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Interference of Components of the Phosphoenolpyruvate Phosphotransferase System with the Central Virulence Gene Regulator PrfA of *Listeria monocytogenes*▽

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Analysis of *Listeria monocytogenes ptsH*, *hprK*, and *ccpA* mutants defective in carbon catabolite repression (CCR) control revealed significant alterations in the expression of PrfA-dependent genes. The *hprK* mutant showed high up-regulation of PrfA-dependent virulence genes upon growth in glucose-containing medium whereas expression of these genes was even slightly down-regulated in the *ccpA* mutant compared to the wild-type strain. The *ptsH* mutant could only grow in a rich culture medium, and here the PrfA-dependent genes were up-regulated as in the *hprK* mutant. As expected, HPr-Ser-P was not produced in the *hprK* and *ptsH* mutants and synthesized at a similar level in the *ccpA* mutant as in the wild-type strain. However, no direct correlation was found between the level of HPr-Ser-P or HPr-His-P and PrfA activity when *L. monocytogenes* was grown in minimal medium with different phosphotransferase system (PTS) carbohydrates. Comparison of the transcript profiles of the *hprK* and *ccpA* mutants with that of the wild-type strain indicates that the up-regulation of the PrfA-dependent virulence genes in the *hprK* mutant correlates with the down-regulation of genes known to be controlled by the efficiency of PTS-mediated glucose transport. Furthermore, growth in the presence of the non-PTS substrate glycerol results in high PrfA activity. These data suggest that it is not the component(s) of the CCR or the common PTS pathway but, rather, the component(s) of subsequent steps that seem to be involved in the modulation of PrfA activity.

Listeria monocytogenes, a gram-positive, facultative intracellular human pathogen, escapes from the primary phagosome, replicates efficiently in the host cell's cytosol, and spreads from cell to cell. These processes, which are of major importance for pathogenesis of an *L. monocytogenes* infection, require several, well-characterized virulence factors (for recent reviews, see references 17, 32 and 63), like internalins (InlA, InlB, and InlC) for active internalization into nonprofessional phagocytic cells, listeriolysin O (LLO) for the release from the phagosomal compartment into the cytosol, or ActA for intra- and intercellular mobility. The genes that encode these and other virulence factors are either clustered or dispersed on the listerial chromosome (31, 63).

Most listerial virulence genes are regulated by the central transcription factor PrfA (8, 36, 41). This regulator belongs to the Crp/Fnr family of transcription activators; it recognizes as a dimer a conserved 14-bp sequence of dyad symmetry ("PrfA box") located about −40 bp 5′ upstream of the transcription start site of PrfA-regulated promoters (22, 34, 40, 55). The synthesis of PrfA is autoregulated by a PrfA-dependent promoter in front of a bicistronic operon comprising the genes *plcA* and *prfA* (11, 21, 41). In addition, two promoters, P1 and P2, in front of *prfA* itself constitutively transcribe this gene at a low level (21, 22, 51, 64). This small amount of PrfA is essential for initiating transcription at the autoregulated *plcA* promoter. As recently shown (29), the nontranscribed 5′ region of the mRNA starting at P1 can form a secondary structure which acts as a thermoswitch for the translation of this *prfA* transcript, resulting in PrfA synthesis only above 30°C.

Furthermore, there is evidence for the interaction of PrfA with other listerial factors that modulate its activity (6, 7, 16, 19). Mutations within specific regions of PrfA have been obtained (45, 52, 56, 64, 65, 68) which lead to a permanently active state of PrfA; e.g., the addition of activated charcoal to brain heart infusion (BHI) medium or incubation in minimal essential medium (MEM) does not cause further activation, and cellobiose no longer inhibits the activity of these mutant PrfA proteins (termed PrfA*).

Readily metabolizable sugars which are taken up by phosphoenolpyruvate (PEP)-dependent phosphotransferase systems (PTS), like glucose, mannose, and especially cellobiose, strongly inhibit the activity of PrfA (23, 42, 46, 51), suggesting that component(s) involved in these pathways or the connected carbon catabolite repression (CCR) system interfere with PrfA, thereby modulating its activity. CCR control in gram-positive bacteria of low G+C content depends on several components. The *c*atabolite *c*ontrol *p*rotein A (CcpA), the activity of which is modulated by different cofactors, regulates gene expression by different modes (5, 26, 44). A major cofactor of CcpA is HPr phosphorylated at Ser46. The HPr protein (encoded by *ptsH*) is a central component of all PTS pathways and possesses two phosphorylation sites. During active PTS-medi-

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TABLE 1. Oligonucleotides used to confirm integration of the vectors pSM1-3 in the genes *ccpA*, *hprK*, and *ptsH*

Oligonucleotide	Sequence (5′→3′)	Target DNA
ccpA-1	TACAATGGTAGGTAGAGCC	Upstream region of *ccpA*
ccpA-2	TTGTTCTGAAATACGTTCGCC	*ccpA*
hprK-1	CTAAATAATATTTTGTAGCGAACAG	Upstream region of *hprK*
hprK-2	AAGTCTGCTTTCTAAGTAATTCG	*hprK*
ptsH-1	TCAATAATGTCTCCAACATGTGC	Upstream region of *ptsH*
ptsH-2	AGCAGATAAGCTTTCGCAATG	Downstream region of *ptsH*
pLSV101BamHI	AATAAGCTTGGCTGCAGGTC	Vector pLSV101
pLSV101EcoRI	GTTTTCCCAGTCACGACGTT	Vector pLSV101

ated sugar uptake, HPr is phosphorylated (by PEP) at His15. This HPr phosphorylation is catalyzed by enzyme I, encoded by *ptsI* which forms an operon structure with *ptsH* (25, 66). The phosphate group of HPr-His-P is then transferred to the sugar-specific transport component EIIA and further to EIIB. The membrane-associated EIIB activates the sugar translocation via EIIC. EIIA is also involved in other regulatory functions (9). The second phosphorylation site of HPr is Ser46. This phosphorylation is catalyzed by a specific ATP-dependent HPr kinase/phosphorylase (HPrK/P) (48, 49) which is stimulated by intermediates of the glycolytic pathway, in particular, by fructose 1,6-bisphosphate. Glucose starvation, increased concentration of inorganic phosphate, and low concentrations of glycolytic intermediates trigger phosphorylase activity of HPrK/P, leading to dephosphorylation of HPr-Ser-P (20). HPr-Ser-P binds to CcpA, and this complex interacts with the *c*atabolite *r*esponsive *e*lement (Cre-box) located in or near promoter regions (1, 28) of many genes, especially those involved in catabolic pathways (44, 61). In most cases this interaction leads to repression of gene expression. Consequently, these CcpA/HPr-Ser-P-controlled genes are up-regulated in *ccpA*- and *hprK*-deficient mutants. These components, which were studied in great detail particularly in *Bacillus subtilis* (59), have also been identified in *L. monocytogenes* (2, 3, 10, 12, 24), and the same arrangement of *ptsH* and *ptsI* in an operon structure as in *B. subtilis* was reported (12, 13).

A direct influence of the central regulatory CCR protein CcpA on PrfA activity has been ruled out (3), but a recent report describing activation and inhibition of PrfA in appropriate *B. subtilis* mutants suggests that the second key player of CCR control, HPr-Ser-P (14), might be responsible for the modulation of the PrfA activity.

To analyze the interference of components of CCR and PTS with PrfA activity, we characterized *ptsH*, *hprK*, and *ccpA* mutations in *L. monocytogenes* and studied the effect of these mutations on gene expression with emphasis on PrfA-dependent virulence gene expression. Additionally, the phosphorylation status of HPr of *L. monocytogenes* wild-type after growth with different carbon sources was examined. The results suggest that HPr-Ser-P does not directly modulate PrfA activity but, rather, that other component(s) involved in uptake of carbohydrates by PTS permeases may interfere with PrfA.

MATERIALS AND METHODS

General techniques. PCR amplifications, cloning procedures, isolation of chromosomal DNA, and DNA manipulations were carried out according to standard procedures (54). Cycle sequencing was performed using a CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter), and sequencing reactions were run on an XL2000 Beckman Coulter Sequencer. The *Listeria* home page of the Institut Pasteur (http://www.genolist.pasteur.fr/ListiList/) was used for sequence comparison. All oligonucleotides used in this study were synthesized by Sigma-Genosys.

Bacterial strains and growth conditions. The *Escherichia coli* strain XL2-Blue was used for cloning and construction of the mutagenesis vectors. *L. monocytogenes* EGD-e strains and *L. monocytogenes* P14-A (52) were grown under aerobic conditions in brain heart infusion (BHI) medium (Difco), in Luria-Bertani medium (LB), or in chemically defined minimal medium (MM) (50) supplemented with different sugars for *L. monocytogenes* at 37°C or 42°C with antibiotics if required. Erythromycin was used at a concentration of 5 μg/ml for *L. monocytogenes* and at 300 μg/ml for *E. coli*. Fresh stock solutions of carbohydrates (glucose, cellobiose, mannose, and glycerol) were filter sterilized and added to the culture medium at a final concentration of 50 mM. The linear extrapolation method was used to calculate the growth rate, reported as generation time.

Disruption of the *ccpA*, *hprK* (lmo2483), and *ptsH* (lmo1002) gene in *L. monocytogenes*. The insertion mutants were constructed by using *L. monocytogenes* Sv1/2a EGD-e as the parental strain. Insertion mutants were obtained by homologous recombination using constructs derived from the pLSV101 mutagenesis vector (30, 69). Plasmid pLSV101 carries an erythromycin resistance gene, a gram-negative *ori*, and a gram-positive temperature-sensitive origin of replication.

Internal (N-terminal) fragments of 307 bp (*ccpA*), 306 bp (*hprK*), and 146 bp (*ptsH*) were PCR amplified from chromosomal DNA derived from *L. monocytogenes* EGD-e by using the following primer pairs: ccpA-BamHI (TGTTGCA CGGGA**T**CCGAACG), ccpA-EcoRI (AAGTACTTG**G**A**A**TTCTTTATCCTC), hprK-BamHI (ATGACAAAATCGG**A**TCCGGTAAAG), hprK-EcoRI (TGCT GCTAC**G**AATTCTTTCGG), ptsH-BamHI(2) (ATGTAAATTAT**G**GA**T CC**A GCAAG), and ptsH-SalI (GATTTAAG**GTCGACT**TTTTTACC) (sites for the restriction endonucleases are underlined; boldface indicates deviation from original sequence). The purified PCR products were digested with the corresponding restriction enzymes and cloned via the restriction sites into pLSV101 to yield the mutagenesis plasmids pSM1 (*ccpA*), pSM2 (*hprK*), and pSM3 (*ptsH*). These plasmids were transformed by electroporation into *L. monocytogenes* EGD-e, and insertion mutants were obtained by selection on erythromycin at 42°C. Integration of the vectors in the genes *ccpA*, *hprK*, and *ptsH* were confirmed by PCR and sequencing using the oligonucleotides listed in Table 1. Revertants were obtained by subcultivating the insertion mutants at 30°C without erythromycin. Precise excision of the single plasmid insertion was confirmed by PCR analysis and sequencing.

Preparation of supernatant and cellular and surface-associated proteins of *L. monocytogenes* strains. Overnight cultures of *L. monocytogenes* were diluted 1:10 into fresh BHI medium or defined MM and grown to an optical density of 0.6 and 1.0 at 600 nm (OD_{600}), respectively. Each culture was then centrifuged for 10 min at 6,000 rpm at 4°C.

The supernatant (containing LLO and ActA) was precipitated on ice with 10% trichloroacetic acid, pelleted by centrifugation at 6,000 rpm for 30 min at 4°C, and washed twice in acetone. After a washing step, the pellet was resuspended in urea buffer (7 M urea, 2 M thiourea, and dithiothreitol) and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate).

For extraction of cell wall proteins (containing LLO), the bacteria pellet was washed twice in 1× phosphate-buffered saline (PBS) and resuspended in 1.8 ml of 1× PBS with 1% sodium dodecyl sulfate (SDS). Subsequently, the sample was mixed gently for 20 min at room temperature and then centrifuged at 6,000 rpm for 5 min at room temperature. The supernatant was precipitated with trichloroacetic acid as described previously (see above).

For preparation of cellular proteins (containing PrfA and ActA), the pellet was washed twice in 1× PBS, resuspended in cold lysis buffer (1× PBS with

TABLE 2. Oligonucleotides used in real-time RT-PCR

Oligonucleotide	Sequence (5′→3′)	Target DNA
actA-F	AGCAGATGAGTCTTCACCACA	*actA*
actA-R	CCCTGCACTTTTATCAACAATC	*actA*
hly-F	ATGCAATTTCGAGCCTAACCT	*hly*
hly-R	TTATTGTCTTGATTAGTCATAC	*hly*
hpt-F	AAGCGCTAGGATGGAGCACAA	*uhpt*
hpt-R	CAACTGCAATAATCGAGCAAAG	*uhpt*
inlA-F	ATATTAGTATTTGGCAGCGG	*inlA*
inlA-R	TTTTTCCTAAGACCGTCTTC	*inlA*
inlB-F	TTTCTATCAGCCAGTCACTATTGGA	*inlB*
inlB-R	CGCGTCCCTGCTTCTACTTTTGT	*inlB*
inlC-F	CAAATACAGGTGGACTAACTAGA	*inlC*
inlC-R	GATATCCATCTTCCATCTGGGT	*inlC*
lgt-F	CGAGAAGACTGCCATTGCCTATAA	*lgt*
lgt-R	CCCAACACGATACTCCGAAAGTAA	*lgt*
lmo1001-F	GAATTTAAGGATGGCCTTACAGGAA	lmo1001
lmo1001-R	GGTAATTCTTGTGGTTAACCACTGT	lmo1001
lmo1003-F	GGGGAAGCAGTAGGACTTTATCGTA	*ptsI*
lmo1003-R	CACAGATTTTCCGTCCATTCCGGAT	*ptsI*
lmo2480-F	GCGAGAAGACTGCCATTGCCTATAA	lmo2480
lmo2480-R	CCCCAACACGATACTCCGAAGTAA	lmo2480
lmo2481-F	GTATGATACGATTATGCGAGGTCTT	lmo2481
lmo2481-R	AGAAAGAGCCATCTCAATACCTTCA	lmo2481
lmo2484-F	CGACAGCGCTACTAGCGAGTTTTA	lmo2484
lmo2484-R	GCATAATCGCATTGACAACGAAGGT	lmo2484
mpl-F	TTGCTCCAGAGGCCACTACATGT	*mpl*
mpl-R	GATACCACTTTCCCAAACGAAGTG	*mpl*
plcA-F	AATGCATCACTTTCAGGTGTATTAGA	*plcA*
plcA-R	GTTGATTAGTGGTTGGATCCGATAA	*plcA*
plcB-F	TCAAGGAATATATGATGCGGATCAT	*plcB*
plcB-R	CTTTGCTCCTGTTATTTTCGCATTA	*plcB*
prfA-F	CAGGCTACCGCATACGTTATCAAA	*prfA*
prfA-R	AGCCAAGCTTCCCGTTAATCGAAA	*prfA*
rpoB-F	AAGTAACTGGCGGAATCGATA	*rpoB*
rpoB-R	GGAATCCATAGATGGACCGTT	*rpoB*
tyrS-F	TGCCGTTTGCAAATTGGTGGTAGT	*tyrS*
tyrS-R	GATTTCCCAAATTTCGTTCCATCAG	*tyrS*

additional protease inhibitor [Roche]), and transferred into a 2-ml BLUE TUBE (Q-Biogene) filled with silica sand. The tube was shaken six times for 30 s each at speed 6.5 in a bead beater (FP120 Fast Prep cell disrupter; Savant Instruments, Inc.). The cell debris was removed by centrifugation at 14,000 rpm for 30 min at 4°C. Total protein concentrations were determined using a Bio-Rad-Protein Microassay (Bio-Rad).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to standard protocols (33). After SDS-PAGE cytoplasmatic cell surface proteins and proteins from the culture supernatant were subjected to Western blotting onto nitrocellulose membranes, and *L. monocytogenes* proteins were immunodetected using the following *Listeria*-specific rabbit polyclonal antibodies and dilutions: anti-LLO (1:1,000; J. Kreft), anti-ActA (1:1,000 [47]), anti-PrfA (1:750; see below) and anti-HPr (1:3,000; see below).

Cloning and expression of HPr and PrfA and generation of antisera against both proteins. A PCR fragment encoding the open reading frame (ORF) of *ptsH* was amplified with *Pfu* DNA polymerase (Promega) using *L. monocytogenes* EGD-e wild-type (WT) as a template and the primers ptsH-NdeI (5′-GGAGA ATGTAACATATGGAACAAG CAAG-3′, introducing an NdeI site [underlined]) and ptsH-BamHI (5′-TGCTGCGGATCCTTTCAACTCTTT-3′, introducing a BamHI site [underlined]) and subsequently cloned into the pET3c expression vector (Novagen), yielding the plasmid pET3c-HPr. The nucleotide sequence of the amplified sequence was verified by automated sequencing. The pET3c-HPr vector was transformed into BL21(DE3) delta(ptsHIcrr)/pLysS (W. Hillen, University of Erlangen-Nürnberg), and protein purification was carried out following the manufacturer's instructions (pET expression system; Novagen) with the following modifications: HPr was purified according to its molecular weight using a Superdex75 HiLoad 16/60 gel filtration column applied to an ÄKTAprime protein purification system (Amersham Biosciences). Gel filtration was achieved in 20 mM Tris-HCl (pH 7.5) plus 50 mM NaCl at a flow rate of 1 ml/min. Following elution, protein fractions were analyzed by Coomassie staining of SDS-polyacrylamide gels according to the method of Laemmli (33). HPr-containing fractions were pooled, and native HPr protein was finally stored in 20 mM Tris (pH 7.5) and 50 mM NaCl at −80°C.

The N-terminal His-tagged PrfA protein was isolated from a recombinant *E. coli* strain (7). The purification of recombinant PrfA was performed with an ÄKTAprime protein purification system (Amersham Biosciences) and HiTrap Chelating HP columns (Amersham Biosciences) as recommended by the manufacturer. Following elution, protein fractions were analyzed by Coomassie staining of SDS-polyacrylamide gels according to the method of Laemmli (33). PrfA-containing fractions were pooled, and recombinant PrfA protein was finally

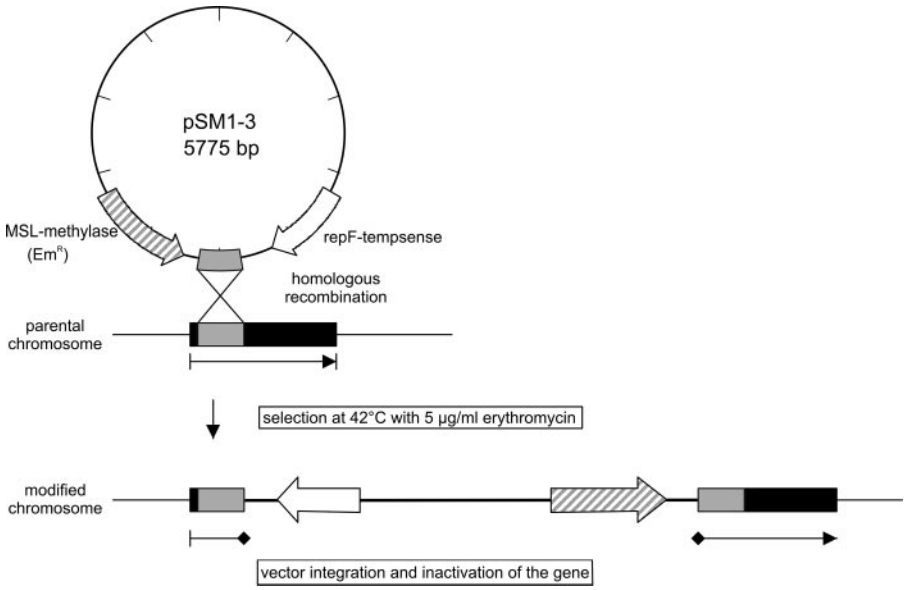

FIG. 1. Schematic presentation of vector integration and inactivation of the genes *ccpA*, *hprK*, and *ptsH*. A fragment of *L. monocytogenes* EGD-e chromosomal DNA was cloned into the multiple cloning site of pLSV101 resulting in the plasmids pSM1 (*ccpA*), pSM2 (*hprK*), and pSM3 (*ptsH*). Replication of pSM1-3 in *Listeria* is stopped by increasing the temperature to 42°C. This selects for events in which pSM1-3 has integrated into the host chromosome by homologous recombination at the point of homology provided by the cloned DNA.

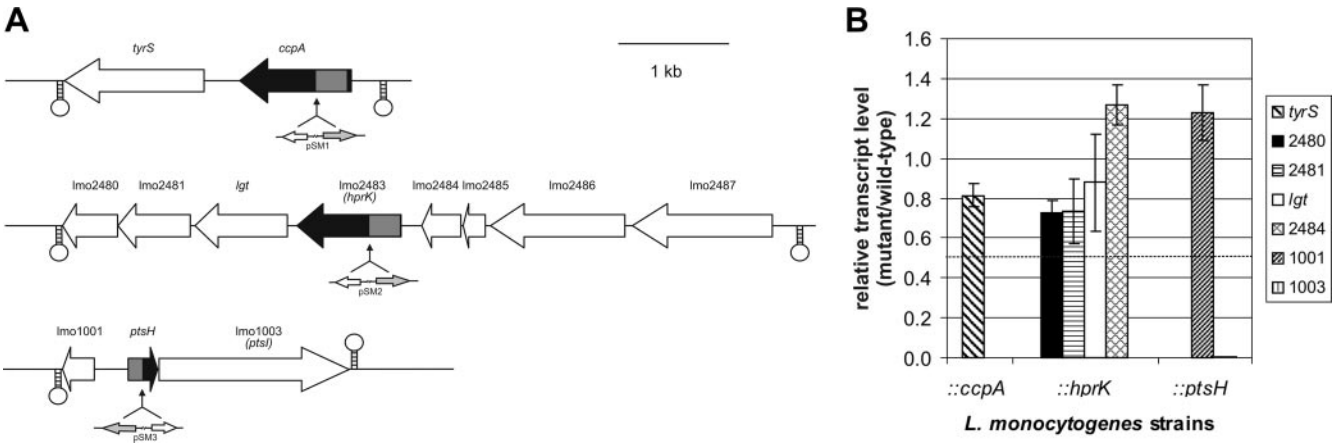

FIG. 2. (A) Map of genetic loci disrupted during the present study with surrounding regions. All genes are drawn approximately to scale by using the *L. monocytogenes* EGD-e genome sequence data (http://genolist.pasteur.fr/ListiList/). The genes disrupted in *L. monocytogenes* EGD-e are indicated in black; adjacent ORFs are shown in white. The sites of pSM1-3 insertion into the corresponding genes are indicated by black arrows. Shaded areas within black arrows depict the regions of the genes cloned into pSM1-3. Stem-loop structures are used to illustrate putative terminator regions. Descriptions of the genes are as follows: *tyrS*, tyrosyl-tRNA synthetase; *ccpA*, catabolite control protein A; lmo2480, similar to acetyltransferase; lmo2481, similar to *B. subtilis* HPr-Ser-P phosphatase; *lgt*, highly similar to prolipoprotein diacylglyceryl transferase; lmo2483, HPr-Ser-P kinase/phosphatase; lmo2484, similar to *B. subtilis* YvlD protein; lmo2485, similar to *B. subtilis* YvlC protein; lmo2486, unknown; lmo2487, similar to *B. subtilis* YvlB protein; lmo1001, similar to *B. subtilis* protein YkvS; *ptsH*, PTS phosphocarrier protein HPr; lmo1003, phosphotransferase system enzyme I. (B) Transcriptional analysis with real-time RT-PCR to study the polar effect of pSM1-3 insertion on the transcription of genes located up- and downstream in the *ccpA* (*tyrS*), *hprK* (lmo2480, lmo2481, *lgt*, and lmo2484), and *ptsH* (lmo1001 and lmo1003) insertion mutants (indicated as ::*ccpA*, ::*hprK*, and ::*ptsH*, respectively) (see panel A). The strains were grown in BHI medium to an OD_{600} of 1.0, where the RNAs were prepared. The relative increases in the expression of the neighboring genes in the mutants compared to the wild-type strain (relative transcript level of mutant/wild-type) are depicted here. The relative expression levels of the genes studied was normalized to the housekeeping gene *rpoB* as described elsewhere (43, 60). The RT-PCR was performed with three independently isolated RNAs from the various strains in duplicate. The values represented here are means of the six obtained values, and the error bars indicate the standard deviations from the means. Relative expression levels of >1.8 or <0.55 (marked by dotted line) were considered to be differentially regulated based on microarray data (for details, see Material and Methods).

stored in 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2.5 mM $CaCl_2$, 1 mM dithiothreitol, and 20% (vol/vol) glycerol at −80°C.

Anti-HPr and anti-PrfA sera were raised in rabbits by using purified HPr and His-tagged PrfA, respectively, as antigens. Aliquots of 150 to 200 μg of the particular protein per injection were mixed with Freud's adjuvant (Difco Laboratories), and after a total of four injections at 2-week intervals, the antisera were obtained. The immunization was performed by Charles River, Kisslegg, Germany.

Determination of HPr and HPr-Ser46/His15-P in the *L. monocytogenes* strains. The strains of *L. monocytogenes* were grown in defined MM supplemented with 50 mM glucose, 50 mM cellobiose, 50 mM mannose, or 50 mM glycerol to OD_{600} of 0.6 and 1.0. Each culture was then centrifuged for 10 min at 6,000 rpm at 4°C.

For the preparation of cellular proteins, the pellet was washed twice with 50 mM Tris-HCl, pH 7.5, and 50 mM EDTA, pH 8.0, buffer and resuspended in the same buffer with additional protease inhibitor (Roche). The cells were disrupted by a FP120 Fast Prep bead beater (see the paragraph on preparation of cellular proteins above), and subsequently total protein concentrations were determined using a Bio-Rad-Protein Microassay. Cell extracts untreated or incubated at 70°C for 10 min to hydrolyze the heat-labile HPr-His15-P were separated on a 15% nondenaturing polyacrylamide gel and immunoblotted using specific rabbit polyclonal antibodies against HPr.

Determination of hemolytic activity. *L. monocytogenes* strains were grown in different media at 37°C to an OD_{600} of 0.6 or 1.0. The hemolytic activity in the supernatants was determined as described previously (53). Briefly, 50 μl of culture supernatant was incubated in 1 ml of 4% sheep erythrocyte suspension for 30 min at 37°C. After incubation the tubes were centrifuged at 2,500 rpm for 5 min at room temperature. Hemolytic activity was estimated at 543 nm using an Ultrospec 2100 Pro photometer (Amersham).

RNA isolation. RNA from the *L. monocytogenes* strains grown to a later growth phase (corresponding to an OD_{600} of 1.0) was extracted using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol with some modifications to lyse the bacteria. Cell pellets were suspended in lysis buffer and placed in a 2-ml BLUE TUBE filled with silica sand (Q-Biogene). The tube was shaken three times for 45 s each, with a 1-min interval on ice in between each shaking, at a speed setting of 6.5 in a FP120 FastPrep bead beater (Savant Instruments, Inc.). Residual DNA was removed on a column with QIAGEN RNase-free DNase.

Microarray hybridization and data analysis. Transcriptome analyses were performed using whole-genome DNA microarrays that contained synthetic 70-mer oligodeoxyribonucleotides covering all ORFs of the *L. monocytogenes* genome. The oligonucleotides (sequences available at http://www.operon.com/arrays/oligosets_listeria.php) were spotted on epoxy-coated glass slides from Quantifoil according to the manufacturer's instructions by T. Chakraborty (Institut für Medizinische Mikrobiologie, Giessen, Germany). Each oligonucleotide was spotted twice on a slide to generate two replicates for each oligonucleotide. A total of two RNA samples was prepared for cDNA labeling and hybridization for each combination. Briefly, equal amounts (40 μg) of the RNAs were used to synthesize cDNA differentially labeled with Cy3-dCTP and Cy5-dCTP (Amersham Pharmacia) during a first-strand reverse transcription reaction with Superscript II RNase H^- reverse transcriptase and 9 μg of random primers (Life Technologies) with dye swap. The two cDNA samples were combined, diluted in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) SDS, hybridized to the microarray, and incubated at 65°C for 16 h. After a washing step, the slides were scanned using ScanArray HT and analyzed using ScanArray express software (Perkin Elmer). Spots were flagged and eliminated from analysis when the signal-to-noise ratio was less than 3 or in obvious instances of high background or stray fluorescent signals. The LOWESS method of normalization (70) was performed for the background corrected median intensity of the spots. We had a total of four slides per experiment from the two biological replicates. The eight normalized ratios per spot were further analyzed with Microsoft Excel and Significance Analysis of Microarrays software for statistical significance (62). To determine the significance of differential expression, RNA was isolated from *L. monocytogenes* wild-type strain grown in BHI medium, and 30 to 40 μg of this RNA was labeled either with Cy3-dCTP or with Cy5-dCTP. The two cDNA probes generated were hybridized onto the same slide, and the data were analyzed as described above. Since the values ranged from 0.6 to 1.6, we considered values of >1.8 and <0.55 to be significant in our experiments (data not shown).

Real-time (RT-PCR). Real-time reverse transcription-PCR (RT-PCR) was conducted on total RNA samples independently isolated as for transcriptome

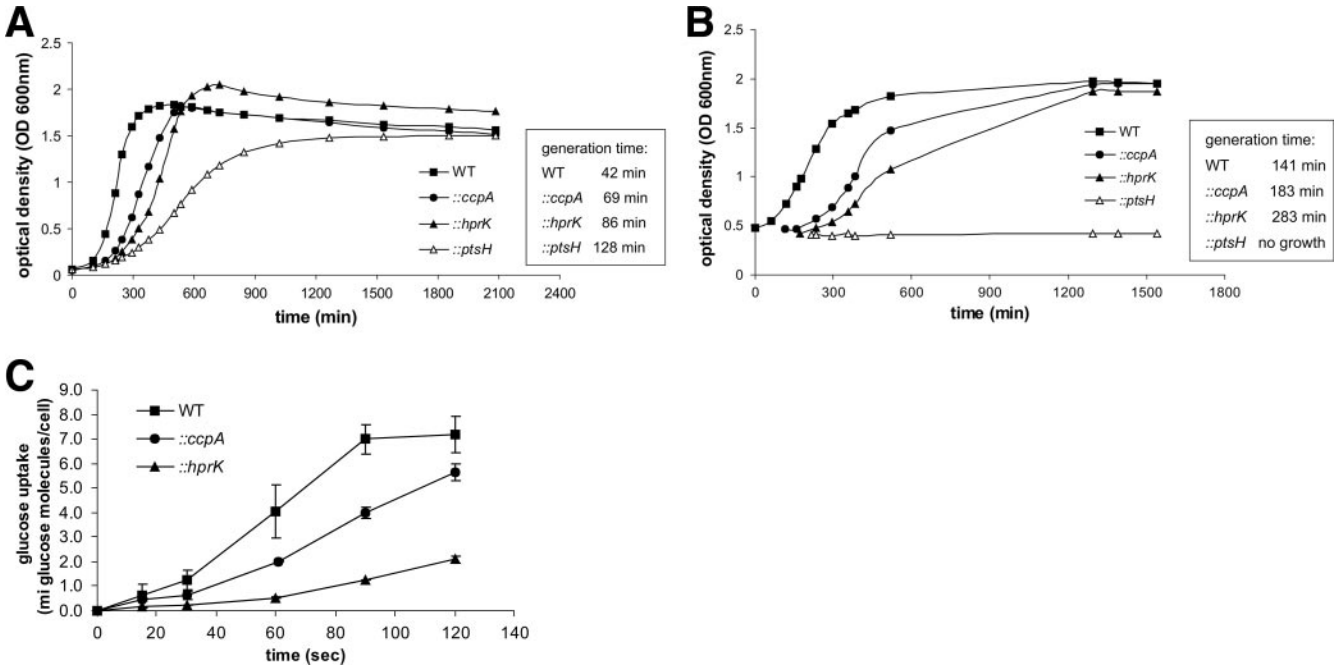

FIG. 3. Growth of *L. monocytogenes* EGD-e wild-type, *ccpA*, *hprK*, and *ptsH* insertion mutants. The generation times of WT and insertion mutants are shown to the right. (A) The strains were grown in BHI medium. (B) Strains were shifted from BHI medium to MM with 50 mM glucose. The cells were grown first in BHI medium to an OD_{600} of 0.5. After centrifugation the cells were washed twice in MM supplemented with 50 mM glucose and then resuspended in MM with 50 mM glucose where growth was determined. The growth curves are representative of four replicates. (C) Glucose uptake in *L. monocytogenes* EGD-e wild-type, the *ccpA*, and *hprK* insertion mutants. To measure the uptake of radioactively labeled D-[U-^{14}C]glucose the strains were grown in LB medium supplemented with 50 mM glucose to an OD_{600} of 1.0. The *y* axis indicates the number (10^6) of molecules of glucose taken up per bacterial cell. The glucose uptake measurements were performed in triplicate, and the error bars indicate standard deviations of the means for the three measurements.

analysis experiments. Before RT-PCR was performed, the absence of DNA from RNA samples was verified by PCR amplification of the genes to be assayed with 1 μg of RNA as template. cDNA synthesis was performed as described above from 5 μg of total RNA. Instead of the labeled nucleotides, equal amounts of each (20 mM) dATP, dCTP, dGTP, and dTTP were used. RT-PCR was done in a final volume of 25 μl. Protocol and cycling conditions were carried out according to the manufacturer's protocol of the qPCRCore Kit for SYBR Green-I (Eurogentec). The relative expression levels of the genes studied were normalized to the housekeeping gene *rpoB* (43, 60). All primers used for real-time RT-PCR are listed in Table 2.

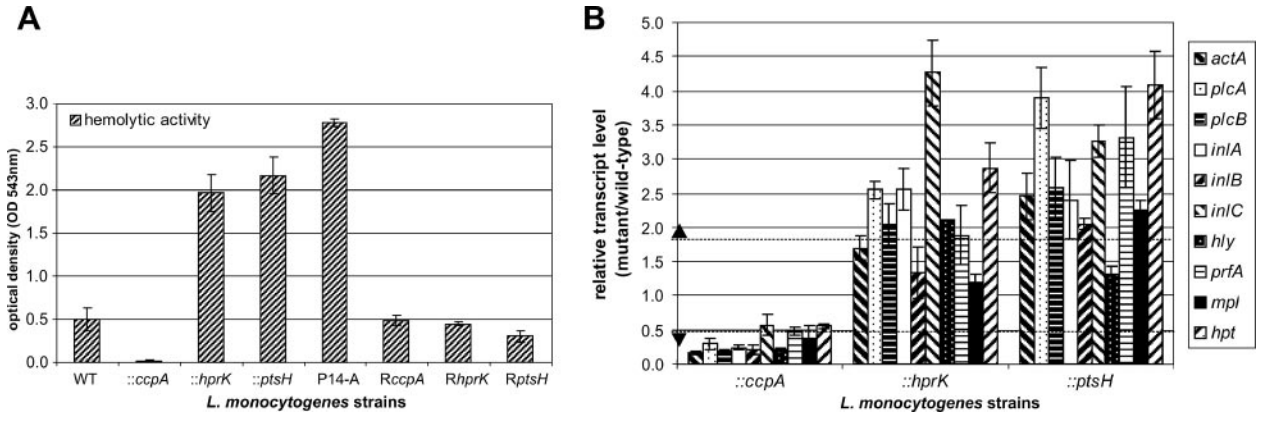

FIG. 4. (A) Hemolytic activity of WT EGD-e, insertion mutants (::*ccpA*, ::*hprK*, and ::*ptsH*), revertants (R*ccpA*, R*hprK*, and R*ptsH*), and the P14-A strain (expressing constitutively active PrfA* due to a G145S exchange [52]) grown in BHI medium to an OD_{600} of 1.0. The hemolytic activity was determined in three independently performed experiments; the error bars indicate standard deviations of the means for the three experiments. (B) Transcriptional analysis of the virulence genes in the insertion mutants *ccpA*, *hprK*, and *ptsH*. The strains were grown in BHI medium to an OD_{600} of 1.0, and RNAs were prepared. The relative changes in the expression of the virulence genes in the mutants compared to the wild-type strain (relative transcript level of mutant/wild-type) are depicted here. The relative expression of the genes studied was normalized to the housekeeping gene *rpoB* as described elsewhere (43, 60). RT-PCR was performed with three independently isolated RNAs from the various strains in duplicate. The values represented here are the means of the six obtained values, and the error bars indicate the standard deviations from the means. Relative expression levels of >1.8 or <0.55 (marked by dotted line) were considered to be differentially regulated based on microarray data (for details, see Material and Methods).

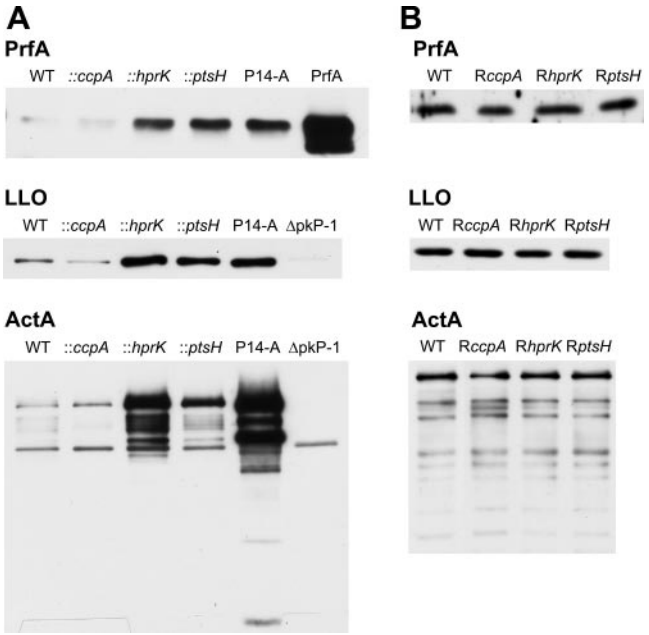

FIG. 5. Western blot analysis of PrfA and PrfA-regulated virulence proteins LLO and ActA. WT EGD-e (A and B), insertion mutants (::*ccpA*, ::*hprK*, and ::*ptsH*) and strain P14-A (A), and revertants (R*ccpA*, R*hprK*, and R*ptsH*) (B) were grown in BHI medium. At an OD_{600} of 1.0 (later growth phase) proteins were prepared, equal amounts of proteins were separated by SDS-PAGE, and equivalent loading of the gels was controlled by Coomassie-staining (data not shown). Loading controls: PrfA, purified PrfA protein; ΔpkP-1, EGD with a deletion in the virulence gene cluster (18).

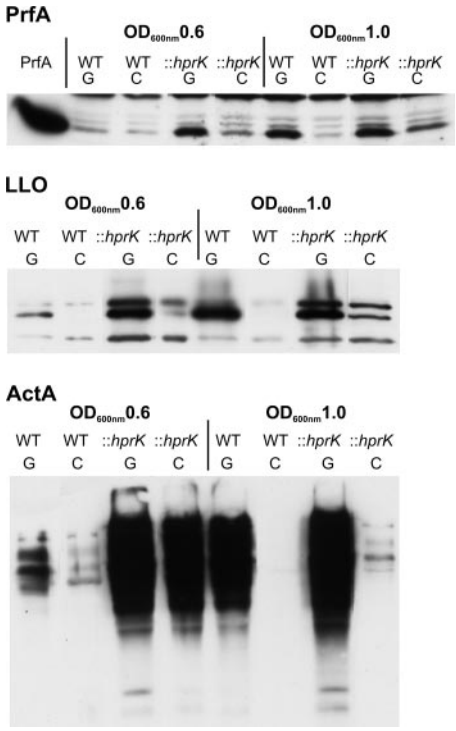

FIG. 6. Western blot analysis of PrfA and PrfA-regulated virulence proteins LLO and ActA. WT EGD-e and the *hprK* insertion mutant (::*hprK*) were grown in MM supplemented with 50 mM glucose (G) or 50 mM cellobiose (C). At OD_{600} values of 0.6 (early growth phase) and 1.0 (later growth phase) proteins were prepared, equal amounts of proteins were separated by SDS-PAGE, and equivalent loading of the gels was controlled by Coomassie-staining (data not shown). PrfA, purified PrfA protein (loading control).

Glucose transport assay. Different *L. monocytogenes* strains were grown in LB medium with 50 mM glucose to an OD_{600} of 1.0. Each culture was then harvested by centrifugation at 5,000 rpm for 3 min at 4°C. The pellet was washed three times in transport buffer (50 mM Tris-HCl [pH 7.2] and 20 mM $MgCl_2$) and resuspended in the same buffer. Labeled D-[U-^{14}C]glucose (2 μCi/ml; Amersham Pharmacia) was mixed with unlabeled sugar D-[U-^{12}C]glucose and added to the cells (final concentration, 2 mM) and incubated at 37°C. Aliquots (50 μl) were taken out at different time points (0 s, 15 s, 30 s, 60 s, 90 s, and 120 s) and filtered rapidly under vacuum through a 0.45-μm-pore-size cellulose nitrate filter (Sartorius). The filters were washed three times with 3 ml of cold saline (0.9% NaCl) and dried for 20 min at 42°C. Radioactivity was determined using a liquid scintillation counter (1214 Rackbeta; PerkinElmer). Additionally, the number of CFU of each sample was determined, and the glucose uptake was calculated for each strain.

Microarray data accession number. The data obtained in this study have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE6028.

RESULTS

Construction and characterization of *ccpA*, *hprK* (lmo2483), and *ptsH* mutants of *L. monocytogenes* EGD-e. Mutations were introduced in the genes *ccpA*, *hprK* (lmo2483), and *ptsH* by insertion of the plasmids pSM1 to pSM3 (pSM1-3) (Fig. 1), each carrying a specific fragment from the 5′ part of the gene. After electroporation of the plasmids into *L. monocytogenes* EGD-e at 30°C, insertion mutants were selected on erythromycin-containing BHI agar plates by incubation at 42°C, as described earlier (69). The insertions were confirmed by PCR and sequence analysis (results not shown). No Em^r colonies were obtained with the EGD-e strain carrying the vector plasmid alone after shift to 42°C. Southern blot analyses confirmed that only a single (specific) insertion had occurred in all three mutants. The absence of CcpA and HPr in the corresponding insertion mutants was shown by immunoblotting with polyclonal antibodies against these proteins. The loss of functional HPrK/P was determined by the absence of HPr phosphorylation at Ser46 by Western blot analysis (data not shown).

All attempts to obtain in-frame deletion mutants of these genes failed although we applied all well-established methods.

The three insertion mutants were stable upon growth in all growth media tested at 37°C while a >95% loss of Em^r, indicative of plasmid excision, was observed after growth at 30°C for 10 generations. The erythromycin-sensitive colonies seem to represent mostly precise revertants, as judged by the growth kinetics in culture medium and the wild-type sequences of the corresponding genes in the colonies tested (see below).

To rule out the effect of erythromycin on the growth rate or protein expression of the insertion mutants, we compared the erythromycin-sensitive *L. monocytogenes* parental strain (WT) with an erythromycin-resistant strain (WT Em^r) bearing pLSV101 inserted in the *int* gene (putative integrase of bacteriophage A118). Insertion in the *int* gene has been shown previously to have no influence on growth and gene expression of *Listeria* (A. Frentzen, unpublished data). Expression levels of PrfA and PrfA-regulated genes in these two strains were

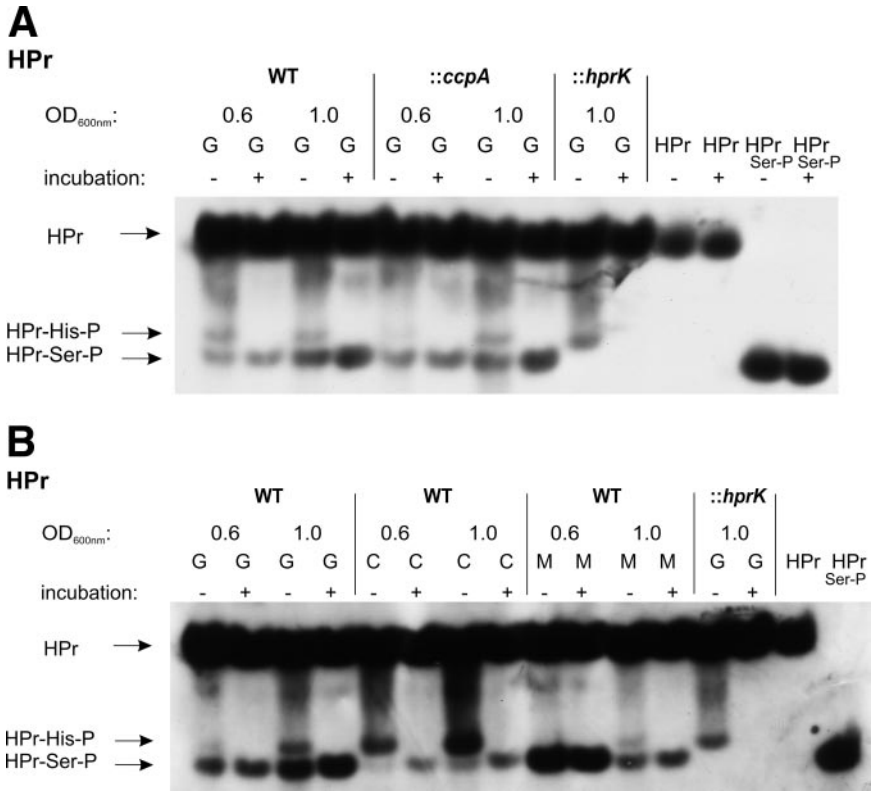

FIG. 7. Western blot analysis of the HPr and its phosphorylated forms (HPr-His15-P or HPr-Ser46-P) to determine the amount of HPr-Ser46-P in *L. monocytogenes* strains. Equal amounts of cell extracts untreated (−) or incubated at 70°C for 10 min (+) to hydrolyze the heat-labile HPr-His15-P were separated on a 15% nondenaturing polyacrylamide gel and immunoblotted using specific rabbit polyclonal antibodies against HPr. The positions of HPr, HPr-Ser46-P, and HPr-His15-P are indicated. Equivalent loading of the gels was controlled by Coomassie-staining (data not shown). (A) Detection of HPr and its phosphorylated forms (HPr-His15-P or HPr-Ser46-P) in WT EGD-e and the *ccpA* mutant grown in MM supplemented with 50 mM glucose to OD_{600}s of 0.6 and 1.0. The *hprK* mutant (control; only able to phosphorylate HPr at His15; grown in MM supplemented with 50 mM glucose to an OD_{600} of 1.0) and purified HPr and HPr-Ser46-P with and without incubation were separated on the nondenaturing polyacrylamide gel to show that the incubation at 70°C for 10 min has no effect on HPr or HPr-Ser46-P. (B) Determination of HPr and its phosphorylated forms (HPr-His15-P or HPr-Ser46-P) in WT EGD-e grown in MM supplemented with 50 mM glucose (G), 50 mM cellobiose (C), or 50 mM mannose (M) to OD_{600}s of 0.6 and 1.0. The *hprK* mutant (control; see panel A) and purified HPr and HPr-Ser46-P were separated on the gel to indicate the different HPr forms. ::*ccpA* and ::*hprK*, insertion mutants of *ccpA* and *hprK*, respectively.

similar after growth with (WT Em^r) or without (WT and WT Em^r) erythromycin (data not shown).

Since the three mutated genes are part of operons (Fig. 2A), we determined the transcript levels of the flanking genes of each of the three mutants by real-time RT-PCR and compared them to those of the corresponding wild-type genes. As shown in Fig. 2B, the insertions in *ccpA* and *hprK* did not significantly reduce the transcript levels of the genes located downstream (*tyrS* in the case of *ccpA* and lmo2480, lmo2481, and *lgt* in the case of *hprK*) or of the gene lmo2484 located upstream of *hprK*. The insertion in *ptsH* appears to completely abolish the transcription of *ptsI* (lmo1003), which is expected as *ptsI* is located downstream of *ptsH* in the *ptsHI* operon (12). However, this strong reduction of *ptsI* transcription in the *ptsH* mutant should not affect the phenotype caused by the *ptsH* mutation since the gene product of *pstI* is enzyme I, which needs HPr (the gene product of *ptsH*) as a substrate. No polar effect was detected on gene expression of lmo1001 located upstream of *ptsH*.

Growth rates of the three mutants were determined in BHI medium (this nutrient-rich medium contains, in addition to glucose, several other poorly defined carbon sources) and in a defined minimal medium (MM) with 50 mM glucose. All three mutants showed a delay in growth in BHI medium (Fig. 3A) which—as expected—was most pronounced for the *ptsH* mutant, which can no longer take up carbohydrates via the PTS pathway. In the glucose-containing minimal medium, the *ccpA* mutant also grew at a slower growth rate than the wild-type strain while the *ptsH* mutant did not grow at all in this medium (Fig. 3B). The *hprK* mutant exhibited an even slower growth rate than the *ccpA* mutant (Fig. 3B), suggesting that the uptake of glucose [or other essential nutrient(s)] might be impaired in this mutant. This assumption is strongly supported by the reduced rate of D-[U-^{14}C]glucose uptake in the *hprK* mutant and (to a lesser extent) in the *ccpA* mutant compared to the wild-type strain (Fig. 3C).

Revertants from each of the three mutants behaved like the wild-type strain with respect to growth and glucose uptake (data not shown).

Expression of PrfA-dependent virulence genes is enhanced in *hprK* and *ptsH* mutants but not in the *ccpA* mutant. When the three mutants and the wild-type strain were grown on blood agar plates, we noticed much stronger hemolytic zones

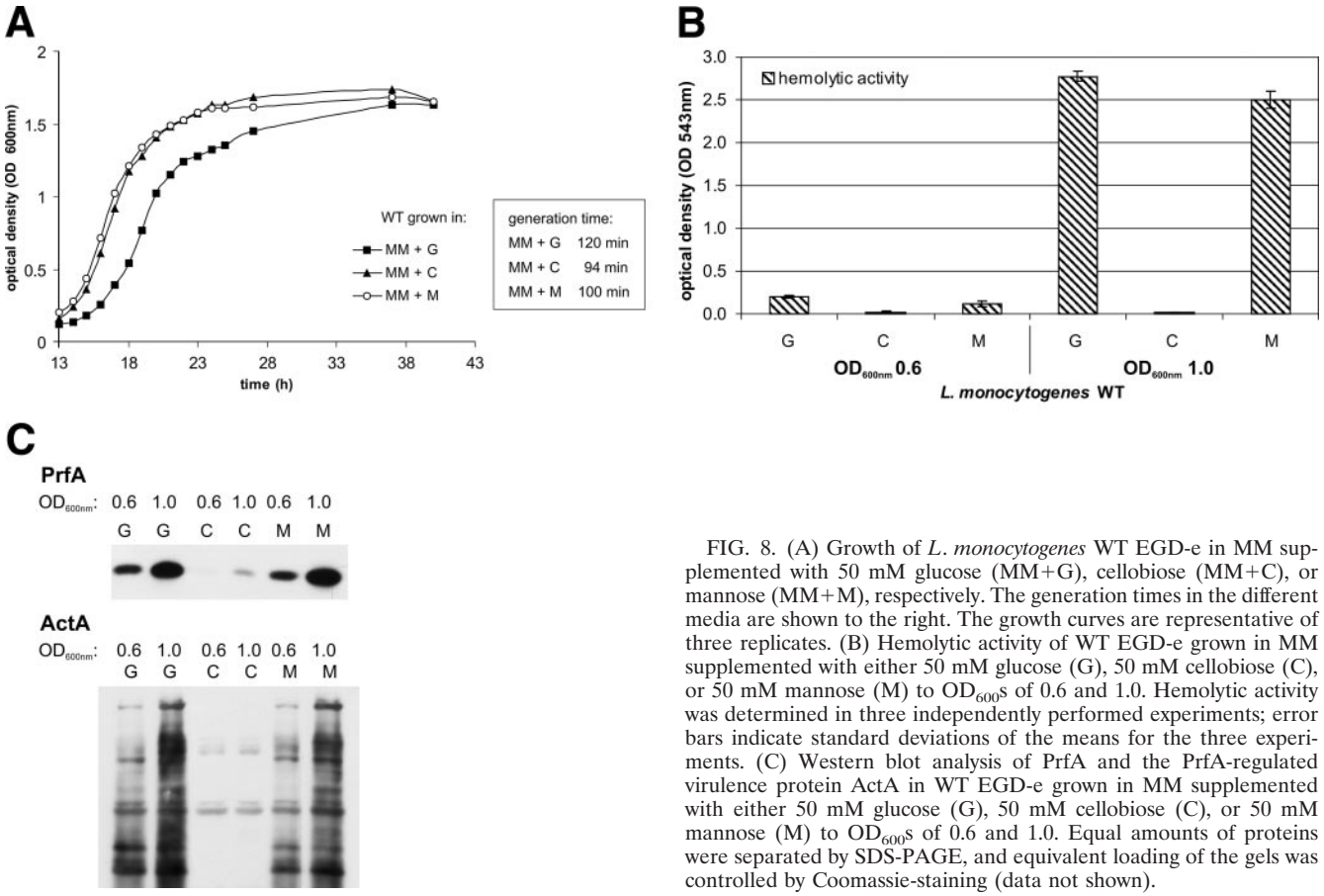

FIG. 8. (A) Growth of *L. monocytogenes* WT EGD-e in MM supplemented with 50 mM glucose (MM+G), cellobiose (MM+C), or mannose (MM+M), respectively. The generation times in the different media are shown to the right. The growth curves are representative of three replicates. (B) Hemolytic activity of WT EGD-e grown in MM supplemented with either 50 mM glucose (G), 50 mM cellobiose (C), or 50 mM mannose (M) to OD_{600}s of 0.6 and 1.0. Hemolytic activity was determined in three independently performed experiments; error bars indicate standard deviations of the means for the three experiments. (C) Western blot analysis of PrfA and the PrfA-regulated virulence protein ActA in WT EGD-e grown in MM supplemented with either 50 mM glucose (G), 50 mM cellobiose (C), or 50 mM mannose (M) to OD_{600}s of 0.6 and 1.0. Equal amounts of proteins were separated by SDS-PAGE, and equivalent loading of the gels was controlled by Coomassie-staining (data not shown).

around the colonies of the *hprK* and *ptsH* mutants than around those of the wild-type strain and the *ccpA* mutant (data not shown), suggesting enhanced synthesis of LLO in the *hprK* and *ptsH* mutants. Measurement of the hemolytic activity in the supernatants of BHI-grown cultures of these strains (arising from secreted LLO), indicated, indeed, an enhanced activity of the PrfA-dependent virulence factor in the *hprK* and *ptsH* mutants compared to the wild-type strain and the *ccpA* mutant (Fig. 4A). Real-time RT-PCR performed with RNA isolated from the three mutants and the wild-type strain (harvested in the exponential growth phase in BHI medium) showed three- to fivefold increases in transcript levels of not only the *hly* gene but also the other PrfA-dependent virulence genes of *L. monocytogenes* (Fig. 4B) in the *hprK* and the *ptsH* mutants compared to the wild-type strain, while transcription of these genes appeared to be slightly reduced in the *ccpA* mutant.

As shown in Fig. 5A, the amounts of PrfA, LLO, and ActA in the *hprK* and *ptsH* mutants measured by immunoblotting also reached higher levels than the wild-type strain and the *ccpA* mutant, and these levels—especially in the case of ActA—were even higher than expected from the increased transcript levels, which may be due to posttranscriptional control of ActA expression (67).

The increased amounts of the PrfA-dependent gene products were similar to results in *L. monocytogenes* P14-A, which expresses a constitutively active PrfA* protein (due to a G145S exchange in PrfA [52]). The parental P14 strain expresses the same amount of all studied PrfA-dependent proteins as the EGD-e wild-type strain (data not shown).

Revertants of all three mutants which had lost the insertions again showed similar hemolytic activity as the wild-type strain (Fig. 4A) and produced wild-type levels of PrfA, LLO, and ActA (Fig. 5B).

In the glucose-containing minimal medium, production of the tested PrfA-dependent virulence factors was rather low in the *L. monocytogenes* wild-type strain during the early exponential growth phase (OD_{600} of 0.6), when balanced growth is expected, but highly enhanced in the late growth phase (OD_{600} of 1.0). In the presence of cellobiose (25 or 50 mM), synthesis of these proteins (Fig. 6) was strongly inhibited after growth of the *L. monocytogenes* strains in this minimal medium.

In the glucose-containing minimal medium, the *hprK* mutant showed enhanced synthesis of PrfA and PrfA-dependent proteins (compared to the wild-type strain) in both growth phases. PrfA activity in this mutant was still inhibited by cellobiose (Fig. 6).

PrfA activity does not correlate with the level of HPr-Ser-P when *L. monocytogenes* is cultured in the presence of different PTS sugars. As expected, the *hprK* mutant was unable to phosphorylate HPr at Ser46 (Fig. 7A). The *ptsH* mutant cannot produce HPr and, hence, also not HPr-Ser-P. In both mutants up-regulation of the PrfA-dependent virulence genes was observed (Fig. 4B). The level of HPr-Ser-P in the *ccpA* mutant was similar to that of the wild-type strain (Fig. 7A). These data

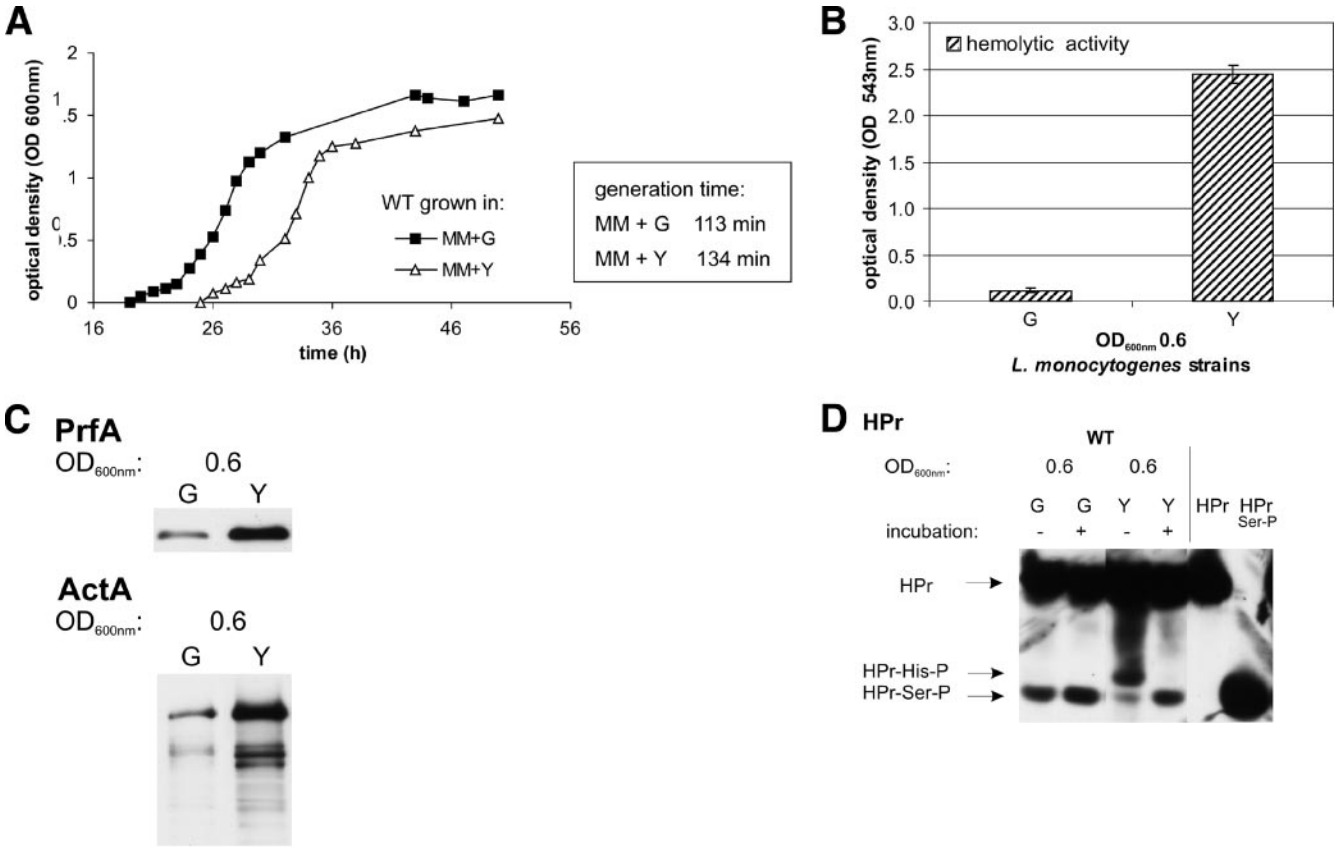

FIG. 9. (A) Growth of *L. monocytogenes* WT EGD-e in MM supplemented with 50 mM glucose (MM+G) or glycerol (MM+Y). The generation times in the different media are shown at right. The growth curves are representative of three replicates. (B) Hemolytic activity of WT EGD-e grown in MM supplemented with either 50 mM glucose (G) or 50 mM glycerol (Y) to an OD_{600} of 0.6. Hemolytic activity was determined in three independently performed experiments; error bars indicate standard deviations of the means for the three experiments. (C) Western blot analysis of the PrfA and PrfA-regulated virulence protein ActA in WT EGD-e grown in MM supplemented with either 50 mM glucose (G) or 50 mM glycerol (Y) to an OD_{600} of 0.6. Equal amounts of proteins were separated by SDS-PAGE, and equivalent loading of the gels was controlled by Coomassie-staining (data not shown). (D) Detection of HPr and its phosphorylated forms (HPr-His15-P or HPr-Ser46-P) in WT EGD-e grown in MM supplemented with 50 mM glucose (G) or 50 mM glycerol (Y) to an OD_{600} of 0.6 (see legend of Fig. 7). As a control, purified HPr and HPr-Ser46-P were separated on the gel to indicate the different HPr forms.

seem to be in line with a recent report (14) which suggests that HPr-Ser-P may be directly or indirectly involved in the negative modulation of PrfA activity.

The presence of β-glucoside cellobiose, a PTS sugar which, similar to mannose, can serve as an efficient carbon source for *L. monocytogenes*, is known to strongly inhibit PrfA activity (46). We therefore compared the level of HPr-Ser-P in *L. monocytogenes* grown in minimal medium with glucose, cellobiose, or mannose (Fig. 7B). *L. monocytogenes* EGD-e grew with these three PTS sugars as carbon sources with generation times of 120 min (glucose), 94 min (cellobiose), and 100 min (mannose) in the minimal medium (Fig. 8A). As expected, hemolytic activity and expression of *prfA* and the PrfA-dependent *actA* gene were much lower in the presence of cellobiose than in the presence of glucose or mannose (Fig. 8B and C). The level of HPr-Ser-P in *L. monocytogenes* grown in minimal medium with cellobiose was, however, even lower than that in *L. monocytogenes* grown in the presence of glucose or mannose, both in the early (OD_{600} of 0.6) and later (OD_{600} of 1.0) growth phases (Fig. 7B). There was no significant difference whether 25 or 50 mM cellobiose was added to the medium.

These data thus do not show an inverse correlation between the level of HPr-Ser-P and PrfA activity in *L. monocytogenes*, as suggested by recent studies (14, 27). The data shown in Fig. 8B and 9B also do not demonstrate a correlation between the level of HPr-His-P and PrfA activity, suggesting that this phosphorylated HPr component is not a direct modulator of PrfA activity either.

Comparative transcript patterns of *hprK* versus WT and *ccpA* versus WT using whole *L. monocytogenes* genome microarrays. As shown in Fig. 3B the growth rate of the *hprK* mutant in glucose-containing minimal medium is strongly impaired compared to the wild-type strain and the *ccpA* mutant (generation time of 283 min versus 141 min). This observation correlates with the reduced rate of D-[U-^{14}C]glucose uptake in the *hprK* mutant (Fig. 3C). Similar to the *ccpA* mutant, the *hprK* mutant is expected to derepress genes and operons which are under CCR control. However, the PrfA-regulated genes are up-regulated in the *hprK* mutant but not in the *ccpA* mutant (Fig. 4B), suggesting that genes other than the typical genes controlled by CCR are differently regulated in the two mutants. The information on these genes was ex-

TABLE 3. Expression of group I genes in *hprK* and *ccpA* insertion mutants relative to *L. monocytogenes* wild type[a]

Gene and operon[b]	Relative expression		Function
	hprK mutant/WT	*ccpA* mutant/WT	
Group Ia (up-regulated)			
lmo0106	9.73	4.22	Similar to transcription regulator
lmo0109	5.33	4.35	Similar to transcriptional regulatory proteins, AraC family
lmo0133	2.81	1.85	Similar to *E. coli* YjdI protein
lmo0297	2.00	1.98	Similar to transcriptional antiterminator (BglG family)
lmo0349	1.92	2.27	Unknown
lmo0384	2.17	2.11	Similar to *B. subtilis* IolB protein
lmo0391	2.27	2.11	Unknown
lmo0392	2.28	2.04	Highly similar to *B. subtilis* YqfA protein
lmo0393	1.94	1.96	Unknown
lmo0422	3.17	1.88	Similar to unknown protein
lmo0425	4.41	3.21	Similar to transcription antiterminator BglG family
lmo0426	2.94	3.48	Similar to PTS fructose-specific enzyme IIA component
lmo0427	3.21	3.26	Similar to PTS fructose-specific enzyme IIB component
lmo0428	3.85	3.02	Similar to PTS fructose-specific enzyme IIC component
lmo0429	3.87	3.81	Similar to sugar hydrolase
lmo0502	1.84	2.94	Similar to putative sugar-phosphate isomerase
lmo0504	1.95	2.16	Unknown
lmo0507	1.85	5.05	Similar to PTS galactitol-specific IIB component
lmo0557	2.52	2.54	Similar to phosphoglycerate mutase
lmo0640	2.32	2.08	Similar to oxidoreductase
lmo0643	2.50	4.48	Similar to putative transaldolase
lmo1042	3.70	3.32	Similar to molybdopterin biosynthesis protein MoeA
lmo1044	4.16	3.55	Similar to molybdopterin converting factor (subunit 2)
lmo1045	4.72	4.31	Similar to molybdopterin converting factor (subunit 1)
lmo1046	5.24	5.28	Similar to molybdenum cofactor biosynthesis protein C
lmo1050	2.34	1.83	Similar to *B. subtilis* YdfE protein
lmo1098	3.16	2.28	Highly similar to TN916 ORF8
lmo1173	4.87	2.56	Similar to two-component sensor histidine kinase
glpD	3.26	5.40	Similar to glycerol-3-phosphate dehydrogenase
lmo1348	4.74	3.09	Similar to aminomethyltransferase
lmo1349	4.81	5.80	Similar to glycine dehydrogenase (decarboxylating) subunit 1
lmo1350	4.50	4.90	Similar to glycine dehydrogenase (decarboxylating) subunit 2
cspL	2.85	3.12	Similar to cold shock protein
lmo1538	5.07	3.22	Similar to glycerol kinase
lmo1539	11.27	5.18	Similar to glycerol uptake facilitator
lmo1867	2.43	2.60	Similar to pyruvate phosphate dikinase
cspD	4.99	3.30	Similar to cold shock protein
lmo1883	1.85	1.88	Similar to chitinases
pflA	2.89	5.49	Similar to pyruvate formate-lyase
lmo1955	2.87	2.52	Similar to integrase/recombinase
lmo2001	3.29	1.89	Similar to PTS mannose-specific enzyme IIC component
lmo2121	3.18	1.89	Similar to maltosephosphorylase
lmo2138	2.17	2.94	Similar to transcription regulator
lmo2175	2.90	1.84	Similar to dehydrogenase
lmo2582	1.95	1.84	Similar to two-component sensor histidine kinase
lmo2584	4.41	1.83	Similar to formate dehydrogenase associated protein
lmo2585	7.76	3.33	Similar to *B. subtilis* YrhD protein
lmo2586	8.18	5.72	Similar to formate dehydrogenase alpha chain
lmo2590	2.31	2.21	Similar to ATP binding proteins
lmo2637	2.27	1.84	Conserved lipoprotein
lmo2646	4.34	5.45	Unknown
lmo2651	6.09	3.28	Similar to mannitol-specific PTS enzyme IIA component
lmo2666	1.83	7.05	Similar to PTS galactitol-specific enzyme IIB component
lmo2732	2.42	2.35	Unknown
lmo2741	1.90	2.24	Similar to drug-efflux transporters
lmo2761	1.85	11.22	Similar to beta-glucosidase
lmo2763	2.19	3.43	Similar to PTS cellobiose-specific enzyme IIC
lmo2764	2.44	11.23	Similar to xylose operon regulatory protein and to glucose kinase
lmo2771	86.23	5.00	Similar to beta-glucosidase
lmo2772	129.24	6.92	Similar to beta-glucoside-specific enzyme IIABC
lmo2774	82.23	6.10	Similar to ABC transporter, ATP-binding protein
bvrA	2.08	2.02	Transcription antiterminator
lmo2799	2.53	3.99	Similar to phosphotransferase system mannitol-specific enzyme IIBC

Continued on facing page

TABLE 3—*Continued*

Gene and operon[b]	Relative expression		Function
	hprK mutant/WT	*ccpA* mutant/WT	
lmo2838	2.53	2.09	Similar to sugar ABC transporter permease protein
lmo2853	2.62	2.14	Highly similar to *B. subtilis* Jag protein
Group Ib (down-regulated)			
lmo0233	0.33	0.29	Similar to DNA repair protein Sms
gbuC	0.29	0.44	Highly similar to glycine betaine ABC transporters (glycine betaine-binding protein)
pykA	0.55	0.49	Highly similar to pyruvate kinases
ilvN	0.51	0.52	Similar to acetolactate synthase (acetohydroxy-acid synthase) (small subunit)
leuC	0.34	0.41	Similar to 3-isopropylmalate dehydratase (large subunit)

[a] Genes up-regulated (group Ia) and down-regulated (group Ib) in *hprK* and *ccpA* insertion mutants relative to (their isogenic) WT *Listeria monocytogenes* EGD-e (grown in BHI medium) were identified by microarray analysis.

[b] Black arrows indicate genes which are probably organized in an operon structure and the direction of transcription based on the *L. monocytogenes* EGD-e genome sequence data (http://genolist.pasteur.fr/ListiList/).

pected to provide some idea of how PrfA may be activated in the *hprK* mutant.

To obtain a more general view on the *L. monocytogenes* genes that are differently regulated in the *hprK* and the *ccpA* mutant, we compared the transcription profiles of these two mutants with that of the wild-type strain using oligonucleotide-based whole-genome microarrays. All strains were cultured in BHI medium. In this complex growth medium, most genes subject to CCR were expected to be repressed in the wild-type strain.

Three groups of differently regulated genes were obtained. In group I are genes that were up- or down-regulated in both the *ccpA* and the *hprK* mutants. These genes are presumably repressed or activated by CcpA/HPr-Ser-P in the presence of glucose.

In group II are genes that were up- or down-regulated in the *ccpA* mutant but not in the *hprK* mutant (with few exceptions). This group of genes is expected to be positively or negatively regulated by CcpA alone, i.e., without HPr-Ser-P as a cofactor.

In group III are genes that were up- or down-regulated in the *hprK* mutant but not in the *ccpA* mutant. We assume that this group of genes is either directly regulated by the level of HPr-Ser-P or by the ratio of HPr-His-P/HPr-Ser-P and hence by the efficiency of glucose uptake. Since the PrfA-regulated genes are also specifically up-regulated in the *hprK* mutant, we expected to observe a correlation between the regulation of the PrfA-dependent genes and other genes that are specifically up- or down-regulated in this mutant.

Group I (Table 3) contains a large number of up-regulated genes or operons (Ia) involved in sugar transport (mainly PTS) and metabolism, ABC transporters, coenzyme metabolism, and synthesis of regulation factors. Many genes of this group are similar or even identical to those previously identified in *B. subtilis* as being subject to repression by CcpA/HPr-Ser-P. There are very few down-regulated genes in this group (Ib), suggesting that only a small number of genes in *L. monocytogenes* are activated by CcpA/HPr-Ser-P.

Among the group II genes being up-regulated in the absence of CcpA only (Table 4, IIa) are notably some genes (operons) which encode several PTS and transcription factors other than those of group Ia. These genes are apparently repressed by CcpA without HPr-Ser-P. A similar number of genes are down-regulated (greater than twofold) in the *ccpA* mutant (IIb) and hence activated in *L. monocytogenes* by CcpA in the presence of glucose; these genes encode mainly metabolic enzymes. Interestingly *inlA* and *inlB* which, like the other PrfA-dependent virulence genes, are up-regulated in the *hprK* mutant (see below) also belong to this set of genes, suggesting that expression of the *inlAB* operon which is under the control of several promoters, including a PrfA-dependent one (37, 57), is also affected directly or indirectly by CcpA.

The number of up-regulated genes (up-regulation greater than twofold) in group III is rather large (Table 5; IIIa) and interestingly contains the PrfA-regulated virulence genes, which accords with the data described above. In addition, this group again comprises genes involved in sugar transport via PTS, carbohydrate metabolism, and transcription factors. Several phage-specific genes belonging to the A118 prophage are also found in group IIIa.

The down-regulated genes (greater than twofold) of group III (IIIb) are predominantly genes involved in the biosynthesis of branched amino acids and pyrimidine and in nitrogen metabolism. Interestingly, several of these genes were previously shown to be down-regulated by impaired glucose uptake in *B. subtilis* (38, 44).

PrfA activity is high when *L. monocytogenes* grows in minimal medium with the non-PTS substrate glycerol as the carbon source. The above data show that PrfA activity is low when *L. monocytogenes* wild-type grows in BHI medium and also in minimal medium in the presence of PTS sugars, particularly in the early growth phase when the glucose uptake is highest (Fig. 5A, 6, and 8). On the other hand, expression of PrfA-regulated genes appears to be induced when glucose uptake is impaired, and expression of genes controlled by the efficiency of PTS-mediated glucose transport is down-regulated (see group III of the *hprK* mutant). Based on the described data the two major components of CCR, CcpA or HPr-Ser-P, do not act as direct modulators of the PrfA activity, although the data do not strictly rule out the possibility that additional factors may modify HPr-Ser-P activity, which could then act as a modulator of PrfA activity.

However, in our opinion the results suggest, rather, that components of the specific PTS-mediated sugar transport may

TABLE 4. Group II genes only up- or down-regulated in a *ccpA* insertion mutant relative to *L. monocytogenes* wild-type[a]

Gene and operon[b]	Relative expression of *ccpA* mutant/WT	Function
Group IIa (up-regulated)		
dnaN	2.68	DNA polymerase III; beta chain
lmo0034	2.45	Similar to PTS, cellobiose-specific IIC component
lmo0119	1.95	Unknown
lmo0121	1.85	Similar to bacteriophage minor tail proteins
lmo0141	2.28	Unknown
lmo0182	1.84	Similar to alpha-xylosidase and alpha-glucosidase
lmo0299	1.87	Similar to PTS beta-glucoside-specific enzyme IIB component
lmo0307	1.83	Unknown
lmo0383	1.88	Highly similar to *B. subtilis* methylmalonate-semialdehyde dehydrogenase IolA
lmo0488	1.80	Similar to transcriptional regulator (LysR family)
lmo0521	1.86	Similar to 6-phospho-beta-glucosidase
lmo0695	2.96	Unknown
dltA	2.10	D-Alanine-activating enzyme (Dae); D-alanine-D-alanyl carrier protein ligase (Dcl)
lmo1188	1.93	Unknown
lmo1597	6.13	Unknown
lmo1645	5.20	Similar to ATP-dependent double-stranded DNA exonuclease SbcC
lmo1647	5.01	Similar to 1-acylglycerol-3-phosphate *O*-acyltransferases
tsf	2.13	Translation elongation factor
lmo1728	2.03	Some similarities to cellobiose-phosphorylase
lmo2226	2.45	Similar to unknown proteins
lmo2426	2.01	Conserved hypothetical proteins
lmo2492	2.13	Unknown
lmo2659	2.37	Similar to ribulose-phosphate 3-epimerase
lmo2660	5.21	Similar to transketolase
lmo2661	3.76	Similar to ribulose-5-phosphate 3-epimerase
lmo2662	9.72	Similar to ribose 5-phosphate epimerase
lmo2663	17.05	Similar to polyol dehydrogenase
lmo2664	6.30	Similar to sorbitol dehydrogenase
lmo2665	7.56	Similar to PTS galactitol-specific enzyme IIC component
lmo2667	4.46	Similar to PTS galactitol-specific enzyme IIA component
lmo2668	13.88	Similar to transcriptional antiterminator (BglG family)
lmo2749	3.34	Similar to glutamine amidotransferase
lmo2762	4.80	Similar to PTS cellobiose-specific enzyme IIB
lmo2781	1.84	Similar to beta-glucosidase
lmo2789	1.87	Unknown
Group IIb (down-regulated)		
lmo0148	0.50	Unknown
lmo0170	0.49	Unknown
lmo0174	0.55	Similar to transposase
inlA	0.23	Internalin A
inlB	0.15	Internalin B
lmo0539	0.24	Similar to tagatose-1,6-diphosphate aldolase
lmo0554	0.25	Similar to NADH-dependent butanol dehydrogenase
lmo0555	0.21	Similar to ditripeptide transporter
lmo0590	0.33	Similar to a fusion of two types of conserved hypothetical protein
lmo0596	0.11	Similar to unknown proteins
lmo0628	0.27	Unknown
lmo0647	0.45	Unknown
lmo0669	0.16	Similar to oxidoreductase
lmo0670	0.37	Unknown
lmo0722	0.14	Similar to pyruvate oxidase
lmo0723	0.43	Similar to metyl-accepting chemotaxis protein
lmo0724	0.31	Similar to *B. subtilis* YvpB protein
lmo0782	0.50	Similar to mannose-specific phosphotransferase system (PTS) component IIC
lmo0784	0.40	Similar to mannose-specific phosphotransferase system (PTS) component IIA
lmo0880	0.30	Similar to wall-associated protein precursor (LPXTG motif)
lmo0912	0.45	Similar to transporters (formate)
lmo1261	0.50	Unknown
opuCD	0.30	Similar to betaine/carnitine/choline ABC transporter (membrane protein)
opuCB	0.36	Similar to glycine betaine/carnitine/choline ABC transporter (membrane protein)
opuCA	0.41	Similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)
lmo1433	0.14	Similar to glutathione reductase
murC	0.54	Similar to UDP-N-acetyl muramate-alanine ligases
lmo1606	0.48	Similar to DNA translocase
lmo2213	0.46	Similar to unknown protein
lmo2230	0.38	Similar to arsenate reductase

Continued on facing page

TABLE 4—*Continued*

Gene and operon[b]	Relative expression of *ccpA* mutant/WT	Function
lmo2231	0.36	Similar to unknown proteins
lmo2571	0.46	Similar to nicotinamidase
lmo2674	0.51	Similar to ribose 5-phosphate epimerase
lmo2683	0.50	Similar to cellobiose phosphotransferase enzyme IIB component

[a] Genes identified by microarray analysis as up-regulated (group IIa) or down-regulated (group IIb) in the *ccpA* insertion mutant relative to (its isogenic) wild-type *Listeria monocytogenes* EGD-e (grown in BHI medium) are shown.

[b] Black arrows indicate genes which are probably organized in an operon structure and the direction of transcription based on the *L. monocytogenes* EGD-e genome sequence data (http://genolist.pasteur.fr/ListiList/).

affect PrfA activity. We therefore decided to study PrfA activity in the presence of the non-PTS carbon source glycerol.

As shown in Fig. 9A, *L. monocytogenes* grew in minimal medium containing 50 mM glycerol—after a longer lag phase—at a similar rate as in the presence of 50 mM glucose. Under these growth conditions, the expression of the genes encoding a glycerol uptake facilitator (lmo1539), glycerol kinase (lmo1538), and glycerol-phosphate dehydrogenase (*glpD*) as well as the genes for gluconeogenesis were highly up-regulated (B. Joseph, personal communication) compared to growth in the presence of glucose or cellobiose.

The amount of PrfA and PrfA activity measured by the hemolytic activity and the amount of ActA (Fig. 9B and C) in *L. monocytogenes* grown to the early (balanced) growth phase (OD_{600} of 0.6) were significantly higher in the presence of the non-PTS sugar glycerol compared to the PTS sugar glucose. In the later growth phase (OD_{600} of 1.0) the enhancement of the PrfA activity was less pronounced in glycerol-containing medium compared to glucose-containing medium (data not shown). However, in the later growth phase PrfA activity is already rather high even in glucose-containing medium.

The amount of HPr-Ser-P and HPr-His-P in *L. monocytogenes* grown in glycerol-containing minimal medium was rather similar to that observed in the presence of cellobiose (Fig. 7B and 9D) although PrfA activity was much higher in the glycerol-containing medium (Fig. 8B and C and 9B and C).

These data again argue against the idea that HPr-Ser-P and HPr-His-P are direct modulators of PrfA activity and rather favor component(s) of subsequent steps (i.e., PTS permeases or others phosphorylated by HPr-His-P) as a negative effector of PrfA activity.

DISCUSSION

In this study the influence of components involved in the CCR control system and (directly or indirectly) in PTS-mediated sugar transport on the activity of the major virulence regulator PrfA of *L. monocytogenes* was studied. Specifically, PrfA-dependent gene expression was determined in *L. monocytogenes* insertion mutants defective in *ccpA* (encoding the central catabolite control protein CcpA), *hprK* (encoding the ATP-dependent HPr kinase-phosphorylase, the central regulator of CCR), and *ptsH* (encoding HPr) when these listerial strains were grown either in nutrient-rich medium (BHI) or in a defined glucose-containing minimal medium.

We could not obtain in-frame deletions in these genes, possibly due to a massive outgrowth of the wild-type strain relative to these mutants even when grown in BHI medium. Nevertheless, the effects on PrfA activity observed with the insertion mutants seem to be a direct consequence of the absence of functional CcpA, HPr, and HPrK/P and not due to polar effects caused by the insertion on the adjacent genes. In contrast to the insertions in *ccpA* and *hprK*, which showed no significant polar effects on the expression of the upstream or downstream located genes, no transcription of *ptsI* (located downstream of *ptsH* in the *ptsHI* operon [12]) was observed apparently as a result of the insertion in *ptsH*. But since enzyme I (the gene product of *ptsI*) acts only on HPr, it is unlikely that this polar effect on *ptsI* expression affects the PrfA activity differently than the *ptsH* mutation alone. Revertants of all three mutants behaved in all tested properties similar to the wild-type strain, thus excluding the possibility that additional, unrecognized mutations might affect the phenotype of the mutants.

In agreement with previous findings (3), the *ccpA* mutation did not enhance PrfA activity. Our studies showed, rather, even a slightly reduced expression of the PrfA-dependent virulence genes compared to the EGD-e wild-type strain, which is in accord with the recent findings of Herro et al. (27).

Highly significant up-regulation of all tested PrfA-dependent virulence genes was observed in the *hprK* and *ptsH* mutants upon growth in glucose-containing medium. The degree of enhanced transcription of most of these genes including *prfA* itself in the two mutants was, however, not as high as that observed in the *L. monocytogenes prfA** mutant (used as standard for a constitutively active PrfA), suggesting that PrfA is not as active in the *hprK* and *ptsH* mutants as in the *prfA** mutant. Besides, in minimal medium cellobiose could still inhibit—albeit at a reduced rate compared to the wild type strain—PrfA-dependent gene expression in the *hprK* mutant but at a lower rate than in the *prfA** mutant (52, 53).

A major difference between the *ptsH* and *hprK* mutants, on one hand, and the *ccpA* mutant, on the other, lies in the capability of these mutants to phosphorylate HPr. Obviously, no phosphorylated HPr can be produced in the *ptsH* mutant since HPr is not synthesized. The loss of HPrK/P activity in the *hprK* mutant restricts HPr phosphorylation to His15 (essential for PTS-mediated sugar transport) while the *ccpA* mutant can still phosphorylate HPr at both positions, His15 and Ser46. The level of HPr-Ser-P in the *ccpA* mutant appears to be similar or even slightly higher than in the wild-type strain and virtually zero in the *hprK* mutant. Increased levels of HPr-Ser-P were also observed in *ccpA* mutants in other gram-positive bacteria, e.g., *Enterococcus faecalis* (35) and *B. subtilis* (39).

TABLE 5. Group III genes only up- or down-regulated in an *hprK* insertion mutant relative to *L. monocytogenes* wild-type[a]

Gene and operon[b]	Relative expression of *hprK* mutant/WT	Function
Group IIIa (up-regulated)[c]		
qoxB	2.06	AA3-600 quinol oxidase subunit I
lmo0049	2.13	Unknown
lmo0071	1.92	Unknown
lmo0094	1.93	Unknown
lmo0096	3.28	Similar to PTS mannose-specific, factor IIAB
lmo0098	2.94	Similar to PTS mannose-specific, factor IID
lmo0100	2.45	Unknown
lmo0102	4.01	Unknown
lmo0105	12.17	Highly similar to chitinase B
lmaA	3.04	antigen A
lmo0120	2.48	Unknown
lmo0122	3.28	Similar to phage proteins
lmo0126	3.70	Unknown
lmo0128	2.69	Similar to a protein from bacteriophage phi-105 (ORF 45)
lmo0130	3.72	Similar to 5-nucleotidase, putative peptidoglycan bound protein (LPXTG motif)
lmo0136	1.97	Similar to oligopeptide ABC transporter, permease protein
lmo0139	1.83	Unknown
plcA	3.26	Phosphatidylinositol-specific phospholipase C
hly	2.04	LLO precursor
plcB	2.03	Phospholipase C
folA	2.31	Highly similar to dihydroneopterin aldolase
lmo0227	2.35	Conserved hypothetical protein
lmo0324	2.43	Unknown
lmo0369	1.83	Conserved hypothetical protein, highly similar to *B. subtilis* YeeI protein
lmo0386	2.12	Similar to *B. subtilis* IolD protein and to acetolactate synthase
lmo0394	2.49	Similar to *L. monocytogenes* extracellular P60 protein
lmo0398	2.05	Similar to phosphotransferase system enzyme IIA
lmo0421	2.52	Similar to rod shape-determining protein RodA
lmo0423	3.15	Similar to RNA polymerase extracytoplasmic function-type sigma factor
inlA	4.00	Internalin A
inlB	2.01	Internalin B
lmo0471	1.82	Unknown
lmo0477	2.92	Putative secreted protein
lmo0501	1.88	Similar to transcription antiterminator BgIG family
lmo0536	2.29	Similar to 6-phospho-beta-glucosidase
lmo0539	2.10	Similar to tagatose-1,6-diphosphate aldolase
lmo0543	2.20	Similar to PTS, glucitol/sorbitol-specific enzyme IIBC component
lmo0610	2.22	Similar to internalin proteins and putative peptidoglycan-bound protein (LPXTG motif)
lmo0632	2.65	Similar to PTS, fructose-specific IIC component
lmo0634	2.04	Similar to an *E. coli* putative tagatose 6-phosphate kinase
lmo0641	1.83	Similar to heavy metal-transporting ATPase
lmo0674	2.14	Unknown
lmo0722	2.59	Similar to pyruvate oxidase
lmo0737	1.82	Unknown
lmo0738	1.81	Similar to PTS beta-glucoside-specific enzyme IIABC component
lmo0782	1.84	Similar to mannose-specific PTS component IIC
lmo0784	2.23	Similar to mannose-specific PTS component IIA
lmo0798	2.19	Similar to lysine-specific permease
uhpT	1.84	Highly similar to hexose phosphate transport protein
lmo0866	2.03	Similar to ATP-dependent RNA helicase
lmo0913	2.17	Similar to succinate semialdehyde dehydrogenase
lmo0915	2.08	Similar to phosphotransferase system enzyme IIC
lmo0918	1.93	Similar to transcription antiterminator BglG family
lmo0994	2.00	Unknown
lmo1048	2.02	Similar to molybdenum cofactor biosynthesis protein B
lmo1105	2.32	Highly similar to TN916 ORF15
lmo1150	1.99	Regulatory protein similar to *Salmonella enterica* serovar Typhimurium PocR protein
lmo1166	1.84	Similar to NADPH-dependent butanol dehydrogenase
lmo1172	2.92	Similar to similar to two-component response regulator

Continued on facing page

TABLE 5—*Continued*

Gene and operon[b]	Relative expression of *hprK* mutant/WT	Function
eutA	3.35	Similar to ethanolamine utilization protein EutA (putative chaperonin)
eutB	2.76	Similar to ethanolamine ammonia-lyase; heavy chain
lmo1177	3.25	Similar to putative carboxysome structural protein (EutL)
lmo1178	6.42	Similar to putative carboxysome structural protein
lmo1179	1.94	Similar to acetaldehyde dehydrogenase/alcohol dehydrogenase
lmo1181	2.06	Similar to cobalamin adenosyl transferase
lmo1185	2.10	Similar to *Salmonella enterica* PduT protein
lmo1190	1.89	Unknown
cbiF	2.38	Similar to precorrin-3 methylase
cbiG	2.46	Similar to cobalamin biosynthesis protein G CbiG
cbiH	2.60	Similar to precorrin methylase
lmo1201	2.17	Similar uroporphyrinogen-III methyltransferase/uroporphyrinogen-III synthase
lmo1254	9.41	Similar to alpha, alpha-phosphotrehalase
lmo1255	9.11	Similar to PTS trehalose-specific enzyme IIBC
lmo1257	2.09	Unknown
lmo1374	1.87	Similar to branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)
tcsA	2.54	$CD4^+$ T-cell-stimulating antigen, lipoprotein
pflB	2.00	Pyruvate formate-lyase
lmo1433	2.41	Similar to glutathione reductase
lmo1471	2.57	Similar to ribosomal protein L11 methyltransferase
lmo1614	1.82	Similar to unknown proteins
lmo1654	1.90	Putative cellsurface protein
lmo1669	1.84	Some similarity to hypothetical proteins
lmo1714	1.98	Unknown
lmo1729	1.85	Similar to beta-glucosidases
lmo1731	1.80	Similar to sugar ABC transporter, permease protein
lmo1732	3.47	Similar to sugar ABC transporter, permease protein
inlC	2.23	Internalin C
lmo1855	1.86	Similar to similar to D-alanyl-D-alanine carboxypeptidases
pnp	1.93	Similar to purine-nucleoside phosphorylase
lmo1999	2.07	Weakly similar to glucosamine-fructose-6-phosphate aminotransferase
cspB	2.07	Similar to major cold-shock protein
lmo2084	2.67	Unknown
lmo2085	2.87	Putative peptidoglycan bound protein (LPXTG motif)
lmo2125	2.83	Similar to maltose/maltodextrin ABC-transporter (binding protein)
lmo2129	2.04	Unknown
lmo2133	2.83	Similar to fructose-1,6-biphosphate aldolase type II
lmo2159	2.98	Similar to oxidoreductase
lmo2160	3.63	Similar to unknown proteins
lmo2161	3.18	Unknown
lmo2163	2.24	Similar to oxidoreductase
lmo2245	3.50	Similar to unknown proteins
lmo2277	1.89	Unknown
lmo2292	2.54	Protein gp11 (bacteriophage A118)
lmo2293	1.83	Protein gp10 (bacteriophage A118)
lmo2294	3.11	Protein gp9 (bacteriophage A118)
lmo2295	2.32	Protein gp8 (bacteriophage A118)
lmo2298	2.53	protein gp4 (bacteriophage A118)
lmo2313	4.21	Similar to a bacteriophage protein
lmo2314	2.49	Unknown
lmo2316	2.72	Similar to site-specific DNA-methyltransferase
lmo2319	1.89	Similar to bacteriophage proteins
lmo2320	2.10	Unknown
lmo2364	3.01	Hypothetical protein
lmo2436	2.70	Similar to transcription antiterminator
lmo2437	2.53	Unknown
lmo2572	1.84	Similar to chain A, dihydrofolate reductase
lmo2573	1.98	Similar to zinc-binding dehydrogenase
lmo2583	2.53	Similar to two-component response regulator
lmo2648	1.96	Similar to phosphotriesterase
lmo2650	3.51	Similar to hypothetical PTS enzyme IIB component
lmo2685	2.21	Similar to cellobiose phosphotransferase enzyme IIA component
lmo2695	2.76	Similar to dihydroxyacetone kinase
lmo2696	2.50	Similar to hypothetical dihydroxyacetone kinase
lmo2697	1.92	Unknown

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TABLE 5—*Continued*

Gene and operon[b]	Relative expression of *hprK* mutant/WT	Function
lmo2707	2.20	Unknown
lmo2735	2.20	Similar to Sucrose phosphorylase
lmo2742	1.97	Unknown
bvrB	2.84	Beta-glucoside-specific phosphotransferase enzyme II ABC component
lmo2796 ↑	2.47	Similar to transcription regulator
lmo2797 |	3.43	Similar to phosphotransferase system mannitol-specific enzyme IIA
lmo2798 |	2.14	Similar to phosphatase
lmo2840	2.25	Similar to sucrose phosphorylase
rpmH	1.98	Ribosomal protein L34
Group IIIb (down-regulated)		
lmo0209	0.50	Unknown
lmo0372	0.53	Similar to beta-glucosidase
lmo0519	0.54	Similar to multidrug resistance protein
lmo0560	0.51	Similar to NADP-specific glutamate dehydrogenase
lmo0593	0.46	Similar to transport proteins (formate?)
lmo1516 |	0.29	Similar to ammonium transporter NrgA
lmo1517↓	0.37	Similar to nitrogen regulatory PII protein
aspS	0.49	Aspartyl-tRNA synthetase
leuB	0.31	Similar to 3-isopropylmalate dehydrogenase
lmo2192 ↑	0.48	Similar to oligopeptide ABC transporter (ATP-binding protein)
lmo2193 |	0.53	Similar to oligopeptide ABC transporter (ATP-binding protein)
lmo2194 |	0.54	Similar to oligopeptide ABC transporter (permease)
lmo2195 |	0.52	Similar to oligopeptide ABC transporter (permease)
lmo2460	0.40	Similar to *B. subtilis* CggR hypothetical transcriptional regulator
lmo2757	0.43	Similar to ATP-dependent DNA helicases

[a] Genes identified by microarray analysis as up-regulated (group IIIa) or down-regulated (group IIIb) in an *hprK* insertion mutant relative to (its isogenic) wild-type *Listeria monocytogenes* EGD-e (grown in BHI medium) are shown.

[b] Black arrows indicate genes which are probably organized in an operon structure and the direction of transcription based the *L. monocytogenes* EGD-e genome sequence data (http://genolist.pasteur.fr/ListiList/).

[c] The genes *actA* and *prfA* are missing from this group because of the nonfunctioning of the oligonucleotides, but the transcriptional up-regulation of these genes was confirmed by real-time RT-PCR.

Thus, there seems to be an inverse correlation between PrfA activity and the cellular HPr-Ser-P concentration in *L. monocytogenes* when cultured in glucose-containing medium. This observation would therefore be in accord with a recent study on PrfA modulation in *B. subtilis* (27). However, when *L. monocytogenes* was grown in minimal medium in the presence of the PTS sugar cellobiose instead of glucose (both sugars in equimolar quantities), the growth rates were similar in both cases, but the level of HPr-Ser-P was even lower in the presence of cellobiose than in the presence of glucose although the PrfA activity was much higher in the latter case. The reason for this reproducible result is presently unclear. Possibly the activity of HPrK, catalyzing synthesis of HPr-Ser-P, is lower in presence of cellobiose than with glucose due to lower levels of the HPrK-activating glycolysis intermediates, such as fructose-1,6-diphosphate or PEP.

These data do not support the notion that HPr-Ser-P acts as a direct negative modulator of PrfA activity. This conclusion is also in line with our recent studies aimed at demonstrating direct binding of purified PrfA with HPr-Ser-P in Biacore assays or inhibition of PrfA-mediated in vitro transcription initiated at several PrfA-dependent promoters by HPr-Ser-P, which were all negative (S. Müller-Altrock, personal communication). However, the data do not rule out the possibility that PrfA activity is inhibited by HPr-Ser-P in combination with an additional factor that may modify HPr-Ser-P activity. Synthesis of this factor could be enhanced when *L. monocytogenes* grows in the presence of cellobiose.

The lack of correlation between the amount of HPr-Ser-P or HPr-His-P and PrfA activity also does not favor the assumption that HPr-His-P may directly phosphorylate PrfA, as shown for the activation of several transcription regulators that contain a conserved PTS regulation domain (58). This is not surprising as a PTS regulation domain (PRD) could not be identified in PrfA.

The comparative transcript profiling carried out with RNAs from the *ccpA* and the *hprK* mutants versus the wild-type strain shows that a rather large number of genes (operons) are up-regulated in both the *ccpA* and the *hprK* mutants compared to the wild-type strain when strains are cultured in BHI medium, suggesting that they were repressed upon growth of *L. monocytogenes* in BHI medium. Many of these genes and operons encode proteins involved in transport and metabolism of various carbohydrates and some transcription factors, and they are likely to be under negative control of the CcpA/HPr-Ser-P complex, as in *B. subtilis* (44). This assumption is further supported by the observation that most of these genes carry typical *cre* sites (CcpA/HPr-Ser-P binding sites) in their upstream regulatory regions. Clearly, the PrfA-regulated virulence genes do not belong to this group of genes, which is in accord with previous findings (3).

There is, however, another set of genes which are up- or down-regulated in the *hprK* but not in the *ccpA* mutant compared to the wild-type strain. All PrfA-controlled genes are found among the up-regulated genes of this group. Among the down-regulated genes are mainly those that were previously termed class II CCR-controlled genes (39, 44). Several of these down-regulated genes are involved in nitrogen metabolism, like *nrgAB* (encoding an ammonium transporter and a PII-like

protein) and in biosynthesis of branched-chain amino acids. Their expression was shown to be reduced when glucose uptake was impaired (38), which is also the case in the *hprK* (and *ptsH*) mutant.

Thus, there seems to be an inverse correlation between the regulation of PrfA-dependent genes and class II CCR-controlled genes; i.e., PrfA-controlled genes are up-regulated (suggesting increased PrfA activity) when PTS-mediated sugar uptake is impaired, whereas the class II CCR-controlled genes are down-regulated under these conditions.

The notion that PTS-mediated sugar transport may modulate PrfA activity can also be deduced from the observation that PrfA and its regulated genes are highly active when *L. monocytogenes* is grown in a minimal medium containing glycerol as the carbon source in comparison to growth in glucose- or cellobiose-containing medium (B. Joseph, unpublished data). Uptake of glycerol is not mediated by PTS, but probably, as in *B. subtilis* (4), by the glycerol uptake facilitator (encoded by lmo1539) which is highly up-regulated in *L. monocytogenes* grown in glycerol-containing medium. Metabolism of glycerol requires glycerol kinase and glycerol-phosphate dehydrogenase (the genes encoding these two enzymes are also highly up-regulated in glycerol-containing minimal medium), and glycerol kinase activation requires phosphorylation by HPr-His-P (15). The amounts of HPr-His-P and HPr-Ser-P are rather similar in the presence of glycerol and cellobiose, despite the much higher expression of PrfA-controlled genes in the presence of glycerol, and thus shows again that there is no correlation between the amount of HPr-Ser-P and PrfA activity (see above).

Glucose, mannose, and especially cellobiose are mediated by several PTS permeases (2, 10), which seem to functionally overlap in part and are either constitutively expressed or substrate induced (R. Ecke, personal communication). It is reasonable to assume that the EIIA and EIIB components of the PEP-PTS mediating transport of glucose, mannose, or cellobiose will be in a mainly nonphosphorylated state during active sugar transport, while these components are expected to be either not expressed or in a phosphorylated state in the presence of glycerol as carbon source. Under the first condition PrfA activity is low while it is high under the latter condition. We therefore propose that it is not the cellular level of HPr-Ser-P, HPr-His-P, or other components of the common PTS pathway that negatively affects PrfA activity but, rather, the phosphorylation status of the component(s) that is phosphorylated by HPr-His-P (i.e., specific PTS permeases or others). We are currently in the process of testing this hypothesis further.

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