

Regulation of *hly* Expression in *Listeria monocytogenes* by Carbon Sources and pH Occurs through Separate Mechanisms Mediated by PrfA

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Expression of the PrfA-controlled virulence gene *hly* (encoding the pore-forming cytolysin listeriolysin) is under negative regulation by readily metabolized carbon sources in *Listeria monocytogenes*. However, the hyperhemolytic strain NCTC 7973 exhibits deregulated *hly* expression in the presence of repressing sugars, raising the possibility that a defect in carbon source regulation is responsible for its anomalous behavior. We show here that the activity of a second glucose-repressed enzyme, α -glucosidase, is 10-fold higher in NCTC 7973 than in 10403S. Using *hly-gus* fusions, we show that the *prfA* allele from NCTC 7973 causes deregulated *hly-gus* expression in the presence of sugars in either the wild-type or the NCTC 7973 background, while the 10403S *prfA* allele restores carbon source regulation. However, the *prfA* genotype does not affect the regulation of α -glucosidase activity by repressing sugars. Of the two mutational differences in PrfA, only a Gly145Ser change is important for regulation of *hly-gus*. Therefore, NCTC 7973 and 10403S have genetic differences in at least two loci: one in *prfA* that affects carbon source regulation of virulence genes and another in an unidentified gene(s) that up-regulates α -glucosidase activity. We also show that the decrease in pH associated with utilization of sugars negatively regulates *hly-gus* expression, although sugars can affect *hly-gus* expression by another mechanism that is independent of pH.

Listeria monocytogenes is a gram-positive, facultative intracellular pathogen and is an important source of food-borne infection in humans (12). Several of the genes required for the pathogenesis of the bacterium are located on a 10-kb region of the chromosome. These genes include *hly* (encoding listeriolysin O, a thiol-activated cytolysin), *plcA* and *plcB* (encoding phosphatidylinositol-specific and phosphatidylcholine-specific lecithinases, respectively), *actA* (encoding a protein required for actin-based motility inside host cells), and *mpl* (encoding a metalloprotease involved in the maturation of the *plcB* gene product) (23, 34, 43). Expression of these genes is controlled by the virulence regulator PrfA. PrfA is a site-specific DNA-binding protein that recognizes the “PrfA box,” a 14-bp region of dyad symmetry in its target promoters (8, 14, 15, 28, 29). The *prfA* gene is also part of the virulence gene cluster and lies downstream of the *plcA* gene. It is expressed both as a monocistronic message from two promoters in the *plcA-prfA* intergenic region and as a bicistronic *plcA-prfA* message from the *plcA* promoter. This results in a positive feedback loop, since the expression of *prfA* is also up-regulated when it activates transcription from the upstream *plcA* promoter (7). PrfA has sequence similarity with cyclic AMP receptor protein (CRP), the global regulator of catabolite control in *Escherichia coli*, and significant structural and functional homology of PrfA with members of the CRP/Fnr family has been demonstrated by site-directed mutagenesis studies with the putative DNA-binding domain of PrfA (25, 41).

Several environmental signals regulate the expression of virulence genes in *L. monocytogenes*, including temperature (27), growth phase (29), composition of the medium (39), and the disaccharide cellobiose (32). It was reported recently that the

effect of cellobiose on *hly* expression is not unique and that several utilizable sugars can down-regulate *hly* expression (30). Furthermore, while the presence of sugars resulted in over 50-fold down-regulation of *hly*, there was no detectable change in the level of PrfA protein itself. Therefore, it was hypothesized that regulation of *hly* expression by sugars is an aspect of global catabolite regulation rather than signature molecule-mediated signal transduction unique to cellobiose (30). Surprisingly, while the effect of utilizable sugars on *hly* expression was seen in three wild-type natural isolates, strain NCTC 7973 did not exhibit the same phenotype. In this strain, only cellobiose was found to repress virulence gene expression, leading to the suggestion that *L. monocytogenes* has at least two sugar-sensing pathways and that NCTC 7973 is a partially deregulated variant with a defect in some aspect of carbon source regulation. Ripio et al. showed recently that utilization of the carbohydrate glucose-1-phosphate in *L. monocytogenes* is coordinately regulated with other virulence factors and is dependent on PrfA (37). These results suggest the existence of important links between regulation of utilizable carbon sources and expression of virulence genes in *L. monocytogenes* and imply that coordinate regulation of these pathways may be a critical aspect of the pathogenicity and virulence of the bacterium.

While there has been much interest recently in sugar uptake mechanisms in *L. monocytogenes* (10, 33), catabolite regulation in this organism has never been studied. It is not known which genes are under catabolite regulation and what the mechanism of their regulation in *Listeria* may be. In this report, we establish that the metabolic enzyme α -glucosidase is under catabolite control in *L. monocytogenes*. We show that in NCTC 7973, a strain that exhibits high-level expression of *hly*, the activity of α -glucosidase also is over 10-fold higher than in the wild-type strain 10403S. However, the elevated activity of α -glucosidase and the sugar-insensitive expression of *hly-gus* in NCTC 7973

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TABLE 1. *L. monocytogenes* strains used in this work

| Strain | Genotype or description | Source or reference |
|-----------|---|---------------------|
| 10403S | Laboratory strain, serotype 1 | 35 |
| NCTC 7973 | Laboratory strain, serotype 1/2a | 30 |
| LO28 | Laboratory strain, serotype 1/2c | 45 |
| EGD | Laboratory strain, serotype 1/2a | 26 |
| AML73 | 10403S <i>hly-gus-neo</i> | 31 |
| JB77 | NCTC 7973 <i>hly-gus-neo</i> | This work |
| JB136 | AML73 <i>prfA::prfA</i> _{10403S} | This work |
| JB137 | JB77 <i>prfA::prfA</i> _{10403S} | This work |
| JB138 | AML73 <i>prfA::prfA</i> ₇₉₇₃ | This work |
| JB139 | JB77 <i>prfA::prfA</i> ₇₉₇₃ | This work |

are due to distinct genetic defects. Furthermore, we show that the decrease in pH associated with utilization of sugars can itself down-regulate *hly-gus* expression. However, sugars affect *hly-gus* expression even if the pH is stringently controlled during growth, suggesting that the regulation of virulence genes by carbon sources involves both pH-dependent and pH-independent components.

MATERIALS AND METHODS

Bacterial strains. The *L. monocytogenes* strains used in this work are listed in Table 1. *E. coli* DH5 α *mcr*[ϕ 80*dlacZ* Δ M15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^-$) *supE44 relA1 deoR* Δ (*lacZYA-argF*)U169 *mcr*] (Gibco BRL) was used for the construction of plasmids. This strain was grown in Luria-Bertani (LB) medium, and ampicillin was added at a concentration of 100 μ g ml⁻¹ for selection.

Cultivation of bacteria. *L. monocytogenes* strains were grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) or LB medium. Overnight cultures were grown in LB medium buffered with 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0) at 37°C with aeration for 12 to 15 h. Unless otherwise specified, overnight cultures were diluted 1:25 into fresh LB medium buffered with 100 mM MOPS (pH 7.4 for β -glucuronidase assay and pH 7.0 for α -glucosidase assay). Where indicated, filter-sterilized sugar supplements were added to cultures at a final concentration of 25 mM. Growth of cultures was monitored at a wavelength of 600 nm on a Shimadzu UV 1201 spectrophotometer. pH measurements were made on a digital ionalyzer (model 601A; Orion Research). For experiments measuring the effect of pH, LB medium was buffered with 100 mM MOPS (pH 7.4 to 6.5) or 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.0).

DNA sequencing. To sequence the entire coding region of the *prfA* gene, a 1,039-bp DNA fragment was PCR amplified from each of the four *L. monocytogenes* strains by using primers PrfA1R (5' AACCTCGGTACCATATACTAACTCT 3') and PrfA4L (5' GTACGCGTTCTAGAAAATGCTTCT 3'). DNA sequencing was carried out by the method of Sanger et al. (40) with the final thermal cycling sequencing system (Promega). Custom-synthesized oligonucleotides were purchased from the University of Georgia Molecular Genetics Facility or from DNAgency, Aston, Pa. Sequencing reactions were carried out on both strands of the PCR product amplified from strains 10403S and NCTC 7973 and on one strand of the product from strains EGD and LO28 (except at the 3' end of the *prfA* coding sequence, where both strands were sequenced).

In vitro manipulations of DNA. Restriction analyses and cloning were done by standard techniques as described before (1). Enzymatic reagents were purchased from New England Biolabs or Boehringer Mannheim and used as specified by the manufacturer. Amplification of DNA by PCR was carried out as previously described (19).

Construction of plasmids and strains for the *prfA* allele exchange. The *E. coli*-*L. monocytogenes* shuttle vector pCON1 was used for cloning and strain construction. pCON1 was constructed by ligating *EcoRI*-*ScaI*-digested pUC18 with pKSV5-T (2) that had been digested with *EcoRI* and *ScaI*. pCON1 has a temperature-sensitive origin of replication, pE194ts, and a chloramphenicol resistance (Cm^r) gene for selection in gram-positive bacteria. A 1,039-bp *prfA* fragment was amplified from either 10403S or NCTC 7973 chromosomal DNA with the primers PrfA1R and PrfA4L. There is an *EcoRI* site in the *prfA* structural gene (GenBank accession no. X61210), and the 3' primer PrfA4L has an *XbaI* cloning site engineered into it. Therefore, digestion of the amplified product with *EcoRI* and *XbaI* resulted in a 790-bp *prfA* fragment lacking the promoter region and the first 19 nucleotides of the *prfA* coding region. This *prfA* fragment was ligated into *EcoRI*-*XbaI*-digested pCON1 and transformed into *E. coli* with ampicillin selection, resulting in plasmids pCON1- Δ *prfA*-10403S and pCON1- Δ *prfA*-7973. The plasmids were transformed into the conjugation donor strain *E. coli* S17-1 (44) and conjugated into either AML73 (10403S *hly-gus-neo*) or JB77 (NCTC 7973 *hly-gus-neo*) as described previously (2), except that cells

were washed once in BHI medium and mating spots were incubated overnight at 30°C. To force chromosomal integration of the vectors, transconjugants were propagated in BHI medium with chloramphenicol (5 μ g ml⁻¹) selection for 3 h at 30°C and then shifted to 41°C for another 3 h. Appropriate dilutions were plated out on BHI agar containing chloramphenicol at 5 μ g ml⁻¹ and incubated for 2 to 3 days at 41°C. Integration of the vector into the chromosome and the two nucleotide changes in the *prfA* sequence were confirmed by PCR amplification of the gene and sequencing of the PCR products.

Construction of *hly-gus* fusions. *hly-gus* fusion strains in either the NCTC 7973 or 10403S background were constructed with the vector pCON1-HGNH (31). pCON1-HGNH has a *gusA* gene (encoding β -glucuronidase) from *E. coli* (22) and a neomycin resistance (Nm^r) cassette (20), flanked by a 489-bp *hly* fragment (5' end) and a 397-bp *hly-mpl* fragment (3' end) on a pCON1 vector backbone. Shifting of *L. monocytogenes* strains carrying the plasmid to the nonpermissive temperature (41°C) with chloramphenicol selection resulted in the chromosomal integration of pCON1-HGNH by homologous recombination between the 5' *hly* fragment and the wild-type *hly* allele on the chromosome. These clones were Nm^r and Cm^r and were nonhemolytic on blood agar plates. Overnight cultures of these merodiploid strains were diluted 1:800 in BHI with only neomycin selection at the permissive temperature (30°C) to select for cells with spontaneous excision of the plasmid. To bring about vector curing after excision of the integrated construct, 1 ml of the saturated culture was diluted into 100 ml of BHI with 5 μ g of neomycin ml⁻¹ and incubated with aeration at the nonpermissive temperature to stationary phase. Appropriate dilutions were plated onto BHI plates with 5 μ g of neomycin ml⁻¹ at 41°C. Individual colonies were then patched onto neomycin, chloramphenicol, or blood agar plates. Excision of the plasmid via homologous recombination on the 3' end of the chromosomal *hly-mpl* genes resulted in clones that had chromosomal *hly-gus* fusions and were Nm^r, Cm^r, and Hly⁻. These clones were tested for β -glucuronidase activity on plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-GlcA) and confirmed by Southern blot hybridization.

Preparation of cell lysates. Samples (10 ml) were collected by centrifugation and washed once with an equal volume of the assay buffer (50 mM potassium phosphate buffer for α -glucosidase and 50 mM sodium phosphate buffer for β -glucuronidase). The cells were resuspended in 2 ml of the same buffer and lysed by sonication on ice three times for 30 s each. The suspension was clarified by centrifugation, and up to 0.05 ml of the supernatant was used for assaying enzyme activity. Protein concentrations in cell lysates were determined by the method of Bradford (6), using a Bio-Rad protein assay, with bovine serum albumin as the standard.

Enzyme assays. α -Glucosidase activity was assayed by using *p*-nitrophenylglucoside as a substrate essentially as described previously (9), except that the increase in absorbance was monitored at 405 nm on a Shimadzu UV-1201 spectrophotometer. β -Glucuronidase activity was assayed as described previously (21), except that the increase in *p*-nitrophenol absorbance was monitored at 405 nm.

RESULTS AND DISCUSSION

Utilization of sugars and catabolite regulation in NCTC 7973. NCTC 7973 is a partially deregulated mutant in which utilizable sugars other than cellobiose do not affect virulence gene expression (30). To test the possibility that NCTC 7973 is generally defective in catabolite regulation, we examined the activity of a metabolic enzyme, α -glucosidase, which is subject to catabolite regulation in other gram-positive bacteria (13, 46). Specific activity of the enzyme in exponentially growing cells was measured. In 10403S, α -glucosidase activity was inducible by maltose (data not shown), and addition of glucose, fructose, or cellobiose to the cultures resulted in approximately fourfold repression of α -glucosidase (Fig. 1). Surprisingly, the specific activity of α -glucosidase was over 10-fold higher in NCTC 7973 than in 10403S, suggesting that a major difference between these two strains might be related to central pathways of catabolite repression. Addition of either glucose, fructose, or cellobiose resulted in a two- to fourfold repression of α -glucosidase activity in NCTC 7973, although the levels still remained severalfold higher than in the wild-type (10403S) control.

We also asked whether there was any difference in the utilization of glucose and cellobiose that might account for the differential effects of these sugars on *hly* expression in NCTC 7973 reported previously (30, 32). As indicated by effects on doubling time and final cell densities reached in the cultures, 10403S and NCTC 7973 could utilize glucose and cellobiose

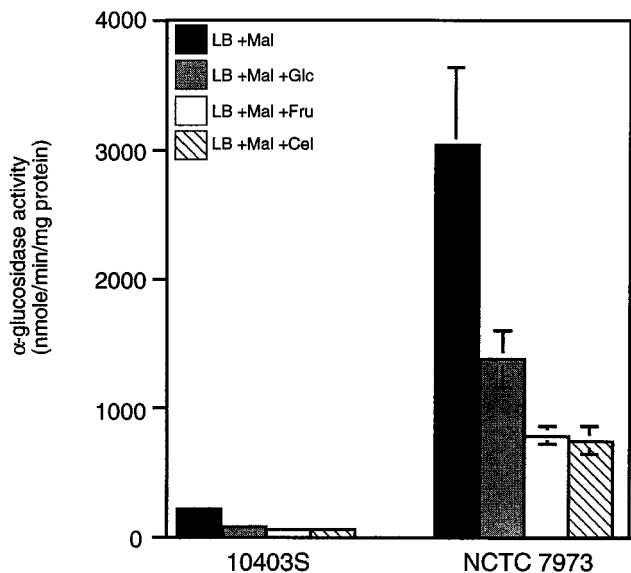


FIG. 1. Catabolite regulation of α -glucosidase in strains 10403S and NCTC 7973. Cells were grown at 37°C in LB medium buffered with 100 mM MOPS (pH 7.0) and supplemented with the indicated sugars at 25 mM. The specific activity of α -glucosidase in exponentially growing cells was measured as described in Materials and Methods and is expressed as nanomoles of product formed minute^{-1} milligram of protein $^{-1}$. Mal, maltose; Glc, glucose; Fru, fructose; Cel, cellobiose. Each sample was analyzed in triplicate, and the data represent the means and standard errors of the means for three independent experiments.

equally well (Fig. 2). However, NCTC 7973 had a longer lag phase and a growth rate approximately 34% lower than those of 10403S in the presence of both sugars. Thus, NCTC 7973 differs from 10403S both in general growth characteristics and in the specific activity of at least one glucose-repressed enzyme but exhibits no obvious defect in the ability to metabolize cellobiose.

Sequence differences between PrfA proteins from NCTC 7973 and other natural isolates. Other groups have noted the presence of mutational differences in the *prfA* alleles of various natural isolates, but the literature is contradictory and the functional significance of these mutations is unclear. With strain EGD as the wild-type reference, the deduced sequence of PrfA from NCTC 7973 was reported by two groups to contain two mutational substitutions, Gly145Ser and Cys229Tyr (4, 38). However, at least one other report suggested that there was only a single amino acid difference in PrfA_{NCTC 7973}, a Cys-to-Tyr change at position 229 (5). Furthermore, the sequence of PrfA from the wild-type strain EGD in the GenBank database (accession no. M55160) is different from that of PrfA_{LO28} (accession no. X61210) at three amino acid residues at the carboxy terminus of the protein. The *prfA* gene from 10403S was not previously sequenced. To resolve the conflicting reports regarding the sequence of PrfA, we amplified and sequenced the *prfA* genes from the four strains used in a previous study (30). Figure 3 shows a partial alignment of the deduced amino acid sequences of PrfA from the four *L. monocytogenes* strains. We were able to confirm the nucleotide changes in codons 145 (GGT to AGT) and 229 (TGT to TAT) in *prfA*_{NCTC 7973} that lead to Gly145Ser and Cys229Tyr substitutions in the PrfA protein as reported recently by Ripio et al. (38). However, we found the *prfA* sequences from LO28, EGD, and 10403S to be identical at the amino acid level, although there was a silent nucleotide change (T to C) at the third position of codon 127 in *prfA*_{10403S}.

Effect of exchanging *prfA* alleles between 10403S and NCTC 7973 on *hly-gus* expression. Ripio et al. reported recently that a Gly-to-Ser substitution at position 145 of PrfA leads to constitutive overexpression of virulence factors in the strain P14-A (belonging to serovar 4b) (38). Interestingly, this mutation in PrfA corresponds to a mutation in CRP that makes its activity independent of cyclic AMP in *E. coli*. Introduction of the *prfA* allele from P14-A on a multicopy vector into the *prfA*-deficient LO28 background resulted in overexpression of virulence genes. However, when the monocistronic wild-type *prfA* allele was introduced into the P14-A background, there was a decrease in expression levels of *hly* and *plcB*, which was attributed to competition between the mutant and wild-type forms of PrfA for their target promoters. Paradoxically, when the wild type bicistronic *plcA-prfA* region was introduced into P14-A, there was no significant reduction in the high levels of expression of virulence genes. Results based on propagation of regulatory factors on multicopy vectors can be misleading for many reasons. Therefore, it remained unclear whether the high levels of virulence gene expression in P14-A could be explained by the PrfA mutation alone or whether there were additional defects in the genetic background responsible for the phenotype. NCTC 7973 has a mutation in PrfA similar to that in P14-A, as well as aberrantly high activity of a glucose-repressed metabolic enzyme. Thus, we wanted to find out whether the sugar-insensitive expression of *hly* in NCTC 7973 was due to the *prfA* allele alone or to additional defects in the genetic background that affected several catabolite-controlled genes, including those of the virulence cluster. To differentiate between these possibilities, we exchanged the *prfA* alleles between the strains 10403S and NCTC 7973 in such a way that a single copy of either the wild-type or mutant allele of *prfA* would be under control of the natural promoter in either genetic background. To achieve this exchange of alleles, we cloned a promoterless, truncated copy of *prfA* from each of the two strains into the temperature-sensitive integrational vector pCON1 and introduced the vector by conjugative transfer into either 10403S or NCTC 7973 containing *hly-gus* transcriptional fusions. Integration of the vector into the chromosome placed either the wild-type or the mutant copy of *prfA* under control of the natural promoter (Fig. 4). The integration was confirmed by amplifying the *prfA* gene by using the primers PrfA1R and PrfA4L, and the mutations were confirmed by sequence analysis of the PCR product.

Introduction of the mutant copy of *prfA* into the 10403S background resulted in 10-fold higher expression of *hly-gus* (Fig. 5). The expression was also completely deregulated in the presence of glucose, while 25 mM cellobiose exerted a modest (twofold) effect. On the other hand, introduction of the wild-type copy of the *prfA* gene into the NCTC 7973 background decreased the level of *hly-gus* in LB medium alone and restored at least partial regulation by glucose and cellobiose. Significantly, we did not detect more than a twofold down-regulation of *hly-gus* by cellobiose in either NCTC 7973 or 10403S expressing *prfA*_{NCTC 7973}. Two previous papers had reported that cellobiose was unique in its ability to down-regulate virulence gene expression in NCTC 7973, raising the possibility that cellobiose has a signal transduction pathway distinct from that of other sugars (30, 32). However, Ripio et al. reported that cellobiose has almost no effect on virulence gene expression in P14-A (38), a result that was quite puzzling since P14-A, like NCTC 7973, has a Gly145Ser substitution in PrfA. While it is not clear why our results with cellobiose are different from those reported previously, we speculate that it could be due to subtle differences in growth conditions or to the elimination of the confounding effects of acidity on *hly-gus*

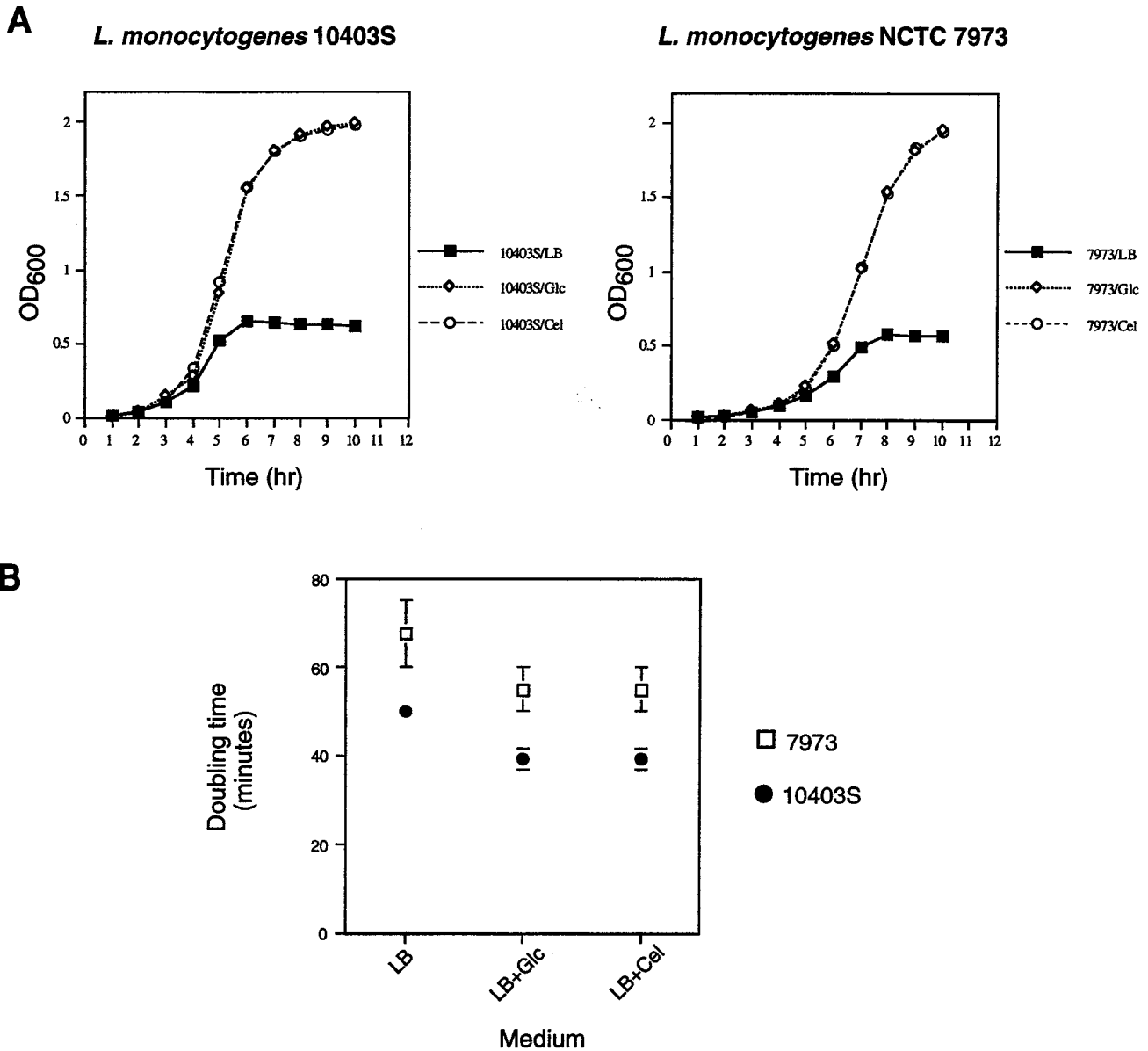


FIG. 2. (A) Growth of 10403S and NCTC 7973 in LB medium supplemented with various sugars. Overnight cultures were grown in LB medium buffered with 100 mM MOPS (pH 7.0) and were diluted 1:25 into fresh medium with or without either glucose or cellobiose at 25 mM. The results of one representative experiment are shown. Similar results were obtained in three independent experiments. OD₆₀₀, optical density at 600 nm. (B) Doubling times of 10403S and NCTC 7973 in buffered LB medium with different carbon sources. Data represent means ± standard errors of the means for three independent experiments.

expression by the stringent control of pH in our experiments. While it is certainly possible that independent sensing pathways exist for cellobiose or other sugars upstream of PrfA, it seems likely that modification of PrfA activity is the final common pathway for regulation by all sugars.

PrfA has been shown to be a structural and functional homolog of CRP, and so the possibility existed that the PrfA mutations, besides causing *hly-gus* overexpression, were also responsible for the up-regulation of α-glucosidase. An analogous situation exists in the opportunistic pathogen *Pseudomo-*



FIG. 3. Alignment of the deduced carboxy-terminal amino acid sequences of PrfA proteins from four *L. monocytogenes* strains. The substitutions in the PrfA sequence from NCTC 7973 are indicated by dots above the alignment. The numbers to the left of the sequences correspond to the position of the first residue in the full-length protein. The helix-turn-helix motif is boxed (41). The three residues at the carboxy terminus of PrfA_{EGD} that were found to be different from the published sequence (28) are indicated by asterisks. Identical residues are shown in white letters on a black background, while divergent residues are in black letters on a white background.

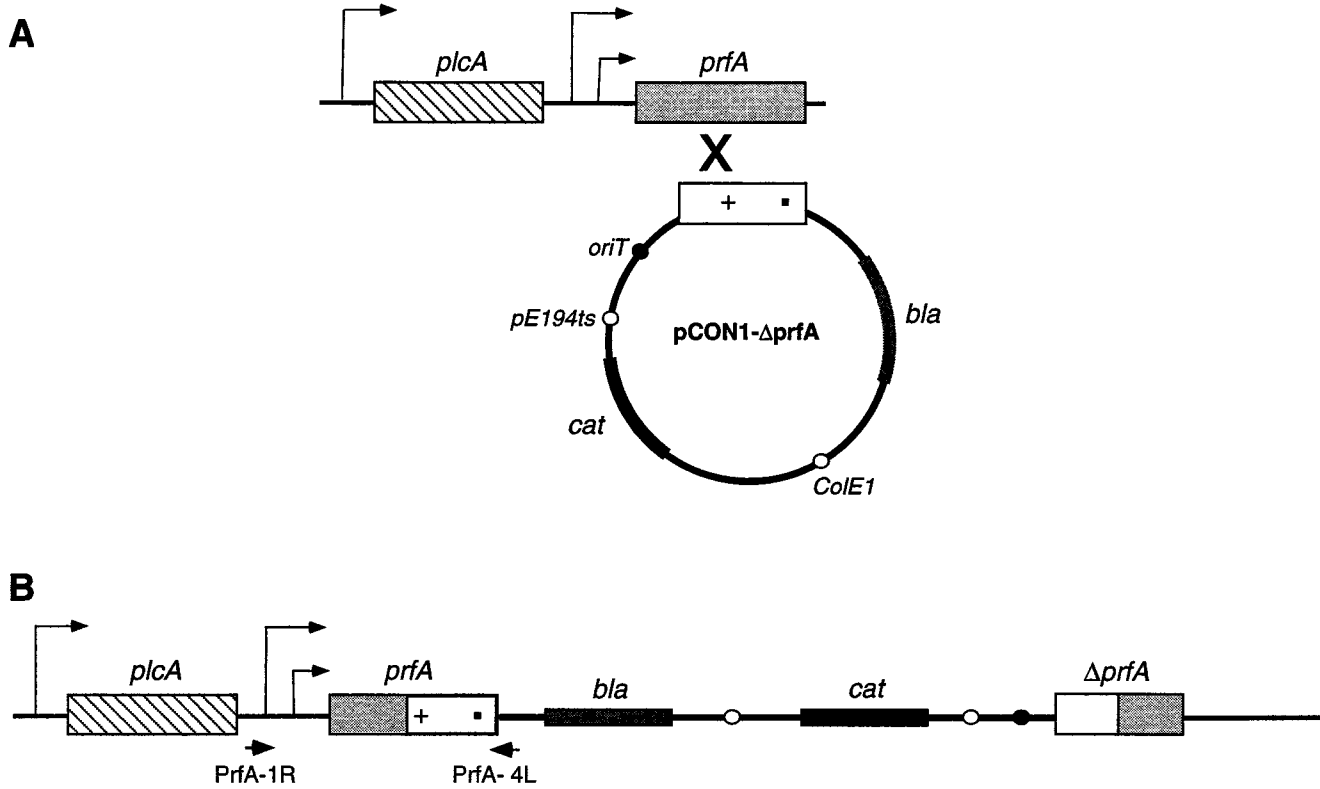


FIG. 4. Strategy for construction of strains for *prfA* allele exchange by integrative replacement. (A) A promoterless, truncated copy of *prfA* from either 10403S or NCTC 7973 was cloned into the integrational vector pCON1 and conjugated into the host strains. (B) Shifting the strains to the nonpermissive temperature resulted in the integration of the temperature-sensitive vector into the chromosome at the *prfA* locus. The two nucleotide changes in the *prfA* sequence in codons 145 and 229 are represented as a cross and a dot, respectively. The integrated copy of *prfA* was amplified with the primers PrfA1R and PrfA4L, and the PCR product was sequenced to confirm the sequence changes. *bla*, β -lactamase gene; *cat*, chloramphenicol acetyltransferase gene; *oriT*, mobilization signal from plasmid RP4; *pE194ts*, replication functions derived from plasmid pE194ts; *ColE1*, replication functions derived from pUC18.

nas aeruginosa, in which the expression of genes encoding exotoxin A and protease is regulated by the *vfr* gene product. Interestingly, the *vfr* gene product, which is also a homolog of CRP, not only controls virulence gene expression in *P. aerugi-*

nosa but also can complement the β -galactosidase- and tryptophanase-deficient phenotypes of an *E. coli crp* deletion mutant (47). To determine whether the regulation of α -glucosidase in NCTC 7973 was related to its *prfA* genotype, we asked whether there was any effect of exchanging the *prfA* alleles on α -glucosidase activity in either the wild-type or the NCTC 7973 background. The levels and regulation of α -glucosidase activity were identical in 10403S containing either the wild-type or mutant *prfA* allele (Fig. 6). Similarly, the levels of the enzyme did not decrease upon introduction of the wild-type copy of *prfA* into NCTC 7973. We conclude from these data that NCTC 7973 has mutations in at least two loci: a defect in *prfA* that causes overexpression of *hly* and a second mutation that up-regulates α -glucosidase activity. While the nature of this second defect in NCTC 7973 is uncertain, we speculate that it could be similar to the *ccrA1* mutation of *Streptomyces coelicolor*, which up-regulates the expression of several catabolite-controlled genes without affecting repression by carbon sources (18).

During the construction of strains for the *prfA* allele exchange by integrative recombination, we obtained several clones that had the vector integrated in the chromosome of 10403S but which showed normal levels and patterns of regulation of *hly-gus* (data not shown). We reasoned that this phenotype could be due to a single recombinational event taking place between codons 145 and 229 in *prfA*, thus leaving the wild-type Gly residue at position 145 but resulting in a Cys-to-Tyr substitution at position 229 of PrfA. The result was confirmed by PCR amplification and sequence analysis of the *prfA*

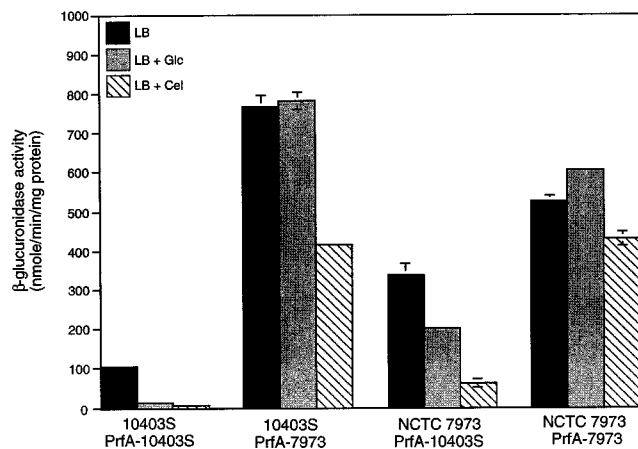


FIG. 5. Effect of exchanging *prfA* alleles between strains 10403S and NCTC 7973 on the regulation of *hly-gus* expression by utilizable sugars. Cells were grown in LB medium buffered with 100 mM MOPS (pH 7.4) with or without either glucose or cellobiose at 25 mM. β -Glucuronidase activity was measured as described in Materials and Methods. Data represent means \pm standard errors of the means for two independent experiments, each done in triplicate. Glc, glucose; Cel, cellobiose.

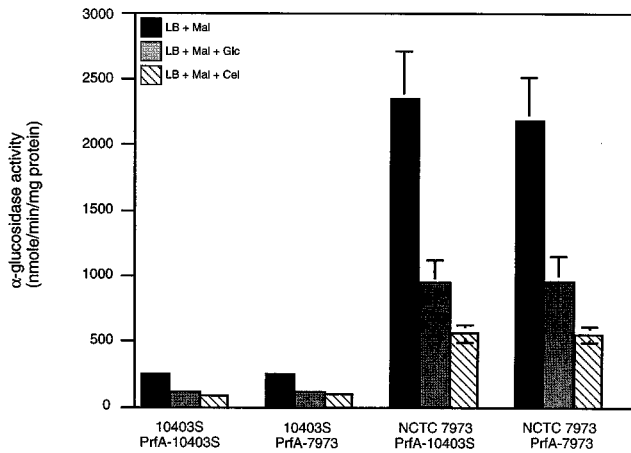


FIG. 6. Effect of exchanging *prfA* alleles between 10403S and NCTC 7973 on the catabolite regulation of α -glucosidase. Cells were grown in LB medium buffered with 100 mM MOPS (pH 7.0) with or without either glucose or cellobiose at 25 mM. Specific activity of α -glucosidase from exponentially growing cells was measured as described in Materials and Methods. Data represent means \pm standard errors of the means for three separate assays from each of two independent experiments. Mal, maltose; Glc, glucose; Cel, cellobiose.

allele from this strain. This finding is consistent with the results of Ripio et al. with the strain P14-A, which contains only the Gly-to-Ser substitution at position 145 of PrfA (38).

Effect of extracellular pH on *hly-gus* expression. During the course of our experiments, we noticed that the final pH of the culture medium buffered with 100 mM MOPS was approximately 6.5 after growth in the presence of utilizable sugars. To test whether low extracellular pH could itself affect expression of *hly-gus* in the absence of sugars, we measured expression in LB medium buffered at various pH values ranging from 7.4 to 6.0. To our surprise, *hly-gus* expression was repressed approximately fourfold at pH 6.5 and eightfold at pH 6.0 relative to

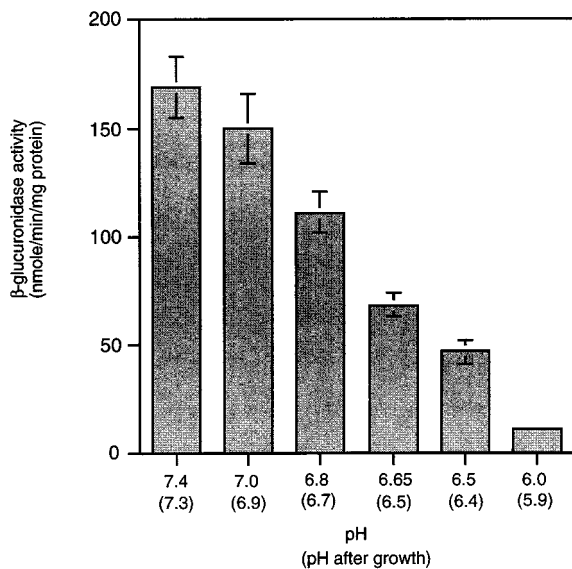


FIG. 7. Effect of pH on the expression of *hly-gus*. Cells were grown in LB medium buffered with either 100 mM MOPS (pH 7.4 to 6.5) or MES (pH 6.0). Samples were collected at 1 h into stationary phase, and β -glucuronidase specific activity in cell lysates was measured. Data represent means \pm standard errors of the means for three independent experiments, each done in triplicate.

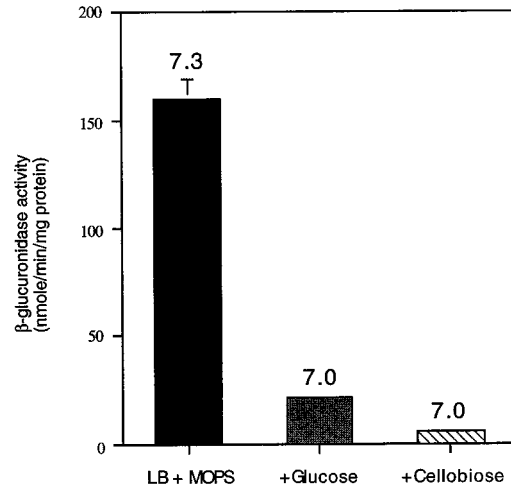


FIG. 8. Sugars regulate *hly-gus* expression by a mechanism that is independent of pH. Cells were grown in LB medium (with 100 mM MOPS, pH 7.4) with or without either glucose or cellobiose at 25 mM. Samples were collected at 1 h into stationary phase, and β -glucuronidase specific activity was measured. The starting pH of each culture was 7.4. The number above each bar represents the pH of the culture at the time samples were collected.

expression at pH 7.4 (Fig. 7). Over this range of pH, there was a significant decrease neither in the growth rates of cultures nor in the basal level of activity of α -glucosidase (data not shown). This result suggests that the regulation of virulence genes by extracellular pH is a specific regulatory effect and not the result of a general down-regulation of gene expression.

The effect of acidic extracellular pH on *hly-gus* expression raised the possibility that the effect of sugars on virulence genes observed by us and by others was due to the decrease in pH and not due to carbon source regulation. To test this possibility, we assayed *hly-gus* expression in medium buffered at pH 7.4 with 100 mM MOPS, so that the final pH of the cultures did not drop below 7.0 (Fig. 8). Under these conditions, the presence of either glucose or cellobiose at 25 mM still resulted in down-regulation of *hly-gus*, confirming that sugars could regulate virulence gene expression by a pH-independent mechanism.

We then asked whether the influence of pH on *hly-gus* was also mediated through PrfA by comparing the expression of *hly-gus* at pH 6.5 and 7.4 in either 10403S or 10403S expressing *prfA*_{NCTC 7973} (Fig. 9). The presence of the *prfA*_{NCTC 7973} allele in a 10403S background resulted in partial deregulation of *hly-gus* at pH 6.5 (1.5-fold repression, compared to nearly 4-fold repression in the wild-type strain). This observation suggests that the effect of pH, like those of sugars and other environmental factors, is mediated by alteration of PrfA activity, possibly by changing the levels or activity of the putative PrfA-associated factor (Paf) (4, 36, 38, 42).

L. monocytogenes requires the *hly* gene product to escape from a vacuole in the mammalian cell (3, 11, 16, 24, 35). Listeriolysin has a pH optimum of 5.5 (17). It is therefore surprising that the expression of *hly* should be shut off at a pH which is optimal for its activity. We speculate that the expression of *hly* takes place at a pH of 7.4, a condition which the bacterium would encounter in the bloodstream, while low pH might be a signal for secretion of preformed listeriolysin, enabling the rapid escape of the bacterium from the vacuole into the cytoplasm of the host cell.

Conclusions. We have demonstrated catabolite regulation of the metabolic enzyme α -glucosidase in *L. monocytogenes* and

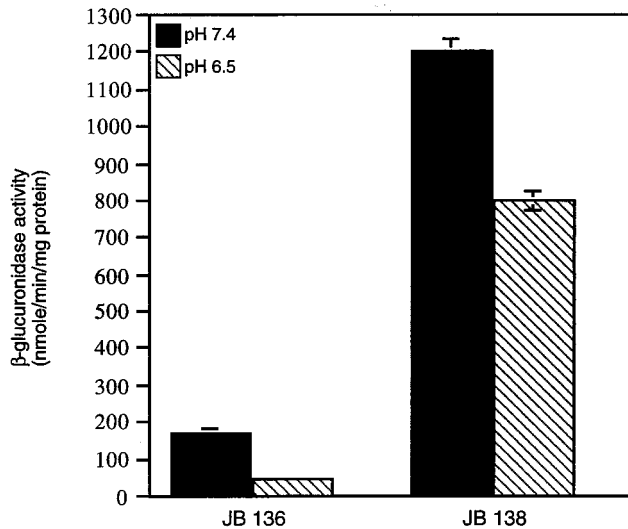


FIG. 9. Effect of *prfA*_{10403S} and *prfA*_{NCTC 7973} alleles on pH regulation of *hly-gus* expression. Culture conditions were as described in the legend to Fig. 7. Results are expressed as nanomoles of *p*-nitrophenol formed minute⁻¹ milligram of protein⁻¹ and represent the means \pm standard errors of the means for two independent experiments, each done in triplicate.

have shown that the specific activity of this enzyme is significantly higher in the hyperhemolytic mutant NCTC 7973 than in 10403S but is still subject to repression by glucose, fructose, and cellobiose. We have confirmed that the sequences of PrfA from three wild-type *L. monocytogenes* strains are identical but that there are two mutations in PrfA_{NCTC 7973}. By introducing a single copy of the mutant *prfA* allele from NCTC 7973 into the wild-type 10403S background, under control of its natural promoter, we have confirmed that the defect in *prfA* is responsible for the sugar-insensitive expression of *hly-gus*. Conversely, introduction of the *prfA* allele from 10403S into the NCTC 7973 background is sufficient to lower expression levels of *hly-gus* and restore at least partial regulation by sugars. However, there is no change in the levels of α -glucosidase activity when either the wild-type or mutant copy of *prfA* is introduced under control of the natural *prfA* promoter in the 10403S or the NCTC 7973 background. These results suggest that NCTC 7973 is anomalous not only with respect to the regulation of PrfA-controlled virulence genes but also in the expression of at least one other metabolic gene. Our results demonstrating the regulation of *hly-gus* by low extracellular pH suggest that the down-regulation of *hly-gus* expression in the presence of sugars may be due to a combination of effects produced by changes in pH or growth rate or by specific regulatory proteins. Therefore, it seems not only that several environmental factors regulate virulence gene expression but that any one factor may affect this regulation by several different mechanisms. Our results also underscore the importance of stringent control of extracellular pH during experiments measuring the effects of utilizable carbon sources on virulence genes. The fact that the Gly145Ser mutation in PrfA results in defective pH regulation suggests that as with several other environmental factors, the final common pathway of regulation of virulence genes by pH may be through modification of PrfA activity. Therefore, our results are consistent with the model of Ripio et al. (38), which proposes that regulation of virulence genes by environmental cues is achieved by alterations in the level or activity of a factor(s) required by PrfA for its activity.

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