

## The *bvr* Locus of *Listeria monocytogenes* Mediates Virulence Gene Repression by $\beta$ -Glucosides

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Received 16 March 1999/Accepted 31 May 1999

The  $\beta$ -glucoside cellobiose has been reported to specifically repress the PrfA-dependent virulence genes *hly* and *plcA* in *Listeria monocytogenes* NCTC 7973. This led to the hypothesis that  $\beta$ -glucosides, sugars of plant origin, may act as signal molecules, preventing the expression of virulence genes if *L. monocytogenes* is living in its natural habitat (soil). In three other laboratory strains (EGD, L028, and 10403S), however, the effect of cellobiose was not unique, and all fermentable carbohydrates repressed *hly*. This suggested that the down-regulation of virulence genes by  $\beta$ -glucosides is not a specific phenomenon but, rather, an aspect of a global regulatory mechanism of catabolite repression (CR). We assessed the effect of carbohydrates on virulence gene expression in a panel of wild-type isolates of *L. monocytogenes* by using the PrfA-dependent phospholipase C gene *plcB* as a reporter. Utilization of any fermentable sugar caused *plcB* repression in wild-type *L. monocytogenes*. However, an EGD variant was identified in which, as in NCTC 7973, *plcB* was only repressed by  $\beta$ -glucosides. Thus, the regulation of *L. monocytogenes* virulence genes by sugars appears to be mediated by two separate mechanisms, one presumably involving a CR pathway and another specifically responding to  $\beta$ -glucosides. We have identified in *L. monocytogenes* a 4-kb operon, *bvrABC*, encoding an antiterminator of the BglG family (*bvrA*), a  $\beta$ -glucoside-specific enzyme II permease component of the phosphoenolpyruvate-sugar phosphotransferase system (*bvrB*), and a putative ADP-ribosylglycohydrolase (*bvrC*). Low-stringency Southern blots showed that this locus is absent from other *Listeria* spp. Transcription of *bvrB* was induced by cellobiose and salicin but not by arbutin. Disruption of the *bvr* operon by replacing part of *bvrAB* with an interposon abolished the repression by cellobiose and salicin but not that by arbutin. Our data indicate that the *bvr* locus encodes a  $\beta$ -glucoside-specific sensor that mediates virulence gene repression upon detection of cellobiose and salicin. Bvr is the first sensory system found in *L. monocytogenes* that is involved in environmental regulation of virulence genes.

The gram-positive, facultative intracellular bacterium *Listeria monocytogenes* causes listeriosis, a severe, often fatal infectious disease of humans and animals. *L. monocytogenes* is an opportunistic pathogen that affects predominantly debilitated individuals with a defective cell-mediated immune response. Its natural habitat is soil, from which it reaches the vertebrate host via the food chain (15, 45). As in many other bacterial pathogens that can live saprophytically in inanimate environments, virulence gene expression is tightly regulated in *L. monocytogenes*. Almost all known listerial virulence determinants are coordinately expressed under the positive control of the PrfA protein, a transcription factor structurally and functionally related to cyclic AMP receptor protein (CRP) (6, 24, 25, 51, 54). A model has been proposed for the regulatory mechanism of PrfA in which, as for CRP, the protein becomes transcriptionally active via an allosteric conformational transition brought about by a putative low-molecular-weight cofactor (41, 54). There is evidence that the PrfA regulon is subject to complex environmental control in *L. monocytogenes*. Viru-

lence genes are expressed at 37°C (i.e., the body temperature of warm-blooded animals) but not at 26°C or below (i.e., environmental temperatures) (10, 27, 41), suggesting that temperature sensing may enable *L. monocytogenes* to detect its transition from the free environment to the animal host. However, an increase in temperature is not sufficient for the full activation of the PrfA regulon (40–42). *L. monocytogenes* produces little or no virulence factors in rich medium (e.g., brain heart infusion [BHI]) at 37°C, but a clear induction of virulence genes takes place at this temperature if BHI is either supplemented with the absorbent activated charcoal (40–43) or replaced by minimal essential medium (4). Clearly, therefore, chemical components of the extracellular environment also play a critical role in regulating the expression of the PrfA regulon in *L. monocytogenes*.

Park and Kroll reported in 1993 (35) that the presence in the growth medium of the  $\beta$ -glucoside cellobiose, but not of other common fermentable carbohydrates, strongly repressed the expression of two genes of the PrfA virulence regulon (*hly*, encoding the hemolysin, and *plcA*, encoding a phosphatidylinositol-specific phospholipase C) in *L. monocytogenes* NCTC 7973. Another  $\beta$ -glucoside, arbutin, was shown to have a similar effect in the same strain (34). In soil, *L. monocytogenes* is thought to utilize decaying vegetation as its primary growth substrate. This is perhaps most clearly illustrated by silage, a fermented vegetable fodder which, if incorrectly made, supports the growth of high numbers of *L. monocytogenes* bacteria

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and causes listeriosis outbreaks in domestic ruminants (52). Both cellobiose and arbutin are unique to the plant kingdom and are presumably abundant in decaying vegetation, so Park and Kroll reasoned that these plant-derived  $\beta$ -glucosides may act as specific "signature molecules," allowing *L. monocytogenes* bacteria to sense that they are present in a soil environment and, consequently, to repress their virulence genes (35). This notion was recently refuted by Milenbachs et al. (32), who found that in *L. monocytogenes* strains other than NCTC 7973 not only cellobiose, but several other readily metabolized sugars, such as glucose or fructose, caused virulence gene down-regulation. They observed that sugar-mediated inhibition of virulence gene expression only occurred in the presence of amounts of carbohydrates sufficient to significantly stimulate growth, indicating that the repression of virulence genes by sugar metabolism in *L. monocytogenes* might result from a general catabolite repression (CR) response (32). This view has been challenged by a recent report from the same group (3), showing that an *L. monocytogenes* homolog of the transcription factor CcpA, an important mediator of CR in low G+C content gram-positive bacteria (21, 46), appears not to be involved in the carbon source regulation of virulence genes. Nevertheless, CR in gram-positive bacteria is still poorly understood, and there are other possible CR mediators and pathways that may account for the repression of virulence genes in *L. monocytogenes*.

In this study we present evidence that reconciles the two hypotheses: that of Park and Kroll (35) suggesting that  $\beta$ -glucosides act as specific "environmental signature" repressor molecules and that of Milenbachs et al. (32) that the regulation of virulence genes by sugars is part of a global CR mechanism.

#### MATERIALS AND METHODS

**Bacterial strains, media, and culture conditions.** Wild-type *L. monocytogenes* strains were from a previously described panel comprising recent clinical and environmental isolates and collection or laboratory strains of various serotypes (41, 42). The strains tested met with the phenotypic criteria of the wild-type *prfA* genotype (41, 42): they exhibited weak to undetectable hemolytic and lecithinase reactions in BHI at 37°C, and the production of the corresponding activities was strongly activated in charcoal-treated medium. *Escherichia coli* DH5 $\alpha$  was used as cloning host. Bacteria were cultured at 37°C in BHI (Difco) or Luria-Bertani (LB) media, with antibiotics if required. Liquid cultures were shaken at 170 rpm. Fresh stock solutions of carbohydrates were filter sterilized and added to the culture medium to a final concentration of 10 to 25 mM. Charcoal-treated BHI was prepared as previously described (42).

**DNA methods.** Restriction and modification enzymes were purchased from Pharmacia and were used according to the manufacturer's instructions. *Listeria* chromosomal DNA was isolated as previously described (20). Specific DNA was amplified with the Expand High Fidelity PCR system (Boehringer Mannheim). PCR products were purified with the Quiaquick Gel Extraction Kit (Qiagen) and were cloned with the Sure Clone Kit (Pharmacia). Plasmid DNA was extracted from *E. coli* with the Plasmid Purification Kit (Qiagen). Sequences were determined from both strands of plasmid DNA with an Applied Biosystems 377 apparatus. For Southern blot analysis, DNA fragments were subjected to electrophoresis in 1% agarose in Tris-borate-EDTA, denatured, and transferred to nitrocellulose membrane (Schleicher & Schuell) according to standard protocols (1). DNA probes consisted of PCR or restriction fragments internal to the genes of interest. They were radiolabeled with the Boehringer Mannheim Random Primed DNA Labeling Kit and [ $\alpha$ -<sup>32</sup>P]dATP (Amersham). Prehybridization (2 h) and hybridization (16 h) were performed in 6 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 56°C. Filters were then washed twice for 30 min at 45°C each in 5 $\times$  SSC-0.1% sodium dodecyl sulfate and autoradiographed.

**PlcB (lecithinase) activity determinations.** The expression of *plcB* was quantified via the lecithinase activity of its protein product, the wide-substrate-range phospholipase C, PlcB (53), by using a previously described turbidimetric assay (42) and type IV phosphatidylcholine from egg yolk (Sigma) as the substrate. We use *plcB* as a reporter of PrfA-dependent expression (40, 41) because it has several advantages over the *hly* (hemolysin) gene used by others (2, 3, 32). First, unlike *hly*, which is expressed from PrfA-dependent and -independent promoters (12), the expression of *plcB* is driven uniquely by a PrfA-dependent promoter, P<sub>actA</sub> (53). This promoter also has a threshold for activation by PrfA that is higher than that of *hly* (41), which helps eliminate any constitutive background activity. Second, the PlcB assay gives more clearcut, linear results than do

hemolysin determinations (41). PlcB activity was determined in the supernatant of cultures collected when the optical density at 600 nm (OD<sub>600</sub>) reached 2.0. PrfA-dependent virulence gene expression is normally silenced in wild-type *L. monocytogenes* growing in BHI at 37°C (see above) (40–42). Therefore, to enable the repressor effect exerted by sugars on *plcB* expression to be observed, wild-type strains were cultured in charcoal-treated BHI, in which the PrfA regulon is fully activated (40–43).

**Cloning and sequencing of the *bvr* locus.** A 4.4-kb DNA fragment was amplified by PCR from EGD<sup>CR</sup> by using the oligonucleotide B35031 (5'-AGGTTG TGATGTTTATGGAAA), specific for *parB*, and the degenerate primer A35030 (5'-RTTNCCYTCYTCNGTRTATAAYTT; where R = purine, Y = pyrimidine, and N = any base), designed from the *Listeria seeligeri* *kat*-encoded oligopeptide KFYTEEEN (7) (see below and Fig. 2). Several attempts to clone the full-length fragment in *E. coli* by using pUC18 failed, suggesting that this DNA region was toxic for bacteria if present in multiple copies. However, a plasmid was isolated (pKLB3A) that harbored a deletion at the *parB*-proximal part of the 4.4-kb fragment. The 2.1-kb insert of pKLB3A was partially sequenced and two further oligonucleotides, 94C230 (5'-GTTGCTCCTGCAGCCGGTGT-3') and 94C229 (5'-TAATTCCAAGCGCATGTCTT), were designed which, when used with B35031 and A35030, respectively, gave rise to 2.1- and 2.4-kb PCR products. These PCR fragments were inserted into pUC18, yielding the plasmids pKLB3B (B35031-94C230 fragment) and pKLB3C (A35030-94C229 fragment). The entire sequence of the *bvr* locus, encompassed by the *parB* and *kat* genes (see Fig. 2), was determined from the overlapping inserts of pKLB3B and pKLB3C.

**Construction of the *bvrAB:: $\Omega$ Km* mutant.** pKLB3B was digested with *HincII* and blunt-end ligated to the 2.1-kb *SmaI* fragment of pBR322WKm. This fragment contains the  $\Omega$ -Km2 interposon, which bears the *aphA-3* kanamycin resistance gene from Tn1545 and, at both ends, transcription and translation termination signals in each reading frame (36). In the resulting plasmid, pKLB1, the 850-bp fragment between the two *HincII* sites encompassing the 3' end of *bvrA* and the 5'-proximal region of *bvrB* was replaced by the  $\Omega$ -Km2 element (see Fig. 2). The interposon-disrupted *bvr* locus was PCR amplified from pKLB1 by using the primers B35031 and 94C230 (see above). This PCR fragment was then blunt-end ligated into the *SmaI* site of the shuttle vector pLSV1 (55), giving rise to pKLB2. pLSV1 has an erythromycin resistance gene and a thermosensitive origin of replication for gram-positive bacteria. pKLB2 was introduced into *L. monocytogenes* EGD<sup>CR</sup> as previously described (41). Transformants were grown for 6 h at 30°C in BHI broth in the presence of 25  $\mu$ g of kanamycin and 5  $\mu$ g of erythromycin per ml, after which serial dilutions of the culture were plated onto BHI agar plates containing the same antibiotics. The plates were incubated for 48 h at 42°C to select for single crossover events between the chromosome and pKLB2. One of the resulting colonies was grown overnight at 37°C in BHI broth containing 25  $\mu$ g of kanamycin per ml, and appropriate dilutions of this culture were plated onto kanamycin BHI agar. Bacteria in which a second crossover event took place, leading to *bvr* allelic exchange, were selected by checking colonies for the loss of erythromycin resistance. The *bvrAB:: $\Omega$ Km* mutation was confirmed by Southern blot and PCR with appropriate primers.

**RNA procedures.** Total RNA was extracted from mid-exponential-phase cultures of *L. monocytogenes* by using a hot-acid-phenol protocol as described elsewhere (43). The operon structure of the *bvr* locus was investigated by using 5  $\mu$ g of total RNA and the Titan One Tube reverse transcription-PCR (RT-PCR) Kit from Boehringer Mannheim. The effect of  $\beta$ -glucosides on *bvrB* and *plcB* transcription was assessed by semiquantitative RT-PCR as previously described (14). Samples of total RNA (0.5  $\mu$ g/ $\mu$ l) were serially diluted in diethylpyrocarbonate-treated water, and cDNA was synthesized from 2  $\mu$ l of each dilution by using the First-Strand Synthesis Kit from Stratagene. Then, 2- $\mu$ l volumes of each primer extension reaction were subject to 15, 20, 25, and 30 cycles of PCR, and amplified DNA fragments were detected after electrophoresis in 1% agarose by ethidium bromide staining. Appropriate oligonucleotides internal to the genes of interest were used for primer extension and PCR amplification of cDNA. As controls, primers specific for the 16S rRNA and the *sod* gene encoding superoxide dismutase (5) of *L. monocytogenes* were used.

#### RESULTS

**Evidence that cellobiose downregulates virulence gene expression via a specific mechanism.** Milenbachs et al. (32) showed that NCTC 7973 has an anomalous pattern of carbon source regulation, with cellobiose being the only sugar, of a variety of fermentable carbohydrates tested, causing virulence gene repression. This behavior suggests that (i) NCTC 7973 is probably a deregulated mutant with a defect in an aspect of carbon source regulation (2) and that (ii) cellobiose represses virulence genes via a specific pathway that is different from that used by other common carbohydrates.

The observations made with NCTC 7973 should, however, be interpreted with caution. This strain belongs to a class of *L. monocytogenes* mutants, called *prfA*<sup>\*</sup>, that constitutively over-

express the PrfA virulence regulon (42). This phenotype is due to a point mutation in *prfA* leading to the synthesis of a mutant form of the regulatory protein, PrfA\* (Gly145Ser), which binds with an increased affinity to the specific target sequences in the promoter regions of PrfA-regulated genes (41, 54). We (40, 41) and others (2) have presented evidence that the constitutive overexpression of PrfA-dependent genes conferred by the *prfA*\* allele overcomes carbon source regulation in *L. monocytogenes*. Therefore, it is difficult to discern in the NCTC 7973 background whether the sugar-insensitive phenotype results simply from a masking effect exerted by the *prfA*\* mutation alone or from additional mutations in central CR pathways. There is evidence that NCTC 7973 has accumulated other mutations affecting carbohydrate catabolic pathways (2), which may indeed lead to a completely distorted pattern of sugar-mediated virulence gene repression.

The carbon source regulation of virulence genes, on the other hand, has been studied in only a restricted number of *L. monocytogenes* strains. Besides NCTC 7973, only three other laboratory strains of serogroup 1 have been analyzed: EGD, 10403S, and L028 (32). Strains kept in laboratory conditions for a long time may accumulate mutations and exhibit aberrant phenotypes, as illustrated by NCTC 7973 and by at least one other of the strains examined, L028, which also has an anomalous pattern of virulence gene expression (41, 42). We therefore examined additional *L. monocytogenes* strains with a confirmed wild-type PrfA phenotype (see Materials and Methods). The fermentable carbohydrates glucose, fructose, and mannose and the  $\beta$ -glucoside cellobiose were tested for their ability to repress virulence gene expression. Saccharose, which is not utilized by *L. monocytogenes* and consequently does not repress virulence genes (32, 40), was used as a control. The product of the *plcB* gene, the wide-substrate-range phospholipase C, PlcB (or lecithinase), was used to monitor PrfA-dependent virulence gene expression (see Materials and Methods).

Consistent with previous observations with *hly* as a reporter (32), a strong downregulation of *plcB* occurred in all strains with all of the fermentable carbohydrates tested (not shown). There was, however, an exception. The group of wild-type strains tested included two subcultures of *L. monocytogenes* EGD from different laboratories: one was that kept by one of us (J.K.), while the other came from T. Chakraborty (University of Giessen, Giessen, Germany). The latter, termed EGD-e, is the strain whose DNA is being sequenced by the European *Listeria* genome consortium. Interestingly, EGD-e exhibited the normal repression pattern of wild-type strains, but our EGD subculture resembled NCTC 7973 in that *plcB* expression was only downregulated by cellobiose (see Fig. 1). Since our EGD variant (which was otherwise of wild-type *prfA* background) was no longer repressed by glucose and other common fermentable sugars, we presumed it was affected in a central CR pathway and thus called it EGD<sup>CR</sup>.

In contrast to cellobiose and arbutin, the phenolic  $\beta$ -glucoside salicin has been reported to have no downregulating effect on *hly* expression in NCTC 7973 (34). We thus analyzed in more detail whether NCTC 7973 and EGD<sup>CR</sup> had similar defects by assessing *plcB* expression in these two strains grown in the presence of each three  $\beta$ -glucosides. In NCTC 7973, as expected, *plcB* expression was repressed in the presence of cellobiose and arbutin but was not repressed by salicin (Fig. 1). EGD<sup>CR</sup>, however, differed from NCTC 7973 in that virulence gene expression was identically downregulated by the three natural  $\beta$ -glucosides (Fig. 1). An identical pattern was observed in EGD-e (Fig. 1), indicating that the EGD<sup>CR</sup> variant retained a wild-type response to  $\beta$ -glucoside sugars.

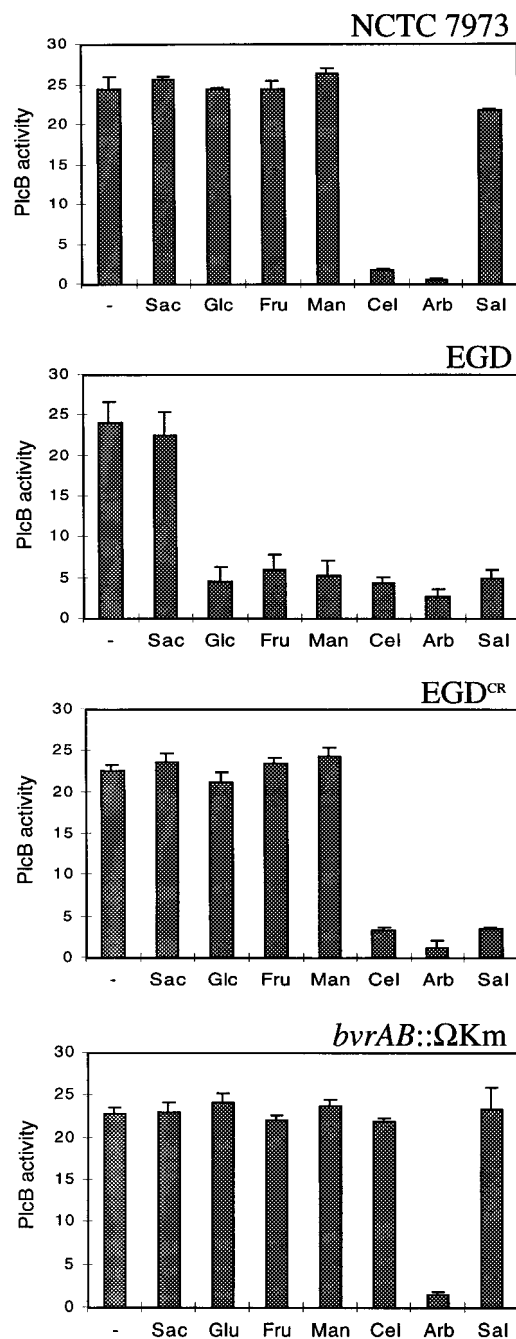


FIG. 1. Patterns of virulence gene (*plcB*) repression in response to sugars in *L. monocytogenes* strains NCTC 7973, EGD, and EGD<sup>CR</sup> and the *bvrAB::Km* mutant (Sac, saccharose; Glc, glucose; Fru, fructose; Man, mannose; Cel, cellobiose; Arb, arbutin; Sal, salicin; -, no sugar). Means for three independent experiments  $\pm$  the standard error are shown.

Overall, these observations suggest: (i) that  $\beta$ -glucosides cause virulence gene repression via a specific regulatory pathway, independent from the CR mechanism presumably used by glucose and other common utilizable sugars; and (ii) that there may also be various sugar-sensing mechanisms, with different substrate specificities, involved in the regulation of virulence genes by  $\beta$ -glucosides.



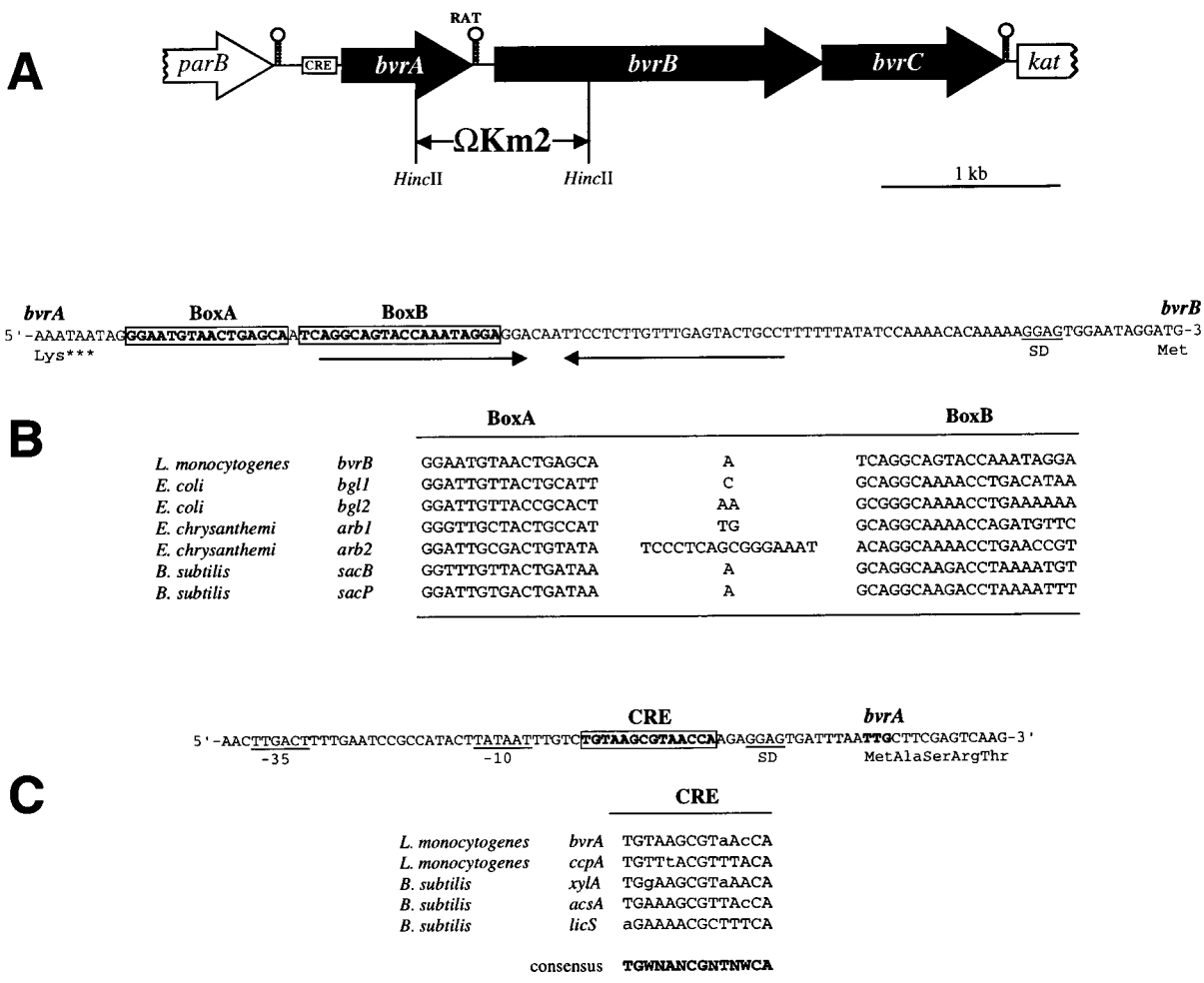


FIG. 2. (A) Scheme of the genetic structure of the *bvr* locus of *L. monocytogenes*. The positions of the putative transcriptional terminators, CRE, and RAT sequences are shown. The *bvr* mutation was created by allele exchange by using a plasmid construct in which a *HincII* fragment encompassing the 3'-terminal third of *bvrA* and the 5'-proximal third of *bvrB* was replaced by the  $\Omega$ Km2 interposon, as indicated. (B) Nucleotide sequence of the intergenic region between *bvrA* and *bvrB* (the respective last and first codons are shown) and position of the putative RAT sequence (boxed), which overlaps the 5' part of a palindromic structure that may act as transcriptional terminator (indicated by inverted arrows). Below, comparison of the putative RAT sequence preceding *bvrB* with known RAT sequences (13). (C) Nucleotide sequence of the 3' region upstream from *bvrA* (its TTG start codon is in boldface type) showing -10 and -35 putative promoter sequences and the CRE-like sequence (boxed). Below, comparison of the putative CRE preceding *bvrA* with the CRE-like element of the *ccpA* promoter region of *L. monocytogenes* (3) and known active CRE sites from gram-positive bacteria (21). Deviations from the CRE consensus sequence (in boldface) are shown in lowercase (N = any nucleotide; W = A or T).

**Cloning and sequence analysis of the *bvr* locus of *L. monocytogenes*.** We have previously reported the cloning and characterization of the *kat* gene encoding the catalase of the non-pathogenic species *L. seeligeri* (20). Upstream from *kat*, separated by a 370-bp intergenic region, we identified in *L. seeligeri* the *parB* locus (7). Like *kat*, *parB* was present in a highly conserved form in all *Listeria* species (7). While attempting to isolate the *L. monocytogenes* homolog of *kat*, we found that, in this species, there was an insertion of about 4 kb between this gene and *parB*. As such genomic differences between pathogenic and nonpathogenic species of the same genus may correspond to virulence-associated chromosomal islands (18, 19), we were interested in characterizing the DNA region between *parB* and *kat* in *L. monocytogenes*. A 4.4-kb fragment comprising the entire *parB-kat* intergenic region of *L. monocytogenes* was amplified by PCR, subcloned, and sequenced (see Materials and Methods for details). It comprised three open reading frames (ORF) of 810, 1,919, and 980 bp arranged in the same orientation (Fig. 2A). The locus was

called *bvrABC* (for  $\beta$ -glucoside-mediated virulence gene repression [see below]).

The largest ORF, *bvrB*, encoded a 640-residue protein with extensive similarity to various permease components (enzyme II complex) of the phosphoenolpyruvate-sugar phosphotransferase system (PTS). The highest degree of similarity of the *bvrB* product, BvrB, was with ArbF from *Erwinia chrysanthemi* (13), BglF from *E. coli* (50), and BglP from *Bacillus subtilis* (26) (Fig. 3B), all of which are involved in the uptake of  $\beta$ -glucosides. BvrB also had the multidomain structure IIBCA, which is characteristic of enzymes II of the subfamily of  $\beta$ -glucoside PTS permeases (8, 28). These permeases are regulated by transcriptional antitermination, brought about by a family of structurally related antiterminator proteins (AT), the prototype of which is BglG from *E. coli* (44, 47). In the  $\beta$ -glucoside PTS operons of the enteric bacteria *E. coli* and *E. chrysanthemi*, the AT genes (*bglG* and *arbG*, respectively) are immediately upstream from the genes encoding the  $\beta$ -glucoside-specific enzymes II. In *B. subtilis*, in contrast, the two genetic



determinants are not located in the same chromosomal region (44). Although *L. monocytogenes* is phylogenetically very closely related to *B. subtilis*, the genetic structure of the *bvrAB* locus was virtually identical to that of the enterobacterial loci *bglGF* and *arbGF*: the predicted *bvrA* product, BvrA (270 amino acids), was very similar to BglG (50) and related ATs from the *E. chrysanthemi* *arb* operon (ArbG) (13) and the *B. subtilis* *bgl* and *sac* regulons (LicT, SacY, and SacT) (9, 11, 48) (Fig. 3A). ATs bind specifically to conserved target sequences called "RAT" (for ribonucleic antiterminator), which overlap the 5' part of stem-loop transcriptional terminator structures present in the leader sequences of the controlled genes or operons, thereby preventing the formation of the terminator and allowing readthrough transcription to the downstream (PTS permease) genes (44). In the 110-bp region between *bvrA* and *bvrB*, we identified a putative RAT sequence for BvrA overlapping a potential *rho*-independent transcriptional terminator (Fig. 2B).

PTS genes are frequently clustered with the corresponding catabolic enzymes in operons (38, 44). In the case of the *E. coli*, *E. chrysanthemi*, and *B. subtilis*  $\beta$ -glucoside-specific PTS operons and regulons, the enzyme II gene is immediately followed by the structural gene of phospho-(P)- $\beta$ -glycosidase (*bglC*, *arbC*, and *bglH*, respectively), which is involved in the further metabolism of the incorporated sugar (13, 26, 50). In the *L. monocytogenes* *bvr* locus, however, no such P- $\beta$ -glucosidase gene was identified downstream from *bvrB*. FASTA searches detected a significant degree of similarity (21.3% identity, 51.5% similarity) between the *bvrC* product, BvrC, and the ADP-ribosylglycohydrolase DraG from *Rhodospirillum rubrum* (16). BvrC (327 amino acids) was also very similar to the proteins encoded by two ORFs present in the genomes of *E. coli* (hypothetical protein b2099: 33.3% identity, 65.2% similarity) and *Methanococcus jannaschii* (DraG-homolog: 25.8% identity, 59.3% similarity). The predicted primary structure of these two polypeptides also showed significant similarity to that of ADP-ribosylglycohydrolases (Fig. 3C).

***bvrB* expression is induced by  $\beta$ -glucosides.** ATs act as positive regulators of bacterial catabolic operons via a regulatory process induced by the substrate. For example, in the absence of  $\beta$ -glucosides, the BglF PTS permease of *E. coli* inactivates BglG by phosphorylating it. Thus, constitutive readthrough transcription of the operon is prevented by terminators preceding the *bglG* and *bglF* genes. However, in the presence of inducer  $\beta$ -glucosides, the phosphate groups are transferred from BglF to the sugar during uptake, creating a backward flow that dephosphorylates BglG. Active BglG prevents termination by binding to RAT sequences and induces the expression of the operon (38, 44, 47). The structure of the *L. monocytogenes* *bvrAB* locus is very similar to that of the *E. coli* *bglGF* locus (see above), suggesting functional similarity. If a similar antitermination mechanism of positive regulation operates in *bvrAB*, then the expression of the putative permease gene *bvrB* should be induced if *L. monocytogenes* is grown in the presence of  $\beta$ -glucosides.

We studied *bvrB* transcription in *L. monocytogenes* in the presence of glucose (control) or the  $\beta$ -glucosides cellobiose, salicin, and arbutin (Fig. 4). In the 5' region immediately upstream from *bvrA*, overlapping a putative promoter, we identified a 14-bp sequence that matched the reported consensus sequence of catabolite responsive elements (CRE) (Fig.

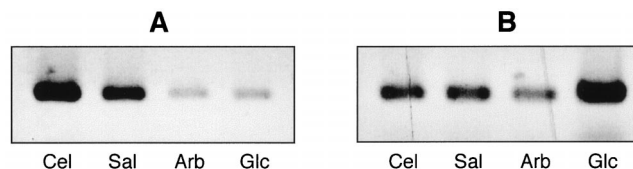


FIG. 4. RT-PCR transcription analysis of *bvrB* (A) and *plcB* (B) expression in EGD<sup>CR</sup> grown in the presence of cellobiose (Cel), salicin (Sal), arbutin (Arb), and glucose (Glc) (control). Note that in panel A, cellobiose and salicin, but not arbutin, upregulate *bvrB*. In panel B, the three  $\beta$ -glucosides repress *plcB*, a finding consistent with the data obtained by using PlcB activity as reporter (Fig. 1).

2C), the *cis*-acting binding sites for the repressor transcription factors (presumably, the CcpA/HPr[Ser-46-P] complex) involved in CR in low-G+C-content, gram-positive bacteria (21, 46). This suggests that the *bvr* locus may be subject to CR if glucose or other readily metabolizable carbon sources are present in the culture medium. Therefore, to avoid the effects of any possible expressional crosstalk between the *bvr* locus and the putative global CR mechanism involved in carbon source regulation, we performed the experiments with EGD<sup>CR</sup>. *bvrB* transcription was semiquantitatively determined by RT-PCR. The level of *bvrB* expression was significantly higher in the presence of cellobiose and salicin (Fig. 4A). These results are consistent with the structural data, which predict that BvrAB is a  $\beta$ -glucoside-specific, substrate-inducible PTS permease system functionally similar to BglEF from *E. coli*. Arbutin, however, did not activate the transcription of *bvrB* (Fig. 4A).

The TGA stop codon of *bvrB* overlapped with the ATG start codon of *bvrC*, suggesting that the two genes are cotranscribed and that the *bvr* locus constitutes an operon. We confirmed this by RT-PCR with total RNA from *L. monocytogenes* grown on cellobiose and oligonucleotide primers specific for *bvrA* and *bvrC* (not shown).

**The *bvr* locus is *L. monocytogenes* specific.** We were interested in determining whether *bvr*-homologous sequences were present elsewhere on the *L. seeligeri* chromosome or if the locus was present in other *Listeria* species. Southern blot analyses were performed with chromosomal DNA from all known *Listeria* species, hybridizing at low stringency with a *bvr* probe. Positive hybridization signals were only obtained with *L. monocytogenes* (Fig. 5). This is consistent with the fact that we have only observed *bvr*-positive PCR reactions with DNA from *L. monocytogenes* isolates.

**The *bvr* locus is involved in  $\beta$ -glucoside-mediated virulence gene repression.** Our structural and functional data suggest that BvrB is a  $\beta$ -glucoside-specific PTS permease. It was therefore possible that the *bvr* locus was somehow involved in  $\beta$ -glucoside-mediated virulence gene repression in *L. monocytogenes*. To test this, we constructed a *bvr* mutant in EGD<sup>CR</sup> by replacing a *HincII* fragment spanning the 3' terminal part of *bvrA*, the *bvrAB* intergenic region, and the 5' part of *bvrB* with the  $\Omega$ -Km2 interposon (Fig. 2A) (see Materials and Methods). RT-PCR analyses indicated that *bvrB* expression was abolished in the *bvrAB:: $\Omega$ Km* mutant (not shown). No transcripts were

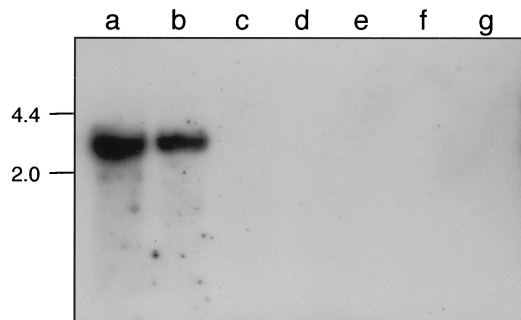


FIG. 5. Detection of *bvr* sequences in *Listeria* spp. by low-stringency Southern blot. Lanes: a, *L. monocytogenes* EGD<sup>CR</sup>; b, *L. monocytogenes* NCTC 7973; c, *L. ivanovii* ATCC 19119; d, *L. seeligeri* SLCC 5921; e, *L. welshimeri* SLCC 5334; f, *L. grayi*. Chromosomal DNA was digested with *Eco*RI. Numbers on the left indicate the size in kilobases.

detected for *bvrC*, demonstrating that the *bvr* locus constitutes an operon.

We studied the effect of the *bvrAB*:: $\Omega$ Km mutation on virulence gene repression by  $\beta$ -glucosides by using *plcB* as a reporter. The repression exerted by cellobiose and salicin was totally abolished in *bvrAB*:: $\Omega$ Km. Arbutin, however, still downregulated *plcB* expression to a level similar to that of the parent strain (Fig. 1). These results were entirely consistent with the fact that *bvrB* expression is induced by cellobiose and salicin but not by arbutin (Fig. 4) and demonstrate that the BvrB PTS permease is involved in virulence gene repression by  $\beta$ -glucosides.

PTS permeases mediate the transport into the bacterial cell of specific carbohydrate substrates, thus initiating their catabolism (28, 38, 46). We therefore investigated whether the *bvr* locus was important for cellobiose and salicin utilization by *L. monocytogenes*. The growth characteristics of *bvrAB*:: $\Omega$ Km and its parent strain were determined in LB broth supplemented with glucose (control), cellobiose, salicin, or arbutin as the only carbon source. LB is a poor growth medium, in which *L. monocytogenes* can hardly grow in the absence of an additional carbon source (maximum OD<sub>578</sub> of 0.5 versus 2.0 in LB plus 10 mM fermentable carbohydrate) (40). Virtually identical growth curves and final bacterial yields were observed for both strains in LB medium with glucose or any one of the three  $\beta$ -glucosides (not shown). No differences were observed when the sugar utilization profiles of the parent and mutant strains were investigated with the API 50 CH system.

## DISCUSSION

The regulatory effect of fermentable sugars on virulence genes of *L. monocytogenes* has recently attracted considerable interest (2, 3, 32, 34, 35). This issue has been investigated in only four laboratory strains, at least two of which (L028 and NCTC 7973) have anomalous patterns of virulence gene expression and even regulatory mutations in *prfA* (41, 42). To unambiguously determine the normal pattern of this type of carbon source regulation in *L. monocytogenes*, we assessed the effect of a selection of carbohydrates on the expression of the PrfA-dependent gene *plcB* by using a panel of well-characterized wild-type strains. Our results confirmed that the repressibility of PrfA-dependent virulence genes is a general characteristic of wild-type *L. monocytogenes* upon growth on any fermentable sugar.

Among the strains tested, we identified a phenotypic variant of strain EGD in which virulence gene expression was still

repressed by the three natural  $\beta$ -glucosides (cellobiose, salicin, and arbutin) but not by other common utilizable sugars such as glucose. We concluded that the repression of virulence genes by sugars is mediated in *L. monocytogenes* by at least two different regulatory mechanisms: one presumably involving a general CR repression pathway, as suggested by Milenbachs et al. (32), which can be eliminated by a spontaneous mutation, and another responding specifically to  $\beta$ -glucosides. In support of this conclusion, we report here the identification of the *bvr* locus of *L. monocytogenes*, which specifically mediates virulence gene repression by  $\beta$ -glucoside sugars.

The *bvr* locus comprises three genes, *bvrABC*, which are expressed as an operon. *bvrAB* code for a putative  $\beta$ -glucoside-specific, substrate-inducible PTS permease system similar to that of the *bgl* and *arb* operons of *E. coli* and *E. chrysanthemi*, respectively, and the *bgl* regulon of *B. subtilis*. Transcription of the enzyme II gene, *bvrB*, was induced by cellobiose and salicin, but not by arbutin, suggesting that the BvrB permease was specific for the two first  $\beta$ -glucosides. Moreover, a knockout mutation of the *bvr* operon totally abolished the repression exerted by cellobiose and salicin, whereas arbutin downregulated virulence genes to the same extent as in the parent strain. These results demonstrate the involvement of the *bvr* locus in virulence gene regulation by  $\beta$ -glucosides, suggesting at the same time that various regulatory mechanisms, with different substrate specificities, may be involved in  $\beta$ -glucoside-mediated virulence gene regulation in *L. monocytogenes*. Arbutin is a major substrate for other bacterial  $\beta$ -glucoside PTS permeases (BglF, ArbF, and BglP from *E. coli*, *E. chrysanthemi*, and *B. subtilis*, respectively) (13, 26, 50) to which BvrB is homologous. Primary structure differences in certain conserved domains may account for the altered substrate specificity of BvrB (Fig. 3B).

It has been previously shown that cellobiose reduces virulence gene expression without affecting the levels of the PrfA protein (32, 39). The only PrfA-dependent virulence genes used to date to assess the effect of cellobiose are *hly* and *plcA* (2, 3, 23, 32, 34, 35). These genes are physically linked, divergent transcriptional units with overlapping promoter regions (31), so the possibility existed that the repressor effect exerted over them by cellobiose was completely unrelated to PrfA and involved a specific interaction with the common promoter region. Here we show that cellobiose and other  $\beta$ -glucosides also repress *plcB*, another gene of the virulence regulon, the expression of which is strictly dependent on PrfA (41). As shown in Fig. 4B, *plcB* is downregulated at the transcriptional level, as are *hly* and *plcA* (23, 32, 35). Thus, the regulatory mechanism triggered by  $\beta$ -glucosides appears to somehow interfere with PrfA.

How does the *bvr* locus brings about  $\beta$ -glucoside-mediated virulence gene regulation? It is well known that sugar transport by PTS permeases initiates a regulatory cascade of CR via PEP-dependent, enzyme I-catalyzed phosphorylation of the His-15 residue of the general PTS protein HPr both in gram-negative and gram-positive bacteria (21, 38, 46). However, our observations with EGD<sup>CR</sup> would rule out the possibility that a general CR pathway is involved in virulence gene repression by  $\beta$ -glucosides. There is extensive functional crosstalk between the various ATs of the *bgl-sac* family, of which there are three well-characterized members in *B. subtilis* (see above) (44). Therefore, one possibility is that BvrB-mediated dephosphorylation and activation of BvrA upon the transport of cellobiose and salicin positively regulate, by transcriptional antitermination, a putative effector system responsible for the inhibition of the PrfA regulon. Another possibility involves the *bvrC*-encoded putative ADP-ribosylglycohydrolase (Fig. 3C). In pro-



karyotes, these enzymes participate in posttranslational regulatory networks involving reversible ADP-ribosylation of target proteins (29) (see the legend to Fig. 3C). Thus, BvrC may well provide the link between the BvrAB  $\beta$ -glucoside-specific PTS permease system and the virulence regulon by posttranslationally controlling, directly or indirectly, PrfA function.

Mutation of the *bvr* locus totally eliminated the repressor effect exerted by cellobiose and salicin without affecting the utilization of these sugars. Clearly, therefore, other sugar transport systems mediating  $\beta$ -glucoside utilization should be present in *L. monocytogenes*. These systems are likely to be conserved in *Listeria* because all species in this genus are able to ferment  $\beta$ -glucosides (reference 33 and unpublished observations). Thus, any role of the *bvr* locus in the uptake of  $\beta$ -glucosides by *L. monocytogenes* would be purely accessory or redundant. *bvr* is present exclusively in *L. monocytogenes*, as a 4-kb chromosomal island inserted between two housekeeping genes, *parB* and *kat*, which are contiguous in the nonpathogenic species *L. seeligeri* (6). This may indicate that the function associated with this locus is relevant only to the biology of the pathogenic species *L. monocytogenes*. Virulence factors are primarily required during infection, and their synthesis outside the host would be a waste of energy, reducing the fitness of the bacterium to successfully compete in the free environment and thereby limiting its potential for transmission. Our results suggest that the *bvr* locus is responsible for a regulatory mechanism that, like thermoregulation, would be aimed at ensuring that virulence genes are shut off outside the vertebrate host. The PTS acts as a signal transduction system in positive chemotaxis in response to the presence of PTS-transported carbohydrates in the extracellular medium (17, 38). It is therefore possible that the Bvr system functions as a sensor mechanism triggering virulence gene silencing upon detection of  $\beta$ -glucosides, sugars specific to the plant kingdom which, as previously suggested by Park and Kroll (35), might be used by *L. monocytogenes* as signal molecules of the soil habitat. It must be noted that the virulence gene regulatory function of the *bvr* locus would have remained cryptic if *L. monocytogenes* were not cultured in PrfA-activating conditions (i.e., culture at 37°C in charcoal-treated BHI). This shows that *bvr* genes are responsible for a repressor mechanism that is superimposed on the positive control pathway that activates the PrfA virulence regulon. The environmental context sensed by *L. monocytogenes* in soil may sometimes become ambiguous, for example, if the ambient temperature rises above 30°C. We believe that in such situations the Bvr system may play a key role as a fail-safe mechanism to avoid any deleterious leaky expression of virulence genes.

#### ACKNOWLEDGMENTS

We thank T. Chakraborty for the gift of the EGD strain kept in his laboratory. N. Montero is acknowledged for excellent technical assistance.

This work was supported by grants from the European Commission (HRCX-CT94-451 and BMH4-CT96-659), the Fondo de Investigación Sanitaria (FIS 94/0043-02), the Dirección General de Investigación de la Comunidad de Madrid (29/97), the Deutsche Forschungsgemeinschaft (SFB 165), and the Fonds der Chemischen Industrie.

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