The Extremely Conserved pyroA Gene of Aspergillus nidulans
Is Required for Pyridoxine Synthesis and Is Required Indirectly for Resistance to Photosensitizers*

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Numerous disparate studies in plants, filamentous fungi, yeast, Archaea, and bacteria have identified one of the most highly conserved proteins (SNZ family) for which no function was previously defined. Members have been implicated in the stress response of plants and yeast and resistance to singlet oxygen toxicity in the plant pathogen Cercospora. However, it is found in some anaerobic bacteria and is absent in some aerobic bacteria. We have cloned the Aspergillus nidulans homologue (pyroA) of this highly conserved gene and define this gene family as encoding an enzyme specifically required for pyridoxine biosynthesis. This realization has enabled us to define a second pathway for pyridoxine biosynthesis. Some bacteria utilize the pdxA pyroxidine bio- synthetic pathway defined in Escherichia coli and others utilize the pyroA pathway. However, Eukarya and Archaea exclusively use the pyroA pathway. We also found that pyridoxine is destroyed in the presence of singlet oxygen, helping to explain the connection to singlet oxygen sensitivity defined in Cercospora. These data bring clarity to the previously confusing data on this gene family. However, a new conundrum now exists; why have highly related bacteria evolved with different pathways for pyridoxine biosynthesis?

The pathway leading to pyridoxine (vitamin B6) synthesis has been largely defined in Escherichia coli through the study of pyridoxine auxotrophic mutants and tracer experiments using radiolabeled precursors (1). Genetic studies indicate that four genes are specifically involved in the synthesis of pyridoxine including pdxA, pdxB, pdxJ, and pdxF (serC) (2–9). Labeling studies demonstrate that two components, 1-deoxy-D-xylulose and 4-hydroxy-D-threonine, serve as the precursors for pyridoxine (10–15). Both pdxB and pdxF (serC) are involved in the synthesis of 4-hydroxy-D-threonine, and it has been proposed that pdxA and pdxJ play a role in generating pyridoxine from 4-hydroxy-D-threonine and 1-deoxy-D-xylulose (15). All four pdx gene products have been identified (9, 16).

Although little work has been done on the pyridoxine biosynthetic pathway in Eukarya (eukaryotes), results of these studies suggest that the pyridoxine biosynthetic pathway is significantly different between Saccharomyces cerevisiae and E. coli because the label of L-[amide-15N]glutamine is incorporated efficiently into pyridoxine in S. cerevisiae but not in E. coli. This indicates that the origin of the nitrogen atom of pyridoxine in S. cerevisiae is different from that of E. coli (17).

In Aspergillus nidulans a pyridoxine auxotrophic mutation termed pyroA defined a gene involved in the biosynthesis of pyridoxine (18). We have sequenced pyroA and show it encodes a highly conserved protein found in all major phylogenetic domains (19) but for which a function was previously unknown. Our data indicate that the function of this highly conserved protein is the synthesis of pyridoxine in a novel pathway that is distinct from the pathway utilized in E. coli, which does not contain this gene. Interestingly, mutation of the pyroA homologue of Cercospora nicotianae (SOR1) causes increased sensitivity to singlet oxygen generated by photosensitizer compounds, and our analysis provides a molecular explanation for this phenomenon.

EXPERIMENTAL PROCEDURES

Strains were grown and maintained as described previously (20) using minimal medium with urea as the nitrogen source except for pyroB100 strains when adenine was utilized (18). For incubation in the light, an F15 CW 8-watt fluorescent bulb was fixed in a 35 °C incubator, and plates were placed directly below the light source at a distance of 12 inches. For incubation in the dark, plates were double-wrapped in heavy duty aluminum foil and placed in the same incubator. Strains employed were OR5 (pyrG89; wa3; pyroA4); R153 (wa3; pyroA4); and SO53 (wa3 ninT23). Strains 2325a (γA2 ade2 pyroB100; riboB2) and 2323b (γA2 ade2 pabaA1; pyroB100) were the kind gift of Herb N. Arst, Jr. Transformation of A. nidulans was as described previously (20). The insert of plasmid p14 (21) was sequenced on both strands to tentatively identify the pyroA encoding region. Overlapping 5’-RACE† PCR was employed using conditions as described by the manufacturer (CLON-TECH, Palo Alto, CA) to define the 5’ and 3’ end of the pyroA transcript. The region defined by RACE PCR was PCR-amplified and cloned directionally into the conditional A. nidulans expression plasmid pAL5 (22) to generate plasmid pPYRO4. Induction and repression of transformants containing pPYRO4 were as described (22). Data bank BLAST searches were performed at the National Center of Biotechnology Information (23).

RESULTS

Molecular Analysis of pyroA—A 2684-base pair PstI-BamHI fragment capable of complementing the pyroA mutation (Fig. 1A) that had previously been identified (21) was sequenced on

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† The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; ORF, open reading frame.
both strands. Analysis of this sequence identified an open reading frame (ORF 1) capable of encoding a peptide of 32.9 kDa. BLAST searches using this peptide sequence did not reveal homologues in organisms with completely or largely sequenced genomes to see if these functions were shared by any bacteria (Table I). Those species having pdsA also contained pdsJ but did not contain pyroA. Results of these searches indicate that no organism contains both pyroA and pdsA or pdsJ, although many obligate parasitic organisms lack pdsA, pdsJ, and pyroA.

**Mutation of pyroA Causes Methylene Blue Photosensitivity**—Because mutation of SOR1, the pyroA homologue of *C. nicotianae*, causes sensitivity to photosensitizing compounds (25) we asked if mutation of *pyroA* in *A. nidulans* generated a similar phenotype. In the light, the photosensitizer methylene blue (5 μM) caused almost complete inhibition of strains containing pyroA4, but it caused much less inhibition in the dark (Fig. 3 and data not shown). No such photosensitizing was apparent for the wild type control strain. Sensitivity of the pyroA4 mutant strain, and resulting transformants were replica plated on repression medium (glucose) and in-ning expression of ORF 2.

![Fig. 1. Complementation of pyroA4 by ORF 2.](image)

**TABLE I**

| Protein Family | pdsA | pdsJ | pyroA |
|----------------|------|------|-------|
| Proteobacteria | +    | +    | -     |
| *Escherichia coli* | +    | +    | -     |
| *Haemophilus influenzae* | -    | -    | +     |
| *Helicobacter pylori* 26695 | +    | +    | -     |
| *Helicobacter pylori* 3999 | +    | +    | -     |
| *Shewanella putrefaciens* | +    | +    | -     |
| Aquificales | Aquifex aeolicus | +    | +    |
| *Bacillus subtilis* | -    | -    | +     |
| *Mycobacterium tuberculosis* | -    | -    | +     |
| Cyanobacteria | *Synechocystis* PCC6803 | +    | +    |

* pdsA and pdsJ were not found in the available sequence of the Archaea or Eukaryota.

* pyroA was identified in *Bacteria* as indicated and in *Archaea* and *Eukaryota*.

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![Image](image)
prevent photosensitizing by methylene blue. The higher level of pyridoxine was able to significantly reverse the toxicity caused by methylene blue and light (Fig. 3).

**Photosensitivity of pyroA4 Strains Is Due to Destruction of Pyridoxine**

The data presented in Fig. 3 indicate that pyroA4 strains cannot grow in the presence of methylene blue and light and that this effect can be suppressed by elevating the level of pyridoxine. We considered the possibility that pyridoxine itself may be photosensitive in the presence of methylene blue and was being destroyed under these conditions. We exposed plates containing 0.5 or 25 μg/ml pyridoxine, with or without methylene blue, to light for 2, 4, 8, or 20 h. The plates were then inoculated with a pyroA4 strain and a pyroA4 strain transformed with the pyroA gene. After inoculation the plates were incubated in the dark for 3 days at 35 °C. The slightly smaller size of GR5 and GR5 transformed with pyroA is caused by the pyrG89 mutation in these strains, which is lacking in strain SO53. MB, methylene blue.

**To confirm that the inability of pyroB100 pyridoxine auxotrophs to grow on plates exposed to light and photosensitizers is due to pyridoxine being destroyed and not an effect specific to pyroA mutations, we tested another mutation in a different gene causing pyridoxine auxotrophy,** pyroB100. Plates were prepared containing normal or extremely elevated concentrations of pyridoxine (from 0.5 to 250 μg/ml) with or without methylene blue. All four plates were exposed to light prior to being inoculated with two pyroB100 mutant strains. The plates were then incubated in total darkness for 3 days. The strains could grow equally well at high or low concentrations of pyridoxine on those plates lacking methylene blue (Fig. 5). However, plates containing methylene blue could not grow on plates containing methylene blue unless a 500-fold excess of pyridoxine was present during exposure to light (Fig. 5). If the plate upon which the pyroB100 strains could not grow was then supple-

**FIG. 2. Alignment of four members of the pyroA family.** PYROA-like proteins from *A. nidulans* (PYROA), rubber tree *Hevea brasiliensis* (plant), *Bacillus subtilis*, and the Archaea *Methanobacterium thermoautotrophicum* are compared using ClustalW. Positions of identity between three members are shaded.

**FIG. 3. Inhibition of pyroA4 strain by methylene blue and light.** Plates containing normal (top) or 50-fold (bottom) pyridoxine were inoculated with a control strain (wild type (Wt) = SO53), a pyroA4 mutant (GR5), and GR5 transformed with the pyroA clone (pyroA) in the absence or presence of methylene blue (5 μM) under the conditions indicated for 3 days at 35 °C. The slightly smaller size of GR5 and GR5 transformed with pyroA is caused by the pyrG89 mutation in these strains, which is lacking in strain SO53. MB, methylene blue.

**FIG. 4. Inhibition of pyroA4 strain on methylene blue plates first exposed to light prior to inoculation and incubation in the dark.** Plates, as labeled, were exposed to light for 20 h. They were then inoculated with a pyroA4 strain (GR5) and the same strain transformed with the pyroA gene. The plates were subsequently incubated in the dark for 3 days at 35 °C. MB, methylene blue.

**Figures 2-4.**
ment with pyridoxine and reinoculated, then the pyroB100 strains could grow normally (data not shown). These data demonstrate that the inability of pyroA4 strains to grow on plates exposed to light and photosensitizers is not a specific effect of the pyroA4 mutation. Rather, it is the effect of these conditions destroying pyridoxine such that pyridoxine auxotrophs are unable to grow.

**Discussion**

A. nidulans is able to grow when supplied with trace elements, inorganic salts, and a simple carbon source. However, this filamentous ascomycete requires pyridoxine for growth when the pyroA gene is mutated. The pyroA4 mutation therefore defines an essential function for pyridoxine biosynthesis. The pyroA gene is shown to encode a member of a highly conserved protein family with members in the *Archaea*, bacteria, and *Eukarya* but for which no function was previously known. We propose this function is an enzymatic step in the biosynthetic pathway to pyridoxine.

The nitrogen atom of pyridoxine has a different origin in *E. coli* compared with *S. cerevisiae*, suggesting that the biosynthetic route to pyridoxine is different between these organisms (17). Two of the genes involved in pyridoxine biosynthesis in *E. coli*, *pxdxA* and *pxdJ*, are highly conserved in some of the bacteria but absent from others. Those bacteria that do not contain *pxdxA* or *pxdJ* instead contain a pyroA homologue. We have been unable to identify genes similar to *pxdxA* or *pxdJ* in the *Archaea* or *Eukarya*, which either contain pyroA homologues or do not contain *pxdxA* or *pxdJ* genes. These genetic and biochemical data strongly argue that there are two pathways to pyridoxine synthesis, one involving the *pxdx* genes and another involving pyroA. Numerous parasitic organisms lack both *pxdx* genes and pyroA and are likely to require pyridoxine to grow. Crown eukaryotes are also unlikely to encode these enzymes but instead need pyridoxine in their diet.

In some *S. cerevisiae* strains there are multiple genes, termed SNZ1, -2, etc., that are homologous to pyroA. Each SNZ gene is adjacent to a member of a second conserved gene family termed SNO genes, suggesting that their functions are linked. Like pyroA, SNO genes are found in all three phylogenetic domains and are distributed in a similar fashion (26, 27). If an organism contains a pyroA homologue then it also contains a SNO-like gene. Because of the tight linkage of pyroA-like genes and SNO genes in the genomes of several organisms and their identical distribution among the *Archaea*, bacteria, and *Eukarya*, it is possible that SNO-related genes function in the pyroA pathway of pyridoxine biosynthesis. Because of low level sequence similarity it has been proposed that SNO proteins are related to glutamine amidotransferase enzymes (26). This proposed function would fit well with the suggestion that SNO proteins play a role in the biosynthesis of pyridoxine, given that glutamine acts as the nitrogen donor for pyridoxine synthesis in yeast.

PyroA-related genes (SNZ genes) have been implicated in the starvation response in *S. cerevisiae* as cells accumulate during the stationary phase. One possibility is that this is due to depletion of the pyridoxine pool, causing an increased requirement for biosynthetic capacity for pyridoxine. It is likely that the biosynthetic pathway to pyridoxine is under negative feedback control and that the genes in this pathway will not be expressed at high levels if sufficient pyridoxine is present. It has also been noted that SNZ mutants are sensitive to 6-azauracil, an inhibitor of purine and pyrimidine biosynthesis (27). This is also consistent with the proposed role for pyroA (SNZ) in pyridoxine biosynthesis, as pyridoxine is a precursor for the pyrimidine moiety of thiamine in *S. cerevisiae* (28).

In another ascomycete fungus, the filamentous plant pathogen *C. nicotiana*, a pyroA homologue (SOR1) has been implicated in singlet oxygen ($^1{\text{O}}_2$) resistance. This fungus produces cercosporin, a compound that generates $^1{\text{O}}_2$ when exposed to light and is therefore called a photosensitizer. Mutation of *SOR1* renders normally resistant strains of *C. nicotiana* sensitive to $^1{\text{O}}_2$-generating compounds such as cercosporin and methylene blue. *S. cerevisiae* SNZ mutants are also sensitive to methylene blue and light. Similarly, our data show that *A. nidulans* pyroA4 mutants are also sensitive to methylene blue and light. However, by exposing plates containing pyridoxine and methylene blue or rose bengal to light prior to inoculation we have found *A. nidulans* pyroA4 mutants unable to grow subsequently on these plates in the dark. PyroA4 strains complemented with the pyroA gene are able to grow on such plates normally. This is not a specific effect caused by the pyroA4 mutation as a mutation in a second gene involved in pyridoxine biosynthesis, pyroB100, also renders cells sensitive to photosensitizers and light. These data indicate that pyridoxine is sensitive to photosensitizers when exposed to light. We predict that other organisms having mutations in pyroA- or pyroB-like genes will also be sensitive to photosensitizers and light as pyridoxine will be destroyed and they will be unable to synthesize pyridoxine. Indeed, any mutation causing pyridoxine auxotrophy should show sensitivity to photosensitizers and light.

The realization that pyroA-like genes are required to synthesize pyridoxine helps to explain some previously confusing aspects of this gene family. The proposed role of *SOR1* in $^1{\text{O}}_2$ resistance in *Cercospora* is at odds with the occurrence of members of this gene family in anaerobic organisms (25). The fact that pyroA is required for pyridoxine biosynthesis explains the occurrence of gene family members in anaerobic organisms, and our data suggest that the role of *SOR1* in $^1{\text{O}}_2$ resistance may be an indirect effect related to the stability of pyridoxine. However, it will be interesting to ascertain if *SOR1* plays an additional role in resistance to photosensitizers apart from supplying pyridoxine for normal growth of *Cercospora*. The distribution of pyroA to a subset of the bacteria can also now be explained if two pathways of pyridoxine biosynthesis exist, one involving pyroA and another the *pxdx* genes. What is less clear is why two highly conserved pathways for pyridoxine biosynthesis have evolved and what evolutionary pressures caused some bacteria to retain pyroA while others evolved instead with *pxdx* genes. The precise enzymatic function of pyroA, *pxdxA*, and *pxdJ* may help to shed some light on this new conundrum.

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