The use of bacterial luciferase for monitoring the environmental regulation of expression of genes encoding virulence factors in *Listeria monocytogenes*

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A promoter probe vector, which utilized the *luxAB* genes from *Vibrio fischeri* as reporters of gene expression, was constructed for use in *Listeria monocytogenes*. Using this system gene expression can be monitored non-destructively and in real-time, simply by measuring cellular bioluminescence. Derivatives of the promoter probe were constructed that contained the cloned promoters from the *hlyA* and *plcA* genes of *L. monocytogenes*. The activity of these promoters was dependent on the transcriptional activator PrfA. Accordingly, in a strain containing an intact copy of the *prfA* gene, expression from both the *hlyA* and *plcA* promoters was 25–45-fold higher than in *prfA* mutants. Heat shock was identified as an environmental signal which induced expression of *hlyA* and *plcA*. Conversely, oxidative stress had no effect upon the expression of the virulence factors. In addition, the composition of the growth media was found to have a dramatic effect upon the expression of *hlyA* and *plcA*, suggesting the presence of an unidentified signal which may regulate induction of expression of virulence genes in *L. monocytogenes*.

Introduction

Listeria monocytogenes is an ubiquitous Gram-positive pathogen responsible for a variety of infections in a wide range of animals and is now recognized as a foodborne pathogen of significance to humans (Farber & Peterkin, 1991). A key factor in the pathogenicity of *L. monocytogenes* is its ability to survive and multiply within macrophages (Mackanness, 1962) and at least two virulence factors have been implicated in this phenomenon. The most widely studied is the listeriolysin O (Cossart & Mengaud, 1989), which disrupts the phagosomal membrane and is essential for virulence (Gaillard *et al.*, 1986; Michel *et al.*, 1990). Listeriolysin is encoded by the *hlyA* gene (Mengaud *et al.*, 1988) and regions located 5' and 3' of this gene also encode virulence factors (Mengaud *et al.*, 1989). Cells containing mutations in the *plcA* gene, which is located upstream of *hlyA* and encodes a phospholipase C (Leimeister-Wächter *et al.*, 1991; Mengaud *et al.*, 1991a), have attenuated virulence (Camilli *et al.*, 1991; Mengaud *et al.*, 1991a). Additionally, a metalloprotease encoded by the *mpl* gene

(Domann *et al.*, 1991; Mengaud *et al.*, 1991b) and a lecithinase encoded by the *plcB* gene (Vazquez-Boland *et al.*, 1992) may play roles in the infection process and in intercellular spread.

Co-ordinated regulation of virulence factors enables the pathogen to respond to the diverse range of environments encountered during the infection process. The *hlyA* and *plcA* genes are located back-to-back on the *L. monocytogenes* chromosome and are transcribed from divergent overlapping promoters (Mengaud *et al.*, 1989). Expression of both these genes is co-ordinately regulated by a pleiotropic activator for virulence gene expression which is encoded by the *prfA* gene (Leimeister-Wächter *et al.*, 1990; Mengaud *et al.*, 1991c). Strains of *L. monocytogenes* which harbour deletions in this gene do not transcribe either the listeriolysin or phospholipase C genes and, accordingly, have reduced virulence (Mengaud *et al.*, 1991c; Chakraborty *et al.*, 1992). Additionally, PrfA is known to co-ordinately regulate the expression of other genes in the virulence chromosomal region (Vazquez-Boland *et al.*, 1992; Chakraborty *et al.*, 1992), the products of which include metalloprotease (*mpl*), lecithinase (*plcB*) and an actin nucleation factor (*cta*).

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Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Characteristics	Origin*
<i>Strains</i>		
<i>L. monocytogenes</i>		
7973	Haemolytic	1
10357	<i>prfA</i> Δ, non-haemolytic	1
HM1	<i>prfA</i> Δ, non-haemolytic	2
<i>Plasmid</i>		
pCK1	Cm ^R Km ^R , broad host range	3
pSB185	pBR322 derivative with promoterless <i>luxAB</i>	4
pSB283	pCK1 derivative with no <i>Bam</i> HI + <i>Eco</i> RI sites	2
pSB285	pCK1 derivative with promoterless <i>luxAB</i>	2
pSB292	Promoter probe vector with <i>luxAB</i> markers	2
pSP13	pSB292 with cloned constitutive promoter	2
pSP38	<i>plcA-luxAB</i> fusion vector	2
pSP39	<i>hlyA-luxAB</i> fusion vector	2

* 1, National Collection of Type Cultures, Colindale, London, UK; 2, this study; 3, Gasson & Anderson (1985); 4, Ahmad & Stewart (1988).

The use of gene fusions now constitutes a well-established method for the study of gene expression and characterization of transcriptional control signals (Silhavy & Beckwith, 1985). Although reporter gene systems have already been constructed for use in *Bacillus* spp. and *Lactobacillus* spp., and these may well operate in *L. monocytogenes*, the only reporter gene systems which have so far been used in this bacterium are a Tn917-based transposon, which has potential use as a *lacZ*-based operon fusion system (Camilli *et al.*, 1990), and a plasmid vector which can be used to generate gene fusions to *lacZ* (Leimeister-Wächter *et al.*, 1992).

This report describes the construction of a bacterial-luciferase-based promoter probe plasmid for use in *L. monocytogenes*. The use of the *luxAB* genes as markers of gene expression conveys the readily selectable phenotype of bioluminescence (Meighen, 1991). Assays for luciferase are simple and do not compromise cellular viability so that the induction of gene expression from a given promoter can be monitored in real-time, *in situ* and in complex environments. The utility of the vector system was demonstrated by constructing *luxAB* fusions to the *hlyA* and *plcA* promoters and this has allowed the identification of environmental signals which regulate the expression of virulence genes in *L. monocytogenes*.

Methods

Bacterial strains, transformations and growth conditions. The bacterial strains used are described in Table 1. For routine growth, *L. monocytogenes* was grown in Tryptone Soy Broth (TSB; Oxoid) at 37 °C. Strains were transformed with plasmid DNA using electroporation as described by Park & Stewart (1990). Selection for plasmids in *L. monocytogenes* was made by incorporating chloramphenicol into growth media at 5 µg ml⁻¹. When the effect of media composition on virulence gene expression was studied, *L. monocytogenes* was grown overnight in Brain Heart Infusion Broth (BHI; Oxoid) or TSB at 37 °C.

The cells were recovered by centrifugation, washed and then resuspended in an equal volume of the new media. This inoculum was diluted 1:50 into fresh media and incubation continued at 37 °C with aeration.

Plasmids and sequencing. The plasmids used and constructed in this study are listed in Table 1. Plasmid constructions were performed in *Escherichia coli* JM101 using standard techniques (Maniatis *et al.*, 1982). The identity of the 464 bp PCR-generated DNA fragment from the intergenic region between *hlyA* and *plcA* was confirmed using a plasmid based dideoxy sequencing system (Bartlett *et al.*, 1986).

PCR reactions. Two 37-mer primers were designed to enable the cloning of the *hlyA-plcA* intergenic region. The primer with homology to the 5' terminus of *hlyA* had the sequence: 5'CGCATGGATCCG-AATTCGTCTCCGTTATAGCTCATCG3'. The non-homologous region, which introduced sites for *Bam*HI and *Sma*I into the end of the PCR product, is underlined and the translational start codon for *hlyA* is shown in bold. The second primer, with homology to the 5' end of *plcA*, had the sequence: 5'GCGATCCCGGGGATCCCATGGTTTCA-CTCTCCTTC3'. The 5' non-homologous region in this primer introduced *Bam*HI and *Sma*I sites and formed an *Nco*I site with the existing DNA sequence. Primers were included in PCR reactions at 20 pmol. Target DNA was prepared from *L. monocytogenes* by the method of Pitcher *et al.* (1989) and used at a concentration of 1 µg ml⁻¹. A commercial *Taq* DNA polymerase and buffer system (Amersham) was used to amplify the target in the presence of deoxynucleotides (50 µM). A total of 26 cycles was used to amplify the target region and each cycle included a 30 s denaturation step at 94 °C and a 30 s annealing step at 55 °C, followed by a 2 min extension step at 72 °C. The 464 bp fragment generated by this procedure was resolved by agarose electrophoresis and recovered from the gel by Prepagene (Bio-Rad). Following digestion with *Bam*HI, the fragment was cloned directly into the corresponding sites of the promoter probe vector, pSB292. The integrity of the *prfA* gene in the various strains of *L. monocytogenes* was determined by PCR using chromosomal DNA from the respective strains as template and the primers: 5'ATG-AACGCTCAAGCAGAAGA3' and 5'TCTGTTGCAGCTCTTCT-TGG3'. Cycling parameters were the same as those described above.

Luciferase assays and stress experiments. To measure steady state gene expression from *hlyA* and *plcA* fusions to *luxAB*, various strains of *L. monocytogenes* were grown overnight in TSB diluted 1:50 into fresh media and grown with agitation to an OD₆₀₀ of 0.4. Simultaneous

luciferase assays and optical density measurements were then taken. For heat shock and oxidative stress experiments, cells were grown similarly, to an optical density of 0.2, when either H₂O₂ was added or samples were transferred to a 49 °C waterbath for 30 min before being returned to 37 °C. Regular measurements of optical density and luciferase were then made. Luciferase activity was assessed *in vivo* by removing 1 ml samples and adding 20 µl 1% (v/v) decanal solution in ethanol. Bioluminescence was assessed immediately in an LKB luminometer. Activity is given by the peak of the light intensity measured.

Results

Construction of a broad host range promoter probe plasmid

Plasmid pCK1 (Gasson & Anderson, 1985) is a broad host range Gram-positive vector which replicates in both *Escherichia coli* and *L. monocytogenes* (Park & Stewart, 1990). This vector contains two unique *Eco*RI and *Bam*HI sites, which were not desirable in the completed promoter probe vector. Consequently, these two sites were removed by digesting the plasmid with the respective enzymes, removing the intervening fragment and filling the cohesive ends. The plasmid formed by ligation of these ends was designated pSB283 (Fig. 1). A promoterless copy of the *Vibrio fischeri luxAB* genes was then introduced into this backbone. The plasmid pSB185 (Ahmad & Stewart, 1988) contains a promoterless copy of the *luxAB* genes but retains the native Shine-Dalgarno sequence (Fig. 1). This is preceded upstream by stop codons in all three reading frames and a unique *Eco*RI site. In addition, the TL transcriptional terminator from λ is located downstream of the *lux* genes. The terminated *lux* genes were removed from this plasmid as a 2500 bp *Hpa*I fragment, generated by partial digestion, and cloned into the unique *Pvu*II site of pSB283. Plasmids containing both orientations of the *lux* cassette were recovered but only one, containing the orientation shown by pSB285, was analysed further. A synthetic oligonucleotide was cloned into the *Eco*RI site of this vector to introduce unique cloning sites for promoter cloning. The completed promoter probe plasmid was designated pSB292 (Fig. 1). In the absence of cloned promoters no luciferase activity was detected when this vector was introduced into *L. monocytogenes* (results not shown).

Cloning of the intergenic region between hlyA and plcA which contains divergent promoters

The *hlyA* and *plcA* genes, which encode two major virulence factors (Mengaud *et al.*, 1988; Leimeister-Wächter *et al.*, 1991), are located on the *L. monocytogenes*

Fig. 1. Construction of the *luxAB* promoter probe vector, pSB292. The hatched area contains the pCK1 origin of replication and the filled area represents the *luxAB Hpa*I fragment cloned into pSB283; TL shows the bacteriophage λ TL transcriptional terminator. Genes encoding ampicillin (Ap), chloramphenicol (Cm) and kanamycin (Km) resistance are shown.

chromosome in a back-to-back arrangement and are transcribed by divergent promoters (Mengaud *et al.*, 1989). The *hlyA* gene is transcribed from two promoters separated by 10 bp, whilst *plcA* is transcribed in the opposite direction from a single promoter. A 14 bp palindromic region is present in the -35 region of these promoters and those of other genes encoding virulence factors, and may be responsible for binding the positive regulator of virulence gene expression. To enable the study of environmental signals, which may regulate virulence gene expression and thus prove the utility of the promoter probe vector, this 464 bp intergenic region was cloned by PCR. Primers for PCR were designed to hybridize to the 5' end of *plcA* and the 5' end of *hlyA*. The 464 bp PCR product defined by these primers was

Fig. 2. Detection of deletions in the *prfA* gene of various strains of *L. monocytogenes* using PCR. Lanes: 1, 2, 3, PCR products obtained by using universal primers to rRNA and DNA from NCTC 7973, NCTC 10357 and HM1 as target; 4, 5, 6, PCR products obtained by using primers to the *prfA* gene and DNA from NCTC 7973, NCTC 10357 and HM1 as target; 7, PCR control performed in the absence of template DNA.

digested with *Bam*HI and cloned into the promoter probe vector pSB292. The identity of the cloned fragment was confirmed by DNA sequencing and was shown to have 96% identity with the published sequence for the *plcA*–*hlyA* intergenic region (Mengaud *et al.*, 1989). The changes in the nucleotide sequence were confined to

regions which were unlikely to be involved in the regulation of gene expression and, therefore, may have represented strain-specific sequence differences that have been observed for the virulence genes (Rasmussen *et al.*, 1991). Depending on the orientation of this fragment, expression of the *luxAB* marker genes could have occurred from either the *plcA* promoter or the tandemly arranged and divergent *hlyA* promoters. The two orientations of the promoter bearing DNA fragment were identified by digesting recombinant plasmids with *Nco*I. In plasmid pSP38, the orientation of the cloned DNA was such that transcription of the *luxAB* genes occurred from the *plcA* promoter and, conversely, in pSP39 expression of the markers was directed by the dual promoters of the *hlyA* gene.

Regulation of hlyA and plcA expression by the product of the prfA gene

The product of the *prfA* gene is a pleiotropic regulator required for virulence gene expression. Strains of *L. monocytogenes* which harbour deletions in this gene do not express *plcA*, *hlyA* or other virulence factors (Mengaud *et al.*, 1991c; Chakraborty *et al.*, 1992) and, consequently, have decreased virulence. To confirm that this regulation occurred at the transcriptional level, and to test the utility of the promoter probe vector, we monitored the activity of the promoters of *hlyA* and *plcA* by assessing luciferase expression from the corresponding promoter probe constructs, both in the absence and presence of the *prfA* gene product. The plasmids pSP38 and pSP39 were introduced into the several strains of *L. monocytogenes* by electroporation (Park & Stewart, 1990). *L. monocytogenes* NCTC 7973 is virulent and haemolytic so is likely to contain the *prfA* gene. Conversely, strain NCTC 10357 (NCTC Type Strain) is avirulent, does not express phospholipase C or haemolysin, and has been shown previously to contain a deleted

Table 2. Regulation of gene expression from *hlyA*–*luxAB* and *plcA*–*luxAB* fusions by the *prfA* gene product

The activities correspond to those obtained in a typical experiment. Similar results were reproducibly obtained in several experiments.

Plasmid*	Strain† . . .	Luciferase activity [light units (OD ₆₀₀) ⁻¹]		
		NCTC 7973 (<i>prfA</i> ⁺)	NCTC 10357 (<i>prfA</i> Δ)	HM1 (<i>prfA</i> Δ)
pSP39 (<i>hlyA</i>)		270	12	6
pSP38 (<i>plcA</i>)		25	2	1
pSP13 (constitutive)		806	616	651

* The promoter activity which is fused to *luxAB* in each plasmid is indicated in parentheses.

† The relevant genotype of each strains is indicated in parentheses.

copy of the *prfA* gene (Leimeister-Wächter *et al.*, 1990). A second *prfA*-deleted strain, HM1 (which arose in the laboratory as a spontaneous non-haemolytic mutant of *L. monocytogenes* 7973) was also used. To confirm the genotype of these strains with respect to *prfA*, two PCR primers were designed to anneal to the 5' and 3' end of the *prfA* gene sequence (Mengaud *et al.*, 1991c). PCR was then performed using these primers and chromosomal DNA from the various strains of *L. monocytogenes* as the target. The presence of an intact copy of *prfA* was confirmed in NCTC 7973 by the generation of an 830 bp DNA fragment (Fig. 2), which was as predicted for an intact *prfA* sequence (Mengaud *et al.*, 1991c). For strains NCTC 10357 and HM1, PCR generated DNA fragments of 270 bp and 340 bp respectively. This confirmed a 560 bp deletion in the *prfA* gene of NCTC 10357 (Mengaud *et al.*, 1991c) and identified a 490 bp deletion in the same gene of the non-haemolytic strain HM1. Interestingly, an identically sized deletion was identified in strain ATCC 43250 (Mengaud *et al.*, 1991c). When universal primers to bacterial 16S ribosomal RNA genes (Lane *et al.*, 1985) were used as positive controls in PCR reactions all target strains produced the expected 500 bp fragment (Fig. 2).

The activities of the *hlyA* and *plcA* promoters were assessed by measuring the bioluminescence from strains containing the *luxAB* fusion plasmids. As expected, in strains NCTC 10357 and HM1, the absence of an intact copy of the *prfA* gene led to low levels of *luxAB* expression from both promoters (Table 2). However, in NCTC 7973, which contains an intact copy of *prfA*, expression of *luxAB* was 22–45-fold higher for the *hlyA* promoters and 12–25-fold higher for the promoter from *plcA*. Interestingly, the promoters from the *hlyA* gene also led to higher expression of *luxAB* than that from *plcA*, as the bioluminescence was 6–11-fold higher in strains containing the *hlyA-luxAB* fusion plasmid (Table 2). This difference was apparent both in the absence and presence of PrfA. Plasmid pSP13 is a pSB292 derivative that contains a cloned promoter from a listerial bacteriophage and which is not environmentally regulated by pH, osmolarity, oxidative stress or temperature (S. F. Park & R. G. Kroll, unpublished data). When this construct was introduced into the strain selection, little difference in *luxAB* expression occurred between strains (Table 2). This confirmed that PrfA acts as an activator of expression for the *hlyA* and *plcA* genes at the transcriptional level and that luciferase activity was correctly reporting gene expression.

Effect of heat shock and oxidative stress on virulence gene expression

The expression of a variety of bacterial virulence factors is regulated by heat shock and other environmental

Fig. 3. Heat-shock-induced gene expression in *L. monocytogenes* from *hlyA-luxAB* and *plcA-luxAB* fusions. Strain NCTC 7973, carrying appropriate plasmids, was subjected to heat shock and luciferase activity was assayed at intervals. (a) pSP13, which contained a constitutively expressed *luxAB* fusion; (b) pSP39, which contained a *hlyA-luxAB* fusion; (c) pSP38, which contained a *plcA-luxAB* fusion. □, No induction; ■, heat shock for 30 min at 49 °C. Similar results were reproducibly obtained in several experiments.

stresses (Mekalanos, 1992). Recently, Sokolovic & Goebel (1989) demonstrated that for particular strains of *L. monocytogenes*, production of listeriolysin O is induced by heat shock at 48 °C. This has only been analysed qualitatively and no information is available regarding the kinetics of the heat shock induction. The effect of heat shock and oxidative stress on the expression of luciferase from the *hlyA*- and *plcA-luxAB* fusions were, therefore, studied.

Above 37 °C, bacterial luciferase is irreversibly denatured in *L. monocytogenes* (S. F. Park & R. G. Kroll,

Table 3. Effect of heat shock and oxidative stress on the expression of *hlyA-luxAB* and *plcA-luxAB* fusions

The activities correspond to those obtained in a typical experiment. Similar results were reproducibly obtained in several experiments. –, Not tested.

Plasmid/strain	Relevant characteristics	Luciferase activity [light units (OD ₆₀₀) ⁻¹]			
		TSB	1 mM-H ₂ O ₂ *	5 mM-H ₂ O ₂ *	49 °C†
pSP13/NCTC 7973	Constitutive <i>luxAB</i> , <i>prfA</i> ⁺	750	881	820	590
pSP13/NCTC 10357	Constitutive <i>luxAB</i> , <i>prfA</i> Δ	800	–	–	766
pSB38/NCTC 7973	<i>plcA-luxAB</i> , <i>prfA</i> ⁺	20	23	24	410
pSB38/NCTC 10357	<i>plcA-luxAB</i> , <i>prfA</i> Δ	1	–	–	0
pSP39/NCTC 7973	<i>hlyA-luxAB</i> , <i>prfA</i> ⁺	275	264	262	3935
pSP39/NCTC 10357	<i>hlyA-luxAB</i> , <i>prfA</i> Δ	71	–	–	67

* Cells were grown to an OD_{600 nm} of 0.2, H₂O₂ was then added and bioluminescence assessed after 3 h.

† Cells were grown to an OD_{600 nm} of 0.2 and then heat-shocked at 49 °C for 30 min. Bioluminescence was then assessed after 3 h.

unpublished data). Accordingly, when a strain containing pSP13, in which *luxAB* expression appears to be constitutively expressed, was subjected to heat shock, there was an initial abolition of cellular bioluminescence. Resynthesis of luciferase following the heat shock occurred after 30 min, and after 90 min the levels were restored to pre-heat-shock values (Fig. 3). When expression of luciferase was dependent on the *hlyA* and *plcA* promoters, low levels of luciferase activity were again apparent at 30 min. After 60 min, however, the expression of luciferase was stimulated from the plasmids pSB38 and pSB39. The levels of expression from the *hlyA* and *plcA* promoters reached a maximum after 120 min and were stimulated by 20-fold and 14-fold respectively. When the same experiment was carried out in strain NCTC 10357, which does not produce PrfA, no induction of virulence gene expression was observed following heat shock (Table 3). Bacterial cells were subjected to oxidative stress by the inclusion of H₂O₂ in the growth media at 1 mM and 5 mM. An initial decrease in bioluminescence occurred, suggesting that the cells were stressed (data not shown). However, following the restoration of luciferase activity, no induction of gene expression from the *hlyA* and *plcA* promoters occurred (Table 3).

Expression of virulence genes is induced following a change in cultural conditions

Cultural conditions are known to influence the expression of listeriolysin (Geoffroy *et al.*, 1989). In this study, *L. monocytogenes* containing the *luxAB* fusion plasmid, pSP39, was routinely cultured in Tryptone Soy Broth when the activity of the *hlyA* promoter was being assessed. Surprisingly, when TSB-grown cells were transferred to Brain Heart Infusion Broth a dramatic

Fig. 4. Induction of *hlyA* expression by a change in cultural conditions. The luciferase activity from strain NCTC 7973, carrying either the *hlyA-luxAB* fusion plasmid pSB39, or pSP13 in which *luxAB* is constitutively expressed, was assayed during growth and transfers into various media. □, pSP39, TSB to TSB; ■, pSB39, TSB to BHI; △, pSP39, BHI to BHI; ○, pSP13, TSB to TSB; ●, pSP13, TSB to BHI.

induction in the activity of the *hlyA* promoter occurred (Fig. 4). The increase in *hlyA* expression was apparent 60 min after the transfer into BHI and reached a maximal level after 200 min. At this point, induced levels of *hlyA* expression were 170-fold greater than those which were measured in TSB. Furthermore, when cells were grown continually in BHI the activity of the *hlyA*

promoter appeared to be permanently induced (Fig. 4). A similar induction was seen for the *plcA* promoter following transfer of cells from TSB to BHI (data not shown). No induction of gene expression occurred when the experiment was repeated with the strain of *L. monocytogenes* which carried the plasmid pSP13, in which expression of *luxAB* is directed by a promoter which appears to be constitutively active (Fig. 4). Moreover, the increase in *hlyA* and *plcA* expression was apparent only in cells containing an intact copy of the *prfA* gene and, accordingly, when strain NCTC 10357 (*prfA*Δ) carrying the plasmids pSP38 or pSP39 was grown in BHI no induction in the expression of virulence genes was detected (data not shown).

Discussion

A promoter probe vector for use in *L. monocytogenes*, which utilized the *luxAB* genes from *V. fischeri* as reporters of gene expression, was constructed. This system allowed the activity of cloned promoters to be assessed readily by measuring cellular bioluminescence. By constructing *hlyA* and *plcA* fusions to *luxAB*, we have confirmed that the expression of the virulence factors, listeriolysin O and phosphatidylinositol-specific phospholipase C, is dependent on the pleiotropic activator of virulence, PrfA, and that regulation occurs at the transcriptional level. When gene fusions are present on multicopy plasmids abnormal regulation of gene expression can occur if regulatory proteins are titrated. The promoter probe system described herein, however, appears to accurately monitor virulence gene expression in *L. monocytogenes* despite the presence of the *luxAB* gene fusions in *trans* and on plasmids. The reason for this may be that plasmids with the pCK1 replicon are maintained at a relatively low copy number (<10 copies per cell; S. F. Park & R. G. Kroll, unpublished results).

During infection, any pathogen must be able to recognize and respond to multiple environmental cues which signal its entry into the host. Accordingly, environmental stress regulates the expression of a number of bacterial virulence factors (Mekalanos, 1992). Heat shock, for example, is known to induce the synthesis of a characteristic set of proteins in *L. monocytogenes*, which include the listeriolysin (Sokolovic & Goebel, 1989; Sokolovic *et al.*, 1990). In this study, the utility of bacterial luciferase as a marker of gene expression has allowed an assessment of the kinetics of heat shock induction of virulence gene expression. The conditions of the heat shock irreversibly denature bacterial luciferase so that any bioluminescence observed after this event must result from re-synthesis of the enzyme. For heat shocked cells of *L. monocytogenes*,

in which luciferase was constitutively expressed, a significant time delay was apparent before luciferase activity was restored to pre-shock levels. Presumably, this lag period represents the time it takes the bacterial cell to recover from the heat shock and resume macromolecular synthesis. The same delay in luciferase expression occurred when gene expression was monitored from the promoters of *hlyA* and *plcA*. After this initial lag, however, expression of these virulence genes was induced by up to 20-fold. Heat shock induction of *hlyA* and *plcA* did not occur in a (*prfA*Δ) mutant strain, but whether or not this effect is regulated by the transcriptional activator, PrfA, is unclear. Recently, Leimeister-Wächter *et al.* (1992) demonstrated that expression of virulence genes in *L. monocytogenes* occurs only when cells are incubated at 37 °C or above. In our experiments, cells were pre-incubated at 37 °C prior to heat shock and would already have been induced for virulence gene expression by growth at this temperature (Leimeister-Wächter *et al.*, 1992). It is possible, therefore, that the induction of *hlyA* and *plcA* expression described herein represents a true heat shock response rather than it being a manifestation of the thermoregulation of virulence gene expression.

Genetic analysis of the microbial stress response has revealed an interlocked regulatory system (Watson, 1990). For example, in *Salmonella typhimurium* many proteins induced by heat shock are also induced by oxidative stress (Morgan *et al.*, 1986), and this phenomenon is thought to represent a co-ordinated response to the multiple stresses which are encountered in the macrophage environment (Buchmeier & Heffron, 1990). In our experiments, however, neither *hlyA* or *plcA* expression was induced in media containing H₂O₂. In contrast, Sokolovic *et al.* (1990) have reported that an activation of listeriolysin expression occurs during oxidative stress. However, this was only modest compared to the heat shock response and required an extended period of pre-adaption before a complete response occurred.

The composition of growth media for *L. monocytogenes* is known to affect the expression of listeriolysin but this phenomenon has never been characterized in detail and has been attributed, previously, to iron concentration (Geoffroy *et al.*, 1989). In this study, we have shown for the first time that the expression of at least two virulence genes, *hlyA* and *plcA*, is dramatically induced following transfer of *L. monocytogenes* from TSB to BHI. It is possible that this change in cultural conditions somehow mimics the transition in environmental parameters which enables *L. monocytogenes* to recognize its entry into host tissue and, as a consequence, leads to induction of expression of virulence factors. The nature of the signal(s) that trigger this increase in *hlyA* and *plcA*

expression is at present unknown, but it is possible to rule out a number of environmental parameters which are known to regulate virulence gene expression in other bacterial pathogens (Mekalanos, 1992). For instance, the TSB and BHI media used in these experiments had equivalent pH values (pH 7.4) and the induction of virulence gene expression, therefore, was not dependent on pH. Similarly, both media were unlikely to have had significantly different osmolarities or concentrations of phosphate. Furthermore, both media are considered to be iron replete, so that it is unlikely that iron limitation was responsible for the induction of *hlyA* and *plcA* expression that we observed. It is possible, therefore, that the effect of media composition on the expression of virulence factors is due to either the presence of unidentified inducers of virulence in BHI and their absence from TSB or, conversely, the existence of a specific repressor of *hlyA* and *plcA* gene expression in TSB. Interestingly, we have also demonstrated that expression of *hlyA* and *plcA* can be induced by heat shock and that this induction in gene expression can occur in TSB. The repression of virulence gene expression during growth in TSB can be overcome, therefore, by additional environmental stimuli which suggests that the regulation of *hlyA* and *plcA* expression can be controlled by multiple environmental signals. A detailed analysis of the media-dependent regulation of *hlyA* and *plcA* expression is currently underway in this laboratory; this may lead to the identification of novel factors that mediate the regulation of expression of virulence genes in *L. monocytogenes*, and which may enable this pathogen to recognize that entry into host tissue has occurred.

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