

The Function of Cytoplasmic Flavin Reductases in the Reduction of Azo Dyes by Bacteria

RAINER RUSS,† JÖRG RAU, AND ANDREAS STOLZ*

Institut für Mikrobiologie, Universität Stuttgart, 70569 Stuttgart, Germany

Received 10 August 1999/Accepted 11 January 2000

A flavin reductase, which is naturally part of the ribonucleotide reductase complex of *Escherichia coli*, acted in cell extracts of recombinant *E. coli* strains under aerobic and anaerobic conditions as an “azo reductase.” The transfer of the recombinant plasmid, which resulted in the constitutive expression of high levels of activity of the flavin reductase, increased the reduction rate for different industrially relevant sulfonated azo dyes in vitro almost 100-fold. The flavin reductase gene (*fre*) was transferred to *Sphingomonas* sp. strain BN6, a bacterial strain able to degrade naphthalenesulfonates under aerobic conditions. The flavin reductase was also synthesized in significant amounts in the *Sphingomonas* strain. The reduction rates for the sulfonated azo compound amaranth were compared for whole cells and cell extracts from both recombinant strains, *E. coli*, and wild-type *Sphingomonas* sp. strain BN6. The whole cells showed less than 2% of the specific activities found with cell extracts. These results suggested that the cytoplasmic anaerobic “azo reductases,” which have been described repeatedly in in vitro systems, are presumably flavin reductases and that in vivo they have insignificant importance in the reduction of sulfonated azo compounds.

Various bacterial strains reduce azo dyes under anaerobic conditions. The most generally accepted hypothesis for this phenomenon is that many bacterial strains possess rather unspecific cytoplasmic enzymes, which act as “azo reductases” and under anaerobic conditions transfer electrons via soluble flavins to the azo dyes (35). We have recently suggested a different mechanism for the unspecific anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and other bacteria. In this system, the reduction of the azo dyes is catalyzed extracellularly by the action of mediator compounds, which are either formed during the metabolism of certain substrates by the bacteria themselves or which may be added externally (e.g., compounds such as anthraquinonesulfonates). These mediators enable the transfer of redox equivalents from the cell membrane of the bacteria to azo dyes. In addition, it was demonstrated that *Sphingomonas* sp. strain BN6 also possesses cytoplasmic azo reductase activities, which could be assayed in vitro with cell extracts under anaerobic conditions (14, 15, 17). It was shown that the reduction rate of whole cells of *Sphingomonas* sp. strain BN6 for amaranth after growth with glucose was $0.3 \mu\text{mol min}^{-1} \text{g}$ of soluble cell protein⁻¹ and could be increased by the addition of anthraquinone-2-sulfonate to $3 \mu\text{mol min}^{-1} \text{g}$ of soluble cell protein⁻¹. In contrast, in vitro with cell extracts or solubilized membrane preparations, specific activities of 20 to $40 \mu\text{mol min}^{-1} \text{g}$ of soluble cell protein⁻¹ were determined (15, 17). A possible explanation for the significantly lower reaction rates of the whole-cell preparations compared to those in the in vitro systems was that in vivo the cytoplasmic azo reductase activity does not function, because the highly polar sulfonated azo dyes are not able to penetrate through the cell membranes. In the present study, we attempted to clarify the question of whether in vivo the intracellular cytoplasmic azo reductase activity is involved at all in

the metabolism of the azo compounds or whether this enzymatic activity is only active under in vitro conditions. Therefore, we increased the intracellular cytoplasmic azo reductase activity by using a genetic engineering approach and compared the resulting recombinant organism in vivo with the parent strain.

MATERIALS AND METHODS

Bacterial strains. The isolation and characterization of *Sphingomonas* sp. strain BN6 have been described before (20). The strain has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany, as DSM 6383. *Escherichia coli* K38 and JM109(DE3) were used for overexpression of the flavin reductase, and *E. coli* S17-1 (32) was used for the conjugative transfer of plasmids to strain BN6.

Culture conditions. *Sphingomonas* sp. strain BN6 was routinely grown in a mineral medium according to the method of Dorn et al. (7) with glucose (15 mM) and naphthalene-2-sulfonate (0.5 mM). For *Sphingomonas* sp. strain BN6(pRJR34), this medium was supplemented with 10 μg of tetracycline per ml. *E. coli* strains were routinely cultured in nutrient broth (NB) or Luria-Bertani (LB) medium. *E. coli* JM109(pRJR34) was grown in LB medium plus 12.5 μg of tetracycline per ml and isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM) to induce the flavin reductase.

Plasmids and DNA manipulation techniques. Plasmid DNA was isolated from *E. coli* by the method of Lee and Rasheed (18). Digestion of DNA with restriction endonucleases (Gibco BRL, Boehringer), electrophoresis, and ligation with T4 DNA ligase (Gibco BRL) were performed according to standard procedures (30). Transformation of *E. coli* was done by the method of Chung et al. (4).

The flavin reductase was produced by using *E. coli* K38(pEE1001) or *E. coli* JM109(pEE1001). Plasmid pEE1001 was constructed by Spyrou et al. (33) by cloning the flavin reductase gene from *E. coli* into the expression vector pTZ18R. In this construct, the flavin reductase gene (*fre*) is under the control of a phage T7 promoter.

For the construction of pRJR34, pEE1001 was cut with *EcoRI* and *HindIII*, the DNA fragment carrying the *fre* gene was isolated, and the ends were filled with the Klenow fragment. The resulting DNA fragment was ligated into the *EcoRV* site of plasmid pJOE890 (for a similar construct, see the study by Altenbuchner et al. [1]). From the resulting plasmid, the *fre* gene was removed by cleavage with *EcoRI* and ligated into the *EcoRI* cleavage site of pRK415 (16). The resulting plasmid was transformed into *E. coli* JM109. From eight of the recombinant clones, the flavin reductase activity was determined. The plasmid from the clone with the highest specific activity (2.4 U/mg of protein) was designated pRJR34 and used for further investigations.

Detection of the *fre* gene from *E. coli* in *Sphingomonas* sp. strain BN6(pRJR34) by PCR. For the PCR, oligonucleotide primers were custom synthesized according to the sequence of the gene described by Spyrou et al. (33). From the nucleotide sequence of the *fre* gene, bases 16 to 38 (forward primer) and 627 to 646 (reverse primer) were used as primers. PCR mixtures (50 μl) for the amplification of DNA contained 50 pmol of each primer, 10 to 20 ng of plasmid

* Corresponding author. Mailing address: Institut für Mikrobiologie, Universität Stuttgart, 70569 Stuttgart, Germany. Phone: 49-711-6855489. Fax: 49-711-6855725. E-mail: Andreas.Stolz@PO.Uni-Stuttgart.DE.

† Present address: Lehrbereich Biotechnologie, Universität Kaiserslautern, 67663 Kaiserslautern, Germany.

TABLE 1. Color index registration numbers, absorption maxima, purity, and calculated molar extinction coefficients of the sulfonated azo dyes used^a

Azo dye	Color index no.	Dye purity (%)	Absorption maximum (nm)	Extinction coefficient (mM ⁻¹ cm ⁻¹)
Acid black 1 (naphthol blue black)	20470	85	618	53.2
Acid black 24	26370	50	560	20.1
Acid black 52 (Palatines fast black)	15711	25	565	63.2
Acid blue 113	26360	70	557	20.1
Acid red 1	18050	60	530	21.1
Direct red 81	28160	50	509	55.2
Direct yellow 4 (brilliant yellow)	24890	70	404	74.2
Direct yellow 50	29025	60	398	38.3
Amaranth (acid red 27)	16185	90	520	22.6

^a The dyes were purchased from 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 at the dye purities indicated.

template DNA (isolated with Pharmacia Flexiprep kits), 0.1 mM (each) deoxynucleotide triphosphate, and 1 U of *Taq* DNA polymerase, polymerase reaction buffer, and MgCl₂ according to the manufacturer (GibcoBRL). The PCR was performed with a touchdown thermocycle program under the following conditions: initial denaturation (95°C, 3.75 min) before addition of the polymerase, 10 cycles with decreasing annealing temperature (60 to 55°C, 30 s), polymerization (72°C, 1 min), and denaturation (95°C, 45 s). Fifteen more cycles with 55°C as the annealing temperature and 72°C as the polymerization temperature were performed before the reaction was stopped, and the reaction products were analyzed by agarose gel electrophoresis. Thus, a fragment of the expected size (630 bp) was detected after amplification of the DNA from *E. coli* JM109(DE3)(pEE1001) and *Sphingomonas* sp. strain BN6(pRJR34), but not with DNA from the wild-type strain, *Sphingomonas* sp. strain BN6.

Anaerobic reduction of azo dyes by whole-cell preparations. *Sphingomonas* sp. strain BN6 was grown aerobically in a mineral medium to the late exponential growth phase (optical density at 546 nm of about 2 to 4) and then transferred to rubber-stoppered serum bottles (100 ml). Oxygen was removed from the medium by at least 15 2-min cycles of evacuation and flushing with nitrogen gas. Glucose (10 mM) and amaranth (1.3 mM) were added anaerobically, and samples were taken at different time intervals. The decrease in the dye concentration was determined by high-performance liquid chromatography as described previously (14).

Preparation of cell extracts. Cell suspensions in 50 mM Tris-HCl (pH 7.5) were disrupted by using a French press (Aminco, Silver Spring, Md.) at 80 MPa. Cell debris was removed by centrifugation at 100,000 × g for 30 min at 4°C. The protein concentration was determined by the method of Bradford (2), with bovine serum albumin as the standard.

Enzyme assays. One unit of enzyme activity was defined as the amount of enzyme that converts 1 μmol of substrate per min.

(i) **Flavin reductase.** The NAD(P)H:flavin oxidoreductase was measured by a modification of the method given by Fontecave et al. (9). In this aerobic assay, the flavin reductase catalyzes the reduction of riboflavin, and the reduced riboflavin is immediately reoxidized by oxygen. Cell extract was added to a solution (final volume, 1 ml) containing 10 μmol of Tris-HCl (pH 7.5), 0.25 μmol of NADPH, and 0.003 μmol of riboflavin. The decrease in *A*₃₄₀ was measured spectrophotometrically. Reaction rates were calculated by using a molar extinction coefficient of 6.3 mM⁻¹ cm⁻¹.

(ii) **Azo reductase.** Azo reductase activity was routinely measured by a modification of the spectrophotometric assay described previously by Haug et al. (14). The anaerobic reaction mixture contained (in 1.6 ml) 70 μmol of Tris-HCl buffer (pH 7.5), 1 μmol of NADH, 0.32 μmol of flavin adenine dinucleotide (FAD),

and 0.1 μmol of amaranth. The stock solutions were made anaerobically by repeated gassing with N₂. The reaction was performed in gastight cuvettes and started by the addition of cell extracts. The decrease in the concentration of amaranth was determined spectrophotometrically at 520 nm, and reaction rates were calculated by using a molar extinction coefficient of 22.6 mM⁻¹ cm⁻¹. In certain experiments, azo dyes different from amaranth were used. The relevant wavelengths and extinction coefficients for these dyes are summarized in Table 1.

Chemicals. All chemicals were obtained from Merck, Fluka, Sigma, or Aldrich, except for Mordant yellow 3, which was kindly provided by Bayer AG.

RESULTS

The flavin reductase from *E. coli* as azo reductase. It was previously suggested that, under anaerobic conditions, unspecific cytoplasmic azo reductases act via the intermediate formation of free reduced flavins (35). This hypothesis has been substantiated by the observation that photochemically generated reduced FAD (FADH₂) reduced the sulfonated azo compound amaranth (10). In this system, the only function of the "cytoplasmic azoreductases" would be the supply of reduced flavins for the subsequent purely chemical reduction of the azo compounds by the reduced flavins. It was therefore reasoned that any flavin reductase [NAD(P)H:flavin oxidoreductase] could also act as an azo reductase. Recently, a flavin reductase gene (*fre*) was cloned. The gene product, which is naturally part of the ribonucleotide reductase complex of *E. coli*, was produced in large amounts with the expression plasmid pEE1001. In this construct, the flavin reductase gene is transcribed from the phage T7 promoter (26, 33). This system was applied to assay the flavin reductase for its azo reductase activity. *E. coli* JM109(DE3)(pEE1001), *E. coli* K38(pEE1001), and, as a negative control, *E. coli* JM109 were grown in LB medium, cell extracts were prepared, and the flavin reductase and the azo reductase activities were determined under anaerobic conditions. Thus, it was confirmed that the *E. coli* strains with the recombinant plasmid really produced the flavin reductase with very high specific activities, and it was demonstrated that the flavin reductase could act under anaerobic conditions as azo reductase (Table 2). Surprisingly, the flavin reductase activity was constitutively expressed in *E. coli* JM109(DE3)(pEE1001), and the activity could not be increased by the addition of IPTG (up to 2 mM). The proposed function of FAD as a mediator compound transferring redox equivalents from the flavin reductase to the azo dye was shown in a control experiment, in which the cell extract was incubated with NADPH and amaranth with or without added FAD. Thus, it was found that, in the absence of externally added FAD, less than 5% of the azo reductase activity could be detected.

Comparison of the cytoplasmic azo reductase activities in *E. coli* K38(pEE1001) and *Sphingomonas* sp. strain BN6 with different industrially relevant azo dyes. It has been previously reported that azo reductase activity was present in cell extracts of *Sphingomonas* sp. strain BN6 (14, 15, 17). Therefore, the azo reductase activities of cell extracts from *E. coli* K38(pEE1001) and *Sphingomonas* sp. strain BN6 were compared and tested for their ability to reduce different industrially relevant azo dyes (Table 3). Thus, it was found that the azo reductase activity in the cell extract of *E. coli* K38(pEE1001) for many

TABLE 2. Specific activities of the flavin reductase and the azo reductase in cell extracts

Enzyme	Sp act (U/mg of protein) of cell extract				
	<i>E. coli</i> JM109	<i>E. coli</i> JM109(DE3)(pEE1001)	<i>E. coli</i> K38(pEE1001)	<i>Sphingomonas</i> sp. strain BN6	<i>Sphingomonas</i> sp. strain BN6(pRJR34)
Flavin reductase	0.09	2.0	4.0	0.008	0.7
Azo reductase	0.007	0.3	1.3	0.004	0.14

TABLE 3. Specific reductive activities of different industrially relevant azo dyes^a

Azo dye	Chemical structure	Specific azo reductase activity with		
		Strain BN6- whole cells (U/g prot.)	Strain BN6- cell extract (U/g prot.)	<i>E.coli</i> K38 (pEE1001)- cell extract (U/g prot.)
Acid Black 1		1.7	7.4	1000
Acid Black 24		<0.05	0.8	<10
Acid Blue 113		ND	0.6	<10
Acid Red 1		0.55	2.9	690
Amaranth		0.8	10	1300
Direct Red 81		1.3	2.4	910
Direct Yellow 4		0.7	<0.1	50
Direct Yellow 50		0.8	1.7	490
Mordant Yellow 3		1.9	1.5	53

^a ND, not done; prot., protein.

azo dyes was more than 100-fold higher than the activities found in cell extracts of strain BN6. The cell extracts from both strains had high relative activities for those dyes which carried in the *ortho* position to the azo group a hydroxyl group and in general had somehow lower activities for dyes with a hydroxy group in the *para* position to the azo bond. The hydrogen of the hydroxy group in the *ortho* position can form an intramolecular hydrogen bridge with the nitrogen atom of the azo group. This reduces the electron density at the azo bond and thus facilitates a reductive cleavage. Furthermore, it was observed that an increasing number of sulfonic acid substituents also resulted in an increased reduction rate. This may be

caused by the electron withdrawal effect of the sulfonic acid substituents, which also reduces the electron density at the azo bond.

Transfer of the flavin reductase gene to *Spingomonas* sp. strain BN6. The plasmid pEE1001, which carries the *fre* gene, is a pBR322 derivative and has only a narrow host range. It has been previously reported that IncP1 plasmids can be conjugatively transferred to *Spingomonas* sp. strain BN6 (28). Therefore, plasmid pRJR34 was constructed, which contains the *fre* gene cloned into the broad-host-range plasmid pRK415 (16). After the preparation of cell extracts from JM109(pRJR34), flavin reductase activities of 1.7 U/mg of protein were found.

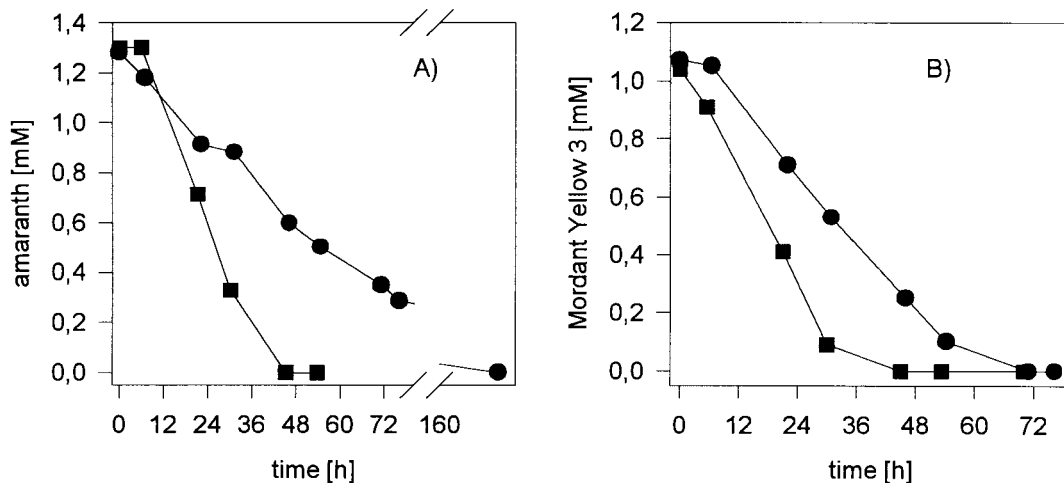


FIG. 1. Reduction of amaranth (A) or Mordant yellow 3 (B) under anaerobic conditions by whole cells of *Spingomonas* sp. strain BN6 (●) or BN6(pRJR34) (■). The cells were grown aerobically in a mineral medium with glucose (10 mM) and naphthalene-2-sulfonate (0.5 mM) [plus 10 μ g of tetracycline per ml for BN6(pRJR34)]. When the cells reached the late-exponential growth phase, 25 ml of the cultures was transferred into serum bottles (100-ml volume) with a nitrogen atmosphere, and glucose (10 mM) and Mordant yellow 3 (1 mM) or amaranth (1.3 mM) were added. At the indicated intervals, aliquots were taken, cells were removed by centrifugation, and the concentrations of the azo dyes were determined by high-performance liquid chromatography.

Thus, the flavin reductase was expressed in this system too. Finally, *E. coli* S17-1 (32) was transformed with plasmid pRJR34, and the resulting transformant was used to conjugatively transfer pRJR34 to *Spingomonas* sp. strain BN6. After selection on a mineral medium with glucose (10 mM) plus tetracycline (5 μ g/ml), a transfer frequency of plasmid (pRJR34) from *E. coli* S17-1 to strain BN6 of almost 100% was found.

Expression of the flavin reductase and the azo reductase activity in *Spingomonas* sp. strain BN6. *Spingomonas* sp. strain BN6(pRJR34) was grown in liquid culture with NB and tetracycline (10 μ g/ml), and cell extracts were prepared. The flavin reductase activity in the recombinant strain was 0.7 U/mg of protein, compared to a flavin reductase activity in wild-type strain BN6 of 0.008 U/mg of protein. The presence of the *fre* gene from *E. coli* in *Spingomonas* sp. strain BN6(pRJR34) was also shown by PCR (see Materials and Methods). These results demonstrated that the recombinant organism indeed expressed the flavin reductase gene originating from *E. coli*. Therefore, whether these cell extracts also showed an increased azo reductase activity under anaerobic conditions was also tested. Thus, it could be demonstrated that the azo reductase activity in the recombinant strain was almost 30-fold higher than that in the wild-type strain (Table 2).

In contrast to the cell-free system, whole cells of *Spingomonas* sp. BN6(pRJR34) showed only an approximately threefold increase in the reduction rate for amaranth and Mordant yellow 3 compared to the wild type BN6 strain (Fig. 1). Furthermore, the specific activities of whole cells (0.002 U mg of protein⁻¹) were significantly lower for strain BN6(pRJR34) than the activities determined with cell extracts from the same strain (0.14 U mg of protein⁻¹). This suggested that either the cell membranes limited the uptake of the highly polar sulfonated azo compounds or the lack of some cofactors (e.g., free flavins) limited the reduction of the azo compounds by whole cells. The addition of flavin mononucleotide (FMN) or FAD to cell suspensions of the recombinant strain BN6(pRJR34) did not significantly increase the azo reductase activity. In contrast, an almost 100-fold increase in the reduction rate was observed after the addition of anthraquinone-2-sulfonate (0.5 mM).

Aerobic function of the flavin reductase as azo reductase.

All of the experiments described above were performed under anaerobic conditions, because it is well known that reduced flavins react rapidly with molecular oxygen. Since the recombinant strains expressed the flavin reductase with high specific activities, we assumed that there could be an azo reductase activity of the flavin reductase as well in the presence of molecular oxygen. Therefore, the azo reductase assays with cell extracts were compared under anaerobic and aerobic conditions. The reactions were analyzed spectrophotometrically at $\lambda = 340$ nm to measure the oxidation of NADH and at $\lambda = 520$ nm to assay the reduction of amaranth (Fig. 2). Thus, it was found that, also under aerobic conditions, some decrease in the concentration of amaranth was observed, although the reaction rates were significant lower than those of the anaerobic

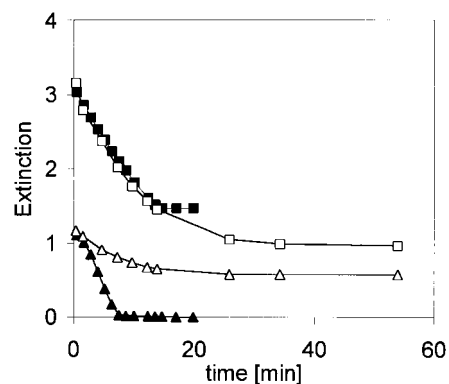


FIG. 2. Reduction of amaranth by cell extracts from *E. coli* K38(pEE1001) under aerobic (□, △) and anaerobic conditions (■, ▲). The reaction mixtures contained (per milliliter) 50 μ mol of NaK phosphate buffer (pH 7.7), 0.4 μ mol of NADH, 0.1 μ mol of FAD, and 0.05 μ mol of amaranth. The reactions were started by the addition of 20 μ l of a cell extract from *E. coli* K38(pEE1001) (88 μ g of protein), and the decrease in absorbance was simultaneously determined spectrophotometrically at $\lambda = 340$ nm (■, □) and $\lambda = 520$ nm (▲, △). For the reaction under anaerobic conditions, all solutions were made anaerobically by repeated gassing with N₂, and the reaction was performed in gastight cuvettes.

control. In contrast, almost identical oxidation rates for NADH were observed under aerobic and anaerobic conditions (Fig. 2). This observation may explain the results of some sporadic reports about the presence of unspecific aerobic azo reductases in bacteria such as *E. coli* (11, 12).

DISCUSSION

There are numerous reports which describe the reduction of azo compounds by bacteria under anaerobic conditions. The main interest in this field has focused on bacteria from the intestine which are involved in the metabolism of azo dyes ingested as food additives, and there are some studies which refer to bacteria isolated from soil or sewage treatment systems (5, 6, 8, 27, 29, 31). The earlier studies were mainly performed with facultatively anaerobic bacteria (e.g., *Proteus vulgaris*, *Streptococcus faecalis*, or *Bacillus* sp.). It had been repeatedly suggested that, in these strains, cytosolic flavin-dependent reductases were responsible for this unspecific reaction and that the low permeability of the cell membranes for the highly polar sulfonated azo compounds would cause the significantly lower reduction rates observed with whole cells compared to those in cell-free systems (19, 27, 36). Because none of these cytosolic azo reductases has ever been purified, until now it has been speculative whether bacterial flavin reductases were responsible for the azo reductase activity observed with bacterial cell extracts. The present study proved that flavin reductases are indeed able to act as azo reductases and that cell extracts containing the cloned flavin reductase decolorized a broad range of industrially relevant azo dyes.

Our results suggested that the reduction of sulfonated azo dyes by reduced flavins formed by cytosolic flavin-dependent azo reductases is mainly observed *in vitro* and *in vivo* is of insignificant importance. This became evident with the recombinant strain BN6(pRJR34), which *in vitro* showed significantly increased azo reductase activities compared to those of the wild type, although *in vivo* only a very small increase in azo reductase activity was found. Theoretically, this could also have been caused by a lack of the required cofactors (NADH and/or free flavins) in the resting cells. This seems to be improbable, because the anaerobically incubated cells should possess a rather high concentration of NADH. There is only limited information available about the free flavin content of bacterial cells. However, it has been observed that recombinant FMN-dependent luciferases are fully functional in different bacterial backgrounds without the concurrent transfer of recombinant FMN reductases (34). This has also been shown recently for strain BN6 (J. Klein, personal communication). This suggests that reduced free flavins are present in various bacteria and also in strain BN6 in quantities which allow the support of enzymatic reactions which require reduced flavins.

A further indication that the flavin-dependent azo-reductases are almost completely laboratory artifacts was shown by the effects of externally added flavins on the rates of azo reduction under anaerobic conditions. Thus, it was found that the addition of FAD to a resting cell suspension of strain BN6 resulted in no significant increase in the rate of azo dye reduction. In contrast, the addition of the same concentration of anthraquinone-2,6-disulfonate resulted in an almost 10-fold increase in the dye reduction rate (17). Similar observations were also made with *Sphingomonas* sp. strain BN6(pRJR34) and *E. coli* JM109(DE3)(pEE1001) (unpublished results). This suggested that the bacterial membranes not only are efficient barriers for the uptake of sulfonated azo dyes, but also are hardly permeable for flavin-containing cofactors and therefore restrict the transfer of reduction equivalents by flavins from the cytosol of

intact cells to sulfonated azo dyes in the culture medium. Thus, in living cells with intact cell membranes, other enzyme systems and/or other redox mediators are presumably responsible for the reduction of azo dyes. In bacteria which possess electron transport systems in their membranes, such as the purely aerobic or facultatively anaerobic bacteria studied in the present report, the transfer of the redox equivalents from the respiratory chain of the cell membranes to appropriate redox mediators could take place directly. If intracellular reductases should be involved in this process, it may be assumed that mediators different from flavin cofactors must be involved. A prerequisite for these mediators would be a higher ability to pass the bacterial membranes than that of flavins.

A different concept for the reduction of sulfonated azo compounds which also does not require a membrane transport of the dyes has been suggested for bacterial strains from the strictly anaerobic microflora of the intestine. Raffi and coworkers isolated different bacteria from the human intestine (e.g., *Eubacterium* sp., *Clostridium* sp., *Butyrivibrio* sp., or *Bacteroides* sp.), which decolorized sulfonated azo dyes during growth on solid or liquid complex media. It was shown that at least part of the azo reductase activities were extracellular, because the culture supernatants were able to decolorize the dyes under anaerobic conditions (24, 25). This extracellular azo reductase activity was due to the activity of specific proteins, because after non-denaturing polyacrylamide gel electrophoresis and activity staining under anaerobic conditions, usually only one distinct protein band in the gels was able to decolorize the dyes (21). In most isolates, flavin compounds were required for azo reductase activity, but in some clostridia, the azo reductase activity was independent of externally added flavins (24). Recently, a DNA fragment from *Clostridium perfringens* was cloned in phage λ gt11, which increased the azo reductase activity of lytic and lysogenic cultures of *E. coli* infected with the recombinant phage (23). The azo reductase from *C. perfringens* was detected by immunoelectron microscopy throughout the cytoplasm and in the vicinity of the cells (22). This suggested that the protein is not a typical extracellular enzyme, but that it is presumably released only from lysed cells. The azo reductase activity from *C. perfringens* is clearly different from the flavin reductase investigated in the present study, because the enzyme activity was described as being independent of that from added flavins, and furthermore, the enzyme was rapidly and irreversibly inactivated by oxygen (24). It is still unclear in this system how the supposed extracellular azoreductases should gain the NADH necessary for the reduction of the azo dyes in their extracellular environment, and there may be some effects from the complex growth media of the bacteria. Furthermore, there are some other publications about the reduction of azo compounds by whole-cell preparations of strictly anaerobic bacteria which suggested the involvement of redox mediators (3, 4). Therefore, it can't be excluded at present that extracellular mediator compounds also participate in the reduction of azo compounds by strictly anaerobic bacteria.

The third possibility for the extracellular reduction of azo dyes caused by microorganisms is the action of reduced inorganic compounds (Fe^{2+} , H_2S), which are formed as end products of certain strictly anaerobic bacteria. Also, in these cases, mediator compounds may have an important function (13, 37).

It may be concluded that, depending on the environmental conditions (or the reaction conditions of a biotechnological process), different reactions catalyzed by bacteria can accomplish the reduction of azo compounds under anaerobic conditions, but that intracellular reactions have only insignificant importance in these reactions.

REFERENCES

1. Altenbuchner, J., P. Viell, and I. Pelletier. 1992. Positive selection vectors based on palindromic DNA sequences. *Methods Enzymol.* **216**:457–466.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
3. Brown, J. P. 1981. Reduction of polymeric azo and nitro dyes by intestinal bacteria. *Appl. Environ. Microbiol.* **41**:1283–1286.
4. Chung, C. T., S. L. Niemela, and R. H. Miller. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. USA* **86**:2172–2175.
5. Chung, K.-T., G. E. Fulk, and M. Egan. 1978. Reduction of azo dyes by intestinal anaerobes. *Appl. Environ. Microbiol.* **35**:558–562.
6. Chung, K.-T., S. E. Stevens, Jr., and C. E. Cerniglia. 1992. The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.* **18**:175–190.
7. Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss. 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. *Arch. Microbiol.* **99**:61–70.
8. Dubin, P., and K. L. Wright. 1975. Reduction of azo food dyes in cultures of *Proteus vulgaris*. *Xenobiotica* **5**:563–571.
9. Fontecave, M., R. Eliasson, and P. Reichard. 1987. NAD(P)H:flavin oxidoreductase of *E. coli*: a ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. *J. Biol. Chem.* **262**:12325–12331.
10. Fujita, M., and J. Peisach. 1982. The stimulation of microsomal azoreduction by flavins. *Biochim. Biophys. Acta* **719**:178–189.
11. Ghosh, D. K., S. Ghosh, P. Sadhukhan, A. Mandal, and J. Chaudhuri. 1993. Purification of two azoreductases from *Escherichia coli* K12. *Indian J. Exp. Biol.* **31**:951–954.
12. Ghosh, D. K., A. Mandal, and J. Chaudhuri. 1992. Purification and partial characterization of two azoreductases from *Shigella dysenteriae* type 1. *FEMS Microbiol. Lett.* **98**:229–234.
13. Glaus, M. A., C. G. Heijman, R. P. Schwarzenbach, and J. Zeyer. 1992. Reduction of nitroaromatic compounds mediated by *Streptomyces* sp. exudates. *Appl. Environ. Microbiol.* **58**:1945–1951.
14. Haug, W., A. Schmidt, B. Nörtemann, D. C. Hempel, A. Stolz, and H.-J. Knackmuss. 1991. Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.* **57**:3144–3149.
15. Keck, A., J. Klein, M. Kudlich, A. Stolz, H.-J. Knackmuss, and R. Mattes. 1997. Reduction of azo dyes by mediators originating in the naphthalene-sulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. *Appl. Environ. Microbiol.* **63**:3684–3690.
16. Keen, K. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**:191–197.
17. Kudlich, M., A. Keck, J. Klein, and A. Stolz. 1997. Localization of the enzyme system involved in the anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiol.* **63**:3691–3694.
18. Lee, S., and S. Rasheed. 1990. A simple procedure for maximum yield of high-quality plasmid DNA. *BioTechniques* **9**:676–679.
19. Mechsner, K., and K. Wuhrmann. 1982. Cell permeability as a rate limiting factor in the microbial reduction of sulfonated azo dyes. *Eur. J. Appl. Microbiol. Biotechnol.* **15**:123–126.
20. Nörtemann, B., J. Baumgarten, H. G. Rast, and H.-J. Knackmuss. 1986. Bacterial communities degrading amino- and hydroxynaphthalene-2-sulfonates. *Appl. Environ. Microbiol.* **52**:1195–1202.
21. Rafii, F., and C. E. Cerniglia. 1990. An anaerobic gel assay for the detection of azoreductase from anaerobic bacteria. *J. Microbiol. Methods* **12**:138–149.
22. Rafii, F., and C. E. Cerniglia. 1993. Localization of the azoreductase of *Clostridium perfringens* by immuno-electron microscopy. *Curr. Microbiol.* **27**:143–145.
23. Rafii, F., and T. Coleman. 1999. Cloning and expression in *Escherichia coli* of an azoreductase gene from *Clostridium perfringens* and comparison with azoreductase genes from other bacteria. *J. Basic Microbiol.* **39**:29–35.
24. Rafii, F., W. Franklin, and C. E. Cerniglia. 1990. Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. *Appl. Environ. Microbiol.* **56**:2146–2151.
25. Rafii, F., J. D. Moore, J. G. H. Ruseler-van Embden, and C. E. Cerniglia. 1995. Bacterial reduction of azo dyes used in foods, drugs and cosmetics. *Microecol. Ther.* **25**:147–156.
26. Reichard, P. 1993. From RNA to DNA, why so many ribonucleotide reductases? *Science* **260**:1773–1777.
27. Roxon, J. J., A. J. Ryan, and S. E. Wright. 1967. Enzymatic reduction of tartrazine by *Proteus vulgaris* from rats. *Food Cosmet. Toxicol.* **5**:645–656.
28. Russ, R., A. Stolz, and H.-J. Knackmuss. 1995. Comparison of the efficiency to degrade naphthalene-2-sulfonate between a mixed culture and an *in-vitro* constructed hybrid strain, p. 114–116. *In R. D. Schmid (ed.), Biochemical engineering 3*. Kurz & Co., Stuttgart, Germany.
29. Ryan, A. J., J. J. Roxon, and A. Sivayavirajana. 1968. Bacterial azo reduction: a metabolic reaction in mammals. *Nature* **219**:854–855.
30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. Scheline, R. R., R. T. Nygaard, and B. Longberg. 1970. Enzymatic reduction of the azo dye, Acid Yellow, by extracts of *Streptococcus faecalis*, isolated from rat intestine. *Food Cosmet. Toxicol.* **8**:55–58.
32. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for *in-vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *BioTechnology* **1**:784–791.
33. Spyrou, G., E. Haggård-Ljungquist, M. Krook, H. Jörnvall, E. Nilsson, and P. Reichard. 1991. Characterization of the flavin reductase gene (*fre*) of *Escherichia coli* and construction of a plasmid for overproduction of the enzyme. *J. Bacteriol.* **173**:3673–3679.
34. Stewart, G. S., and P. Williams. 1992. *lux* genes and the applications of bacterial luminescence. *J. Gen. Microbiol.* **138**:1289–1300.
35. Walker, R. 1970. The metabolism of azo compounds: a review of the literature. *Food Cosmet. Toxicol.* **8**:659–676.
36. Wuhrmann, K., K. Mechsner, and T. Kappeler. 1980. Investigations on rate-determining factors in the microbial reduction of azo dyes. *J. Appl. Microbiol. Biotechnol.* **9**:325–338.
37. Yoo, E. S., J. Libra, and U. Wiesmann. 1999. Untersuchungen zum anaeroben Entfärbungsmechanismus von Azofarbstoffen. *DECHEMA Jahrestagung 1999*:399–400.