Deregulation of *Listeria monocytogenes* virulence gene expression by two distinct and semi-independent pathways

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Expression of the major virulence cluster in *Listeria monocytogenes* is positively regulated by the transcription factor PrfA and is influenced by several environmental factors, including the presence of readily metabolized carbohydrates such as cellobiose and glucose. Although little is understood about the mechanisms through which environmental factors influence expression of the PrfA regulon, evidence for structural and functional similarities of PrfA to the CRP-FNR family of regulatory proteins suggests the possibility that PrfA activity could be modulated by a small molecule ligand. The identity of components of the PrfA-associated regulatory pathway was sought through the isolation of mutants that exhibit high levels of PrfA-controlled gene expression in the presence of cellobiose or glucose. Here are described the properties and preliminary genetic analysis in two different genetic loci, *gcr* and *csr*, both unlinked by general transduction to the major virulence cluster. A mutation in *gcr* deregulates the expression of PrfA-controlled genes in the presence of several repressing sugars and other environmental conditions, a phenotype similar to that of a G145S substitution in PrfA itself. A mutation in the *csr* locus, within *csrA*, results in a cellobiose-specific defect in virulence gene regulation. Gene products encoded by the *csr* locus share homology with proteins involved in the sensing and transport of β-glucosides in other bacteria. Mutations in both *gcr* and *csr* are required for full relief of cellobiose-mediated repression of the PrfA regulon. These results suggest the existence of two semi-independent pathways for cellobiose-mediated repression and further reconcile conflicting reports in previous literature concerning the repressive effects of carbohydrates on virulence gene expression in *L. monocytogenes*.

**INTRODUCTION**

The Gram-positive, facultative intracellular pathogen *Listeria monocytogenes* is an important cause of food-borne infection of humans. It is the causal agent of listeriosis, a serious disease characterized by septicemia, spontaneous abortion and meningoencephalitis (Farber & Peterkin, 1991; Vazquez-Boland et al., 2001). A number of genes specifically involved in the infectious process have been identified; six of these are clustered within a 10 kb region of the bacterial chromosome [reviewed by Kreft & Vazquez-Boland (2001) and Vazquez-Boland et al. (2001)]. The *hly* gene encodes listeriolysin O (LLO), a cholesterol-dependent cytolysin involved in the lysis of the host cell vacuolar membrane. The *plcA* and *plcB* genes encode a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-specificity phospholipase C (PC-PLC), respectively, both of which are involved in vacuolar lysis and cell-to-cell spread. The *actA* gene encodes a protein responsible for actin-based motility within host cells, and *mpl* encodes a metalloprotease necessary for the maturation of PC-PLC. These virulence genes have all been shown to be under positive regulation by the transcriptional activator PrfA (encoded by the *prfA* gene), the sixth member of the 10 kb gene cluster (Chakraborty et al., 1992; Mengaud et al., 1991). PrfA exerts tight control over expression of the genes in this cluster and its presence is absolutely required for

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**Abbreviations:** EMS, ethyl methanesulfonate; PTS, phosphotransferase system; XG, 5-bromo-4-chloro-3-indoyl β-D-glucuronide.
successful infection of the host (Chakraborty et al., 1992; Freitag et al., 1993; Leimeister-Wachter et al., 1990; Mengaud et al., 1991).

PrfA is the only definitive regulator of virulence gene expression identified thus far in L. monocytogenes. PrfA-mediated activation requires binding of the PrfA protein at conserved 14 bp sequences of dyad symmetry found in target promoters (Chakraborty et al., 1992; Freitag et al., 1992, 1993; Leimeister-Wachter et al., 1990; Mengaud et al., 1991), a characteristic shared by the cyclic AMP (cAMP) receptor protein (CRP)-recognition elements (Ripio et al., 1997b). The primary structure of PrfA has limited but significant similarities (approx. 20% amino acid identity, 30% similarity) to CRP and other members of the CRP-FNR family of transcription factors (Kreft et al., 1995; Lampidis et al., 1994). Fundamental functional similarity of the two proteins is most strongly indicated by the properties of a specific PrfA mutant resulting in a glycine to serine substitution at amino acid position 145. PrfA-dependent genes are constitutively overexpressed in this mutant even under environmental conditions that normally down-regulate expression of virulence genes (Behari & Youngman, 1998a; Ripio et al., 1996, 1997a, b). An analogous mutation in CRP leads to a cAMP-independent, constitutively active CRP protein. Thus, it has been proposed that PrfA may require a similar co-factor or some form of post-translational modification for efficient binding and activation of its target promoters (Ripio et al., 1997b; Vega et al., 1998).

Several recent studies have contributed to a better understanding of the mechanisms that mediate regulation of genes under PrfA control (Bockmann et al., 2000; Dickneite et al., 1998; Herler et al., 2001; Lalic-Multhaler et al., 2001; Renzoni et al., 1997, 1999; Shetron-Rama et al., 2002; Williams et al., 2000). Park & Kroll (1993) originally reported that the disaccharide cellobiose is the only one of many carbohydrates tested that has a repressive effect on the expression of two virulence genes, hly and plcA, in L. monocytogenes strain NCTC 7873. These results led to the proposal that cellobiose functions as a specific signature molecule providing L. monocytogenes bacteria with a mechanism for sensing their environment (Park & Kroll, 1993). However, it was subsequently demonstrated in experiments utilizing three other L. monocytogenes wild-type isolates that cellobiose is not unique in its repressive effect (Milenbachs et al., 1997). In these isolates, growth in the presence of several other readily metabolized sugars was found to significantly down-regulate virulence gene expression. PrfA protein levels were unaffected by growth in the presence of these sugars, suggesting either that the activity of PrfA may be subject to regulatory modulation (e.g. via a covalent modification or allosteric interaction) or that some additional unidentified regulatory factor may be involved (Milenbachs et al., 1997; Renzoni et al., 1997).

Recently, gene products encoded by the bvrABC locus have been implicated in the repression of L. monocytogenes virulence gene expression in response to the β-glucosides cellobiose and salicin (Brehm et al., 1999). The bvrABC locus encodes an anti-terminator of the BglG family (bvrA), a β-glucoside-specific enzyme II permease component of the phosphoenolpyruvate-sugar phosphotransferase system (PTS) (bvrB) and a putative ADP-riboseglycohydrolase (bvrC). The mechanisms mediating BvABC-dependent repression of virulence gene expression in the presence of cellobiose have not been elucidated, but mutations within this locus do not prevent utilization of the sugar by L. monocytogenes. A second locus has also been implicated in cellobiose-mediated repression of hly expression, but the gene products that mediate repression were not well defined (Huillet et al., 1999).

In this work, we identify a novel locus involved in cellobiose-dependent repression of virulence gene expression in L. monocytogenes and also provide genetic evidence for regulatory factors in L. monocytogenes other than PrfA that are involved in virulence gene regulation in response to diverse environmental stimuli. We have characterized two independent ethyl methanesulfonate (EMS)-generated mutations, both unlinked to the prfA gene, which result in deregulation of virulence gene expression. One mutation deregulates hly expression in the presence of several repressing sugars and under environmental conditions that normally down-regulate hly, including low temperature. The second mutation alleviates repression by cellobiose via the truncation of a putative multi-domain regulatory protein with homology to Bacillus subtilis LeVR, which regulates bacterial utilization of levans (polymers of fructose) (Debarbouille et al., 1991). Complete relief of cellobiose repression in L. monocytogenes requires both mutations. However, even in the double mutant, regulation of the catabolite control protein (CcpA)-controlled enzyme α-glucosidase remains intact. These results are consistent with a model in which cellobiose acts through at least two semi-independent pathways to repress virulence gene expression. The results also support the earlier proposal by Park & Kroll (1993) that cellobiose may play a role in virulence gene regulation distinct from that of other readily metabolized sugars. Either or both of these hypothesized pathways could act through a co-factor that modulates PrfA activity.

METHODS

Bacterial strains and growth conditions. Escherichia coli DH5α, DH5α MCR, HB101 (Gibco-BRL) and S-17-1 were utilized for all plasmid constructions. Plasmid-containing strains were grown in Luria–Bertani (LB) medium, and ampicillin was added to a concentration of 100 μg ml⁻¹ for selection. L. monocytogenes strains were grown in brain–heart infusion (BHI) (Difco Laboratories) or on LB medium. For cultivation of L. monocytogenes strains, overnight cultures were grown to saturation in BHI medium and diluted 1:50 into either LB or BHI medium. All LB medium was buffered with 100 mM MOPS to pH 7.4. For experiments measuring the effects of pH, LB was buffered with 100 mM MOPS (pH 6.5–7.4) or 100 mM MES (pH 5.6–6.5). Where indicated, filter-sterilized carbohydrate supplements were added to media at a final concentration of...
Construction of pCON1-HGNG and hly-gusA transcriptional fusions. The E. coli–L. monocytogenes shuttle vector pCON1-HGNG, used to create a stable hly-gusA transcriptional fusion in L. monocytogenes 10403S, was constructed as follows. An internal fragment of the hly gene was PCR-amplified from the 10403S chromosome with the oligonucleotides AAAMly3250R (5'-CACTCGGATCCGATTTCCCAATTGGGAAATTTGG-3') and AAAMly3762L (5'-AAGCTTGTTTGATTTGGTAACCGAAAGTAGCG-3'). Each primer contains two mismatches, indicated by bold type, which create BamHI and SalI sites (underlined), respectively. This 512 bp fragment was digested with BamHI and SalI and ligated into the vector pMLK117 (Karow & Piggot, 1995) which contains a promoterless copy of the gusA gene (encoding β-glucuronidase) from E. coli and a neomycin-resistance cassette, creating the vector pMLK117-hly. This vector was digested with BamHI and HindIII and the resulting fragment (containing the hly fragment, the promoterless gus gene and the neomycin cassette) was ligated into BamHI/HindIII-digested pCON1 (Freitag, 2000), creating pCON1-HGNG. Propagation of pCON-1 in Gram-positive bacteria depends upon a temperature-sensitive origin of replication derived from pE194ts, and it carries a chloramphenicol-resistance (Cm') marker for selection in Gram-positive bacteria. A fragment spanning the intergenic region between the hly and mpl gene was PCR-amplified from the 10403S chromosome with primers AAAMly4143R (5'-CATCGGATCCGATTTCCCAATTGGGAAATTTGG-3') and AAAMly3762L (5'-AAGCTTGTTTGATTTGGTAACCGAAAGTAGCG-3'). AAAMly6470L contains two mismatches in its sequence, indicated in bold type, which create a NarI site (underlined). This 527 bp fragment was digested with HindIII and NarI and ligated into HindIII/NarI-digested pCON1-HGNG, creating pCON1-HGNG. pCON1-HGNG was transformed into the E. coli donor strain S-17-1 and conjugated into L. monocytogenes 10403S as described previously (Behari & Youngman, 1998a). 10403S(pCON1-HGNG) was initially grown at 30°C until mid-exponential phase and then shifted to the non-permissive temperature (42°C) with selection for Cm'. These growth conditions selected for the homologous recombination of pCON1-HGNG into the 10403S chromosome at the hly locus. Resultant clones were Cm', Nm' and non-haemolytic on blood agar plates (Hly'). To select for cells where spontaneous excision of the undesired pCON1 sequences had occurred, several isolates were diluted 1:1000 from overnight cultures into BHI medium containing 5 µg neomycin ml⁻¹ and grown at the permissive temperature (30°C) for 24 h. For subsequent curing of the vector, stationary-phase cultures were diluted 1:100 into pre-warmed BHI medium containing 5 µg neomycin ml⁻¹ and grown at 42°C for 24 h. Ten-fold serial dilutions of these cultures were plated onto pre-warmed BHI agar containing neomycin and incubated at 42°C for 48 h. Colonies were tested for vector integration on neomycin/chloramphenicol/blood agar plates. Desired colonies, in which the second recombination event at the intergenic region between hly and mpl occurred, were neomycin-resistant (Nm'), Cm' and Hly', and were blue when tested for β-glucuronidase activity on plates containing the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (XG) (US Biologicals). This strain was named AML73. Constructs were confirmed by PCR amplification and Southern blot hybridization.

EMS mutagenesis of AML73. Bacteria harvested from mid-exponential-phase cultures (OD₅₉₀ 0.5) of AML73 grown in 50 ml BHI medium were washed twice in 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3) and resuspended in 25 ml PBS. An aliquot (2.5 ml) of a prewarmed EMS (Sigma) solution (1 mM EMS/24 ml PBS) was mixed with an equal volume of resuspended cells and incubated at 37°C for various lengths of time spanning 0–75 min. Each sample was washed twice and resuspended in 2.5 ml PBS. Colony titres were determined and samples exhibiting 90–95% killing were used for mutational analysis. Three independent EMS libraries were generated, and a total of 1×10⁶, 3.75×10⁶ and 6.25×10⁶ colonies were screened from these libraries.

Generalized transduction. Generalized transduction for linkage analysis and transposon tagging procedures were carried out as described by Hodgson (2000). To generate phage lysates, 100 µl of bacteriophage U153 dilutions made from a high-titre stock (10⁹ p.f.u.) was mixed with 100 µl of mid-exponential-phase BHI culture of the L. monocytogenes donor strain grown at 30°C and incubated for 40 min at room temperature. Three millilitres of molten LB agar plus 10 mM CaCl₂ and 10 mM MgSO₄ were added to the mix and poured onto LB plates containing 10 mM CaCl₂ and 10 mM MgSO₄. Plates were incubated overnight at room temperature. Lysates were harvested from just-confluent plates by adding 5 ml sterile TM buffer (8.0 mM MgSO₄, 10 mM Tris/HCl, pH 8.0) and the recovered lysate was filter-sterilized to remove bacteria. The bacteriophage titres were determined as p.f.u. ml⁻¹. To transduce L. monocytogenes, 10⁶ p.f.u. of the bacteriophage grown on the appropriate donor strain was mixed with 10⁹ mid-exponential-phase recipient cells, and the mixture was incubated at room temperature for 40 min. To select for Nm' transductants, the mixture was plated directly onto BHI agar containing 10 mM sodium citrate (pH 7.5) and 5 µg neomycin ml⁻¹. To select for erythromycin-resistant (Em') transductants, 2.5 ml BHI molten top agar containing 10 mM sodium citrate (pH 7.5) to which 100 µl of 10 µg ml⁻¹ erythromycin had been previously added was mixed with the cells and bacteriophages, and the mixture was poured onto BHI agar containing 10 mM sodium citrate (pH 7.5). The plates were incubated for 2 h at 37°C for induction of erm expression before another 2.5 ml BHI top agar containing 10 mM sodium citrate, 40 µl erythromycin (1 µg ml⁻¹) and 40 µl lincomycin (25 µg ml⁻¹) was added (lincomycin prevents the growth of spontaneous Em' colonies). Plates were incubated for 48 h at 37°C.

Preparation of cell lysates. Samples (10 ml) from mid-exponential-phase cultures were collected and washed once in an equal volume of 50 mM potassium phosphate buffer. The cells were resuspended in 2–3 ml of the same buffer and lysed three times by sonication (on ice) for 30 s each. Debris was clarified by centrifugation and 0.05 ml of the clarified supernatant was used for assay of x-glucosidase activity [units of which are nmol substrate hydrolysed (mg protein)⁻¹ min⁻¹]. Protein concentrations in cell lysates were determined by the method of Bradford (1976) using a Bio-Rad protein assay, with BSA as the standard.

Enzyme assays. For plate assays, β-glucuronidase activity was estimated by intensity of blue colour of bacteria spotted onto buffered LB plates containing 50 µg XG ml⁻¹, with or without cellobiose or glucose each at a concentration of 25 mM. For liquid assays, β-glucuronidase activity was measured from late-exponential-phase cultures using 4-methylumbelliferone-β-D-glucuronic acid trihydrate (US Biologicals) as a substrate. The activity was determined essentially by the fluorescence assay of Youngman (1987), except that 0.1% Triton X-100 was added to the assay buffer to enhance bacterial permeability to the substrate. x-Glucosidase-specific activity, using p-nitrophenyl x-D-glucopyranoside (Sigma) as a substrate, was measured from mid-exponential-phase cultures and was assayed as described previously (Behari & Youngman, 1998b), except that the increase in absorbance was monitored at 405 nm on a Shimadzu UV-1201 spectrophotometer. Leucithinase activity of the plcB gene product was measured on egg yolk agar plates. Five microlitres of mid-exponential-phase cultures were spotted onto LB plates (± sugars) topped with 3 ml LB agar containing 50% of an equal volume egg yolk/1× PBS solution. Following incubation at 37°C,
plates were examined for precipitation of degraded egg yolk. The degree of lecithinase activity was estimated from the size of the zone (in mm) of degraded egg yolk precipitate surrounding the spots.

Mapping of csr mutation and construction of an isogenic csr mutant strain. The csr mutation was tagged with linked transposon insertions as described by Kaiser (1984). A U153 lysate was prepared from a population of wild-type bacteria carrying a library of random Tn917 insertions and this lysate was used to transduce AML1142 to Em'. Transductants were isolated and tested by restoration of wild-type regulation by sugars using buffered LB plates containing erythromycin, 50 μg XG ml⁻¹ and either 25 mM cellobiose or 25 mM glucose. Among 2000 AML1142 transductants tested, one was found that no longer deregulated in the presence of cellobiose. Repeated efforts to tag the csr mutation using Tn917 were unsuccessful. Following Tn917-tagging of the csr mutation, the exact site of Tn917 insertion was determined by DNA sequencing of the transposon–chromosome junction site which had been cloned as described previously (Camilli et al., 1990). DNA sequencing of double-stranded plasmid DNA was performed by the SBRI Genome Center using an oligonucleotide primer complementary to a sequence 83 bp from the lacZ-proximal end of Tn917. Based on sequence comparisons with the L. monocytogenes EGDe genome (Glaser et al., 2001), Tn917 was found to have inserted within the open reading frame (ORF) designated lmo1716. Based on the estimates obtained from transduction experiments regarding the linkage of csr to the Tn917-encoded erythromycin-resistance gene, chromosomal regions within 5–7 kb of the transposon insertion were PCR-amplified from wild-type and csr mutant strains and sequenced to identify the csr mutation. A substitution of a T for a C was found in lmo1716 at position 1687 of a 2679 bp ORF designated lmo1721. This mutation was not present in PCR products derived from the wild-type strain, and it was confirmed by DNA sequencing of two independent PCR products.

Reconstruction of the csr mutation in AML73. Primers 1721-A (5'-GGGGATCCCGAGATCAATGAAAG-3') and 1721-B (5'-GGGAAATTCCAGAAGTATCATGTTGTA-3') were used to amplify a 1.2-kb fragment containing the csr mutation as well as 600 bp of flanking DNA on each side of the mutation and to introduce BamHI and EcoRI restriction sites (underlined) for subsequent cloning. The resulting PCR product was digested with BamHI and EcoRI and ligated into appropriately digested pKSV7 (Smith & Youngman, 1992), then transformed into competent E. coli DH5α. The resulting plasmid, pNF1024, was introduced into AML73 by electroporation and the csr mutation was introduced into the chromosome by allelic exchange as described previously (Camilli et al., 1993). The presence of the csr mutation within the chromosome in the correct location was confirmed by PCR amplification of genomic DNA and digestion of the PCR product with Rsal, a restriction site created by the point mutation, and by sequencing of the PCR products.

RESULTS

Isolation of L. monocytogenes hly expression mutants

To facilitate the isolation of deregulated L. monocytogenes mutants, we constructed a strain in which the expression of the gus gene of E. coli was controlled by Phly, the promoter for hly, a strongly expressed gene of the PrfA regulon (Moors et al., 1999). This was accomplished using the temperature-sensitive vector pCON1-HGNH (Fig. 1a), in which an hly-gus fusion with an adjacent neo gene that is selectable in L. monocytogenes is flanked by a portion of hly and the hly–mpl intergenic region. These flanking regions provide homology for integrative recombination into the virulence cluster. The pCON1-HGNH vector was transferred from E. coli to L. monocytogenes strain 10403S by conjugation and integrants were selected for neomycin-resistance at a temperature non-permissive for autonomous replication of the vector. Subsequent vector excision and curing steps resulted in strain AML73, in which the hly–gus fusion is stably integrated at the hly locus (Fig. 1b). As anticipated, AML73 colonies were dark blue on plates containing the substrate XG, but completely white in the presence of 25 mM cellobiose or glucose (Fig. 1c). The ability of the hly–gus fusion to provide a sensitive visual assay for expression of PrfA-controlled genes was found to be far superior to that of comparable lacZ fusions which suffer from background due to an endogenous β-galactosidase activity present in L. monocytogenes.

To obtain mutants deregulated for hly expression, EMS-treated AML73 bacteria were plated on buffered LB agar containing either cellobiose or glucose and examined for a deregulated phenotype (blue colour). Distinctly blue colonies arose at a frequency of about 10⁻² under both conditions. Two candidates from cellobiose-containing plates (AML1142, AML1143) and one from glucose-containing plates (AML1141) (Table 1) were chosen for further study. Expression of hly–gus in all three mutants was deregulated on both cellobiose and glucose (Fig. 1c) as well as several other sugars utilized by L. monocytogenes, including fructose, trehalose and maltose (data not shown), suggesting a general defect in catabolite regulation of hly expression. The extent of hly deregulation in each mutant was also evaluated quantitatively by assay in liquid culture (Fig. 2). Expression of hly–gus in all mutants was at least twofold higher in LB alone and at least 15-fold higher in the presence of either sugar relative to the wild-type parent, indicating that hly–gus was constitutively overexpressed in these mutants.

Mutations in AML1142 and AML1143 are unlinked to prfA

To determine whether the mutant phenotypes of AML1141, AML1142 and AML1143 were caused by genetic changes in genes other than prfA, the hly–gus/neo cassette present in the mutant strain was transferred to a wild-type strain (10403S) by generalized transduction with phage U153 (Hodgson, 2000). Because the phage has a packaging capacity of approximately 40 kb and prfA is only 3 kb from hly in the virulence cluster, it was expected that any mutations in prfA would be very tightly linked to the neo-containing cassette. Indeed, 100% (178/178) of the Nz' transductants obtained with a donor lysate prepared from AML1141 were found to display the deregulated phenotype (Table 2). Subsequent DNA sequence analysis (data not shown) revealed that the prfA gene in AML1141 contained a mutation that resulted in a glycine to serine substitution at position 145, identical to a change previously reported to
cause a deregulated phenotype (Ripio et al., 1997b). In contrast, none of the Nm’ transductants obtained with lysates prepared from AML1142 or AML1143 displayed a deregulated phenotype (Table 2) indicating that the mutants contained one or more genetic changes unlinked to the virulence cluster.

Fig. 1. Strategy for construction of hly–gus transcriptional fusion strain by allelic replacement, and expression of this fusion in wild-type and deregulated mutants. (a) A portion of the 10403S virulence regulon is depicted undergoing a homologous recombination event with the vector pCON1-HGNH (see Methods). The recombination event resulted in the replacement of a functional hly gene with an hly–gus transcriptional fusion and integration of an independently expressed neomycin cassette, generating strain AML73. (b) Arrows indicate direction of transcription; bla, β-lactamase gene; cat, chloramphenicol acetyltransferase gene; oriT, origin of transfer sequences from plasmid RP4; pE194ts, replication functions from plasmid pE194ts; ColE1, replication functions from pUC18. Pv, PvuII; P, PstI; Sc, ScaI. (c) Five microlitres of mid-exponential-phase cultures of AML73, AML1141, AML1142 and AML1143 were spotted onto buffered LB plates containing XG (50 μg ml⁻¹) with or without cellobiose or glucose (25 mM each). Spots were incubated for 24 h at 37 °C.
Mutant AML1142 contains genetically separable mutations

To characterize genetically the mutations responsible for the deregulated phenotype of AML1142 and AML1143, we sought to ‘tag’ the mutations with linked transposon insertions as described by Kaiser (1984). This was accomplished by producing a U153 phage lysate on a population of wild-type bacteria containing a library of random Tn917 insertions and using this lysate to transduce AML1142 to Emr (the Tn-associated drug-resistance marker). Transductants were isolated and tested for restoration of wild-type regulation by sugars. Among approximately 2000 AML1142 transductants tested, we found one that was no longer deregulated in the presence of cellobiose (white on XG plates containing cellobiose). However, when this transductant was tested on XG plates containing glucose, it was unexpectedly found to retain a deregulated phenotype similar to that of the original AML1142 mutant. This result suggested that the AML1142 mutant might contain more than a single mutation that contributed to the deregulated phenotype. To investigate the possibility that the transductant might be a contaminant, we transduced AML1142 with a U153 phage lysate produced from the white transductant (e.g. AML133), selecting again for Emr. In this back-cross to the mutant parent, we found that 62 % (124/200) of the resulting Emr transductants were white on XG plates containing cellobiose. Like the original AML1142 transductant, however, these back-cross transductants were still blue on XG plates containing glucose or other readily metabolized sugars, indicating that they retained a generally deregulated phenotype for sugars other than cellobiose. This result confirmed that the original AML1142 mutant contained mutations in at least two separable genetic loci and that one of these mutations was not required for deregulation in the presence of glucose and other sugars (except cellobiose). The simplest hypothesis consistent with the results was that AML1142 contained mutations in two loci, which we refer to as csr (cellobiose-specific regulation) and gcr (general catabolite regulation), as indicated schematically in Fig. 3. According to this hypothesis, the specific Tn917 insertion (indicated as chr::Tn917a) recovered in the original AML1142 transductant is 62 % linked by transduction to csr, but not detectably linked to the virulence cluster: transductants of

<table>
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<th>Strain</th>
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<th>Source or reference</th>
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<td>Freitag et al. (1993)</td>
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<tr>
<td>NCTC 7973</td>
<td>Laboratory strain, serotype 1/2a</td>
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<tr>
<td>AML1141†</td>
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</tr>
<tr>
<td>AML1142†</td>
<td>AML73 gcr–csr–</td>
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</tr>
<tr>
<td>AML1143†</td>
<td>AML73 gcr–csr– (presumptive)</td>
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<td>AML132‡‡</td>
<td>AML73 gcr–csr– chr:::Tn917a</td>
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<tr>
<td>AML934§</td>
<td>AML73 gcr–csrA+ (Q562X)</td>
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†Mutant generated by EMS mutagenesis.
‡Strain contains a Tn917 insertion with an erythromycin-resistance marker linked to the csr gene.
§Introduction of the Q562X mutation into AML73 by allelic exchange.

Fig. 2. Deregulation of hly–gus expression in EMS-generated mutants. AML73, AML1142, AML1143 and AML1141 cultures were grown to early-stationary phase in buffered LB with or without either cellobiose or glucose (25 mM each). β-Glucuronidase (GUS) activity was measured as described in Methods. Data represent the mean of at least three independent experiments; error bars indicate the SEM. Open bars, no sugar; solid bars, plus cellobiose; patterned bars, plus glucose.
AML1142 that are white on cellobiose would have the genotype $gcr^{-}csr^{+}$ chr::Tn$_{917a}$ (e.g. AML133) and blue transductants would have the genotype $gcr^{-}csr^{-}$ chr::Tn$_{917a}$ (e.g. AML132).

To determine whether mutations in both $gcr$ and $csr$ contribute to the cellobiose-deregulation phenotype of AML1142, we prepared a U153 lysate on a blue transductant (e.g. AML132) and used it to transduce AML73 to Em$^r$. Progeny were scored for blue colour on XG plates containing cellobiose. Interestingly, 68% of the transductants (136/200) were slightly blue (e.g. AML134) but much less intensely blue than the presumptive double mutant AML132; the remaining 32% were white. This result indicates that a mutation in $csr$ is necessary but not sufficient for full relief of cellobiose-mediated repression. The absence of any progeny dark blue on cellobiose indicates further that $csr$ and $gcr$ are not detectably linked to each other. A summary of the qualitative phenotypes associated with the hypothesized genotypes discussed above is presented in Table 2.

Table 2. Results of transduction crosses

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<th>Donor</th>
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<td>0</td>
</tr>
<tr>
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<td>AML1142</td>
<td>2000§</td>
<td>0</td>
<td>0</td>
<td>1999§</td>
</tr>
<tr>
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<td>AML1142</td>
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<tr>
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<td>AML73</td>
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<tr>
<td>Tn917 library‡</td>
<td>AML1143</td>
<td>2500§</td>
<td>0</td>
<td>0</td>
<td>2500§</td>
</tr>
<tr>
<td>AML133‡</td>
<td>AML1143</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>66</td>
</tr>
</tbody>
</table>

*Number of progeny displaying dark-blue (DB), light-blue (LB) or white (W) colour on LB XG plates containing the indicated sugar.
†The hly–gus/neo cassette (Nm$^r$) was transferred from donor to recipient strain.
‡A Tn917 insertion (Em$^r$) was transferred from donor to recipient strain.
§Approximate numbers.

To evaluate the phenotypes associated with $csr$ and $gcr$ mutations quantitatively, single and double mutants were assayed for $\beta$-glucuronidase activity during growth in buffered LB broth in the presence or absence of glucose or cellobiose. The results confirmed that mutations in both $csr$ and $gcr$ are required for full derepression in the presence of cellobiose, but that a mutation in $gcr$ alone is sufficient for derepression in the presence of glucose (Fig. 4).

Mutant AML1143 was also transduced to Em$^r$ with a U153 lysate prepared on bacteria containing a library of Tn$_{917}$ insertions in the wild-type background; among approximately 2500 transductants examined, none were white on XG plates containing cellobiose. However, when AML1143 was transduced to Em$^r$ with a U153 lysate prepared on a...
strain (AML133) with the presumptive genotype (gcr− csr+ chr::Tn917a), 67% of the progeny were white on XG plates containing cellobiose (Table 2). Because AML1143 and AML1142 arose from the same population of EMS-treated bacteria, we conclude that they are probably siblings that contain identical mutations at the gcr and csr loci.

**Not all glucose-repressed genes are deregulated in AML1142**

Because mutations in AML1142 resulted in deregulation of hly expression in the presence of several repressing sugars, it was possible that these mutations might also affect general mechanisms of catabolite control in *L. monocytogenes*. Although little is known about catabolite control mechanisms in *L. monocytogenes*, a ccpA homologue has been identified and shown to mediate at least some aspects of catabolite control, including glucose repression of a-glucosidase activity (Behari & Youngman, 1998b). To determine whether the mutations in AML1142 affected the CcpA-mediated control pathway, we assayed a-glucosidase levels from wild-type and mutant bacteria grown in LB medium plus 25 mM maltose (for the induction of a-glucosidase) with or without cellobiose or glucose. We found that the level of a-glucosidase activity in AML1142 was slightly higher than wild-type levels in the absence of cellobiose or glucose, but did not significantly differ from that of wild-type in the presence of either sugar. This indicates that while the mutations in AML1142 significantly affected carbon-source regulation of hly–gus expression in *L. monocytogenes*, they had little effect on the regulation of a known member of the CcpA-controlled regulon, nor presumably on CcpA-mediated catabolite regulation in general. However, since mechanisms of catabolite repression are still poorly understood in *L. monocytogenes*, the possibility remains that virulence gene regulation occurs through some unknown general mechanism of catabolite control.

**The gcr and csr mutations deregulate expression of other PrfA-controlled genes**

To determine whether the mutant phenotypes associated with gcr and csr mutations were hly-specific, or if they affected PrfA-dependent gene expression in general, we measured the activity of lecithinase, a broad-specificity phospholipase C and the product of the PrfA-controlled plcB gene. The plcB gene is expressed independently of the hly gene, allowing determination of whether the mutations affect transcription from other PrfA-dependent promoters. Lecithinase activity was assayed directly by measuring the level of lecithin degradation exhibited by each mutant when spotted onto egg yolk plates (Table 3). Mid-exponential-phase cultures were spotted onto egg yolk plates with or
with either cellobiose or glucose. After 48 h incubation at 37 °C, plates were examined for precipitation of degraded egg yolk, and the degree of lecithinase activity was estimated from the size of the zone of precipitate surrounding the spots. The AML1142 double mutants (gcr− csr−) generated large zones of precipitate on all plates, indicating that plcB expression was deregulated in the presence of both cellobiose and glucose. In AML133 (gcr− csr− chr::Tn917a), expression was deregulated on glucose plates only; in AML134 (gcr+ csr− chr::Tn917a), normal regulation of plcB expression was observed on glucose plates, but a slightly deregulated phenotype was observed on cellobiose plates. These results indicate that the csr and gcr mutations had a general deregulatory effect on at least two genes within the PrfA virulence regulon.

**Table 3. Effect of gcr and csr mutations on the regulation of lecithinase (PlcB) activity by sugars**

Samples (5 µl) of AML73, AML1142, AML133 or AML134 cultures grown to mid-exponential phase were spotted onto egg yolk plates plus either cellobiose or glucose (25 mM each).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lecithinase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No sugar</td>
</tr>
<tr>
<td>AML73 (gcr+ csr+)</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>AML1142 (gcr− csr−)</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>AML133 (gcr− csr− chr::Tn917a)</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>AML134 (gcr+ csr− chr::Tn917a)</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

*Values correspond to the size of the zone of precipitated egg yolk surrounding each spot after 48 h incubation at 37 °C. Data are reported in millimetres and represent the mean ± SEM from three independent experiments.

**Mutations in gcr but not csr affect other environmental responses**

Several other environmental factors in addition to the presence of readily metabolized carbon sources have been shown to have an inhibitory effect on virulence gene expression in *L. monocytogenes*, including growth at low temperature (30 °C) and low pH (6.0). The inhibition of *L. monocytogenes* virulence gene expression at low temperature is thought to be mediated through a secondary structure motif present in the untranslated mRNA of prfA which masks the prfA ribosome binding region at low temperatures (Johansson *et al.*, 2002). Interestingly, in *L. monocytogenes* strains containing the constitutively activated PrfA* mutant (PrfA G145S), virulence gene expression is derepressed in response to low temperature or low pH. To determine whether mutation in gcr or csr acts more broadly to deregulate virulence gene expression, we assayed β-glucuronidase activity in mutant bacteria grown at low temperature or at low pH (Fig. 6). Results in Fig. 6(a) show that in AML73, hly–gus expression was approximately 100-fold lower when grown at 30 °C compared to 37 °C. However, when a mutation in gcr was present (AML133), growth at low temperature had only a twofold repressive effect on hly–gus regulation. When a mutation in csr was present (AML134), regulation of hly–gus at low temperatures was identical to that of AML73, indicating that the csr gene is strictly involved in cellobiose-specific regulation of virulence genes. A similar pattern of regulation was seen when cultures were grown at low pH (Fig. 6b), providing further evidence that deregulation of virulence genes due to the mutation in gcr is not carbon-source specific. Overall, the gcr mutation resulted in a phenotype similar to that produced by a G145S mutation in PrfA, providing evidence that additional factors besides PrfA can modulate *L. monocytogenes* gene expression in response to environmental cues.

**The csr mutation maps within a putative multi-domain transcriptional regulator**

The distance of the csr mutation from the site of the Tn917 transposon insertion tag was estimated based on the frequency of co-transduction of the mutant phenotype with the transposon antibiotic-resistance marker. The frequency of co-transduction of the Tn917-based Em' gene and the csr phenotype was 62%, indicating that the csr mutation mapped within approximately 6 kb of the Tn917 insertion. Regions of *L. monocytogenes* genomic DNA encompassing areas within 5–7 kb on either side of the Tn917 transposon insertion were PCR-amplified from wild-type and csr mutant strains using the published *L. monocytogenes* genome as a guide (Glaser *et al.*, 2001). The presence of a C to T transition mutation that introduced a stop codon within the coding sequence of *lmo1721* was detected only in the csr mutant strain. This mutation truncated the predicted *lmo1721*-encoded gene product (892 aa) at residue 562, removing the C-terminal third of the protein. A BLAST search using *lmo1721* indicates that the predicted *lmo1721*-encoded gene product has domains homologous to both GxxG- and PTS-dependent regulators, a structure shared by the LevR transcriptional regulator of *B. subtilis* (Debarbouille *et al.*, 1991) to which the *lmo1721*-encoded gene product shares homology (34% identity,

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LevR controls the expression of the levanase operon, which encodes a fructose-specific phosphoenolpyruvate-sugar PTS and a levanase, which hydrolyses fructose polymers and sucrose (Martin-Verstraete et al., 1998). It is possible therefore that *lmol721*, which we now designate as *csrA*, regulates a similar PTS involved in the transport and utilization of cellobiose.

To confirm that the mutation within *csrA* was sufficient to confer the deregulated *hly* expression phenotype in response to cellobiose, the C to T mutation that truncates CsrA at amino acid 562 was introduced into the AML73 parent strain and the resulting mutant AML934 was assayed for *hly*-dependent β-glucuronidase expression in the presence and absence of cellobiose. In the AML73 parent strain, the expression of *hly-gus* in the presence of cellobiose was reduced to only 6% (±1.4% SE) of the levels observed in the absence of cellobiose. AML134, containing the original *csr* mutation, retained expression levels that were 21% (±1.0% SE) of those observed in the absence of cellobiose, and the introduction of the *csrA* mutation into AML73 was sufficient to increase the levels of *hly* expression in the presence of cellobiose (20% ±2.4% SE) to those observed for the original AML134 mutant strain. These experiments confirm the role of this mutation in the partial alleviation of repression of *hly* expression in response to cellobiose.

**DISCUSSION**

Work by Park & Kroll (1993) using the natural isolate NCTC 7973 established that cellobiose is a potent repressor of PrfA-controlled gene expression in *L. monocytogenes*. Subsequent investigation (Milenbachs et al., 1997) established that several readily metabolized sugars repressed virulence gene expression in most natural isolates of *L. monocytogenes*. It has been suggested that down-regulation of virulence genes by cellobiose and other sugars might represent an aspect of more general pathways of catabolite repression. NCTC 7973 has been interpreted as a partially deregulated mutant in which some of the influence of these pathways was absent. In the present study, we were able to reconcile these conflicting hypotheses and show that complete abrogation of virulence gene regulation by cellobiose requires mutations in two unlinked genetic loci, referred to as *gcr* and *csr*. Strains carrying mutations in *gcr* alone retain cellobiose-mediated repression, but lose repression mediated by other sugars. This mutant phenotype closely resembles the behaviour of NCTC 7973 and supports the earlier suggestion that this strain is a partially deregulated variant (Milenbachs et al., 1997). The results also indicate that *gcr* may play a role in a general pathway of virulence gene regulation, possibly by influencing levels of a co-factor required by PrfA for efficient binding to target DNA sequences. Strains carrying a mutation in *csrA* alone exhibit partial relief of cellobiose repression. It is only when both mutations are present that cellobiose repression is eliminated entirely.

The introduction of a stop codon mutation at position 563 within the *csrA* coding sequences was sufficient to confer the partial relief of cellobiose repression observed in the AML134 mutant strain. The *csrA* gene product shares...
homology with a family of multi-domain transcriptional regulatory proteins, including LevR of *B. subtilis*, which controls the expression of a fructose-specific PTS and an extracellular levanase, which hydrolyses fructose polymers and sucrose (Martin *et al.*, 1987; Martin-Verstraete *et al.*, 1990). LevR is a multi-domain protein, with its N-terminal domain similar to the NifA/NtrC transcriptional activator family and a C-terminal domain similar to the regulatory domain similar to the NifA/NtrC transcriptional activator (1990). LevR is a multi-domain protein, with its N-terminal genome sequence (Glaser *et al.*), 1998). Based on the EGDe *L. monocytogenes* genome sequence (Glaser *et al.*, 2001), the *csrA* gene is followed by a transcriptional terminator. Downstream of *csrA* is a series of ORFs whose predicted gene products share homology with a PTS lichenan-specific enzyme IIB component (*lmo1720*) and a PTS lichenan-specific enzyme IIA component (*lmo1719*). Lichenan, like cellobiose, is a β-glucoside, and it is possible that this putative transport system responds to the presence of lichenan and/or cellobiose, as does the *lic* operon of *B. subtilis* (Tobisch *et al.*, 1997). The *csrA* mutation may therefore prevent the expression of the associated PTS gene products and thus eliminate the expression of a β-glucoside-specific sensor that mediates virulence gene repression in response to cellobiose. Such an effect would be similar to that proposed for the disruption of the *bvr* locus of *L. monocytogenes*, also reported to contribute to the repression of virulence gene expression by β-glucosides (Brehm *et al.*, 1999).

The results presented here imply that cellobiose can influence the expression of virulence genes by at least two separate, semi-independent pathways. One of these pathways (the one affected by gcr mutations) mediates the repressive effects of all readily metabolized sugars and probably represents a global pathway for catabolite repression. If so, however, our results and those of Behari & Youngman (1998b) indicate that the *L. monocytogenes CcpA* protein is probably not involved. The other pathway is cellobiose (or β-glucoside) specific and is influenced by both *csr*- and *bvr*-encoded gene products. Nevertheless, the *csr* pathway (and perhaps *bvr*) is not completely independent of the gcr pathway, since mutations in *csr* are necessary but not sufficient for full relief from cellobiose repression. Although we cannot provide a mechanistic description of these pathways, earlier work suggests that they both act through PrfA, as indicated schematically in Fig. 7. Moreover, since previous work has demonstrated that levels of PrfA protein do not change in the presence of repressing carbon sources (Milenbachs *et al.*, 1997; Renzoni *et al.*, 1997), it is likely that both pathways converge upon PrfA either through a covalent modification of the protein or through the synthesis of a co-inducer molecule that modifies the DNA-binding activity of PrfA.

Of final note is the ability of the gcr mutation to alleviate low-temperature-mediated repression of *L. monocytogenes* virulence gene expression. This alleviation either occurs independently of the regulation imposed by the presence of the *prfA* mRNA thermosensor (Johansson *et al.*, 2002) or may be due to the production of a factor that alters the stability of the thermosensor secondary structure to facilitate translation of the *prfA* message. A third alternative mechanism might act through the induction of *prfA* expression via the *prfAP2* promoter, the transcripts of which would lack the thermosensor structure (Freitag & Portnoy, 1994). The results described here highlight the variety of mechanisms used by *L. monocytogenes* to sense its environment and to regulate the expression of gene products necessary for bacterial survival both inside and outside of host cells.

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**Fig. 7.** Model for pathways mediating virulence gene regulation in *L. monocytogenes*. Although specific receptors (ovals) and other bacterial membrane features are depicted, the exact mechanisms by which the environmental factors are detected or enter the cell are unknown. Regulation by each factor is shown to act through either the *gcr*-mediated pathway or the *csr*-bvr-mediated pathway. The *csr*-bvr-mediated pathway is presumably a β-glucoside uptake system that has a dual role in virulence gene regulation. The *gcr*-mediated pathway may represent a more general pathway controlling virulence gene regulation and the *gcr* gene product may influence the levels of a co-factor required by PrfA for its function as a transcriptional activator. Alternatively, the *gcr* gene product may act as a repressor protein inhibiting PrfA function. Both pathways probably converge and act on the PrfA protein specifically to regulate its conversion from an active to an inactive form and inhibit its ability to activate PrfA-dependent gene expression.
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REFERENCES


