Functional complementation between the *PDX1* vitamin B_6 biosynthetic gene of *Cercospora nicotianae* and *pdxJ* of *Escherichia coli*

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Abstract The pathway for de novo vitamin B_6 biosynthesis has been characterized in *Escherichia coli*, however plants, fungi, archaebacteria, and most bacteria utilize an alternative pathway. Two unique genes of the alternative pathway, *PDX1* and *PDX2*, have been described. *PDX2* encodes a glutaminase, however the enzymatic function of the product encoded by *PDX1* is not known. We conducted reciprocal transformation experiments to determine if there was functional homology between the *E. coli pdxA* and *pdxJ* genes and *PDX1* of *Cercospora nicotianae*. Although expression of *pdxJ* and *pdxA* in *C. nicotianae pdx1* mutants, either separately or together, failed to complement the pyridoxine mutation in this fungus, expression of *PDX1* restored pyridoxine prototrophy to the *E. coli pdxJ* mutant. Expression of *PDX1* in the *E. coli pdxA* mutant restored very limited ability to grow on medium lacking pyridoxine. We conclude that the *PDX1* gene of the alternative B_6 pathway encodes a protein responsible for synthesis of the pyridoxine ring.

Key words: *SNZ*; *YaaD*; *SOR1*; *PYROA*; Pyridoxal-5′-phosphate; Pyridoxamine

1. Introduction

The term vitamin B_6 is used to refer collectively to the compound pyridoxine and its vitameric forms, pyridoxal, pyridoxamine, and their phosphorylated derivatives. Vitamin B_6 is required by all organisms and plays an essential role as a co-factor for enzymatic reactions. Plants, fungi, bacteria, archaebacteria, and protists synthesize pyridoxine. Animals and some highly specialized obligate pathogens obtain it nutritionally. All organisms have an efficient salvage pathway that interconverts between the various vitamers and their phosphorylated derivatives, but only the pyridoxine-synthesizing organisms listed above contain the de novo biosynthetic pathway [1–3].

A de novo pathway for B_6 synthesis was extensively characterized in *Escherichia coli* [2,4–7]. Two genes, *pdxB* and *serC*, encode enzymes for synthesis of 4-phosphohydroxy-L-threonine, one of the two precursors of the pyridoxine molecule. The second precursor is 1-deoxy-D-xylulose-5-phosphate, produced via transketolases also involved in thiamine and isoprenoid synthesis. These two precursors are joined together via the action of the products of the *pdxA* and *pdxJ* genes to form the pyridine ring of pyridoxine-5′-phosphate [2,8,9]. The product of the *pdxA* gene (4-phosphohydroxythreonine dehydrogenase) has been proposed to mediate the oxidation and decarboxylation of 4-phosphohydroxy-L-threonine to form an unstable intermediate, 3-hydroxy-1-aminoacetone-3-phosphate [10]. The *pdxJ* product (pyridoxine 5′-phosphate synthase) then catalyzes the ring closure reaction between this proposed intermediate and 1-deoxy-D-xylulose-5-phosphate to produce pyridoxine 5′-phosphate.

Work in our laboratory on the fungus *Cercospora nicotianae* identified two genes, *PDX1* and *PDX2*, that are required for de novo biosynthesis of pyridoxine but are completely unrelated in sequence to the known *E. coli* biosynthetic genes [11,12]. Independently, two other groups identified *PDX1* and *PDX2* homologues in *Aspergillus nidulans* and *Bacillus subtilis* as B_6 biosynthetic genes [13,14]. Sequence database and phylogenetic analyses demonstrated that fungi, plants, archaebacteria, and some eubacteria contain homologues to *PDX1* and *PDX2* and lack homologues to the *E. coli pdxA* and *pdxJ* genes, whereas only members of the γ subdivision of the proteobacteria contain *pdxA* and *pdxJ* and lack homologues to *PDX1* and *PDX2* [3,11,12]. These results demonstrated that most organisms utilize a de novo pathway for pyridoxine synthesis that is distinct from the one in *E. coli*. Consistent with the existence of two pathways, Tanaka et al. [15] showed that the nitrogen moiety in pyridoxine is derived from glutamine in four fungi and two prokaryotes (*Staphylococcus* and *Bacillus*), whereas it is derived from glutamate in *Pseudomonas*, *Enterobacter*, and *E. coli*.

Very recently, the enzymatic function of the product of *PDX2* has been demonstrated. Dong and co-workers [16] expressed the *Saccharomyces cerevisiae PDX2* homologue (*SNO1*) in an *E. coli* expression system, and directly assayed glutamine hydrolyzing activity. Independently, Bauer and co-workers [17] determined the crystal structure of the *B. subtilis* PDX2 homologue (YaaE), and showed it to be most similar to HisH, a glutaminase involved in histidine biosynthesis. These observations confirmed the earlier prediction, based on sequence motifs, that *PDX2* encodes a glutamine amidotransferase [18], and are consistent with the identity of glutamine as the nitrogen donor [15].

In contrast to *PDX2*, sequence analysis of the predicted PDX1 protein provides no clues to its function. Two indepen-

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dent research groups have proposed, based on two-hybrid analysis in yeast [19] and structural similarities of the *B. subtilis* homologues to the HisH–HisF complex [17], that the PDX1 and PDX2 proteins interact and form a complex. This prediction was recently confirmed by Dong and co-workers [16] who showed by affinity chromatography that the yeast proteins form a complex. They also proposed that the complex functions as a glutamine amidotransferase, with PDX2 acting as the glutaminase. However, *PDX1* and *PDX2* are the only unique genes identified in the alternative pathway, arguing that *PDX1* has an additional function.

The purpose of this research was to use cross-complementation analysis to determine if there is functional homology between the products encoded by the *C. nicotianae PDX1* gene and the *E. coli pdxA* and *pdxJ* genes, which would provide clues to the function of PDX1. Our research indicates a functional relationship between *PDX1* and *pdxJ*, suggesting that PDX1 is involved in the pyridoxine ring closure reaction.

2. Materials and methods

2.1. Fungal strains, cultural conditions and transformation

Wild type *C. nicotianae* strain ATCC #18366 and the *pdx1* mutant strain CS8 [20,21] were maintained on malt medium [22] at 28°C in the dark. The vectors pHYGPRO and pBarGPE1 [23] were used to transform *C. nicotianae* for expression of *pdxA* and *pdxJ* (see vector construction below). Mycelial protoplasts from *C. nicotianae* were prepared and transformed as previously described [24,25]. Transformants were selected on medium containing 200 μg/ml hygromycin (pHYGPRO) and/or 50 μg/ml bialaphos (pBarGPEI). Experiments to determine pyridoxine auxotrophy used a minimal medium [22] containing bacteriological agar (Sigma, St. Louis, MO, USA) with and without the addition of 1 μg/ml pyridoxine. Growth was determined by transferring fungal mycelium as a toothpick point inoculation and measuring the increase in colony diameter after 4 days at 28°C.

2.2. E. coli *strains, culture conditions, and transformation*

E. coli strains and clones were provided by Malcolm E. Winkler, University of Indiana, Bloomington, IN, USA. Strains NU812 and TX1918 are mutant, respectively, in *pdxA* [4] and *pdxJ* [7], and are derived, respectively, from parent strains NU426 and NU816. Both mutants carry a kanamycin resistance marker. The *pdxA* and *pdxJ* genes were provided on plasmids pNU244 and pNU199, respectively, both containing an ampicillin resistance marker. Standard methods [26] were used for transformation of *E. coli* with vector pBluescript II KS^+ for expression of *PDX1* (see vector construction below). Transformants were initially screened on minimal Vogel–Bonner 1XE medium (E medium) containing 0.01 mM $FeSO_4$ [27] with and without 1 μg/ml pyridoxine. To quantify growth, strains were grown in 5 ml of liquid E medium+1 μg/ml pyridoxine overnight at 37°C with shaking at 200 rpm. One ml of culture was centrifuged at 5220 RCF for 5 min and the pellet resuspended in 2 ml deionized H_2O. The density of the suspension was adjusted to an OD of 0.6 at 600 nm. Ten and 100 μl of each inoculum suspension was plated onto plates of solid E medium with and without 1 μg/ml pyridoxine. Cultures were incubated for 24 and 48 h at 37°C. Bacterial lawns were washed from the plates with 2 ml of deionized H_2O, diluted 1:20, and the OD_{600} of the diluted suspension determined.

2.3. Gene cloning

The *C. nicotianae PDX1* gene was amplified from cosmid clone 18E1 [25] using *Taq* DNA polymerase (Promega, Madison, WI, USA) with an annealing temperature of 60°C for 30 cycles. *PDX1* was amplified utilizing primers 5′-ATGGCCTGTAACGGAACTTC-3′ and 5′-TGGCTGGTAGATGCTGCAAA-3′. The amplified gene was ligated into the Sma site of the *E. coli* vector pBluescript II KS^+ (Stratagene, La Jolla, CA, USA) behind the β-galactosidase promoter according to the manufacturer's recommendations. The resulting construct was sequenced and used for transformation of *E. coli* mutants NU812 and TX1918. The transformants were selected on media containing ampicillin and kanamycin.

For cloning of *pdxA* and *pdxJ* into fungal vectors, primers were designed using sequence data in the National Center for Biotechnology Information (NCBI) database. The *pdxA* gene was amplified from strain NU1350 containing the pNU244 plasmid using primers 5′-GCGCCTACGTTAAAATCCTGA-3′ and 5′-CTAAGTGGCCCTG-GTGGACT-3′. The amplified gene was ligated into the Sma site of fungal vector pBarGPE1 behind the constitutive *A. nidulans gpdA* (glyceraldehyde-3-phosphate) promoter [28]. This vector contains the complete phosphinothricin acetyltransferase *bar* gene [29] used as a selectable marker behind the *trpC* promoter, conferring resistance to bialaphos. The *pdxJ* gene was amplified from strain NU1062 containing plasmid pNU199 using primers 5′-CCTAAGCGAACGG-TGAAAAC-3′ and 5′-TCCACAATATCCGTGCCTAA-3′. The fungal transformation vector, pHYGPRO, was constructed by utilizing pGEM-3Zf(+) (Promega) and inserting a fragment containing the hygromycin B phosphotransferase (*hph*) gene from *E. coli* [30] into the Nde site, allowing for selection with hygromycin. The amplified *pdxJ* was cloned into the vector behind Promoter 1 from pBar3 [29]. The pHYGPRO+pdxJ and pBarGPE1+pdxA constructs were sequenced, and used for transformation of the *C. nicotianae pdx1* (CS8) mutant. Transformants were selected and screened as described above. All plasmids were manipulated in *E. coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) and placed in LB medium as recommended. Standard methods were used for endonuclease digestion, ligation, construction of plasmids, and *E. coli* transformation [26].

2.4. Transgene expression

Transgene expression was determined by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from lyophilized fungal tissue using Tri-Reagent (Sigma) following the manufacturer's protocol and adding a second phenol–chloroform extraction. RNA was resuspended in RNAsecure (Ambion, Austin, TX, USA) and DNase-treated twice with DNA-*free* (Ambion) for 1 h. RT-PCR was performed in a ratio of 1 ng RNA per μl reaction mix using the Access RT-PCR System (Promega). Gene-specific primers (0.2 pM/μl final concentration) used (from IDT) were: *pdxA* 5′-CGG-TCGAACTGGTTGTTTG-3′ and 5′-CACTGATGTGCGAATAAA-GG-3′; *pdxJ* 5′-TGAAGATCGCCGTCACATTAC-3′ and 5′-GTC-ATCACTGCACGACCAATA-3′. Thermocycler parameters for the reverse transcription reaction were 48°C for 45 min, 94°C for 2 min, followed by 30 cycles of PCR at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min.

3. Results and discussion

E. coli pdxA and *pdxJ* mutants were transformed with the *C. nicotianae PDX1* gene, and the resulting transformants were tested for pyridoxine auxotrophy. Plates of E medium with or without pyridoxine were inoculated with 10 or 100 μl of inoculum, and the resulting growth assayed spectrophotometrically at 24 and 48 h (Fig. 1). All strains (wild type, mutants, transformants) grew on E medium+pyridoxine and reached similar levels of growth within 24 h (Fig. 1, top). On medium lacking pyridoxine, wild type strains grew within 24 h, whereas the *pdxA* and *pdxJ* mutants failed to grow at either inoculum concentration up to 48 h (Fig. 1, bottom). *PDX1* clearly restored pyridoxine prototrophy to the *pdxJ* mutant, although growth was slower than that of the wild type strain. By contrast, growth of the *pdxA* mutant transformed with *PDX1* was very poor and never reached wild type levels even within 48 h. The differential ability of *PDX1* to complement the *pdxA* and *pdxJ* mutants argues against the possibility that expression of PDX1 simply by-passes the normal *E. coli* pathway through catalyzing a reaction using other substrates present in *E. coli*, and supports the hypothesis that PDX1 and pdxJ have functional homology.

Our complementation results are consistent with a hypothesis that the PDX1 protein utilizes the normal PdxJ precur-

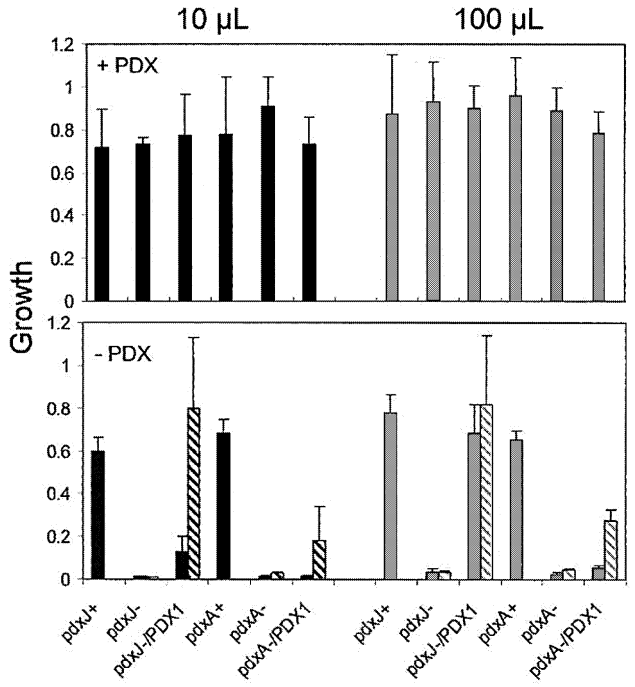

Fig. 1. Growth of *E. coli pdx* mutants transformed with the *C. nicotianae PDX1* gene. Strains were inoculated onto minimal E medium plates with 1 µg/ml pyridoxine (+PDX) or E medium alone (−PDX). Cultures started with 10 µl (black bars, left) or 100 µl (gray bars, right) of inoculum and incubated for 24 (solid bars) or 48 (hatched bars) h. Growth was assayed as described in Section 2. Strains tested were the wild type parent of the *pdxJ* mutant (pdxJ+), the *pdxJ* mutant (pdxJ−), the *pdxJ* mutant transformed with *PDX1* (pdxJ−/PDX1), the wild type parent of the *pdxA* mutant (pdxA+), the *pdxA* mutant (pdxA−), and the *pdxA* mutant transformed with *PDX1* (pdxA−/PDX1). Growth of wild type strains or of cultures grown on E medium+pyridoxine were assayed only at 24 h. Data shown are the means of two independent experiments. Error bars represent S.E.M.

sors, but has a lower affinity for them. All evidence to date supports the conclusion that the precursor substrates specific for the different biosynthetic pathways utilized by PDX1 and PdxJ are different. Tracer labeling studies in two yeasts, *S. cerevisiae* and *Candida utilis*, support a pentose or pentulose precursor for the C_5 unit and an intact triose for the C_3 unit [31,32] rather than the 1-deoxy-D-xylulose-5-phosphate and 4-phosphohydroxy-L-threonine precursors in *E. coli*. Consistent with the above, *serC*, required for production of 4-phosphohydroxy-L-threonine in *E. coli* is not required for B_6 synthesis in *B. subtilis* [33].

Interestingly, recent structural studies of the PdxJ protein have demonstrated significant similarities to earlier predictions about the PDX1 family of proteins. Garrido-Franco and co-workers [34] identified the PdxJ protein as a β/α-barrel protein that contains two different phosphate binding sites. Earlier, Galperin and Koonin [18] predicted, based on sequence motifs, that the PDX1 group of proteins were β/α-barrel proteins with a single phosphate binding site.

Reciprocal experiments were also conducted to express the *E. coli pdxA* and *pdxJ* genes in the *C. nicotianae pdx1* mutant. Surprisingly, none of the transformants were restored to pyridoxine prototrophy, either by transformation with *pdxJ* alone, or in combination with *pdxA* (Fig. 2). RT-PCR experiments showed the expected 0.5- and 0.8-kb bands for *pdxA* and *pdxJ*, respectively, confirming expression of the transgenes in the transformants (Fig. 3). Thus lack of complementation was not due to lack of expression of the transgenes.

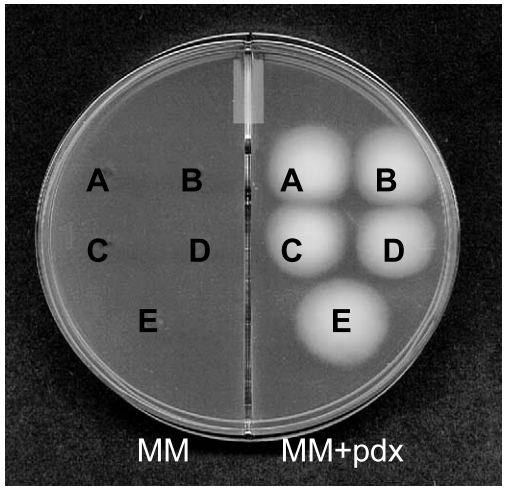

Fig. 2. *C. nicotianae pdx1* mutant transformed with *E. coli pdx* genes grown on minimal medium (MM, left) and minimal medium plus 1 µg/ml pyridoxine (MM+pdx, right). Strains transformed with: (A) *pdxA*, (B) *pdxA* vector control, (C) *pdxJ*, (D) *pdxJ* vector control, and (E) double transformant with *pdxA* and *pdxJ*.

Thus, although *PDX1* complements the *pdxJ* mutation, *pdxJ*, either alone or in combination with *pdxA*, cannot complement the *pdx1* mutation. This lack of complementation may be due to the inability of the *E. coli* proteins to utilize the substrates of PDX1 and the lack of the normal *E. coli* substrates in *C. nicotianae*. There is no evidence at this time that fungi produce the normal substrates for the *E. coli* PdxA and PdxJ proteins. The C_5 intermediate, 1-deoxy-D-xylulose 5-phosphate, is a component of the non-mevalonate pathway for isoprenoid synthesis that operates in bacteria, green algae and plant chloroplasts [35], but we have been unable to find a report of its presence in fungi, or of 4-phosphohydroxy-L-threonine. Thus it is likely that *C. nicotianae* lacks the precursors necessary for functioning of PdxA and PdxJ.

In summary, we have shown that the *PDX1* gene of *C. nicotianae* complements the *E. coli pdxJ* mutation. These results support the conclusion that the PDX1 protein is involved in the formation of the pyridoxine ring.

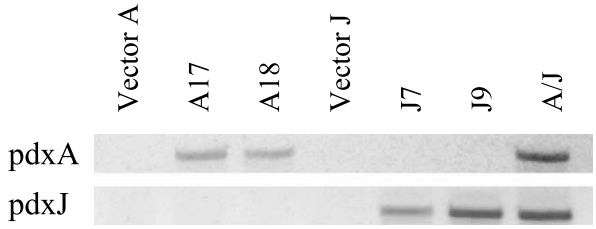

Fig. 3. Expression of the *E. coli pdxA* and *pdxJ* transgenes in *C. nicotianae pdx1* mutant. RNA was extracted from the fungal tissue and amplified by RT-PCR. Strains tested are: vector A = mutant transformed with plasmid pBarGPE1; A17, A18 = two independent transformants with pBarGPE1+pdxA; vector J = mutant transformed with plasmid pHYGPRO; J7, J9 = two independent transformants with pHYGPRO+pdxJ; A/J = mutant transformed with both pBarGPE1+pdxA and pHYGPRO+pdxJ.

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