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Glucose-1-Phosphate Utilization by *Listeria monocytogenes* Is PrfA Dependent and Coordinately Expressed with Virulence Factors

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Virulence genes of the facultative intracellular pathogen *Listeria monocytogenes* are coordinately regulated by the activator protein PrfA, encoded by *prfA*, a member of the cyclic AMP receptor protein family of bacterial transcription factors. We found that *prfA mutants that constitutively overexpress the virulence regulon due to a Gly145Ser substitution in PrfA (M.-T. Ripio, G. Domínguez-Bernal, M. Lara, M. Suárez, and J.-A. Vázquez-Boland, J. Bacteriol. 179:1533–1540, 1997) rapidly utilized glucose-1-phosphate (G-1-P) as a carbon source for growth, in contrast to wild-type strains, which characteristically do not. Wild-type strains acquired the capacity for readily metabolizing G-1-P upon exposure to environmental conditions that activate the expression of *prfA* and PrfA-dependent virulence genes (i.e., culture at 37°C in charcoal-treated medium). In these strains, G-1-P utilization followed an expressional pattern identical to that of virulence genes controlled by PrfA, with repression at 20°C. Tn*917* insertions in *L. monocytogenes* mutants selected for G-1-P utilization deficiency mapped to the *plcA-prfA* operon, a Δ*prfA* strain was totally unable to utilize G-1-P, and *trans* complementation with *prfA* constructs restored the ability to efficiently metabolize and grow on G-1-P to these mutants. Thus, G-1-P utilization by *L. monocytogenes* is under the tight positive control of the central virulence regulator, PrfA, and is coexpressed with PrfA-dependent pathogenicity determinants. It was recently reported that readily utilized carbohydrates, such as glucose or cellobiose, repress virulence genes in *L. monocytogenes*. We confirmed this but, interestingly, found that G-1-P does not inhibit expression of the PrfA regulon, indicating that this sugar follows a catabolic pathway that bypasses the repressor mechanism triggered by other readily metabolized carbon sources. PrfA dependence and coexpression with virulence genes suggest that utilization of exogenous G-1-P may be relevant to *Listeria* pathogenesis. G-1-P is the precursor metabolite and primary degradation product of glycogen and is therefore available within the mammalian cell. Based on our results, we hypothesize that G-1-P could play an important role as a growth substrate for intracellular *Listeria*.**

The only known virulence regulator in *Listeria* is the PrfA protein, a transcriptional activator with structural and functional similarities to the cyclic AMP receptor protein (CRP) (7, 20, 28, 36, 38). Its structural gene, *prfA*, is present in a chromosomal pathogenicity island which comprises a cluster of determinants involved in major virulence functions, namely, the hemolysin gene *hly*, the phospholipase genes *plcA* and *plcB*, and the actin-polymerizing surface protein gene *actA* (see references 19, 32, and 40 for reviews of the molecular determinants of listerial pathogenicity and references 5 and 18 for reviews of PrfA). All the genes of this cluster, including *prfA* itself, are tightly regulated by PrfA (7, 28). Other virulence genes located elsewhere on the listerial chromosome are also under the control of PrfA, for example, the members of the internalin multigene family *inlAB* (9, 24) and *inlC* (10), and there is experimental evidence that additional, as yet unidentified listerial genetic determinants are PrfA regulated (41).

Based on similarities with CRP, a model for PrfA-mediated virulence gene regulation has recently been proposed in which PrfA switches between transcriptionally inactive and active forms upon interaction with a low-molecular-weight cofactor (36). This unidentified cofactor is predicted to be responsible for transducing activatory signals from the environment to the PrfA system. These signals may include elevated temperature (22) and appropriate changes in the composition of the extracellular medium (4, 36, 37), which could indicate to *Listeria monocytogenes* its location inside a mammalian host. *prfA* is transcribed either monocistronically in a PrfA-independent manner or as a PrfA-dependent bicistronic *plcA-prfA* mRNA, thereby creating an autoregulatory loop essential for the normal expression of the virulence regulon (12, 22, 28). According to the model, transition of PrfA molecules synthesized from the monocistronic *prfA* transcript into the active form activates the *prfA* transcriptional amplification loop, concomitantly triggering an abrupt upregulation of the remaining virulence genes (36). There is a second level of regulation based on the differential response of the PrfA-dependent promoters according to structural differences in the target DNA sequences to which PrfA binds (3, 39).

All the PrfA-regulated genes identified so far code for conventional virulence factors, i.e., surface proteins involved in cytoadhesion, invasion, and host cell actin-based intracellular motility and direct cell-to-cell spread (*inlAB* and *actA*) and membrane-damaging toxins or enzymes that mediate phagosomal escape or cytotoxicity (*hly*, *plcA*, and *plcB*) (1, 9, 13, 19, 40, 43). In this paper we report that glucose-1-phosphate (G-1-P) is utilized by *L. monocytogenes* under the tight positive control of PrfA, the central regulator of listerial virulence, and that catabolism of this sugar is coordinately expressed with virulence genes. This is the first demonstration that the tran-

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TABLE 1. Bacterial strains[a]

Strain	Description[b]	Phenotype (Hly/PlcB)[c]	G-1-P acidification test[d]	Source and/or reference(s)
L. monocytogenes				
P14	Serovar 4b, phenotype 1, wild-type *prfA*	+/(+)	−	36, 37
EGD	Serovar 1/2a, phenotype 1, wild-type *prfA*	+/(+)	−	36, 37
P7	Serovar 4b, phenotype 1, wild-type *prfA*	+/(+)	−	36
P14-A	Phenotype 2, *prfA** mutant from P14	++++/++++	+	36, 37
EGD-A	Phenotype 2, *prfA** mutant from EGD	+++/+++	+	36, 37
NCTC 7973	Serovar 1/2a, phenotype 2, *prfA** mutant	+++/+++	+	36, 37
SLCC 2373	Serovar 3a, phenotype 2, *prfA** mutant	+++/+++	+	36, 37
CLIP 545	Serovar 4b, phenotype 2, *prfA** mutant	++++/++++	+	36, 37
CO244	Serovar 3a, phenotype 2, *prfA** mutant	+++/+++	+	36
L028	Serovar 1/2c, phenotype 3, wild-type *prfA*	++/+	−	36, 37
MS5	Tn*917*-induced, PrfA-deficient mutant from P14-A	+/(+)	−	This work, 36
MS5(pMK4)	MS5 with the bifunctional plasmid vector pMK4	+/(+)	−	36, 42
MS5(pRWT)	MS5 complemented with wild-type *prfA* in pMK4	+/(+)	−	36
MS5(pRG145S)	MS5 complemented with mutant *prfA** allele in pMK4	++++/++++	+	36
B1E7[e]	Tn*917*-induced, PrfA-deficient mutant from P14	(+)/−	−	This work
B2A7[e]	Tn*917*-induced, PrfA-deficient mutant from P14	(+)/−	−	This work
IB6[e]	Tn*917*-induced, PrfA-deficient mutant from P14	(+)/−	−	This work
OD43[e,f]	Tn*917-lac*-induced, PrfA-deficient mutant from L028	(+)/−	−	P. Cossart, 28, 36
Δ*prfA*	*prfA* deletion mutant from L028	(+)/−	−	P. Cossart, 36, 39
Δ*prfA*(pMK4)	Δ*prfA* with the bifunctional plasmid vector pMK4	(+)/−	−	36, 42
Δ*prfA*(pRWT)	Δ*prfA* complemented with wild-type *prfA* in pMK4	+/(+)	−	36
Δ*prfA*(pRG145S)	Δ*prfA* complemented with mutant *prfA** allele in pMK4	++++/++++	+	36
L. innocua ATCC 33090	Type strain, serovar 6a	−/−	−	Collection
L. ivanovii				
ATCC 19119	Type strain, serovar 5	++++/++++	+	Collection, 37
8/6	Tn*1545*-induced, PrfA-deficient mutant from ATCC 19119	++[g]/−	−	J. Kreft, 20
D23	Field isolate, serovar 5	++++/++++	+	This work

[a] Strains were routinely grown at 37°C in BHI (Difco Laboratories) broth or agar supplemented with appropriate antibiotics as necessary.
[b] Phenotypes 1, 2, and 3, which we previously described in *L. monocytogenes* according to the expression levels of hemolysin and phospholipase C (lecithinase) (36, 37), are as follows: 1 (or wild-type), weak hemolytic and lecithinase activities; 2, hyperhemolytic and hyperlecithinase variant; 3, moderately hyperhemolytic and hyperlecithinase variant.
[c] Hemolytic (Hly) and phospholipase C/lecithinase (PlcB) activities on agar plates. Symbols: + to ++++, different degrees of positive reaction; (+), weak or doubtful reaction; −, negative reaction.
[d] Acid production from G-1-P after 24 h of incubation at 37°C as determined in charcoal-free phenol red medium or by API *Listeria*.
[e] See Fig. 1.
[f] Transposon insertion in this mutant is located 4 bp upstream from the P1*prfA* transcription start point (28) (Fig. 1).
[g] This hemolytic activity is attributable to the sphingomyelinase, which is not regulated by PrfA.

scriptional activator PrfA, like CRP in *Escherichia coli* (17), controls a metabolic function in *Listeria.*

Virulence factor-overexpressing mutants of *L. monocytogenes* are positive in the G-1-P utilization test. We recently described a variant phenotype in *L. monocytogenes*, designated type 2, characterized by the constitutive overexpression of PrfA-dependent virulence genes under culture conditions in which virulence factor expression is normally downregulated. The variant strains produce strong hemolytic and phospholipase (lecithinase) activities, and this distinguishes them from wild-type, or type 1, strains characterized by low-to-undetectable levels of PrfA-dependent virulence factor expression (37). The abnormal phenotype is due to a point mutation in codon 145 of *prfA*, leading to a Gly→Ser substitution in PrfA (36). This amino acid change is in a PrfA region highly similar to the D α-helix of CRP, where similar substitutions originate *crp** mutants which synthesize a cofactor-independent, transcriptionally active form of CRP (17, 36). By analogy with CRP, the Gly145Ser substitution in PrfA (which we now call the PrfA* mutation) presumably blocks the regulatory protein in a transcriptionally active conformation (36). The model *prfA** variant is P14-A, a spontaneous mutant of P14, a wild-type clinical isolate of serovar 4b (Table 1). Other than the differences in PrfA-dependent virulence factor expression, P14-A is phenotypically indistinguishable from its isogenic parent strain, P14 (37).

P14 and P14-A were tested with the API *Listeria* identification system (Biomérieux). Surprisingly, we found that the two strains gave different results in the G-1-P acidification test: P14 scored negative, which is the expected result for *L. monocytogenes* (2), whereas the mutant, P14-A, was clearly positive (Tables 1 and 2).

Acid production from G-1-P is one of the markers used by the API *Listeria* system to differentiate *Listeria ivanovii*, most (79 to 92%) of the strains of which acidify this sugar, from the other *Listeria* species, which do not (2). Like P14-A and the other *prfA** variants of *L. monocytogenes*, *L. ivanovii* constitutively expresses high levels of hemolysin and lecithinase (37). Thus, the ability to utilize G-1-P might be linked to virulence factor overexpression in *Listeria*. To test this, all the remaining *prfA** variants of *L. monocytogenes* we had identified (Table 1) were investigated for acid production from G-1-P, together with additional wild-type strains. The latter were, as expected, G-1-P negative, whereas all the hyperhemolytic variants gave positive reactions (Table 1).

An exception was L028, which in spite of being moderately

TABLE 2. Influence of temperature and medium composition on G-1-P and glucose utilization capacities of *L. monocytogenes* and *L. innocua*[a]

Species and strain	Sugar utilization			
	G-1-P		Glucose	
	20°C	37°C	20°C	37°C
L. monocytogenes				
P14	−/−[b]	−[c]/+	+/+	+/+
P14-A	+/+	+/+	+/+	+/+
L. innocua	−/−	−/−	+/+	+/+

[a] Sugar acidification tests were performed in phenol red medium, and the results shown are those recorded after 24 h of incubation (which remained constant after prolonged incubation unless otherwise indicated). The pattern of expression of G-1-P utilization is perfectly concordant with that of PrfA-dependent virulence genes (see the text). The strains were grown on charcoal-free medium (reaction shown before the slash) and on charcoal-treated medium (reaction shown after the slash). +, positive reaction; −, negative reaction.

[b] Positive reaction after 65 h of incubation.

[c] Positive reaction after 48 h of incubation.

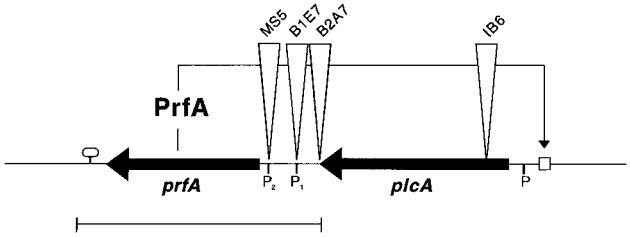

FIG. 1. Schematic representation of the *plcA-prfA* operon (adapted from Ripio et al. [36]) and location of transposon insertions (triangles) in G-1-P utilization-deficient mutants of *L. monocytogenes*. Known promoters are indicated (P). In mutants MS5 and B1E7 the Tn*917* insertion took place 4 bp upstream from the P2- and P1*prfA* transcription start sites, respectively, as determined by cloning and sequencing of the chromosomal region flanking the transposon. In the previously described mutant OD43, which was isolated from L028 after Tn*917-lac* mutagenesis by selecting for virulence factor (PlcB or lecithinase) expression deficiency (28), the transposon was inserted at the same position as in B1E7. In mutants B2A7 and IB6, Tn*917* was located between the 3′ end of *plcA* and the P1*prfA* promoter and in the 5′ region of *plcA*, respectively, as determined by PCR mapping. Note that in all these mutants the transposon insertions interrupt the PrfA-dependent *plcA-prfA* bicistronic transcript responsible for the PrfA autoamplification loop (the PrfA box of the P*plcA* promoter is indicated by a square), thereby causing PrfA deficiency in spite of the *prfA* gene being intact. The segment at the bottom represents the DNA fragment which was inserted into pMK4 in the *prfA* constructs used in the complementation experiments (Fig. 2).

hyperhemolytic was G-1-P negative (Table 1). L028 is a laboratory strain which displays a particular phenotype, designated type 3, characterized by intermediate levels of hemolysin and lecithinase expression (37), but which has a wild-type *prfA* (36).

Thus, in *L. monocytogenes* a positive G-1-P acidification test appears to be specifically associated with the virulence factor-overexpressing phenotype of the *prfA** mutants.

G-1-P utilization by *L. monocytogenes* is coexpressed with virulence factors. Our observations suggest that G-1-P utilization and virulence factors are coexpressed in *Listeria*. If so, wild-type strains of *L. monocytogenes* should acquire the capacity to utilize G-1-P when cultured under conditions in which the PrfA-dependent virulence regulon is fully upregulated. At 37°C the expression of listerial virulence factors is induced, whereas at 20°C or below it is repressed (22). However, culture at 37°C is not sufficient for maximal expression of PrfA-dependent virulence genes, which also requires an adequate extracellular medium composition, e.g., that resulting from the addition of activated charcoal to the medium (37). Under these conditions, wild-type strains, which normally do not (or at most very poorly) express virulence factors at 37°C in normal medium, produce large amounts of hemolysin and lecithinase, up to levels similar to those produced by *prfA** mutants in normal medium (36, 37). We therefore investigated G-1-P utilization by *L. monocytogenes* at 20 and 37°C in phenol red broth (Biomérieux) with and without 0.2% charcoal.

The results for P14 and P14-A after 24 h of incubation are shown in Table 2. Other wild-type strains and *prfA** mutants gave similar results. In normal medium P14 was, as expected, negative at both temperatures. In charcoal-treated medium this strain remained negative for G-1-P utilization at 20°C but gave a clearly positive acidification reaction at 37°C. The *prfA** mutant P14-A was G-1-P positive at both temperatures regardless of whether charcoal was present in the medium (Table 2). This expression pattern of G-1-P utilization is perfectly concordant with that of PrfA-dependent virulence genes (22, 36, 37).

No differences between wild-type strains and virulence factor-overexpressing mutants were observed in the capacity for metabolizing glucose (Table 2), cellobiose, or other carbohydrates normally fermented by *L. monocytogenes* (not shown). Moreover, the apathogenic species *Listeria innocua*, which lacks the entire virulence gene cluster, including the *prfA* gene (15), was completely incapable of utilizing G-1-P, although, like all *Listeria* spp., it efficiently metabolized glucose at 20 and 37°C (Table 2).

These results indicate that certain listerial genetic determinants important for G-1-P metabolism are coordinately regulated with virulence genes under the control of PrfA.

G-1-P utilization by *L. monocytogenes* is PrfA dependent. A bank of 2,000 transposon-induced mutants was generated from P14-A with the thermosensitive plasmid pTV1 carrying Tn*917* (44). Insertion mutants were screened for G-1-P utilization deficiency and glucose utilization proficiency with the sugar acidification assay described above performed in sterile U-form microtiter plates. Mutants failing to acidify G-1-P after 24 h at 37°C were selected. One such mutant, MS5, harboring a single copy of Tn*917* as determined by Southern blotting, was isolated. The transposon in MS5 was inserted into the *plcA-prfA* intergenic region, 4 bp upstream from the transcriptional start point of the P2*prfA* promoter (Fig. 1). Transposon mutants with the same phenotype as MS5 were also isolated from the parent strain, P14, by screening for G-1-P deficiency in charcoal-treated medium. Again, the Tn*917* insertions mapped within the *plcA-prfA* region (Fig. 1), confirming the importance of this locus in G-1-P utilization.

A *prfA* background can be complemented by *prfA* provided in *trans* on a plasmid (3, 28, 36). Two previously described (36) *prfA* constructs in the *E. coli-Bacillus* or *Listeria* shuttle vector pMK4 (42) were used to complement the PrfA-deficient mutant MS5 (Table 1): pRG145S, carrying the *prfA** (Gly145Ser) mutant allele from P14-A, and pRWT, with wild-type *prfA* from P14. Complementation with these *prfA* alleles conferred on MS5 the ability to utilize G-1-P with a pattern similar to that of the corresponding natural *prfA* background: like P14-A, MS5(pRG145S) was able to acidify G-1-P within 24 h in normal (charcoal-free) medium, whereas MS5(pRWT) behaved similarly to the wild-type strain, P14, i.e., it utilized the sugar within 24 h only in charcoal-treated medium (Table 1 and Fig. 2). These results were entirely consistent with those obtained when PrfA-dependent expression was measured by determining the activities of the *hly* or *plcB* gene products (36) and

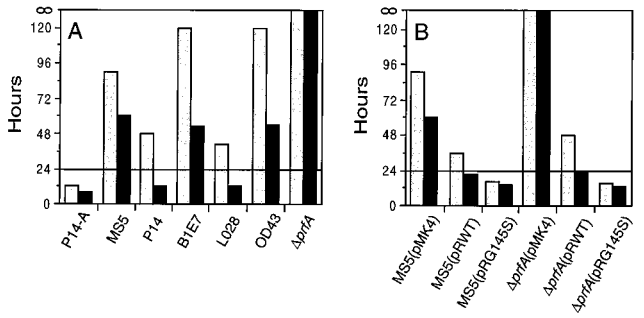

FIG. 2. G-1-P utilization efficiency by PrfA-proficient strains and PrfA-deficient mutants of *L. monocytogenes* (A) and by PrfA-deficient mutants *trans* complemented with *prfA* (B), as determined by the time required to acidify the medium at 37°C (gray bars, normal medium; black bars, charcoal-treated medium). Cognate PrfA-proficient strains and PrfA-deficient mutants are as follows: P14-A and MS5; P14 and B1E7; L028, OD43, and Δ*prfA* (see Table 1, Fig. 1, and the text for details). *prfA* constructs (in the plasmid vector pMK4) are as follows: pRG145S, with the *prfA** allele from P14-A, and pRWT, with the wild-type *prfA* from P14. Note the following: (i) the *prfA** variant P14-A utilizes G-1-P in normal medium much more efficiently than P14 or L028 with wild-type *prfA*; (ii) the latter strains utilize G-1-P in charcoal-treated medium as efficiently as P14-A in normal medium; (iii) the transposon-generated, PrfA-deficient mutants MS5, B1E7, and OD43, which have intact *prfA* genes (Fig. 1), are drastically delayed in G-1-P utilization, whereas the deletion mutant Δ*prfA* is completely unable to acidify G-1-P; (iv) complementation of PrfA-deficient mutants with pRG145S, but not with the cloning vector pMK4 alone, restores (MS5) or confers (Δ*prfA*) the same ability as P14-A to efficiently utilize G-1-P in normal medium; and (v) mutants complemented with pRWT display a G-1-P utilization pattern similar to that of the strains with natural, wild-type *prfA* background (i.e., P14 or L028). The horizontal line represents the 24-h incubation threshold, which delimits positive (below) and negative (above) scores in Tables 1 and 2. ∞, >6 weeks of incubation.

demonstrated that G-1-P utilization by *L. monocytogenes* is positively controlled by the virulence regulator, PrfA.

Although it is G-1-P negative after 24 h, P14 metabolized the sugar after prolonged incubation at 37°C in normal medium (Table 2 and Fig. 2). Similarly, neither MS5 nor the other G-1-P-deficient transposon mutants we selected (e.g., B1E7) had totally lost the ability to acidify G-1-P: sugar utilization was severely impaired but not abolished (Fig. 2). These mutants, like P14, had an intact *prfA* gene (Fig. 1) and therefore could still express residual, low levels of PrfA protein (36). To determine the extent of the PrfA dependence of G-1-P utilization by *L. monocytogenes*, we made use of two previously described PrfA-deficient mutants from L028: OD43, similar to MS5 or B1E7 in that it has a Tn*917-lac* insertion in the *plcA-prfA* intergenic region (28) (Fig. 1), and Δ*prfA*, from which virtually all of the regulatory gene was deleted (39) (Table 1). Strain L028 displayed a G-1-P utilization pattern similar to that of P14, and OD43 behaved exactly like MS5 or B1E7, but Δ*prfA* was totally unable to utilize G-1-P even in charcoal-treated medium (Fig. 2). Complementation with the two *prfA* constructs conferred on Δ*prfA* the ability to utilize G-1-P with patterns identical to those observed with MS5 (Fig. 2), which confirmed that G-1-P utilization by *L. monocytogenes* is strictly dependent on PrfA.

Metabolism of G-1-P efficiently promotes growth of *L. monocytogenes*. We investigated whether G-1-P, like other easily metabolizable carbon sources, promotes growth of *L. monocytogenes*. Experiments were performed at 37°C in Luria-Bertani broth (LB) (Oxoid), a poor growth medium for *L. monocytogenes* (maximal optical density at 578 nm [OD_{578}] is around 0.5 compared to 2 to 2.5 in rich medium, i.e., brain heart infusion [BHI]). Overnight *Listeria* cultures in LB were diluted 1:200 in fresh medium with and without supplementation with 10 mM G-1-P or glucose, and bacterial growth kinetics were monitored by measuring the OD_{578}.

Growth of P14-A in the presence of G-1-P was as rapid as that on glucose (Fig. 3A). However, final yields of P14-A grown on G-1-P were significantly higher ($OD_{578} > 2.5$ [usually around 2.7 to 3.0]) than those on glucose ($OD_{578} = 2.0$) (Fig. 3). This was attributable to a growth-stimulating effect of the phosphate moiety of G-1-P, since growth in LB plus glucose supplemented with 10 mM phosphate was as high as that in LB plus G-1-P (not shown). Growth of *L. monocytogenes* in LB plus G-1-P was virtually identical to that on BHI (not shown).

The mutant MS5 with and without pRG145S complementation was similarly tested. As expected, MS5 behaved like P14-A in LB plus glucose (final yields, 2.0 OD_{578} units), but its growth in LB plus G-1-P was as poor as that in LB alone, showing it was selectively deficient in G-1-P utilization (Fig. 3B). Introduction of pRG145S restored the growth capacity in LB plus G-1-P to a level identical to that of P14-A (Fig. 3B).

Unlike other readily metabolized sugars, G-1-P does not repress virulence gene expression. Milenbachs and colleagues (29) recently reported that sugars readily utilized by *L. monocytogenes*, such as glucose or cellobiose, which substantially promote bacterial growth when added to the culture medium, downregulate the expression of the PrfA-controlled virulence gene *hly*. These observations were difficult to reconcile with

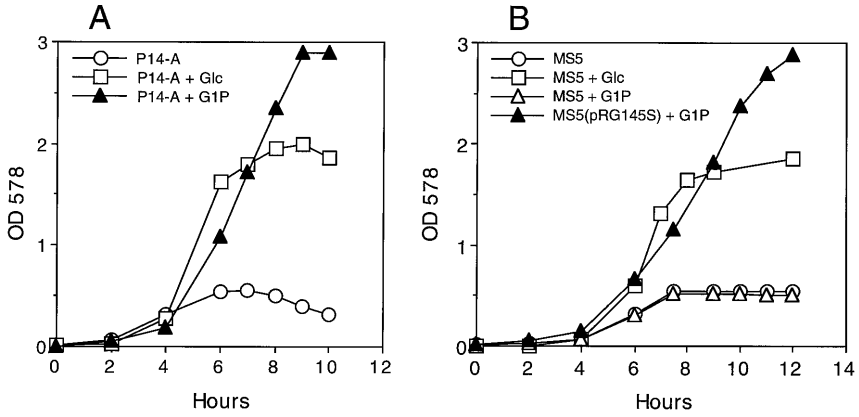

FIG. 3. Growth curves of P14-A (A) and its PrfA-deficient mutant MS5 (with or without pRG145S complementation) (B) in LB medium alone or supplemented with 10 mM glucose (Glc) or glucose-1-phosphate (G1P).

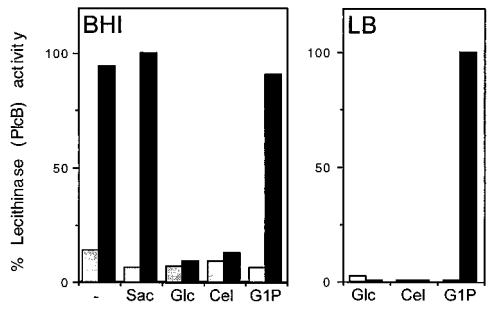

FIG. 4. *plcB* expression in the *L. monocytogenes* wild-type strain, P14, in normal (gray bars) and charcoal-treated (black bars) BHI or LB medium without (−) or with supplementation with 25 mM saccharose (Sac), glucose (Glc), cellobiose (Cel), or glucose-1-phosphate (G1P). *plcB* expression was measured by determining the lecithinase activity in the culture supernatant after 8 h of incubation at 37°C; results are expressed as relative activities (percentage) with respect to the highest value obtained (which was set at 100%). In the right panel, data from nonsupplemented LB or LB plus the nonmetabolizable saccharose are not shown because listerial growth is very poor in LB in the absence of a readily utilizable carbon source (Fig. 3), and therefore the results obtained were not comparable.

our results, which show that the capacity for efficiently metabolizing the sugar G-1-P is coexpressed with the genes of the PrfA regulon and requires the PrfA system to be activated. It was therefore interesting to compare the effects of glucose, cellobiose, and G-1-P on PrfA-dependent virulence gene expression in *L. monocytogenes.*

P14 and several other wild-type strains were tested in BHI and LB media supplemented with 25 mM the above-mentioned sugars (29), with or without 0.2% charcoal. The disaccharide saccharose was included as a control, as it is not metabolized by *L. monocytogenes* and consequently does not inhibit virulence genes (29). *plcB* was used as the reporter gene to assess PrfA-dependent virulence gene expression. For this, *plcB* offers the following advantages over *hly*: (i) the activity of its gene product, the wide-substrate-range phospholipase C (43), can be easily and accurately quantified in the culture supernatant by means of a turbidimetrical lecithinase assay (37), which in our hands gives more linear and reproducible results than hemolysin titration (36, 37); and (ii) results are also more clear-cut (36), since unlike *hly*, the promoters from which it is transcribed are strictly PrfA-dependent and have higher thresholds of activation by PrfA (3, 5, 12, 39). Media were inoculated 1/200 with overnight cultures in the corresponding base medium, and *plcB* expression was measured after 8 h of incubation at 37°C.

To detect significant *plcB* expression in a wild-type strain requires conditions of maximal upregulation of the PrfA system (36, 37); consequently, meaningful results were only observed with P14 in charcoal-treated medium (Fig. 4). In agreement with Milenbachs et al. (29), utilization of glucose and cellobiose significantly (up to 200-fold) inhibited *plcB* expression. By contrast, and very interestingly, metabolization of G-1-P did not inhibit *plcB* expression at all. As expected, no inhibitory effect was observed with the nonmetabolizable saccharose. Similar results were obtained in assays for *hly* expression (not shown), and other wild-type strains of *L. monocytogenes* behaved similarly to P14 (not shown).

Neither G-1-P, glucose, nor cellobiose significantly inhibited *plcB* expression in P14-A or other *prfA** mutants in normal medium (not shown). This suggests that the constitutively activated form of PrfA that is presumably synthesized by these mutants (36) relieves them from carbohydrate-mediated virulence gene repression. In the *prfA** mutant NCTC 7973, however, *plcB* expression was inhibited by cellobiose although not by the other sugars tested (not shown), corroborating previous observations that this strain behaves anomalously (29).

Why does G-1-P, in contrast to other easily utilized sugars, cause no repression of the PrfA-dependent virulence genes? According to Milenbachs et al., sugar-mediated inhibition of virulence genes only occurs in the presence of sufficient carbohydrate to produce growth enhancement (29), suggesting that repression of the PrfA-dependent regulon results from a global regulatory mechanism of sugar metabolism probably related to catabolite repression. Preferentially utilized sugars that trigger catabolite repression are mostly translocated into the bacterial cell via the phosphoenolpyruvate-dependent sugar transport system (PTS), and glucose and the β-glucoside cellobiose are PTS-transported sugars in both gram-negative and gram-positive bacteria (21, 23, 31, 33). The phosphate sugar G-1-P is not expected to be PTS transported (PTS-mediated translocation of a carbohydrate is coupled to its phosphorylation [23, 33], and G-1-P is one of the first metabolites that results from glucose uptake via the PTS [11]); thus, the most likely explanation for the lack of virulence gene inhibition is that G-1-P is taken up by *L. monocytogenes* in an unmodified form through a non-PTS permease. This is the case, for example, with *E. coli*, in which the non-PTS permease UhpT mediates direct import of G-1-P (35). The UhpT permease uses a phosphate antiport mechanism (35), and consistent with this we observed a significant increase of the free inorganic phosphate (P_i) concentration in the culture medium upon utilization of G-1-P by *L. monocytogenes* (6). However, this increase in extracellular P_i might also be a consequence of another mechanism of G-1-P uptake involving a G-1-P phosphatase, like that encoded by *agp* in *E. coli*, which dephosphorylates G-1-P in the periplasm (34). In this case the resulting free glucose would be taken up by the PTS and induce repression of virulence genes, which argues against this alternative explanation. We are currently investigating the molecular basis of PrfA-dependent G-1-P utilization by *L. monocytogenes* and the concomitant nonrepression of the PrfA regulon.

Possible role of PrfA-dependent utilization of G-1-P in *Listeria* virulence. Many virulence genes in pathogenic bacteria are organized in extensive regulatory networks controlled by a transcription factor whose expression is in turn modulated by environmental signals. Coregulation with known virulence factors is therefore a major criterion for the identification of potential new virulence determinants (27, 30). In this paper we show that G-1-P utilization by *L. monocytogenes* is positively coregulated with virulence factors in response to environmental conditions that trigger the activation of the PrfA regulon and that this function is executed under the tight positive control of the central virulence regulator, PrfA. Moreover, we also show that G-1-P utilization efficiently promotes bacterial growth without repressing virulence genes, in contrast to the effects of other easily fermentable sugars. These observations are strong arguments in favor of a role for G-1-P utilization in listerial virulence. Indeed, the ability to utilize G-1-P is also expressed by *L. ivanovii*, another pathogenic species of the genus, but is totally absent from the apathogenic species *L. innocua*, and a PrfA-deficient, avirulent mutant of *L. ivanovii* was also impaired in G-1-P utilization (Tables 1 and 2) (6).

What could be the role of G-1-P utilization in virulence? *Listeria* spp. are invasive pathogens which, after escape from the phagocytic vacuole, rapidly multiply in the cytoplasm of many cell types (19, 40). Only a small number of intracellular bacterial pathogens (i.e., *Listeria*, *Shigella*, and *Rickettsia*) have adopted the mammalian cytoplasm as a multiplication site, and

there is increasing experimental evidence that this milieu is not as permissive for bacterial growth as currently believed (discussed in reference 14). Possibly therefore, intracellular parasites require special adaptations to proliferate in the cytoplasm, and PrfA-dependent utilization of G-1-P by pathogenic *Listeria* might be one of them. G-1-P is the precursor metabolite and primary breakdown product of glycogen, and turnover of this storage polysaccharide is high in the mammalian cell (25, 26). G-1-P is therefore constantly released to the mammalian cytoplasm, where, according to our results, it could be utilized as an exogenous source of carbon, phosphate, and energy by intracellular bacteria. Interestingly, hepatocytes are among the mammalian cells that have the highest content (up to 8 to 10% [wet weight]) of glycogen, and active intracytoplasmic multiplication within the liver parenchyma is a key step in the pathogenesis of *Listeria* infection (8, 13, 40). Moreover, G-1-P is a precursor for the biosynthesis of the glucose-teichoic acid of the gram-positive cell wall (11); thus, incorporation of unmodified G-1-P could contribute to improving the fitness of *L. monocytogenes* for growing within the highly competitive environment of infected host tissues.

It is indeed very tempting to assign to PrfA-dependent utilization of G-1-P an important role in the intracytoplasmic proliferation of *L. monocytogenes*. This requires that *prfA* expression be activated in the cytoplasm, which appears to be the case, since transcription of PrfA-dependent virulence genes, such as *actA* (4), *plcA* (16), and *inlC* (10), has been shown to be induced in this compartment. It would also require *L. monocytogenes* to use G-1-P as a carbon source without triggering the downregulation of the PrfA system, and we show here that this is also true. Work is in progress in our laboratory to assess the significance of PrfA-dependent G-1-P utilization by *Listeria* in vivo.

M.-T.R. and K.B. contributed equally to this work.

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