Azo dyes are regarded as pollutants because they are not readily reduced under aerobic conditions. Bacillus sp. OY1-2 transforms azo dyes into colorless compounds, and this reduction is mediated by a reductase activity for the azo group in the presence of NADPH. A 1.2-kbp EcoRI fragment containing the gene that encodes azoreductase was cloned by screening the genomic library of Bacillus sp. OY1-2 with digoxigenin-labeled probe designed from the N-terminal amino acid sequence of the purified enzyme. An open reading frame encoding the azoreductase, consisting of 178 amino acids, was predicted from the nucleotide sequence. In addition, because only a Bacillus subtilis hypothetical protein was discovered in the public databases (with an amino acid identity of 52.8%), the gene encoding the azoreductase cloned in this study was predicted to be a member of a novel family of reductases. Southern blot analysis revealed that the azoreductase gene exists as a single copy gene on a chromosome. Escherichia coli-expressing recombinant azoreductase gave a ten times greater reducing activity toward azo dyes than the original Bacillus sp. OY1-2. In addition, the expressed azoreductase purified from the recombinant E. coli lysate by Red-Sepharose affinity chromatography showed a similar activity and specificity as the native enzyme. This is the first report describing the sequencing and characterization of a gene encoding the azo dye-reducing enzyme, azoreductase, from aerobic bacteria and its expression in E. coli.

Molecular Cloning and Characterization of the Gene Coding for Azoreductase from Bacillus sp. OY1-2 Isolated from Soil*

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Experimental Procedures

Bacterial Strains, Plasmids, and Culture Conditions—Azo dye-degrading bacteria isolated from soil near a waste-water plant from a textile factory were identified as Bacillus sp. OY1-2 based on biological characterization (9). This strain can grow in brain-heart infusion broth (Difco). E. coli strains C600hfl and XLI-Blue were cultured in Luria broth consisting of 10 g of bacitryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl per liter. The E. coli strains GI724 and GI618 were cultured in RMG medium consisting of 40 g of cazamino acids (Difco), 5 g of glycerol, 1 mM MgCl2, 6 g of Na2HPO4, 3 g of KH2PO4, 0.5 g of NaCl, 1 g of NH4Cl per liter. Recombinant proteins were expressed by E. coli strains GI724 or GI618 in LB medium consisting of 0.4 g of casamino acid, 5 g of glucose, 1 mM MgCl2, 6 g of Na2HPO4, 3 g of KH2PO4, 0.5 g of NaCl, 1 g of NH4Cl per liter. A phage vector λgt10 was used for the construction of the genomic library. The plasmid pUTBluetR/V (Novagen, Inc.) and pUC18 were used for the subcloning of genes. The plasmids pTrx-Fus (Invitrogen Co.) and pTrcNd (reconstructed from pQE30 (Qiagen Inc.) were used for expression of recombinant azoreductase.

N-terminal Amino Acid Sequence of Native and Recombinant Azoreductase—Samples of native and recombinant azoreductase were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad). The blotted protein strips were used for amino acid sequencing on a PE Biosystems 470/120A protein sequencer.

Construction of Bacillus sp. OY1-2 Genomic DNA Library—Bacillus
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This paper is available on line at http://www.jbc.org
Azoreductase Gene Cloned from Bacillus sp.

**FIG. 1. Reduction of Methyl Red by azoreductase.** Methyl Red was treated with crude azoreductase solution in the presence of β-NADPH. Products were analyzed by gas chromatography and identified as dimethyl p-phenylenediamine and o-aminobenzoic acid (9).

Sp. OYI-2 genomic DNA was prepared by mechanical disruption as described previously (11). Briefly, bacterial pellet from 5 ml of liquid culture was suspended in 0.5 ml of lysis buffer consisting of 0.3 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 6 mM EDTA. The cell suspension was transferred into a conical 2-ml screw-cap vial, which is one-fourth filled with 0.17-mM-activated starch gel beads. Cells were disrupted by vigorous shaking with 0.5 ml of chloroform on a Mini-Bead cell disrupter (BioMack Products, Bartlesville, OK) for 5 min. DNA in the upper layer after centrifugation was further purified by phenol/chloroform extraction, concentrated by ethanol precipitation, and dissolved in 300 μl of TE buffer consisting of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The purified genomic DNA was completely digested with EcoRI and ligated into the EcoRI site of pgt10. Genomic library constructs were introduced into E. coli strain C600hfl by means of in vitro packaging using gigapack plus (Stratagene).

**Generation of Probe for Screening—**The N-terminal amino acid sequence was used to design oligonucleotide primers for amplifying the DNA fragment encoding the N-terminal of azoreductase (Fig. 2A). The reaction mixture (50 μl) consisted of long and accurate (LA) PCR buuffer II (Mg2+-free); 2.5 mM MgCl2; 200 mM each dATP, dCTP, dGTP, and dTTP; 10 ng of DNA from Bacillus sp. OYI-2; 1.25 units of Takara LA Taq DNA polymerase (Takara Shuzo Co., Ltd., Japan); and 0.5 mM each primer AZR-1 and AZR-2 (Fig. 2A). PCR was carried out for 30 cycles in a Takara Thermal Cycler Personal (Takara Shuzo Co., Ltd.) with each cycle consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and extension for 1 min at 72 °C. The PCR product was extracted from the gel after separation on a 1% agarose gel and was directly subcloned into the pTZ18R vector. The subclones were sequenced as shown in Fig. 2 by the dideoxy chain termination method with a Model 310 genetic analyzer.

**Southern Blot Hybridization—**Detection of the restriction DNA fragment carrying the azoreductase gene was performed according to Southern (14). One μg of genomic DNA was completely digested with restriction enzymes, separated on a 0.7% agarose gel, and vacuum-transferred to Nitran 13N nylon filters. The filters were prehybridized in ExpressHyb hybridization solution at 68 °C for 30 min followed by hybridization with the same solution containing a 10 ng/ml Dig-labeled 1.2-kbp EcoRI DNA fragment carrying the whole coding region of azoreductase. After hybridization, the filters were washed for 5 min in 2× SSC and 0.1% SDS at room temperature followed by washing for 15 min in 0.2× SSC and 0.1% SDS at 48 °C. The hybridized probe was detected after 30 min of incubation at room temperature with alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab; Roche Diagnostics Co.) diluted 1:5000. The enzyme-catalyzed color reaction was carried out using a nitro blue tetrazolium salt (NBT/5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Wako Pure Chemical Industries, Japan) in Buffer 3 consisting of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl2. The DNA inserts in the positive clones were subcloned into the EcoRI site of pUC18 for further characterization. The EcoRI fragment in the subclone was digested with SpH1, NlaIV, or HincII; further subcloned in pUC18; and sequenced as shown in Fig. 2 by the dyeoxy chain terminalization method with a Model 310 genetic analyzer.

**Database Search—**Protein and DNA sequences with homology to the deduced amino acid sequence of the azoreductase ORF were searched using BLAST from the National Center for Biological Information. **Southern Blot Hybridization—**Detection of the restriction DNA fragment carrying the azoreductase gene was performed according to Southern (14). One μg of genomic DNA was completely digested with restriction enzymes, separated on a 0.7% agarose gel, and vacuum-transferred to Nitran 13N nylon filters. The filters were prehybridized in ExpressHyb hybridization solution at 68 °C for 30 min followed by hybridization with the same solution containing a 10 ng/ml Dig-labeled 1.2-kbp EcoRI DNA fragment carrying the whole coding region of azoreductase. After hybridization, the filters were washed for 5 min with 2× SSC and 0.1% SDS at room temperature followed by washing twice with 2× SSC/0.1% SDS for 15 min at 68 °C. The hybridized Dig-labeled probe on the filters were detected by alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by color development using NBT/BCIP as substrates in Buffer 3.

**Expression of Azoreductase in E. coli—**The entire open reading frame of azoreductase was amplified by PCR. Briefly, the reaction mixture (50 μl) consisted of LA-PCR buffer II (Mg2+-free); 2.5 mM MgCl2; 200 mM each dATP, dCTP, dGTP, and dTTP; 10 ng plasmid pT7B-1R5–8; 1.25 units of Takara LA Taq DNA polymerase, and 0.5 μM each of primers AZR-rec-S-Nde (CATATGAAAATCTGTTATTAAC) and AZR-rec-E-Xba (TCTTAGACGAGATGATTGCTC). PCR was carried out for 30 cycles in a Takara Thermal Cycler Personal, with each cycle consisting of denaturation for 30 s at 94 °C annealing for 30 s at 55 °C, and extension for 1 min at 72 °C. The PCR product was extracted from the gel after separation on a 1% agarose gel electrophoresis and subcloned into pETBlue/R/E for confirmation of the nucleotide sequence and then transferred into expression vectors pTrx-Fus or pTrcNde after digestion by NdeI and XbaI. Expression of reductase in pTrx-Fus system was performed by adding tryptophan at a concentration of 0.1 mg/ml in medium. Expression in pTrcNde was performed by adding isopropyl-β-D-thio-galactopyranoside (IPTG) at a concentration of 1 mM in Luria-Bertani medium. Cells from 10 ml of induced culture were suspended in
0.5 ml of 20 mM sodium phosphate buffer, pH 7.0, lysed by two cycles of freezing at −80 °C and thawed at 37 °C followed by sonication (15 s, 70% output, 10×). The supernatants from a 9000 × g, 30 min centrifugation were used directly for enzyme assay or SDS-PAGE analysis.

**Purification of Recombinant Azoreductase by Red-Sepharose CL-6B**—The cells from 200 ml of culture were suspended in 20 ml of 20 mM sodium-phosphate buffer, pH 7.0 and lysed by freezing and thawing followed by sonication (15 s, 70% output, 10×). After centrifugation at 9000 × g for 30 min, the supernatant was applied to a Red-Sepharose CL-6B column (Amersham Pharmacia Biotech) followed by washing with 20 ml sodium phosphate buffer, pH 7.0. The recombinant azoreductase was eluted from the column with 10 mM β-NADH. The eluate was dialyzed against two changes of 1000 volumes of 20 mM sodium phosphate buffer, pH 7.0 and used for enzyme assay.

**Enzyme Assays**—Azo dye-reducing activity was analyzed by measuring the decrease in optical density at suitable wavelengths with a Hitachi U 3300 spectrophotometer at various temperatures basically according to Pasti-Grigsby et al. (15). The reaction mixture in a total volume of 1.0 ml consisted of various concentrations of azo dyes (Rocceline, Solar Orange, Sumifix Black B; shown in Fig. 3) in 20 mM sodium phosphate buffer, pH 7.0, and bacterial lysates or purified enzyme. The reaction mixture was preincubated for 5 min at the assay temperature, and the reaction was started by the addition of 25 μl of various concentrations of β-NADPH. The enzymatic activities were measured by the decrease in optical density at optimal wavelengths. The enzyme activity was expressed as the amount of reduced dye per min with 1 mg of enzyme. Kinetic parameters for the reduction of each dye by native and recombinant azoreductases were estimated by nonlinear regression analysis according to Shimada et al. (16).

**RESULTS AND DISCUSSION**

We have been interested in biological reduction of azo dyes under aerobic conditions. The utilization of azo dye-degrading microorganisms is very important for generating an efficient bioreactor for waste-water treatment plants in industries that use azo dyes. In addition, these enzymes can be applied for white discharge printing of cloths. In the course of our study, we have isolated three bacterial strains that can reduce azo dyes under aerobic conditions. A constitutively expressed enzyme in *Bacillus* sp. OY1-2 (one of the isolated strains) was purified from bacterial cell lysates and characterized with respect to reduction of different kinds of azo dyes in the presence of β-NADPH (10). Subsequently, we cloned and characterized the gene encoding this protein activity.

**Cloning of the Gene Encoding the Azoreductase**—To clone the azoreductase gene of *Bacillus* sp. OY1-2, the N-terminal amino acid sequence of purified azoreductase was determined. Because there was neither significant amino acid nor nucleotide homology to the N-terminal amino acid sequence in the databases, this protein was determined to be novel. We attempted to clone the gene encoding this enzyme by means of screening the genomic library of *Bacillus* sp. OY1-2 with digoxigenin-labeled probe amplified by PCR using primers designed from the N-terminal amino acid sequence of azoreductase (Fig. 2A). A 0.1-kbp DNA fragment was amplified by PCR using primers AZR-1 and AZR-2. The PCR product carrying the DNA fragment encoding the N-terminal part of azoreductase was extracted from the agarose gel, directly subcloned into the pT7Blue(R/T) vector, and sequenced (Fig. 2B). A Dig-labeled hybridization probe was synthesized by PCR using the plasmid carrying the azoreductase N-terminal part as a template. A genomic library of *Bacillus* sp. OY1-2 was constructed with the *Eco*RI-digested phage vector λgt10 by ligation with the completely *Eco*RI-digested fragments of total genomic DNA. Transfection of the host *E. coli* C600hfl by the *in vitro* packaged library gave independent clones from 1.1 × 10^6 plaques. By using Dig-labeled probe, seven clones with inserts of the same length were isolated from the 2 × 10^6 plaques. Inserts in these seven clones were amplified by PCR and sequenced. One of the clones (λgt10-AZR5) was then subcloned into the *E. coli* vector pUC18 (pUC18-AZR5–8) for further analysis. The entire sequence of the 1.2-kbp insert was determined according to the sequencing strategy shown in Fig. 4, and an ORF was found in this fragment (Fig. 5) using the specific nucleotide sequence as a probe. An inframe initiation codon (ATG) was located at nucleotide 32, as indicated in Fig. 5 (boxed), and a putative Shine-Dalgarno sequence GGAG (17) (Fig. 5, double underline) was found in its upstream region. The deduced amino acid sequence from this initiation codon showed good agreement with the N-terminal amino acid sequence determined from the purified native azoreductase (Fig. 5, underline). In addition, the molecular mass of the azoreductase product calculated from the gene encoding azoreductase was 19,423 Da, which exhibited good agreement with the molecular mass of 20 kDa of the purified azoreductase (10). Thus we concluded that the ORF found in this 1.2-kbp *Eco*RI DNA fragment coded for the azoreductase. However, as the upstream region of this clone was only 31 bp, the sequence of the putative promoter region could not be identified. When we searched for homologs from other organisms in the DNA and protein databases, an ORF of *Bacillus subtilis* (function unknown) was obtained with an amino acid sequence homology of 52.8% (GenBank™/EBI database, accession no. Y14079), although it was not present in...

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**Fig. 3. Structure of azo dyes used for reduction assay.** A, Rocceline; C.I., color index name. B, Solar Orange. C, Sumifix Black B.
Azoreductase Gene Cloned from Bacillus sp.

**Southern Blot Hybridization**—Fragments of genomic DNA generated by digestion with restriction enzymes were separated on a 0.7% agarose gel, transferred onto the nylon filter, and hybridized with the 1.2-kbp EcoRI fragment of genomic DNA carrying the entire open reading frame of azoreductase. The probe hybridized to DNA fragments of length 8.0, 20, 25, 6.6, 5.0, 1.2, and 11 kbp cut by restriction enzymes BamHI, EcoRI, HindIII, PstI, SalI, SmaI, and XhoI, respectively (Fig. 7). The probe hybridized to only one band for each restriction enzyme tested, indicating that the azoreductase gene exists as a single copy gene on the chromosome.

**Expression of the Azoreductase in E. coli**—The entire open reading frame of the azoreductase gene was amplified by PCR using pUC18-AZR5–8 as a template, was inserted into expression vectors pTrx-Fus and pTrcNd, and was transformed into E. coli to express recombinant azoreductase (Fig. 8A). The expression of azoreductase was observed not in pTrcNd but in pTrx-Fus (data not shown). The reason that the azoreductase was expressed only by the pTrx-Fus system is not clear although it might be attributed to the different promoters as follows: the PL-Trp promoter used in pTrx-Fus can be controlled strictly by λcI repressor and tryptophan starvation, whereas the control of the trc promoter (fusion promoter of T5 phage promoter and lactose operator, inducible by addition of IPTG) used in pTrcNd is more leaky than the PL-Trp promoter. The expressed azoreductase may have a negative effect on the growth or survival of E. coli and inhibit the colony formation of transformed E. coli in the pTrcNd system.

The expressed azoreductase in plasmid (pTrx-Fus) in E. coli GI724 and GI618 was analyzed by SDS-PAGE to identify the protein with a molecular mass of 20 kDa (Fig. 8B). The 20-kDa protein on SDS-PAGE was then transferred onto a polyvinylidene membrane and subjected to N-terminal amino acid sequence determination. The resulting N-terminal amino acid sequence corresponds to the N-terminal amino acid sequence of native azoreductase. These results indicate that the recombinant protein obtained in this study was the same as the native azoreductase obtained from Bacillus sp. OY1-2.

**Azo Dye-degrading Activity of Crude Recombinant Azoreductase**—The E. coli GI724 and GI618 expressing recombinant azoreductase were lysed by freezing and thawing followed by sonication. Recovery of supernatant fractions following centrifugation and SDS-PAGE analysis revealed larger amounts of recombinant azoreductase in the GI618 supernatant than the GI724 samples (data not shown). The activity of azoreductase in 5 μl of the sonicate supernatant (containing 0.1 mg of protein) of GI618 was compared with that of original Bacillus sp. OY1-2. The azo dyes Roccblin, Solar Orange, and Sumifix Black B were used as substrates for this assay. These compounds were reduced into a colorless mixture by the azoreductase in the sonic supernatant of GI618. The azoreductase activity in the GI618 supernatant was ten times or more greater than that of Bacillus sp. OY1-2 (Fig. 9). These results indicate that the recombinant azoreductase expressed in E. coli contains a large amount of azoreductase activity.

**Purification of Recombinant Azoreductase from E. coli**—The recombinant azoreductase was purified from the sonicate supernatant of GI618 bacterial lysate by means of affinity purification by Red-Sepharose column chromatography. The recombinant azoreductase was eluted with 10 mM β-NADPH. The azoreductase activity in the GI618 supernatant was ten times or more greater than that of Bacillus sp. OY1-2 (Fig. 10A). This result indicates that the recombinant azoreductase expressed in E. coli contains a large amount of azoreductase activity.

**Purification of Recombinant Azoreductase from E. coli**—The recombinant azoreductase was purified from the supernatant of GI618 bacterial lysate by means of affinity purification by Red-Sepharose column chromatography. The recombinant azoreductase was eluted with 10 mM β-NADPH (Fig. 10A) and was purified in one step of column chromatography (Fig. 10B). In contrast, our standard protocol for purifying the azoreductase from the lysate of Bacillus sp. OY1-2 required four steps of column chromatography (DEAE-cell-
urofine, Blue-cellurofine, and Red-Sepharose followed by gel filtration, Ref. 10). However, as the amount of expressed recombinant azoreductase in *E. coli* in this system was very large, only the Red-Sepharose column chromatography was necessary to obtain purified azoreductase of the same quality.

Thus, the azoreductase expression system designed in this study may make it possible to obtain large amounts of purified azoreductase in a very simple manner.

Azo Dye-reducing Activity of Recombinant Azoreductase—The eluate obtained by NADH elution from Red-Sepharose was then dialyzed against 20 mM sodium phosphate buffer, pH 7.0 and used in the azoreductase enzyme assay. The enzyme assay was performed at different reaction temperatures from 20 to 85 °C in a reaction mixture containing 20 μM Roccellin and 250 μM β-NADPH. The maximum specific activity of recombinant azoreductase (7.60 μmoles/min/mg protein) was achieved at 50 °C whereas that of native enzyme (11.7 μmoles/min/mg protein) was achieved at 70 °C (Fig. 11). The maximal specific activity of recombinant azoreductase was 1.54 times lower and the optimal temperature was 20 °C lower than that of the native form. These results indicate that the temperature stability of recombinant azoreductase was lower than that of the native enzyme. The reason why the temperature stability of
recombinant enzyme declined was not defined in this study, but will be elucidated in future studies.

Kinetic analysis was performed with different concentrations of substrates at 25 °C in a reaction mixture consisting of 20 mM sodium phosphate buffer, pH 7.0, 20 mM $\beta$-NADPH, and native and recombinant enzymes. The value of $V_{\text{max}}$ and $K_m$ for three dyes were estimated by nonlinear regression analysis (Fig. 12). The $V_{\text{max}}/K_m$ value of recombinant azoreductase for Roccelline was 3.03, which is very close to 2.78 of the native enzyme. Similar observations were found with other substrates (Sumifix Black B, 0.15 and 0.14; Solar Orange, 0.013 and 0.014; by native and recombinant azoreductase, respectively). The efficiency ($V_{\text{max}}/K_m$) for Roccelline was about 20- and 200-fold greater than for Sumifix Black B and Solar Orange, respectively, indicating that the former substrate was better than the others. Considering the structure of dyes used in this study (Fig. 3), the compounds with paired naphthalene groups coupled with the azo group may serve as good substrates. This should be clarified in future studies.

Heiss et al. (21) described the cloning of DNA from a Rhodococcus strain that confers the ability of decolorizing azo dyes. However, no characterization of the gene was presented in his study. Recently, the azoreductase gene was cloned from an anaerobic bacteria Clostridium perfringens (Agt11 genomic library) by means of screening with an antibody raised against purified azoreductase (10). Rafii and Coleman (10) observed azoreductase activity in cell lysates of lytic and lysogenic E. coli cultures infected with recombinant phage. In addition, they have demonstrated the existence of a similar gene in some anaerobic bacteria with Southern hybridization techniques. As the nucleotide sequence of the azoreductase of C. perfringens was not presented in the study, it is not clear whether the enzyme involved in the reduction of azo dye in C. perfringens has some homology with the azoreductase gene cloned in this study. This should be investigated in the future. However, with regard to waste-water treatment plant construction using azoreductases, reduction of azo dyes under the aerobic conditions reported in this study is more practical. Thus, the azoreductase gene cloned and characterized in this study may be a good candidate for construction of a bioreactor for the treatment of azo dye-containing waste-water.

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