Overexpression of PrfA Leads to Growth Inhibition of *Listeria monocytogenes* in Glucose-Containing Culture Media by Interfering with Glucose Uptake

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Listeria monocytogenes strains expressing high levels of the virulence regulator PrfA (mutant PrfA* or wild-type PrfA) show strong growth inhibition in minimal media when they are supplemented with glucose but not when they are supplemented with glucose-6-phosphate compared to the growth of isogenic strains expressing low levels of PrfA. A significantly reduced rate of glucose uptake was observed in a PrfA*-overexpressing strain growing in LB supplemented with glucose. Comparative transcriptome analyses were performed with RNA isolated from a *prfA* mutant and an isogenic strain carrying multiple copies of *prfA* or *prfA** on a plasmid. These analyses revealed that in addition to high transcriptional up-regulation of the known PrfA-regulated virulence genes (group I), there was less pronounced up-regulation of the expression of several phage and metabolic genes (group II) and there was strong down-regulation of several genes involved mainly in carbon and nitrogen metabolism in the PrfA*-overexpressing strain (group III). Among the latter genes are the *nrgAB*, *gltAB*, and *glnRA* operons (involved in nitrogen metabolism), the *ilvB* operon (involved in biosynthesis of the branched-chain amino acids), and genes for some ABC transporters. Most of the down-regulated genes have been shown previously to belong to a class of genes in *Bacillus subtilis* whose expression is negatively affected by impaired glucose uptake. Our results lead to the conclusion that excess PrfA (or PrfA*) interferes with a component(s) essential for phosphotransferase system-mediated glucose transport.

Listeria monocytogenes is a gram-positive, food-borne pathogen that causes severe diseases in newborns, pregnant women, and immunocompromised individuals (44). Listerial virulence genes, their differential regulation, and the interactions of the encoded virulence factors with host cell processes have been extensively studied in the past (for recent reviews see references 7 and 57). The known virulence genes encode products involved in adherence to and internalization by the host cell (*inlA*, *inlB*, *inlC*), escape from vacuoles (*hly*, *plcA*, *plcB*), intracellular replication (*hpt*), and cellular movement (*actA*). The major transcriptional factor which regulates the expression of these virulence genes is PrfA (6, 10, 23, 27). Recent transcriptome studies have shown that PrfA also affects the expression of additional genes (31), the promoters of which do not possess the features of typical PrfA-dependent promoters (30). In addition, promoters of these genes do not show PrfA-dependent transcription in vitro (39), suggesting that PrfA influences the expression of these genes more indirectly, possibly by interacting with other regulatory factors.

Based on the high level of similarity of PrfA to the cyclic AMP (cAMP) receptor protein Crp of *Escherichia coli*, this central virulence regulator of *L. monocytogenes* belongs to the Crp/Fnr family of bacterial transcription activators (26) and shares two functionally important structural features with

members of this protein family: a helix-turn-helix DNA-binding motif in the C-terminal region and several short antiparallel β -strands in the N-terminal half, forming a β -roll structure (45). The PrfA protein facilitates specific binding to its target site, the so-called PrfA box (consensus sequence, TTAACANNTGTTAA), via the C-terminal helix-turn-helix motif, and affinity is weakened when this target sequence diverges from the perfect palindromic sequence (17, 46). The precise function of the β -roll structure in PrfA is still not known. In Crp of *E. coli* this structure is essential for binding of the activating cofactor cAMP. However, cAMP is not produced by gram-positive bacteria, and the cAMP-binding site of Crp is less conserved in PrfA (12, 58). There is evidence, however, that PrfA may also bind an additional factor(s) (9, 41), thereby modulating its activity. Ripio and colleagues (43) isolated a constitutively active *prfA* mutant (designated *prfA**). Subsequently, several other *prfA** mutations have been identified (47, 59, 62). The mutant PrfA* proteins do not respond to conditions which negatively affect PrfA activity, such as low temperature (31) and especially the presence of certain phosphotransferase system (PTS) carbohydrates, including glucose, fructose, mannose, and especially cellobiose (2, 34, 37), whose uptake is mediated by phosphoenolpyruvate-sugar phosphotransferase systems, suggesting that PrfA may interfere either with components of PTS and/or with the connected carbon catabolite repression (CCR) control of *L. monocytogenes*.

CCR controls, in addition to many genes involved in carbon and nitrogen metabolism (for recent reviews see references 53 and 54), the expression of virulence genes in several pathogenic bacteria (22, 32, 51) and developmental genes involved in the initiation of sporulation in *Bacillus subtilis* (16). CCR control in gram-positive bacteria having low G+C contents de-

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TABLE 1. Bacterial strains used in this study

<i>L. monocytogenes</i> strain or genotype	Description	Source or reference
EGD	Wild type	S. H. E. Kaufmann (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany)
EGD(pERL3)	Wild-type strain with pERL3, Em ^r	This study
$\Delta prfA$	Isogenic <i>prfA</i> deletion mutant of EGD	9
PKP1	EGD with deletion in the virulence cluster	49
PKP1(pPrfA*)	PKP1 with pERL3/pPrfA*, Em ^r	This study
EGD(pPrfA*)	Wild-type strain with pERL3/pPrfA*, Em ^r	This study
$\Delta prfA$ (pPrfA*)	$\Delta prfA$ with pERL3/pPrfA*, Em ^r	This study
$\Delta prfA$ (pPrfA)	$\Delta prfA$ with pERL3/pPrfA, Em ^r	This study
$\Delta prfA$ (pERL3)	Isogenic <i>prfA</i> deletion mutant with pERL3, Em ^r	This study
P14	Serovar 4b, human clinical isolate	42
P14-A	Variant of P14 with Gly-145-Ser substitution, hyperactive PrfA phenotype	42
P14(pERL3)	P14 with pERL3, Em ^r	This study
P14(pPrfA*)	P14 with pERL3/pPrfA*, Em ^r	This study
P14-A(pERL3)	P14A with pERL3, Em ^r	This study
P14-A(pPrfA*)	P14A with pERL3/pPrfA*, Em ^r	This study

depends on the regulator protein CcpA (catabolite control protein A). This protein, a member of the LacI/GalR family of bacterial regulatory proteins, regulates the expression of genes by binding to the catabolite responsive element (cre box) located in or near the promoter regions (about 200 genes in *B. subtilis*) (21, 36). CcpA activity is modulated by different cofactors, which lead to different modes of gene regulation (3, 20, 36). A major cofactor of CcpA is HPr phosphorylated at Ser46. The HPr protein is a basic component of all PTSs. During PTS-mediated sugar uptake, HPr (encoded by *ptsH*) is phosphorylated (by phosphoenolpyruvate) at His15 by enzyme I. HPr-His15-P triggers phosphorylation of the sugar-specific transport component EIIA and then EIIB; the latter component activates the sugar translocation via EIIC. EIIA is also involved in other regulatory functions (53). HPr can also be phosphorylated or dephosphorylated at Ser46 by a specific ATP-dependent HPr-kinase/phosphorylase (HPrK/P) (33, 40). This phosphorylation of HPr is stimulated by intermediates of the glycolytic pathway, particularly fructose-1,6-bisphosphate. Glucose starvation, an increase in the concentration of inorganic phosphate, and low concentrations of glycolytic intermediates trigger the phosphorylase activity of HPrK/P, leading to dephosphorylation of HPr-Ser-P (15, 33). The CcpA/HPr-Ser46-P complex leads to repression of many catabolic genes (8, 19, 52, 54). These genes are consequently up-regulated in *ccpA*- and *hprK*-deficient mutants (3, 36).

However, a *ccpA*-deficient mutant of *B. subtilis* also exhibits impaired glucose transport (61) and down-regulation of the transcription of several genes that are essential for C metabolism and N metabolism (3, 14, 28, 29, 48, 54). Typical members of this group of genes that are down-regulated by a limited glucose supply are involved in the biosynthesis of the branched amino acids (encoded by the *ilvB* operon), in glycolysis (*gapA* operon), and in nitrogen metabolism (e.g., glutamate synthetase) (*gltAB*). The genome sequence of *L. monocytogenes* (19) contains orthologs of all essential components involved in PTS and CCR control in *B. subtilis*, suggesting that the mechanisms of these global systems are similar in *L. monocytogenes* and *B. subtilis*. However, it has been shown that PrfA-dependent virulence gene expression is not affected in a *ccpA* mutant

of *L. monocytogenes*, which rules out direct CCR control of either *prfA* gene expression or possible PrfA modulating factors (1).

In this study we analyzed the molecular basis for the observed strong growth inhibition of *L. monocytogenes* overexpressing PrfA* (PrfA) when the organism was growing in a glucose-containing minimal medium. We found that excess PrfA* reduces the rate of glucose uptake. By comparing the gene expression patterns of a *prfA* deletion mutant and an isogenic strain overexpressing PrfA*, we identified in the PrfA*-overexpressing strain, besides the highly up-regulated PrfA-dependent virulence genes, several down-regulated genes involved in C and N metabolism that belong to the group of genes which respond to a limited glucose supply in *B. subtilis* (36). The data suggest that overproduced PrfA (PrfA*) interferes with components of the glucose uptake PTS(s).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All strains were grown at 37°C with shaking at 190 rpm either in brain heart infusion broth (BHI) (Difco), in Luria-Bertani broth (LB) supplemented with 50 mM glucose (LB-Glc) or with glucose-6-phosphate (LB-G-6-P) when required, or in a chemically defined minimal medium (MM-Glc) for *L. monocytogenes* (38). Erythromycin at a concentration of 5 µg/ml was used for strains harboring different constructs of the pERL3 plasmid. For activation of PrfA, the resin Amberlite XAD was added to LB-Glc at a final concentration of 1% (wt/vol). To determine growth curves, aliquots were removed at regular intervals and the optical density at 600 nm (OD₆₀₀) was determined using a spectrophotometer. All growth experiments were performed at least four times independently.

Construction of plasmids pPrfA* and pPrfA. The *prfA** and *prfA* genes of *L. monocytogenes* strains P14-A (42) and EGD, respectively, were amplified by PCR using primers 5'-TAA CAT ATA TTA TGT CGA CAA AAA AAG GGT TAG-3' and 5'-GTT CAT GAA AAT GCT GCA GTA AGT TCT TTA TTC G-3' engineered with SalI and PstI sites (underlined) and were cloned into the SalI and PstI sites of the multicopy vector pERL3, resulting in plasmids pPrfA* and pPrfA. pPrfA* and pPrfA contained the entire structural gene *prfA** and *prfA*, respectively, with the upstream PprfA₁ and PprfA₂ promoters (18). Standard cloning protocols were used, and transformants were selected on agar plates containing 5 µg/ml erythromycin. DNA sequences of the inserted genes were determined using the dideoxy chain termination method with a CEQ 2000 dye terminator cycle sequencing Quick Start kit (Beckman Coulter).

Preparation of supernatant and cellular proteins of *L. monocytogenes*. Overnight cultures of *L. monocytogenes* grown in BHI were washed twice in MM-Glc,

inoculated into fresh MM-Glc, and grown to an optical density at 600 nm of 1.0. Each culture was then centrifuged for 10 min at 6,000 rpm at 4°C. The supernatant 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 washing, the pellet was resuspended in urea buffer (7 M urea, 2 M thiourea, dithiothreitol) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). For preparation of cellular proteins, the pellet was washed twice in 1× phosphate-buffered saline and resuspended in cold lysis buffer (1× phosphate-buffered saline with additional protease inhibitor [Roche]) and placed in a 2-ml BLUE TUBE (Q-Biogene) filled with silica sand. The tube was shaken six times for 30 s each time at speed 6.5 with 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. The total protein concentration was determined by the Bio-Rad protein microassay.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using standard protocols (24). After SDS-PAGE, cytoplasmic proteins and proteins from culture supernatant were Western blotted onto nitrocellulose membranes, and listeriolysin O, ActA, and PrfA were immunodetected using specific rabbit polyclonal antibodies.

Determination of hemolytic activity. *L. monocytogenes* was grown in the appropriate medium at 37°C to an optical density at 600 nm of 1.0. Culture supernatants were assayed for hemolytic activity as described previously (43). Twenty-five microliters of the culture supernatant was incubated in 1 ml of a 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. The hemolytic activity was measured by determining the OD₅₄₃.

Extraction of RNA from *L. monocytogenes* strains. RNA was extracted from *L. monocytogenes* strains grown in MM-Glc as follows. A cell pellet was suspended directly in lysis buffer (QIAGEN) and placed in a 2-ml BLUE TUBE that was filled with silica sand (Q-Biogene). The tube was shaken three times for 45 s each time (with 1 min of incubation on ice between treatments) at speed 6.5 with a bead beater (FP120 FastPrep cell disrupter; Savant Instruments, Inc.). After centrifugation at 13,000 × g for 5 min, the supernatant was transferred to a fresh tube, and 1 ml of Trizol reagent (Gibco BRL) was added. The sample was incubated for 5 min at room temperature. Total RNA was extracted once with chloroform and precipitated in 0.7 volume of isopropanol. After washing with 70% ethanol, the RNA pellet was dissolved in sterile diethyl pyrocarbonate-treated water and then purified further by using an RNeasy mini kit (QIAGEN) according to the manufacturer's protocol and quantified based on the absorbance at 260 and 280 nm.

Microarray analysis. Transcriptome analyses were performed using whole-genome DNA microarrays that contained synthetic 70-mer oligodeoxyribonucleotides covering all open reading frames (ORFs) of the *L. monocytogenes* genome. The oligonucleotides (Operon Co.) were spotted on epoxy-coated glass slides from Quantifoil according to the manufacturer's instructions at the microarray facility of Institut für Hygiene und Mikrobiologie, Würzburg, Germany. Each oligonucleotide was spotted four times on a slide to generate four replicates for each oligonucleotide on a slide. Three independent RNA preparations obtained from the conditions tested were pooled, and six equal aliquots (15 to 20 µg) of total RNA from the strains were used to synthesize cDNA differentially labeled with Cy3-dCTP and Cy5-dCTP (Amersham Pharmacia, Freiburg, Germany) during a first-strand reverse transcription (RT) reaction with Superscript II RNase H⁻ reverse transcriptase and 9 µg random primers (Life Technologies, Karlsruhe, Germany). Dye swapping was performed as follows. Three cDNA samples from one strain were generated using Cy3-dCTP, and three cDNA samples were generated using Cy5-dCTP; cDNA samples from the other strain investigated were generated similarly. Two differentially labeled cDNA samples were combined, diluted with 3× SSC–0.1% (wt/vol) sodium dodecyl sulfate (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), hybridized to a microarray slide, and incubated at 65°C for 16 h. Six such slides were hybridized using the probes generated. After washing according to the manufacturer's protocol (Quantifoil), the slides were scanned using ScanArray HT and were analyzed using the ScanArray express software (Perkin-Elmer, Boston, MA). Spots were flagged and eliminated from the analysis when the signal-to-noise ratio was less than 3 or in obvious instances when there were high background or stray fluorescent signals. The LOWESS method of normalization (63) was performed for the background-corrected median intensity of the spots. The 24 normalized ratios for each gene resulting from the six slides were also analyzed with Microsoft Excel (Microsoft, Redmond, WA) and the SAM software for statistical significance (56).

Real-time RT-PCR. RT-PCR was performed with independently isolated total RNA, such as the RNA used for transcriptome analysis experiments. Before RT-PCR was performed, the absence of DNA from RNA samples was verified

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

Oligonucleotide ^a	Sequence (5'–3')	Gene
rpoB (F)	GCGGATGAAGAGGATAATTACG	<i>rpoB</i>
rpoB (R)	GGAATCCATAGATGGACCGTTA	<i>rpoB</i>
glnR (F)	TCTCACTGCTAGACAAATTCGC	<i>glnR</i>
glnR (R)	CTCTGGCTCAGGTCCTAAGTTA	<i>glnR</i>
glnA (F)	GCGGTATGGTTCGACGCAAAAA	<i>glnA</i>
glnA (R)	CAGGGGCGAAAGCTAACGAATA	<i>glnA</i>
lmo1516 (F)	GCGGTATGGTTCGACGCAAAAA	<i>lmo1516</i>
lmo1516 (R)	CAGGGGCGAAAGCTAACGAATA	<i>lmo1516</i>
lmo1517 (F)	ATCGAAATTATTACTCGCCCAA	<i>lmo1517</i>
lmo1517 (R)	GTACCCCGATAAAGTTCAATAA	<i>lmo1517</i>
lmo1734 (F)	GAGGCGGAAAGTCGTTATATTGA	<i>lmo1734</i>
lmo1734 (R)	AGGCTGTTCCCGGAAATAATT	<i>lmo1734</i>
lmo1849 (F)	GGGCGAGGTAAATCAACCTTAT	<i>lmo1849</i>
lmo1849 (R)	GTGCCAGCAAAACCATATCAA	<i>lmo1849</i>
leuA (F)	CTTGCTTCCATTAAACACCACA	<i>leuA</i>
leuA (R)	CGAATTCGGTTGGGTTAGTATA	<i>leuA</i>

^a F, forward; R, reverse.

by PCR amplification of the genes to be assayed with 1 µg RNA as the template. cDNA synthesis was performed as described above by using 5 µg total RNA. Instead of the labeled nucleotides, 20 mM dATP, 20 mM dCTP, 20 mM dGTP, and 20 mM dTTP were used. RT-PCR was performed in a 20-µl (final volume) reaction mixture. The protocol and cycling conditions used were the protocol and cycling conditions recommended by the manufacturer for a qPCRCore kit for SYBR Green I (Eurogentec). The oligonucleotides used are listed in Table 2. For quantification of RT-PCR data, a standard curve was established by using serial dilutions of an *rpoB* PCR fragment as the template in an RT-PCR, which served as an external standard. Normalization of all results was performed by establishing a normalization factor as follows. For each strain in all the growth media tested, the *rpoB* expression was determined using *rpoB*-specific primers. By setting the *rpoB* ratio equal to 1, a normalization factor for all strain combinations in all media tested was calculated and used to normalize all data sets. The specificity of all amplicons was confirmed using melting curves. Final means and standard deviation were calculated based on the ratio of the results of four independent RT-PCRs for each strain combination and growth condition.

Glucose transport assay. Different *L. monocytogenes* strains were grown in LB-Glc and harvested by centrifugation at 5,000 rpm for 3 min at 4°C. Each pellet was washed three times with transport buffer (50 mM Tris HCl [pH 7.2], 20 mM MgCl₂) and resuspended in the same buffer. Labeled D-[U-¹⁴C]glucose (2 µCi/ml; Amersham Pharmacia) was mixed with unlabeled D-[U-¹²C]glucose and added to the cells (final concentration, 2 mM), and the preparation was incubated at 37°C. Aliquots (50 µl) were taken at different times (15 s, 30 s, 60 s, 90 s, 120 s, and 180 s) and filtered rapidly under a vacuum through 0.45-µm-pore-size cellulose nitrate filters (Sartorius). The filters were washed three times with 3 ml cold saline (0.9% NaCl) and dried for 20 min at 42°C. Radioactivity was determined by liquid scintillation counting (Beckmann). Additionally, the number of CFU in each sample was determined, and the glucose uptake was calculated for each strain.

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 GSE4414.

RESULTS

Growth inhibition of *L. monocytogenes* mutant overexpressing PrfA* or wild-type PrfA cultured in glucose-containing minimal medium. *L. monocytogenes* EGD strains (wild type and the isogenic $\Delta prfA$ deletion mutant) overexpressing constitutively active PrfA (due to a mutation in *prfA* leading to a G145S substitution, designated PrfA*) encoded by the multicopy plasmid pPrfA* showed strong growth inhibition upon cultivation in a defined minimal medium (MM-Glc) (38) or in LB-Glc compared to the growth of the same strains carrying only the vector pERL3 (Fig. 1a and b). Both culture media

FIG. 1. (a) Growth of EGD(pERL3), EGD(pPrfA*), $\Delta prfA$ (pERL3), $\Delta prfA$ (pPrfA), and $\Delta prfA$ (pPrfA*) strains in MM-Glc. (Inset) Western blot results for PrfA for EGD(pERL3) (lane I), $\Delta prfA$ (pPrfA) (lane II), and $\Delta prfA$ (pPrfA*) (lane III) strains. All strains were grown in MM-Glc and harvested at an OD₆₀₀ of 1. (b) Growth of $\Delta prfA$ (pERL3), $\Delta prfA$ (pPrfA), and $\Delta prfA$ (pPrfA*) strains in LB-Glc. (c) Growth of PKP1 and PKP1(pPrfA*) in MM-Glc. The growth curves are representative of four replicates.

contained 50 mM glucose as a carbon source. As shown in Fig. 1a, highly significant growth inhibition was also observed when the *prfA* mutant contained high numbers of wild-type *prfA* (plasmid pPrfA). However, growth inhibition was more pronounced for the $\Delta prfA$ (pPrfA*) strain than for the $\Delta prfA$ (pPrfA) strain, although these two strains produced almost equal amounts of the PrfA (PrfA*) protein and, as expected, the wild-type strain with the control plasmid contained far less PrfA (Fig. 1a, inset). Similar growth inhibition of the strain containing pPrfA* was also observed in LB-Glc with strains P14

FIG. 2. (a) Growth of P14(pERL3), P14(pPrfA*), P14-A(pERL3), and P14-A(pPrfA*) in LB-Glc. (Inset) Western blot results for PrfA for purified PrfA (positive control) (lane I), P14-A(pERL3), P14(pERL3) (lane II), P14(pPrfA*) (lane IV), and P14-A(pPrfA*) (lane V). The strains were grown in LB-Glc and harvested at an OD₆₀₀ of 1. (b) Growth of P14(pERL3), P14(pPrfA*), P14-A(pERL3), and P14-A(pPrfA*) in LB-G-6-P. (c) Growth of $\Delta prfA$ (pPrfA) and $\Delta prfA$ (pPrfA*) strains in the presence or absence of Amberlite XAD in LB-G-6-P. The growth curves are representative of four replicates.

and P14-A (Fig. 2a), which were also used in this study (these strains were isogenic and hyperactive PrfA-producing variants of *L. monocytogenes* [42]). The PrfA in these strains was quantified by Western blot analysis, and the P14-A strain with and without overexpression of PrfA* contained far larger amounts of PrfA than the isogenic P14 strain contained (Fig. 2a, inset). The larger amount of PrfA in P14-A(pPrfA*) than in P14(pPrfA*) was due to the presence of the chromosomal copy of *prfA*, which resulted in autoregulation. There was no difference between the growth rate of wild-type strain EGD and the growth rate of the isogenic $\Delta prfA$ mutant when the organisms were grown in MM-

Glc (Fig. 1a). In BHI only marginal differences in the growth rates of these strains were observed (data not shown).

To test whether the growth inhibition observed in the presence of excess PrfA* (PrfA) was caused by overexpression of the products encoded by the listerial virulence genes which are under the control of PrfA and hence produced at high concentrations, especially in the presence of PrfA* (42) (see below), we introduced pPrfA* into *L. monocytogenes* mutant PKP1, in which the *prfA* virulence gene cluster is deleted (49). When overproducing PrfA*, this strain showed growth inhibition in MM-Glc similar to that of wild-type strain EGD or $\Delta prfA$ carrying pPrfA* (Fig. 1c). A possible effect of overproduction of other PrfA-dependent gene products on growth inhibition could also be ruled out (mutants with deletions in *inlAB* and *inlC* and in *hpt* were tested) (data not shown).

Growth inhibition of the PrfA*-overexpressing strains was also observed when the culture medium was supplemented with 50 mM fructose, 50 mM mannose, or 25 mM cellobiose instead of 50 mM glucose, but not when glucose was replaced by the non-PTS carbon source adenine (data not shown).

***L. monocytogenes* overexpressing PrfA* shows no growth inhibition with glucose-6-phosphate as a carbon source.** To test whether the observed inhibition of growth by overexpressed PrfA (PrfA*) in glucose-containing minimal medium was caused by interference of PrfA with PTS-mediated glucose transport or subsequent steps linked to glucose metabolism, we replaced glucose with glucose-6-phosphate (G-6-P) as the carbon source. G-6-P is taken up by *L. monocytogenes* via the non-PTS permease Hpt, whose gene, designated *hpt* or *uhpT*, is under PrfA control (7); however, the subsequent catabolism should be the same. Growth experiments were carried out with the EGD strains mentioned above and, in addition, with *L. monocytogenes* strains P14 and P14-A, which carry single copies of the wild-type *prfA* and mutant *prfA** genes in the chromosome (42). The bacteria had to be cultivated in LB-G-6-P, as MM-G-6-P did not allow growth of *L. monocytogenes* in the presence of G-6-P. Without added glucose there was only limited growth in LB (up to an OD₆₀₀ of 0.5) of the *L. monocytogenes* wild-type strains (EGD and P14) (data not shown), which was obviously due to residual, undetermined PTS sugars in LB as a *ptsH* mutant of *L. monocytogenes* (which is unable to produce HPr and hence also is unable to take up PTS sugars) does not grow at all in LB (Mertins, unpublished data).

It is interesting that in LB with G-6-P not even the $\Delta prfA$ (pPrfA) strain expressing rather large amounts of wild-type PrfA was able to grow efficiently. However, growth of this strain was readily observed when Amberlite XAD (a resin that activates PrfA [13; M. Rauch, unpublished data]) was added to the medium (Fig. 2c), indicating that a component(s) of LB strongly suppresses PrfA activity. As shown in Fig. 2b, excess PrfA* did not inhibit but rather enhanced the growth of *L. monocytogenes* in G-6-P-containing LB, in contrast to the growth in glucose-containing LB (Fig. 2a). Neither the $\Delta prfA$ (pPrfA*) strain nor P14(pPrfA*) and P14-A(pPrfA*) showed growth inhibition in G-6-P-containing LB compared to the growth of the $\Delta prfA$ (pPrfA) strain or strains P14 and P14-A carrying the vector pERL3 alone. These data suggest that excess PrfA (or PrfA*) interferes with components of PTS-mediated glucose uptake but not with Hpt-mediated uptake of G-6-P. The data indicate that interference of overex-

FIG. 3. Glucose uptake in $\Delta prfA$ (pPrfA*) and $\Delta prfA$ (pERL3) strains. To measure the uptake of radioactively labeled D-[U-¹⁴C]glucose, the strains were grown in LB supplemented with 50 mM glucose to an OD₆₀₀ 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.

pressed PrfA* or overexpressed and activated wild-type PrfA with subsequent steps linked to the metabolism of the G-6-P is not the cause of growth inhibition. To further support this conclusion, we determined directly the rate of uptake of ¹⁴C-labeled glucose in EGD $\Delta prfA$ (pERL3) and $\Delta prfA$ (pPrfA*) strains when they were growing in LB-Glc. Figure 3 shows that high levels of PrfA* indeed inhibited glucose uptake significantly under the conditions tested.

Transcriptome analysis reveals up- and down-regulated genes in the presence of excess PrfA* after growth of *L. monocytogenes* in MM-Glc. To determine the influence of excess PrfA* (PrfA) on gene expression, we compared the expression profiles of the *prfA* deletion mutant carrying only the vector [$\Delta prfA$ (pERL3)] and the same strain with pPrfA* after growth of these strains in MM-Glc. To do this, we used microarrays containing oligonucleotides of all ORFs identified in the genome sequence of *L. monocytogenes* (19). RNA was prepared from the two strains grown either for the same amount of time [in this case the OD₆₀₀ of the $\Delta prfA$ (pERL3) strain reached 1.0 and that of the $\Delta prfA$ (pPrfA*) strain reached 0.3] or to the same cell density (in this case both cultures were harvested at an OD₆₀₀ of 1.0). In the first case the two strains were in similar logarithmic growth states (the set of RNAs from these cultures is referred to below as "set 1 RNA"), while in the second case the $\Delta prfA$ (pPrfA*) strain was already close to the stationary phase ("set 2 RNA"). Equal amounts of set 1 and set 2 total RNAs were labeled with Cy3-dCTP and Cy5-dCTP, respectively, and hybridized to the microarrays. Each experiment was carried out with three independently prepared RNAs and with six microarray slides. Since each oligonucleotide was spotted four times on each slide, 24 normalized background-corrected median intensity ratios for the two probes were obtained for each ORF for a given strain combination. Only the genes for which there were at least 12 good replicates based on the quality control criteria used and which showed significant two-fold differential regulation as determined by SAM (56) were considered further. Expression of some of the differently regulated genes was confirmed further by real-time PCR (see below). With this set of data we identified three groups of

TABLE 3. Genes regulated differently in $\Delta prfA$ (pPrfA*) and $\Delta prfA$ (pERL3) strains, as identified by microarray analysis^a

Gene	Ratio	Function ^b
Strains grown for the same amount of time		
Group I		
<i>prfA^c</i>	5.8	Listeriolysin positive regulatory protein
<i>hly^c</i>	13.6	Listeriolysin O precursor
<i>mpl^{c,d}</i>	19.3	Zinc metalloproteinase precursor
<i>actA^c</i>	19.6	Actin assembly-inducing protein precursor
<i>plcB^{c,d}</i>	23.2	Phospholipase C
<i>lmo0206</i>	7.9	Unknown
<i>inlA^{c,d}</i>	6.9	Internalin A
<i>inlB^c</i>	4.4	Internalin B
<i>lmo0752</i>	4.0	Putative haloacetate dehalogenase (p)
<i>hpt^{c,d}</i>	17.4	Hexose phosphate transport protein (pr)
<i>inlC</i>	4.2	Internalin C
<i>plsX</i>	4.3	PlsX protein involved in fatty acid/phospholipid synthesis (p)
Group II		
<i>lmo0207</i>	3.6	Lipoprotein
<i>lmo0212^e</i>	3.0	Unknown
<i>pth</i>	2.1	Peptidyl-tRNA hydrolase (p)
<i>lmo0227</i>	2.5	Conserved hypothetical protein
<i>lmo0276</i>	2.1	Conserved hypothetical protein
<i>lmo0279^d</i>	2.4	Anaerobic ribonucleoside triphosphate reductase (pr)
<i>lmo0422</i>	2.1	Unknown
<i>lmo0485</i>	2.0	Unknown
<i>lmo0604^e</i>	3.0	Similar to <i>B. subtilis</i> YvIA protein
<i>lmo0759</i>	2.4	Unknown
<i>lmo0901^d</i>	2.9	PTS, cellobiose-specific IIC component (p)
<i>dltD</i>	2.3	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
<i>dltC</i>	2.4	D-Alanyl carrier protein
<i>dltB</i>	2.6	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
<i>dltA</i>	2.6	D-Alanine-activating enzyme (Dae), D-alanine-D-alanyl carrier protein ligase (Dcl)
<i>lmo0991</i>	2.1	Conserved hypothetical protein
<i>lmo1007</i>	2.5	Unknown
<i>lmo1481</i>	2.1	Unknown
<i>comEC</i>	2.5	ComEC specifically required for DNA uptake but not for binding (p)
<i>comEB</i>	3.0	Similar to <i>B. subtilis</i> ComEB protein
<i>lmo1741^f</i>	2.5	Two-component sensor histidine kinase (p)
<i>adeC^f</i>	3.4	Adenine deaminases (p)
<i>lmo1743^f</i>	2.9	Unknown
<i>lmo1744^f</i>	3.0	Unknown
<i>lmo1745^{d,f}</i>	2.9	Two-component response regulator (p)
<i>lmo1746^f</i>	3.6	ABC transporter (permease) (p)
<i>lmo1752</i>	2.0	Unknown
<i>lmo1761</i>	3.0	Putative sodium-dependent transporter (p)
<i>purQ</i>	2.4	Phosphoribosylformylglycinamide synthetase I
<i>purC</i>	2.0	Phosphoribosylaminoimidazole succinocarboxamide synthetase
<i>purB</i>	2.5	Adenylosuccinate lyase
<i>fabG</i>	3.8	Similar to 3-ketoacyl-acyl carrier protein reductase
<i>fabD</i>	3.2	Malonyl coenzyme A-acyl carrier protein transacylase (p)
<i>fhbB</i>	2.6	Ferrichrome ABC transporter (permease) (p)
<i>lmo1962</i>	2.3	Transcription regulators (TetR family) (p)
<i>lmo2104</i>	2.9	Unknown
<i>lmo2105</i>	2.1	Ferrous iron transport protein B (p)
<i>lmo2115^e</i>	2.0	ABC transporter (permease) (p)
<i>lmo2151</i>	2.0	Unknown
<i>lmo2152</i>	2.2	Thioredoxin (p)
<i>lmo2153</i>	2.4	Flavodoxin (p)
<i>lmo2154</i>	2.9	Ribonucleoside diphosphate reductase, subunit beta (p)
<i>lmo2155</i>	2.2	Ribonucleoside diphosphate reductase, subunit alpha (p)
<i>lmo2156^c</i>	2.3	Unknown
<i>lmo2159</i>	2.4	Oxidoreductase (p)
<i>lmo2160</i>	3.6	Unknown
<i>lmo2177^c</i>	2.3	Unknown
<i>lmo2181</i>	3.2	Unknown
<i>lmo2184</i>	3.1	Ferrichrome ABC transporter (binding protein) (p)
<i>lmo2313^c</i>	2.1	Bacteriophage protein
<i>lmo2320^c</i>	2.2	Unknown
<i>lmo2322^c</i>	2.0	Protein gp44 (bacteriophage A118)
<i>lmo2323^c</i>	2.0	Protein gp43 (bacteriophage A118)
<i>lmo2423</i>	2.4	Conserved hypothetical protein
<i>lmo2437</i>	2.9	Unknown
<i>lmo2518</i>	2.2	Similar to <i>B. subtilis</i> putative transcriptional regulator LytR
<i>lmo2522</i>	2.6	Hypothetical cell wall-binding protein from <i>B. subtilis</i> (p)
<i>lmo2566</i>	2.0	Unknown
<i>rpsL</i>	2.0	Ribosomal protein S12

Continued on following page

TABLE 3—Continued

Gene	Ratio	Function ^b
Group III		
lmo0310	0.5	Unknown
lmo0312	0.4	Unknown
lmo0798	0.5	Lysine-specific permease (p)
<i>fri</i>	0.4	Nonheme iron-binding ferritin
<i>gbuA^d</i>	0.5	Glycine betaine ABC transporter (ATP-binding protein) (pr)
<i>gbuB</i>	0.4	Glycine betaine ABC transporters (permease) (pr)
<i>gbuC</i>	0.3	Glycine betaine ABC transporters (glycine betaine-binding protein) (pr)
<i>pheS</i>	0.5	Phenylalanyl-tRNA synthetase alpha subunit
<i>pheT^d</i>	0.5	Phenylalanyl-tRNA synthetase beta subunit
lmo1223 ^d	0.5	ABC transporter, ATP-binding proteins (p)
<i>glnA</i>	0.4	Glutamine synthetases (pr)
lmo1423 ^d	0.5	Unknown
lmo1424	0.3	Manganese transport NRAMP proteins (p)
lmo1433 ^d	0.5	Glutathione reductase (p)
lmo1516 ^d	0.3	Ammonium transporter NrgA (p)
lmo1517	0.3	Nitrogen regulatory PII protein (p)
lmo1625	0.4	Putative transporters (p)
lmo1626	0.4	Unknown
<i>trpA</i>	0.4	Tryptophan synthase (alpha subunit) (pr)
<i>trpB</i>	0.3	Tryptophan synthase (beta subunit) (pr)
<i>trpF</i>	0.3	Phosphoribosyl anthranilate isomerase
<i>trpC</i>	0.3	Indole-3-glycerol phosphate synthases (pr)
<i>trpD</i>	0.4	Anthranilate phosphoribosyltransferase (pr)
<i>trpG</i>	0.3	Anthranilate synthase beta subunit (pr)
<i>trpE</i>	0.3	Anthranilate synthase alpha subunit (pr)
lmo1651	0.5	ABC transporter (ATP-binding protein) (p)
lmo1652	0.5	ABC transporter (ATP-binding protein) (p)
lmo1720	0.5	PTS lichenan-specific enzyme IIB component (p)
lmo1733	0.2	Glutamate synthase (small subunit) (p)
lmo1734 ^d	0.2	Glutamate synthase (large subunit) (p)
lmo1847 ^d	0.3	Adhesion binding proteins and lipoproteins (ABC transporter) (p)
lmo1848 ^d	0.2	Metal cation ABC transporter, permease (p)
lmo1849	0.3	Metal cation ABC transporter, ATP-binding proteins (p)
lmo1926	0.5	Chorismate mutase (p)
<i>ilvD^d</i>	0.3	Dihydroxy acid dehydratase (p)
<i>ilvB</i>	0.3	Acetolactate synthase (acetohydroxy acid synthase) (large subunit) (p)
<i>ilvN</i>	0.4	Acetolactate synthase (acetohydroxy acid synthase) (small subunit) (p)
<i>ilvC</i>	0.3	Keto acid reductoisomerase (acetohydroxy acid isomeroreductase) (p)
<i>leuA</i>	0.3	2-Isopropylmalate synthase (p)
<i>leuB</i>	0.3	3-Isopropylmalate dehydrogenase (p)
<i>leuC</i>	0.4	3-Isopropylmalate dehydratase (large subunit) (p)
<i>leuD</i>	0.4	3-Isopropylmalate dehydratase (small subunit) (p)
<i>ilvA</i>	0.4	Threonine dehydratase (p)
lmo2196 ^d	0.4	Pheromone ABC transporter (binding protein) (p)
lmo2410	0.5	Unknown
<i>eno</i>	0.5	Enolase (pr)
<i>pgm^d</i>	0.5	Phosphoglycerate mutase (pr)
<i>pgk</i>	0.5	Phosphoglycerate kinase (pr)
lmo2637	0.5	Conserved lipoprotein
lmo2638	0.5	NADH dehydrogenase (p)
lmo2683 ^d	0.3	Cellobiose phosphotransferase enzyme IIB component (p)
lmo2691	0.5	Autolysin, <i>N</i> -acetylmuramidase (p)
lmo2720	0.5	Acetate coenzyme A ligase (p)
lmo2753	0.5	Unknown
<i>kat</i>	0.5	Catalase
Strains grown to the same density		
Group I		
<i>prfA^c</i>	4.4	Listeriolysin positive regulatory protein
<i>hly^c</i>	8.2	Listeriolysin O precursor
<i>mpl^{c,d}</i>	15.7	Zinc metalloproteinase precursor
<i>actA^c</i>	6.7	Actin assembly-inducing protein precursor
<i>plcB^{c,d}</i>	12.1	Phospholipase C
lmo0206	21.7	Unknown
lmo0207	8.0	Lipoprotein
<i>inlA^{c,d}</i>	10.5	Internalin A
<i>inlB^c</i>	7.1	Internalin B
<i>hpt^{c,d}</i>	13.0	Hexose phosphate transport protein (pr)
<i>inlC^c</i>	7.0	Internalin C
Group II		
<i>lmaD</i>	2.7	Antigen D (p)
<i>lmaC^d</i>	2.9	Antigen C (p)
<i>lmaB</i>	3.2	Antigen B

Continued on following page

TABLE 3—Continued

Gene	Ratio	Function ^b
<i>lmaA</i>	3.8	Antigen A
<i>lmo0119</i>	3.0	Unknown
<i>lmo0120</i>	2.5	Unknown
<i>lmo0121</i>	2.8	Bacteriophage minor tail proteins (p)
<i>lmo0122</i>	3.1	Phage proteins (p)
<i>lmo0123</i>	2.3	Protein gp18 from bacteriophage A118 (p)
<i>lmo0124</i>	2.3	Unknown
<i>lmo0125</i>	2.1	Unknown
<i>lmo0126</i>	2.3	Unknown
<i>lmo0128</i>	2.3	Protein from bacteriophage phi-105 (ORF 45) (p)
<i>lmo0129</i>	2.2	Autolysin, <i>N</i> -acetylmuramoyl-L-alanine amidase (p)
<i>lmo0212^c</i>	2.1	Unknown
<i>lmo0560^d</i>	2.6	NADP-specific glutamate dehydrogenase (p)
<i>lmo0604^e</i>	3.6	Similar to <i>B. subtilis</i> YvIA protein
<i>lmo1257</i>	2.0	Unknown
<i>lmo1634</i>	2.4	Alcohol-acetaldehyde dehydrogenase (p)
<i>lmo2115^e</i>	2.0	ABC transporter (permease) (p)
<i>lmo2156</i>	3.5	Unknown
<i>lmo2177^e</i>	3.1	Unknown
<i>lmo2219</i>	2.4	Posttranslocation molecular chaperone (p)
<i>lmo2280</i>	2.2	Protein gp23 (bacteriophage A118)
<i>lmo2304</i>	2.3	Protein gp65 (bacteriophage A118)
<i>lmo2305</i>	2.4	Unknown
<i>lmo2306</i>	2.4	Phage protein (p)
<i>lmo2308</i>	2.1	Single-stranded DNA-binding protein (p)
<i>lmo2309</i>	2.3	Unknown
<i>lmo2311</i>	2.2	Unknown
<i>lmo2312</i>	2.4	Unknown
<i>lmo2313^e</i>	2.2	Bacteriophage protein (p)
<i>lmo2317</i>	2.4	Protein gp49 (bacteriophage A118) (p)
<i>lmo2319</i>	2.2	Bacteriophage proteins (p)
<i>lmo2320^e</i>	2.7	Unknown
<i>lmo2321</i>	3.1	Unknown
<i>lmo2322^e</i>	2.5	Protein gp44 (bacteriophage A118)
<i>lmo2323^e</i>	2.0	Protein gp43 (bacteriophage A118)
<i>lmo2324</i>	2.5	Antirepressor (bacteriophage A118) (p)
<i>lmo2325</i>	2.3	Unknown
<i>lmo2327</i>	2.6	Unknown
<i>lmo2328</i>	2.2	Transcription regulator (p)
<i>lmo2437</i>	2.4	Unknown
<i>lmo2439</i>	2.1	Unknown
<i>lmo2581</i>	3.0	Conserved hypothetical protein
Group III		
<i>lmo0726</i>	0.4	Hypothetical CDS
<i>lmo0727</i>	0.4	L-Glutamine-D-fructose-6-phosphate amidotransferase (p)
<i>gbuA^d</i>	0.5	Glycine betaine ABC transporter (ATP binding) (pr)
<i>gbuC</i>	0.5	Glycine betaine ABC transporters (glycine betaine) (pr)
<i>glnR^d</i>	0.3	Glutamine synthetase repressor (p)
<i>glnA</i>	0.2	Glutamine synthetases (pr)
<i>lmo1516^d</i>	0.1	Ammonium transporter NrgA (p)
<i>lmo1517</i>	0.1	Nitrogen regulatory PII protein (p)
<i>lmo1518</i>	0.2	Unknown
<i>lmo1625</i>	0.5	Putative transporters (p)
<i>lmo1626</i>	0.5	Unknown
<i>trpB^d</i>	0.4	Tryptophan synthase (beta subunit) (pr)
<i>trpF^d</i>	0.4	Phosphoribosyl anthranilate isomerase
<i>trpG</i>	0.4	Anthranilate synthase beta subunit (pr)
<i>lmo2101^d</i>	0.3	Protein required for pyridoxine synthesis (p)
<i>lmo2102^d</i>	0.4	Unknown
<i>lmo2196^d</i>	0.4	Pheromone ABC transporter (binding protein) (p)

^a RNA was prepared from the two strains grown for the same amount of time or to the same cell density. Genes are grouped according to the pattern of differential regulation in the PrfA*-expressing strain, as follows: group I, genes up-regulated at least fourfold; group II, genes up-regulated two- to threefold; and group III, genes down-regulated at least twofold.

^b (p) and (pr) indicate putative and probable functions of the genes, respectively, as annotated in <http://genolist.pasteur.fr/ListiList/>.

^c Gene which contains a putative PrfA box in the promoter region with a maximum of one mismatch compared to the consensus sequence TTAACANNTGTAA. *plcA* is missing from group I because of the degraded oligonucleotide from the oligonucleotide set used to prepare the slides, but its transcriptional up-regulation was confirmed by real-time RT-PCR (data not shown).

^d Gene which contains a putative cre box in the promoter region with up to two mismatches based on the consensus sequence for *B. subtilis*, as reported recently (35).

^e Group II gene commonly up-regulated in both sets of RNA (same time and same cell density).

^f Gene belonging to the cluster coding for a two-component system as described by Mandin et al. (31).

genes that were differently regulated in the two strains and whose expression was hence influenced by excess PrfA* (PrfA).

Genes belonging to group I were highly up-regulated in the

$\Delta prfA$ (pPrfA*) strain (more than fourfold) (Table 3), and these genes included *prfA* and the virulence genes (i.e., *hly*, *mpl*, *actA*, *plcB*, *inlAB*, *inlC*, and *hpt*); transcription of these genes is known to be activated by PrfA in vivo (for a recent

FIG. 4. (a) Real-time quantitative RT-PCR analysis of selected genes belonging to group III (Table 3) using different *L. monocytogenes* strain combinations, including $\Delta prfA$ (pPrfA*) and $\Delta prfA$, $\Delta prfA$ (pPrfA*) and $\Delta prfA$ (pPrfA), and $\Delta prfA$ (pPrfA) and $\Delta prfA$ strains. The black bars indicate the means of four values for normalized, $\log_2$ -transformed ratios observed after growth in MM-Glc. The gray bars indicate the means of four values for normalized, $\log_2$ -transformed ratios observed after growth in BHI. The error bars indicate the standard deviations for the four separate experiments. (b) Real-time quantitative RT-PCR analysis of selected genes belonging to group III (Table 3) using different *L. monocytogenes* strain combinations, including EGD(pPrfA*) and $\Delta prfA$, EGD(pPrfA*) and wild-type EGD, and wild-type EGD and $\Delta prfA$ strains. The black bars indicate the means of four values for normalized, $\log_2$ -transformed ratios observed after growth in MM-Glc. The gray bars indicate the means of four values for normalized, $\log_2$ -transformed ratios observed after growth in BHI. The error bars indicate the standard deviations for the four separate experiments. WT, wild type.

review see reference 57) and in vitro (25, 30). The expression of lmo0206 and lmo0207, which are located in an operon together with *mpl*, *actA*, and *plcB*, was also up-regulated. Both ORFs were therefore included in group I. As expected, these genes were found in set 1 and set 2 RNAs; the up-regulation of these genes in the $\Delta prfA$ (pPrfA*) strain was more pronounced with set 1 RNA than with set 2 RNA.

Group II comprised genes whose expression was moderately increased (two- to threefold) in the $\Delta prfA$ (pPrfA*) strain compared to the expression in the isogenic strain carrying only the empty vector. In contrast to the genes in group I, none of the genes in this group contained a characteristic PrfA box in the upstream regulatory regions. Only a few of these genes were identified with set 1 and set 2 RNAs (Table 3). The functions of these common genes are largely unknown, and several of the genes are genes of the integrated bacteriophage A118. Up-regulation of these phage genes was more pronounced with set 2 RNA. Some of the other genes in group II, which were observed mainly with set 1 RNA, are genes that belong to operons involved in cell wall modifications (lmo0971 to lmo0974), in a two-component system (encoded by the lmo1741-lmo1746 operon), in fatty acid biosynthesis (lmo1805 to lmo1809), and in reduction of ribonucleosides (lmo2151 to lmo2155).

Genes belonging to group III were very significantly down-regulated (two- to fivefold) in the $\Delta prfA$ (pPrfA*) strain and were best identified with set 1 RNA. In addition to some unknown products, these genes encode mainly enzymes involved in glycolysis (*eno*, *pgm*, *pgk*), in biosynthetic pathways (*ilvB* operon, *trp* operon), and in N metabolism or regulation of this metabolism (lmo1733 and lmo1734-*[gltAB]*, encoding the small and large subunits of glutamate synthase; lmo1516 and lmo1517; encoding the ammonium transporter NrgA and the PII-like protein NrgB; *glnA*, encoding the glutamine synthase). Other interesting genes include *kat* (catalase), genes for a PTS specific for β -glucosides, and genes encoding two ABC transporters specific for glycine betaine (lmo1015 and lmo1016) and for metal cations (lmo1848 and lmo1849). Again, none of the group III genes have a putative PrfA box in the 5' upstream regulatory region (Table 3).

Confirmation of microarray data for the group III genes by quantitative real-time RT-PCR. Real-time RT-PCR was employed for selected genes to confirm the differential regulation observed in the transcriptome analyses. These experiments were performed with set 1 RNA preparations isolated independently of the preparations used for the transcriptome analysis described above. In particular, we analyzed the interesting group III genes *glnR*, *glnA*, lmo1516 and lmo1517 (*nrgAB*), lmo1734 (*gltA*), lmo1849, and *leuA* (Fig. 4). To further show that excess wild-type PrfA down-regulates these genes as well as PrfA*, we performed RT-PCR with RNAs isolated from the $\Delta prfA$ (pERL3), $\Delta prfA$ (pPrfA*), and $\Delta prfA$ (pPrfA) strains after growth in MM-Glc. The results obtained confirmed that overproduced wild-type PrfA qualitatively had the same effect as PrfA* on the expression of the group III genes tested (Fig. 4a). Similar results were obtained when we used the wild-type EGD strain instead of the $\Delta prfA$ mutant (Fig. 4b). These results suggest that the expression of these genes is inhibited by excess PrfA* and wild-type PrfA. Furthermore, Fig. 4a and 4b show that these genes were not differentially regulated when

FIG. 5. Hemolytic activity (a) and amounts of listeriolysin (b) and ActA protein (c) as determined by Western blot analysis of $\Delta prfA$ (pPrfA) and $\Delta prfA$ (pPrfA*) strains grown in minimal medium supplemented with 50 mM glucose (MM-Glc) (bars I and III and lanes I and III) or with 50 mM glucose and 25 mM cellobiose (MM-Glc-Cel) (bars II and IV and lanes II and IV).

the strains were grown in BHI, supporting the observation that there was no growth difference between the strains in BHI.

Effect of PrfA on the expression of group III genes is dependent on the amount and activity of PrfA. As shown in Fig. 1a, the amounts of wild-type PrfA and PrfA* in the $\Delta prfA$ (pPrfA) and $\Delta prfA$ (pPrfA*) strains, respectively, are similar. However, as recently shown (13), the activities of the two PrfA species can still vary significantly despite similar concentrations. To confirm the difference in activity for the two strains when they were grown in MM-Glc, we measured the hemolytic activities (determined entirely by the PrfA-controlled *hly* gene) of the two strains in the presence and absence of cellobiose. This β -glucoside is known to strongly inhibit the activity of wild-type PrfA but not the activity of PrfA* (43). As shown in Fig. 5a, the hemolytic activity was fivefold higher in the $\Delta prfA$ (pPrfA*) strain than in the $\Delta prfA$ (pPrfA) strain in MM-Glc in the absence of cellobiose, and the hemolytic activity was strongly inhibited by cellobiose in the $\Delta prfA$ (pPrfA) strain but not in the $\Delta prfA$ (pPrfA*) strain. Similar results were obtained when we determined the amounts of listeriolysin O and ActA under these conditions (Fig. 5b and 5c).

To test the effect of the PrfA concentration and activity on expression of the group I to III genes, we compared the expression profiles of the $\Delta prfA$ strains overexpressing PrfA* or wild-type PrfA grown in MM-Glc. The two strains exhibited reduced growth rates in MM-Glc (albeit to different extents) compared to the growth rate of the $\Delta prfA$ strain not producing PrfA (Fig. 1a). Again, both strains were harvested at the same time, and RNA was isolated as described above. As shown in Table 4, the expression pattern for the

TABLE 4. Genes differentially regulated in $\Delta prfA$ (pPrfA*) and $\Delta prfA$ (pPrfA) strains, as identified by microarray analysis^a

Gene	Ratio	Function ^b
Group I		
<i>hly^c</i>	4.4	Listeriolysin O precursor
<i>mpl^{c,d}</i>	7.2	Zinc metalloproteinase precursor
<i>actA^c</i>	4.9	Actin assembly-inducing protein precursor
<i>plcB^{c,d}</i>	5.5	Phospholipase C
<i>lmo0206</i>	7.1	Unknown
<i>lmo0212</i>	5.1	Unknown
<i>hpt^{c,d}</i>	7.5	Hexose phosphate transport protein (pr)
<i>inlC^c</i>	8.4	Internalin C
<i>lmo2186</i>	5.7	Unknown
Group II		
<i>lmaD</i>	2.6	Antigen D (p)
<i>lmaC^d</i>	3.3	Antigen C (p)
<i>lmaB</i>	3.8	Antigen B
<i>lmaA</i>	3.1	Antigen A
<i>lmo0119</i>	2.4	Unknown
<i>lmo0121</i>	2.8	Bacteriophage minor tail proteins (p)
<i>lmo0122</i>	3.4	Phage proteins (p)
<i>lmo0123</i>	2.3	Protein gp18 from bacteriophage A118 (p)
<i>lmo0124</i>	2.2	Unknown
<i>lmo0125</i>	2.8	Unknown
<i>lmo0126</i>	2.3	Unknown
<i>lmo0127</i>	2.0	Protein gp20 from bacteriophage A118 (p)
<i>lmo0128</i>	2.2	Protein from bacteriophage phi-105 (ORF 45) (p)
<i>lmo0129</i>	2.1	Autolysin, <i>N</i> -acetylmuramoyl-L-alanine amidase (p)
<i>lmo0139</i>	2.7	Unknown
<i>lmo0207</i>	3.9	Lipoprotein (p)
<i>pth</i>	2.7	Peptidyl-tRNA hydrolase (p)
<i>lmo0230</i>	2.3	Similar to <i>B. subtilis</i> YacH protein
<i>lmo0279^d</i>	2.2	Anaerobic ribonucleoside triphosphate (pr)
<i>lmo0421</i>	2.7	Rod shape-determining protein RodA (p)
<i>lmo0422</i>	2.5	Unknown
<i>lmo0423</i>	2.9	RNA polymerase ECF-type sigma factor (p)
<i>inlA^d</i>	3.2	Internalin A
<i>inlB</i>	2.0	Internalin B
<i>lmo0504</i>	3.2	Unknown
<i>lmo0604</i>	2.7	Similar to <i>B. subtilis</i> YvIA protein
<i>lmo0647</i>	2.3	Unknown
<i>lmo0752</i>	2.0	Putative haloacetate dehalogenase (p)
<i>dltD</i>	2.7	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
<i>dltC</i>	2.9	D-Alanyl carrier protein
<i>dltB</i>	3.5	DltB protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid
<i>dltA</i>	3.4	D-Alanine-activating enzyme (Dae), D-alanine-D-alanyl carrier protein
<i>clpE</i>	2.6	ATP-dependent protease
<i>cadA</i>	2.6	Cadmium resistance protein
<i>lmo1250</i>	3.1	Antibiotic resistance protein (p)
<i>lmo1303</i>	2.8	Similar to <i>B. subtilis</i> YneA protein
<i>comEC</i>	2.0	Putative integral membrane protein ComEC specific (p)
<i>comEB</i>	2.3	Similar to <i>B. subtilis</i> ComEB protein
<i>lmo1741^e</i>	2.0	Two-component sensor histidine kinase (p)
<i>adeC^c</i>	2.4	Adenine deaminases (pr)
<i>lmo1745^{d,e}</i>	2.0	Two-component response regulator (p)
<i>lmo1746^e</i>		ABC transporter (permease) (p)
<i>lmo1752</i>	2.0	Unknown
<i>smc</i>	2.0	Smc protein essential for chromosome condensation (p)
<i>fabG</i>	2.2	3-Ketoacyl-acyl carrier protein reductase (p)
<i>fabD</i>	2.0	Malonyl coenzyme A-acyl carrier protein transacylase (p)
<i>plsX</i>	2.6	PlsX protein involved in fatty acid/phospholipid (p)
<i>lmo2104</i>	2.6	Unknown
<i>lmo2114</i>	3.0	ABC transporter (ATP-binding protein) (p)
<i>lmo2115</i>	2.1	ABC transporter (permease) (p)
<i>lmo2154</i>	2.7	Ribonucleoside diphosphate reductase, subunit (p)
<i>lmo2155</i>	2.0	Ribonucleoside diphosphate reductase, subunit (p)
<i>lmo2156</i>	2.5	Unknown
<i>lmo2177</i>	2.3	Unknown
<i>lmo2181</i>	3.7	Unknown

Continued on following page

TABLE 4—Continued

Gene	Ratio	Function ^b
lmo2304	2.2	Protein gp65 (bacteriophage A118)
lmo2308	2.1	Single-stranded DNA-binding protein (p)
lmo2318	2.2	Unknown
lmo2321	2.1	Unknown
lmo2322	2.3	Protein gp44 (bacteriophage A118)
lmo2323	2.2	Protein gp43 (bacteriophage A118)
lmo2416	2.2	Unknown
lmo2437	2.8	Unknown
lmo2439	2.5	Unknown
lmo2522	2.0	Hypothetical cell wall-binding protein from <i>B. subtilis</i> (p)
lmo2676	2.1	UV damage repair protein (p)
lmo2687	2.2	Cell division protein FtsW (p)
lmo2689	2.3	Mg ²⁺ transport ATPase (pr)
lmo2751	2.0	ABC transporter, ATP-binding protein (p)
Group III		
lmo0099	0.5	Unknown
lmo0152	0.4	Oligopeptide ABC transporter-binding protein (p)
lmo0153 ^d	0.5	Probable high-affinity zinc ABC transporter (p)
lmo0283	0.4	ABC transporter permease protein (p)
lmo0284 ^d	0.4	ABC transporter (ATP-binding protein) (p)
lmo0641	0.5	Heavy metal-transporting ATPase (p)
lmo0708	0.5	Hypothetical flagellar protein (p)
lmo0709	0.3	Unknown
lmo0713	0.5	Flagellar basal body M-ring protein FlhF (p)
lmo0724 ^d	0.4	Similar to <i>B. subtilis</i> YvpB protein
lmo0731	0.4	Unknown
gbuA ^d	0.5	Glycine betaine ABC transporter (ATP binding) (pr)
gbuB	0.4	Glycine betaine ABC transporters (permeability) (pr)
lmo1046	0.5	Molybdenum cofactor biosynthesis protein C (p)
lmo1118	0.5	Unknown
pheS	0.5	Phenylalanyl-tRNA synthetase alpha subunit
glnR ^d	0.5	Glutamine synthetase repressor (p)
glnA	0.3	Glutamine synthetases (pr)
lmo1423 ^d	0.4	Unknown
lmo1424	0.4	Manganese transport NRAMP proteins (p)
lmo1516 ^d	0.1	Ammonium transporter NrgA (p)
lmo1517	0.2	Nitrogen regulatory PII protein (p)
lmo1518	0.2	Unknown
aspS ^d	0.5	Aspartyl-tRNA synthetase
lmo1733	0.2	Glutamate synthase (small subunit) (p)
lmo1734 ^d	0.3	Glutamate synthase (large subunit) (p)
lmo1847 ^d	0.4	Adhesion-binding proteins and lipoproteins (p)
lmo1848 ^d	0.2	Metal cation ABC transporter (permease protein) (p)
lmo1849	0.5	Metal cation ABC transporter, ATP-binding protein (p)
ilvD ^d	0.4	Dihydroxy acid dehydratase (p)
ilvN	0.5	Acetolactate synthase (acetohydroxy acid synthase) (p)
leuB	0.5	3-Isopropylmalate dehydrogenase (p)
lmo2196 ^d	0.4	Pheromone ABC transporter (binding protein) (p)
kat	0.5	Catalase

^a RNA was prepared from the two strains grown for the same amount of time. Genes are grouped according to the pattern of differential regulation in the PrfA*-expressing strain, as follows: group I, genes up-regulated at least fourfold; group II, genes up-regulated two- to threefold; and group III, genes down-regulated at least twofold.

^b (p) and (pr) indicate putative and probable functions of the genes, respectively, as annotated in <http://genolist.pasteur.fr/ListiList/>.

^c Gene which contains a putative PrfA box in the promoter region with a maximum of one mismatch compared to the consensus sequence TTAACANNTGTAA. *plcA* is missing from group I because of the degraded oligonucleotide from the oligonucleotide set used to prepare the slides, but its transcriptional up-regulation was confirmed by real-time RT-PCR (data not shown).

^d Gene which contains a putative cre box in the promoter region with up to two mismatches based on the consensus sequence for *B. subtilis*, as reported recently (35).

^e Gene belonging to the cluster coding for a two-component system, as described by Mandin et al. (31).

$\Delta prfA(pPrfA^*)$ - $\Delta prfA(pPrfA)$ strain combination was quite similar to that for the $\Delta prfA(pPrfA^*)$ - $\Delta prfA(pERL3)$ strain combination (Table 3); although the change was much greater for the latter combination, the results suggested that not only the amount but also the activity of PrfA is responsible for the strength of the growth inhibition and the differential gene expression in these strains caused by excess PrfA* or wild-type PrfA.

DISCUSSION

In this report we show that the growth of *L. monocytogenes* strains (EGD wild-type strain or the isogenic $\Delta prfA$ mutant) overproducing PrfA* is strongly inhibited in a defined minimal medium containing glucose as the carbon source (MM-Glc) compared to the growth of the wild-type EGD strain or the *prfA* null mutant. Greater growth inhibition was observed in

the presence of excess hyperactive PrfA* than in the presence of wild-type PrfA protein (when roughly equal amounts were used), suggesting that not only the amount but also the PrfA activity (which is considerably higher in PrfA*) is decisive for the inhibition. The growth rates of all of the strains were only marginally different in rich culture medium (BHI). The data clearly show that the growth inhibition is not caused by overproduction of the products of the PrfA-controlled virulence genes but correlates with reduced PTS-mediated glucose transport that is apparently caused by overexpressed PrfA (PrfA*). Since the growth of *L. monocytogenes* in the presence of glucose-6-phosphate taken up by the PrfA-dependent Hpt transporter (7) is as efficient as the growth in the presence of glucose and is not inhibited (but rather is enhanced) by excess PrfA (PrfA*), we concluded that excess PrfA (PrfA*) interferes with a component(s) of PTS-mediated glucose transport rather than with components of the subsequent steps linked to the metabolism, as the later steps should be similar in both cases.

A comparison of the expression profiles of the $\Delta prfA$ strain overexpressing PrfA* and the same strain not expressing PrfA showed that after growth in MM-Glc there was transcriptional up- and down-regulation of several genes that can be divided into three groups. Group I contains all known PrfA-dependent virulence genes, including the genes encoding LIPI-I (57), *inlAB*, *inlC*, and *hpt*. Up-regulation of these genes clearly correlates with the activity of PrfA, since greater up-regulation was observed with the hyperactive molecule PrfA* than with equal amounts of wild-type PrfA, which is in accord with other reports (13). It is interesting that expression of none of the genes which were recently reported to be also under PrfA control, like *bsh*, *bilE*, or *vip* (5, 11, 50), was found to be up-regulated under these conditions, suggesting that these genes are controlled differently by PrfA than the "classic" PrfA-regulated virulence genes mentioned above.

The up-regulation of the group II genes was less pronounced than the up-regulation of the group I genes and was observed mainly with RNAs from the $\Delta prfA$ and $\Delta prfA(pPrfA^*)$ strains when they were grown for the same amount of time (set 1 RNA), which reflected the actual growth inhibition by excess PrfA* (PrfA). Only a few of these genes were also found to be up-regulated with RNAs from bacteria grown to the same cell density (set 2 RNA), when both cultures reached similar growth phases (although at very different times [Fig. 1a]). Among the group II genes, which do not contain typical PrfA boxes in the regulatory regions preceding the genes, is the *lmo1741-lmo1746* operon, which contains the genes for a two-component system, VirR (*lmo1745*) and VirS (*lmo1741*). VirR was recently identified as a response regulator that is critical for *L. monocytogenes* virulence (31). Interestingly, most of the genes that were shown by Mandin et al. to be under the control of VirR are also group II genes. The fact that up-regulation of these genes was also observed when the comparative transcription profiles of the $\Delta prfA(pPrfA^*)$ and $\Delta prfA(pPrfA)$ strains were examined suggests that the induced *virR* expression correlates not only with the amount of PrfA but also with the PrfA activity. Growth inhibition and up-regulation of group II genes by excess PrfA and PrfA*, respectively, thus behave similarly, suggesting that both events may be caused by the impaired glucose uptake due to excess PrfA* (PrfA). Other genes in this

group (better observed with set 2 RNA) belong to the integrated A118 bacteriophage, and expression of these genes may be induced by nutrient stress.

Among the most strongly down-regulated genes belonging to group III are genes that were also found to be down-regulated under glucose-limited conditions in *B. subtilis*, i.e., the *gltAB* and *ilvB* operons (3, 14, 28, 29, 48, 54). It is therefore likely that the observed down-regulation of these genes and also of the other genes involved in N metabolism (i.e., *ngrAB* and *glnAR*) in PrfA* (PrfA)-overproducing *L. monocytogenes* is also caused by the impaired glucose transport. The same explanation may also apply to down-regulation of the *eno* and *pgm* genes, which are involved in the lower part of glycolysis and have also been shown to be down-regulated in *B. subtilis* under glucose-limited conditions (3). The strong down-regulation of the *trp* operon observed in the presence of excess PrfA* has not been observed in *B. subtilis* under glucose-limited conditions. Tryptophan biosynthesis depends strongly on intermediates of the pentose phosphate cycle (erythrose-4-phosphate and ribose-5-phosphate). Perhaps the reduced glucose concentration in the presence of a high level of PrfA* slows down the pentose phosphate pathway, which in turn may lead to reduced expression of the *trp* operon. Similar to the up-regulation of group II genes, the down-regulation of group III genes was most obvious with RNAs from listerial cells that were grown for the same amount of time (set 1 RNA; reflecting the actual growth inhibition by excess PrfA) and less obvious with RNAs from cells that were grown to the same cell density (set 2 RNA). As expected, there was not a significant difference in the up-regulated group I genes with these two RNA sets.

We think that the impaired glucose uptake is primarily responsible for the observed growth inhibition, while the down-regulation of the group III genes (and possibly also the up-regulation of group II genes) may be primarily a consequence of the reduced glucose uptake by excess PrfA*. Particularly the down-regulation of the *ilvB* and *glnAR* operons is surprising in this context, since the MM-Glc medium used contained leucine, isoleucine, valine, and glutamine in addition to other amino acids (38). The results thus indicate either that the genes for biosynthesis of the branched amino acids and glutamine are not repressed in logarithmically growing *L. monocytogenes* by the presence of these amino acids in the culture medium or that these amino acids are not efficiently taken up by *L. monocytogenes* and hence their intracellular levels are too low for repression of their biosynthesis operons.

The precise mechanism for the down-regulation of these genes under glucose-limited conditions in *B. subtilis* is not understood yet. It has been claimed that an intracellular metabolite that accumulates during active PTS-mediated glucose transport may be required for induction of these genes (3). Recent reports (48, 55) have shown that regulation of the *ilvB* operon, which belongs to this class of genes, involves, in addition to CcpA (there is a cre site in front of the first gene of this operon), other global regulators which respond to nutrient limitation, like CodY and TnrA.

Recently, Mandin and coworkers (31) reported comparative gene expression profiles for *L. monocytogenes* EGDe producing different levels of PrfA (and PrfA*). These authors showed that in addition to expression of the known PrfA-dependent virulence genes, there was up-regulation of SigB-controlled

genes in the presence of PrfA, which we did not observe in our study. Although the data of the two studies cannot be directly compared due to the different culture media and strains used, it is interesting that expression of SigB is induced in *B. subtilis* under various stress conditions, including a limited glucose supply (60). Thus, PrfA-mediated inhibition of glucose transport may also cause induction of the SigB-regulated genes by PrfA under the culture conditions used by Mandin and co-workers. This conclusion is in agreement with the observation described by these authors that induction of the SigB-regulated genes is strongly enhanced in strain P14-A, which produces much more PrfA than the EGDe strain produces.

Our data led us to conclude that high concentrations of wild-type PrfA and of PrfA* inhibit PTS-mediated glucose transport probably by binding to a component essential for this process. Several previous studies (2, 4, 13, 34, 37) have shown that PrfA activity is inhibited by an unknown mechanism when *L. monocytogenes* is cultured in the presence of PTS sugars, like glucose and especially cellobiose. Both observations may be based on the interaction of PrfA with a common component involved in the PTS-mediated uptake of these sugars. Assuming that the concentration of this component in the bacterial cell is constant, the interaction should lead to inhibition of wild-type PrfA activity but (due to the altered structure [12], not to inhibition of PrfA* activity) at a low PrfA concentration. At a high PrfA concentration this component is titrated out by PrfA (and even better by PrfA*), leading to inhibition of PTS-mediated sugar uptake.

ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (grant SFB 479-B1) and the Fonds der Chemischen Industrie. B.J. was a recipient of a postdoctoral fellowship from the "Europäisches Graduiertenkolleg 587/2," and A.K.M. was supported by the "STUDIENSTIFTUNG des deutschen Volkes."

We are grateful to M. Frosch and Anja Schramm for help with the microarray facility.

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