Sequence Analysis and Initial Characterization of Two Isozymes of Hydroxylaminobenzene Mutase from Pseudomonas pseudoalcaligenes JS45

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Pseudomonas pseudoalcaligenes JS45 grows on nitrobenzene by a partially reductive pathway in which the intermediate hydroxylaminobenzene is enzymatically rearranged to 2-aminophenol by hydroxylaminobenzene mutase (HAB mutase). The properties of the enzyme, the reaction mechanism, and the evolutionary origin of the gene(s) encoding the enzyme are unknown. In this study, two open reading frames (habA and habB), each encoding an HAB mutase enzyme, were cloned from a P. pseudoalcaligenes JS45 genomic library and sequenced. The open reading frames encoding HabA and HabB are separated by 2.5 kb and are divergently transcribed. The deduced amino acid sequences of HabA and HabB are 44% identical. The HAB mutase specific activities in crude extracts of Escherichia coli clones synthesizing either HabA or HabB were similar to the specific activities of extracts of strain JS45 grown on nitrobenzene. HAB mutase activity in E. coli extracts containing HabB withstood heating at 85°C for 10 min, but extracts containing HabA were inactivated when they were heated at temperatures above 60°C. HAB mutase activity in extracts of P. pseudoalcaligenes JS45 grown on nitrobenzene exhibited intermediate temperature stability. Although both the habA and the habB gene conferred HAB mutase activity when they were separately cloned and expressed in E. coli, reverse transcriptase PCR analysis indicated that only habA is transcribed in P. pseudoalcaligenes JS45. A mutant strain derived from strain JS45 in which the habA gene was disrupted was unable to grow on nitrobenzene, which provided physiological evidence that HAB is involved in the degradation of nitrobenzene. A strain in which habB was disrupted grew on nitrobenzene. Gene Rv3078 of Mycobacterium tuberculosis H37Rv encodes a protein whose deduced amino acid sequence is 52% identical to the HabB amino acid sequence. E. coli containing M. tuberculosis gene Rv3078 cloned into pUC18 exhibited low levels of HAB mutase activity. Sequences that exhibit similarity to transposable element sequences are present between habA and habB, as well as downstream of habB, which suggests that horizontal gene transfer resulted in acquisition of one or both of the hab genes.

In recent years a number of microorganisms with the ability to utilize mononuclear nitroaromatic compounds as sources of nitrogen, carbon, and energy have been isolated and studied in pure culture. Although the degradative pathways of the organisms differ, three basic strategies are employed to prepare the aromatic ring for cleavage. Two of the pathways include an initial attack on the aromatic ring by dioxygenase enzymes and produce catecholic intermediates that are further degraded by classical ring fission pathways (14, 36, 37). The third mechanism used to prepare the ring for cleavage begins with partial reduction of a nitro group by nitroreductase enzymes to form aromatic hydroxylamo compounds. The hydroxylamo products are rearranged to aminophenols that serve as ring fission substrates (26, 33, 34, 38).

Pseudomonas pseudoalcaligenes JS45 grows on nitrobenzene as a sole source of carbon, nitrogen, and energy (26). Nitrobenzene is first reduced to hydroxylaminobenzene by nitroreductase (Fig. 1) (26, 35). Hydroxylaminobenzene is rearranged to 2-aminophenol by hydroxylaminobenzene mutase (HAB mutase). 2-Aminophenol 1,6-dioxygenase, a member of the type II dioxygenase family (8, 40), catalyzes an extradiol cleavage of the aromatic ring to produce 2-amino-3-methyl-1,4-cyclohexadiene (1, 22, 41). 2-Aminomuconic acid 6-semialdehyde is subsequently oxidized to 2-amino-3-methyl-1,4-cyclohexadiene (15, 17). Ammonia is released from 2-aminomuconate by a deaminase (16, 17), and the resulting 4-oxalocrotonic acid is broken down to compounds that enter central metabolism. Recently, Park et al. (29) reported the isolation of a strain of Pseudomonas putida that degrades nitrobenzene by the same partially reductive pathway.

The mechanism of HAB mutase-catalyzed conversion of hydroxylaminobenzene to 2-aminophenol is known. The reaction is analogous to the nonenzymatic acid-catalyzed Bamberger rearrangement of hydroxylaminobenzene to 4-aminophenol (2, 7), but HAB mutase produces 2-aminophenol almost exclusively (26). Two other strains of bacteria that rearrange hydroxylaminoaromatic intermediates during the degradation of nitroaromatic compounds have been reported. The mutase enzymes of these isolates differ somewhat from the HAB mutase of P. pseudoalcaligenes JS45 in their biochemical properties (33, 34, 38). The mutase of Mycobacterium sp. strain HL 4-NNT-1, which grows on p-nitrotoluene but not on nitrobenzene, also produces 2-aminophenol from hydroxylaminobenzene (38). In contrast,Ralstonia eutropha JMP134, which grows on 3-nitrophenol but not on nitrobenzene, contains a
For induction of the Rv3078 gene in pUC18 and the cosmid MTCY22D7 (accession number Z83866) containing gene pSE380 (Invitrogen, Carlsbad, Calif.) were used for subcloning and expression in 25 g of ampicillin per ml, 25 g of chloramphenicol per ml, 10 g of kanamycin per ml, or 10 g of streptomycin per ml, was added to the medium. pUC18 (25) and pSE380 (Invitrogen, Carlsbad, Calif.) were used for subcloning and expression in E. coli. Cosmid MTCY22D7 (accession number ZS3866) containing gene Rv3078 (5) was a gift from K. Eiglermeier of the Institute Pasteur, Paris, France. For induction of the Rv3078 gene in pUC18 and the habA gene in pSE380 in E. coli, cultures were grown in LB to an A600 of 0.5. IPTG (isopropyl-ß-D-thiogalactopyranoside) was added to a final concentration of 1 mM. The cells were harvested when the A600 of the culture reached 1.8.

Reagents and enzymes. All enzymes and molecular biology kits were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.) and were used according to the manufacturer's instructions. Protein concentrations were determined by the Bradford method (3) by using the Coomassie Protein Plus assay reagent (Pierce, Rockford, Ill.) with bovine serum albumin as the standard. Sodium pentacyanoamine ferrocate (PCF) and 2-aminophenol were purchased from Aldrich (Milwaukee, Wis.). Hydroxylaminobenzene, which was provided by S. Nishino, was prepared as previously described (26). All molecular biology reagents were prepared by previously described methods and were of the highest purity commercially available (12, 32).

FIG. 1. Initial steps in the enzyme-catalyzed pathway of nitrobenzene degradation by P. pseudoalcaligenes JS45 contrasted with the chemical Bamberger rearrangement of hydroxylaminobenzene to 4-aminophenol (not observed in JS45).

Mutagenesis of the habA and habB genes. Plasmids pNBZ108 and pNBZ130, which expressed HAB mutase activity, were digested with the enzymes indicated in Fig. 2. The ends of the digested plasmids were filled in by using the Klenow enzyme (32), and the blunt-ended linear plasmids were religated, which resulted in frameshift mutations. Transformants were screened for loss of the original restriction site and for the ability to produce 2-aminophenol from nitrobenzene as described above.

Strains of P. pseudoalcaligenes JS45 in which the habA or habB gene was disrupted with an antibiotic resistance cassette were constructed. For disruption of habA, a 610-bp EcoRV fragment containing habA was cloned into the Smal site of pUC18, which yielded plasmid pNBZ118. The habA gene in plasmid pNBZ118 was disrupted by introducing the gentamicin-resistance gene from p34S-Gm (9) as a XbaI fragment into the unique SpeI site in the habA gene. The disrupted habA gene was moved into suicide plasmid pJP503 (30) as an EcoRI-XbaI fragment. The resulting construct was mobilized into strain JS45 via E. coli S17-1 (30). The natural resistance of P. pseudoalcaligenes JS45 to chloramphenicol was used to select against the E. coli donor. Six hundred Cm' Gm' exconjugants were selected and then screened for loss of plasmid-borne kanamycin resistance. Genomic DNA was prepared (11) from wild-type strain JS45 and five Cm' Km' strains. Southern blotting and hybridization analysis revealed that four of the five Cm' Km' isolates contained only the disrupted copy of habA. One such strain, designated JS45ΔA, was characterized further. For disruption of the habB gene, a 966-bp SalI-SstI fragment containing habB was subcloned from pNBZ139 into pJP503 (30) prepared by digestion with SalI and SstI. The habB gene was disrupted by cloning the streptomycin resistance gene from p34S-Sm2 (9) as an XmaI fragment into the unique PatI site in the habB gene. The disrupted habB gene was moved into suicide plasmid pJP503 (30) as an EcoRI-XbaI fragment. The resulting construct was mobilized into strain JS45 via E. coli S17-1 (30). Five hundred Cm' Sm' exconjugants were selected and then screened for loss of plasmid-borne kanamycin resistance. Four of nine Sm' Km' isolates were found to contain the disrupted copy of habB when they were examined by hybridization. One such strain, JS45ΔB, was characterized further.

Southern blotting and hybridization analysis were performed using digoxigenin random primed labeled probes and detected with the CSPD chemiluminescent detection system as described by the manufacturer (Roche Molecular Biochemicals).

RT-PCR analysis. Total RNA was prepared from P. pseudoalcaligenes JS45 grown in LB or in mineral medium supplemented with nitrobenzene by using a Qiagen RNeasy Mini kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. DNA was removed from the RNA preparation by two on-column treatments with RNase-free DNase. Reverse transcription PCR (RT-PCR) was performed by using the ProStar HF single-tube RT-PCR system (Stratagene, LaJolla, Calif.) according to the manufacturer's protocols with first-
strand synthesis at 42°C for 20 min. We used the following primers (Macromolecular Structure Facility, Michigan State University, East Lansing): 5'-TTCTKCTCGGKCTGSTGACYGG (forward primer that binds to habA and habB), 5'-COTGAGATACCGATAAGAAG (reverse primer specific for habA), and 5'-CCGGAGCATCCGTGTCG (reverse primer specific for habB). The annealing temperature during amplification was 55°C. The predicted sizes of the amplification products were 368 bp for habA and 400 bp for habB. The RT-PCR products were subjected to electrophoresis on a 2.5% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator (wavelength, 300 nm).

Preparation of crude extracts. Cells were suspended in 25 mM potassium phosphate buffer (pH 7.0) and broken by two passages through a French pressure cell at 135,000 kPa. The resulting suspension was centrifuged at 10,000 × g for 20 min, at 30,000 × g for 30 min (in the presence or absence of Triton X-100 [reduced]), or at 105,000 × g for 60 min. The supernatant fluid was removed, and the pellet was suspended in a volume of buffer equal to the volume of the supernatant.

Enzyme assays. HAB mutase activity was determined spectrophotometrically at room temperature by monitoring the increase in absorbance at 283 nm corresponding to the formation of 2-aminophenol (in less than 1 min) was recorded.

Preparation of crude extracts.

Preparation of crude extracts.

Enzyme assays.

RESULTS

Identification of two HAB mutase genes in P. pseudoalcaligenes JS45. A P. pseudoalcaligenes JS45 genomic library containing 1,087 clones (8) was screened for HAB mutase activity. Four HAB mutase-positive clones were identified. Complete digestion of the 48-kb HAB mutase-positive cosmid pNBZ100 with PstI and ligation into pUC18 yielded recombinant plasmids containing 1-kb (pNBZ108) and 5.7-kb (pNBZ130) inserts that conferred HAB mutase activity on E. coli (Fig. 2). A 1.8-kb SphI fragment was cloned from pNBZ130 into pUC18. The resulting plasmid (pNBZ139) conferred HAB mutase activity on E. coli (Fig. 2).

Sequence analysis. The nucleotide sequence of the 8,134-bp region shown in Fig. 2 was determined. Sequence analysis revealed that plasmids pNBZ108 and pNBZ139, which confer HAB mutase activity on E. coli, contain single complete open reading frames that are 408 bp (habA) and 495 bp (habB) long, respectively. Both open reading frames were preceded by a putative ribosome binding site, and no alternate start sites were present. The habA and habB genes had significantly different G+C contents (53 and 71%, respectively). The open reading frames encode proteins with deduced molecular masses of 14.6 kDa (HabA) and 17.0 kDa (HabB), which are 44% identical. The predicted isoelectric point of HabA is 9.7, and the predicted isoelectric point of HabB is 10.9. The two hab genes are separated by 2.5 kb and are transcribed divergently. The PstI fragments of pNBZ108 and pNBZ130 resulted in HAB mutase activity in E. coli when they were cloned in either orientation into pUC18, suggesting that transcription is directed by promoters on the cloned fragments. HAB mutase activity was no longer detectable when frameshift mutations were introduced at the SpeI and SalI sites within habA or the KpnI site within habB (Fig. 2).

No protein in the SWISSPROT database exhibited significant amino acid sequence similarity to either HabA or HabB. Gene Rv3078 of cosmids MTCY22D7 from a M. tuberculosis H37Rv genomic library (5) exhibited significant DNA sequence identity to habB (74% identity over a 134-nucleotide region and additional short regions of identity). The deduced amino acid sequence of Rv3078 was 51% identical to the HabA amino acid sequence and 52% identical to the HabB amino acid sequence, and there were no gaps in the alignments (Fig. 3).

Due to the levels of sequence identity between the M. tuberculosis H37Rv Rv3078 gene product and the P. pseudoalcaligenes JS45 hab genes, we tested the possibility that the Rv3078 gene product had HAB mutase activity. Although E. coli DH5α containing cosmids MTCY22D7 from a M. tuberculosis H37Rv genomic library (5) exhibited significant DNA sequence identity to habB (74% identity over a 134-nucleotide region and additional short regions of identity). The deduced amino acid sequence of Rv3078 was 51% identical to the HabA amino acid sequence and 52% identical to the HabB amino acid sequence, and there were no gaps in the alignments (Fig. 3).

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Analysis of the nucleotide sequence surrounding the habA and habB genes of strain JS45 showed that these genes are associated with transposable genetic elements. The deduced amino acid sequence of an open reading frame between habA and habB (Fig. 2) was similar to the amino acid sequence of a transposase from IS801 (48% similarity and 22% identity over a 476-bp aligned region). In addition, an inverted repeat approximately 2 kb downstream of habA was an exact match with the 38-bp inverted repeat of Tn5501. The nucleotide sequence from this inverted repeat to the end of the sequenced region was 95% identical to the sequence of Tn5501 of plasmid pGHI1 (18, 21). This region included the atpR gene (encoding a resolvase) and part of the tnpA gene (encoding a transposase). As in Tn5501, the 3′ end of atpR overlapped the 5′ end of tnpA.

Disruption of the hab genes and RT-PCR analysis. In order to establish a physiological role for HAB mutase in the degradation of nitrobenzene by P. pseudoalcaligenes JS45, we constructed strains in which the genomic copy of habA or habB was disrupted. Wild-type strain JS45 and mutant strains JS45ΔA (habA minus) and JS45ΔB (habB minus) were tested for the ability to grow on solid media with nitrobenzene provided in the vapor phase as the sole carbon and energy source. Unlike the wild-type strain, strain JS45ΔA was not able to grow on nitrobenzene. Strain JS45ΔB grew on nitrobenzene. In addition, only habB was amplified by RT-PCR when we used template RNA prepared from P. pseudoalcaligenes JS45 grown in either LB or mineral medium supplemented with nitrobenzene (Fig. 4).

Properties of HAB mutase in E. coli. The habA and habB genes could be expressed in E. coli at levels that resulted in HAB mutase specific activity similar to the specific activity observed in extracts of nitrobenzene-grown strain JS45. When E. coli cells containing pNBZ108 (habA) and E. coli cells containing pNBZ130 (habB) were grown in LB and harvested during late log phase, the HAB mutase specific activities in cell extracts were 0.18 and 5.6 μmol min⁻¹ mg of protein⁻¹, respectively. By comparison, the HAB mutase specific activity was 5.8 μmol min⁻¹ mg of protein⁻¹ in extracts prepared from nitrobenzene-grown P. pseudoalcaligenes JS45. The relatively low level of HAB mutase activity in E. coli expressing habA on plasmid pNBZ108 prompted us to construct a more efficient expression clone. A 610-bp EcoRV fragment containing habA was cloned into expression vector pSE380. The HAB mutase activity in extracts prepared from IPTG-induced cultures of E. coli containing the pSE380-habA construct was 2.7 μmol min⁻¹ mg⁻¹. The M. tuberculosis Rv3078 gene resulted in HAB mutase activity of 0.078 μmol min⁻¹ mg of protein⁻¹ in extracts prepared from IPTG-induced cultures of E. coli pMB3078.

HAB mutase activity was found in both the supernatant and the pellet after centrifugation (Table 1). Ultracentrifugation at 105,000 × g did not cause all of the activity to sediment into the pellet, which suggested that HAB mutase is not an integral membrane protein. Inclusion of the surfactant Triton X-100 in the lysis buffer caused more of the activity to partition into the pellet. Samples obtained from wild-type JS45 and samples obtained from E. coli clones behaved similarly, which suggested that the lack of solubility was not an artifact of expression in E. coli but an intrinsic property of the enzyme.

The thermostabilities of the HAB mutase isozymes differed. HAB mutase activity in E. coli extracts containing HabB withstood heating at 85°C for 10 min, whereas extracts containing HabA were inactivated when they were heated at temperatures above 60°C (Fig. 5). The thermostability of HAB mutase activity in extracts prepared from nitrobenzene-grown strain JS45 was intermediate to the thermostabilities of HabA and HabB synthesized separately in E. coli. The enzyme activities of HabA and HabB were relatively constant at pH values between 6.5 and 8.0 in crude extracts.

**DISCUSSION**

Two genes that encode HAB mutase isozymes were cloned from P. pseudoalcaligenes JS45 and expressed in E. coli, yet only one of the genes was expressed in strain JS45. RT-PCR analysis indicated that habA, but not habB, is expressed in strain JS45. The presence of habA transcripts in cells from cultures of P. pseudoalcaligenes JS45 grown in both minimal medium supplemented with nitrobenzene and a rich medium is not surprising. There is little difference in HAB mutase specific activity between succinate-grown and nitrobenzene-grown P. pseudoalcaligenes JS45.
pseudoalcaligenes JS45 (data not shown). Similar HAB mutase activities were also observed in succinate-grown and nitrobenzene-grown P. putida HS12 (28). Although habB encodes a gene product that is capable of HAB mutase activity when it is synthesized in E. coli, the results of RT-PCR analysis clearly indicate that habB is not expressed in strain JS45. Although there may be conditions under which habB transcription is induced, strain JS45 does not grow on other nitroaromatic compounds that have been tested (26; J. K. Davis, C. C. Somerville, and J. C. Spain, unpublished data).

The phenotypes of the habA-minus and habB-minus strains are consistent with the results of the RT-PCR transcriptional analysis. In particular, the inability of strain JS45ΔA (habA minus) to grow on nitrobenzene is consistent with the lack of habB transcription in P. pseudoalcaligenes JS45. These results establish that HabA plays a physiological role in the degradation of nitrobenzene by strain JS45. The role of HabB has not been determined yet.

The regulation of habA and habB expression was not examined in this study, but differences in the regulation of hab gene expression were observed in both E. coli and P. pseudoalcaligenes. The difference in HAB mutase activity between E. coli containing pNBZ108 (habA) and E. coli containing pNBZ130 (habB) was most likely due to differences in regulation of the native promoters since the levels of mutase activity were measured in the absence of IPTG induction. Although a higher level of habB expression compared to the level of habA expression in E. coli is the opposite of what was observed in P. pseudoalcaligenes JS45, both results suggest that there are significant differences in the regulation of habA and habB. The regulation of hab gene expression in strain JS45 is currently being investigated.

The thermostability of the HAB mutase activity in extracts prepared from P. pseudoalcaligenes JS45 was intermediate to the thermostabilities of the individual isozymes expressed in E. coli. This suggests that both isozymes are expressed in P. pseudoalcaligenes JS45 or that a host factor affects the stability of one or both of the isozymes. The latter explanation appears to be correct, since RT-PCR results indicated that habA, but not habB, is expressed in strain JS45 and strain JS45ΔA was not able to grow on nitrobenzene.

The presence of HAB mutase activity in both the soluble and particulate fractions of extracts of both P. pseudoalcaligenes JS45 and E. coli synthesizing HAB mutase suggests that HAB mutase is membrane associated or forms aggregates upon cell lysis. It is unlikely that HAB mutase is an integral membrane protein because significant enzyme activity remains in the soluble fraction after centrifugation at 105,000 g. The high percentage of hydrophobic amino acids (54% for HabA and 49% for HabB) and the results of an analysis of hydrophobicity in which the Kyte-Doolittle scale was used (20) (data not shown) indicate that aggregation of the HAB mutase proteins or a peripheral membrane association is likely.

Mutase reactions involving rearrangement of hydroxylamino compounds have been found in other organisms as well. Mycobacterium sp. strain HL 4-NT-1, which was isolated from soil, grows on p-nitrotoluene by first reducing it to p-hydroxylaminotholueone. p-Hydroxylaminotholueone is rearranged to 6-amino-m-cresol in a reaction catalyzed by a mutase (38). Resting cells of Mycobacterium sp. strain HL 4-NT-1 induced with p-nitrotoluene produce 2-aminophenol from hydroxylaminobenzene, although strain HL 4-NT-1 does not grow on nitrobenzene. A mutase is also involved in the growth of R. eutropha JMP134 on 3-nitrophenol. The enzyme 3-hydroxylaminophenol mutase catalyzes the rearrangement of 3-hydroxylaminophenol to 2-aminophenol in this organism.

### Table 1. Distribution of HAB mutase activity in supernatant and pellet fractions after centrifugation

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Strain JS45</th>
<th>HabA</th>
<th>HabB</th>
<th>Rv3078</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broken cells 10,000 × g</td>
<td>8.1 100</td>
<td>0.33 100</td>
<td>10.1 100</td>
<td>0.11 100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>4.6 57</td>
<td>0.21 64</td>
<td>6.1 60</td>
<td>0.057 52</td>
</tr>
<tr>
<td>Pellet</td>
<td>3.4 42</td>
<td>0.13 39</td>
<td>4.3 43</td>
<td>0.047 43</td>
</tr>
<tr>
<td>Supernatant</td>
<td>3.5 43</td>
<td>0.12 36</td>
<td>4.7 47</td>
<td>0.058 53</td>
</tr>
<tr>
<td>Pellet</td>
<td>4.2 52</td>
<td>0.20 61</td>
<td>6.2 61</td>
<td>0.060 55</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.42 5</td>
<td>0.02 6</td>
<td>1.1 11</td>
<td>0.016 15</td>
</tr>
<tr>
<td>Pellet</td>
<td>5.8 72</td>
<td>0.24 73</td>
<td>7.8 77</td>
<td>0.050 45</td>
</tr>
<tr>
<td>Supernatant</td>
<td>3.1 38</td>
<td>0.13 39</td>
<td>2.9 29</td>
<td>0.042 38</td>
</tr>
<tr>
<td>Pellet</td>
<td>3.0 37</td>
<td>0.15 45</td>
<td>5.9 58</td>
<td>0.060 55</td>
</tr>
</tbody>
</table>

a The protein concentrations in broken cells were 1.4 mg · ml⁻¹ for JS45, 1.8 mg · ml⁻¹ for HabA, 1.8 mg · ml⁻¹ for HabB, and 1.5 mg · ml⁻¹ for Rv3078. The volume of each sample was 1 ml.
phenol to aminohydroquinone (33, 34). Unlike the mutase enzymes of \textit{P. pseudoalcaligenes} JS45 and \textit{Myco bacterium} sp. strain HL 4-NT-1, which produce predominantly 2-aminophenol from hydroxylaminobenzene (26), resting cells of induced \textit{R. eutropha} JMP134 convert hydroxylaminobenzene to a mixture of 2- and 4-aminophenols, in which 4-aminophenol is the predominant isomer (33). Thus, the enzyme of strain JS45 appears to be functionally more similar to the enzyme of the gram-positive bacterium than to the enzyme of the gram-negative bacterium \textit{R. eutropha} strain HL 4-NT-1, which produce predominantly 2-aminophenol. Furthermore, the deduced amino acid sequences of the N-terminal sequence (34) of the \textit{R. eutropha} mutase. The sequences of the mutase genes of \textit{R. eutropha} JMP134 and \textit{Myco bacterium} sp. strain HL 4-NT-1 have not been determined; thus, their similarity to \textit{habA}, \textit{habB}, and \textit{Rv}3078 is not known.

The function of the \textit{Rv}3078 gene product in \textit{M. tuberculosis} is not known, but the HAB mutase enzyme catalyzed by this enzyme could be involved in resistance to certain antibiotics. Some antibiotics used in the treatment of mycobacterial infections are nitroaromatic compounds. Such antibiotics must be activated by reduction to hydroxylamino compounds by nitroreductase (10, 19). One possible function of HAB mutase could be to convert the extremely toxic hydroxylamino product into a less harmful aminophenol.

Recruitment of the genes encoding the HAB mutase has been the key step in assembling a functional partially reductive pathway that enables bacteria to grow with nitrobenzene as a sole source of carbon, nitrogen, and energy. Nitroreductase enzymes are widespread (36, 37), and the enzymes involved in the ring fission reaction and the subsequent metabolism of 2-aminophenol are related to the enzymes involved in other meta fission pathways (8, 40, 41). \textit{Pseudomonas} sp. strain AP-3 is able to use 2-aminophenol as a sole source of carbon and nitrogen but has not been reported to grow on nitrobenzene (1, 40), presumably due to the lack of HAB mutase in this strain. Thus, the search for the origin of the HAB mutase genes is critical to understanding the mechanism involved in the evolution of nitrobenzene degradation and other novel catabolic capabilities in natural ecosystems in response to xenobiotic compounds. The origin of the genes encoding HAB mutase is not known, but several lines of evidence suggest that one or both of the \textit{hab} genes were recently acquired by \textit{P. pseudoalcaligenes} JS45. The significant differences among the G+C contents of \textit{habA} (53%), \textit{habB} (71%), and \textit{P. pseudoalcaligenes} (62 to 64%) (27) suggest that the genes encoding the two HAB mutase isozymes may have evolved separately and in hosts other than \textit{P. pseudoalcaligenes}. Furthermore, the genes encoding HAB mutase are not closely linked to the genes encoding either 2-aminophenol 1,6-dioxygenase (\textit{amnB}amnA) or 2-aminomucronie 6-semialdehyde dehydrogenase (\textit{amnC}) of \textit{P. pseudoalcaligenes} JS45 (8, 15; Davis et al., unpublished data). Thus, the genes encoding HAB mutase could have been acquired separately from the genes encoding the lower-pathway enzymes. In addition, Park and Kim recently reported that the gene encoding HAB mutase in \textit{P. putida} HS12 is on a plasmid that is different from the plasmid containing the genes encoding the other enzymes involved in degradation of nitrobenzene (28). The close proximity of the HAB mutase genes to sequences nearly identical to \textit{Tn}5501 sequences (18, 21) and to an open reading frame that is similar to the sequence of the \textit{IS801} transposase (32) suggests that these genes are associated with a transposon. \textit{Tn}5501 was originally identified in \textit{P. putida} on mobilizable plasmid \textit{pPGH1}, which also encodes genes for phenol degradation. Transposonlike sequences similar to \textit{IS801} have also been identified in association with genes that encode enzymes involved in degradation of aromatic compounds in other bacteria (23). Recent acquisition of one or both of the \textit{hab} genes by \textit{P. pseudoalcaligenes} JS45 by horizontal gene transfer involving transposition remains a distinct possibility.

Although the genes involved in degradation of nitrobenzene have been localized to plasmids in \textit{P. putida} HS12 (28), there is no evidence that the genes encoding HAB mutase in \textit{P. pseudoalcaligenes} JS45 reside on plasmids. In contrast to the situation in \textit{P. putida} HS12, the ability to degrade nitrobenzene is very stable in \textit{P. pseudoalcaligenes} JS45, even when cultures are grown in the presence of curing agents (Davis et al., unpublished data). In addition, attempts to demonstrate the existence of plasmids by employing the procedure used by Park and Kim for strain HS12 (28) were unsuccessful.

The availability of the sequences of the HAB mutase genes of \textit{P. pseudoalcaligenes} JS45 and \textit{M. tuberculosis} H37Rv plus the very recent cloning of the HAB mutase gene from \textit{P. putida} HS12 (28) should facilitate studies of the catalytic mechanism of the enzymes, as well as studies of their evolution and ecological roles.

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