Molecular Cloning and Characterization of the Gene Coding for the Aerobic Azoreductase from *Xenophilus azovorans* KF46F

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The gene coding for an aerobic azoreductase was cloned from *Xenophilus azovorans* KF46F (formerly *Pseudo-

* MATERIALS AND METHODS

**Bacterial strains, media, and plasmids.** *X. azovorans* KF46 was originally isolated from a soil inoculum after a prolonged enrichment with carboxy-Orange II [1-(4'-carboxyphenylazo)-2-naphthol] as sole source of carbon and energy (27).
AEROCBIC AZOREDUCTASE FROM X. AZOVORANS KF46F

TABLE 1. Color index registration numbers, absorption maxima, purity, and calculated molar extinction coefficients of sulfonated azo dyes used in this study.

| Azo dye                        | CI number | Dye content (%) | Absorption max (nm) | Extinction coefficient (mM⁻¹ cm⁻¹) |
|--------------------------------|-----------|-----------------|---------------------|-----------------------------------|
| Acid Orange 7 (Orange II)      | 15510     | 95              | 482                 | 18.2                              |
| Mordant Violet 5 (Violet N)    | 15670     | NS              | 529                 | 10.7                              |
| Acid Orange 8                  | 15575     | 65              | 466                 | 27.4                              |
| Acid Orange 12 (CrocceOrange G)| 15970     | 70              | 482                 | 24.7                              |
| Acid Red 66 (Ponceau BS)       | 26905     | NS              | 502                 | 22.9                              |
| Acid Red 88                    | 15620     | 75              | 500                 | 7.8                               |
| Food Yellow 3 (Sunset Yellow F6D)| 15905   | NS              | 480                 | 19.8                              |
| Solvent Yellow 14 (Sudan I)    | 12055     | 55              | 557                 | 8.7                               |
| Solvent Orange 7 (Sudan II)    | 12140     | 90              | 559                 | 4.3                               |
| Solvent Red 23 (Sudan III)     | 26100     | NS              | 512                 | 2.3                               |
| Solvent Red 24 (Sudan IV)      | 26105     | NS              | 515                 | 5.7                               |
| Acid Orange 10 (Orange G)      | 16250     | NS              | 477                 | 20.7                              |
| Acid Red 18 (Neococin)         | 16255     | NS              | 507                 | 19.9                              |
| Acid Red 27 (Amaranth)         | 16185     | 90              | 520                 | 22.6                              |
| Acid Black 52 (Palatine Fast Black WAN)| 15711   | 25              | 656                 | 13.2                              |
| Acid Red 151                   | 26900     | 40              | 486                 | 6.9                               |
| 1-(2-Pyridyldiaz)-2-naphthol   | NS        | 445             | 7.7                  |
| Calconcarboxylic acid          | NS        | 555             | 3.2                  |
| Calmagite                      | NS        | 539             | 9.9                  |

*The dyes were purchased from Fluka, Aldrich, or Sigma and not further purified. The absorption maxima were determined in NaK phosphate buffer (pH 7.4, 54 mM). The molar extinction coefficients were calculated using the dye purities indicated. If the dye purity was not indicated by the supplier, it was assumed that the preparations consisted of pure dye.

# Determination of molecular weight
The relative molecular mass of the native enzyme was determined by gel filtration using a Superdex 75 prep-grade column (Amersham Pharmacia Biotech) and appropriate standard proteins.

# Protein purification, isolation of peptides, and sequencing of peptides and N termini
The digestion of the azoreductase by trypsin (Sigma, Deisenhofen, Germany) and the subsequent separation of tryptic digests by reversed-phase high-pressure liquid chromatography (HPLC) were performed as described previously (43). The digestion of the enzyme with endoproteinase Glu-C (Sigma) was performed in Na-phosphate buffer (pH 7.8) in order to ensure a proteolytic cleavage of the enzyme on the carboxyl-side of glutamate or aspartate residues. For the digestions, 38 or 23 μg, respectively, of the purified azoreductase was incubated with 1.5 μg or 1 μg of trypsin or endoproteinase C, respectively. The digests were incubated for 24 h at 37°C, and the individual peptides were purified by reverse-phase HPLC. The amino acid sequences were determined by automated Edman degradation using an Applied Biosystems model 491 sequencer.

# DNA manipulation techniques
The genomic DNA was prepared as described by Ausubel et al. (3). Plasmid DNA from E. coli DH5α was isolated with the Bacteria Maxi Prep Kit (Amersham Pharmacia Biotech) or the Qiagen Spin Miniprep Kit (Qiagen, Hilden, Germany). Digestion of DNA with restriction endonucleases (Gibco BRL, New England Biolabs, Frankfurt, Germany), electrophoresis, purification, and ligation with T4 DNA ligase (Gibco BRL) were performed according to the standard procedures (39). Transformation of E. coli was done by the method of Inoue et al. (20). For cloning of PCR products a T vector was prepared as described by Marchuk et al. (30).

# PCR
Oligonucleotides were custom synthesized according to the known or deduced sequences of the amino-terminal amino acid sequence and various internal peptides. PCR mixtures (50 μl) for the amplification of genomic DNA contained 50 μM of each primer, 0.1 μg of genomic template DNA, a 0.1 mM concentration of each deoxynucleoside triphosphate, 0 to 7.5% (vol/vol) dimethyl sulfoxide, 1.5 mM MgCl₂, 0.7 U of Taq DNA polymerase, and the corresponding reaction buffer (Gibco BRL).

For the amplification reaction with the primers deduced from the amino terminus and the internal peptides, the following PCR program was used: an initial denaturation (95°C, 3 min; addition of the Taq polymerase after 2 min) was followed by 29 cycles consisting of an annealing temperature of 50°C (1.5 min), a polymerization step (72°C, 2 min), and denaturation (95°C, 40 s). The last amplification step was extended to 10 min. The PCR products were initially cloned into the T-tailed EcoRV-site of pBlueScript II Ks (+) (30).
DNA sequencing and nucleotide sequence analysis. The DNA sequence was determined by dideoxy chain termination with double-stranded DNA of clones and overlapping subclones in an automated DNA sequencing system (ALFexpress-Sequencer; Amersham Pharmacia Biotech) with fluorescein labeled primers or nucleotides.

Sequence analysis, database searches, and comparisons were done with the PCGene software package, release 6.85, and the BLAST search at the National Center for Biotechnology Information (NCBI). The alignment of the azoreductases was obtained with the program CLUSTAL using the default parameters.

Expression of the azoreductase in E. coli. For expression in E. coli, azoB was inserted into pET11a (44) under the control of the phage T7 promoter. The DNA segment encompassing azoB was amplified by PCR with simultaneous introduction of an NdeI site upstream and a BamHI site downstream of azoB. The following oligonucleotide primers were used for the amplification: 5'¬ATG ACA TAT GAT TCT GGT CGT CGG AGG AAC-3' and 5'¬TGG ATC CGA CGG CAT GCG CAG CAT C. The amplified products were cleaved with NdeI and BamHI and ligated into pET11a. E. coli DH5α was transformed with the resulting plasmids. The plasmids were subsequently isolated and introduced into E. coli BL21(DE3)pLysS by transformation.

Chemicals. The azo dyes and all other chemicals were obtained from Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Sigma, and Gerbu Biotech (Gaiberg, Germany). The azo dyes Mordant Yellow 3 and 1-(4-hydroxyphenylazo)-2-naphthol-6-sulfonate were kindly provided by Bayer AG (Leverkusen, Germany) and K. Bredereck (University of Stuttgart, respectively. The oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany).

Nucleotide sequence accession number. The nucleotide sequence of the 5,782-bp SsrI fragment was deposited in GenBank under accession number AF466104.

RESULTS

Purification of the aerobic azoreductase. Xenophilus azovorans KF46F was grown in liquid culture on a mineral medium with 4-hydroxybenzoate (15 mM) and Orange II (0.2 mM) in order to achieve an optimal expression of the azoreductase. The azoreductase was purified about 400-fold, giving a specific activity of 10.8 U/mg of protein (Table 2). The purified enzyme gave a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of approximately 30,000. This estimation was confirmed by the amino acid sequence of the protein deduced from the nucleotide sequence (see below). Surprisingly, the gel filtration indicated a molecular weight of the holoenzyme of less than 20,000. Therefore it can be assumed that the enzyme is a monomer which somehow behaved extraordinary during gel filtration. The results obtained agreed sufficiently with the results obtained previously by Zimmermann et al. (49) after a different purification procedure using the original strain KF46, who described the enzyme as a monomer with a molecular weight of 30,000.

Determination of the NH2-terminal and some internal amino acid sequences. The NH2-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation (Table 3). To obtain more sequence information, the purified protein was digested with trypsin and the fragments were separated by HPLC. Thus, the sequences of two fragments were determined (Table 4), which obviously overlapped each other and thus allowed us to deduce a peptide consisting of 26 amino acids. The azoreductase was also digested with endoprotease Glu-C, which resulted in two additional amino acid sequences (Table 4).

Cloning of the azoreductase gene. The amino acid sequences of two peptides served for the design of oligonucleotide primers (Table 4). Using these primers and genomic DNA of strain KF46F as a template, an approximately 0.2-kb DNA fragment was amplified. This PCR product was cloned into a pBluescript II KS(+) T vector and sequenced. In the deduced amino acid sequence the amino acid sequence of a part of fragment A (Table 4) was found which had not been used for the design of the oligonucleotide primers. It was therefore deduced that indeed a fragment of the azoreductase gene from strain KF46F had been amplified. The PCR fragment was labeled using digoxigenin-labeled dUTP residues and used as a probe to identify by Southern hybridization the complete azoreductase gene. Thus, hybridization signals were found with an approximately 6-kb SsrI fragment, a 3-kb EcoRI fragment, and a 2-kb Srl fragment from the total DNA of strain KF46F. Finally, the probe was used to identify the complete gene in a size-selected gene bank obtained by SsrI digestion of the genomic DNA of strain KF46F. This plasmid carrying an approximately 6-kb SsrI fragment cloned into plasmid pBluescript II SK(+) was designated pBlue-OII-S3-59.

Determination of the nucleotide sequence of the azoreductase gene and the surrounding DNA fragments. The DNA sequence of the insert in plasmid pBlue-OII-S3-59 was deter-

| Protein or peptide | Amino acid sequencea | Deduced primer sequence |
|--------------------|----------------------|------------------------|
| Amino terminus      | MILVVGGTGTI          | 5'-GA(T/C)-AA(A/G)-GTI-TT(T/C)-GTN-GT-3' |
| A (trypsin digest)  | EEVDKVVFVYTPLVPDOVMR | 5'-CA(A/G)-CCN-GGN-TT(T/C)-TT(T/C)-ATG-3' or TGGATCC |
| B (endoprotease Glu-C digest) | SGMAWTVPQGFFM | 5'-GT(T/C)-GGN-CCN-AA(A/G)-AA(A/G)-TAC-5' |
| C (endoprotease Glu-C digest) | LYLALPYPGLAVLDTVPKVTRGLPA | |

a Segments used for the design of oligonucleotides for PCR are underlined.

TABLE 2. Purification of aerobic azoreductase from X. azovorans KF46F

| Purification step | Total protein (mg) | Sp act (U/mg) | Total activity (U) | Recovery (%) | Purification (fold) |
|-------------------|--------------------|---------------|-------------------|--------------|---------------------|
| Crude extract     | 402                | 0.027         | 10.9              | 100          | 1                   |
| Red Sepharose CL-6B | 11               | 1.10          | 12.2              | 111          | 41                  |
| Octyl-Sepharose + ultrafiltration | 0.7           | 4.5         | 3.3               | 30           | 165                 |
| Gel filtration    | 0.09               | 10.8          | 1.0               | 9            | 400                 |

TABLE 3. Sequences of the amino terminus, tryptic peptides, and deduced primers

| Protein or peptide | Amino acid sequencea | Deduced primer sequence |
|--------------------|----------------------|------------------------|
| Amino terminus      | MILVVGGTGTI          | 5'-GA(T/C)-AA(A/G)-GTI-TT(T/C)-GTN-GT-3' |
| A (trypsin digest)  | EEVDKVVFVYTPLVPDOVMR | 5'-CA(A/G)-CCN-GGN-TT(T/C)-TT(T/C)-ATG-3' or TGGATCC |
| B (endoprotease Glu-C digest) | SGMAWTVPQGFFM | 5'-GT(T/C)-GGN-CCN-AA(A/G)-AA(A/G)-TAC-5' |
| C (endoprotease Glu-C digest) | LYLALPYPGLAVLDTVPKVTRGLPA | |

a Segments used for the design of oligonucleotides for PCR are underlined.
mined and a continuous DNA fragment of 5,782 bp sequenced. The gene for the azoreductase (azoB) was unequivocally identified on the cloned fragment by the presence of the amino-terminal region and the internal peptides determined by Edman degradation (Fig. 1). The gene encoded a protein consisting of 281 amino acids, which corresponded to a molecular mass of 30,278 Da. An NAD(P)H binding site could be clearly identified near the amino terminus of the deduced protein sequence. The presence of an arginine residue as the 10th conserved amino acid sequence in this NAD(P)H-binding site indicated that the azoreductase binds in vivo preferentially NADPH (34). This corresponds with the biochemical data of Zimmermann et al. (49), who determined Km values of the azoreductase for NADPH and NADH of 5 and 180 μM, respectively. A BLAST search using the deduced amino acid sequence of azoB did not identify proteins with significantly similar sequences. The highest degrees of sequence identities (24.2 to 16.4%) were found with a hypothetical protein (P39315) in Arabidopsis thaliana (P52577). In all these cases the major regions of sequence identity were within the highly conserved NAD(P)H-binding region. No significant sequence similarity was detected with the recently described aerobic azoreductase from Bacillus sp. OY1-2 (AB032601 [46]). Because these sequence comparisons did not give any indications about the putative evolutionary origin of the azoreductase, also the DNA sequences upstream and downstream of azoB were analyzed. Two putative regions of homology with sequences deposited at the NCBI were found downstream of the azoreductase gene, but these sequences demonstrated sequence similarities only for parts of the reference sequences deposited at the NCBI and are proba-
bly also not parts of open reading frames in the DNA from strain KF46F (Table 4).

**Expression of the azoreductase in *E. coli.* The azoreductase gene was amplified by PCR from the genomic DNA of strain KF46F using a set of primers which created new *NdeI* and *BamHI* restriction sites and was functionally expressed in *E. coli* using a phage T7-promoter system. Cell extracts were prepared from cultures of *E. coli* BL21(DE3)pLysS carrying the expression plasmid, which had been induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). After 6 h of induction an azoreductase activity of 1.3 U/mg of protein was obtained, which was almost 50 times higher than the activity observed in cell extracts of wild-type *E. coli*.

**Comparison of the in vivo- and in vitro-reduction rates for azo dyes by the recombinant *E. coli* strain.** It has been repeatedly suggested that the metabolism of sulfonated azo dyes would be restricted by the limited permeability of bacterial cell membranes for the highly polar sulfonated azo dyes (13, 17, 37, 47). Because a recombinant azoreductase is now available, it was attempted to experimentally verify this proposal. The recombinant strain *E. coli* BL21(DE3)pLysSpET-OII-Ex9 was grown in Luria-Bertani medium, and the azoreductase gene was induced by the addition of IPTG. Then the culture was split into two parts, and from one part of the cells a cell extract was prepared. Finally, the azoreductase activities of the resting cells and a cell extract prepared from the same cells were compared. Thus, it was observed that the cell extract demonstrated an azoreductase activity of about 0.8 U/mg of protein. In contrast, for the resting cell suspension no detectable azoreductase activity was found. When resting cells were stirred on a vortex mixer with toluene (25 μl per ml of cell suspension) for 3 min prior to the assay, a low but clearly measurable decolorization activity was detected (0.0012 U/mg of protein), which was increased approximately 10 times by the addition of NADPH (1 mM) to the whole-cell assay. These experiments suggested that for the recombinant *E. coli* strain the missing transport of the sulfonated dye into the cells was indeed a limiting factor which prevented the metabolism of the sulfonated azo dye by whole cells. Furthermore, it is apparent that under the test conditions using resting cells also the NADPH supply may become limiting for the reduction of azo dyes.

**Substrate specificity of the azoreductase.** The previous biochemical characterization of the azoreductase from strain KF46 demonstrated that the enzyme converted a wide range of specifically synthesized model compounds, which contained a hydroxy group in ortho position towards the azo group (49). Because azo dyes with this basic structure are rather widely used as industrial dyestuff, it was tested in the present study if the recombinantly expressed azoreductase would also convert industrially relevant dyes (Table 5). These experiments demonstrated that the azoreductase decolorized several azo dyes carrying a hydroxy group in the 2 position of the naphthol ring and confirmed previous suggestions (49) that electron-withdrawing groups on the phenyl ring accelerated the reactions, while a charged functional group in proximity to the azo group or the presence of a second polar group interfered with the reaction. This was especially evident when those (few) dyes were analyzed which fulfilled the basic structural requirement of the enzyme (a hydroxy group in ortho position to the azo group) but which were not converted. In almost all of these substrates two sulfonic acid groups were attached to the naphthalene ring system (Table 5).

**DISCUSSION**

This paper is the second example of the analysis of an aerobic azoreductase on the genetic level. Recently, Suzuki et al. (46) cloned an aerobic azoreductase from a *Bacillus* strain (OY1-2). This strain was originally isolated for its ability to decolorize low concentrations (0.02%) of the azo dye Acid Red 88 [C.I. 15620, 2‘-hydroxy-(1,1’-azidonaphthalene-4-sulfonate)] while growing on agar plates on a nutrient medium (45). Both azoreductases from *Bacillus* sp. strain OY1-2 and *X. azovorans* KF46F were able to decolorize Acid Red 88 and Acid Orange 7, but unfortunately only very limited conclusions can be drawn about the substrate specificity of the azoreductase from *Bacillus* sp. strain OY1-2 because with this enzyme mainly proprietary reactive dyes of unpublished structure have been tested (45). Similarly to the azoreductase studied in the present paper, also the azoreductase from *Bacillus* sp. strain OY1-2 was found to be a rather small monomeric protein with a molecular mass of approximately 20 kDa (compared to about 30 kDa for the enzyme from strain KF46F) (46). Surprisingly, no other similarities were observed among both aerobic azoreductases. Thus, a sequence alignment of both azoreductases did not show any noticeable homology between the deduced amino acid sequences. Furthermore, it was suggested that the azoreductase from *Bacillus* sp. strain OY1-2 contains an NAD(P)H-binding motif (GXGXXG) at positions 106 to 111 of the protein and not at the amino terminus as found in the present study for the enzyme from *X. azovorans* KF46F.

The existence of two nonhomologous aerobic azoreductases is somehow surprising, because of the great problems that have been observed when trying to isolate microorganisms with the ability to grow aerobically with sulfonated azo dyes (4, 25, 26, 27). On the other hand, it had been shown that it is easy to isolate bacteria with rather simple azo compounds such as 4,4‘-dicarboxyazobenzene (19, 32), which form aromatic amines after the reductive cleavage that can be mineralized aerobically by many bacteria. Thus, it may be possible that the major

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**TABLE 4. Regions of sequence similarity between deduced peptides from the sequenced DNA fragment from *X. azovorans* KF46F and sequences previously deposited at NCBI**

| Deduced peptide | Position in clone sequence | Size (aa) | Probable function of product | Source | % Identity (aa) | Reference for homologous proteins |
|-----------------|---------------------------|----------|------------------------------|--------|----------------|----------------------------------|
| 1               | 4530-3949                 | 194      | 3-Oxoacyl (acyl-carrier protein) reductase | *Bacillus subtilis* | 45 (79–246) | P51831                           |
| 2               | 3980-2109                 | 593      | Hypothetical protein         | *Methanococcus jannaschii* | 37 (14–617) | Q58010                           |

*a Percentage of amino acids that are identical when sequences were aligned with sequences listed in the GenBank database of the NCBI facilities.

*b* aa, amino acids.
TABLE 5. Relative activities of the azoreductase from *X. azovorans* KF46F with different azo dyes

| Azo dye        | Structure | Relative activity (%) | Azo dye        | Structure | Relative activity (%) | Azo dye        | Structure | Relative activity (%) |
|----------------|-----------|-----------------------|----------------|-----------|-----------------------|----------------|-----------|-----------------------|
| Acid Orange 7  | ![Structure of Orange II](image1) | 100                   | Acid Red 88    | ![Structure of Sudan II](image2) | 29                   | Solvent Orange 7* | ![Structure of Sudan II](image2) | 33                   |
| (Orange II)    |           |                       | Calconcarboxylic acid | ![Structure](image3) | 3                    | Food Yellow 3 (Sunset Yellow FDC 6) | ![Structure](image4) | 0.8                  |
| 1-(2-Pyridylazo) -2-naphthol* | ![Structure](image5) | 980                   | Calmagite      | ![Structure](image6) | 6                    | Acid Orange 12  | ![Structure](image7) | 17                   |
| Mordant Violet 5 | ![Structure](image8) | 3                     |                |           |                       | Solvent Yellow 14* | ![Structure](image9) | 92                   |
| (Violet N)     |           |                       |                |           |                       | (Croceinorange G) |           |                       |
| Acid Orange 8  | ![Structure](image10) | 36                    | Acid Red 66    | ![Structure](image11) | 2                    | Solvent Red 23* | ![Structure](image12) | 8                    |
| (Ponceau BS)   |           |                       | (Ponceau BS)   |           |                       | (Sudan III)     |           |                       |
| Acid Orange 10 | ![Structure](image13) | 0                     | Neucoccin      | ![Structure](image14) | 0                    | Amaranth       | ![Structure](image15) | 0                    |
| (Orange G)     |           |                       |                |           |                       |                |           |                       |
| Acid Black 52  | ![Structure](image16) | 0                     | Acid Red 151   | ![Structure](image17) | 0                    |                |           |                       |
| (Palatine Fast Black WAN) | ![Structure](image18) |                       |                |           |                       |                |           |                       |

*The tests contained in 1 ml 87 μmol of potassium phosphate buffer (pH 7.1), 1 μmol NADH, 25 nmol of the respective azo compounds, and cell extracts (0.15 mg/ml) from *E. coli* BL21(DE3)pLysSεET-OII-Ex9. The enzyme activities were determined at the absorption maxima of the respective dyes indicated in Table 1. The dyes were added from aqueous or ethanolic (*) stock solutions. The reaction rates are expressed as percentages of that for Orange II (100%). The activities with the dyes added from aqueous stock solutions were related to the activity of the enzyme with Orange II in a purely aqueous system (0.57 U/mg of protein), and those with the dyes from the ethanolic stock solutions with activity for Orange II in the presence of 1% (vol/vol) ethanol (0.14 U/mg of protein) were taken, respectively, as 100% enzyme activity.*

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problem in the isolation of aerobic bacteria with the ability to degrade the commercially important sulfonated azo dyes is either the limited transport of the dyes into the cells or the high reactivity of many of the *ortho*-aminohydroxyaromatics which are formed after the reductive cleavage of the azo dyes. These cleavage products can escape a productive degradation because of their spontaneous auto-oxidation reactions and can also harm the cells by futile redox cycles or the formation of addition products between the quinoneimines or quinones and various cell constituents (18, 23).

A rather astonishing observation from the enzymatic and molecular studies about aerobic azoreductases is that the enzyme from strain KF46F [and also the aerobic azoreductases from *P. kulae* (formerly *Pseudomonas* sp.) K22 and *Bacillus* strain OY1-2] are rather simple polypeptides which do not contain any metal ions or enzyme bound cofactors (46, 48, 49). This is surprising because the complete reduction of the azo compounds to the respective amines requires 2 mol of NAD(P)H and thus a rather complex coordinated four-electron reduction, which may occur either by a simultaneous reduction process or by two subsequent coordinated two-electron transfers. This may indicate that the azoreductases mainly catalyze the reduction of the azo dyes to the corresponding hydrazo compounds. These may then be reduced spontaneously in the presence of NAD(P)H to the corresponding amines or undergo a spontaneous disproportionation to an aminoquinone and an aminoaromatic compound, as previously suggested for the chemical or anaerobic reduction of azo compounds (13, 16). This mechanism would require a rapid spontaneous reduction of the aminoquinones (or the naphthaquinones formed from the hydrolysis of the aminoquinones) to the aminohydroxy (or dihydroxy) compounds, because the enzyme demonstrates a rather fixed ratio of 2 mol of NAD(P)H consumed per mol of azo compound cleaved (49).

The experiments with the recombinant *E. coli* strain gave some further indications that the metabolism of sulfonated azo dyes is apparently limited by the transport of the highly charged dyes into the microbial cells. This has already previously been suggested for the intracellular reduction of sulfonated azo dyes under anaerobic conditions (13, 17, 37, 38, 47) and has now been substantiated also for the aerobic metabolism of this class of compounds. This suggests that microbial strains with the ability to decolorize sulfonated azo dyes intracellularly will require not only the presence of azoreductases but also a transport system(s) which allows the uptake of the sulfonated dyes into the cells. Currently there is no information available about transport systems for sulfonated azo dyes, but there are some reports about specific transport systems which are involved in the transport of other kinds of sulfonated substrates into bacterial cells (e.g., *p*-toluenesulfonate, taurine or alkanesulfonates) (14, 21, 29). Functionally similar transport systems are expected to exist also in bacteria (e.g., *Hydrogenophaga intermedia* S1 or *Sphingomonas xenophaga* BN6), which are able to grow aerobically on aminobenzene- or aminonaphthalenesulfonates, which are structural elements of many sulfonated azo dyes (12, 15, 41). Furthermore, it is clear that whole cells of strain KF46 are able to take up Orange II and to reduce this sulfonated dye in vivo (27). In order to construct recombinant organisms with the ability to decolorize sulfonated azo dyes in vivo, it therefore may be necessary to transfer the gene for the aerobic azo reductase into bacterial strains which are able to grow with sulfonated aromatics.

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