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Construction of *Escherichia coli* Strains for Conversion of Nitroacetophenones to *ortho*-Aminophenols

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The predominant bacterial pathway for nitrobenzene (NB) degradation uses an NB nitroreductase and hydroxylaminobenzene (HAB) mutase to form the ring-fission substrate *ortho*-aminophenol. We tested the hypothesis that constructed strains might accumulate the aminophenols from nitroacetophenones and other nitroaromatic compounds. We constructed a recombinant plasmid carrying NB nitroreductase (*nbzA*) and HAB mutase A (*habA*) genes, both from *Pseudomonas pseudoalcaligenes* JS45, and expressed the enzymes in *Escherichia coli* JS995. IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cells of strain JS995 rapidly and stoichiometrically converted NB to 2-aminophenol, 2-nitroacetophenone (2NAP) to 2-amino-3-hydroxyacetophenone (2AHAP), and 3-nitroacetophenone (3NAP) to 3-amino-2-hydroxyacetophenone (3AHAP). We constructed another recombinant plasmid containing the nitroreductase gene (*nfs1*) from *Enterobacter cloacae* and *habA* from strain JS45 and expressed the enzymes in *E. coli* JS996. Strain JS996 converted NB to 2-aminophenol, 2-nitrotoluene to 2-amino-3-methylphenol, 3-nitrotoluene to 2-amino-4-methylphenol, 4-nitrobiphenyl ether to 4-amino-5-phenoxyphenol, and 1-nitronaphthalene to 2-amino-1-naphthol. In larger-scale biotransformations catalyzed by strain JS995, 75% of the 2NAP transformed was converted to 2AHAP, whereas 3AHAP was produced stoichiometrically from 3NAP. The final yields of the aminophenols after extraction and recovery were >64%. The biocatalytic synthesis of *ortho*-aminophenols from nitroacetophenones suggests that strain JS995 may be useful in the biocatalytic production of a variety of substituted *ortho*-aminophenols from the corresponding nitroaromatic compounds.

2-Amino-3-hydroxyacetophenone (2AHAP) is an *ortho*-aminophenol of considerable biological and industrial significance. 2AHAP is an intermediate in oxidative metabolism of tryptophan in mammals (5, 15, 16) and is a key monomer for the synthesis of 3-amino-4,5-diacetylphenoxazone, a dimer similar to the chromophore contained in actinomycin (1). A glucoside having excellent UV absorbing and scattering properties in the human lens is derived from 2AHAP (37) and is potentially useful in the synthesis of a variety of compounds such as cosmetic humectants, antioxidants, and tyrosinase activity inhibitors (36). 2AHAP is a precursor for the synthesis of benzoxaprofen, a nonsteroidal anti-inflammatory agent (9), and also a possible precursor for the synthesis of acetal glucosides which are the allelo chemicals in some plants (8). 3-Amino-2-hydroxyacetophenone (3AHAP) is a key feedstock for making 2-acetyl-6-[4-(4-phenylbutoxyl)benzoyl]aminophenol, an important pharmaceutical amide (12). *o*-Aminophenols are also important building blocks for synthesis of polybenzoxazole polymers (23, 35) with potential aerospace applications (4, 7, 9–11, 40) and biologically active compounds (21, 38). Because the above aminophenols are difficult to synthesize chemically and are consequently not commercially available, a biocatalytic synthesis of the compounds would be very useful.

In certain bacteria, the metabolism of nitroaromatic compounds occurs via the partial reduction of the nitro group to yield arylhydroxylamine intermediates which are subsequently converted to aminophenols (17, 26–28, 30, 34, 41) or 1,2-dihydroxyl aromatic compounds (14). *Pseudomonas pseudoalcaligenes* JS45 grows on nitrobenzene (NB) as the sole carbon and nitrogen source by a partially reductive catabolic pathway (26). NB is reduced by NB nitroreductase to hydroxylaminobenzene (HAB), and HAB mutase then catalyzes the rearrangement of HAB to 2-aminophenol (2AP), which serves as the substrate for *meta*-ring cleavage. It was previously shown that a combination of NB nitroreductase and HAB mutase from strain JS45 can catalyze the production of *ortho*-aminophenols from nitroaromatic compounds (25). In preliminary studies, partially purified NB nitroreductase and HAB mutase A from strain JS45 transformed 2-nitroacetophenone (2NAP). The use of purified or immobilized enzymes is problematic, however, because the cofactor, NADPH, is consumed in the reaction. Furthermore, the presence of a ring-cleavage dioxygenase in NB-grown cells of JS45 or cell extracts limits its usefulness for the production of aminophenols. As an alternative approach, we constructed a strain with genes encoding NB nitroreductase (*nbzA*) and HAB mutase A (*habA*) from JS45 using the pET-21a(+)–*Escherichia coli* C43(DE3) expression system. We also constructed a strain containing the nitroreductase (*nfs1*) gene from *Enterobacter cloacae* and *habA* from strain JS45 using the pSE380-*E. coli* JM109 expression system. The *E. cloacae* nitroreductase was chosen because it is also a member of a larger family of type I oxygen-insensitive nitroreductases, capable of reducing a variety of structurally diverse nitroaromatic compounds (3). We then compared the potentials of the constructed strains in the biocatalytic production of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Escherichia coli		
C43(DE3)	Derivative of strain BL21(DE3)	22
JS995	Strain C43(DE3) carrying recombinant plasmid pJS490	This study
JM109	F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE33 thi-1 gyrA96 relA1* Δ(*lac-proAB*)	Invitrogen
JS996	Strain JM109 carrying recombinant plasmid pJS491	This study
JS999	Strain C43(DE3) carrying recombinant plasmid pJS489	This study
Plasmids		
pET-21a(+)	Expression vector; Ap^r *lacI*^{q} T7 promoter with multiple cloning sites	Novagen
pJS489	pET-21a(+) containing *nbzA* from strain JS45	This study
pJS490	pET-21a(+) containing *nbzA* and *habA* from strain JS45	This study
pRK1	pET-24d(+) containing *nfs1* from *E. cloacae*	20
pSE380::*habA*	pSE380 from Invitrogen containing *habA* from strain JS45	6
pJS491	pSE380 containing *nfsI* from *E. cloacae* and *habA* from strain JS45	This study

aminophenols that are difficult to synthesize by traditional organic chemistry.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions, and chemicals. The bacterial strains and plasmids used in this study are listed in Table 1. Cultures of *P. pseudoalcaligenes* JS45 were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) or on NB as described previously (26). *E. coli* strains were routinely grown in Luria-Bertani (LB) broth on horizontal shakers at 250 rpm and 37°C. *E. coli* BL21(DE3) harboring pRK1 expressing the *nfs1* gene from *E. cloacae*, provided by Anne-Frances Miller, was grown as previously described (20). During IPTG (isopropyl-β-D-thiogalactopyranoside) induction, 2× TY (tryptone-yeast extract) medium (22, 29) was used for growing strain JS995 while strain JS996 was grown in 2× LB medium with 1% glycerol. When necessary, ampicillin at 100 to 200 μg ml^{-1} was added to the medium. All the nitroaromatic compounds and 2-aminoacetophenone (2AAP) were purchased from Aldrich (Milwaukee, Wis.), and other chemicals were analytical grade.

DNA manipulations. Standard techniques were used for preparation of genomic DNA from JS45, isolation of plasmid DNA, cloning, and transformations (13, 29). All enzymes and molecular biology kits were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.). Restriction endonuclease digestion and ligation with T4 DNA ligase were performed according to the instructions of the manufacturer. The QIAquick gel extraction kit (Qiagen, Valencia, Calif.) was used for the recovery of DNA fragments from agarose gels.

Construction of strain JS995. PCR was used to amplify the *nbzA* gene (685 bp) from the strain JS45 genome (G. Zylstra, unpublished data). The forward primer 5′-CAGA*CATATG*CCGACCAGCCCGTTC-3′ with an *Nde*I site (shown in italics) and the reverse primer 5′-GTGA*GGATCC*TGGTAATTGCTGAAA**CTA**-3′ containing a *Bam*HI site (shown in italics) and a stop site (shown in boldface) were obtained from Integrated DNA Technology, Coralville, Iowa. PCR (25 cycles) conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension for 2 min at 72°C; initial denaturation was for 3 min, and final elongation was for 5 min. The PCR product was gel purified, treated with *Nde*I and *Bam*HI, and then ligated into *Nde*I- and *Bam*HI-digested pET-21a(+). The coding sequence of NB nitroreductase was thus joined to the initiation codon at the *Nde*I site of the vector. The resulting plasmid, designated pJS489, was transferred to *E. coli* JM109 to check for the presence of the insert and subsequently into *E. coli* C43(DE3) for expression of *nbzA*.

The recombinant plasmid pSE380::*habA* (6) was used as the template for PCR amplification of the *habA* gene (408 bp; GenBank accession no. AF028594). The primers used were 5′-CAGTC*GAATTC***AAGGAGA**TCACATT**ATG**-3′ (*Eco*RI site in italics, and ribosome-binding site and start site in boldface) and 5′-GAT C*AAGCTT***A****CTA**ACGTAGGATACCG-3′ (*Hin*dIII site in italics and stop site in boldface). The gel-purified PCR product was double digested with *Eco*RI and *Hin*dIII and ligated with similarly treated pJS489. The resulting construct (pJS490) which couples *nbzA* and *habA* under the control of the T7 promoter of pET-21a(+) was introduced into *E. coli* C43(DE3), and the strain was designated JS995.

Construction of strain JS996. The *nfs1* gene of *E. cloacae* was isolated from the recombinant plasmid pRK1 by using PCR with the primers 5′-ATTAGA*G AATTC***CAGGAG**TTGTT**ATG**GATATCATTTCTGTCGC-3′ (*Eco*RI site in italics and ribosome-binding site and start site in boldface) and 5′-ATTA*CCC GGG***TCA**GCACTCGGTCACAATCG-3′ (*Xma*I site in italics and stop site in boldface). The amplicon (650 bp) and the recombinant plasmid pSE380::*habA* were cleaved with *Eco*RI and *Xma*I to allow subcloning of *nfs1* upstream from *habA*. The resulting plasmid (pJS491) was transferred to *E. coli* JM109, and the strain was designated JS996.

Induction of the recombinant enzymes and enzyme activities in cell extracts. Cells of *E. coli* strains carrying the recombinant plasmids were grown at 37°C in 250 ml of 2× TY medium or 2× LB medium with 1% glycerol containing 100 μg of ampicillin/ml in an incubator shaker until the cultures reached an A_{600} of 0.8. The cells were induced with 1 mM IPTG by incubating strain JS995 at 30°C and strain JS996 at 37°C with shaking for 16 h. The cells were harvested, washed twice in 20 mM potassium phosphate buffer (pH 8), and used directly in whole-cell transformation assays or for the preparation of cell extracts.

Cells were broken by two passages through a French pressure cell at 1,379 MPa. The resulting lysate was centrifuged at 17,000 × *g* for 20 min at 4°C, and the supernatant was used for enzyme assays. Reductase activity against various nitroaromatic compounds was determined spectrophotometrically by measuring the initial rate of NADPH disappearance (32). The activity of HAB mutase A was measured spectrophotometrically by monitoring the increase in absorbance at 283 nm, which indicates the formation of 2AP from HAB (6).

Whole-cell biotransformations. Initially, IPTG-induced cells of strain JS996 were suspended to an A_{600} of 2 in 20 mM potassium phosphate buffer (pH 7.0), and transformations of NB, 2-nitrotoluene, 3-nitrotoluene, 4-nitrobiphenyl ether, and 1-nitronaphthalene were performed at 37°C. For comparison of the two strains in whole-cell biotransformations, induced cells were suspended to an A_{600} of 0.75 in 25 ml of 50 mM potassium phosphate buffer, pH 8, with glucose (1%, wt/vol). The reaction was initiated by the addition of 100 μM NB to the cell suspension and incubation at 30°C with shaking. Samples of the duplicate reaction mixtures were removed at intervals, added to equal volumes of acetonitrile on ice to stop the reaction, centrifuged at 13,000 × *g* for 2 min, and analyzed by high-pressure liquid chromatography (HPLC) for product formation.

Transformation of nitroacetophenones by strain JS995 for isolation and purification of the corresponding aminophenols was carried out in 250 ml of 50 mM phosphate buffer, pH 8, containing 1% (wt/vol) glucose. Washed induced cells were suspended in the buffer to an A_{600} of 6.8 and incubated at 30°C with shaking. 2NAP or 3NAP (500 mM in ethanol) was added periodically over 4 h, and incubation proceeded for 6.5 h to provide a total of 120 and 105 mg of substrate, respectively. At the end of the incubation period, the cells were removed by centrifugation, and the pH of the supernatant was adjusted to 6.5. The solution was extracted with three 100-ml volumes of diethyl ether. The ether extracts were combined and concentrated to 30 ml under a stream of nitrogen. Aminophenols were back extracted with 50 ml of NaOH (5%, wt/vol), and the organic phase containing the amine was discarded. The pH of the aqueous phase was adjusted to 6.5, and the solution was extracted three times with 50 ml of diethyl ether. The ether was dried over sodium sulfate and concentrated to 5 ml under nitrogen. One milliliter of water was added, and the product crystallized as the remaining ether was evaporated under nitrogen.

Analytical methods. HPLC was performed on an Alltima phenyl column (5 μm; 250 by 4.6 mm; Alltech, Deerfield, Ill.) with an HP 1040 M diode array

TABLE 2. Specific activities of recombinant nitroreductases in cell extracts of *E. coli* strains[a]

Substrate	NbzA[b]	NR[c]
NB	2.5	0.1
2NAP	1.6	0.1
3NAP	2.1	0.2
4-Nitroacetophenone	1.7	0.9
2-Nitrobenzaldehyde	2.3	0.7
3-Nitrobenzaldehyde	2.0	0.3
4-Nitrobenzaldehyde	0.1	<0.1
2-Nitrobenzoic acid	0.3	<0.1
3-Nitrobenzoic acid	0.3	0.1
4-Nitrobenzoic acid	0.7	0.1
2-Nitrotoluene	1.4	<0.1
3-Nitrotoluene	1.9	0.1
4-Nitrotoluene	0.1	<0.1
DNB	2.9	5.1
4-Nitrobiphenyl ether	0.8	0.2
TNT	2.6	11.1
4-Nitrotrifluorotoluene	0.4	0.1
4-Nitrobenzothiozole	2.2	0.2
2,4-Dinitrotoluene	1.1	1.7
2,6-Dinitrotoluene	2.3	0.1
2-Amino-4-nitrophenol	0.1	<0.1
1-Chloro-4-nitrobenzene	0.4	0.2

[a] Specific activities are shown as micromoles of NADPH oxidized minute^{-1} milligram of protein^{-1}. Values are averages of duplicates; the deviation was <10%.
[b] *E. coli* JS995.
[c] *E. coli* JS996. NR, nitroreductase.

detector (Hewlett-Packard, Wilmington, Del.) at a wavelength of 235 nm. The mobile phase was acetonitrile–13.5 mM trifluoroacetic acid (40:60 for 2NAP and its metabolites or 25:75 for 3NAP and its products) at a flow rate of 1.0 ml min^{-1}. Purified 2AHAP or 3AHAP was used to construct a linear calibration curve for quantification by HPLC. NB, 2-nitrotoluene, 3-nitrotoluene, 4-nitrobiphenyl ether, and 1-nitronaphthalene and the aminophenols formed were separated on a Supelcosil LC-ABZ+Plus column (250 by 4.6 mm; Supelco, Bellefonte, Pa.) with acetonitrile-water (50:50) at 1.0 ml min^{-1}.

2AHAP or 3AHAP and their *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Alltech Associates, Inc.) or *n*-butylboronic acid derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS) with an Agilent 5973 mass spectrometer (Agilent Technologies, Inc., Palo Alto, Calif.) and an Agilent Chemstation model 6890N gas chromatograph equipped with an HP-5 MS capillary column (30 m by 0.25 mm by 0.25-μm film thickness; Hewlett-Packard) as described previously (25). Helium was the carrier gas at a constant flow rate of 0.8 ml min^{-1}. The initial column temperature was 90°C for 5 min, was increased at 20°C min^{-1} to 280°C, and was isothermal for 8 min. Nuclear magnetic resonance (NMR) analysis of the 2AHAP was performed by T. Gedris of the NMR Laboratory, Chemistry Department, Florida State University, Tallahassee. NMR analysis of 3AHAP was done at the University of Florida, Gainesville. Protein concentrations were determined by the bicinchoninic acid assay (32) with the BCA kit (Pierce, Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Expression of reductases and HAB mutase A with the recombinant systems. The activities of the nitroreductases towards the selected nitroaromatic compounds differed substantially between the two strains (Table 2). No nitroreductase activity toward NB or 2NAP was detected in extracts prepared from induced cells of the host strains carrying only the vectors. The specific activity of the recombinant NB nitroreductase expressed in strain JS995 with NB as the substrate was similar to that reported previously (2.4 U mg of protein^{-1}) in wild-type JS45 (33) and significantly higher than that (0.27 U mg of protein^{-1}) in NB-grown *Pseudomonas putida* HS12 (28). The NB nitroreductase in cell extracts of strain JS995 catalyzed rapid transformation (specific activity of >1.1 U mg of protein^{-1}) of 13 out of 22 nitroaromatic compounds tested. Transformation rates did not seem to be affected by the position of the nitro group relative to the other substituent for the mononitro compounds. Both 1,3-dinitrobenzene (DNB) and 2,4,6-trinitrotoluene (TNT) were better substrates for NB nitroreductase than was NB, the growth substrate for strain JS45. The specific activity of *E. cloacae* nitroreductase expressed in strain JS996 was relatively low toward NB. It was very active, however, with TNT and DNB. The nitroreductase activities toward DNB and TNT in the control host strain containing vector alone were 0.10 and 0.30 U mg of protein^{-1}. Both nitroreductases reduce NB to the four-electron reduction product, HAB (19, 33).

The specific activity of HabA, which plays a physiological role in the degradation of NB by strain JS45 (6), was markedly higher (7.5 U mg of protein^{-1}) in strain JS995 than in strain JS996 (0.3 U mg of protein^{-1}), wild-type JS45 (5.80 U mg of protein^{-1}) (6), or strain HS12 (1.5 U mg of protein^{-1}) (28). The expression of HAB mutase A in strain JS995 is probably facilitated by the host's ability to produce a network of internal membranes in which the overexpressed membrane proteins accumulate (39). Similarly, using the host strain *E. coli* C43(DE3), Arechaga et al. (2) have demonstrated the abundant overproduction of subunit b of *E. coli* F_1F_o ATP synthase accompanied by the proliferation of intracellular membranes without formation of inclusion bodies. Even though the expression levels of the enzymes in strain JS995 are higher than those in strain JS996, the different substrate preferences of the nitroreductase in strain JS996 might make it very useful.

Initially, to determine the potential of strain JS996 in the production of aminophenols, resting-cell experiments were performed by incubating IPTG-induced cells with various nitroaromatic compounds. The rates of substrate transformation were 2.0, 1.62, 1.13, 0.93, and 0.47 nmol min^{-1} mg of protein^{-1} for NB, 3-nitrotoluene, 4-nitrobiphenyl ether, 1-nitronaphthalene, and 2-nitrotoluene, respectively, during the initial 10 min of reaction. The reaction products were 2AP, 2-amino-4-methylphenol, 4-amino-5-phenoxyphenol, 2-amino-1-naphthol, and 2-amino-3-methylphenol, respectively, and the corresponding conversion efficiencies at the end of 30 min of incubation were 98.6, 63.8, 100, 53.6, and 27%. The results suggested that the recombinant strain could be used to convert a variety of nitroaromatic compounds to the corresponding aminophenols, but the rates were low and optimization of the system would be necessary prior to practical application.

Strains JS995 and JS996 were compared for their abilities to produce 2AP from NB in a time course experiment performed at 30°C. The conversion of NB was substantially faster in strain JS995 than in strain JS996 (Fig. 1). The specific activities for transformation of NB and production of 2AP during the initial 10 min of reaction with strain JS995 were 13.4 and 12.5 nmol min^{-1} mg of protein^{-1}, respectively, while those with strain JS996 were 5.4 and 5.0 nmol min^{-1} mg of protein^{-1}, respectively. The results taken with the data in Table 2 related to substrate range suggest that strain JS995 expressing *nbzA* and *habA* genes both derived from strain JS45 would be a more suitable biocatalyst for synthesis of *ortho*-aminophenols from the corresponding nitroaromatic compounds.

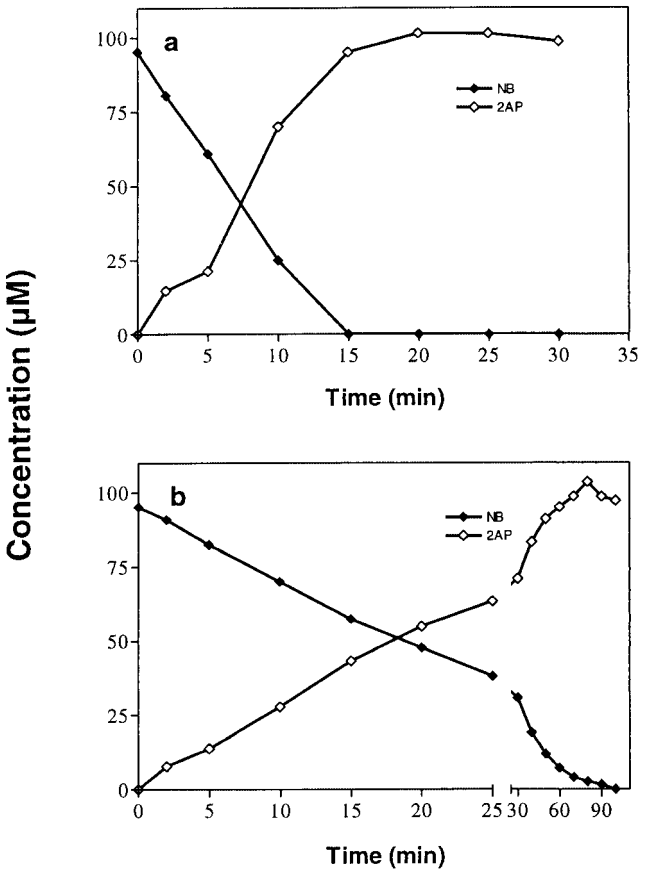

FIG. 1. Transformation of NB by whole cells of *E. coli* JS995 (a) and JS996 (b). NB was added to a cell suspension (A_{600} of 0.75) in 50 mM potassium phosphate buffer, pH 8.0, containing 1% glucose. The reaction mixture was monitored by HPLC for production of 2AP from NB.

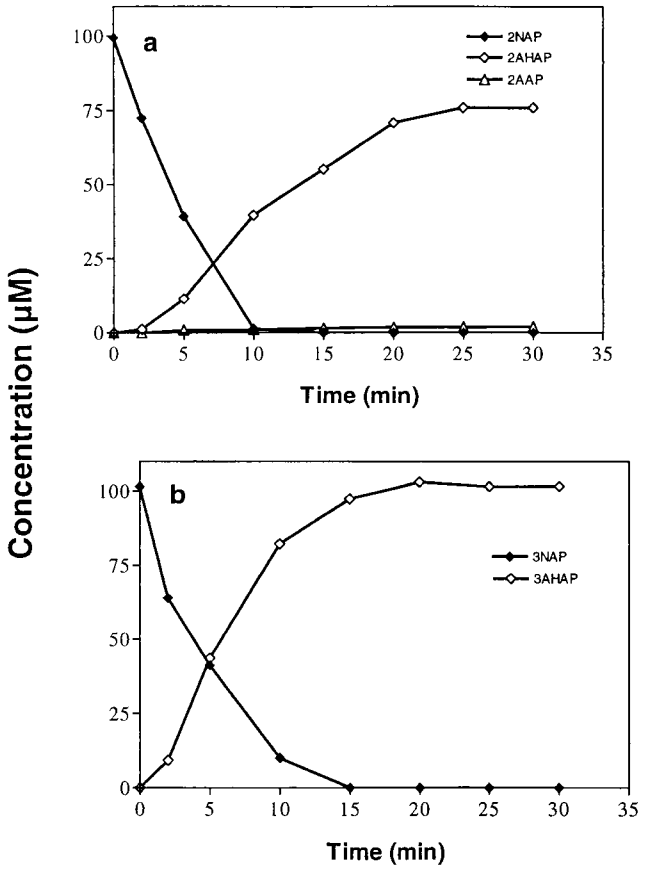

FIG. 2. Transformation of 2NAP (a) and 3NAP (b) by whole cells of strain JS995. Substrate was added to a cell suspension (A_{600} of 0.75) in 50 mM potassium phosphate buffer, pH 8.0, containing 1% glucose. Production of aminophenol or 2AAP from the nitroacetophenone was monitored by HPLC.

Biotransformation of nitroacetophenones. When IPTG-induced cells of strain JS995 were incubated with 2NAP in the absence of glucose, the transformation rate was very low, and only 50% of the added substrate was transformed at the end of 110 min of incubation (data not shown). Under similar conditions, addition of glucose (1%) resulted in a rapid and extensive transformation of 2NAP, yielding a single major product tentatively identified as 2AHAP and 2AAP as a minor product within about 10 min (Fig. 2a). The specific activity for the transformation of 2NAP during the initial 10 min of reaction in the presence of glucose was 17.6 nmol min^{-1} mg of $protein^{-1}$. The difference in specific activities between whole-cell assays and cell extracts of the constructed strain (Table 2) could be due to limitations in uptake of the substrate into the cells or availability of reducing equivalents in the whole-cell system. The results also indicate that a source for generating the required reduced cofactor for the initial reduction of 2NAP is necessary to support the transformation by whole cells. During transformation, there was a transient accumulation of a product with an HPLC reaction time (R_t) of 7.96 min and absorption maxima at 206 and 320 nm under acidic conditions. Incubation of cells of strain JS999 carrying only the *nbzA* gene with 2NAP led to accumulation of the same compound as the major product (data not shown). The product formed by the catalytic activity of the NB nitroreductase is most likely the hydroxylamino derivative of 2NAP which subsequently acts as a substrate for the HAB mutase A. The transient accumulation of the intermediate accounts for the delay between the disappearance of the substrate and the appearance of the final product (Fig. 2).

The reaction was carried out on a larger scale, and the major product was extracted for rigorous identification. The light-brown crystals of 2AHAP had a melting temperature of 180 to 183°C (reported elsewhere as 185 to 187°C [8]). The absorption maxima of 2AHAP in methanol were 232, 270, and 375 nm; the reported values are 233, 270, and 378 nm (16). Analysis of the product by GC-MS revealed a compound (R_t of 8.32 min) with a parent ion at *m/z* 151 consistent with the expected mass of 2AHAP and a base peak at 136, which indicates the loss of a CH_3 group (M-15) (Fig. 3a). The other predominant ions at *m/z* 108 (M-43), 80 (M-71), and 53 (M-98) were consistent with losses of exocyclic CO, endocyclic CO, and CNH, respectively. The BSTFA-derivatized product (R_t of 7.57 min) yielded a parent ion at *m/z* 223 and is consistent with derivatization of a single substituent of 2AHAP (data not shown). Both the amino and hydroxyl substituents can be derivatized by

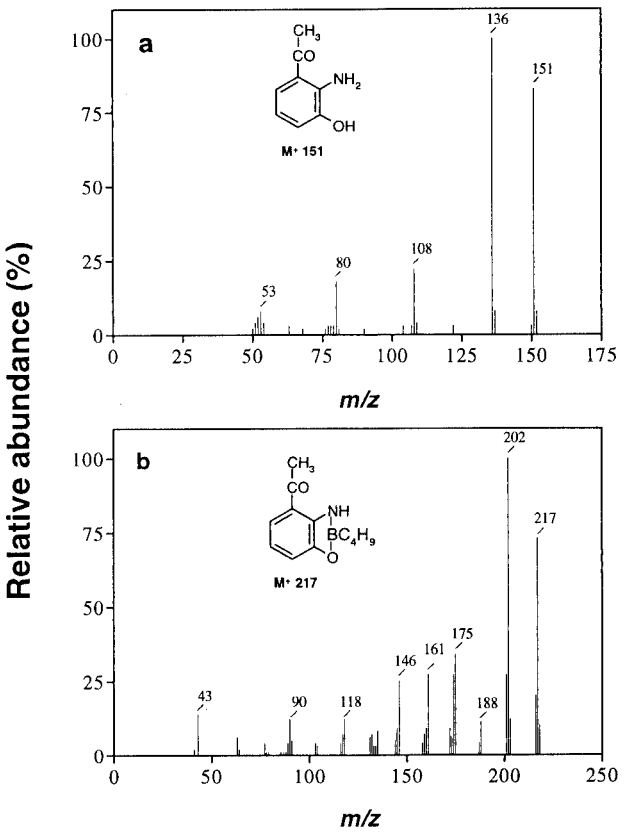

FIG. 3. Mass spectra of the end product from 2NAP transformation by strain JS995 (a) and the end product derivatized with *n*-butylboronic acid (b).

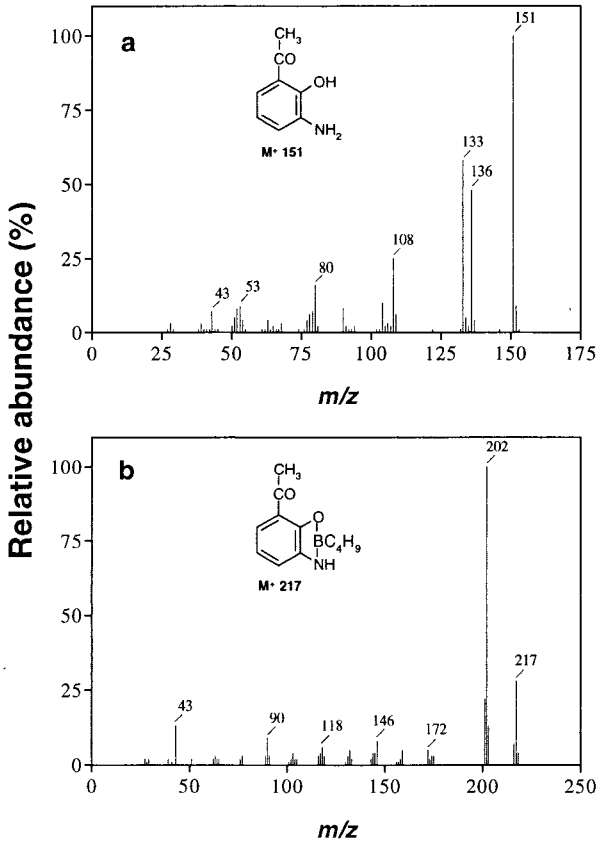

FIG. 4. Mass spectra of the end product from 3NAP transformation by strain JS995 (a) and the end product derivatized with *n*-butylboronic acid (b).

BSTFA, but the hydroxyl group is more reactive (18). Other fragment ions at *m/z* 208 (M-15), 192 (M-31), 166 (M-57), 150 (M-73), and 73 (M-150) correspond to the loss of CH_3, NH_2, CN, CH_3 and $C_8H_8NO_2$, respectively. To verify the position of the substituents, the product was derivatized with *n*-butylboronic acid. The only product formed had a GC-MS R_t of 8.96 min, with a parent ion at *m/z* 217 and a base peak at 202 (Fig. 3b). The results clearly indicate the presence of vicinal functional groups with reactive protons and a structure consistent with rearrangement of a hydroxylamine to the corresponding aminophenol. Based upon mass spectra and NMR data (Fig. 3) (8), the major product from 2NAP was identified conclusively as 2AHAP.

During larger-scale transformation by strain JS995, 3 mM 2NAP yielded 2.2 mM 2AHAP; thus, the conversion efficiency of the reaction was 75%. No attempt was made to optimize the transformation, extraction, and recovery procedure. The only other detectable product, accounting for <10% of the substrate transformed, was 2AAP. It was, however, not clear whether nonspecific reductases of *E. coli* or the NB nitroreductase and HAB mutase A in strain JS995 were responsible for the formation of 2AAP. During solvent extraction, 2AAP partitioned into the diethyl ether phase at a pH of 13.4 and thus could be separated from 2AHAP, which remained in the aqueous phase. The aminophenol was stable in neutral solution at 4°C for more than 8 months.

By using several steps in a complex chemical process with 2-nitro-3-methoxyacetophenone as an intermediate, Kaseda et al. (16) synthesized 2AHAP with a yield of less than 13%. Escobar et al. (8), however, reported a 96% yield of 2AHAP by catalytic hydrogenation of 3-hydroxy-2-nitroacetophenone, an intermediate synthesized initially by a chemical process. The results presented here indicate that whole cells of strain JS995 catalyze the rapid conversion of 2NAP to 2AHAP in good yield and in a single reaction.

Strain JS995 also transformed 3NAP very rapidly to a single product tentatively identified as 3AHAP in stoichiometric amounts (Fig. 2b). A metabolite formed transiently (R_t of 5.56 min) during the conversion of 3NAP and disappeared with the concomitant accumulation of 3AHAP. The intermediate is likely to be the hydroxylaminoacetophenone based on analogy with the above results during conversion of 2NAP to 2AHAP. The reaction was carried out on a larger scale, and the product was extracted for detailed analysis. The melting point of the brown crystalline product was 185 to 187°C, and the absorption maxima in methanol were 231, 274, and 360 nm. The mass spectrum of the compound (R_t of 7.19 min) revealed a parent ion at *m/z* 151, a base peak at 136 (M-15), and other fragment ions at *m/z* 133 (M-18), 108 (M-43), 80 (M-71), and 53 (M-98), corresponding to the loss of H_2O, exocyclic CO, endocyclic CO, and CNH, respectively (Fig. 4a). As with 2AHAP, derivatization of the product from 3NAP with *n*-butylboronic acid

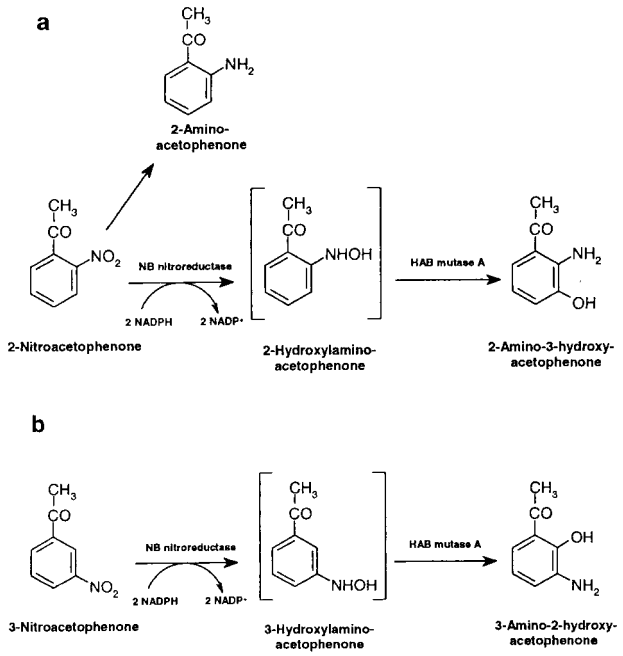

FIG. 5. Conversion of 2NAP to 2AHAP (a) and 3NAP to 3AHAP (b) catalyzed by strain JS995.

yielded a single compound with a GC R_t of 8.44 min, a parent ion at *m/z* 217 consistent with the expected mass of the product derivatized at adjacent hydroxyl and amino groups, and a base peak at 202 (Fig. 4b). The NMR analysis (dimethyl sulfoxide-d_6) of the product formed from 3NAP revealed the following data: ^{1}H NMR, δ 2.60 (s, 3H, CH_3), 6.70 (dd, 1H, H_5), 6.88 (dd, 1H, H_4), 7.12 (dd, 1H, H_6), 4.99 (s, 1H, OH), 3.32 (s, 2H, NH and OH), and ^{13}C NMR, δ 27.1 (CH_3), 119.6 (C-5), 133.1 (C-1), 119.6 (C-4), 118.8 (C-6), 162.7 (C-2) 149.5 (C-3), and 206.6 (CO). The results are consistent with the structure of 3AHAP (24).

A Japanese patent (KOKAI publication no. 95144/1991) describes the chemical synthesis of 3AHAP from *p*-chlorophenol by a multistep process involving acetylation followed by Fries rearrangement, nitration, and catalytic reduction (24). The stoichiometric conversion of 3NAP to 3AHAP by the biocatalyst in a single reaction provides a substantial improvement over the multistep process. The 2- and 3-hydroxylaminoacetophenone intermediates were immediately detected in the reaction mixture, and their presence may account for the discrepancy between the amount of substrate transformed and that of aminophenol produced, especially during the early time points of the transformation (Fig. 2). Hydroxylamino aromatic compounds can be unstable in the presence of oxygen. The instability could account for the relatively lower conversion efficiency with 2NAP.

Partially purified NB nitroreductase and HAB mutase from strain JS45 were used previously for synthesis of 2-amino-5-phenoxyphenol from 4-nitrobiphenyl ether (25). The process is efficient but slow and requires regeneration of NADPH consumed during the transformation. Due to the high cost of cofactor-dependent purified enzyme reactions, whole cells are often used for synthetic reactions that require cofactors (31). It is generally easier and less expensive to regenerate cofactors in metabolically active cells by adding cheap and readily available carbon sources such as glucose. The results presented in this study (Fig. 5) indicate that strain JS995 has a considerable potential in synthesizing *ortho*-aminophenols with glucose as an inexpensive substrate to provide energy for the process. In addition, construction of *E. coli* strains expressing both nitroreductase and HAB mutase overcomes problems associated with the use of purified enzymes or wild-type strains for biosynthesis of *o*-aminophenols. The regiospecificities of the reactions are remarkable and would be difficult to achieve with traditional organic chemistry. Production of other novel aminophenols from nitroaromatic compounds using the strain will be the subject of future research.

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