Published in final edited form as:

Protein Expr Purif. 2004 April ; 34(2): 302–310. doi:10.1016/j.pep.2003.12.016.

Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*

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Abstract

Azo dyes represent a major class of synthetic colorants that are ubiquitous in foods and consumer products. *Enterococcus faecalis* is a predominant member of the human gastrointestinal microflora. Strain ATCC 19433 grew in the presence of azo dyes and metabolized them to colorless products. A gene encoding a putative FMN-dependent aerobic azoreductase that shares 34% identity with azoreductase (AcpD) of *Escherichia coli* has been identified in this strain. The gene in *E. faecalis*, designated as *azoA*, encoded a protein of 208 amino acids with a calculated isoelectric point of 4.8. AzoA was heterologously overexpressed in *E. coli* with a strong band of 23 kDa on SDS-PAGE. The purified recombinant enzyme was a homodimer with a molecular weight of 43 kDa, probably containing one molecule of FMN per dimer. AzoA required FMN and NADH, but not NADPH, as a preferred electron donor for its activity. The apparent K_m values for both NADH and 2-[4-(dimethylamino)phenylazo]benzoic acid (Methyl red) substrates were 0.14 and 0.024mM, respectively. The apparent V_{max} was 86.2 μ M/min/mg protein. The enzyme was not only able to decolorize Methyl red, but was also able to convert sulfonated azo dyes Orange II, Amarant, Ponceau BS, and Ponceau S. AzoA is the first aerobic azoreductase to be identified and characterized from human intestinal gram-positive bacteria.

Keywords

Enterococcus faecalis; Aerobic azoreductase; Azo dye; Human intestinal microflora

Azo dyes, which are characterized by one or more $R_1-N=N-R_2$ bonds, are the most important group of commercially used synthetic dyes. They are found widely in textiles, plastics, pharmaceuticals, food, and cosmetic products [1,2]. These dyes are reduced to aromatic amines by enzymatic mechanisms and some of the aryl amines are presumed carcinogens. Several bacterial strains have been shown to reduce azo compounds under anaerobic conditions [1,2]. Early studies demonstrated that azo reduction under anaerobic conditions may represent a fortuitous, nonenzymatic reduction by enzymatically generated reduced flavins, whereas inhibition by oxygen is due to regeneration of the oxidized form [3]. The abilities of cytosolic flavin reductases to act in vitro as azoreductases were recently

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demonstrated using a recombinant flavin reductase in different genetic backgrounds [4]. In the aerobic metabolism of azo dyes, bacterial strains were mainly grown aerobically with complex media and then incubated without shaking in the presence of different azo dyes [2]. Several bacterial strains isolated from soil, which aerobically decolorize azo dyes, have been previously described [5–8]. Recently, the genes encoding a few aerobic azoreductases have been cloned from *Bacillus sp.* OY1-2 [7], *Escherichia coli* [9], *Xenophilus azovorans* KF46F [10], and *Pigmentiphaga kullae* K24 [11]. The deduced protein sequences from these cloned genes do not show significant homologies with each other and probably evolved from different origins.

Human intestinal microflora are exposed to azo dyes both from environmental contamination and from the use of these dyes in foods, drugs, and cosmetics. These microflora play an important role in the metabolism of xenobiotics including azo dyes with azo reduction being the most important reaction related to the toxicity and mutagenicity of these compounds [1]. The initial reduction of benzidine-based azo dyes is the result of the azoreductase activity of intestinal microbiota. These metabolites such as benzidine, 4-aminobiphenyl, monoacetylbenzidine, and acetylamino-biphenyl were mutagenic in the *Salmonella*/microsome mutagenicity test system [12–16]. With the exception of the azoreductase gene cloned from *E. coli* [9], no other aerobic azoreductases from the intestinal microflora, which is composed of a large number of gram-positive and gram-negative bacteria, have been demonstrated.

Enterococci are the predominant gram-positive cocci of the human intestinal flora [17,18]. In some instances, they are the cause of a variety of infections including endocarditis, neonatal, central nervous system, respiratory tract, and skin infections [19,20]. Enterococci have also garnered much attention due to their increasing resistance to antibiotics in nosocomial infections [21]. Enterococcal infections have increased with the enhanced use of broad-spectrum antibiotics. In humans, about 90% of the enterococcal infections are caused by *Enterococcus faecalis* [22–24]. Among several bacterial species isolated from rat caecal contents, cell-free extracts of *E. faecalis* possessed the highest specific azoreductase activity [25].

The focus of our research is to investigate the biodegradation and bioconversion of pigments in food products, drugs, tattoos, and permanent makeup by the intestinal microflora. In addition, we are determining the physicochemical properties of azoreductases and elucidating the role of the intestinal microflora in the metabolism of these compounds to potentially genotoxic intermediates. In this study, we describe the cloning and identification of a gene, *azoA*, encoding an aerobic FMN-dependent azoreductase from *E. faecalis*. Characterization of the purified AzoA overexpressed in *E. coli* demonstrated that this azoreductase was able to decolorize the azo dye 2-[4-(dimethylamino)phenylazo] benzoic acid (Methyl red), as well as a wide range of more structurally complex sulfonated azo dyes.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Enterococcus faecalis ATCC 19433 was grown in brain heart infusion (BHI) broth or on BHI agar plates and used for inoculum and genomic DNA preparation. *E. coli* TOP10F' (Invitrogen), NovaBlue (DE3) (Novagen), and BL21-Gold(DE3)pLysS (Stratagene) were used for recombinant DNA studies. *E. coli* strains were cultured at 37 °C in Luria–Bertani (LB) medium with appropriate antibiotics (50 µg/ml). The plasmids pCR2.1-TOPO (Invitrogen) and pET-11a (Stratagene) were used for cloning and expression, respectively.

Culture conditions for *E. faecalis* to grow in various azo dyes

Enterococcus faecalis from a BHI agar plate was inoculated into 20 ml BHI medium supplemented with individual azo dye at a final concentration of 0.2mM and incubated at 37 °C for 20 h without shaking in air. The cells were harvested by centrifugation at 5000g. The supernatants were used to assay the azo dye reduction by measuring residual absorption at the appropriate wavelength for each azo dye, as described in the enzyme assay description below. The cells were suspended in 1ml of 25mM sodium phosphate buffer, pH 7.1, and disrupted mechanically with sea-sand using a Vortex Gene2 (Scientific Industries) for 15 min at 4 °C. The mixtures were then centrifuged at 14,000g for 10 min at 4 °C and the supernatant fraction was collected and used as cell-free extract.

Cloning of *E. faecalis* *azoA* gene and expression of AzoA in *E. coli*

Genomic DNA of *E. faecalis* was isolated essentially as described by Wang et al. [26]. Plasmids from *E. coli* Top10F' and NovaBlue (DE3) were isolated using a Qiaprep Spin Miniprep kit (Qiagen). A DNA fragment containing the putative *E. faecalis* *azoA* was obtained by PCR with the genomic DNA of *E. faecalis* used as template. The forward primer included a *Nde*I site before the start codon: 5'-gattcatatgtcaaaattattagttg-3' (Efs-forward). The reverse primer included a *Bam*HI site downstream of *azoA*: 5'-cgggatccgctttatgccgcagc taag-3' (Efs-reverse). PCR was performed in a Mastercycler gradient (Eppendorf). The amplification conditions were one cycle of 95 °C for 3 min, 30 cycles with each cycle including 30 s of melting at 95 °C, 30 s of annealing at 50 °C, and 60 s of extension at 72 °C, and one final extension cycle at 72 °C for 5 min. The PCR products were examined by 1% agarose gel electrophoresis.

The PCR products amplified from the genomic DNA were directly cloned into pCR2.1-TOPO vector and sequenced. For over-expression of native AzoA in *E. coli*, the amplified PCR products were cleaved with *Nde*I and *Bam*HI (New England BioLabs). The digested DNA was purified from the agarose gel and ligated into pET-11a with a rapid DNA ligation kit (Roche). *E. coli* NovaBlue (DE3) was transformed with the resulting plasmids. The plasmids (pAZOA) were subsequently isolated and introduced into *E. coli* BL21-Gold(DE3)pLysS by transformation. DNA sequence analysis, translation, and alignment with related genes and proteins were carried out using the Lasergene program (Version 5, DNASTAR). The GenBank program BLAST was utilized to find similar genes or proteins.

Enzyme assays

Azoreductase activity was assayed by measuring the decrease in optical density at suitable wavelengths with a Hewlett–Packard 8453 UV–visible spectrophotometer at room temperature (23 °C). A typical reaction mixture (2.0 ml) contained 25mM potassium phosphate buffer (pH 7.1), 25 μ M azo dye, 0.1mM NADH, 10 μ M FMN, and a suitable amount of enzyme. The reaction was initiated by addition of the enzyme. Initial velocity was determined by monitoring the change in the amount of substrate in the first 2 min in a glass cuvette of 1.0-cm light path. The following molar absorption coefficients were used: 23,360M⁻¹ cm⁻¹ (Methyl red at 430 nm), 18,200M⁻¹ cm⁻¹ (Orange II at 482 nm), 22,600M⁻¹ cm⁻¹ (Amaranth at 520 nm), 20,700M⁻¹ cm⁻¹ (Orange G at 477 nm), 22,900M⁻¹ cm⁻¹ (Ponceau BS at 502 nm) [9,10], and 33,470M⁻¹ cm⁻¹ (Ponceau S at 520 nm). One unit (U) of enzyme activity was defined as the amount of enzyme required to degrade 1 μ M azo dye per min. Menadione reductase activity was assayed by employing a similar procedure as above using a molar absorption coefficient of 5060M⁻¹ cm⁻¹ (NADH at 342 nm). Proteins were quantified using the bicinchoninic acid assay (Pierce) with bovine serum albumin (BSA) as the standard.

Enzyme purification

Proteins were purified at -4 °C by using an AKTApurifier 10 system with UNICORN 4.10 software (Amersham Biosciences). A single colony of *E. coli* BL21-Gold(DE3)pLysS harboring pAZOA grown on LB–ampicillin–chloramphenicol plate media was inoculated into a flask containing 50 ml LB–ampicillin–chloramphenicol (50 μ g/ml of each antibiotic) broth. The culture was shaken (250 rpm) at 37 °C overnight. The next morning, the culture was added to 1 liter LB broth containing no selection antibiotics. The culture was shaken at 250 rpm at 37 °C for 2 h. Isopropyl-1-thio- β -galactopyranoside (IPTG, 1mM) was added and the culture was incubated for another 2.5 h. Cells were harvested by centrifugation (5000g, 10 min), washed with 50 ml buffer containing 25mM Tris–HCl (pH 7.5), and resuspended in 80 ml of the same buffer. The cells were then disrupted by freezing and thawing followed by 5 min sonication at 4 °C with a Vibracell VCX 400 model sonifier (Sonics and Materials). Cell debris was removed by centrifugation at 12,000g for 10 min. Forty milliliters of the supernatant (399.6 mg protein) was applied to a HiPrep 16/10 Q XL anion-exchange column (1.6 $\times$ 10 cm, Amersham Biosciences) equilibrated with the same buffer at a linear flow rate of 120 ml/h. Elution with 100 ml of the starting buffer at the same flow rate removed unbound proteins. This was followed by elution with 240 ml of a NaCl gradient from 0 to 1.0M with a fraction size of 3ml in the same buffer. Peak fractions of azoreductase activity eluted around 0.25M NaCl (20 ml) were pooled and concentrated to a volume of about 8 ml by using an Amicon Ultra-15 (Millipore). The sample was dialyzed and buffered with 25mM Tris–HCl (pH 8.0). The sample was then applied in 2ml portions to a Mono QHR 5/5 column (0.5 $\times$ 5 cm, Amersham Biosciences) equilibrated with 25mM Tris–HCl (pH 8.0) buffer at a linear flow rate of 0.5 ml/min. The column was washed with 5ml of the starting buffer and eluted with 10ml of a NaCl gradient from 0 to 1.0M in the same buffer at the same flow rate. Fractions of 0.5 ml were collected and those containing major enzyme activity were pooled (1.0 ml, around 0.35M NaCl). Final purification was achieved by gel filtration on a HiLoad 16/60 Superdex 75 pre grade column (1.6 $\times$ 60 cm, Amersham Biosciences) equilibrated and run with 25mM potassium phosphate (pH 7.1), containing

0.1M NaCl at a linear flow rate of 1 ml/min. The enzyme solution in 1ml portions was injected into the column through a sample loop. Fractions of 2ml were collected, and those containing major enzyme activity were combined (6 ml) and stored at -20°C . BSA (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Amersham Biosciences) were used as the standards.

Preparation of apo-enzyme

One volume of 10mM potassium phosphate (pH 7.1) containing 2.0M ammonium sulfate was mixed with one volume of the purified dimeric enzyme (5 mg) from above. Five milliliters of the sample was applied to a HiPrep 16/10 Phenyl FF hydrophobic interaction column (1.6×10 cm, Amersham Biosciences) equilibrated with the same buffer plus 1.0M ammonium sulfate at a linear flow rate of 2 ml/min. Four hundred milliliters of a linear gradient of ammonium sulfate from 1.0 to 0M in the same buffer was applied at the same flow rate. This was followed by further elution with 100 ml of the buffer. The fractions (30 ml) containing apo-enzyme were concentrated and stored at -20°C .

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and N-terminal amino acid sequencing of the protein

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in Laemmli's buffer [27] with 12.5% polyacrylamide. Perfect protein markers (Novagen) were used. Electrophoresis was performed in a Hoefer SE 260 Mighty Small II Mini Vertical Unit (Amersham Biosciences). Gels were stained for proteins with Coomassie brilliant blue R-250 (Bio-Rad). Amino acid sequencing was done with protein bands from SDS–PAGE. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane in a Hoefer TE77 Semi-Dry Transfer Unit (Amersham Biosciences). The transferred proteins were visualized by Coomassie staining and excised with a razor blade. N-terminal amino acid sequencing was performed on an Applied Biosystems model 477A gas-phase sequencer equipped with an automatic on-line phenylthiohydantoin analyzer.

Apparent kinetic constants of the purified AzoA

Initial velocities of the enzymatic reaction were performed by varying the concentrations of one substrate, Methyl red (from 0.005 to 0.04mM) or NADH (from 0.05 to 0.4 mM), while the concentration of the other substrate was kept constant (NADH: 0.4mM or Methyl red: 0.04 mM). Apparent K_m and V_{max} values were obtained from Lineweaver–Burk plots.

Nucleotide sequence accession number

The nucleotide sequence of *azoA* of *E. faecalis* ATCC 19433 has been assigned the GenBank Accession No. AY422207.

Results

Comparison of degradation of various azo dyes and azoreductase production by *E. faecalis*

After 20 h cultivation, all azo dyes were reduced at various rates in BHI medium by *E. faecalis* strain ATCC 19433 (Table 1). Methyl red was reduced very quickly in the culture and the dye color disappeared in 12 h. Orange G was reduced much more slowly than the other dyes in 20 h. The azoreductases in *E. faecalis* appeared constitutive. The specific activities of the azoreductase in the cultures were increased 15–74% with various azo dyes added. However, total protein concentrations of the cell-extracts from the induced cultures were lower than those of the cultures without dye, which indicated that the azo dyes might inhibit the growth of the bacterium.

Identification of the *E. faecalis* azoA gene

Recently, an azoreductase gene (*acpD*) of *E. coli* was identified and partially characterized [9]. Therefore, we searched the *E. faecalis* V583 genome sequence in the GenBank released by The Institute for Genomic Research for similar genes encoding proteins by using the *E. coli* AcpD [9]. The search identified a single *acpD* like gene encoding a 208 amino acid residue, 23,220 Da hypothetical protein with a calculated isoelectric point of 4.8 (Fig. 1). The protein displayed 34% primary structure identity and had the similar size with that of *E. coli* acpD (201 amino acid residues). Six deletion and/or insertion differences between these two protein sequences were found. The putative azoreductase gene of *E. faecalis* was amplified by PCR using a pair of primers (Efs-forward and -reverse) yielding a DNA band of about 800 bp on agarose gel from the purified genomic DNA of *E. faecalis*. The amplified DNA fragment contained the open reading frame (ORF) as well as a short sequence downstream of the stop codon. It was directly ligated to TA cloning vector, pCR2.1-TOPO. Sequencing of the insert revealed that it contained 808 bp DNA fragment with a complete ORF encoding a protein (AzoA). There are only three base pair differences between *azoA* from *E. faecalis* ATCC 19433 and the corresponding gene from *E. faecalis* V583. However, both of the translated protein sequences were identical.

Expression of *E. faecalis* AzoA in *E. coli*

For expression of the azoreductase gene, the PCR amplified fragment was inserted via its unique *Nde*I and *Bam*HI restriction sites into corresponding sites of pET-11a. We tried to express recombinant azoreductase as a native enzyme and no other modification of the gene was performed in the construction of the expression vector. The enzyme was functionally expressed in *E. coli* using a phage T7-promoter system. Cell extracts were prepared from cultures of *E. coli* BL21-Gold(DE3) pLysS carrying the expression plasmid, which had been induced by the addition of IPTG after 2.5 h induction. The cell extract supernatant from the induced culture displayed a bright yellow color and an elevated level of azoreductase activity was detected in the sample as compared with those of noninduced culture, indicating that the enzyme was induced and overexpressed in the host. This expression system was particularly useful, since the cells grew and expressed the flavoprotein continuously without disruption of cellular growth.

Purification of the recombinant *E. faecalis* AzoA expressed in *E. coli*

The recombinant azoreductase of *E. faecalis* (AzoA) was purified by consecutive chromatography using anion exchangers HiPrep Q XL and Mono Q followed by a gel filtration step on HiLoad Superdex 75. As the enzyme was overexpressed in *E. coli*, several grams of wet weight cells were needed to purify the protein in useful quantities. The purification procedure is summarized in Table 2. The enzyme was purified about 7.7-fold with a yield of 10.9%, which allows the proportion of the enzyme to be estimated at 13.0% of the protein present in the crude cell extract. The specific activity of the final purified *E. faecalis* AzoA was 63.9 U/mg protein using Methyl red as substrate. The molecular mass of the purified azoreductase was found to be 23 kDa as shown by SDS-PAGE (Fig. 2). However, when determined by gel filtration chromatography on a HiLoad Superdex 75 column, it had an approximate size of 43 kDa. The purified protein on SDS-PAGE was transferred onto PVDF membrane and subjected to N-terminal sequence. The first 10 amino acid residues (SKLLVVK AHP) were determined and found to be identical to the 10 residues shown for the polypeptide sequence, beginning with the second position in the N-terminal sequence, translated from the azoreductase ORF of *E. faecalis*.

Enterococcus faecalis AzoA has FMN as a prosthetic group

The solution of the purified azoreductase was yellow and this coloration was resistant to dialysis after several chromatography steps. The absorption spectrum of the purified AzoA was measured using a Hewlett-Packard 8453 UV-visible spectrophotometer (Fig. 3) [28]. The absorption spectrum in the visible-light region of AzoA (29.5 μ M) was similar to that of free FMN (32.5 μ M) but was shifted about 7 nm (from 445 to 462 nm) toward to the long wavelength, which is a typical pattern of flavoprotein absorption [28], indicating that AzoA is a flavoprotein tightly associated with FMN. On the basis of spectroscopy and protein molecular weight, the molar ratio between FMN and the AzoA protein was estimated at 1.1, thus probably suggesting that 1M of FMN binds to 1M AzoA (homodimer).

Flavin removal

The purified dimeric recombinant azoreductase of *E. faecalis* (AzoA) was dissociated by removing the prosthetic FMN group. The FMN was eluted out first by gradually decreasing ionic strength to decrease hydrophobic interactions in the HiPrep 16/10 Phenyl FF column. Finally, the apoenzyme was collected from the column by passing the buffer without ammonium sulfate through the column. Size of the purified apoenzyme was determined on the gel filtration column to be approximately 22 kDa.

Apparent kinetic constants of *E. faecalis* AzoA

Enzymatic reactions were performed by varying the concentrations of one substrate (Methyl red or NADH) and fixing the other substrate concentration. Apparent K_m and V_{max} values were obtained from Lineweaver-Burk plots. The results are shown in Table 3. K_m values of 0.14 and 0.024mM and V_{max} values of 86.2 and 89.2 U/mg protein for NADH and Methyl red, respectively, were obtained. The K_m of *E. faecalis* with Methyl red as the substrate is similar to K_m of the azoreductase from *E. coli* (AcpD) (0.018mM) [11] and *Enterobacter*

agglomerans (0.029mM) [8], but K_m of *E. faecalis* with NADH as the substrate is much higher than those of *E. coli* (AcpD) (0.032mM) and *E. agglomerans* (0.059mM) [8].

Substrate specificity of *E. faecalis* AzoA

Analysis of the purified AzoA indicated the enzyme used NADH but not NADPH for Methyl red reduction (Table 4). AzoA required FMN for its activity while FAD was much less effective. Riboflavin had no capability of mediating oxidation of the NADH. The electron acceptor specificity spectrum of *E. faecalis* AzoA was similar to that of *E. coli* azoreductase (AcpD) [9]. A number of synthetic model azo compounds were used in testing the substrate specificity of the enzyme (Table 4, Fig. 4). The enzyme displayed the highest activity with Methyl red. The enzyme also degraded sulfonated azo dyes Orange II, Amaranth, Ponceau BS, and Ponceau S.

Discussion

More than 3000 azo dyes are used in the textile, printing, paper manufacturing, pharmaceutical, cosmetics, and food industries. When these compounds enter the human body through ingestion, they are metabolized to aromatic amines by the intestinal microflora [1]. Aromatic amines, such as benzidine, induce urinary bladder cancer in humans and tumors in some experimental animals [29,30]. The human gastrointestinal tract harbors a complex microflora comprising of 400 to 500 species [31]. Enterococci are the predominant gram-positive cocci in human stools with *E. faecalis* being the most common *Enterococcus* species found [31–33]. The aim of this study was to provide insight into the aerobic reduction of azo dyes by these predominant gram-positive cocci.

Cell extracts of *E. faecalis* have been shown to possess higher specific azoreductase activity than those of several other intestinal bacteria [25]. Our studies demonstrated that *E. faecalis* was able to grow and metabolize several azo dyes, although the inhibitory effects of some dyes as reflected in a decrease in bacterial growth were found. There was no detectable azoreductase activity in supernatants of the cultures, which implies that the enzyme is not secreted outside the cells.

Although *E. faecalis* had higher azoreductase activity than that of other tested intestinal bacteria, it is still a very difficult and time-consuming process to purify sufficient amount of protein from the bacterium for biological characterization of the enzyme. Therefore, we adopted a new approach using homology comparison, direct cloning, and overexpression of genes coding for hypothetical proteins. We were able to identify and express a functional azoreductase gene from *E. faecalis* (AzoA) in *E. coli*. Besides using *E. coli* AcpD, two other azoreductase sequences from *Bacillus* sp. OY1-2 [7] and *X. azovorans* KF46F [10] were also employed for a homology search of the *E. faecalis* genome sequence. However, there was no significant homology found indicating there were probably no genes similar to them in *E. faecalis*.

The N-terminal methionine of the recombinant *E. faecalis* AzoA was cleaved by methionine aminopeptidase in the *E. coli* cells as is common for heterologous protein expressed in *E. coli* host [34]. The N-terminal sequence of the purified enzyme started from the second

position of the deduced protein sequence. This indicated that the purified recombinant *E. faecalis* AzoA was an intact protein when it was expressed in *E. coli* and could be similar to the corresponding native enzyme produced in *E. faecalis*. The purified recombinant *E. faecalis* AzoA was a homodimeric protein, which was similar to the azoreductase from *E. coli* (AcpD) [9] but different from the other three cloned monomeric azoreductases in soil bacteria [7,10,11]. Possibly one molecule of *E. faecalis* AzoA was associated with one FMN. This is similar to a homodimer flavin protein containing one molecule of FMN per dimer from *Selenomonas ruminantium* [35] and different than nitroreductases containing two molecules of FMN per homodimer from *E. coli* [28,36]. Although azoreductase from *E. coli* (AcpD) was a dimeric and activity of the enzyme is FMN dependent, there was no indication that FMN was associated with the enzyme [9]. On hydrophobic interaction chromatography, the yellow compound of FMN was eluted gradually with decrease of ammonium sulfate concentration, indicating a non-covalent association with the protein. The prosthetic FMN in the enzyme may hold dimeric subunits together. Azoreductases from *Bacillus* sp. OY1-2 [7], *X. azovorans* [10], and *P. kullae* [11] exist as monomers and enzyme bound-FMN or FAD was not observed.

Substrate specificity of *E. faecalis* AzoA indicated that the enzyme not only reduced azo dye Methyl red, but also decolorized more structurally diverse sulfonated azo dyes. Accessibility of azo dyes to azoreductase is dependent on the chemical structures of the dyes. It was suggested that electron-withdrawing groups on the phenyl ring accelerated reduction of the azo bond, while a charged functional group in proximity to the azo group or the presence of a second polar group interfered with the reaction [10]. This could be the reason that there was no detectable activity against Orange G by *E. faecalis* AzoA. So far, none of the known aerobic azoreductases examined were able to convert Orange G [10]. Since Orange G in the *E. faecalis* culture partially disappeared, it is possible that other enzymes or mechanisms are involved in the conversion of the pigment. The deduced protein sequence of *E. faecalis* AzoA has moderately significant homology (34%) to that of *E. coli* AcpD. *E. coli* AcpD, originally detected as a Methyl red azoreductase, was not able to convert Ponceau SX [9], which indicated it had no ability to convert sulfonated azo dyes. The differences in substrate specificity between *E. faecalis* AzoA and *E. coli* AcpD might reflect the sequence differences and gaps between them. Although there is no sequence homology between *E. faecalis* AzoA and *X. azovorans* KF46F Orange II azoreductase, both of them share some similarity on their substrate specificity. *E. faecalis* AzoA possess activity to reduce menadione, which seems to be common among azo reductases and nitroreductases containing a flavin prosthetic group [9,28,36].

In conclusion, our results demonstrated that the intestinal gram-positive bacterium *E. faecalis* contained the gene encoding aerobic azoreductase. The enzyme was a homodimeric protein and strictly dependent on FMN for its activity. The properties of the purified recombinant *E. faecalis* AzoA indicated that the enzyme had a broad spectrum of substrate specificity, which was capable of degrading a wide variety of azo dyes. This implies that besides unspecific anaerobic reduction of azo dyes, true azoreductase, which is not sensitive to oxygen, may also play an important role in the conversion of the azo dyes in the gastrointestinal tract.

Acknowledgments

We thank Latriana H. Robertson and Donald H. Paine for technical help, Dr. Christopher A. Elkins for very useful discussions, and Drs. Robert D. Wagner and Rajesh Nayak for their critical review of the manuscript.

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AzoA  MSKLLVKAHPLTKEESRSVRALETFLASYRETNPSDEIEILDVYAPETNMPEIDEELLS 60
      MSK+LV+K+  L    S+S + + F+ +RE + +DEI + D+ A    +P +D EL+
acpD  MSKVLVLKSSILAGY-SQSNQLSDYFVEQWREKHSAD EITVRDLAA--NPIPVL D GELV- 56

AzoA  AWGALRAGAAFETLSENQQQKVARFNELTDQFLSADKVVIANPMWNLNVPTRLKAWVDTI 120
      GALR  A    L+  QQ+ +A  +EL  + + D +VIA PM+N N+ T+LK + D +
acpD  --GALRPDA--PLTTRQQEALALSDELIAELKAHDVIVIAAPMYNFNISTQLKNYFDLV 112

AzoA  NVAGKTFQYTAEGPKPLTSGKKALHIQSNGGFYEG--KDFASQYIKAILNFIGVDQVDGL 178
      AG TF+YT GP+ L +GKKA+ I S GG ++    D + Y+  L FIG+ V +
acpD  ARAGVTFRYTENGPEGLVTGKKAIVITSRGGIHKDGPTDLVTPYLPSTFLGFIGITDVKEV 172

AzoA  FIEGIDHFDPRA 190
      F EGI + P+ A
AcpD  FAEGIAYGPEMA 184

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Fig. 1.

Comparison of the deduced amino acid sequences of *E. faecalis* AzoA (top) and *E. coli* acpD (bottom). Amino acid residues with different degrees of conservation (+) are indicated.

Fig. 2. SDS-PAGE of the purified AzoA of *E. faecalis* expressed in *E. coli*. Lane 1, protein molecular mass standards; lane 2, crude cell extract (40 µg); and lane 3, the purified AzoA (3 µg).

Fig. 3. Comparison between spectra of *E. faecalis* AzoA and free FMN. After the final step purification by gel filtration, the absorption spectrum of the purified AzoA was measured. Solid and dotted lines, respectively, show spectra of AzoA at 29.5 μM and 32.5 μM free FMN in 50mM potassium phosphate buffer (pH 7.0) containing 100mM NaCl.

Fig. 4.
Structures of azo dyes used for reduction assays and cultures.

Table 1Decolorization of various dyes by *E. faecalis* and azoreductase activity^a

Dye	Dye remaining after 20 h (%)	Total activity (U × 10 ⁻² /20 ml culture)	Total protein of cell extract (mg/20 ml culture)	Specific activity (U × 10 ⁻² /mg protein) of the cell extract
Methyl red	0.6	16.4	8.6	1.81
Orange II	4.9	10.1	6.9	1.39
Orange G	35.9	9.12	7.3	1.20
Amaranth	0.5	8.26	5.8	1.37
Ponceau BS	11.1	9.58	6.9	1.33
Ponceau S	4.1	9.26	5.3	1.67
None	—	16.4	15	1.04

^aDye concentration in the cultures: 0.2mM.

Table 2Purification of recombinant AzoA of *E. faecalis* expressed in *E. coli*^a

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)
Crude cell-extract	400	3320	8.3	100
HiPrep Q XL	85.7	2320	27.1	70
Mono Q	26.7	1160	43.4	35
HiLoad Superdex 75	10.9	697	63.9	21

^aActivities were determined with Methyl red as substrate.

Table 3

Michaelis constants (K_m) and maximal velocities (V_{max}) of recombinant AzoA of *E. faecalis* expressed in *E. coli*^a

Substrate	K_m (mM)	V_{max} (U/mg protein)
NADH	0.14	86.2
Methyl red	0.024	89.2

^aThe kinetic constants are apparent values.

Table 4Substrate specificity of recombinant AzoA of *E. faecalis* expressed in *E. coli*^a

Substrate	Cofactors	Specific activity (U/mg protein)
Methyl red	FMN, NADH	58.4
Methyl red	FAD, NADH	3.1
Methyl red	FMN, NADPH ^b	ND ^c
Methyl red	None, NADH	ND
Methyl red	Riboflavin, NADH	ND
Orange II	FMN, NADH	1.92
Orange G	FMN, NADH	ND
Amaranth	FMN, NADH	0.28
Ponceau BS	FMN, NADH	5.9
Ponceau S	FMN, NADH	0.22
Menadione	FMN, NADH	140.2

^a A monomeric AzoA (apoenzyme), which did not containing FMN, separated from the purified dimeric form of the enzyme by using a hydrophobic column was employed in the substrate specificity study.

^b 0.1, 0.2, and 0.4mM NADPH were used in the assays.

^c ND, not detected.