Novel Listerial Glycerol Dehydrogenase- and Phosphoenolpyruvate-Dependent Dihydroxyacetone Kinase System Connected to the Pentose Phosphate Pathway

Céline Monniot,^{a,b} Arthur Constant Zébré,^{a,b} Francine Moussan Désirée Aké,^{a,b} Josef Deutscher,^{a,b,c} and Eliane Milohanic^{a,b}

INRA, UMR1319 Microbiologie de l'Alimentation au Service de la Santé Humaine, Jouy en Josas, France^a; AgroParisTech, Microbiologie de l'Alimentation au Service de la Santé Humaine, Jouy en Josas, France^b; and CNRS, SNC9130 Microbiologie de l'Alimentation au Service de la Santé Humaine, Jouy en Josas, France^c

Several bacteria use glycerol dehydrogenase to transform glycerol into dihydroxyacetone (Dha). Dha is subsequently converted into Dha phosphate (Dha-P) by an ATP- or phosphoenolpyruvate (PEP)-dependent Dha kinase. *Listeria innocua* possesses two potential PEP-dependent Dha kinases. One is encoded by 3 of the 11 genes forming the glycerol (*gol*) operon. This operon also contains *gold* (*lin0362*), which codes for a new type of Dha-forming NAD⁺-dependent glycerol dehydrogenase. The subsequent metabolism of Dha requires its phosphorylation via the PEP:sugar phosphotransferase system components enzyme I, HPr, and EIIA^{Dha}-2 (*Lin0369*). P~EIIA^{Dha}-2 transfers its phosphoryl group to DhaL-2, which phosphorylates Dha bound to DhaK-2. The resulting Dha-P is probably metabolized mainly via the pentose phosphate pathway, because two genes of the *gol* operon encode proteins resembling transketolases and transaldolases. In addition, purified *Lin0363* and *Lin0364* exhibit ribose-5-P isomerase (RipB) and triosephosphate isomerase activities, respectively. The latter enzyme converts part of the Dha-P into glyceraldehyde-3-P, which, together with Dha-P, is metabolized via gluconeogenesis to form fructose-6-P. Together with another glyceraldehyde-3-P molecule, the transketolase transforms fructose-6-P into intermediates of the pentose phosphate pathway. The *gol* operon is preceded by *golR*, transcribed in the opposite orientation and encoding a DeoR-type repressor. Its inactivation causes the constitutive but glucose-repressible expression of the entire *gol* operon, including the last gene, encoding a pediocin immunity-like (PedB-like) protein. Its elevated level of synthesis in the *golR* mutant causes slightly increased immunity against pediocin PA-1 compared to the wild-type strain or a *pedB*-like deletion mutant.

Bacteria usually take up glycerol via facilitated diffusion catalyzed by the glycerol facilitator GlpF. Depending on whether they are exposed to aerobic or anaerobic conditions, some bacteria, such as *Escherichia coli*, either first phosphorylate intracellular glycerol to glycerol-3-P by the enzyme glycerol kinase GlpK (10, 36) or first oxidize it to dihydroxyacetone (Dha) by means of a glycerol dehydrogenase (7). In the latter case, Dha is subsequently converted into dihydroxyacetone phosphate (Dha-P) by a Dha kinase (DhaK). Many proteobacteria, such as *Citrobacter freundii* (42) and *Klebsiella pneumoniae* (25), contain an approximately 60-kDa DhaK composed of two domains. The N-terminal domain covalently binds Dha by forming a hemiaminal bond with the Nε2 of a histidyl residue (19), whereas the C-terminal domain binds the phosphoryl donor ATP. ATP-requiring Dha kinases are also found in plants, fishes, and mammals. Most firmicutes, many actinobacteria, and several enterobacteria possess a similar Dha kinase, which, however, is split into two subunits, DhaK and DhaL. DhaK covalently binds Dha by forming a hemiaminal bond similar to the N-terminal domain of fused DhaKs (19, 43). DhaL corresponds to the C-terminal domain of fused Dha kinases and carries ADP or ATP in the active site. The most significant difference between the two types of Dha kinases is that in the case of the split enzyme, ADP remains tightly bound to DhaL after Dha phosphorylation and is therefore only very slowly replaced with an ATP molecule (3). ADP therefore rather functions as a cofactor, and for efficient Dha phosphorylation, it needs to be rephosphorylated to ATP, which is achieved in a phosphoenolpyruvate (PEP)-dependent reaction catalyzed by proteins of the PEP:sugar phosphotransferase system (PTS).

PTS proteins catalyze the uptake and concomitant phosphor-

ylation of carbohydrates and also carry out numerous regulatory functions (12). The PTS is usually composed of four soluble proteins or protein domains, which form a phosphorylation cascade. Enzyme I (EI) autophosphorylates with PEP at a histidyl residue and transfers the phosphoryl group to HPr, which in turn phosphorylates one of usually several sugar-specific EIIs present in a bacterium. Finally, an EIIB of the same sugar specificity accepts the phosphoryl group from P~EIIA and transfers it to a sugar molecule bound to the corresponding membrane-spanning EIIC (in some cases, EIIC and EIID). The phosphorylation of the PTS proteins occurs at conserved histidyl residues, except for most EIIBs, which are phosphorylated at a cysteal residue (34). While the phosphorylation of PTS substrates is coupled to their transport, Dha is not transported by the PTS and requires PTS components only for its phosphorylation. The number and domain organization of the PTS proteins participating in Dha phosphorylation vary depending on the organism (18). Operons containing the *dhaK* and *dhaL* genes for a Dha kinase composed of two distinct proteins also contain *dhaM*, a gene encoding an EIIA of the mannose class PTS called EIIA^{Dha}. Two domains resembling HPr (HPr^{Dha}) and the N-terminal part of EI (the first approximately 230 amino acids), containing the autophosphorylation but

Received 7 May 2012 Accepted 2 July 2012

Published ahead of print 6 July 2012

Address correspondence to Josef Deutscher, Josef.Deutscher@grignon.inra.fr.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JB.00801-12

not the PEP binding site (EI^{Dha}), are sometimes fused to EIIA^{Dha}. This is the case, for example, for the PEP-dependent Dha phosphorylation systems of *E. coli* (21, 35) and *K. pneumoniae*. In these Dha kinase systems, the phosphoryl transfer occurs mainly from PEP via the common PTS components EI and HPr to the truncated EI^{Dha} and the HPr^{Dha} domains present in DhaM before it is finally transferred to the EIIA^{Dha} domain (21). The direct phosphorylation of HPr^{Dha} by the general PTS protein EI probably occurs in some actinobacteria (*Corynebacterium diphtheriae*, *Arthrobacter chlorophenolicus*, and *Leifsonia xyli*), which contain only HPr^{Dha} fused to the EIIA^{Dha} domain. DhaM of another actinobacterium, *Mycobacterium smegmatis* (MSMEG_2121), contains an entire EI fused to the EIIA^{Dha} and HPr^{Dha} domains and therefore might itself bind PEP and autophosphorylate. Finally, most firmicutes and several actinobacteria (streptomycetes and propionibacteria) possess a DhaM protein composed of only EIIA^{Dha}, which therefore was proposed previously to become directly phosphorylated by the general PTS components EI and HPr (49). Only very few archaea contain PTS components functional in carbohydrate transport and phosphorylation. Nevertheless, a split Dha kinase and the general PTS components EI, HPr, and EIIA^{Dha}, required for its phosphorylation, are also present in some archaea, such as *Haloferax volcanii*, *Haloquadratum walsbyi*, and *Halorubrum lacusprofundi* (49). After its phosphorylation, EIIA^{Dha} transfers the phosphoryl group to the ADP molecule in DhaL (21), and the resulting ATP:DhaL holoenzyme phosphorylates Dha bound to DhaK. The dependence of Dha phosphorylation on a functional DhaM was demonstrated previously for *E. coli*, where the replacement of the phosphorylatable His in any one of the three DhaM domains led to a loss of Dha phosphorylation (21).

Some proteobacteria, such as *K. pneumoniae*, contain an ATP-dependent (25) and a split PEP-requiring Dha kinase. Interestingly, the *K. pneumoniae* genes encoding the PEP- and ATP-dependent Dha kinases are located in the same operon. In addition, glycerol kinase often efficiently phosphorylates Dha, as was shown previously for the *Enterococcus faecalis* enzyme (13), whereas Dha kinases discriminate between glycerol and Dha, owing probably to the hemiaminal bond formed with Dha. Glycerol lacks a keto group and therefore cannot form a hemiaminal bond. Dha utilization by an *E. coli* glpK mutant depends strictly on PEP and a functional EI. Dha was therefore erroneously thought not only to be phosphorylated but also to be transported by the PTS (24). However, the uptake of extracellular Dha probably occurs via facilitated diffusion catalyzed by the glycerol facilitator GlpF or homologues of GlpF. In several bacteria, such as *Lactococcus lactis* and a few streptococci, a gene encoding a protein with significant similarity to GlpF is associated with the *dha* operon. Enterococci and clostridia also contain in the *dha* operon a gene encoding a glycerol dehydrogenase resembling the *E. coli* glycerol dehydrogenase GldA (13). Glycerol is converted by GldA into dihydroxyacetone, which is subsequently phosphorylated to Dha-P (6, 40). Enterococci possess a glycerol dehydrogenase/DhaKLM system as well as GlpK (48) and the glycerol-3-P oxidase GlpO (8). In strain JH2-2, both pathways contribute to glycerol utilization under aerobic conditions. However, under anaerobic conditions, only the glycerol dehydrogenase/DhaKLM system was reported to be operative (6).

In numerous bacteria, the GlpK-mediated metabolism of glycerol also depends on the general PTS components EI and HPr. In

enterobacteria, unphosphorylated EIIA^{Glc} was shown previously to interact with GlpK and to inhibit its activity, whereas the phosphorylation of EIIA^{Glc} by EI and HPr prevents its inhibitory effect on GlpK activity (23). A different mechanism of regulation is operative in most Gram-positive bacteria. In these organisms, the phosphorylation of GlpK by P~His-HPr at a conserved histidyl residue is required for GlpK activation, and the absence of phosphorylation leaves the enzyme about 10-fold less active (11). As a consequence, in both types of organisms, mutants defective in EI or HPr phosphorylate glycerol only very slowly. This mode of regulation was called inducer exclusion, because glycerol-3-P, the inducer of the *glp* regulon, is barely formed when the use of glucose or other rapidly metabolizable carbon sources leads to a dephosphorylation of the PTS components (12).

For the utilization of glycerol and/or Dha, *Listeria innocua* possesses two PEP-dependent Dha systems and a glpFK operon. The genes *glpFK* and the *dha-1* operon encoding the common DhaK, DhaL, and EIIA^{Dha} (DhaM) components (19) are found in all listerial species, including the pathogen *Listeria monocytogenes*. The second set of *dha*-like genes (*dha-2*) is part of a large transcription unit containing, in addition to *dhaKLM-2*, seven or eight other genes, one of which (*golD*) encodes a novel glycerol dehydrogenase. This operon is present in 5 of the 7 listerial species for which genome sequences have been determined (it is absent from *Listeria grayi* and *Listeria marthii*). Here we demonstrate that EI and HPr together with *L. innocua* EIIA^{Dha-2}, DhaK-2, and DhaL-2 can efficiently phosphorylate Dha formed from glycerol by the enzyme GolD. The established or deduced activities of the other enzymes encoded by this operon suggest that they catalyze the metabolism of glycerol mainly via the pentose phosphate pathway in the sense of the Calvin cycle, i.e., the production of ribulose-5-P from the two triosephosphates Dha-P and glyceraldehyde-3-P.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. innocua* strain CLIP 11262 and mutants derived from it as well as *L. monocytogenes* strain EGD-e were grown in brain heart infusion (BHI) broth or in Luria-Bertani (LB) medium supplemented with either 50 mM glucose, glycerol, or Dha. *L. monocytogenes* strain EGD-e was also grown in minimal medium complemented with glucose or glycerol (38). *E. coli* strain NM522 (Stratagene) was used for cloning experiments and for protein overexpression. NM522 transformed with pQE30 (Qiagen) harboring various genes of the *gol* operon was grown at 37°C under agitation in LB medium supplemented with 100 µg/ml ampicillin.

RNA isolation, reverse transcriptase, and quantitative reverse transcriptase PCR experiments. The extraction of total RNA from *L. monocytogenes* EGD-e and various *L. innocua* strains was performed on cells grown to the exponential phase in 30 ml LB or minimal medium in the presence or absence of 50 mM glucose, glycerol, or Dha, and cells were collected by centrifugation. RNA from the different strains was extracted by using the RNeasy minikit (Qiagen). Residual genomic DNA was digested with the Turbo DNA-free kit (Ambion). Reverse transcription (RT) experiments were performed by using Ready-To-Go RT-PCR beads (GE Healthcare) by incubating 1 µg of total RNA with 2 µg of random hexamers for 30 min at 42°C to allow cDNA synthesis. For the amplification of intergenic regions during RT experiments, primers were designed based on the coding sequence of the selected genes (Table 1). RT-PCR amplification of the following fragments covering one or several intergenic regions was carried out: 805 bp for *lin0359-lin0360*, 908 bp for *lin0360-lin0362*, 329 bp for *lin0362-lin0363*, 901 bp for *lin0363-lin0364*, 562 bp for *lin0364-lin0365*, 994 bp for *lin0366-lin0369*, and 325 bp for the coding region of *lin0359*. The RT-PCR products were analyzed by electro-

TABLE 1 List of primers used in this study

Primer	Sequence ^a	Use
rpoB_FRT	GCGGATGAAGAGGATAATTACG	RT-PCR
rpoB_RRT	GGAATCCATAGATGGACCGTT	RT-PCR
DhaM-MK	CAATCTCTCGTTAATCCATCC	RT-PCR
DhaK-MK	CTTCTACTGCTTCATACCC	RT-PCR
DhaK-KL	GGAATCAAACGTACTTCCATCG	RT-PCR
DhaL-KL	GTAAGGTTGCAATATCGGG	RT-PCR
DhaK-RTF	CGAGCAATCGGTACATCTTGATCC	RT-PCR
lin0363-RTR	GTTCTGAACCCATTTTCGTCAAC	RT-PCR
lin0363-RTF	CGCCGTTTCGCTACTTGATAC	RT-PCR
lin0362-RTR	CGACTTGAAGTGCGAGCG	RT-PCR
lin0362-RTF	CTCTTTTTAGTAAGTGACG	RT-PCR
lin0360-RTR	CCAAAGCGAGAAAGCCATTG	RT-PCR
lin0360-RTF	GCGCAGCAAAAGCGAAAACCGG	RT-PCR
PedB1	GAAAAAGAATACTCCAGAGGC	RT-PCR
PedB2	GCTTGGAATTGATTGGTC	RT-PCR
GolRint1 (BamHI)	TACGGATCCAAAATAACAAAATAACTGTACC	<i>golR</i> insertion
GolRint2 (EcoRI)	TAAGAATTCAAATCGGTTAAGCCTTCTTCC	<i>golR</i> insertion
GolR_ext3'	GTATGATCTAGAAGCGCAACC	Confirmation of <i>golR</i> mutant
GolR_ext5'	GAAATCATTGATAGGTAAGG	Confirmation of <i>golR</i> mutant
pedB1_BamHI	TCTGGATCCGAAGTACACC	<i>pedB</i> deletion
pedB1_SalI	TGGAAATTGATTGTCGACGAATAAATCTATAAGTGCC	<i>pedB</i> deletion
pedB2_SalI	ATAGATTTATTCGTCGACAATCAATTTCCAAGC	<i>pedB</i> deletion
pedB2_NcoI	CAACCATGGAAAGGAGAAAATCACATG	<i>pedB</i> deletion
pedBext5'	GCAAAAGCGAAAACCGG	<i>pedB</i> mutant verification
pedBext3'	TGTTAGTTAAAGTTTTTG	<i>pedB</i> mutant verification
DhaM_BamHI	AGCGGATCCATGATAAGTATTGTTTTAG	DhaM-2 purification
DhaM_SalI	AATGTCGACTTATTACTTTTTAGTGC	DhaM-2 purification
DhaK_BamHI	GGAGGATCCATGAGACGTTTAGTGAATG	DhaK-2 purification
DhaK_SalI	TTAGTCGACCAAGTAGTAATTTAGTCCAC	DhaK-2 purification
DhaL_BamHI	GGAGGATCCATGAGCGAATTAGTTATGGATAGCG	DhaL-2 purification
DhaL_SalI	AAGGTCGACCGCATAAATCAGACCGC	DhaL-2 purification
tpi_like_BamHI	GTCGGATCCATGCGTAAACCCCTTAGTTGG	Tpi-2 purification
tpi_like_SalI	ATTGTCGACCTAACTCATCCTTTCGTTTTG	Tpi-2 purification
lin0362_BamHI	TAAGGATCCATGACTTTTTAAAGGTTTT	Lin0362 purification
lin0362_PstI	AAGCTGCAGACTTTATTTAATCGTATAACCGC	Lin0362 purification
lin0363_SalI	TGAGTCGACATGAAATCGCAATTGG	Lin0363 purification
lin0363_PstI	CCACTGCAGTTTTTATCTATTTTTTTGG	Lin0363 purification
rpoB-F2	CAGAACTGGTGAAATTATCGC	Quantitative PCR
rpoB-R2	TAGATTGAACGAGTACGCTATCTT	Quantitative PCR
QDhaKF	AATGCAGATCCAATCGAACT	Quantitative PCR <i>dhaK</i> -2
QDhaKR	CCAAGACGATTCCAGAGCC	Quantitative PCR <i>dhaK</i> -2
QPedBF	GTCGAATTAGAAGCTCGTTCTG	Quantitative PCR <i>pedB</i> -like
QPedBR	TGCTTGGAATTGATTGGTCAC	Quantitative PCR <i>pedB</i> -like
DpQE	GTTCTGAGGTCATTACTGG	Sequencing pQE-30 inserts
FpQE	CGGATAACAATTCACACAG	Sequencing pQE-30 inserts
pMAD_5'	GCGAGAAGAATCATAATGGG	Sequencing pMAD inserts
pMAD_3'	GTTACACATTAAGTAGACAG	Sequencing pMAD inserts

^a Restriction sites are indicated in boldface type.

phoresis on 1% agarose gels. RNA extracts were tested for DNA contamination by preincubating the reaction mixture at 95°C for 10 min to inactivate the reverse transcriptase before the addition of hexamers and RNA. The expression of the *gol* operons of *L. innocua* and *L. monocytogenes* was determined by quantitative RT-PCR experiments on a LightCycler instrument (Roche) by using primers specific for either the *dhaK*-2 or the *pedB*-like gene (Table 1) and SuperScript III reverse transcriptase (Invitrogen), according to the protocol provided for the LightCycler Faststart DNA Master SYBR green I kit (Roche). Data were processed with Roche Molecular Biochemicals LightCycler software.

Construction of lin0370 (*golR*) and lin0359 (*pedB*-like) mutants. In order to construct a *pedB*-like deletion mutant, the regions upstream and downstream of the *pedB*-like gene (446 and 511 bp, respectively) were

amplified by PCR by using *L. innocua* DNA as the template and the two primer pairs pedB1_BamHI/pedB1_SalI and pedB2_SalI/pedB2_NcoI (Table 1). The two amplified DNA fragments were cut with the indicated restriction enzymes and cloned into plasmid pMAD (2) digested with the corresponding enzymes. The correct sequence of the two amplified DNA fragments was verified by DNA sequencing. pMAD-derived plasmid pMAD-*pedB*-like was subsequently used to transform *L. innocua* strain CLIP 11262 by electroporation. A *pedB*-like deletion mutant was obtained by double recombination according to a previously described protocol established for *L. monocytogenes* (1). The correct deletion of the *pedB*-like gene was verified by carrying out PCR amplification using primers pedBext3' and pedBext5' and DNA sequencing of the amplified DNA fragment.

In order to construct a *golR* (*lin0370*) disruption mutant, an internal 526-bp fragment of *golR* was amplified by PCR by using *L. innocua* DNA as the template and primers *GolRint1* and *GolRint2* (Table 1). The amplified DNA fragment was digested with the restriction enzymes *Bam*HI and *Eco*RI and cloned into plasmid pMAD cut with the same enzymes. The correct sequence of the insert in plasmid pMAD-*golR* was verified by DNA sequencing. pMAD-*golR* was subsequently used to transform *L. innocua* strain CLIP 11262 by electroporation. A *golR* insertion mutant was obtained by single recombination according to a previously described protocol (1). The correct insertion of pMAD-*golR* into this mutant was verified by carrying out PCR amplification with primers *GolR_ext3'* and *GolR_ext5'*, followed by DNA sequencing of the amplified DNA fragment.

Overproduction and purification of proteins. His-tagged HPr and EI from *Bacillus subtilis* were overproduced and purified by using plasmids pAG2 and pAG3, respectively (18). The genes encoding the *L. innocua* proteins DhaK-2, DhaL-2, EIIA^{Dha}-2, Lin0362 (*GolD*), Lin0363 (*RpiB*), and Lin0364 (triosephosphate isomerase 2 [*Tpi-2*]) were first amplified by PCR by using appropriate primers to introduce the restriction sites *Bam*HI and *Sal*I (*Bam*HI and *Pst*I for Lin0364) at the ends of the amplified DNA fragments (Table 1), which allowed their insertion into the His tag expression vector pQE30 (Qiagen) cut with the two corresponding restriction enzymes. The resulting plasmids were used to transform *E. coli* strain NM522. The expression of the various genes by induction with isopropyl- β -D-thiogalactopyranoside (IPTG) and the purification of the His-tagged proteins by ion chelate affinity chromatography were carried out as previously described (18).

Separation of phosphorylated and unphosphorylated proteins by electrophoresis on polyacrylamide-urea gels. The phosphoryl group transfer from PEP to EI (P~EI), to HPr (P~His₁₅-HPr), and, finally, to DhaM-2 (P~EIIA^{Dha}-2) was monitored by using reaction mixtures containing 50 mM Tris-HCl (pH 7.4), 1 mM PEP, 5 mM MgCl₂, 3 μ g EI, and 3 μ g HPr. These assay mixtures were preincubated for 20 min at 37°C to allow the efficient phosphorylation of HPr before DhaM-2 was added, and incubation was continued for another 20 min at 37°C. Reaction mixtures lacking one or two of the PTS proteins were also prepared. Phosphorylated and unphosphorylated proteins were separated by electrophoresis on 10% polyacrylamide-8 M urea gels (30). After electrophoresis, gels were stained with Coomassie brilliant blue.

Spectrophotometric assay of Dha phosphorylation. In order to measure the PEP-dependent phosphorylation of Dha catalyzed by EI, HPr, DhaM-2, DhaK-2, and DhaL-2, we set up a coupled spectrophotometric assay. Dha-P formed by the PEP-requiring phosphorylation cascade is converted into glycerol-3-P by the enzyme glycerol-3-P dehydrogenase (Sigma). This reaction requires NADH, and its conversion to NAD⁺ was monitored by measuring the decrease of the absorbance at 340 nm (*A*₃₄₀) with a Kontron Bio-Tek spectrophotometer over a time period of 60 min. The 700- μ l reaction mixtures contained 75 mM Tris-HCl (pH 7.4), 6 mM PEP, 1 mM NADH, 6 mM Dha, 10 mM MgCl₂, 1 U of glycerol-3-P dehydrogenase, 30 μ g EI, 35 μ g HPr, and 30 μ g of the DhaK-2, DhaL-2, and DhaM-2 proteins. Reaction mixtures lacking any one of the three PTS proteins or DhaK or DhaL were prepared. After preincubation for 10 min at room temperature, the lacking protein was added, and the decrease of the *A*₃₄₀ was monitored for 60 min and compared to that of a reaction mixture which had not been complemented. A control experiment with all proteins but without Dha was also carried out.

Spectrophotometric assays of glycerol dehydrogenase, triosephosphate isomerase, ribose-5-P isomerase, and ribulose-5-P 3-epimerase activities. The glycerol dehydrogenase activity of Lin0362 (*GolD*) was measured by using a 700- μ l assay mixture containing 75 mM Tris-HCl (pH 7.4), 1 mM NAD⁺ or NADP⁺, 1.2 mM MgCl₂ or MnCl₂, and 1.2 mM glycerol. After preincubation for 5 to 10 min at room temperature, 40 μ g of purified *GolD* was added, and the increase of the *A*₃₄₀ was monitored with a Kontron Bio-Tek spectrophotometer. We also measured the reverse dihydroxyacetone reductase activity of *GolD* by replacing NAD⁺

with NADH and by replacing glycerol with Dha. The reverse reaction was monitored by measuring the decrease of the *A*₃₄₀. All necessary control experiments were also carried out.

In order to measure the activity of *Tpi-2*, we set up another coupled spectrophotometric assay. We started from fructose-1,6-bisphosphate (FBP), which is split by the enzyme FBP aldolase into glyceraldehyde-3-P and Dha-P. As mentioned above, Dha-P is converted into glycerol-3-P by the enzyme glycerol-3-P dehydrogenase in an NADH-consuming reaction. Once all the FBP-derived Dha-P had been converted into glycerol-3-P (no further decrease of the *A*₃₄₀), we added either commercially available triosephosphate isomerase (Sigma) or purified *L. innocua* *Tpi-2*. Triosephosphate isomerase converts glyceraldehyde-3-P into Dha-P, and as a consequence, we expected a second decrease of the *A*₃₄₀. The reaction mixture contained 90 mM Tris-HCl (pH 7.4), 1 mM NADH, 0.2 mM FBP, 1 U of glycerol-3-P dehydrogenase, and 0.3 U of FBP aldolase. Under the reaction conditions employed, we observed a decrease of the *A*₃₄₀ of about 0.7 in the absence of *Tpi*. When either 1.5 U of commercially available *Tpi* or 20 μ g of *L. innocua* *Tpi-2* was added to the reaction mixture, a second decrease of the *A*₃₄₀ of about 0.7 was observed, which did not occur when Tris-HCl (pH 7.4) was added in place of the *Tpi* enzymes.

Finally, the ribose-5-P isomerase activity was measured by using a 700- μ l assay mixture containing 50 mM Tris-HCl (pH 7.4), 0.5 mM NADH, 1.2 mM MgCl₂, and 0.5 mM ribose-5-P. After preincubation for 5 to 10 min at room temperature, 6 μ g of purified Lin0363 (*RpiB*) was added, which was expected to transform ribose-5-P into ribulose-5-P. In order to monitor the formation of ribulose-5-P, the samples also contained 18 μ g of *Lactobacillus casei* ribitol-5-P dehydrogenase, which transforms ribulose-5-P into ribitol-5-P in an NADH-consuming reaction; the decrease of the absorbance at 340 nm was therefore monitored. Because ribitol-5-P dehydrogenase is not commercially available, the gene LCABL_39250 from the presumed *L. casei* BL23 ribitol operon (32) was cloned into plasmid pQE30, and the His-tagged protein was purified. The presumed ribitol-5-P dehydrogenase activity of the *L. casei* enzyme was confirmed with ribulose-5-P and NADH as substrates before it was used in the ribose-5-P isomerase assay (A. Bourand and J. Deutscher, unpublished results). To determine whether Lin0363 might possess ribulose-5-P 3-epimerase activity (transformation of xylulose-5-P into ribulose-5-P), we carried out a similar assay by replacing ribose-5-P with xylulose-5-P. All other conditions were identical to those used for the ribose-5-P isomerase assay. All necessary control experiments were also carried out.

Bacteriocin assay. Bacteriocin activity was measured by using a microtiter plate assay system as previously described (33). Five different purified pediocin-like (class IIa) bacteriocins, kindly provided by Jon Nissen-Meyer, were tested: the pediocin PA-1 K20R variant from *Pediococcus acidilactici* (22), curvacin A from *Lactobacillus curvatus*, leucocin A from *Leuconostoc mesenteroides*, sakacin P from *Lactobacillus sakei*, and enterocin A from *Enterococcus faecium*. Immunity against these bacteriocins was tested with wild-type *L. innocua*, the *golR*-disrupted mutant, and the *pedB*-like deletion mutant. Each well of the microtiter plate contained 200 μ l of BHI broth, either the indicator strain (wild type) or one of the two mutants at an *A*₆₀₀ of about 0.01, as well as a specific concentration of one of the bacteriocins tested. The bacteriocin concentrations ranged from 25 nM to 0.05 nM, with a 2-fold difference for each dilution step. The microtiter plates containing the bacterial cultures were incubated for 12 h at 30°C. The growth of the wild-type strain and the two mutants was monitored spectrophotometrically by measuring the absorbance at 600 nm every 10 min with a microtiter plate reader. The MIC, which is defined as the concentration of a bacteriocin that inhibits growth by 50%, was determined for the wild type and the two mutant strains. The experiment was repeated twice.

RESULTS AND DISCUSSION

***Listeria innocua* contains two potential PEP-dependent Dha kinases.** A BLAST search on the ListiList Web server with the three proteins of the *L. lactis* Dha phosphorylation system (49) revealed

FIG 1 Schematic representation of the *lin0359-0369* (*gol*) operon and the gene encoding its repressor Lin0370 (GolR). Gray arrows indicate the *dha-2* genes, and arrows filled with a motif indicate all other genes for which the function has been determined by *in vitro* or *in vivo* assays.

that *L. innocua* CLIP 11262 and *L. monocytogenes* EGD-e possess two operons encoding homologues of the lactococcal proteins. One operon (*dha-1*) has the same gene order as that of *L. lactis* (*dhaK-1*, *dhaL-1*, and *dhaM-1*; *lin2843-lin2845*; and *lmo2695-lmo2697*). The encoded proteins exhibit 61, 53, and 48% sequence identities, respectively, to their *L. lactis* homologues. The second set of *dha*-like genes seems to be part of a large operon (*dha-2*) and exhibits a different gene order (*dhaM-2*, *dhaK-2*, and *dhaL-2*), with two genes encoding presumed membrane proteins of unknown function inserted between *lin0369* (*dhaM-2*) and *lin0366* (*dhaK-2*) for *L. innocua* (between *lmo0351* and *lmo0348* for *L. monocytogenes*) (**Fig. 1**). The Dha-2 proteins show between 32 and 40% sequence identity compared to their *L. lactis* homologues. The expression of the *dha-2* genes of *L. monocytogenes* was reported to be upregulated during growth on glycerol, especially during late log phase (between 30- and 180-fold upregulation compared to glucose-grown cells), whereas the *dha-1* operon was only slightly induced (26). A similar observation was made during adaptation to a soil environment, where an 8-fold upregulation was detected for the *dha-2* genes (37), whereas the expression level of the *dha-1* operon was only 2-fold elevated.

Six other genes also seemed to be cotranscribed with the *dha-2* genes. They encode a protein with significant similarity to triose-phosphate isomerase (Tpi-2 [*lin0364*]) and proteins resembling enzymes of the pentose phosphate pathway: *lin0360* encodes a presumed transketolase, *lin0361* encodes a transaldolase, *lin0363* encodes a RpiB-type ribose-5-P isomerase (44), and *lin0362* encodes enzymes resembling the short-chain dehydrogenase/reductase family. The last gene in the operon (*lin0359*) encodes a protein with 48% similarity to the pediocin immunity protein from *P. acidilactici*.

By carrying out RT-PCR with 6 different primer pairs (**Table 1**), we showed that in the corresponding *L. innocua* mRNA transcript, each of the 11 genes of the presumed *dha* operon is linked to its two neighboring genes, except for the distal *pedB*-like (*lin0359*) and *dhaM-2* (*lin0369*) cistrons (data not shown). These results strongly suggest that the 11 *L. innocua* genes are organized into an operon (**Fig. 1**), which is in good agreement with previously re-

ported *L. monocytogenes* transcriptome data acquired during soil adaptation (37), where all genes, except for the *pedB*-like gene, were reported to be transiently upregulated after 15 and 30 min of adaptation to soil extracts or soil microcosms. During growth on glycerol to the late log phase, 7 of the 11 *L. monocytogenes* genes were also reported to be upregulated (26).

Glycerol-inducible expression of the *lmo0341-0351* operon in *L. monocytogenes* and constitutive expression of the *lin0359-0369* operon in the *lin0370* mutant. In order to test whether the expression of the *lin0359-lin0369* operon can also be induced in defined growth media, we carried out quantitative RT-PCR experiments. We tested the expression of the *lin0359-0369* operon after growth in LB medium and LB medium containing 50 mM Dha, glycerol, or glucose. We carried out quantitative RT-PCR by using two primer pairs allowing the amplification of a 153-bp *dhaK-2* DNA fragment and a 175-bp *pedB*-like DNA fragment. A small amount of *dha* mRNA was formed when the cells were grown in LB medium without an additional carbon source (**Table 2**). Even less *dha* mRNA was detected when the cells were grown in LB medium containing glucose, glycerol, or Dha. Apparently neither glycerol nor Dha induced *lin0359-0369* expression during growth in LB medium. Unfortunately, *L. innocua* strain CLIP 11262 grows very poorly in minimal medium, even when complemented with glucose. Quantitative RT-PCR experiments were therefore carried out with *L. monocytogenes* strain EGD-e, which grows well in minimal medium, by employing the same *dhaK*- and *pedB*-specific primer pairs as those used for *L. innocua* (100% identity to the corresponding *L. monocytogenes* sequences). When *L. monocytogenes* EGD-e was grown in minimal medium with glucose, no significant expression of the *dha-2* operon (*lmo0341-0351*) was observed. However, after reaching the stationary phase, glycerol-grown cells exhibited elevated expression levels of the *dhaK-2* (500-fold) and the *pedB*-like (8-fold) genes (**Table 3**) compared to those exhibited by cells grown on glucose. No growth was observed in minimal medium containing Dha as the sole carbon source. These results are in agreement with data from transcriptome studies with glycerol-grown *L. monocytogenes* EGD-e cells, where a strong induction (up to 180-fold) of the genes of the

TABLE 2 Relative expression levels of the *dhaK-2* and *pedB*-like genes determined by quantitative RT-PCR for *L. innocua* wild-type strain CLIP 11262 and the *golR* mutant derived from it after growth in LB medium containing 50 mM glucose, glycerol, or Dha

Strain	Mean relative expression level (SD) ^a							
	LB		LB + Glc		LB + Gly		LB + Dha	
	<i>dhaK-2</i>	<i>pedB</i> -like	<i>dhaK-2</i>	<i>pedB</i> -like	<i>dhaK-2</i>	<i>pedB</i> -like	<i>dhaK-2</i>	<i>pedB</i> -like
CLIP 11262	0.044 (0.036)	0.027 (0.007)	0.001 (0.001)	0.016 (0.012)	0.005 (0.002)	0.013 (0.009)	0.001	0.011 (0.009)
<i>lin0370</i> (<i>golR</i>)	1 ^b	1 ^b	0.065 (0.024)	0.111 (0.074)	1.133 (0.423)	0.817 (0.318)	0.680 (0.337)	0.774 (0.117)

^a Mean values and standard deviations (in parentheses) calculated from results obtained with at least four independent RNA preparations are shown.

^b Transcript levels were calculated relative to the LB medium-grown *golR* strain, which was set to 1.

TABLE 3 Relative expression levels of the *dhaK*- and *pedB*-like genes determined by quantitative RT-PCR of *L. monocytogenes* wild-type strain EGD-e grown in minimal medium containing 50 mM glucose or glycerol

Strain	Mean relative expression level (SD) ^a			
	MM glucose		MM glycerol	
	<i>dhaK</i> -2	<i>pedB</i> -like	<i>dhaK</i> -2	<i>pedB</i> -like
EGD-e	0.002 (0.001)	0.132 (0.094)	1	1

^a Mean values and standard deviations (in parentheses) calculated from the results obtained with at least four independent RNA preparations are shown. Transcript levels were calculated relative to the value obtained for glycerol-grown cells, which was set to 1. MM, minimal medium.

lmo0341-0351 operon by glycerol was also found when the cells entered stationary phase (26). The *dha*-1 operon is not induced by glycerol, and its inducer is unknown.

The *lin0359-0369* operon is preceded by the *lin0370* gene (*lmo0352* in *L. monocytogenes*), oriented in the opposite direction and encoding a presumed transcription repressor of the DeoR family, which we suspected to control the expression of the operon. We therefore inactivated *lin0370* and tested the effect of this mutation on the expression of the *lin0359-0369* operon by quantitative RT-PCR. The inactivation of *lin0370* caused 20- and 40-fold-elevated expression levels of the *dhaK*-2 and *pedB*-like genes, respectively, after growth in LB medium compared to the wild-type strain (Table 2). A similar increased expression level of the *lin0359-0369* operon was observed when the *lin0370* repressor mutant was grown in LB medium with glycerol or Dha. The presence of glucose strongly inhibited the constitutive expression of the *dhaK*-2 and *pedB*-like genes in the *lin0370* mutant (Table 2). This repression is probably due to catabolite control protein A/P-Ser-HPr-mediated carbon catabolite repression (12, 17), because the first gene of the *lin0359-0369* operon contains an almost perfect target site (catabolite response element, TGGAAGCGCTA TCT) for the catabolite repressor complex in its coding sequence.

***lin0367* and *lin0368* encode proteins of unknown function, and *lin0362* encodes a new type of glycerol dehydrogenase.** For 3 of the 11 proteins encoded by the *dha*-2 operon, a sequence comparison did not allow the prediction of a function. Two of them are small presumed membrane proteins encoded by *lin0367* and *lin0368*, and homologues are present in most other listeriae. Homologues are also found in members of the orders *Thermoanaerobacterales*, *Lactobacillales*, and *Clostridiales*, where they are usually associated either with a gene encoding a predicted 6-P- β -glucosidase or with a glycerol (*glp*) or *dha* operon. For example, in *Halan-aerobium praevalens*, two homologous genes are located in front of the *glpK* and *glpO* genes. In *Tepidanaerobacter* sp. strain Re1, the genes for these two proteins are located in an operon exhibiting the same gene order as that of the *B. subtilis* glycerol operon (*glpPFKD*—antiterminator, glycerol facilitator, glycerol kinase, and glycerol-3-P dehydrogenase) (5), with the exception that *glpF* is replaced with the two genes encoding the small membrane proteins. This organization suggests that in *Tepidanaerobacter* sp. Re1, the two small membrane proteins might catalyze glycerol uptake either via facilitated diffusion or active transport.

The gene *lin0362* encodes a predicted short-chain dehydrogenase/reductase of unknown specificity. In order to determine the substrate of Lin0362, we purified the His-tagged protein as de-

scribed in Materials and Methods. Enzymes of this family usually catalyze the reversible transformation of HCOH groups in the second position of polyols or related compounds into keto groups. Examples are the *Rhodobacter sphaeroides* sorbitol dehydrogenase/sorbose reductase (41) or the *Gluconobacter oxydans* xylitol dehydrogenase/xylulose reductase (45). We therefore tested the possibility that Lin0362 might catalyze the NADH- or NADPH-dependent reduction of xylulose-5-P or ribulose-5-P, two intermediates of the pentose phosphate pathway, into xylitol-5-P or ribitol-5-P, respectively. However, no such activity could be measured with the purified protein (data not shown). Because glycerol also contains an oxidizable HCOH group in its second position and because this triol induces the expression of the *L. monocytogenes dha*-2 operon (*lmo0341-0351*) (Table 3) (26), we tested the possibility that the protein Lin0362 might be a novel type of glycerol dehydrogenase/dihydroxyacetone reductase. When we tested this hypothesis, we indeed found that Lin0362 catalyzes the NAD⁺-requiring transformation of glycerol into Dha as well as the reverse reaction, the NADH-dependent reduction of Dha to glycerol. As observed previously for other alcohol dehydrogenase/keto reductase-catalyzed reactions, the equilibrium was shifted toward the formation of glycerol and NAD⁺, because the standard free energy strongly favors the formation of the alcohol (4). As a consequence, the formation of NADH during glycerol dehydrogenation rapidly slowed, owing to the preferred backward reaction. In addition, the initial rate of the NADH-dependent reduction of Dha was found to be nearly 10 times higher than the rate of the NAD⁺-dependent oxidation of glycerol (data not shown). For the efficient oxidation of glycerol inside the cells, the Dha formed will therefore have to be rapidly dissimilated, most likely by phosphorylation to Dha-P (Fig. 2, top right). The enzyme Lin0362 specifically uses NAD⁺ and NADH, because no activity was observed when its two functions were tested with NADP⁺ and NADPH, respectively. Divalent cations were necessary for both activities of Lin0362, and the Mg²⁺ and Mn²⁺ ions had similar stimulating effects, whereas the enzyme was inactive in the presence of Zn²⁺.

Similar to the *dha* operon in most enterococci and clostridia (6, 40), the *L. innocua lin0359-lin0369* and *L. monocytogenes lmo0341-lmo0351* operons contain a gene encoding a protein with NAD⁺-dependent glycerol dehydrogenase activity. However, while the enterococcal and clostridial enzymes exhibit between 40 to 60% sequence identity compared to glycerol dehydrogenase (GldA) from firmicutes (31) or enterobacteria (46), the listerial protein is, to the best of our knowledge, the first member of the short-chain dehydrogenase/reductase family exhibiting glycerol dehydrogenase/dihydroxyacetone reductase activity. The *L. innocua lin0359-lin0369* and *L. monocytogenes lmo0341-lmo0351* operons are therefore likely to encode a glycerol utilization system, which in the first step transforms glycerol into dihydroxyacetone, which is subsequently probably converted into Dha-P by the DhaKLM-2 proteins (Fig. 2). We therefore called Lin0362 GoD, for glycerol dehydrogenase, and the genes *lin0359* to *lin0369* the *gol* operon.

In vitro reconstitution of PEP-dependent Dha phosphorylation. Based on the above-described results, we wanted to test whether the DhaKLM-2 proteins would indeed be able to phosphorylate Dha. For that purpose, we purified EI and HPr from *B. subtilis* and the three Dha-2 proteins from *L. innocua*. We first tested whether EI and HPr would be able to phosphorylate DhaM-2 (EIADha-2), encoded by *lin0369*. After the incubation of

FIG 2 Proposed model of glycerol metabolism by the enzymes encoded by the *L. innocua* *gol* operon. Glycerol taken up via facilitated diffusion possibly catalyzed by the small membrane proteins Lin0367 and Lin0368 or by GlpF encoded by the *glpFK* operon is intracellularly oxidized to Dha by the enzyme glycerol dehydrogenase (Gld). The PEP-requiring PTS phosphorylation cascade composed of EI, HPr, and EIIA^{Dha}-2 is connected to DhaL-2::ADP, which is converted to DhaL-2::ATP and subsequently catalyzes the phosphorylation of Dha bound to DhaK-2. Tpi-2 catalyzes the interconversion of Dha-P and glyceraldehyde-3-P (Gap). The two triosephosphates are condensed to FBP, which is subsequently converted into fructose-6-P (F-6-P). The enzyme encoded by the *lin0360* gene resembles transketolases and probably allows entry into the pentose phosphate pathway (PPP) by catalyzing the formation of xylulose-5-P and erythrose-4-P from F-6-P and glyceraldehyde-3-P.

the three PTS proteins in the presence of PEP as the phosphoryl donor, the samples were separated on a 10% polyacrylamide gel containing 8 M urea. This gel system often allows the separation of phosphorylated and unphosphorylated proteins (30). Indeed, HPr incubated with EI and PEP migrates much faster than unphosphorylated HPr (Fig. 3, compare lanes 1 and 2). When the EI^{Dha}-2 protein was incubated with PEP, EI, and HPr (Fig. 3, lane 4), it also exhibited a slightly higher electrophoretic mobility than the EI^{Dha}-2 protein incubated with PEP and HPr (Fig. 3, lane 3) or only PEP (Fig. 3, lane 5). These results established that the EI^{Dha}-2 protein becomes phosphorylated by PEP, EI, and HPr.

We next wanted to test whether Dha would become phosphorylated when the DhaK-2 and DhaL-2 proteins were included in the phosphorylation assay. We set up a coupled spectrophotometric test by also adding glycerol-3-P dehydrogenase to the assay mix-

ture, which we expected to convert the P-Dha formed by the DhaKLM-2 system into glycerol-3-P in an NADH-consuming reaction. We indeed observed a decrease of the A_{340} in the sample when all proteins were present (Fig. 4), whereas no change of the A_{340} occurred when any one of the five proteins was lacking (see “-DhaK” in Fig. 4; identical straight lines were obtained when either one of the other proteins was lacking). These results confirm that Dha is indeed the substrate for this second Dha kinase system. Our results also confirm that the phosphoryl group transfer from PEP to Dha occurs in five steps, as depicted in Fig. 2 (left). While the oxidation of glycerol by Gold is reversible, and the equilibrium is shifted toward glycerol and NAD^+ , the phosphorylation of Dha by the PEP-dependent Dha kinase is almost irreversible owing to the high free energy of PEP. The efficient Dha kinase reaction therefore also provides the driving force for the glycerol dehydrogenase reaction.

When we carried out the spectrophotometric assays, we usually preincubated the assay mixture for 5 min before the reaction was started by the addition of the lacking enzyme. Interestingly, when HPr or DhaL was used to start the reaction, NADH consumption occurred at a significantly reduced rate (about one-third) compared to the reaction velocity obtained with the other three “starter” proteins (Fig. 4). A possible explanation for this unexpected behavior might be that during preincubation, different protein complexes are preformed, depending on which enzyme is lacking. The formation of a strong complex between EIIA^{Dha} and DhaL has indeed been reported for the *L. lactis* proteins, and the crystal structure of the transient complex has been determined (49). In addition, HPr is known to form transient complexes with EI and EIIA^{Man} (EIIA^{Man} belongs to the same EIIA family as EIIA^{Dha}), and the solution structures of the corresponding *E. coli* protein complexes have been determined (20, 47). Because slow Dha phosphorylation was observed when either HPr or DhaL was absent during preincubation, it is likely that the perturbation of the formation of the HPr and DhaL complexes with EIIA^{Dha} might be responsible for the slower Dha phosphorylation.

The protein encoded by *lin0364* exhibits triosephosphate isomerase activity. Like most firmicutes, *L. innocua* possesses an operon with genes encoding triosephosphate isomerase and three or four other enzymes of the lower part of the Embden-Meyerhof-

FIG 3 Phosphorylation of DhaM-2 (EIIa^{Dha}) detected by electrophoresis on an 8 M urea–10% polyacrylamide gel. All samples were loaded onto the gel in a volume of 25 μ l containing 50 mM Tris-HCl (pH 7.4), 1 mM PEP, and 5 mM MgCl₂, and the proteins indicated for each lane: 1 μ g of EI, 3 μ g of HPr, and 3 μ g of DhaM-2. Samples were incubated at 37°C as described in Materials and Methods. The drastic change of the migration behavior caused by the phosphorylation of HPr was described previously (30) (compare lanes 1 and 2). The difference in electrophoretic mobility between DhaM-2 and P~DhaM-2 is less pronounced but still clearly visible (compare lanes 3, 4, and 5).

FIG 4 Spectrophotometric assays of Dha phosphorylation. Spectrophotometric assays were carried out at room temperature by monitoring the decrease in the amount of NADH at an A_{340} over a time period of 60 min by using a Kontron Bio-Tek spectrophotometer. All samples consisted of 700- μ l reaction mixtures containing 75 mM Tris-HCl (pH 7.4), 6 mM PEP, 1 mM NADH, 6 mM Dha, 10 mM $MgCl_2$, and 1 U of glycerol-3-P dehydrogenase. The following five proteins were assumed to be required for Dha phosphorylation: EI, HPr, DhaK-2, DhaL-2, and DhaM-2 (EIIA^{Dha}). Samples lacking any one of the above-mentioned five proteins were prepared in duplicates and preincubated for 10 min before the lacking protein was added to one of the two samples. The decrease of the A_{340} was monitored for both samples. All complemented samples exhibited a more or less rapid decrease of the A_{340} . For the control assays, we show only the sample lacking DhaK-2 (–DhaK-2), because a similar straight line was obtained for all other “noncomplemented” samples. These results confirm that all five bacterial proteins are necessary for Dha phosphorylation.

Parnas pathway. However, as mentioned above, the sequence of the protein encoded by *lin0364* also exhibits significant similarity to triosephosphate isomerases (29% identity to *L. innocua* triosephosphate isomerase of the *gap-pgk-tpi-pgm-eno* operon). We therefore overproduced and purified the *lin0364*-encoded protein and tested whether it indeed possesses a triosephosphate isomerase function. For that purpose, we used a spectrophotometric assay which starts from FBP, which is split by the enzyme aldolase into glyceraldehyde-3-P and Dha-P. The latter product is immediately reduced to glycerol-3-P in an NADH-dependent reaction catalyzed by the enzyme glycerol-3-P dehydrogenase, which was also included in the assay mixture. Once NADH consumption had stopped (no further decrease of the A_{340}), commercially available triosephosphate isomerase or the purified *lin0364*-encoded protein was added to the reaction mixture. In both cases, this led to a second phase of NADH consumption and, therefore, to a further decrease of the A_{340} , which was of an amplitude ($\Delta A_{340} = 0.7$) identical to that of the first observed decrease (data not shown). This result confirmed that the glyceraldehyde-3-P formed during the aldolase reaction in amounts identical to that of Dha-P is converted by the *lin0364*-encoded protein into Dha-P before it is reduced to glycerol-3-P. The *lin0364*-encoded protein therefore possesses triosephosphate isomerase activity, and we called the enzyme triosephosphate isomerase 2 and the corresponding gene *tpi-2*.

Lin0363 exhibits ribose-5-P isomerase (RpiB) activity. The Dha-P formed by the Dha kinase system DhaK-2/DhaL-2/EIIA^{Dha}-2 is probably partially converted by triosephosphate isomerase 2 into glyceraldehyde-3-P. These two glycolytic intermediates might subsequently be fused by the enzyme aldolase, thereby forming FBP, which might be further converted to fructose-6-P. Together with glyceraldehyde-3-P, this glycolytic intermediate is probably transformed into xylulose-5-P and erythrose-4-P by the enzyme transketolase (Lin0360), which provides the entry point into the pentose phosphate pathway (Fig. 2, bottom right). It should be noted that in addition to Lin0360 and Lin0361, listeriae usually possess two other transketolase and transaldolase

homologues. In three additional steps catalyzed by three enzymes encoded within the *gol* operon, the end product of the pentose phosphate pathway, ribulose-5-P, will be formed: transaldolase (Lin0361) produces sedoheptulose-7-P and glyceraldehyde-3-P from erythrose-4-P and fructose-6-P, transketolase (Lin0360) forms ribose-5-P and xylulose-5-P from sedoheptulose-7-P and glyceraldehyde-3-P, and finally, ribose-5-P isomerase B (RpiB [Lin0363]) converts ribose-5-P into ribulose-5-P.

In order to support this concept of glycerol metabolism, we purified the presumed ribose-5-P isomerase B encoded by *lin0363* and tested its function as described in Materials and Methods. It indeed exhibited the expected ribose-5-P isomerase activity by catalyzing the interconversion of ribulose-5-P and ribose-5-P (data not shown). The only enzyme lacking in the *gol* operon-encoded pentose phosphate pathway is ribulose-5-P 3-epimerase, which converts the xylulose-5-P formed during the two transketolase reactions into ribulose-5-P. We therefore tested whether Lin0363 might also possess ribulose-5-P 3-epimerase activity. However, no such activity was exhibited by Lin0363. It is therefore likely that one or several of the five ribulose-5-P 3-epimerase homologues encoded by the listerial genome will complete the pentose phosphate pathway during glycerol utilization via the Dha-2 system.

What is the function of the PedB-like protein encoded by *lin0359*? The *gol* operon seems to be specific for listeriae, because we detected operons encoding triosephosphate isomerase 2, the novel type of glycerol dehydrogenase GolD, and enzymes of the Dha kinase system and the pentose phosphate pathway in only four other listerial species, including *L. monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, and *Listeria welshimeri*. The *gol* operon is lacking in the *L. grayi* and *L. marthii* strains, for which the genome sequences have been determined, as well as in *L. ivanovii* strain PAM55, while it is present in *L. ivanovii* strain FSL F6-596. When present, the Dha-2 proteins of these organisms exhibit between 83% and 96% sequence identities compared to the corresponding *L. innocua* proteins. While the *gol* operons of *L. seeligeri*, *L. ivanovii*, and *L. welshimeri* lack the distal *pedB*-like gene, it is present in

the *gol* operon of all three sequenced *L. innocua* strains. *L. monocytogenes* takes an intermediary position because out of the 27 strains for which the genomes have been sequenced, only 12 contain a *pedB*-like gene in their *gol* operons.

The mode of action of the immunity proteins has not yet been entirely elucidated, but each immunity protein seems to act more or less specifically against its corresponding bacteriocin (14). Interestingly, an extracellular loop of the glucose/mannose-specific EIIC PTS component seems to be involved in the specific targeting by class IIa bacteriocins (28, 39), and the corresponding mutants therefore exhibit resistance to these bacteriocins (9). Organisms producing bacteriocins frequently contain the bacteriocin gene and the corresponding immunity gene in the same operon. Similar to *lin0359* (*pedB*-like), immunity genes have been acquired by bacteria that are the targets of the bacteriocin. The protein encoded by *lin0359* exhibits 36% sequence identity to *Lactobacillus gasseri* PedB and 33% sequence identity to PedB from *P. acidilactici*, with the latter providing immunity against pediocin PA-1. Two obvious questions were therefore whether the listerial PedB-like protein would protect against pediocin or any other known member of the class IIa bacteriocins and why the *pedB*-like gene is attached to the *gol* operon in *L. innocua* and about half of the *L. monocytogenes* strains.

In order to get an answer to the first question, we tried to determine whether the PedB-like protein would provide immunity against some of the most common pediocin-like bacteriocins (class IIa bacteriocins), including pediocin PA-1 from *P. acidilactici* (22), curvacin A, leucocin A, sakacin P, and enterocin A. Their effects on the growth of wild-type *L. innocua* and of mutants carrying either a *golR* disruption (expresses *pedB* constitutively) or a *pedB* deletion were tested. The concentration of each bacteriocin leading to 50% growth inhibition (MIC) was determined. We observed that only the pediocin PA-1 K20R variant had a *pedB*-related effect. Compared to wild-type *L. innocua* and the Δ *pedB*-like mutant, the strong constitutive expression of the *pedB*-like gene in the *golR* mutant rendered it reproducibly about 2 times less sensitive to the presence of pediocin PA-1 (Fig. 5). For all other bacteriocins tested, the observed MIC was the same for the wild type and the two mutant strains (data not shown). The weak protective effect against pediocin PA-1 suggests that pediocin PA-1 is not the physiologically relevant bacteriocin against which the listerial PedB-like protein provides immunity. It probably protects against a bacteriocin more or less closely related to pediocin PA-1. There are several bacteriocins that exhibit only 1 or 2 amino acid exchanges compared to the 44 residues of pediocin PA-1, such as pediocin Ach from *P. acidilactici*, the pediocin produced by *Pediococcus* sp. strain CRA51, or the coagulin CoaA from *Bacillus coagulans* (29). The corresponding PedB proteins protecting against these pediocins are nearly identical to PedB from *P. acidilactici*, whereas the PedB-like protein encoded by the listerial *gol* operon exhibits only 48% sequence similarity. It is therefore likely that the PedB-like protein protects against a bacteriocin more distantly related to pediocin PA-1. Among these bacteriocins, we have tested curvacin A, leucocin A, sakacin P, and enterocin A (16). However, there exist many more which were not at our disposal, such as mundticin from *Enterococcus mundtii*, avicin from *Enterococcus avium*, or piscicidin from *Carnobacterium maltaromaticum*. Again, none of the corresponding immunity proteins produced by the above-mentioned organisms exhibits more than 40% sequence identity to the listerial PedB-like protein. We

FIG 5 Pediocin immunity assays. The immunity of the *L. innocua* wild-type strain (wt), the *golR* mutant, and the *pedB*-like mutant against the pediocin PA-1 K20R variant from *Pediococcus acidilactici* (22) was determined. For that purpose, the growth of the three strains in microtiter plates containing BHI broth and the indicated pediocin PA-1 concentrations was monitored over a time period of 12 h by measuring the A_{600} with a microtiter plate reader. The mean values from three independent experiments and the calculated standard deviations are presented. The growth of the *pedB*-like mutant was strongly reduced at 0.78 nM pediocin PA-1. At 1.54 nM pediocin PA-1, the growth of the wild-type strain was completely inhibited, whereas at this concentration, the *golR* mutant was still able to reach about half the maximal A_{600} .

therefore favor the idea that the bacteriocin to which the listerial PedB-like protein provides immunity has not yet been discovered. In order to protect itself, the organism synthesizing this bacteriocin is likely to produce an immunity protein nearly identical to the listerial PedB-like protein encoded by the *gol* operon.

Regarding the second question, we hypothesize that some listerial species probably fused the *pedB*-like gene to the *gol* operon because it is induced by glycerol during growth in soil (37). The organism producing the corresponding bacteriocin might therefore also be a soil bacterium. Interestingly, all serotype 4b *L. monocytogenes* strains, which are the most virulent strains, lack the PedB-like protein, thus reflecting that serotype 4b strains are probably more adapted to proliferation in host cells than to saprophytic life. Although glycerol is one of the preferred carbon sources during the growth of *L. monocytogenes* within host cells (15), the *gol* operon is not induced during intracellular growth (27). In contrast, the expressions of the *glpFK* genes *lmo1538* and *lmo1539* are upregulated during intracellular growth, and their inactivation leads to a significant attenuation of listerial virulence. Glycerol metabolism during the saprophytic life of *L. monocytogenes* seems to be mediated via the enzymes encoded by both the *gol* and the *glpFK* operons, whereas during intracellular growth, it is probably achieved only by the *glpFK* gene products. How *L. monocytogenes* distinguishes between glycerol present in the soil and glycerol encountered in host cells is not understood. Glycerol phosphorylated by GlpK is probably metabolized mainly via the Embden-Meyerhof-Parnas pathway, whereas glycerol first oxidized to Dha by Gold is probably metabolized mainly via the pentose phosphate pathway. The two modes of glycerol metabolism therefore respond to different metabolic requirements.

ACKNOWLEDGMENTS

We thank Jon Nissen-Meyer for providing us with the bacteriocins pediocin PA-1 K20R, curvacin A, leucocin A, sakacin P, and enterocin A and for his helpful advice; Alexa Bourand for providing purified His-tagged *L.*

casei ribitol-5-P dehydrogenase; and Axel Hartke and Rana Herro for fruitful discussions.

This research was supported by Agence National de la Recherche grant ANR-09-BLANC-0273-01.

REFERENCES

- Aké FMD, Joyet P, Deutscher J, Milohanic E. 2011. Mutational analysis of glucose transport regulation and glucose-mediated virulence gene repression in *Listeria monocytogenes*. *Mol. Microbiol.* 81:274–293.
- Arnaud M, Chastanet A, Débarbouillé M. 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl. Environ. Microbiol.* 70:6887–6891.
- Bächler C, Flükiger-Brühwiler K, Schneider P, Bähler P, Erni B. 2005. From ATP as substrate to ADP as coenzyme: functional evolution of the nucleotide binding subunit of dihydroxyacetone kinase. *J. Biol. Chem.* 280:18321–18325.
- Baumberg JP, Jürgensen JJ, Bardwell K. 1933. The coupled redox potential of the lactate-enzyme-pyruvate system. *J. Gen. Physiol.* 16:961–976.
- Beijer L, Nilsson RP, Holmberg C, Rutberg L. 1993. The *glpP* and *glpF* genes of the glycerol regulon in *Bacillus subtilis*. *J. Gen. Microbiol.* 139:349–359.
- Bizzini A, et al. 2010. Glycerol is metabolized in a complex and strain-dependent manner in *Enterococcus faecalis*. *J. Bacteriol.* 192:779–785.
- Cintolesi A, Clomburg JM, Rigou V, Zygourakis K, Gonzalez R. 2012. Quantitative analysis of the fermentative metabolism of glycerol in *Escherichia coli*. *Biotechnol. Bioeng.* 109:187–198.
- Colussi T, et al. 2008. Structure of α -glycerophosphate oxidase from *Streptococcus* sp.: a template for the mitochondrial α -glycerophosphate dehydrogenase. *Biochemistry* 47:965–977.
- Dalet K, Cenatiempo Y, Cossart P, Héchard Y. 2001. A σ^{54} -dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. *Microbiology* 147:3263–3269.
- Darbon E, Servant P, Poncet S, Deutscher J. 2002. Antitermination by GlpP, catabolite repression via CcpA and inducer exclusion triggered by P-GlpK dephosphorylation control *Bacillus subtilis* *glpFK* expression. *Mol. Microbiol.* 43:1039–1052.
- Deutscher J, Bauer B, Sauerwald H. 1993. Regulation of glycerol metabolism in *Enterococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation of glycerol kinase catalyzed by enzyme I and HPr of the phosphotransferase system. *J. Bacteriol.* 175:3730–3733.
- Deutscher J, Francke C, Postma PW. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* 70:939–1031.
- Deutscher J, Sauerwald H. 1986. Stimulation of dihydroxyacetone and glycerol kinase activity in *Streptococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation catalyzed by enzyme I and HPr of the phosphotransferase system. *J. Bacteriol.* 166:829–836.
- Diep DB, Skaugen M, Salehian Z, Holo H, Nes IF. 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc. Natl. Acad. Sci. U. S. A.* 104:2384–2389.
- Eylert E, et al. 2008. Carbon metabolism of *Listeria monocytogenes* growing inside macrophages. *Mol. Microbiol.* 69:1008–1017.
- Fimland G, Johnsen L, Dalhus B, Nissen-Meyer J. 2005. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *J. Pept. Sci.* 11:688–696.
- Fujita Y, Miwa Y, Galinier A, Deutscher J. 1995. Specific recognition of the *Bacillus subtilis* *gnt cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. *Mol. Microbiol.* 17:953–960.
- Galiniér A, et al. 1997. The *Bacillus subtilis* *crh* gene encodes a HPr-like protein involved in carbon catabolite repression. *Proc. Natl. Acad. Sci. U. S. A.* 94:8439–8444.
- Garcia-Alles LF, et al. 2004. Phosphoenolpyruvate- and ATP-dependent dihydroxyacetone kinases: covalent substrate-binding and kinetic mechanism. *Biochemistry* 43:13037–13045.
- Garrett DS, Seok YJ, Peterkofsky A, Gronenborn AM, Clore GM. 1999. Solution structure of the 40,000 M_r phosphoryl transfer complex between the N-terminal domain of enzyme I and HPr. *Nat. Struct. Biol.* 6:166–173.
- Gutknecht R, Beutler R, Garcia-Alles LF, Baumann U, Erni B. 2001. The dihydroxyacetone kinase of *Escherichia coli* utilizes a phosphoprotein instead of ATP as phosphoryl donor. *EMBO J.* 15:2480–2486.
- Haugen HS, Fimland G, Nissen-Meyer J. 2011. Mutational analysis of residues in the helical region of the class IIa bacteriocin pediocin PA-1. *Appl. Environ. Microbiol.* 77:1966–1972.
- Hurley JH, et al. 1993. Structure of the regulatory complex of *Escherichia coli* III^{Glc} with glycerol kinase. *Science* 259:673–677.
- Jin RZ, Lin ECC. 1984. An inducible phosphoenolpyruvate:dihydroxyacetone phosphotransferase system in *Escherichia coli*. *J. Gen. Microbiol.* 130:83–88.
- Johnson EA, Burke SK, Forage RG, Lin EC. 1984. Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumoniae*. *J. Bacteriol.* 160:55–60.
- Joseph B, et al. 2008. Glycerol metabolism and PrfA activity in *Listeria monocytogenes*. *J. Bacteriol.* 190:5412–5430.
- Joseph B, et al. 2006. Identification of *Listeria monocytogenes* genes contributing to intracellular replication by expression profiling and mutant screening. *J. Bacteriol.* 188:556–568.
- Kjos M, Nes IF, Diep DB. 2011. Mechanisms of resistance to bacteriocins targeting the mannose phosphotransferase system. *Appl. Environ. Microbiol.* 77:3335–3342.
- Le Marrec C, Hyronimus B, Bressollier P, Verneuil B, Urdaci MC. 2000. Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I₄. *Appl. Environ. Microbiol.* 66:5213–5220.
- Lindner C, Galinier A, Hecker M, Deutscher J. 1999. Regulation of the activity of the *Bacillus subtilis* antiterminator LicT by multiple PEP-dependent, enzyme I- and HPr-catalysed phosphorylation. *Mol. Microbiol.* 31:995–1006.
- Mallinder PR, Pritchard A, Moir A. 1992. Cloning and characterization of a gene from *Bacillus stearothermophilus* var. non-diastaticus encoding a glycerol dehydrogenase. *Gene* 110:9–16.
- Mazé A, et al. 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. *J. Bacteriol.* 192:2647–2648.
- Nissen-Meyer J, Holo H, Håvarstein LS, Sletten K, Nes IF. 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J. Bacteriol.* 174:5686–5692.
- Pas HH, et al. 1991. ³¹P NMR demonstration of phosphocysteine as a catalytic intermediate on the *Escherichia coli* phosphotransferase system EII^{Mtl}. *J. Biol. Chem.* 266:6690–6692.
- Paulsen IT, Reizer J, Jin RZ, Lin ECC, Saier MH, Jr. 2000. Functional genomic studies of dihydroxyacetone utilization in *Escherichia coli*. *Microbiology* 146:2343–2344.
- Pettigrew DW, Ma DP, Conrad CA, Johnson JR. 1988. *Escherichia coli* glycerol kinase. Cloning and sequencing of the *glpK* gene and the primary structure of the enzyme. *J. Biol. Chem.* 263:135–139.
- Piveteau P, Depret G, Pivato B, Garmyn D, Hartmann A. 2011. Changes in gene expression during adaptation of *Listeria monocytogenes* to the soil environment. *PLoS One* 6:e24881. doi:10.1371/journal.pone.0024881.
- Premaratne RJ, Lin WJ, Johnson EA. 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 57:3046–3048.
- Ramath M, Arous S, Gravesen A, Hastings JW, Héchard Y. 2004. Expression of *mptC* of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. *Microbiology* 150:2663–2668.
- Raynaud C, et al. 2011. Molecular characterization of the glycerol-oxidative pathway of *Clostridium butyricum* VPI 1718. *J. Bacteriol.* 193:3127–3134.
- Schauder S, Schneider KH, Giffhorn F. 1995. Polyol metabolism of *Rhodobacter sphaeroides*: biochemical characterization of a short-chain sorbitol dehydrogenase. *Microbiology* 141:1857–1863.
- Siebold C, Arnold I, Garcia-Alles LF, Baumann U, Erni B. 2003. Crystal structure of the *Citrobacter freundii* dihydroxyacetone kinase reveals an eight-stranded α -helical barrel ATP-binding domain. *J. Biol. Chem.* 278:48236–48244.
- Siebold C, Garcia-Alles LF, Erni B, Baumann U. 2003. A mechanism of covalent substrate binding in the X-ray structure of subunit K of the *Escherichia coli* dihydroxyacetone kinase. *Proc. Natl. Acad. Sci. U. S. A.* 100:8188–8192.
- Sørensen KI, Hove-Jensen B. 1996. Ribose catabolism of *Escherichia coli*: characterization of the *rpiB* gene encoding ribose phosphate isomerase B and of the *rpiR* gene, which is involved in regulation of *rpiB* expression. *J. Bacteriol.* 178:1003–1011.
- Sugiyama M, Suzuki S, Tonouchi N, Yokozeki K. 2003. Cloning of the xylitol dehydrogenase gene from *Gluconobacter oxydans* and improved production of xylitol from D-arabitol. *Biosci. Biotechnol. Biochem.* 67:584–591.

46. Truniger V, Boos W. 1994. Mapping and cloning of *gldA*, the structural gene of the *Escherichia coli* glycerol dehydrogenase. *J. Bacteriol.* **176**:1796–1800.
47. Williams DC, Jr, Cai M, Suh JY, Peterkofsky A, Clore GM. 2005. Solution NMR structure of the 48-kDa IIA^{Mannose}-HPr complex of the *Escherichia coli* mannose phosphotransferase system. *J. Biol. Chem.* **280**: 20775–20784.
48. Yeh JJ, et al. 2004. Structures of enterococcal glycerol kinase in the absence and presence of glycerol: correlation of conformation to substrate binding and a mechanism of activation by phosphorylation. *Biochemistry* **43**:362–373.
49. Zurbriggen A, et al. 2008. X-ray structures of the three *Lactococcus lactis* dihydroxyacetone kinase subunits and of a transient intersubunit complex. *J. Biol. Chem.* **283**:35789–35796.