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Role of PdxR in the activation of vitamin B_6 biosynthesis in *Listeria monocytogenes*

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SUMMARY

Listeria monocytogenes PdxR is a member of the poorly characterized but widespread group of MocR/GabR-type chimeric bacterial proteins that have DNA-binding and aminotransferase-like domains. Using mutational analysis, real-time RT-PCR, transcriptional fusions, gel-shift assays, DNase I footprinting, and *in vitro* transcription, it was shown that PdxR is a direct activator of the *pdxST* operon, transcribed divergently from *pdxR* and responsible for the *de novo* synthesis of pyridoxal 5′-phosphate (PLP), the major active form of vitamin B_6. PLP acts as an anti-activator of PdxR and is the only effector required to reduce the activity of PdxR. PdxR is also a negative autoregulator, and its ability to repress is increased by PLP. A dyad-symmetry sequence, which overlaps the −35 region of the *pdxS* promoter and lies downstream of the *pdxR* transcription start point, serves as an important element of the PdxR-binding site. Unexpectedly, some mutations in this activator-binding site, disrupting the dyad-symmetry element, caused constitutive, B_6-independent expression from the *pdxS* promoter. The data suggest that PdxR-like proteins, for which PLP plays just a signaling role, form a separate functional group among the MocR/GabR-type proteins.

Keywords

Listeria monocytogenes; vitamin B_6 biosynthesis; pyridoxal 5′-phosphate biosynthesis; MocR/GabR subfamily; GntR family of transcriptional regulators

INTRODUCTION

Chimeric bacterial proteins of the MocR/GabR subfamily belong to the GntR family of transcriptional regulators and are composed of a short N-terminal helix-turn-helix-containing domain (a putative DNA-binding region) of the GntR type and a long C-terminal domain that is similar to full-length aminotransferases (Belitsky and Sonenshein, 2002; Rigali *et al.*, 2002). Aminotransferases are ubiquitous enzymes of nitrogen metabolism that require pyridoxal 5′-phosphate (PLP), the major biologically active form of vitamin B_6, as an essential cofactor for catalysis (Eliot and Kirsch, 2004; Schneider *et al.*, 2000).

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The MocR/GabR subfamily includes at present thousands of proteins from both Gram-negative and Gram-positive bacteria (Bramucci *et al.*, 2011), but only a handful of its members have been characterized functionally and shown to be DNA-binding proteins that activate transcription of their target genes (Belitsky, 2004a; Belitsky and Sonenshein, 2002; El Qaidi *et al.*, 2013; Jochmann *et al.*, 2011; Wiethaus *et al.*, 2008). One member of the family, *Bacillus subtilis* GabR, has been described in detail (Belitsky, 2004a), and its crystal structure has been determined (Edayathumangalam *et al.*, 2013). The structural fold of the C-terminal domain of GabR is indeed very similar to the fold of many aminotransferases (fold type I), and PLP appears to be a tightly-bound covalently-linked component of the protein, similar to the case of aminotransferases and other PLP-dependent enzymes (Edayathumangalam et al., 2013; Eliot and Kirsch, 2004; Grishin *et al.*, 1995; Schneider et al., 2000).

GabR is a transcriptional activator of genes for γ-aminobutyrate (GABA) utilization, which requires the presence of GABA, in addition to PLP, for activation of its target genes (Belitsky, 2004a; Belitsky and Sonenshein, 2002). GabR apparently performs an aminotransferase-like partial reaction between GABA and PLP that is required for its activation as a transcriptional regulator (Belitsky, 2004a).

Three MocR/GabR-type proteins from various bacteria, called PdxR proteins, have been shown to be required for the expression of genes of PLP biosynthesis (El Qaidi et al., 2013; Jochmann et al., 2011; Magarvey *et al.*, 2001). PLP is considered to be one of the most versatile cofactors and is essential, in addition to aminotransferases, for activity of numerous other metabolic enzymes, such as amino acid racemases, decarboxylases, aminomutases, and many others (Percudani and Peracchi, 2003; Schneider et al., 2000). Ability of bacterial cells to synthesize PLP is important for virulence of such pathogens as *Mycobacterium tuberculosis* and *Helicobacter pylori* (Dick *et al.*, 2010; Grubman *et al.*, 2010).

The functional role and ability of PdxR proteins to respond to vitamin B_6 availability in the medium (El Qaidi et al., 2013; Jochmann et al., 2011) suggest that one or more of the B_6 vitamers may be a simple effector of these proteins. To characterize further the mechanism by which PdxR proteins regulate expression of their target genes, I studied a putative PdxR-like protein, Lmo2100, of *Listeria monocytogenes*, a Gram-positive, facultative intracellular bacterium and an important food-borne bacterial pathogen that can cause severe disease in mammals and birds (Vazquez-Boland *et al.*, 2001). In this work, it was shown that *L. monocytogenes* PdxR is a transcriptional activator of the *pdxST* genes responsible for *de novo* PLP biosynthesis. PdxR is inactivated by direct interaction with PLP and, in contrast to GabR, does not require any other effectors for activity.

RESULTS

Phenotype of the *pdxST* null mutant

The products of the *L. monocytogenes lmo2101* and *lmo2102* genes (Glaser *et al.*, 2001) are highly similar (82 and 52% identity, respectively) to the products of the *pdxS* and *pdxT* genes of *B. subtilis* (Belitsky, 2004b), which encode two subunits of PLP synthase, the only dedicated enzyme of *de novo* PLP biosynthesis in many bacteria and other organisms

(Fitzpatrick *et al.*, 2007). Hence these genes were renamed as *pdxS* and *pdxT*, respectively. The open reading frames of the two listerial genes are separated by only 4 bp, and they form a single transcriptional unit (Toledo-Arana *et al.*, 2009). A deletion-insertion mutation, replacing both the *pdxS* and *pdxT* genes with a spectinomycin-resistance cassette, was constructed and introduced into the chromosome of *L. monocytogenes* strain EGD-e as described in Experimental Procedures. No growth of the resulting strain, BLM1 (Δ*pdxST::spc*), was observed in a defined medium (see Experimental Procedures) either in liquid culture or on plates. The growth was fully restored by addition of ≥1 μM pyridoxal (PL) or by complementation of the mutation (Fig. 1 and data not shown). It was concluded that the *pdxST* genes of *L. monocytogenes* encode a functional PLP synthase, consistent with the known ability of this bacterium to synthesize vitamin B_6 (Welshimer, 1963). Apparently, as in all other bacteria, only one *de novo* pathway of PLP synthesis is present in *L. monocytogenes*.

Phenotype of the *pdxR* null mutant

A putative transcriptional regulator of the MocR/GabR subfamily (Belitsky and Sonenshein, 2002; Rigali et al., 2002) is encoded by the *lmo2100* gene that is adjacent to and transcribed divergently from the *L. monocytogenes pdxS* gene (the two genes are separated by 125 bp) (Glaser et al., 2001). A deletion-insertion mutation, replacing about 0.3 kb of the *lmo2100* gene with a spectinomycin-resistance cassette, was constructed and introduced into the chromosome of *L. monocytogenes*. Strain BLM7 (Δ*lmo2100::spc*) required PL for growth in a manner similar to that of the *pdxST* null mutant; complementation of the mutation alleviated the PL requirement (Fig. 1). Therefore, the product of the *lmo2100* gene appears to be required for *pdxST* expression, suggesting that it acts as a positive regulator of *pdxST* transcription. The gene was renamed *pdxR*; it encodes a 481-amino acid protein, which contains, as do other members of the MocR/GabR subfamily, a N-terminal putative DNA-binding domain and a C-terminal aminotransferase-like domain.

Transcriptional regulation of *pdxS* and *pdxR* in *L. monocytogenes*

As determined by real-time RT-PCR experiments, *pdxS* expression in the rich Brain Heart Infusion medium (BHI) required the presence of PdxR and was reduced 8-fold in the presence of PL (Table 1). Thus, PdxR behaves indeed as an activator of *pdxS* expression, and PL or a derivative thereof serves as a likely anti-activator of PdxR. Two other members of the MocR/GabR subfamily, also named PdxR, were shown recently to be B_6-responsive activators of the *pdxST* genes in *Corynebacterium glutamicum* and *Streptococcus pneumoniae* cells (El Qaidi et al., 2013; Jochmann et al., 2011).

pdxR expression was about 10-fold less efficient than expression of *pdxS* but was increased 3-fold in the *pdxST* null mutant in the absence of PL (Table 1), apparently as a result of B_6 limitation experienced by the mutant cells. In contrast, it was reduced 2- to 6-fold in the presence of PL both in wild-type and the mutant. Thus, the efficiency of both *pdxS* and *pdxR* expression negatively correlated with the abundance of B_6. As the *pdxR* gene was altered by the deletion-insertion mutation, the autoregulation of *pdxR* in the *pdxR* mutant could not be faithfully tested by real-time RT-PCR and was analyzed using transcriptional fusions in *B. subtilis* cells as a surrogate host (see below).

Transcription start points of *pdxS* and *pdxR*

Using a RACE approach (Frohman, 1994), two adjacent nucleotides, A and C, located 36 and 37 bp upstream of the *pdxS* initiation codon, respectively, were identified as apparent 5′ ends of the *pdxS* mRNA (Fig. 2)(for convenience, the A36 nucleotide was assigned the +1 position). The sequences TTTTTA and TATCAT with three and one mismatches to the consensus −35 and −10 regions of σ^A-dependent promoters of most bacteria, respectively, and a 17-bp spacer region can be identified upstream of the apparent *pdxS* transcription start points (Fig. 2). In another RACE experiment, a pyrimidine nucleotide C66, located 66 bp upstream of the *pdxR* initiation codon, was identified as the apparent 5′ end of the *pdxR* mRNA. The sequences TTGAAA and TAAGCT with one and three mismatches to the consensus −35 and −10 regions, respectively, and a 17-bp spacer region can be identified upstream of the apparent *pdxR* transcription start point (Fig. 2). Due to the nature of the RACE experiment, the possibility that A65 serves as an alternative transcription start point for *pdxR* cannot be excluded. The +1 positions of the *pdxR* and *pdxS* genes are separated by only 23 bp, and the −10 promoter regions of the two genes overlap (Fig. 2).

PdxR is a DNA-binding protein

A C-terminally His_6-tagged form of PdxR was purified to near homogeneity (see Experimental Procedures). In a gel-shift experiment, PdxR was able to bind a DNA fragment containing the entire *pdxRS* regulatory region with an apparent equilibrium dissociation constant (K_D) of ~100 nM (Fig. 3A). (K_D reflects the PdxR concentration needed to shift 50% of DNA fragments under conditions of vast PdxR excess over DNA). Binding of PdxR appeared to be strongly increased (see below) in the presence of PLP ($K_D \approx 1.6$ nM), but was not affected by other B_6 vitamers, PL, pyridoxine, or pyridoxamine 5′-phosphate (Fig. 3B and data not shown). The apparent ability of PLP to interact with PdxR is consistent with the presence of an aminotransferase-like PLP-binding domain in PdxR and with the *in vivo* observation that PL, a precursor of PLP, affected the ability of PdxR to activate *pdxS* expression. PdxR-DNA complexes with the same mobility were formed in the presence and absence of PLP, indicating that PLP does not affect the stoichiometry of PdxR binding (Figs. 3A and 3B). No binding to the regulatory region of *L. monocytogenes prfA* gene was detected even at 800 nM PdxR, with or without PLP, indicating specificity of PdxR interaction with *pdxRS* (data not shown).

DNase I footprinting experiments showed that PdxR protected 30- to 35-nt regions on the bottom and top DNA strands from positions −72 to −43 or −66 to −32, respectively, with respect to the +1 position of the *pdxS* gene (Figs. 2, 4A, and 4B). The differences in the protected regions on the two strands reflect the known non-uniform and staggered pattern of DNase I cleavage. The protected region overlaps the −35 region of the *pdxS* promoter and lies downstream of the *pdxR* transcription start point. Two nucleotides, at positions −64 and −39 of the bottom strand, became hypersensitive to DNase I digestion, indicating an alteration of DNA conformation (often associated with bending) upon PdxR binding (Fig. 4C); the nucleotide at position −52 of the bottom strand, remained sensitive to DNase I digestion, though the flanking nucleotides became protected. Similar changes were detected for the top strand of *pdxRS* DNA (Fig. 4B).

In the presence of PLP, some repositioning of PdxR apparently occurred, as detected by an additional short area of protection around position −78 on the bottom stand (better seen at positions −77 and −74 of the top strand), the disappearance of the hypersensitive band at position −39 and appearance of a new hypersensitive band at position −51 of the bottom strand (Fig. 4). The observed repositioning of PdxR correlates with the strong positive effects of PLP on PdxR- DNA complex formation observed in the gel-shift experiments. Surprisingly, however, using the footprinting approach, it was found that PdxR affinity for DNA was high even in the absence of PLP and was not increased by more than 2-fold in the presence of PLP (Fig. 4, Table 2).

The important difference between footprinting and gel-shift experiments is that free fragments of digested DNA or protein-DNA complexes are loaded on the gel, respectively. Therefore, the gel-shift experiments can reveal the actual affinity of proteins for DNA under tested conditions only if the protein-DNA complexes remain stable during the electrophoresis. Considering that low K_D values (i.e., high affinities) determined by the two approaches in the presence of PLP were similar (Table 2), it was concluded that a higher K_D value observed in the gel-shift experiments in the absence of PLP reflects not the actual lower affinity of PdxR for DNA, but rather a greater instability of protein-DNA complexes formed by PLP-free PdxR, compared to complexes formed by PLP-bound PdxR, during their loading onto the gel and/or electrophoresis. Despite this rather unusual caveat of the gel-shift experiments, they are fully consistent with the notion, derived from footprinting experiments, that PLP induces a conformational change in PdxR. This conformational change leads to repositioning of PdxR on DNA that correlates with the loss of PdxR's ability to activate *pdxS* transcription *in vivo* and *in vitro* (see the next section) and also causes stabilization of the PdxR-DNA complex in gel-shift experiments. The repositioning of PdxR is rather subtle, does not involve any significant movement along the DNA strand, and probably affects DNA bending.

No effect of PLP or only a small effect of high concentrations of PLP on DNA binding was found previously in gel-shift experiments for *C. glutamicum* and *S. pneumoniae* PdxRs (El Qaidi et al., 2013; Jochmann et al., 2011). However, very low affinities of PdxR for DNA were observed in both cases; no footprinting experiments were reported.

PdxR is a PLP-responsive transcriptional activator of *pdxS in vitro*

In vitro transcription analysis of *pdxS* and *pdxR* expression was performed in run-off experiments using *Escherichia coli* RNA polymerase and a PCR-generated DNA fragment containing the entire *pdxRS* intergenic region. No expression from the *pdxS* promoter was detected in the absence of PdxR (Fig. 5A). Purified PdxR strongly activated transcription from the *pdxS* promoter. Addition of PLP reduced, in a concentration-dependent manner, formation of the *pdxS* transcript (Fig. 5B). PdxR also reduced expression from the *pdxR* promoter either in the presence or absence of PLP, indicating that it serves as a negative autoregulator (Fig. 5)(see also below). Very similar results were obtained with purified *B. subtilis* RNA polymerase (data not shown). Other B_6 vitamers, PL, pyridoxine, pyridoxamine, and pyridoxamine 5′-phosphate, did not affect the activity of PdxR *in vitro* (data not shown). The addition of any one of 20 proteinogenic amino acids or various other

amino group-containing compounds, including non-proteinogenic amino acids, polyamines, and amino sugars, did not affect the ability of PLP to reduce the PdxR-mediated activation of *pdxS* transcription (data not shown). Thus, PdxR appears to be a direct activator of *pdxS* and PLP acts as a direct anti-activator of PdxR and appears to be its only effector.

Transcriptional regulation of the *pdxS* and *pdxR* promoters in *B. subtilis*

A bidirectional *pdxS-lacZ* and *pdxR-gusA* transcriptional fusion containing the entire *L. monocytogenes pdxR-pdxS* intergenic region was constructed and integrated at the *amyE* chromosomal locus of *B. subtilis* as a prototrophic surrogate host; the *B. subtilis* cells do not contain a *pdxR*-like gene upstream of the intrinsic chromosomal *pdxST* genes, and the latter are not subject to B_6-dependent regulation *in vivo* (unpublished results). Though expression of the *pdxR-gusA* fusion was readily detected, only extremely low expression of the *pdxS-lacZ* fusion was observed; no effect of medium composition on the expression of the fusions was observed in the absence of PdxR (Table 3 and data not shown, strain BB2306).

When an intact copy of the *L. monocytogenes pdxR* gene with its own promoter was integrated at the ectopic *sacA* locus of the *B. subtilis* chromosome, much higher expression of the *pdxS-lacZ* fusion was found in glucose-ammonium minimal medium (Table 3, strain BB3311). This confirms that PdxR is necessary and sufficient for activation of the *pdxS* promoter and indicates that none of the seven members of the MocR/GabR subfamily (GabR, YdfD, YisV, YdeF, YdeL, YhdI, YcxD) or any other protein present in *B. subtilis* cells is able to substitute for PdxR. The activity of the *pdxS-lacZ* fusion increased an additional 36-fold under conditions of B_6 limitation created by growing a *pdxT* null mutant, lacking one of the subunits of PLP synthase, in glucose-ammonium medium (Belitsky, 2004b)(Table 3, strain BB3630). Expression from the *pdxS* promoter was also elevated about 10-fold in Casamino acids-containing medium in a wild-type strain (Table 3), and that level of expression was increased a further 2.3- to 2.8-fold, to between 866 and 1,030 units, in rich media, such as L-broth, DS medium, or BHI. Increased expression from the *pdxS* promoter was suppressed when PL was present in the medium (Table 3 and data not shown). These results are fully consistent with the data obtained by real-time RT-PCR in *L. monocytogenes* and the notion that PdxR is a direct positive regulator of *pdxS* transcription, whose activity is decreased by PLP, which is formed intracellularly from exogenous PL. Moreover, the level of *pdxS-lacZ* expression can apparently be used as a reporter for the intracellular concentration of PLP and implies that the latter is reduced in media, containing amino acids and peptides, compared to the PLP concentration in minimal medium. This reduction is likely due to the intensive consumption of PLP during amino acid catabolism.

It should be noted that expression from the *pdxS* promoter is much higher in the presence, than in the absence of PdxR, even in PL-containing medium (Tables 1 and 3). This apparently reflects the ability of PLP-bound PdxR for low-level activation of the *pdxS* promoter and is in fact consistent with the *in vitro* transcription data (Fig. 5B).

Expression of the *pdxR-gusA* fusion was reduced >10-fold in the presence of PdxR, confirming the *in vitro* data showing that PdxR is a negative autoregulator. This negative autoregulation was less pronounced, i.e., expression from the *pdxR* promoter was increased, if the cells were experiencing B_6 limitation in the presence of Casamino acids or due to the

pdxT mutation, but was restored again in the presence of PL (Table 3), similar to the effects observed in *L. monocytogenes* cells (Table 1). It was concluded that after repositioning on DNA, PLP-bound PdxR not only loses the ability to activate *pdxS* but also becomes a stronger repressor of *pdxR*, i.e., PLP acts as anti-activator of PdxR with respect to *pdxS* expression and as a co-repressor of PdxR with respect to negative autoregulation. Both roles of PLP make sense because, in the presence of excess PLP, cells need expression of neither the *de novo* PLP-biosynthetic pathway, nor the activator of this pathway. For unknown reasons, the effect of PLP on the ability of PdxR to autoregulate its expression was observed only *in vivo* (both in *L. monocytogenes* and *B. subtilis*); in the transcription experiments *in vitro* even free PdxR was able to repress the *pdxR* promoter (Fig. 5A).

Mutational analysis of the PdxR-binding site

A perfect dyad-symmetry sequence, AATTGGATG-N10-CATCCAATT, was detected within the PdxR-binding site from positions -69 to -42 (with respect to the +1 position of *pdxS*) (Fig. 2). This dyad-symmetry element is highly conserved in the *pdxRS* intergenic regions of seven other *Listeria* spp. (Fig. 6). Single mutations were introduced into each of the 9-bp arms of this dyad symmetry. The p1 mutation, affecting position −65 of the distal arm, significantly reduced the ability of PdxR to activate *pdxS* expression and almost completely prevented negative autoregulation of *pdxR* (Table 3). A gel- shift experiment revealed that the apparent ability of PdxR to bind the *pdxRSp1* regulatory region was virtually abolished (K_D >1.6 μM) both in the absence and presence of PLP (Figs. 3C and 3D). The strong negative effect of the p1 mutation on PdxR binding was confirmed by a footprinting experiment (Fig. 7A, Table 2). It was concluded that the integrity of the distal arm is essential for efficient binding of PdxR and therefore for both activation of the *pdxS* promoter and repression of the *pdxR* promoter.

The p2 mutation at position −46 in the proximal arm of the PdxR-binding site, which is symmetrical to p1, (Fig. 2) had a very different and unusual phenotype. The mutation only slightly affected high *pdxS-lacZ* expression during B_6 limitation (i.e., in the Casamino acids-containing medium or in the *pdxT* mutant cells), but almost completely prevented the reduction of *pdxS* expression observed in glucose-ammonium medium or when PL was added (Table 3, strains BB3313 and BB3632). In other words, the p2 mutation almost entirely abolished the ability of PL to inactivate PdxR and led to the B_6-independent, constitutive expression of *pdxS*. This constitutive expression was still fully dependent on PdxR availability (Table 3, strains BB3309 and BB3313).

In a gel-shift experiment, the p2 mutation had only a small effect on the formation of unstable complexes of DNA with PdxR in the absence of PLP but completely prevented the stabilization of such complexes in the presence of PLP; in fact, the apparent K_D was reduced 4- fold to ~1.2 μM by PLP addition (Figs. 3E and 3F, Table 2). Importantly, a DNase I protection experiment once again showed that the apparent low affinities of PdxR for p2-containing DNA, both in the absence and presence of PLP, were artifacts of gel-shift experiments, and therefore the p2 mutation did not actually impair PdxR binding (Figs. 7B and 7C, Table 2). However, the inability of PLP to stabilize the *pdxRSp2*-PdxR complex in a gel-shift assay correlated with the inability of PL to reduce *pdxSp2-lacZ* expression *in vivo*

(Table 3) and indicated a different mode of PdxR binding to p2-containing DNA. Indeed, the DNase I protection experiment revealed that, in the presence of the p2 mutation, additional residues of the bottom strand, at positions −41 and −40, became protected when PdxR was added. Importantly, the footprinting pattern was not affected by PLP addition and no protection of the −78 region was observed on either strand (Figs. 4C, 7B, and 7D). With regard to positions −39 and −51 and the −78 region, DNA containing the p2 mutation showed the same pattern of protection by free and PLP-bound PdxR as did wild-type DNA in the presence of free PdxR. In all these cases, the resulting protein-DNA complex contained PdxR in a position and conformation that were appropriate for transcription activation from the *pdxS* promoter. This particular positioning and conformation of PdxR apparently make the complexes with DNA very unstable in a gel-shift experiment.

Considering the unique phenotype of the p2 mutation, another single mutation, p4, was independently introduced at position −47 in the proximal arm of the PdxR-binding site, adjacent to the position of the p2 mutation (Fig. 2). The two single mutations had almost identical phenotypes *in vivo* (Table 3). The effects of the p4 mutation on PdxR binding were also very similar to the effects of the p2 mutation (Figs. 3G, 3H, 7C, and 7D), consistent with their similar phenotypes *in vivo*.

To summarize, in the absence of PLP, PdxR molecules interact with the wild-type DNA in a way that allows RNA polymerase to initiate *pdxS* transcription (Fig. 8). PdxR molecules, which interact with the p2- (or p4)-containing DNA, appear to be locked in a similar position; this positioning of PdxR on the mutant DNA does not change even in the presence of PLP. The constitutive phenotype of the p2- or p4-containing strains indicates that the PLP-induced alteration of PdxR conformation does not prevent transcription activation per se, but rather leads to the altered positioning of PdxR on the wild-type DNA, which is not compatible with the activation of the *pdxS* promoter (Fig. 8). As far as I know, the p2 and p4 mutations are the first examples of mutations in the activator-binding site, which prevent anti-activation by a low-molecular weight effector and allow constitutive expression of the target genes.

Interestingly, the ability of the p2 mutation to confer constitutive expression from the *pdxS* promoter was abolished in the presence of the p1 mutation (i.e., low expression from the *pdxSp1/p2* promoter was further strongly reduced in the presence of PL) and is, thus, dependent on the integrity of the distal arm of the PdxR-binding site (Table 3). Incidentally, the combination of the p1 and p2 mutations restored the perfect inverted repeat within the PdxR-binding region (Fig. 2). Because the activity of the p1/p2 promoter remained low, it is the exact sequence of the element and not the dyad symmetry per se that is important for productive interaction with PdxR.

In accord with the observation that PLP-bound PdxR binds to the *pdxRSp2* and *pdxRSp4* fragments in a manner similar to that of binding of free PdxR to wild-type DNA, both the p2 and p4 mutations abolished the ability of PLP-bound PdxR to repress the *pdxR* promoter (Table 3). Somewhat paradoxically, some ability to repress the *pdxR* promoter containing the p2 or p4 mutation was regained under conditions of B_6 limitation, indicating that PdxR was still able to interact with PLP and the resulting change in PdxR conformation (or an

undetected change in PdxR positioning) affected repression. Additionally, all three mutations, p1, p2, and p4, led to a 2.4- to 3.8-fold increase in the level of *pdxR* expression in the absence of PdxR, apparently affecting the intrinsic ability of RNA polymerase to initiate transcription from the *pdxR* promoter (Table 3); the reason for this effect remains unknown.

One more single mutation, p3, was introduced at the conserved −76 position in the region of the PdxR-binding site that is specifically protected on wild-type DNA only in the presence of PLP (Fig. 2). This mutation did not affect the ability of PdxR to regulate expression of the *pdxS- lacZ* or *pdxR-gusA* fusions; expression of the latter was reduced ~3-fold under all conditions apparently due to the location of the p3 mutation within the *pdxR* ribosomal binding site (data not shown).

DISCUSSION

It has been shown that *L. monocytogenes* PdxR, a member of the MocR/GabR subfamily of the GntR family of transcriptional regulators, is an activator of the *pdxST* genes responsible for the *de novo* biosynthesis of PLP. A similar function in other bacteria was previously demonstrated or suggested for three more members of this subfamily, also called PdxR (El Qaidi et al., 2013; Jochmann et al., 2011; Magarvey et al., 2001). It was shown here that *L. monocytogenes* PdxR responds directly to PLP, which serves as the only effector of the protein. The binding of PLP to *L. monocytogenes* PdxR is sufficient to cause a likely conformational change and repositioning of the protein on DNA, which affect the ability of the protein both to activate and repress (Fig. 8). As far as I know, the requirement for a B_6 vitamer for modulation of activity of a transcriptional regulator is unprecedented among bacteria.

From the mutational analysis, it was concluded that the distal arm of the dyad-symmetry element found within the *L. monocytogenes* PdxR-binding site is essential for efficient binding of the protein and the proximal arm appears to be required for such an interaction with PdxR that allows the protein to respond to the presence of PLP. Based on their similarity to aminotransferases, the MocR/GabR subfamily members were hypothesized to form head-to-tail dimers and, therefore, bind to direct DNA repeats (Rigali et al., 2002). The head-to-tail dimer configuration of such proteins was confirmed for *B. subtilis* GabR (Edayathumangalam et al., 2013) and is likely to be true for PdxR. In this respect it is worth noting that a 9-bp sequence, AATTGGcTG, directly repeating the distal arm of the dyad-symmetry element (with one mismatch indicated in lowercase), can be found 15 bp downstream of the first repeat and still within the PdxR-binding site; this sequence overlaps the proximal arm of the dyad symmetry by 4 bp (Fig. 2 and 6). The role of this direct repeat in PdxR binding was not analyzed. Sites similar to these inversely and directly repeated 9-bp sequences were not detected in the binding regions of other characterized proteins of the MocR/GabR subfamily (Belitsky, 2004a; El Qaidi et al., 2013; Jochmann et al., 2011; Wiethaus et al., 2008). Similarly to PdxR, GabR binds to an extended region of DNA, which overlaps the −35 promoter region of the target gene (Belitsky, 2004a).

The signaling role of PLP with respect to PdxR activity is in drastic contrast to the mode of regulation of the activity of GabR, the best characterized member of the MocR/GabR

subfamily. In addition to PLP, GabR requires another low-molecular weight compound, GABA, for modulation of its activity (Belitsky, 2004a). Moreover, GabR binds PLP through a covalent Schiff-base linkage, in which the ε-amino group of Lys312 forms an imine bond with the aldehyde group of PLP (Edayathumangalam et al., 2013). PLP appears to act as a tightly bound intrinsic component of GabR, akin to the cofactor role, which PLP plays in aminotransferases and other PLP-dependent enzymes (Eliot and Kirsch, 2004; Schneider et al., 2000). Furthermore, the data strongly indicate that to achieve its active state, GabR performs a transimination reaction between PLP and GABA, in which the imine linkage between Lys312 and PLP is broken and a new imine bond is formed between PLP, which remains bound to GabR non-covalently, and GABA (Belitsky, 2004a). A similar cofactor-like role of PLP is possible for other members of the MocR/GabR-like proteins. For instance, to activate TauR, PLP may interact covalently with taurine, a likely TauR effector (Wiethaus et al., 2008). For such proteins that are not involved in regulation of PLP metabolism, it is hypothesized that the apparent conformational change, which causes their activation, is associated not with PLP binding per se but with binding of another low-molecular compound, such as GABA or taurine, and with the subsequent modification of both this compound and PLP.

It is likely that *L. monocytogenes* PdxR and other similar proteins, which regulate genes involved in PLP biosynthesis, form a special group within the MocR/GabR subfamily. Members of the PdxR group, like *L. monocytogenes* PdxR and in contrast to the proteins of the GabR-like group, are hypothesized to respond only to PLP and not to employ additional low-molecular weight compounds for modulation of their activity. In accord with this suggestion, some PdxR proteins do not have the conserved lysine residue that forms a covalent Schiff-base bond with PLP in GabR and aminotransferases (Bramucci et al., 2011; El Qaidi et al., 2013; Jochmann et al., 2011). Assuming that different PdxR proteins interact with PLP in a similar way, it is suggested that for all of them PLP acts as a simple, low-molecular weight effector that causes a conformational change upon non-covalent binding. The aminotransferase-like domain of such proteins, in contrast to GabR-like proteins, is likely to serve only as a PLP-binding domain and does not perform any chemical modification of PLP.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture media

All *L. monocytogenes* and *B. subtilis* strains constructed in this study were derivatives of strains EGD-e (Glaser et al., 2001) or SMY (Zeigler *et al.*, 2008), respectively, and are described in Table S1 and in the text. *E. coli* strain JM107 (Yanisch-Perron *et al.*, 1985) or its *pcnB80* derivative (Lopilato *et al.*, 1986) was used for isolation of plasmids. Cells were grown in Brain Heart Infusion medium (BHI) or, for experiment in Fig. 1, in defined medium (LDM), for *L. monocytogenes*; in DS nutrient broth medium or TSS minimal medium with 0.5% glucose as the carbon source and 0.2% NH_4Cl as nitrogen source (Fouet and Sonenshein, 1990), for *B. subtilis*; and in L broth (Miller, 1972), for *E. coli*. The same media with addition of agar were used for growth of bacteria on plates. LDM was based on published recipes (Premaratne *et al.*, 1991; Welshimer, 1963) and on the composition of

TSS (for K_2HPO_4, $MgSO_4$ x7H_2O, and iron citrate) and DS [for $Ca(NO_3)_2$] media and had the following 18 components: MOPS-HCl (pH 7.5), 50 mM; K_2HPO_4, 2 mM; $MgSO_4$ x7H_2O, 0.81 mM (0.02%); a mixture of $FeCl_3$ and Na_3-citrate x2H_2O, 0.04 g l^{-1} each (0.004%); $Ca(NO_3)_2$, 0.5 mM; glucose, 27.8 mM (0.5%); NH_4Cl, 37.4 mM (0.2%); seven amino acids (Leu, Ile, Val, Cys, Met, Arg-HCl, His-HCl) at 100 μg ml^{-1}, each; and four vitamins (in μg ml^{-1}): biotin, 0.5; riboflavin, 0.5; thiamine-HCl, 1; and lipoic acid, 0.005.

The following antibiotics were used when appropriate (in μg ml^{-1}): ampicillin, 50–100; chloramphenicol, 20; tetracycline, 10; spectinomycin, 50; and nalidixic acid, 50, for *E. coli* strains; chloramphenicol, 5 or 10; and spectinomycin, 100, for *L. monocytogenes* strains; and chloramphenicol, 2.5; neomycin, 2.5; tetracycline, 15; and spectinomycin, 50, for *B. subtilis* strains.

General molecular genetic methods

Methods for common DNA manipulations, *E. coli* electroporation, isolation of *B. subtilis* chromosomal DNA, transformation of *B. subtilis* cells, and sequence analysis were as previously described (Belitsky and Sonenshein, 1998; Belitsky and Sonenshein, 2008). Chromosomal DNA of *L. monocytogenes* was isolated as for *B. subtilis*, but the cells were disrupted using 0.1 mm silica beads and a Mini-BeadBeater (Biospec Products) for two 30-sec cycles at the maximal setting. All oligonucleotides used in this work are described in Table S2. Chromosomal DNA of *L. monocytogenes* or plasmids constructed in this work was used as template for PCR. All cloned PCR-generated fragments were verified by sequencing at the Tufts University Core Facility.

Construction of *pdxST* and *pdxR* null mutants

A Δ*pdxST::spc* fragment, in which 97% (1.42 kb) of the *pdxS* and *pdxT* open reading frames was replaced by a 1.1-kb spectinomycin-resistance cassette, was constructed by a two-step overlapping PCR method (Wach, 1996). First, 5′- and 3′-flanking fragments were generated by using oBB130 and oBB131 or oBB132 and oBB133 as primers, respectively. Primers oBB131 and oBB132 contain sequences that match the ends of the *spc* gene from pJL73 (LeDeaux and Grossman, 1995). In the second step, the two PCR products were used as long primers for splicing PCR with pJL73 as template. The spliced product was further amplified using oBB130 and oBB133. The final PCR product was digested with SpeI (a site within *pdxR*) and SacI and cloned in a temperature-sensitive conjugative plasmid pCON-1 (Smith and Youngman, 1992), digested with XbaI and SacI. The orientation of the *spc* cassette in the resulting plasmid, pBB1212, was the same as the orientation of the *pdxST* genes.

To make a Δ*pdxR::spc* construct, we first deleted a HindIII site from the polylinker of pCON-1 by digesting the plasmid with HindIII, blunt ending the resulting fragment with the DNA polymerase I Klenow fragment, and self-ligating the fragment to obtain pBB1315. The fragment containing the entire *pdxR* gene was synthesized by PCR using oBB131 and oBB168 as primers. The PCR fragment was digested with PstI and XbaI, cloned in pLG103 (Belitsky and Sonenshein, 2002), and then recloned as an EcoRI-XbaI fragment in pBB1315 to generate pBB1323. To construct pBB1324, a 0.28-kb internal HindIII-SpeI fragment of

pdxR was replaced with a 1.1-kb *spc* cassette (in the orientation opposite to *pdxR*) that was cut from pJL73 with the same two enzymes.

pBB1212 (*cat* Δ*pdxST::spc*) and pBB1324 (*cat* Δ*pdxR::spc*) were introduced into an *E. coli* conjugation donor strain, DW1030/RK231.7 (Tet^r) (Thompson and Malamy, 1990) (plasmid RK231.7 is a Kan^s derivative of RK231 obtained by A. Baughn). The two resulting strains were conjugated at 30°C with *L. monocytogenes* strain EGD-e, as a plasmid recipient, on BHI agar plates, selecting for chloramphenicol and spectinomycin resistance and using nalidixic acid to counterselect the donor strains. The transconjugants were passaged several times in liquid BHI under selective conditions at 41°C to allow integration of pCON-1 derivatives into the chromosome, and the resulting integrants were passaged several times at non-selective conditions (i.e., in the absence of chloramphenicol but in the presence of spectinomycin and 10 μM of PL) at 30°C to allow for plasmid excision and allelic replacement. Spectinomycin- resistant colonies were isolated at 41°C in the presence of PL and screened for the loss of chloramphenicol-resistance. The replacement of the chromosomal genes by the deletion- insertion-containing alleles in strains BLM1 (*pdxST*) and BLM7 (*pdxR*) was searched for by comparing the sizes of PCR fragments from the wild-type and mutant *pdxRST* chromosomal loci; the loss of the plasmid was confirmed in separate PCR reactions using vector-specific primers.

Complementation of the *pdxST* and *pdxR* mutations

A PCR product containing the entire *pdxST* genes was synthesized by PCR using oBB164 and oBB133 as primers and cut with BclI and HindIII. The resulting 1.88-kb fragment was cloned between the BamHI and HindIII sites of a low copy-number vector pHP13 (Haima *et al.*, 1987). A 1.59-kb PCR product containing the entire *pdxR* gene was synthesized by PCR using oBB131 and oBB681 as primers, cut with EcoRI and BamHI, and cloned in pHP13. pHP13 and its derivatives were introduced into *L. monocytogenes* cells by electroporation (Monk *et al.*, 2008).

Isolation of RNA

Samples of 1 to 3 ml of *L. monocytogenes* cells growing exponentially in BHI with or without 100 μM PL were collected at the $OD_{600}\approx0.6$ by mixing with an equal volume of an 1:1 (vol/vol) mixture of ethanol/acetone (−20°C) and kept at −80°C until further use. The cells were pelleted, washed with 1 ml of 10 mM tris-HCl (pH 8.0) - 1 mM EDTA buffer, and resuspended in 0.8 ml of the TRI Reagent. The cells were disrupted using a beadbeater as described above, and RNA was purified using the Direct-zol RNA kit (Zymo Research). For real-time RT-PCR experiments, purified RNA (~10 μg) was further treated with Turbo DNA- free DNase I (Ambion) according to the manufacturer's instructions. RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Real-time RT-PCR

cDNA was synthesized starting from 1 μg of RNA using random hexamer primers and SuperScript II reverse transcriptase (Invitrogen) per the manufacturer's instructions. The SYBR Green I dye-based PCR reactions were performed according to the manufacturer's instructions using a LightCycler 480 (Roche Applied Science). The reactions were done in a

total volume of 20 μl and contained 4 μl of 6- to 10-fold diluted cDNA or control RNA samples. Primer pairs oBB657-oBB658 or oBB660-oBB661 were used for detection of *pdxS* or *pdxR* transcripts, respectively. The *rpoB* transcript, detected with primers oBB667 and oBB668, was used for normalization. All primers were designed using the PrimerQuest tool (Integrated DNA Technologies). Serial dilutions of *L. monocytogenes* chromosomal DNA (from 3.2 to 10,000 pg per reaction) were used to create calibration curves for each transcript.

Determination of transcription start points using rapid amplification of cDNA ends (RACE)

cDNA samples were synthesized from 1 μg of RNA in 20-μl reactions using 2 pmol of *pdxS*- or *pdxR*-specific primers, oBB154 or oBB130, respectively, and SuperScript II reverse transcriptase (Invitrogen) per the manufacturer's instructions. 3′ poly(A) tails were added using terminal transferase (New England Biolabs), and cDNA was purified using the PCR Purification kit (Qiagen) and eluted in a 50-μl volume. First-round PCR products were generated using 2 μl of purified cDNA as template, universal anchor primer oKZ69, and primers oBB658 or oBB660 specific for the *pdxS* and *pdxR* genes, respectively. The *pdxS*-specifc product was sequenced directly using primer oBB659. The *pdxR*-specific product (0.05 μl) was PCR-amplified again using the universal amplification primer oKZ70 and the *pdxR*-specific primer oBB164 and then sequenced using oBB164. The nucleotide(s) at the junction between the gene-specific sequence and the stretch of A nucleotides, generated from sequencing the poly(A) tail, was assumed to be an apparent 5′ end of mRNA (Frohman, 1994).

Overexpression and purification of PdxR

A PCR product containing the entire *pdxR* gene with six histidine codons at the 3′ end was synthesized by PCR using oBB162 and oBB163 as primers and cloned downstream of the *ara* promoter between the EcoRI and SphI sites of the expression vector pBAD18 (Guzman *et al.*, 1995).

The resulting plasmid, pBB1266, was introduced into *E. coli* strain LMG194 (Δ*ara714*) (Guzman et al., 1995), and expression of PdxR was induced in L broth (at $OD_{600}\approx0.3$) by adding L-arabinose to 0.2% and incubation of the cells for an additional 4 hours. PdxR-His_6 was purified to almost homogeneity as described previously for GabR (Belitsky, 2004a). Elution from the Ni^{2+}-affinity column (His·Bind resin; Novagen) was with a buffer containing 385 mM imidazole.

In vitro transcription

Assays with *E. coli* σ^A-containing RNA polymerase holoenzyme (Epicentre) or a mixture of purified *B. subtilis* RNA polymerase and σ^A factor were performed *in vitro* as described (Belitsky, 2004a; Belitsky and Sonenshein, 2011b). Various amounts of PdxR with or without 100 μM PLP were added to 10-μl reactions as specified in figure legends. A 281-bp *pdxRS* promoter fragment was synthesized, using pBB1279 as a PCR template and oBB164 and a vector-specific oligonucletide oBB102 as primers, and used as template for *in vitro* transcription.

Construction of *pdxS* and *pdxR* transcriptional fusions

The 0.23-kb fragment, containing the entire 125-bp *pdxRS* intergenic region, was synthesized by PCR using primers oBB164 and oBB131, digested with BclI and PstI, and cloned between the BglII and SbfI sites of the integrative plasmid pLG103 (Belitsky and Sonenshein, 2002). The resulting plasmid, pBB1279, carries the bidirectional *pdxSp*$^+$-*lacZ* and *pdxRp*$^+$-*gusA* fusion.

Mutations in the *pdxRS* regulatory region were introduced by two-step overlapping PCR. In the first step, PCR products containing the 5′ part of the *pdxRS* regulatory region were synthesized by using oBB164 and mutagenic oligonucleotides, specified in Table S2, as primers. In a similar manner, PCR products containing the 3′ part of the *pdxRS* regulatory region were synthesized by using mutagenic oligonucleotides, specified in Table S2, and a vector-specific oligonucleotide oBB253 as primers. In the case of the double mutation p1/p2, plasmid pBB1648 (*pdxRSp2*) was used as template for PCR. The appropriate pairs of PCR products were used in a second, splicing step of PCR mutagenesis as overlapping templates to generate modified fragments containing the entire *pdxRS* regulatory region; oligonucleotides oBB164 and oBB253 served as primers. The spliced PCR products were cloned in pLG103, as described above, to construct pBB1648 (*pdxRSp1*), pBB1649 (*pdxRSp2*), pBB1650 (*pdxRSp3*), pBB1781 (*pdxRSp4*), and pBB1782 (*pdxRSp1/p2*).

B. subtilis strains carrying various *lacZ* and *gusA* fusions at the *amyE* locus were isolated after transforming strain BB1888 (*lacA*) or BB2511 (*amyE::spc lacA*) with the appropriate plasmids, by selecting for resistance to chloramphenicol conferred by the plasmids. The transformants were screened for the Amy$^-$ phenotype (Belitsky and Sonenshein, 1998) or loss of the spectinomycin-resistance phenotype, which indicated a double crossover, homologous recombination event.

Integration of *pdxR* into the *B. subtilis* chromosome

The same *pdxR*-containing fragment that was used to generate pBB1323 was cloned between the XbaI and EcoRI sites of the integrative plasmid pSac-Kan (Middleton and Hofmeister, 2004) in strain JM107 *pcnB80* as a host. The resulting plasmid, pBB1647, was introduced into *B. subtilis* strain BB3304 (*sacA::cat*) to create strain BB3305 [*sacA::(neo pdxR*$^+$)].

Labeling of DNA fragments

The 338-bp PCR products containing the entire *pdxRS* intergenic region were synthesized using vector-specific oligonucleotides oBB252 and oBB102 as primers and pBB1279 or its mutant derivatives as template. One of the primers for each PCR reaction was labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. The labeled PCR products were purified on an 8% nondenaturing polyacrylamide gel or used without purification. oBB252 and oBB102 start 62 bp upstream or 47 bp downstream, respectively, of the BclI and SbfI sites that were used for cloning.

Gel shift assays and DNase I protection experiments

Incubation of CodY with the ^{32}P-labeled promoter fragments was performed in a binding buffer containing 20 mM Tris-Cl (pH 8.0) - 50 mM KCl - 2 mM $MgCl_2$ - 5% glycerol - 0.5 mM EDTA - 1 mM DTT - 0.05% Nonidet P-40 - 25 μg ml^{-1} sonicated salmon sperm DNA. Samples (11 μl) containing varying amounts of PdxR and less than 1 fmole of DNA were incubated for 16 min at room temperature and separated on 8% nondenaturing 50 mM Tris - 384 mM glycine - 1 mM EDTA polyacrylamide gels in 35 mM Hepes - 43 mM imidazole buffer. In some experiments, B_6 vitamers were present in the incubation mixture.

For DNase I protection experiments, samples containing 20–40 fmoles of labeled DNA were incubated with PdxR as described above. One μl of the binding buffer containing 0.1–0.2 U RQ1 DNase I (Promega), 10 mM $MgCl_2$ and 20 mM $CaCl_2$ was then added, followed by addition, after 1 min, of 4 μl of 20 mM EDTA-95% formamide dye solution and subsequent heating of the samples at 80°C for 5 min. The samples were loaded without further purification on 7 M urea - 6% polyacrylamide DNA sequencing gels. The G+A sequencing ladder, generated according to a published procedure by boiling the appropriate samples of labeled DNA for 20 min (Liu and Hong, 1998), served to locate precisely the protected region.

The gels were dried, and the radioactive bands were detected and quantified using storage screens, an Applied Biosystems PhosphorImager, and ImageQuant software (GE Healthcare).

Enzyme assays

β-Galactosidase and β-glucuronidase specific activities were determined as described previously (Belitsky *et al.*, 1995).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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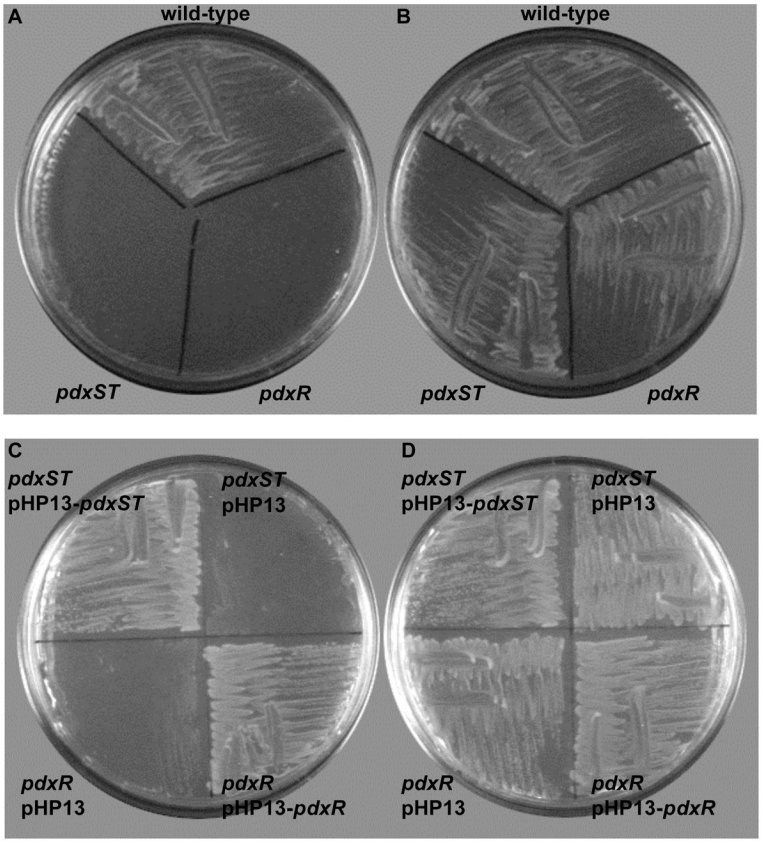

Fig. 1. Growth of *L. monocytogenes* wild-type and mutant strains

Cells were grown on LDM agar plates without (A, C) or with (B, D) 10 μM PL.

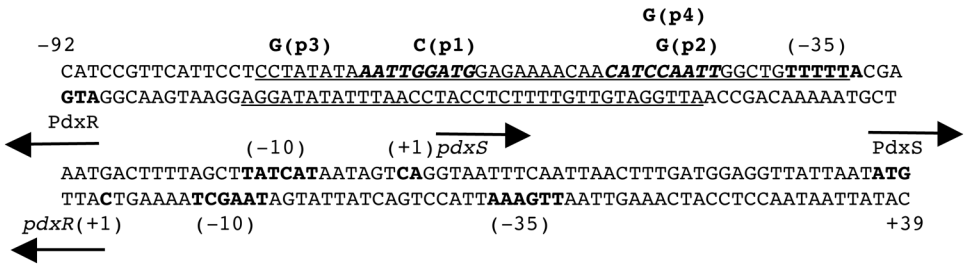

Fig. 2. The double-stranded sequence of the *pdxRS* regulatory region

The likely initiation codons, −10 and −35 promoter regions, and the +1 gene positions are in boldface. The directions of transcription and translation are indicated by the arrows. The sequences protected by PdxR on the top and bottom strands in DNase I footprinting experiments are underlined. The dyad-symmetry sequence is in boldface and italicized. The coordinates of the 5′ and 3′ ends of the sequence with respect to the +1 position of the *pdxS* gene and the locations of the p1 to p4 mutations are indicated.

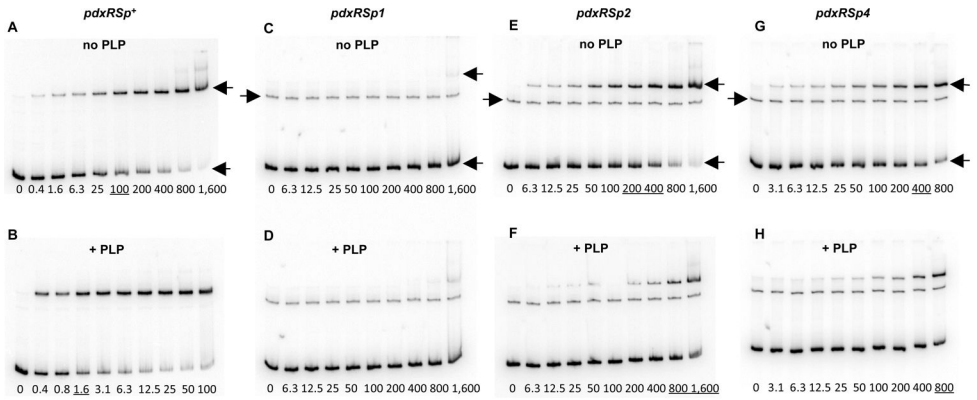

Fig. 3. Binding of PdxR to the *pdxRS* regulatory region as detected by a gel-shift assay

Radioactively labeled *pdxRSp*$^+$ (A, B), *pdxRSp1* (C, D), *pdxRSp2* (E, F), and *pdxRSp4* (G, H) DNA fragments were incubated with increasing amounts of purified PdxR without (A, C, E, G) or with (B, D, F, H) 100 μM PLP. PdxR monomer concentrations used (nM) are indicated below each lane, and the concentrations, which are needed to shift ~50% of DNA fragments, are underlined. The left-pointing arrows indicate the bands corresponding to unbound DNA and the PdxR-DNA complex, and the right-pointing arrows indicate the bands of unspecific DNA present in some experiments. Each gel-shift assay was repeated at least two times.

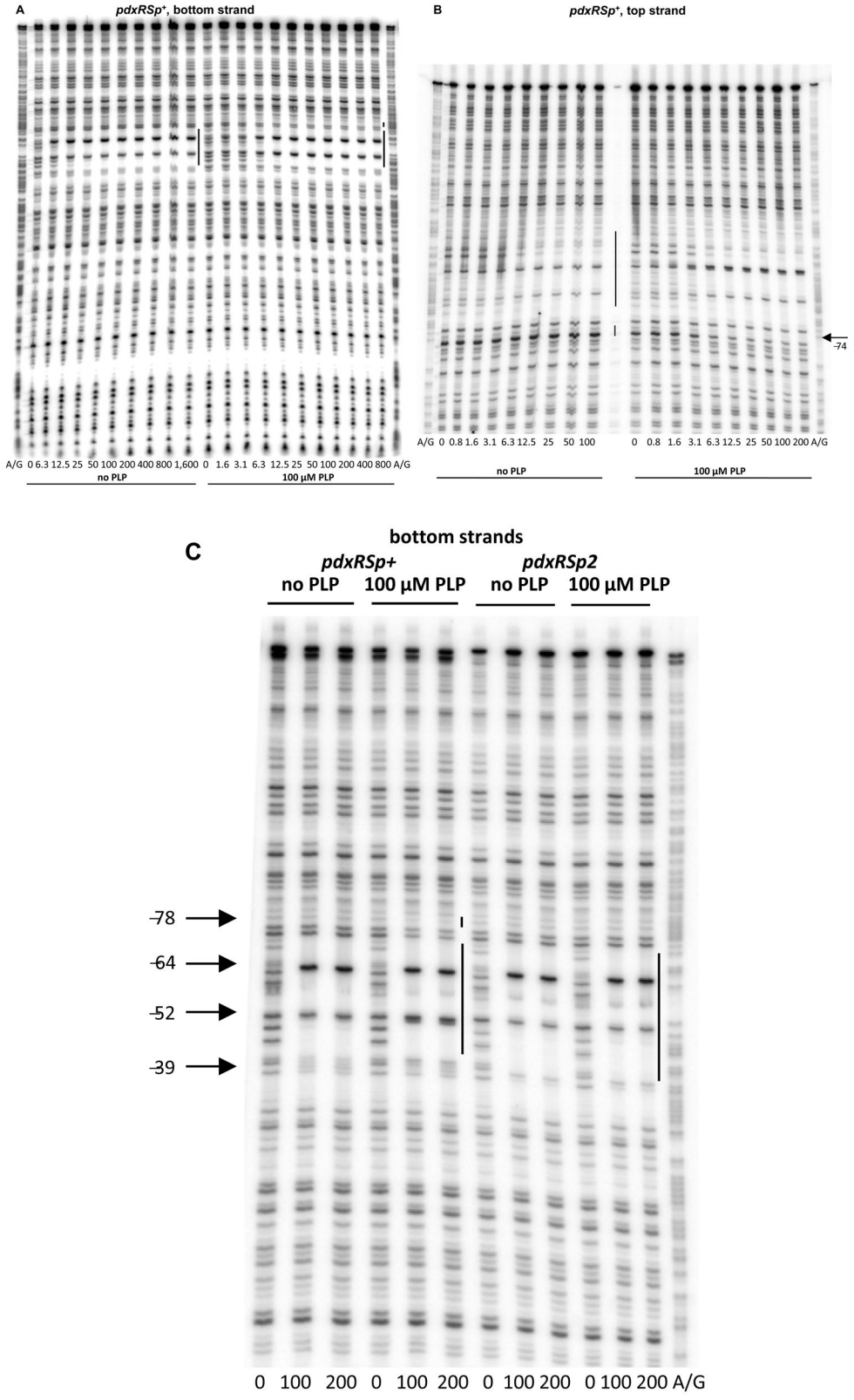

Fig. 4. DNase I footprinting analysis of PdxR binding to the *pdxRS* regulatory region

pdxRSp^{+} (A, B, C) or *pdxRSp2* (C) DNA fragments, radioactively labeled on the bottom (A, C) or top (B) strand, were incubated with increasing concentrations of purified PdxR and in the absence or presence of 100 μM PLP. PdxR monomer concentrations used (nM) are indicated below each lane. The corresponding A + G sequencing ladders are shown in the left and right lanes. The protected areas are indicated by the vertical lines. The positions of some bands (with respect to the +1 position of *pdxS*) are shown by arrows.

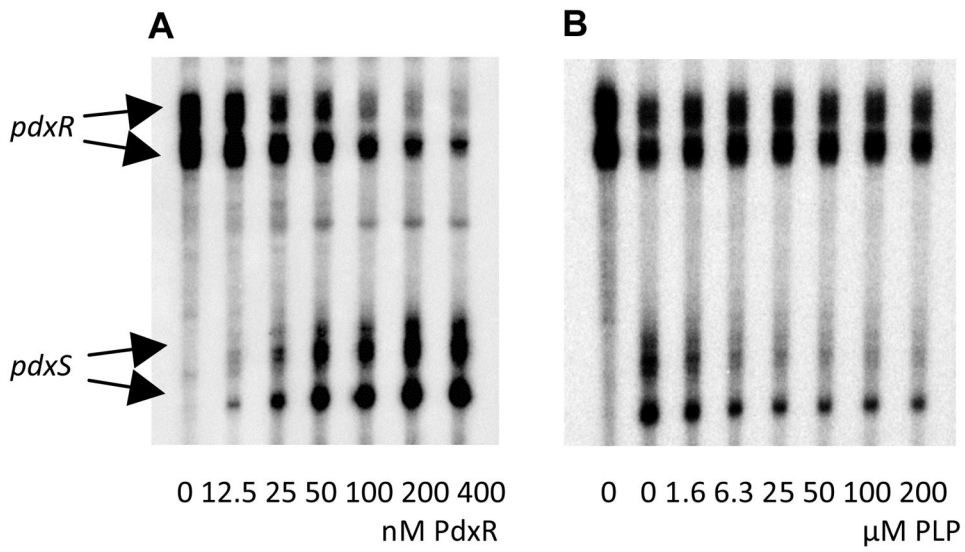

Fig. 5. *In vitro* transcription from the *pdxRS* regulatory region

The *pdxRSp*$^{+}$ PCR fragment was transcribed at 37°C for 20 min using *E. coli* RNA polymerase. (A) The reactions contained increasing amounts of PdxR and no PLP. PdxR monomer concentrations used (nM) are indicated below each lane. (B) The reactions contained no PdxR (first lane) or 200 nM PdxR and increasing concentrations of PLP as indicated below each lane.

Multiple transcripts formed from the *pdxR* and *pdxS* promoters apparently reflect RNA polymerase pausing at sites downstream from the corresponding transcription start points. Identity of the transcripts was proved by using *pdxRS* templates truncated at either the *pdxR* or *pdxS* end, which led to the formation of shorter corresponding transcripts (data not shown).

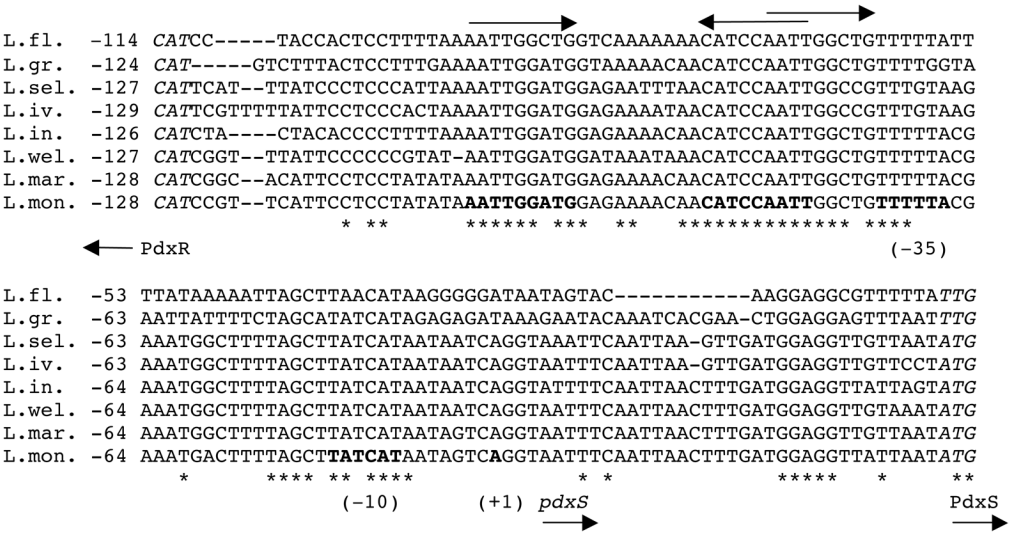

Fig. 6. Multiple alignment of the *pdxRS* intergenic regions from different *Listeria* species

Nucleotide sequences of the entire *pdxRS* intergenic regions from eight *Listeria* strains were aligned using the CLUSTAL Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences are flanked by the initiation codons (italicized) of the *pdxR* and *pdxS* genes at the 5′ and 3′ ends, respectively. The likely −10 and −35 promoter regions and the +1 position of the *L. monocytogenes pdxS* are in boldface. The dyad-symmetry sequence (in boldface) and the direct repeat within the PdxR-binding site are shown by arrows. Identical nucleotides in all genomes are indicated by asterisks. The numbering is with respect to the initiation codons of the *pdxS* genes. The accession numbers for the sequences analyzed are: *L. fleischmannii* (L.fl.) - NZ_ALWW01000008.1, *L. grayi* (L.gr.) - NZ_GL538353, *L. seeligeri* (L.sel.) - ADXK01001019, *L. ivanovii* (L.iv.) - ADXI01001082, *L. innocua* (L.in.) - AL596171, *L. welshimeri* (L.wel.) - NC_008555.1, *L. marthii* (L.mar.) - ADXF01000815.1, *L. monocytogenes* (L.mon.) - AL591982.1.

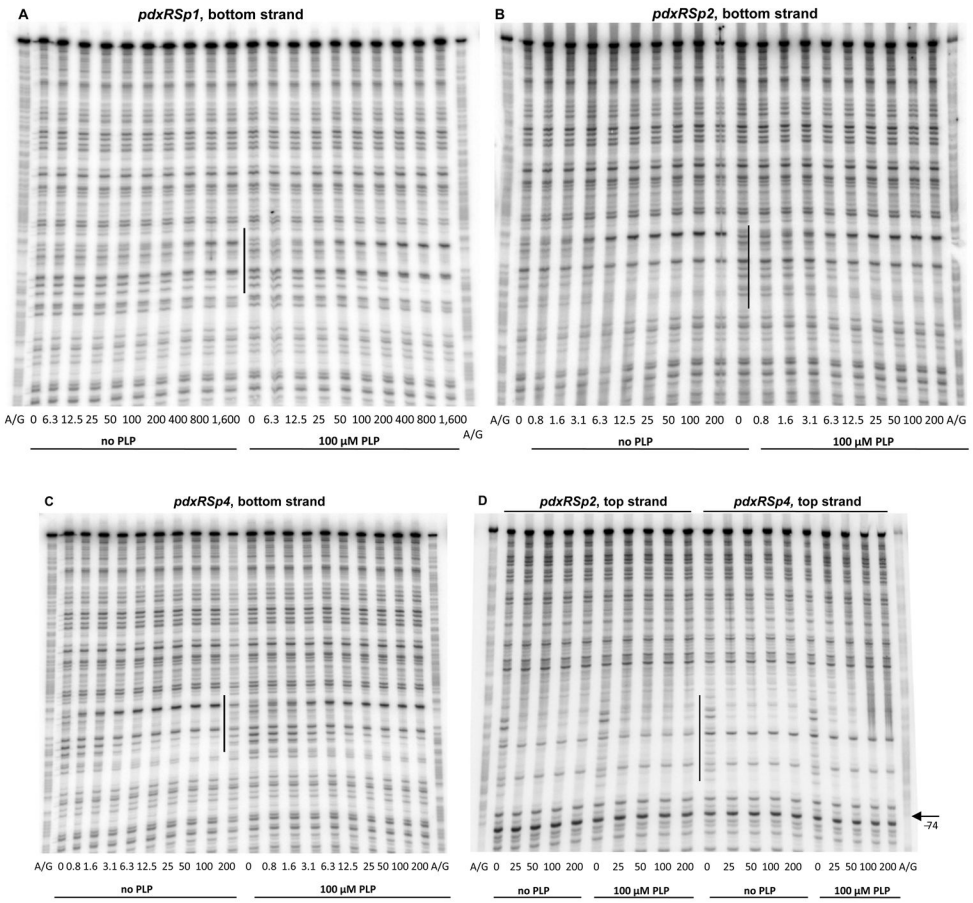

Fig. 7. DNase I footprinting analysis of PdxR binding to the mutant *pdxRS* regulatory regions

pdxRSp1 (A), *pdxRSp2* (B, D), and *pdxRSp4* (C, D) DNA fragments, radioactively labeled on the bottom (A, B, C) or top (D) strand, were incubated with increasing concentrations of purified PdxR and in the absence or presence of 100 μM PLP. PdxR monomer concentrations used (nM) are indicated below each lane. The corresponding A + G sequencing ladder is shown in the left and right lanes. The protected areas are indicated by vertical lines.

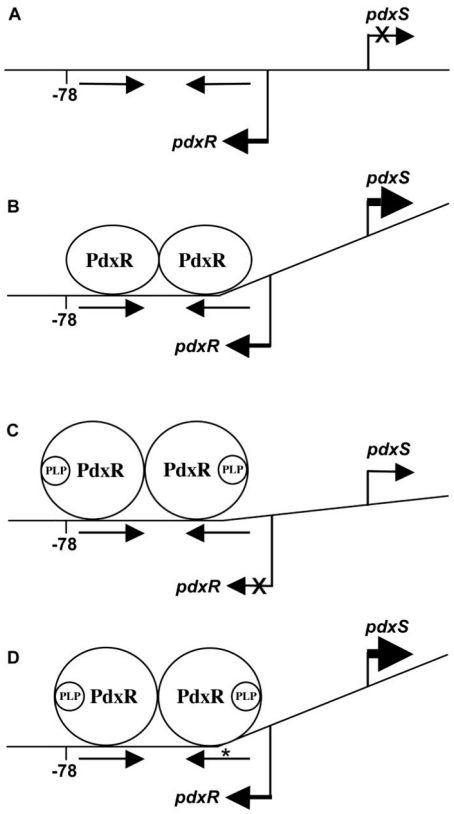

Fig. 8. A model of PdxR interaction with the wild-type and mutant *pdxRS* regulatory regions

(A) wild-type DNA, no PdxR; (B) wild-type DNA plus PdxR; (C) wild-type DNA plus PdxR and PLP; (D) p2- or p4-containing DNA plus PdxR and PLP. The model (not to scale) assumes that a PdxR dimer binds to the dyad-symmetry element (indicated by straight arrows) and includes hypothetical PLP-dependent bending induced by PdxR. Bent arrows show the start points and directions of transcription. An asterisk indicates the location of the adjacent p2 and p4 mutations. The position -78 (with respect to *pdxS*), protected by the PLP-bound form of PdxR on wild-type DNA, is shown.

Table 1

Expression of the *pdxS* and *pdxR* genes in *L. monocytogenes*

Genotype	Additions to the medium	*pdxS* expression		*pdxR* expression	
		copies	%	copies	%
wild-type	none	1.45 ± 0.32	100.0	0.141 ± 0.039	100.0
	PL	0.18 ± 0.051	12.4	0.076 ± 0.014	53.5
pdxR	none	0.013 ± 0.002	0.92	NA[a]	NA
	PL	0.014 ± 0.003	0.96	NA	NA
pdxST	none	NA	NA	0.43 ± 0.060	305.0
	PL	NA	NA	0.078 ± 0.018	55.3

Cells were grown in BHI with or without 100 μM PL. In the absence of PL, the mutant strains grew as fast as the wild-type strain or more slowly, depending on the batch of the medium. The data are presented as the number of copies of the *pdxS* or *pdxR* transcript per a copy of the *rpoB* transcript. All values are averages plus/minus standard deviation from at least two experiments.

[a]NA - not applicable. The transcript abundance could not be reliably determined due to the presence of a deletion-insertion in the corresponding gene.

Table 2

Summary of promoter activities and PdxR affinity for different *pdxRS* alleles

Genotype	PL/PLP[a]	β-gal (*pdxS-lacZ*)	β-gluc (*pdxR-gusA*)	PdxR K_D	
				gel-shift	footprinting
pdxRSp$^+$	−	369	1.23	100	3.1–6.3
	+	27.1	0.20	1.6	3.1–6.3
pdxRSp1	−	52.7	19.5	>1,600	~1,600
	+	12.4	14.0	>1,600	~400
pdxRSp2	−	1,230	3.19	300	~3.1
	+	805	12.2	1,200	~6.3
pdxRSp4	−	1,100	2.50	400	~3.1
	+	605	9.16	800	~3.1

Specific activities of β-galactosidase and β-glucuronidase in TSS medium, enriched with Casamino Acids, were taken from Table 3 and are expressed in Miller units. The values of apparent dissociation constants (K_D) were determined from Fig. 3, 4, and 7 and are expressed as nanomolar concentration of monomeric PdxR. For footprinting experiments, K_D is approximated as the PdxR concentration needed to decrease the intensity of protected DNA bands two-fold under conditions of vast PdxR excess over DNA.

[a] indicates the presence of PL in the growth medium or PLP in the DNA-binding reactions.

Table 3

Expression of the bidirectional *pdxRS* fusion in *B. subtilis* cells

Strain	Genotype	*pdxRS* promoter region	Additions to the medium	β-galactosidase (*pdxS-lacZ*)	β-glucuronidase (*pdxR-gusA*)
BB2306	wild-type	p^+	Cas. acids	0.41 ± 0.026	7.92 ± 0.16
BB3311	*pdxR*$^+$		none	35.2 ± 0.35	0.62 ± 0.064
			PL	24.2 ± 1.84	0.53 ± 0.021
			Cas. acids	369.0 ± 66.8	1.23 ± 0.15
			Cas. acids + PL	27.1 ± 8.27	0.20 ± 0.007
BB3630	*pdxT pdxR*$^+$		none	1260.0 ± 82.1	6.14 ± 0.39
			PL	30.7 ± 1.91	0.54 ± 0.021
BB3308	wild-type	*p1*	Cas. acids	0.38 ± 0.007	18.8 ± 0.42
BB3312	*pdxR*$^+$		Cas. acids	52.7 ± 12.2	19.5 ± 0.14
			Cas. acids + PL	12.4 ± 1.32	14.0 ± 0.40
BB3631	*pdxT pdxR*$^+$		none	163.0 ± 30.3	38.1 ± 3.89
			PL	19.0 ± 0.57	20.9 ± 0.57
BB3309	wild-type	*p2*	Cas. acids	0.47 ± 0.021	30.7 ± 0.42
BB3313	*pdxR*$^+$		none	1090.0 ± 136.0	16.1 ± 0.14
			Cas. acids	1230.0 ± 14.7	3.19 ± 0.021
			Cas. acids + PL	805.0 ± 35.7	12.2 ± 0.42
BB3632	*pdxT pdxR*$^+$		none	1970.0 ± 206.0	3.66 ± 0.44
			PL	999.0 ± 134.0	16.6 ± 0.46
BB3747	wild-type	*p4*	Cas. acids	0.52 ± 0.035	20.0 ± 0.064
BB3749	*pdxR*$^+$		none	873.0 ± 164.0	12.7 ± 0.21
			Cas. acids	1,100.0 ± 161.0	2.50 ± 0.35
			Cas. acids + PL	605.0 ± 180.0	9.16 ± 0.69
BB3751	*pdxT pdxR*$^+$		none	1,810.0 ± 30.1	3.50 ± 0.31
			PL	737.0 ± 81.5	13.1 ± 0.021
BB3748	wild-type	*p1/p2*	Cas. acids	0.40 ± 0.064	13.0 ± 2.13
BB3750	*pdxR*$^+$		Cas. acids	65.2 ± 0.35	15.7 ± 0.85
			Cas. acids + PL	7.09 ± 1.15	13.3 ± 1.41
BB3752	*pdxT pdxR*$^+$		none	220.0 ± 18.3	35.4 ± 5.16

Strain	Genotype	*pdxRS* promoter region	Additions to the medium	β-galactosidase (*pdxS-lacZ*)	β-glucuronidase (*pdxR-gusA*)
			PL	8.43 ± 1.88	26.9 ± 1.74

Cells were grown in TSS glucose-ammonium medium with or without 0.5% vitamin-free Casamino Acids (Difco) and 100 μM PL. β-Galactosidase and β-glucuronidase specific activities were assayed and expressed in Miller units. All values are averages plus/minus standard deviation from at least two experiments.