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Isolation of *PDX2*, a Second Novel Gene in the Pyridoxine Biosynthesis Pathway of Eukaryotes, Archaebacteria, and a Subset of Eubacteria

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In this paper we describe the isolation of a second gene in the newly identified pyridoxine biosynthesis pathway of archaebacteria, some eubacteria, fungi, and plants. Although pyridoxine biosynthesis has been thoroughly examined in *Escherichia coli*, recent characterization of the *Cercospora nicotianae* biosynthesis gene *PDX1* led to the discovery that most organisms contain a pyridoxine synthesis gene not found in *E. coli*. *PDX2* was isolated by a degenerate primer strategy based on conserved sequences of a gene specific to *PDX1*-containing organisms. The role of *PDX2* in pyridoxine biosynthesis was confirmed by complementation of two *C. nicotianae* pyridoxine auxotrophs not mutant in *PDX1*. Also, targeted gene replacement of *PDX2* in *C. nicotianae* results in pyridoxine auxotrophy. Comparable to *PDX1*, *PDX2* homologues are not found in any of the organisms with homologues to the *E. coli* pyridoxine genes, but are found in the same archaebacteria, eubacteria, fungi, and plants that contain *PDX1* homologues. PDX2 proteins are less well conserved than their PDX1 counterparts but contain several protein motifs that are conserved throughout all PDX2 proteins.

Recent work in our laboratory with the filamentous, phytopathogenic fungus *Cercospora nicotianae* revealed that a highly conserved group of gene homologues found in eubacteria, archaebacteria, fungi, and plants play a role in a divergent pyridoxine (vitamin B_6) biosynthesis pathway (8). *PDX1* was originally identified as a gene required for resistance of this fungus to a singlet-oxygen-generating toxin, cercosporin, which it produces to parasitize plants (9, 10). During characterization of this gene, however, we discovered that it rescued both *C. nicotianae* and *Aspergillus flavus* pyridoxine auxotrophs to prototrophy (8). This observation was subsequently confirmed in *Aspergillus nidulans*, in which a *PDX1* homologue, *PYROA*, also rescued pyridoxine auxotrophy (24). Interestingly, despite this direct evidence for the involvement of *PDX1* homologues in pyridoxine synthesis, *PDX1* shows no homology to any of the known *Escherichia coli* pyridoxine biosynthesis genes or to any gene in the completely sequenced *E. coli* genome. Database analysis determined that organisms with homologues to the *E. coli* genes (some eubacteria) lacked *PDX1* homologues and that organisms with *PDX1* homologues (other eubacteria, archaebacteria, fungi, and plants) lacked homologues to the *E. coli* genes. These data suggested that a divergence in the pyridoxine synthesis pathway occurred sometime during the evolution of the eubacteria (8).

The advent of genomic and other large-scale sequencing projects allows homology comparisons on an organismal level. *Saccharomyces cerevisiae* contains three unlinked *PDX1* homologues, one of which (*SNZ1*, for snooze) was extensively studied because its expression increases dramatically during stationary phase (5). Analyses by Galperin and Koonin (12) uncovered that *PDX1*-containing organisms also contained a copy of a second homologous gene and that three of these organisms (*S. cerevisiae* and the eubacteria *Bacillus subtilis* and *Haemophilus influenzae*) all encode this second gene in close physical proximity to their *PDX1* homologues. These researchers hypothesized that the protein encoded by this second open reading frame (ORF) (which they named *SNZB*) possesses glutamine amidotransferase activity. Clusters of orthologous groups of protein analysis performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/COG/) compares protein sequences encoded in 21 complete genomes from 17 major phylogenetic lineages. This analysis supports the supposition that *PDX1* and *SNZB* are functionally linked because it indicates that there is only one other gene with the same organismal distribution pattern as *PDX1*, and that gene is *SNZB* (19, 29, 30). Padilla et al. (25) further characterized the yeast *SNZB* homologues, dubbing them *SNO* (Snz-proximal ORF), and showed that all three *SNZ*/*SNO* pairs were coordinately regulated during growth and nutrient limitation. They also expressed both proteins from one of the gene pairs using a yeast two-hybrid system and showed that the proteins interact.

Despite the above results, there is no direct evidence for a role of SNZB/SNO homologues in pyridoxine synthesis. We have five UV-generated mutant strains of *C. nicotianae* that are pyridoxine auxotrophs (8, 16, 18). Three of these strains can be restored at high frequency to prototrophy by transformation with *PDX1*, while the two remaining strains, CS2 (CS for cercosporin sensitive) and CS7, have wild-type *PDX1* genes (9, 10), suggesting that they are mutant in a different gene in the biosynthesis pathway. In light of the above data, it seemed reasonable to isolate a *C. nicotianae SNZB*/*SNO* homologue and test its involvement in pyridoxine metabolism. Here we report the successful isolation of the *C. nicotianae SNZB*/*SNO* homologue and confirmation of its role in pyridoxine biosynthesis via both targeted gene disruption and the ability to rescue our *C. nicotianae* non-*PDX1* pyridoxine mutants to prototrophy.

MATERIALS AND METHODS

Strains, cultural conditions, and fungal transformations. The wild-type (ATCC 18366) and pyridoxine auxotrophic (CS2, CS7, CS8, and 9714-1) *C. nicotianae* strains used in this study were described previously (8, 16, 18).

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Briefly, CS2, CS7, and CS8 were generated by UV mutagenesis, while 9714-1 is a *pdx1* null strain derived via targeted gene replacement. All strains were maintained on malt medium at 28°C. Experiments to determine pyridoxine auxotrophy or prototrophy used a minimal medium (17), bacteriological agar (Sigma), and supplementation, when necessary, with pyridoxine to a final concentration of 1 μg/ml. Cercosporin resistance was assayed by growth on CM medium (17) supplemented with 10 μM cercosporin. In both cases growth was assayed by transferring fungal mycelium as a toothpick point inoculation and measuring increase in colony diameter after 4 days of growth at 28°C. Cercosporin resistance assays were conducted in a lighted growth chamber (45 to 55 μEinsteins/m^{-2}/s). *C. nicotianae* strains were transformed as previously described (9, 11).

Cloning of PDX2. Degenerate primers for amplification of an internal portion of the *C. nicotianae PDX2* gene were designed by the CODEHOP (consensus-degenerate hybrid oligonucleotide primers) program (http://blocks.fhcrc.org/blocks/codehop.html) (27), based on protein sequences from *Neurospora crassa, Schizosaccharomyces pombe*, and the three yeast homologues. The 5′ and 3′ primers were, respectively, GACCAACATAAACCTACTTGGGGTACNTGYGCNGG and GATCAATAATTTCTTCAATAACAGGAGCNCKDATRAA (see Fig. 4A for regions of the protein to which these correspond). Amplification was performed using Amplitaq Gold (PE Biosystems), an annealing temperature of 57°C, and 50 cycles of amplification. Because the amplification reaction also contained a high-molecular-weight fragment unlikely to correspond to the desired product, an approximately 200-bp band was gel purified and cloned in pGEM-T-Easy (Promega). Sequence analysis confirmed that the cloned fragment corresponded to the desired product.

The entire gene plus flanking regions was recovered using inverse PCR. Southern hybridization analysis was used to determine which restriction enzymes digested the *C. nicotianae* genome into fragments suitable for inverse PCR amplification. *Eco*RI-digested DNA was then self-ligated at dilute concentrations and used as a template for an inverse PCR amplification. When a fragment of the expected size was amplified, it was cloned into pGEM-T-Easy (Promega) for sequence analysis. After sufficient sequence was obtained to encompass the entire ORF plus upstream promoter and downstream sequences, primers were designed to amplify the intact gene from *C. nicotianae* genomic DNA using the high-fidelity DNA *Pfu* polymerase (Promega). After addition of an A residue to both ends, the amplification product was cloned into pGEM-T-Easy for sequence analysis. It was then recovered from pGEM-T-Easy for cloning into the fungal vector pBARKS1 (28) for transformation into *C. nicotianae* mutant strains.

Gene disruption. To produce a gene disruption construct, the cloned inverse PCR product was recovered from pGEM-T-Easy and cloned into the fungal transformation vector pBARKS1. Because the *PDX2* gene is located at either end of the recovered fragment, ligation into pBARKS1 produced a gene disruption construct with the entire vector interrupting the *PDX2* ORF.

PCR amplification of *PDX2* from mutant strains CS2 and CS7 and RT-PCR. The *PDX2* ORFs were amplified from CS2 and CS7 genomic DNA with the high-fidelity *Pfu* DNA polymerase using conditions described by the manufacturer (Promega). Reverse transcription (RT)-PCR was performed using the Access RT-PCR kit (Promega) according to the manufacturer's specifications.

Manipulations of nucleic acids. Fungal genomic DNA was extracted as described (36), digested with restriction enzymes, and electrophoresed though 0.8% agarose prior to transfer to Magnagraph membrane (Osmonics). Probes were generated via PCR incorporation of digoxigenin-dUTP using either degenerate primers or, for the full-length *PDX2* probe, the primers that span the start and stop codons, as shown in Fig. 1. Hybridizations were carried out in aqueous buffer at 65°C and washes at high stringency. Standard methods were used for the construction of plasmids and transformation into *E. coli* strain DH5α.

DNA sequence analysis. DNA sequence analysis was performed at the Molecular Genetics Facility at the University of Georgia, Athens. Homology searches were performed at the National Center for Biotechnology Information with the Blast network service (1). Both strands of the entire wild-type gene were sequenced, while one strand of RT-PCR products or PDX2 products from mutants was analyzed. Multiple alignments were performed using the European Bioinformatics Institute Clustal W Service (http://www2.ebi.ac.uk/clustalw) (33).

Nucleotide and protein sequence accession numbers. The GenBank accession number for the *C. nicotianae PDX2* gene is AF294268. The protein sequences shown in Fig. 4 include *Neurospora crassa* (GenBank AW713653), *S. cerevisiae* homologues (Swiss-Prot P53823, Swiss-Prot Q03144, and Swiss-Prot P43544), *S. pombe* (GenBank AL132798), *Sulfolobus solfataricus* (GenBank Y18930), *Pyrococcus horikoshii* (GenBank AP000006), *Bacillus subtilis* (Swiss-Prot P37527), *Mycobacterium leprae* (GenBank U00011), and wheat (*Triticum aestivum*) (GenBank BE217011).

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   1  GGCAATCAAT GCAGCGTGCA CAACTACGCT GTGCTTGGTG CGCCGCCGGT CATCGATTCT
  61  GGAGTCCCGA AAACGTGATC GGCGCAGCAT TCCCGAATCC TGTCTCTCTT CATCCTCACA
 121  ATTCCTCTTC CAGCACGCCG CCAGCCAGAT GCACGCGGTC GTGACGATGT TGGTGTGACG
 181  GGACTGCCTC ATGCATCGCC CGCCTGGTCG ATAGTAGGCA TCACAGAATG CGAGCAGAGA
 241  ACATGTGTCG AAGAATCATG CCCGTTCAGC ATCCGATCGA GTGTGTAGAA CCCACTTTCC
 301  TCAGCTGTCC TATTCCTCCG TCTGCGCGTC ATTTGTGCAT CTCTCCTCCT CCACCAAGAC
 361  GCCATCGACA ATGACTTCGC GCCCTATCGG ACCAAACCGC TGCGAGTCCA TCTCTGTAGC
 421  GACCATTTTC GTGACTCACT CCCGCGGCCA AGCGAGCAGC ATTCCGTTCT AGTACCCTCA
 481  CATCGCACCC GCCAATGCAC ATTCCCGGCG ACACGACCAC ACC
 524  ATGACAGGCT CCCACTCCTC CCACTCCCTC ACCGTCGGCG TGCTGGCCCT CCCAAGGCGCC
      M  T  G  S  H  S  S  H  S  L  T  V  G  V  L  A  L  Q  G  A      20
 584  TTCATCGAGC ACATCACCCT CCTCCGACAA GCCGCGCCGG CACTGACTGC CGGGTACGGA  CS7
      F  I  E  H  I  T  L  L  R  Q  A  A  P  A  L  T  A  G  Y  G      40
 644  GTCCACTTCA CCTTCATTGA GGTCAGGACG CCCGAACAGC TGGACCGATG CGACGCTCTC
      V  H  F  T  F  I  E  V  R  T  P  E  Q  L  D  R  C  D  A  L      60
 704  ATCCTGCCCG GAGGCGAGAG CACCGCCATC TCGCTCATCG CCGAACGCTG CGGCCTGCTC
      I  L  P  G  G  E  S  T  A  I  S  L  I  A  E  R  C  G  L  L      80
 764  GAACCGCTGC GAAACTTTGT CAAATGGCAA CCGTCGTCCC ACATGGGGAA CATGCGCGGG  CS2
      E  P  L  R  N  F  V  K  W  Q  R  R  P  T  W  G  T  C  A  G     100
 824  CTCATTTTGC TGGCTGAGGA AGCGAACAAG AGCAAGGCGA CAGGGCAAGA GTTGATCGGA
      L  I  L  L  A  E  E  A  N  K  S  K  A  T  G  Q  E  L  I  G     120
 884  GGTCTGGACG TGCGGGTTCA GCGTAATTAC TTTGGCCGAC AAGTCGAGTC TTTCGAAGCA
      G  L  D  V  R  V  Q  R  N  Y  F  G  R  Q  V  E  S  F  E  A     140
 944  GCGCTGCAAC TGCCCTTCCT CGGACCCGAT CCCTTCCACT CCGTATTCAT CCGCGCACCA
      A  L  Q  L  P  F  L  G  P  D  P  F  H  S  V  F  I  R  A  P     160
1004  GTGGTAGAGA ACATTCTGGC GTCGTCCGCC AAAGATGTCA CGACGGAGAT TGTAGAGAAG
      V  V  E  N  I  L  A  S  S  A  K  D  V  T  T  E  I  V  E  K     180
1064  AGTGCCGGCG AAAGCAAGGC AGTTCGACCC AGCATGCCCA ACCGAGCAGA CACCATCTCT
      S  A  G  E  S  K  A  V  R  P  S  M  P  N  R  A  D  T  I  S     200
1124  GCCCCACAGA TAAAGGCGAC CTCAGCACCG GTAGAGATCC TGGGGCGACT GCCCGGAAGG
      A  P  Q  I  K  A  T  S  A  P  V  E  I  L  G  R  L  P  G  R     220
1184  GCAAAGGCGA TCAAAGACAAGACGAGCACGGCGGAAGAGCT GGGAGAGGAGGGCGATATT
      A  K  A  I  K  D  K  T  S  T  A  E  E  L  G  E  E  G  D  I     240
1244  GTCGCTGTGAAGCAGGGCAACGTTTTTGGCACATCCTTCCACCCCGAGTTGACCGGCGAT
      V  A  V  K  Q  G  N  V  F  G  T  S  F  H  P  E  L  T  G  D     260
1304  GACAGAATACACGCCTGGTGGTTGAGGGAAGTCATCAAGAGCAAGCAGGCCACTTGA
      D  R  I  H  A  W  W  L  R  E  V  I  K  S  K  Q  A  T  *        278
1361  ACAAATGCGG GACAACGCAT GCTCATGAAC AAAATACAAC GCGGGAGACG CCAAGTCTGT
1421  GGACATGGTG AACCCACAGA ACGATCCCTC TGCTGGAATG GACTCTTTCC TTCCAACCTG
1481  CCTGCAACCC CTGCCTCGAA ACAAGGGACA CCCCTCCTCC TCCTCTCACA CTGCTCACCC
1541  CTGGTACCGG CATCGAGTTC GGCGTGTTCG GCAG
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FIG. 1. Nucleotide sequence of *C. nicotianae PDX2* gene. The nucleotides shown are sufficient to complement the two non-*pdx1* pyridoxine auxotrophs, CS2 and CS7. The nucleotides in italics at either end represent the primers used to amplify this sequence from *C. nicotianae* genomic DNA. The underlined nucleotides are the primers used to amplify the RT-PCR product and to label the coding sequence with digoxigenin-11-dUTP. The putative AP-1 recognition sequence is shown in bold. The boxed nucleotides represent the regions of the CS2 and CS7 *pdx2* genes that are missing a single nucleotide. The bold italic amino acid residues are identical in all known PDX2 proteins, while the additional amino acid residues shown in bold type are completely conserved only among fungal PDX2 proteins.

RESULTS

Amplification of *PDX2*. Using degenerate primers (see Materials and Methods and Fig. 4A), a fragment comparable in size to fragments amplified from both *N. crassa* and yeast *SNO* clones was amplified from *C. nicotianae* genomic DNA and cloned. After sequence analysis revealed it to encode a partial protein with strong homology to the *SNO* homologues of other fungi, the entire ORF and flanking regions were recovered using inverse PCR. Sufficient sequence was determined from the inverse PCR product for designing primers to amplify and clone a functional ca. 1,600-bp gene using the high-fidelity thermostable *Pfu* DNA polymerase (Fig. 1). Sequence analysis revealed that this fragment contains an uninterrupted ORF of 843 nucleotides encoding a putative polypeptide of 278 amino acid residues. This apparent lack of introns was confirmed by amplification, cloning, and sequence analysis of an RT-PCR product. No canonical promoter sequences are found in the region upstream of the *PDX2* ORF. There is, however, an AP-1 response recognition site approximately 100 bp 5′ of the initiation codon (35).

Complementation of pyridoxine auxotrophs CS2 and CS7. The entire *PDX2* coding sequence plus flanking regions (Fig. 1) was cloned into the fungal transformation vector pBARKS1

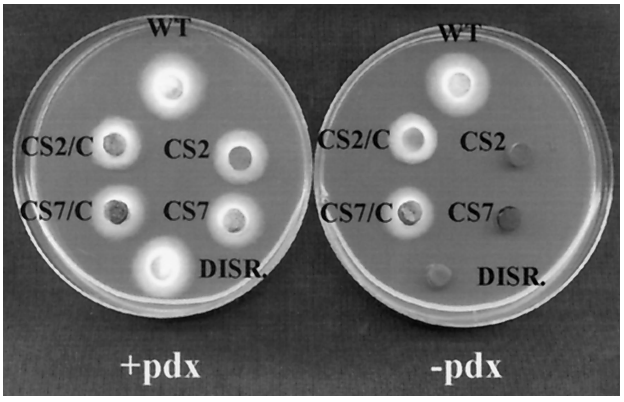

FIG. 2. Growth of wild-type (WT) *C. nicotianae, pdx2* mutant strains CS2 and CS7, a *pdx2* gene disruption mutant (DISR.), and *pdx2* mutant strains complemented by transformation with the *PDX2* gene (CS2/C and CS7/C) on minimal medium with (+pdx) and without (−pdx) pyridoxine. Fungal plugs (6 mm) were placed mycelium side down and incubated for 4 days at 28°C.

(28) for transformation into the two *C. nicotianae* pyridoxine auxotrophic strains (CS2 and CS7) that are not *pdx1* mutants. Ten and 25 bialaphos-resistant transformants of CS2 and CS7, respectively, were tested for ability to grow on minimal medium without pyridoxine (Fig. 2). Eighty percent of CS2 and 76% of CS7 transformants containing *PDX2* grew to between 9 and 100% of wild-type growth on unsupplemented minimal medium. Neither of the parental strains nor any of the two CS2 or eight CS7 colonies transformed with the vector could grow on minimal medium. In previous work, we tested 40 CS2 and 46 CS7 strains transformed with the vector, and none of these vector-transformed strains exhibited even 1% of wild-type growth when tested for complementation (9).

Targeted gene disruption of *PDX2*. The inverse PCR product containing *PDX2* was recovered from pGEM-T-Easy and cloned into pBARKS1. Standard gene disruption constructs that rely on double-crossover events for successful gene replacement generally contain an antibiotic resistance gene within the gene being disrupted. This plasmid (Fig. 3A) corresponds to a standard gene disruption construct except that the entire vector containing an antibiotic resistance marker interrupts *PDX2* and its flanking regions. The disruption construct was transformed into the *C. nicotianae* wild-type strain, and bialaphos-resistant transformants were screened on minimal medium. One transformant of 758 tested was unable to grow on minimal medium, but could be rescued to wild-type growth by supplementation of the medium with pyridoxine (Fig. 2). Southern analysis (Fig. 3B) confirmed that this strain contained a disrupted *pdx2* gene.

PDX2 protein sequence. Figure 4 shows a comparison of the *C. nicotianae* PDX2 protein sequence and all available complete fungal homologues (Fig. 4A) and representative, homologues from other taxa (archaebacteria, eubacteria, and plants) (Fig. 4B). The *C. nicotianae* protein has a region towards the C

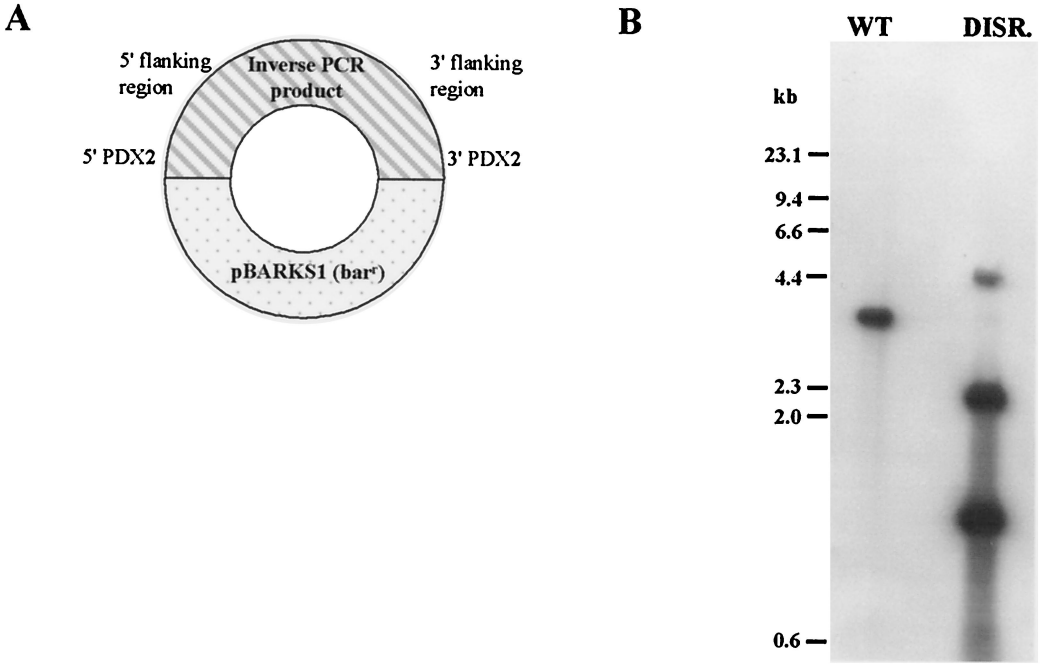

FIG. 3. Disruption of *PDX2*. (A) Schematic describing the disruption plasmid. A clone was constructed in which the *PDX2* inverse PCR product, which consists of the 5′ and 3′ ends of *PDX2* separated by both 5′ and 3′ flanking sequences, was ligated to pBARKS1, a bialophos resistance-encoding vector. Standard gene disruption constructs that rely on double-crossover events for successful gene replacement generally contain an antibiotic resistance gene within the gene being disrupted. This plasmid corresponds to a standard gene disruption construct except that the entire vector containing the antibiotic resistance marker has been used to interrupt *PDX2*. (B) Southern hybridization analysis of a *pdx2*-disrupted strain. *Eco*RI-digested DNA from the wild-type (WT) and a *pdx2*-disrupted (DISR.) strains was probed with a *PDX2*-specific probe spanning the entire ORF. In the wild-type strain, the entire ORF is contained on a single *Eco*RI fragment, which is split into three parts in the gene disruption strain.

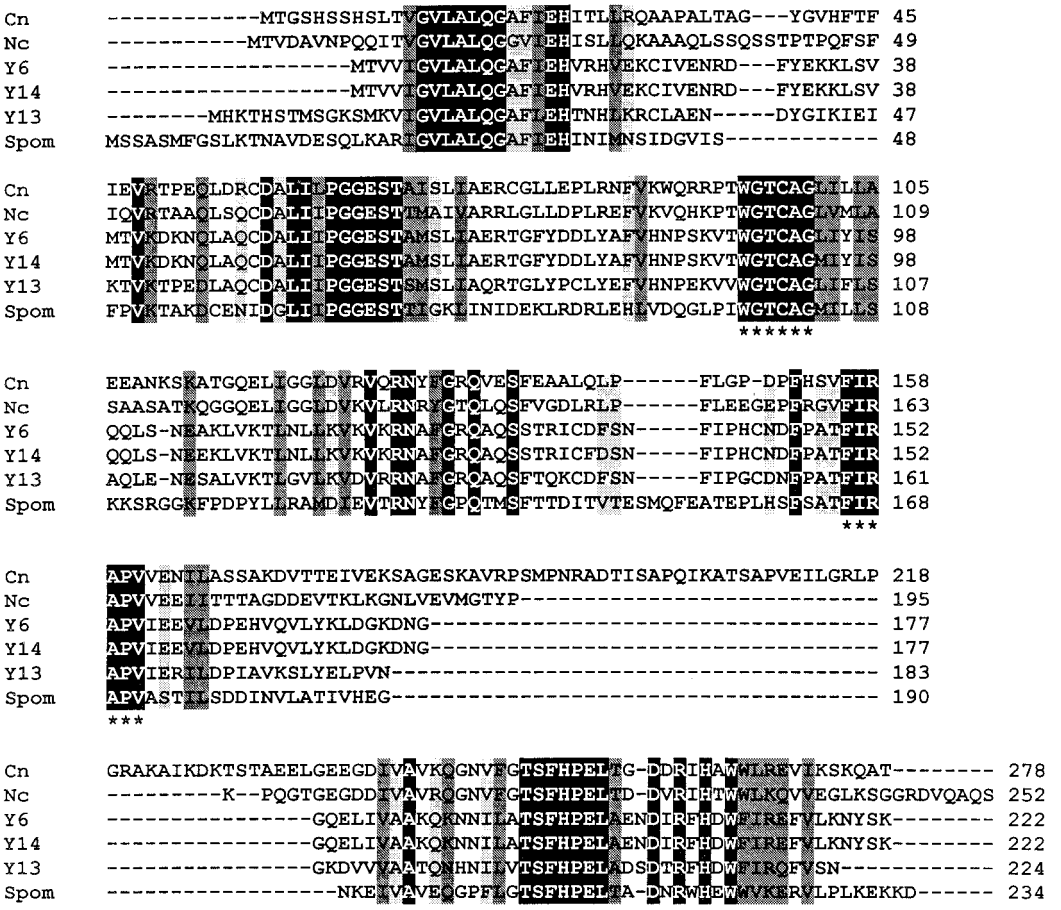

FIG. 4. Comparison of the predicted amino acid sequences of *PDX2* and homologues. (A) Comparison of all fungal PDX2 homologues. Species include *C. nicotianae* (Cn), *Neurospora crassa* (Nc), *S. cerevisiae* homologue from chromosome 6 (Y6), *S. cerevisiae* homologue from chromosome 14 (Y14), *S. cerevisiae* homologue from chromosome 13 (Y13), and *S. pombe* (Spom). The protein sequences from which the degenerate primers were derived are indicated by asterisks. (B) Comparison of the *C. nicotianae* (Cn) PDX2 protein sequence with representative sequences from other major taxa, including the archaebacteria *Sulfolobus solfataricus* (Sulf) and *Pyrococcus horikoshii* (Pyroc), the eubacteria *Bacillus subtilis* (Bsubt) and *Mycobacterium leprae* (Myclep), and the plant wheat (*Triticum aestivum*). The black-boxed residues with white letters indicate identical residues in all proteins, while the darker and lighter shading indicates, respectively, conserved substitutions and semiconserved substitutions.

terminus that is not found in any of the other proteins. Not surprisingly, the fungal proteins exhibit the strongest homology, while the other proteins are distinctly less well conserved. All regions conserved in the fungal proteins, however, are also found in the PDX2 protein sequences from more distantly related taxa, with the regions of homology tending towards conservative and semiconservative substitutions in the latter group. Two regions are highly conserved across all taxa, the PGGEST motif found at *C. nicotianae* residues 63 to 68 and the FHPE(LT) motif at *C. nicotianae* residues 253 to 258 (Fig. 1).

***PDX2* sequences from mutants.** The gene ORFs from mutant strains CS2 and CS7 were amplified from each strain using the high-fidelity thermostable *Pfu* DNA polymerase and cloned for sequence analysis. Two independent amplification reactions were used to generate two independent clones from each strain. One strand of each clone was sequenced. The sequences from the two independent clones from each strain matched and showed that both the CS2 and CS7 *pdx2* genes were missing a single nucleotide, leading to aberrant and truncated proteins of 13 and 12.4 kDa, respectively, in contrast to the wild-type protein of 30.1 kDa. The CS2 ORF is missing one of three cytosine residues at nucleotides 800 to 802, while the CS7 ORF is missing one of a guanosine residue triplet at nucleotides 635 to 637 (Fig. 1). The sequences of the resulting proteins diverge from the wild type at the altered codons (amino acid residues 38 and 93 for CS7 and CS2, respectively), converge with one another at amino acid residue 93, and terminate after 117 amino acid residues at the same stop codon. The CS7 protein contains only the first highly conserved domain (GVLALQGA), while the CS2 protein also contains the second (PGGEST) conserved domain.

Taxonomic distribution of *PDX2* homologues. Database searches were performed with the *C. nicotianae PDX2* gene against GenBank and the finished and unfinished microbial databases available at the Institute for Genomic Research web site (http;//www.tigr.org). As with *PDX1*, no organism containing homologues to the *E. coli* pyridoxine genes encodes a *PDX2* homologue. A list of organisms encoding *PDX2* homologues is shown in Table 1. *PDX2* homologues are encoded by archaebacteria, fungi, plants, and eubacteria of the same groups that that encode *PDX1* homologues. Nearly all of the

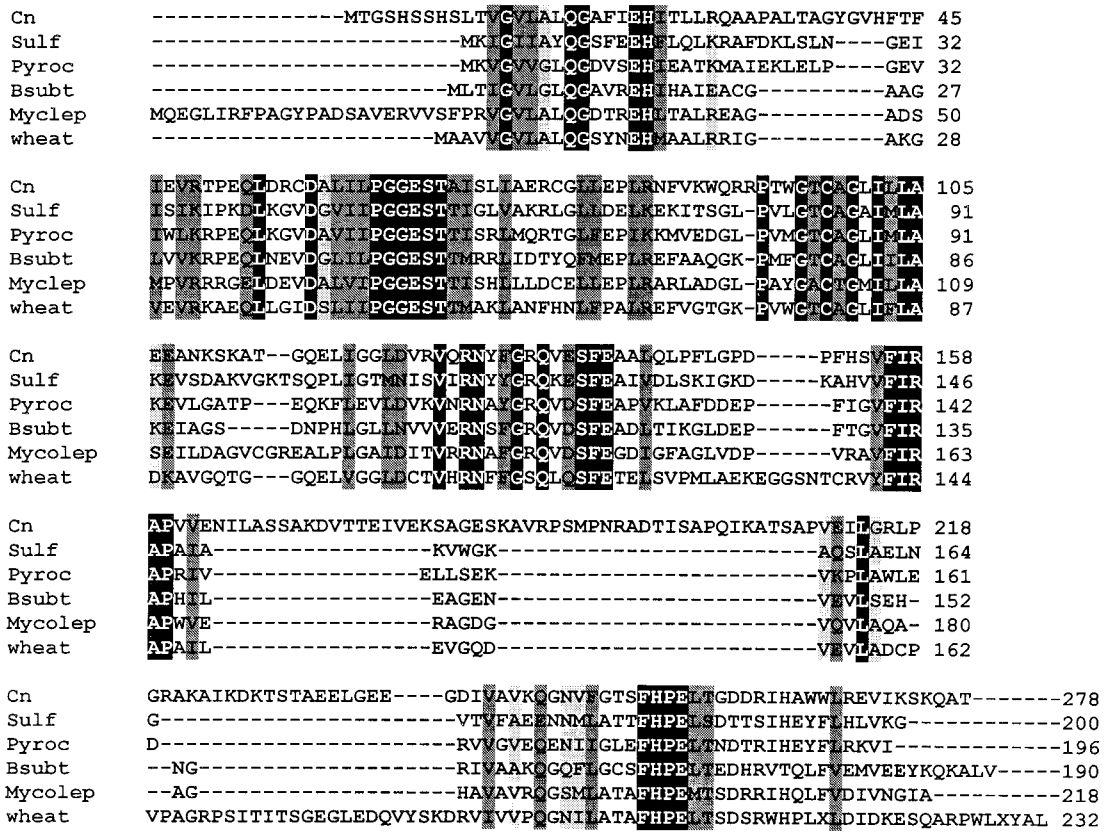

FIG. 4—*Continued.*

organisms listed in Table 1 also have *PDX1* homologues in the databases or are in the same genus as organisms with homologues. The only exceptions are a single plant (soybean, *Glycine max*) and a single eubacterium (*Dehalococcoides ethenogenes*), neither of which has been completely sequenced.

Transformation of *pdx1* mutant strain CS8 and *pdx1* null strain 9714-1 with *PDX2*. The same construct used to transform and complement *pdx2* mutant strains CS2 and CS7 was also used to transform two *C. nicotianae pdx1* mutant strains. CS8 is the UV-derived strain (16, 18) whose complementation led to the identification of *PDX1*; strain 9714-1 was derived via targeted gene replacement and is null for *PDX1* (10). In a previous study we showed that *PDX1* can complement the two *pdx2* mutant strains CS2 and CS7 at low frequency. Whereas *pdx1* mutant strains were complemented at rates of 82 to 100%, CS2 and CS7 were complemented at rates of 35 and 11%, respectively (10). Because this study was conducted prior to our discovery that *PDX1* was involved in pyridoxine synthesis, we used resistance to cercosporin as the measure of complementation. Pyridoxine is consumed during its quenching reaction with cercosporin-generated singlet oxygen, and therefore growth of a transformant in the presence of light and cercosporin indicates that the strain is capable of continued pyridoxine synthesis. In order to directly compare the effect of transformation of *PDX2* into *pdx1* mutant strains with the previously published converse experiment, CS8 and 9714-1 *PDX2* transformants were tested for complementation on CM medium supplemented with 10 μM cercosporin. None of the 59 *PDX2* transformants of the *pdx1* null strain 9714-1 were able to grow in our assay. One of the 59 CS8 *PDX2* transformants (1.7%) did grow in the presence of cercosporin, but only to 45% of the level of the wild-type strain.

DISCUSSION

In this work we provide evidence for a second gene involved in a recently described divergent pathway for pyridoxine biosynthesis. Using a PCR-based degenerate priming strategy, we isolated a *C. nicotianae* homologue of the yeast *SNZB*/*SNO* gene. Complementation of two *C. nicotianae* pyridoxine biosynthesis mutants with this gene demonstrated that it is required for pyridoxine biosynthesis. Sequencing of the *pdx2* genes in these mutants revealed that both encode altered and truncated proteins, leading to their mutant phenotypes. Gene disruption experiments further corroborated the complementation studies and sequence analysis, and we have named this gene *PDX2* in recognition of its newly defined role. Not surprisingly, database analysis revealed that *PDX2* homologues exhibit the same distribution pattern as the *PDX1* group of genes, the only other defined gene in this pathway. Additionally, while PDX2 proteins are not as well conserved as PDX1 proteins, several PDX2 domains are conserved across diverse phylogenetic groups.

In contrast to the situation in *E. coli*, very little is known about the PDX1/PDX2 biochemical pathway. ^{15}N-labeling experiments suggested that the nitrogen of yeast pyridoxine orig-

TABLE 1. Occurrence of PDX2 homologues in organisms of diverse taxa[a]

Plants
Arabidopsis thaliana
Glycine max
Hordeum vulgare
Lycopersicon esculentum
Medicago truncatula
Mesembryanthemum crystallinum
Oryza sativa
Triticum aestivum
Zea mays
Fungi
Aspergillus nidulans
Candida albicans
Neurospora crassa
Saccharomyces cerevisiae
Schizosaccharomyces pombe
Archaebacteria
Aeropyrum pernix
Archaeoglobus fulgidus
Methanobacterium thermoautotrophicum
Methanococcus jannaschii
Pyrococcus abyssi
Pyrococcus horikoshii
Sulfolobus solfataricus
Eubacteria
Bacillus anthracis
Bacillus stearothermophilus
Bacillus subtilis
Clostridium acetobutylicum
Corynebacterium diphtheriae
Dehalococcoides ethenogenes
Deinococcus radiodurans
Haemophilus ducreyi
Haemophilus influenzae
Mycobacterium avium
Mycobacterium bovis
Mycobacterium leprae
Mycobacterium tuberculosis
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus pneumoniae
Streptomyces coelicolor
Thermotoga maritima

[a] Organisms in boldface have been completely sequenced.

inates with the amide moiety of glutamine (32), whereas in *E. coli*, glutamic acid provides the nitrogen (13, 14). Consistent with this, independent sequence motif analysis suggested that the PDX2 protein possesses glutamine amidotransferase activity (12). Data from *Aspergillus nidulans*, however, suggest that ammonium may be the source of the nitrogen atom (2, 3). Recent biochemical work with the eubacterium *Rhizobium meliloti* demonstrated that its pyridoxine biosynthesis pathway differs from that of *E. coli* (31). In *E. coli*, pyridoxine 5′-phosphate is formed via condensation of 4-phosphohydroxy-L-threonine and 1-deoxy-D-xylulose-5-phosphate (6, 7, 20), and 4-phosphohydroxy-L-threonine is formed by a four-step process from erythrose 4-phosphate. In *R. meliloti*, the latter compound is derived instead from condensation of glycine and glycoaldehyde. In *E. coli*, 4-hydroxy-L-threonine may be formed from glycine and glycoaldehyde, but only if glycoaldehyde is supplied. Unfortunately, no information is currently available on whether *R. meliloti* encodes the *E. coli* or *PDX1-PDX2* homologues. We are currently engaged in experiments to determine if our *C. nicotianae PDX1* and *PDX2* genes and the *E. coli pdxA* and *pdxJ* genes (encoding the enzymes catalyzing the final condensation step between 1-deoxy-D-xylulose-5-phosphate and 4-phosphohydroxy-L-threonine) can functionally substitute for each other. In addition, we also intend to look at expression of pyridoxine genes in *A. nidulans*. Further data from these studies as well as from *R. meliloti* should provide valuable information on the actual roles of the PDX1 and PDX2 proteins and differentiate whether there are two completely unrelated pathways or the biochemical pathway is conserved and different proteins perform comparable enzymatic functions in different organisms.

It is currently unknown whether other genes are involved in *C. nicotianae* pyridoxine biosynthesis in addition to *PDX1* and *PDX2*. Because genomic analyses strongly suggest that *PDX1* and *PDX2* are alone in their organismal distribution, it seems unlikely that there are other genes unique to this metabolic pathway. In *B. subtilis* and *H. influenzae*, the *PDX1* and *PDX2* homologues are in the same operon; however, the other flanking genes (e.g., seryl-tRNA synthetase and cytidylate kinase 2) have no obvious relationship to pyridoxine biosynthesis. It is possible that genes required for other metabolic pathways play a dual role in *C. nicotianae* pyridoxine synthesis, as is found in *E. coli* (21, 37). If so, a mutation in such a gene may be lethal. In a screen of over 11,000 UV-irradiated protoplasts regenerated on complete medium, only 5 pyridoxine auxotrophs were found (8, 16, 18), and all can be complemented to wild-type phenotype with either *PDX1* or *PDX2*. Mutagenesis of any other gene in the pathway, even if also required for some other function, should nevertheless cause pyridoxine auxotrophy. All three yeast *PDX2* homologues are found no further than 449 bp from their *PDX1* cohorts, and the *N. crassa PDX1* and *PDX2* homologues are also closely linked (3a), and we initially attempted to isolate the *C. nicotianae PDX2* by sequencing approximately 5 kb of DNA flanking either side of *PDX1*. Our sequencing efforts, however, have led us to conclude that the two known pyridoxine genes are unlinked in *C. nicotianae*, as they are in *A. nidulans* (http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/). Gene clustering can be a useful tool for identifying pathway genes in fungi (15, 22, 26), but will not have utility in this case.

Previous complementation studies suggested that increasing *PDX1* copy number can at least partially compensate for *PDX2* mutation. The two *pdx2* auxotrophs, CS2 and CS7, were transformed with complete and truncated versions of *PDX1* and also with vector alone. Only intact copies of PDX1 could restore either CS2 or CS7 to prototrophy and only at a greatly reduced rate (9, 10). Altogether, 11 and 35% of CS7 and CS2 transformants, respectively, were complemented to pyridoxine prototrophy with *PDX1*, compared to an 82 to 100% complementation rate of three *pdx1* mutant strains. This poorer rate of complementation and the lack of mutations in the CS2 and CS7 *PDX1* genes led us to conclude these two strains were defective in a different part of the same pyridoxine pathway.

In this work we performed the corollary experiment, rescue of *pdx1* mutant strains by transformation with *PDX2*, and showed that this reverse complementation does not work. Only a single *PDX2* transformant of the mutant strain CS8 (1.7%) showed any rescue of the mutant phenotype, and that strain could only grow to 45% of wild-type levels. It is possible that

increased amounts of PDX1 protein yield an enhanced level of substrate for partially functional PDX2 proteins. Alternatively, the two-hybrid analysis described by Padilla et al. (23) indicates that PDX1 and PDX2 proteins interact, suggesting that increased dosage of PDX1 stabilizes defective PDX2 proteins. The exact function of these proteins, however, has yet to be defined.

The sequence analysis of the *pdx2* mutations in CS2 and CS7 revealed that both strains contain frameshift mutations leading to the translation of severely truncated proteins. While the wild-type protein contains 278 amino acid residues, both mutant strains are predicted to encode proteins of 117 amino acid residues. The CS2 protein diverges from the wild type at amino acid 93, while the CS7 protein diverges at residue 38. Nevertheless, the conserved domains that remain, one in the CS7 protein and two in the CS2 protein, must be sufficient for partial function. Otherwise, transformation of these strains with *PDX1* would not result in rescue of their auxotrophy.

We have also found a response element for an AP-1-like transcription factor in both the *C. nicotianae PDX1* and *PDX2* genes. The yeast species *S. cerevisiae, S. pombe*, and *Kluyveromyces lactis* all encode AP-1-like transcription factors essential for their response to oxidative stress (4, 34, 35), and in *S. pombe* the *PDX1* homologue is upregulated by overexpression of its AP-1-like transcription factor (M. W. Toone, personal communication). *PDX1* and *PDX2* were originally discovered during a search for genes involved in resistance to an active-oxygen-generating phototoxin, cercosporin. In organisms as diverse as rubbertree and *S. cerevisiae, PDX1* homologue expression has also been linked to the presence of reactive oxygen species (5, 14, 21). The role of the AP-1 response pathway in cercosporin resistance is not yet clear. In yeast species, gene disruption and overexpression experiments have been valuable in delineating the biological role of their AP-1-like transcription factors. Comparable approaches should prove equally valuable in *C. nicotianae* to define the role of pyridoxine in oxidative stress response.

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