, 1999; Moors *et al*., 1999; Renzoni *et al*., 1999; Shetron-Rama *et al*., 2002; 2003). This activation pathway is important for an individual bacterium that has invaded a host cell as it allows it to survive intracellularly, to multiply in the cytosol and to spread to neighbouring cells before the infected cell is detected and eliminated by the immune system. However, some of the PrfA-dependent virulence factors, such as the membrane-damaging proteins PlcA, PlcB and, particularly, LLO, are potentially cytotoxic so that their unrestricted production can be deleterious for the host cell. This is supported by the observation that *prfA** mutants, in which PrfA-dependent expression is constitutively switched on, are more cytotoxic than is wild-type *L. monocytogenes* (Shetron-Rama *et al*., 2003; our unpublished data). Indeed, there is evidence that LLO has evolved specific molecular adaptations to limit its cytotoxic effect, such as a low pH optimum, which restricts its pore-forming activity to the acidic vacuolar compartment (Jones *et al*., 1996), or the presence of a PEST-like sequence, which reduces the cytosolic half-life of the toxin (Decatur and Portnoy, 2000). The autorepressor-mediated negative regulatory pathway may be an additional control mechanism used by *L. monocytogenes* to limit at the transcriptional level the production of cytotoxic virulence factors in those host cells in which the intracellular bacterial population has exceeded a critical threshold.

Bacterial gene expression is modulated not only by exogenous environmental cues but also by autocrine mechanisms involving the sensing of small signalling molecules released by the bacteria themselves during growth. This type of regulation is referred to as 'quorum sensing' as it allows bacteria to co-ordinate their gene responses as a function of population density. Quorum sensing is typically mediated by autoinducer molecules and serves to activate, in general during the transition to the stationary phase, functions that are only effective when carried out in unison by a population of bacteria, such as bioluminescence, biofilm formation, antibiotic production, competence, mating or, in pathogenic bacteria, virulence factor production (Kievit and Iglewski, 2000; Miller and Bassler, 2001; Winzer and Williams, 2001). Thus, in the extracellular pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*, quorum-sensing regulation is believed to prevent the early release of toxic virulence factors and the triggering of inflammatory and immune responses during the initial phases of infection, when the bacterial load is still too low to compete with host defences (Kievit and Iglewski, 2000; Novick, 2003). This classical concept of quorum sensing has evolved to accommodate new examples of bacterial cell–cell communication in which the gene responses are also regulated at low population density (as appears to be the case here with *Listeria*), and the signalling molecules not only activate but repress their target genes, including virulence genes (Kanamaru *et al*., 2000; Kievit and Iglewski, 2000; Koiv and Mae, 2001; Pearson, 2002; Zhu *et al*., 2002). Recently, the *virB* operon from *Brucella melitensis* and *Brucella suis* has been shown to be repressed by quorum sensing during the late exponential growth phase. This operon, encoding a type IV secretion system, is required for survival during the early stages of infection *in vitro* within macrophages and *in vivo* in mice (Taminiau *et al*., 2002). *Brucella* is a quasi-obligate intracellular pathogen and *Listeria* almost never leaves the cytosolic compartment once it enters into a host. The fact that, in both intracellular bacteria, central virulence pathways are repressed by quorum sensing suggests an intriguing idea: that, in intracellular pathogens, this type of regulation has evolved primarily to repress, rather than to activate, virulence genes in order to limit the possible damage to the host cell caused by the bacterial products required to colonize the intracellular niche. Ultimately, an autorepressor in an intracellular pathogen would serve the same purpose as an autoinducer in an extracellular pathogen, as it would also prevent the early presentation of bacterial antigens to the immune system.

Recently, Shetron-Rama *et al*. (2003) have reported in *L. monocytogenes* the generation by chemical mutagenesis of virulence gene-overexpressing mutations that map outside the *prfA* locus. These mutations could be targeting genetic determinants involved in the synthesis of the autorepressor substance. Work is in progress in our laboratory to isolate the autorepressor from the culture supernatant of *L. monocytogenes* and to elucidate its role in *L. monocytogenes* physiology and pathogenesis.

Experimental procedures

Bacterial strains, media and culture conditions

The *L. monocytogenes* strains P14, NCTC 10527 (serovar 4b) and EGD (serovar 1/2a) were used in this study (Ripio *et al*., 1996; 1997; Ermolaeva *et al*., 1999). The three strains respond similarly to charcoal, although higher levels of PrfA-dependent virulence factor production are generally reached with NCTC 10527. PAM 374 is a Δ*prfA* derivative of P14 that has been *trans*complemented with the plasmid pH*prfA*. The Δ*prfA* mutant lacks 229 of the 237 amino acids of PrfA and was constructed by allelic exchange as described previously (Suárez *et al*., 2001). pH*prfA* was constructed by inserting a polymerase chain reaction (PCR)-generated DNA fragment containing the *prfA* gene (plus the *plcA–prfA* intergenic region carrying the monocistronic *prfA* promoters) from P14

(Ripio *et al*., 1997) into the *Escherichia coli–Bacillus*/*Listeria* shuttle vector, pHPS9 (Haima *et al*., 1990). The *prfA** isogenic derivatives of P14 (strain PAM 50) and NCTC 10527 (PAM 26) are described in detail elsewhere (Ripio *et al*., 1997; Vega *et al*., 2004). Brain–heart infusion (BHI; Difco) was heat sterilized (121°C for 15 min) in the autoclave. For some experiments, BHI was filter sterilized using 0.2 μm pore size membranes (Millipore) (Fig. 2b). Spent *L. monocytogenes* BHI cultures (100 ml) were centrifuged (10 000 r.p.m. for 10 min at 4°C) to remove the bacteria, filter sterilized and freeze dried. Conditioned BHI was prepared by reconstituting the spent culture supernatant lyophilisate in 50 ml of fresh sterile BHI. Charcoal-supplemented medium (0.2% w/v for broth cultures and 0.5% for agar plates) was made by adding appropriate amounts of a heat-sterilized 5% (w/v) stock suspension of activated charcoal powder (Merck) in ultrapure water to sterile BHI or conditioned BHI medium. Amberlite™-based resins (XAD-4, IRA-904, IRC-50) (Sigma) and Diaion HP-20 (Mitsubishi Chemical Industries) were added at 1% (w/v) to the BHI before autoclaving. Except where stated otherwise, broth media were inoculated at 1:100 using an overnight *L. monocytogenes* BHI culture and incubated at 37°C with rotary shaking (185 r.p.m.). Transwell cell culture vessels (Costar) were filled with sterile BHI (lower chamber) and BHIC medium (permeable insert) supplemented with an appropriate amount of charcoal to reach 0.2% (w/v) relative to the total volume of medium in the two chambers. They were inoculated in the lower chamber as indicated above and incubated at 37°C with rotary shaking at 100 r.p.m.

Quantification of PrfA-dependent gene expression using the plcB *gene reporter system*

Bacteria were cultured in 10–20 ml of medium in 50 ml flasks until the stationary phase (16–18 h) and the activity of the *plcB* gene product, the PC-PLC PlcB, was quantified in the culture supernatant using a previously described turbidimetric assay and type IV phosphatidylcholine from egg yolk (Sigma) as substrate (Ripio *et al*., 1996; 1997). PC-PLC units are defined as the amount of enzyme causing an increase of 0.1 absorbance units at 510 nm after incubating the reaction mixture for 12 h at 37°C. For semi-quantitative PC-PLC assays in solid medium, 5% (v/v) of an emulsion prepared by adding one fresh egg yolk to 100 ml of sterile saline solution was added to sterile melted BHI agar stabilized at 45–48°C.

SDS-PAGE and Western immunoblotting

The secreted *L. monocytogenes* proteins present in 10 ml of a cell-free culture supernatant were precipitated on ice for 1 h with 10% trichloracetic acid, pelleted at 14 000 r.p.m. for 10 min at 4°C, washed twice with 70% ethanol, dissolved in 100 μl of 1× Laemmli sample buffer and boiled for 5 min. Soluble intracellular *L. monocytogenes* proteins were extracted after disruption of the bacterial cell wall with a mortar and Al_2O_3, followed by ultrasonication and centrifugation, as described previously (Vega *et al*., 1998). Protein concentration was determined by the Bradford method using a kit from Bio-Rad. The proteins were separated by SDS-PAGE in 12% acrylamide gels and visualized by staining with Coomassie Brilliant Blue R-250. For Western blot analysis, proteins were transferred electrophoretically from SDS-PAGE gels onto nitrocellulose membranes (Amersham) using a Mini-Protean cuvette (Bio-Rad) and immunodetected using previously described monoclonal (ActA, PlcB) (Niebuhr *et al*., 1993; Suárez *et al*., 2001) or polyclonal (PrfA, LLO, PlcA) (Vega *et al*., 1998; Ermolaeva *et al*., 1999) primary antibodies (diluted 1:600 to 1:1000), peroxidase-conjugated secondary antibodies (Bio-Rad) and 4-chloro-1-naphthol or the Super-Signal chemiluminescent kit (Pierce).

RNA procedures

Extraction of total RNA from *L. monocytogenes* and slot-blot transcription analysis was performed as described previously using radiolabelled PCR-generated DNA fragments internal to the genes as probes (Ripio *et al*., 1996; 1997). Transcription was quantified by densitometric scanning of the autoradiograms using the NIH IMAGE version 1.63 public domain analysis software (National Institutes of Health Division of Computer Research and Technology, USA). For reverse transcriptase PCR (RT-PCR) transcription analyses, total RNA was extracted from 1.5 ml of *L. monocytogenes* cultures using the Gram-Cracker® and RNAwiz® kits (Ambion), according to the manufacturer's recommendations. Precipitated RNA was dissolved in 100 μl of diethyl pyrocarbonate-treated distilled water, and 50 U of DNase I (Boehringer Mannheim) and RNase inhibitor (Ambion) was added. Quantitative RT-PCR was carried out as described previously (Brehm *et al*., 1999) with the First-Strand cDNA synthesis kit (Amersham). The activity of the P*actA* promoter was detected with oligonucleotide primers actms-5 AAAGAGAACACGC CAATAGCTAA (for RT and PCR) and actms-7 CATGCAGT CAGCAAGATTAGT (for PCR), and that of the P*iap* promoter with the primers msp60c-2 ACCACGTGAGAAATTCCGCT (for RT and PCR) and msp60a-1 ATGTCATGGAATAATT TATCT (for PCR). The PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide and analysed quantitatively with GelDoc 2000 imaging system (Bio-Rad).

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