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Reduction of Nitroaromatic Compounds Mediated by *Streptomyces* sp. Exudates

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Exudates from *Streptomyces griseoflavus* Tü 2484 effectively mediated electron transfer between hydrogen sulfide and various nitrobenzenes. In general, pseudo-first-order kinetics were observed, except for the initial phase of the reaction at higher pH values. Under fixed pH and E_h conditions, linear free energy relationships were found between the logarithms of the reaction rate constants and the one-electron reduction potentials of the nitroaromatic compounds. No competition was observed between various compounds. Comparison of the results of this study with the results of experiments conducted with model quinones and an iron porphyrin suggest that the secondary metabolites cinnaquinone and dicinnaquinone, excreted by strain Tü 2484 on the order of 100 mg/liter, are responsible for the catalytic activity of the exudate. Further support for this hypothesis comes from the facts that the catalytic activity of the exudate became prominent only after the growth phase of the microorganisms and that the mediating substances have a molecular weight of less than 3,000.

Many xenobiotic organic compounds exhibit functional groups that render them susceptible to reductive transformation (10). Such compounds include polyhalogenated hydrocarbons (20), nitroaromatic compounds (7, 16), azo compounds (23), and compounds that exhibit a sulfoxide or sulfone group (19). Since in many cases the products of such redox reactions are of similar or even greater environmental concern than the parent compounds, information on the mechanisms and rates of these processes is of great interest. There is growing evidence that in anaerobic environments such compounds are reduced not only by biological (i.e., microbial) but also by abiotic processes (10).

Aside from biological electron donors, the most abundant natural reductants include reduced inorganic forms of iron and sulfur, such as iron(II) sulfides, iron(II) carbonates, iron(II) and iron(III) oxides, and hydrogen sulfide (17). Although some of these reductants have been found to react with reducible organic pollutants, including, for example, the reaction of hydrogen sulfide with substituted nitrobenzenes (15), the reaction rates are, in general, much too slow to account for the extremely rapid transformation rates often observed in natural systems. For example, for reduction of the nitro groups of parathion (21) or methyl parathion (24) in anaerobic soils and sediments, half-lives of only seconds to minutes have been determined. Since these half-lives are much shorter than those commonly found with bacterial cultures, even at very high cell counts, highly reactive reductants must be available. These reductants may not be present in abundance but may play the role of electron transfer mediators (Fig. 1); that is, after electron transfer to the xenobiotic compound, they may again be reduced rapidly by the bulk reductants present (15).

In biological systems, species that act as mediators for electron transfer include quinoid-type compounds and a variety of transition metal complexes. Such species have been found to react with various reducible organic compounds in homogeneous aqueous solution, for example, with polyhalogenated hydrocarbons (3, 6) and with nitroaromatic compounds (15, 18). It has also been demonstrated that natural organic matter from carbon-rich groundwaters mediates the reduction of substituted nitrobenzenes (5). Likely sources and/or precursors of electron transfer mediators in natural systems are components of decaying organisms and of natural organic matter. There is, however, only limited information available on the structures and abundance of such species in natural systems. It is also largely unknown to what extent secondary metabolites of microorganisms contribute to the bulk of electron transfer mediators present in natural systems.

Here we report the results of laboratory studies in which the exudate of a bacterial culture, *Streptomyces griseoflavus* Tü 2484, was investigated for the potential to mediate reduction of nitrobenzenes in homogeneous aqueous solutions containing hydrogen sulfide as a bulk electron donor. This genus was chosen for its well-known ability to excrete a variety of secondary metabolites in fairly high amounts and for its broad occurrence in soil. Cells of strain Tü 2484 growing aerobically on medium DSM-65–NO_3 were unable to reduce 4-chloronitrobenzene (4-Cl-NB) in the presence of air within the observation time of a few days. In contrast, the 0.2-μm-pore-size filtrate of such a culture collected after several days of growth mediated the conversion of 4-Cl-NB to 4-chloroaniline in a solution containing hydrogen sulfide. Therefore, we concentrated our observations on the reduction of a series of substituted nitrobenzenes in a homogeneous aqueous solution containing exudate of the bacterial culture and hydrogen sulfide. The results of this study are discussed in the light of an earlier report in which we described the reduction of substituted nitrobenzenes in the presence of model mediators including two quinones and an iron porphyrin (15).

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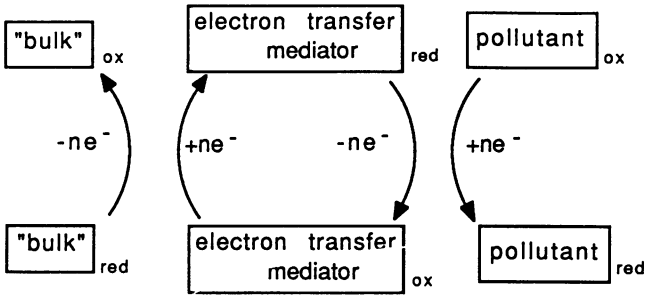

FIG. 1. Proposed role of reductants as electron transfer mediators in nitro group reduction. ne⁻, number of electrons; ox, oxidized; red, reduced.

MATERIALS AND METHODS

Chemicals. 2-(*N*-Morpholino)ethanesulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and disodium sulfide were obtained from Fluka AG, Buchs, Switzerland. The sources and abbreviations of the various nitro compounds are summarized in Table 1. All chemicals were of the highest purity available and were used as received.

Bacterial strain. *S. griseoflavus* Tü 2484 was a gift from H. Zähner, Eberhard-Karls Universität, Tübingen, Germany.

Medium. The basal medium used in this study is known as DSM-65 (4). It consists of 0.4% glucose, 0.4% yeast extract, and 1% malt extract. To this basal medium, 1 M $NaNO_3$ was added to a final concentration of 2 mM. Before autoclaving (20 min at 120°C), the pH was set at 7.20 (±0.05) with 2 M NaOH. This medium is referred to as DSM-65–NO_3.

Growth conditions. The cultures were incubated in baffled 1-liter Erlenmeyer flasks filled to a maximum of about 400 ml. The cultures were incubated aerobically at 30°C on a rotary shaker (100 rpm).

Determination of growth. Dry weight was measured by filtering 2.90 ml of culture through a cellulose nitrate filter (0.2-μm pore size; Sartorius GmbH, Göttingen, Germany) which had been dried at 105°C for 24 h. After being washed twice with 5 ml of water, the samples were dried at 105°C for 24 h. Other parameters for determination of growth had turned out to be impracticable: turbidity because of the size of cell aggregates, protein content because of the high background of the nutrient medium and contamination from the filters, and wet weight because of large fluctuations in the measurements.

TABLE 1. Substrates and analytical conditions

Compound	Abbreviation	Internal standard	Methanol/water ratio (%)[a]	λ (nm)[b]
Nitrobenzene[c]	NB	4-Me-NB	65/35	280
2-Methylnitrobenzene[c]	2-Me-NB	NB	65/35	280
3-Methylnitrobenzene[c]	3-Me-NB	NB	65/35	280
4-Methylnitrobenzene[c]	4-Me-NB	NB	65/35	280
2-Chloronitrobenzene[c]	2-Cl-NB	4-Cl-NB	65/35	254
3-Chloronitrobenzene[c]	3-Cl-NB	2-Cl-NB	65/35	254
4-Chloronitrobenzene[c]	4-Cl-NB	2-Cl-NB	65/35	254
2-Acetylnitrobenzene[d]	2-Ac-NB	NB	50/50	254
3-Acetylnitrobenzene[d]	3-Ac-NB	NB	60/40	280
4-Acetylnitrobenzene[d]	4-Ac-NB	NB	60/40	280

[a] Ratio (vol/vol) of methanol to water in the mobile phase.
[b] Wavelength of HPLC detection.
[c] Obtained from Fluka AG.
[d] Obtained from Merck.

Culture filtrate. Cultures of strain Tü 2484 were grown on DSM-65–NO_3 for about 2 weeks. The cells were removed from the filtrate first by centrifugation at 8,000 × *g* and then by filtration of the supernatant through a 0.2-μm-pore-size filter. The filtrate was stored aseptically at room temperature in the dark. The catalytic activity of the filtrate stored under these conditions remained unchanged for up to 40 days. However, a 5-month-old filtrate exhibited only half of its initial activity.

Ultrafiltration and heat treatment of the culture filtrate. An ultrafilter with a 3,000-Da molecular size cutoff (Centricon-3; Grace Company, Danvers, Mass.) was used for ultrafiltration of the culture filtrate.

The culture filtrate was boiled for about 15 min in 50-ml serum flasks. A needle in the butyl stopper prevented overpressure. After having cooled to room temperature, the evaporated water (determined as loss of weight) was balanced by adding the corresponding amount of distilled water to the flask.

UV and visible spectra. The UV and visible spectra of filtered cultures or media were measured in 1.0-cm cuvettes on a spectrophotometer (Uvicon 810; Kontron Instruments, Zürich, Switzerland) with water as the reference.

Assay to determine the reduction rates of nitrobenzenes. Reduction of substituted nitrobenzenes was carried out in the dark at 25°C in 25, 50, or 100-ml serum flasks. These flasks were sealed with black butyl rubber stoppers (Bellco, Vineland, N.J.) in the case of fast reactions (half-lives of less than 1 h). Because about 1% of the nitro compounds was lost per hour in or through these stoppers, it was necessary to use Teflon-coated stoppers (Wheaton, Millville, N.J.) for experiments in which nitroreduction continued for several hours. Teflon-coated stoppers showed no adsorption of nitroaromatic compounds. Their disadvantage, which consists of slightly increased oxygen leakage, was diminished by applying a small amount of high-vacuum grease (Dow Corning) to the contact area between the stopper and the glass vessel.

The assay was performed by rendering a mixture of distilled water, buffer (potassium phosphate, HEPES, or MES, depending on the pH; 1 M stock solutions), and culture filtrate (or sterile DSM-65–NO_3 for controls) anaerobic by alternatively evacuating and purging the container (five times) with N_2 and then adding NaHS (0.5 M stock solution, pH 8). After incubation for 15 min (allowing the sulfide to reduce the mediators), the reaction was started by adding the nitro compound (1 mM aqueous or 100 mM methanol stock solution). The final concentrations were 50 to 100 mM for buffer (depending on the required buffer capacity), about 5 mM for total sulfide, and about 100 μM for the nitro compounds. The final concentration of the culture filtrate (or DSM-65–NO_3) depended on the reactivity of the assayed nitro compound and ranged from 1 to 91% (vol/vol).

Because of the impossibility of determining the concentration of active components in the exudate, the volumetric part of the filtrate or medium in the reduction assay is given as a percentage (vol/vol). Throughout this report, the terms filtrate and medium, stand for the 0.2-μm-pore-size filtrate of a culture of strain Tü 2484 usually collected after 2 weeks of incubation and for sterile DSM-65–NO_3, respectively.

The apparent redox potential of a hydrogen sulfide buffer in the pH range considered may be calculated with the equation $E_h(pH[S^2]_{tot}) = E_h^0 + (2.303RT/2F)\log\{[H^+]([H^+] + K_{H_2S})/[S^{2-}]_{tot}\}$ (14) with a pK_{H_2S} of 7 and an $E_h^0(S + 2e^-$

$+ 2H^+ = H_2S_{aq}$) of 0.144 V (17). It is −0.192 V for pH 7.0 and a 5 mM $[H_2S]_{tot}$. It should be emphasized that in experiments in which the reaction rates were compared directly, the amount of total sulfide was always equal.

The reaction rate was monitored by subsequent sampling at appropriate time intervals with a nitrogen-filled syringe. Samples of 1 ml were extracted with 1 ml of ethyl acetate after addition of 1 ml of a 0.1 mM (aqueous solution) concentration of the internal standard (Table 1). The ethyl acetate extract was analyzed by high-performance liquid chromatography (HPLC). In cases of formation of an emulsion, centrifugation in Eppendorf tubes produced good phase separation. For quantitative determination of the hydroxlamines, it was important to analyze the sample immediately. In the extraction mixture, 30% of 4-chlorohydroxylamine was converted to the corresponding aniline within 10 h at room temperature.

HPLC analyses. HPLC analyses were performed on an RP-18 reverse-phase column (LiChrocart stainless steel cartouche; 125 by 4 mm; 5-μm-diameter spheres; Merck, Darmstadt, Germany) connected to a series 4 liquid chromatograph pumping system (Perkin-Elmer AG, Ueberlingen, Germany) supplemented with a Rheodyne 7125 injector and a 6-μl injection loop, a Uvikon 430 variable-wavelength UV detector (Kontron AG, Zürich, Switzerland), and a Perkin-Elmer LCI-100 computing integrator. The flow rate was set at 1.0 ml/min. The mobile phase consisted of a mixture of methanol and water, both containing 0.1 M hydroxylammonium chloride buffer (pH 6.0) at 10%. The composition of the mobile phase and the wavelengths of detection are summarized in Table 1.

Calculation of pseudo-first-order rate constants. Pseudo-first-order rate constants can be calculated from a plot of ln $[ArNO_2]_t/[ArNO_2]_{t_0}$ versus time by two linear regression methods (2), one without and the other with a weighting factor (giving the points at high reaction times less weight). The latter method is thus applicable over longer time scales. As the relative error in $[ArNO_2]_t$ is increased by long reaction times, we used the method without a weighting factor and included only values of ln $[ArNO_2]_t/[ArNO_2]_{t_0}$ greater than −3.

RESULTS

Reaction kinetics and reaction products. In neutral aqueous solutions, nitroaromatic compounds ($ArNO_2$) are usually reduced to anilines ($ArNH_2$) in three steps with nitroso (ArNO) and hydroxylamino (ArNHOH) species as intermediates (11):

$$ArNO_2 \xrightarrow[-H_2O]{2e^- + 2H^+} ArNO \xrightarrow{2e^- + 2H^+} ArNHOH \xrightarrow[-H_2O]{2e^- + 2H^+} ArNH_2 \quad (1)$$

As illustrated by Fig. 2 for 4-Cl-NB in a solution containing hydrogen sulfide and filtrate (and thus also medium), the rate of disappearance of a given nitroaromatic compound ($ArNO_2$) could be described by a pseudo-first-order rate law:

$$\text{Rate} = -(d[ArNO_2]_t)/dt = k_{obs}[ArNO_2]_t \quad (2)$$

and thus,

$$\ln([ArNO_2]_t/[ArNO_2]_{t_0}) = -k_{obs} \cdot t \quad (3)$$

$[ArNO_2]_{t_0}$ and $[ArNO_2]_t$ are the concentrations of the nitro compound at time zero and time t, respectively, and k_{obs} is the observed pseudo-first-order rate constant under the given conditions. Figure 2 also shows that the reaction of

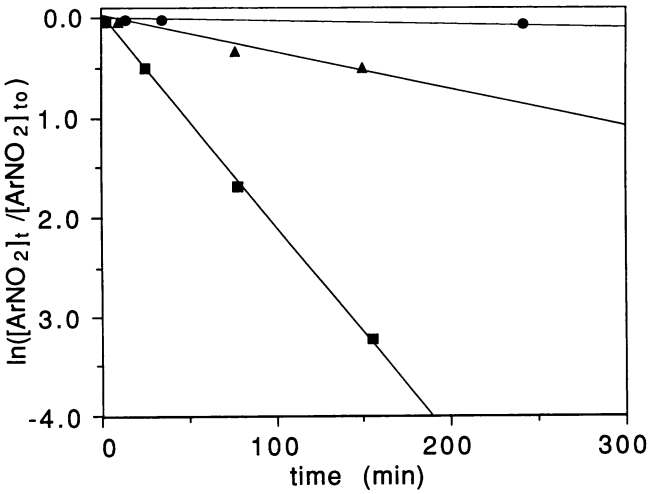

FIG. 2. Plot of ln $([ArNO_2]_t/[ArNO_2]_{t_0})$ versus time for reduction of 100 μM 4-Cl-NB catalyzed by a filtrate of a strain Tü 2484 culture (■), by the sterile growth medium DSM-65-NO_3 (▲), and by a buffer (●) consisting of $[PO_4^{3-}]_{tot}$ of 55 mM and $[S^{2-}]_{tot}$ of 4.15 mM (pH 6.9). Filtrate and medium were added at a concentration of 75% (vol/vol).

4-Cl-NB with hydrogen sulfide alone was very slow but that the presence of medium increased the reaction rate. Hence, for quantification of the effect on the reaction rate of the substances exudated only by the microorganisms, a corrected reaction rate constant, k_{obs} (exudate), was calculated:

$$k_{obs}\text{ (exudate)} = k_{obs}\text{ (filtrate)} - k_{obs}\text{ (medium)} \quad (4)$$

k_{obs} (filtrate) and k_{obs} (medium) are the pseudo-first-order rate constants for the disappearance of the nitroaromatic compound in solutions containing medium plus exudate (filtrate) and medium alone, respectively.

Figure 3 shows that under given conditions (constant pH,

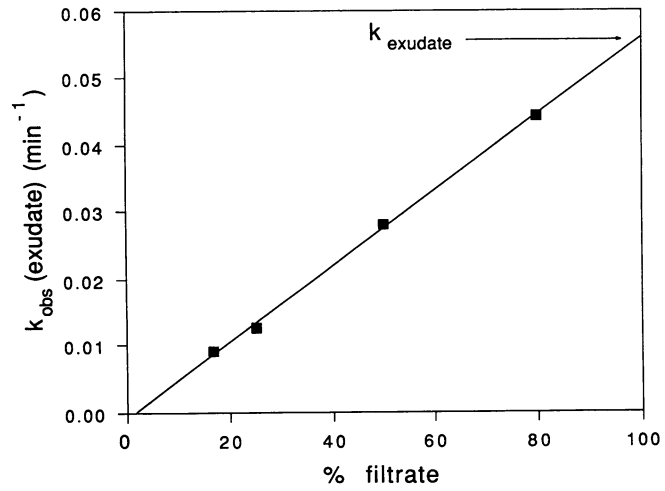

FIG. 3. Plot of k_{obs} (exudate) versus the concentration of filtrate (percent [vol/vol]). The conditions were $[PO_4^{3-}]_{tot}$ of 55 mM and $[S^{2-}]_{tot}$ of 5.0 mM (pH 7.20). This plot allowed for normalization of k_{obs} (exudate) values obtained at various filtrate concentrations. Because the filtrate always has to be diluted in the reduction assays, $k_{exudate}$ could only be calculated by extrapolation to 100% filtrate, as indicated by the arrow.

TABLE 2. One-electron reduction potentials and normalized pseudo-first-order reaction rate constants of several nitro compounds for reduction mediated by strain Tü 2484 exudate[a]

Compound[b]	$E_h^{'1}$ (mV)[c]	$k_{exudate}$ (min^{-1})
NB	−0.485	8.75×10^{-3}
2-Me-NB	−0.590	8.30×10^{-5}
3-Me-NB	−0.475	8.15×10^{-3}
4-Me-NB	−0.500	3.34×10^{-3}
2-Cl-NB	−0.485	2.05×10^{-2}
3-Cl-NB	−0.405	1.23×10^{-1}
4-Cl-NB	−0.450	5.05×10^{-2}
2-Ac-NB	−0.470	7.99×10^{-3}
3-Ac-NB	−0.405	2.18×10^{-1}
4-Ac-NB	−0.360	5.30×10^{-1}

[a] The assay consisted of: 91% filtrate or medium, $[S^{2-}]_{tot}$ of 4.9 mM, $[PO_4^{3-}]_{tot}$ of 49 mM, and $[ArNO_2]_{tot}$ of 100 μM (pH 7.0). The filtrate or medium concentration in the experiments varied as follows: 4-Cl-NB, 32%; 3-Cl-NB and 3-Ac-NB, 12%; 2-Cl-NB, 9%; 4-Ac-NB, 1%. Note that all of the assays were conducted with filtrate from the same batch.

[b] For definitions of abbreviations, see Table 1.

[c] Standard reduction potential (pH 7) for transfer of the first electron, taken from references 8, 12, 13, 15, and 22.

temperature, total concentration of H_2S) k_{obs} (exudate) was linearly related to the concentration of filtrate (exudate plus medium, expressed as a percentage [vol/vol]). For comparison of experiments conducted at different filtrate concentrations, a normalized (to 100% filtrate) pseudo-first-order reaction rate, $k_{exudate}$, can therefore be defined as follows:

$$k_{exudate} = [k_{obs} \text{ (exudate)} \cdot 100]/\% \text{ filtrate added} \quad (5)$$

For all of the model compounds listed in Table 1, significant accumulation of the corresponding hydroxylamines was observed; these were subsequently transformed to the corresponding anilines (cf. equation 1). Through mass balance calculations and by the first-order time course for the disappearance of the nitro compound, it can be shown that no nitroso compound (which could not be separated from the nitro compound by our HPLC analysis) accumulated in the reaction mixture in significant amounts. This is consistent with the results of Dunnivant et al. (5), who found that nitroso compounds were extremely unstable in aqueous solutions containing hydrogen sulfide and natural organic matter.

Effects of substituents on reaction rates. Table 2 summarizes $k_{exudate}$ values for the 10 model compounds at pH 7.0 and a 5 mM total sulfide concentration. Note that these results were obtained from one single batch of exudate, since the absolute $k_{exudate}$ values varied by a factor 2 to 3 from one batch to another. However, very similar relative reaction constants were measured throughout all of the batches.

For all of the model compounds, pseudo-first-order reaction kinetics were observed. Nitroaromatic compounds with an electron-withdrawing substituent (such as the *para*-acetyl group) reacted relatively quickly, whereas those compounds with an electron-donating substituent (such as the *ortho*-methyl group) reacted relatively slowly. For details of the linear free energy relationship between $k_{exudate}$ and the one-electron reduction potential of each of the individual model compounds, see the Discussion.

Effect of pH on reaction kinetics. As illustrated in Fig. 4 for 4-Cl-NB, in some cases at higher pH values (pH >7) in the initial phase of the reaction, the reaction rate increased with increasing time until a final reaction rate (characterized by k_{obs}) was reached. The extent of this nonlinear behavior (which looks somewhat like a lag phase) depended on the type of compound (i.e., type of substituent), and it generally became more prominent with increasing pH. In the pH range considered, it has been shown that no lag phase was observed when, after complete reduction of the substrate, the solution was respiked with the nitro compound.

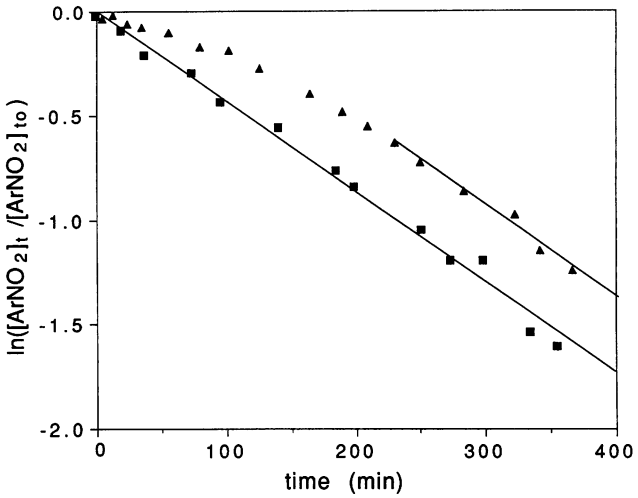

FIG. 4. Difference in reaction course between the first (▲) and second (■) spikes of 100 μM 4-Cl-NB added to a reaction medium containing 30% filtrate, $[S^{2-}]_{tot}$ of 5.0 mM, and $[HEPES]_{tot}$ of 100 mM (pH 7.9). Note that the straight line for the first spike obtained by linear regression with the last six points exhibits the same slope as the straight line for the second spike drawn over the whole reaction course. This illustrative example shows that it is possible to determine k_{obs} (exudate) at a time of 2 half-lives for reactions with a lag phase.

In Fig. 5, $k_{exudate}$ values for 4-Cl-NB are plotted as a function of pH. Note that at pH values of >7, the rate constants were determined after the lag phase, which had assumably ended after about 2 half-lives (half-life = ln [2]/

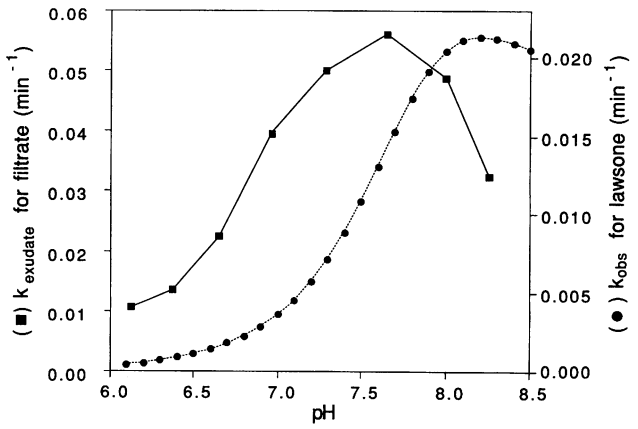

FIG. 5. Plot of $k_{exudate}$ versus pH for reduction of 4-Cl-NB mediated by 27% filtrate (■) in 4 mM sulfide. The dashed line represents the same type of reaction but mediated by the naphthoquinone lawsone in a similar reduction assay (15). These points (●) were not experimentally determined but were calculated from the molar fraction of the singly and doubly deprotonated lawsone species and their corresponding second-order rate constants. Since the second-order rate constant for doubly deprotonated lawsone can be looked at only as a rough estimate, this calculation might become erroneous in the region of pH >8.

TABLE 3. Relative reaction rates for reduction of the three chloronitrobenzenes at different pH values[a]

Compound[b]	Relative reaction rate at a pH of:		
	6.33	7.00	7.77
2-Cl-NB	0.2	0.4	0.4
3-Cl-NB	1.4	2.5	2.1
4-Cl-NB	1.0	1.0	1.0

[a] Reaction conditions: 67% filtrate or medium, $[S^{2-}]_{tot}$ of 5 mM, and [MES] or [HEPES] of 100 mM.
[b] For definitions of abbreviations, see Table 1.

$k_{exudate}$). Since this assumption was not tested for pH values of >8, the $k_{exudate}$ values in this pH region (last point in Fig. 5) may have been underestimated.

The relative reaction rates of the three chloronitrobenzene isomers at three different pH values are given in Table 3. As can be seen, the relative reaction rates did not differ much in the pH range between 6 and 8.

Competition between 2-chloro-, 3-chloro-, and 4-chloronitrobenzene. A mixture of the three chloro isomers, each at 30 μM, was reduced through mediation by filtrate at pH 7. The observed rate constant of each isomer in this ternary mixture was compared with the rate constant obtained in an experiment with each single compound. No significant difference could be found, except for 3-Cl-NB, which showed a 5% reduction of k_{obs} in the ternary mixture. Furthermore, the disappearance of each isomer followed first-order kinetics, which would not be the case if a single compound were preferentially reduced.

Production and some characteristics of active exudate components. During growth of a culture of strain Tü 2484 on DSM-65–NO_3, a change from yellow to red was always observed, which could be the result of production of active electron transfer mediators. In Fig. 6, the dry weight of the culture, the activity of the exudate (expressed as the half-life of the disappearance of 4-Cl-NB), and the A_{500} of the filtrate

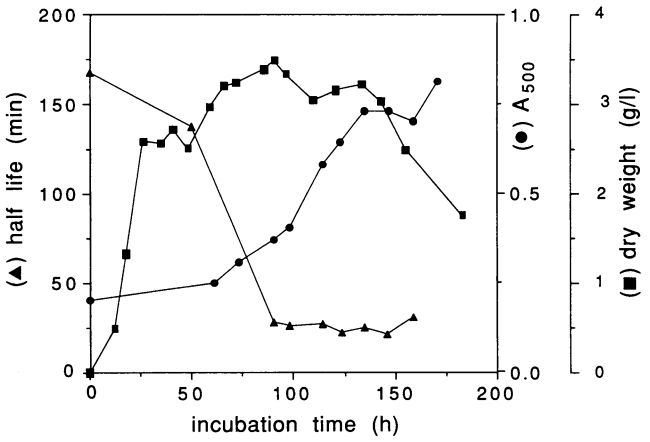

FIG. 6. Time course of the dry weight of a strain Tü 2484 culture growing on DSM-65–NO_3 and the A_{500} and catalytic activity (given as half-life [=ln $2/k_{exudate}$]) of its 0.2-μm-pore-size filtrate. The dry-weight data are from a separate experiment; however, this does not influence the observation that catalyst formation occurred after the exponential growth phase, since every culture reached a biomass plateau after 2 days. The reduction assay was performed with 83.5% filtrate and 4-Cl-NB as the react and but at a total sulfide concentration of only 4.15 mM.

TABLE 4. Characteristic reaction behavior during reduction of aromatic nitro compounds by sulfide mediated by different types of catalysts

Reference (catalyst)	LFER[a] slope (a)	Reaction rate order[b]	Competition between chloroisomers	Lag phase at pH >7
15 (quinones)	~1	Same	No	Yes[c]
15 (iron porphyrin)	~0.5[d]	Different	Yes	No[c]
This work	~1	Same	No	Yes

[a] LFER, linear free energy relationship.
[b] Order for the three chloroisomers at pHs 6, 7, and 8.
[c] Reference 13a.
[d] Except for the *ortho*-substituted compounds, for which the slope was slightly increased.

are given as a function of incubation time. This illustrative example shows that the major amount of the active exudate component(s) was produced after exponential growth of the organisms. The finding that A_{500} increased continuously, even after the activity of the exudate had leveled off, suggested that production of a red substance(s) was an independent process. This hypothesis was supported by results of experiments in which the A_{500} was monitored in filter-sterilized aliquots of growing cultures which had been taken at different incubation times. In the presence of oxygen, A_{500} increased continuously in these sterile samples, while under anoxic conditions (sample purged with nitrogen) absorption remained almost constant (data not shown).

Additional experiments were conducted to get some information on the nature of the active components in the exudate. (i) Boiling of the culture filtrate for 15 min resulted in only a 25% decrease in activity. (ii) It was found by ultrafiltration (exclusion size, 3,000 Da) that the major portion (>70%) of the active components exhibited a molecular mass of less than 3,000 Da.

DISCUSSION

With this study it has been demonstrated that in the presence of a bulk electron donor such as hydrogen sulfide, certain components of the exudate of a culture of strain Tü 2484 effectively mediated the reduction of nitroaromatic compounds. The results of the ultrafiltration experiments and the fact that boiling of the culture filtrate did not significantly reduce its activity support the idea that those exudate components responsible for enhancement of the reduction rates of the nitroaromatic compounds were low-molecular-weight secondary metabolites excreted primarily after exponential growth of the culture (Fig. 6). Because no method for analyzing the composition of the exudate was available to us at the beginning of this work, we tried to get more information on the nature of the electron transfer mediators by comparing the reaction kinetics found with the exudate with those obtained with model mediators. This kind of approach seems all the more interesting as under field conditions the observed reduction of nitro groups is most probably a sum of several abiologically and microbially mediated reactions. These processes are difficult to separate in laboratory systems without disturbing individual reactions. For this reason, relatively great emphasis is given to the discussion of the reaction behavior of the 10 model nitro compounds (especially the aspects of Table 4) in this section.

A comparison of the reaction kinetics obtained with the strain Tü 2484 exudate with those obtained with the model

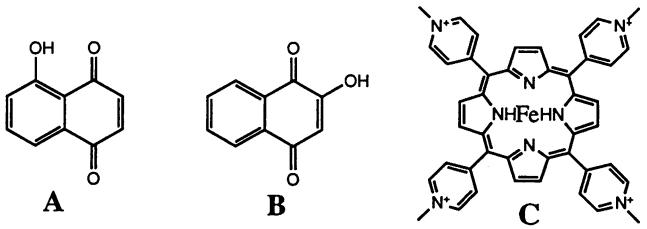

FIG. 7. Structures of model mediators 8-hydroxy-1,4-naphthoquinone (A), 2-hydroxy-1,4-naphthoquinone (B), and tetra(*N*-methylpyridyl)-iron porphyrin (C).

mediators 8-hydroxy-1,4-naphthoquinone (juglone, Fig. 7A) and 2-hydroxy-1,4-naphthoquinone (lawsone, Fig. 7B) and tetra(*N*-methylpyridyl)-iron porphyrin (Fig. 7C) in the report of Schwarzenbach et al. (15) is summarized in Table 4.

From this table a primary conclusion of this report can be drawn: the reaction behavior of the exudate matches that of the model quinones and not that of the iron porphyrin. The most important prerequisite for such a matrix to become a useful tool in outlining the dominant reductants in a complex system is the mechanistic understanding of the individual observations. A detailed discussion of the points listed in Table 4 would go beyond the scope of this report. Therefore, only a short summary of the most important aspects is given in the following; see reference 15 for more details.

The fact that pseudo-first-order kinetics were obtained over a large substrate concentration range (Fig. 2) suggests that rereduction of the electron transfer mediator(s) present in the exudate was fast compared with the reaction of the electron transfer mediator with the nitroaromatic compounds (Fig. 1). Figure 8 shows a plot of the log($k_{exudate}$) values of the various substituted nitrobenzenes listed in Table 2 versus their one-electron reduction potentials [$E_h'^1(ArNO_2)$] divided by 0.059 V (Table 2). Note that $E_h'^1(ArNO_2)$ is defined as the reduction potential for transfer of the first electron to the nitro group:

$$ArNO_2 + e^- = ArNO_2^- \quad (6)$$

As can be seen from Fig. 8, although there is some scatter in the data, a reasonably linear correlation was found between the reactivity and one-electron reduction potential of the compounds:

$$\log (k_{exudate}) = a \cdot [E_h'^1(ArNO_2)/0.059\ V] + b \quad (7)$$

Linear regression analysis of the data in Table 2 yielded a slope (a) of 0.985 and an intercept (b) of 5.95 ($r^2 = 0.957$). From this linear free energy relationship two conclusions can be drawn. (i) The fact that log($k_{exudate}$) correlates linearly with $E_h'^1(ArNO_2)$ suggests that for the cases in which pseudo-first-order kinetics were observed (i.e., in all cases except during the lag phases), transfer of the first electron from the mediator to the nitroaromatic compound (equation 6) was rate determining. (ii) The slope of close to 1 in the linear free energy relationship (equation 7) indicates that this electron transfer occurred by an outer-sphere mechanism.

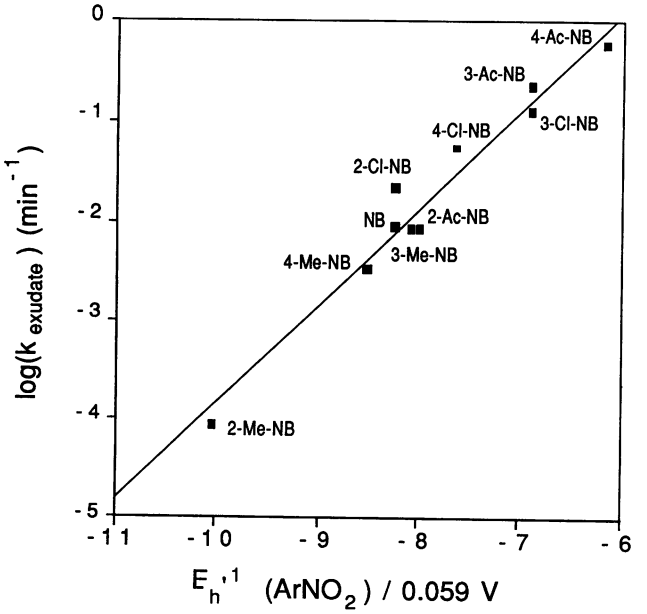

FIG. 8. Plot of log ($k_{exudate}$) versus one-electron reduction potential divided by 0.059 V for the nitrobenzenes listed in Table 2. The assay consisted of $[PO_4^{3-}]_{tot}$ of 49 mM, $[S^{2-}]_{tot}$ of 4.9 mM, and $[ArNO_2]_{t_0}$ of 100 μM (pH 7.0) at a temperature of 23°C. The experimental filtrate and medium concentration can be taken from Table 2, footnote *a*. For definitions of abbreviations, see Table 1.

The close similarity (Fig. 5) in reaction behavior between the active exudate compounds and the model quinones compared with the iron porphyrin investigated by Schwarzenbach et al. (15) is also evident in the pH dependence of the reaction kinetics, whereby one has to consider that, in the case of the exudate, the values of the parameters that control the pH dependence of the reaction rates are unknown. These parameters are primarily the molar fraction of reduced species and secondarily the degrees of protonation of the differently protonated species and the apparent redox potential of each of them, which in turn determines the individual second-order reaction constants. Because of these uncertainties, one cannot directly postulate a structural relationship between the exudate components and the model quinones. However, a decrease in the reaction rate at high pH values, as found for the exudate of strain Tü 2484, is predicted to be highly improbable for transition metal complexes, since their apparent redox potential is expected to decrease with increasing pH in the same manner as that of hydrogen sulfide.

An exact understanding of the lag phase has yet to be achieved. Dunnivant et al. (5) have proposed that in the case of nitroaromatic compounds reacting with hydroquinone mono- or biphenolate species at high pH, transfer of the second electron, which in addition requires a protonation step, might become rate determining. They found lag phases of up to several hours or even days with natural organic matter components as electron-transferring mediators. Since they can exclude the involvement of microorganisms (which can also be stated for the experiments with strain Tü 2484 exudate), their findings, together with ours, are also very interesting from another point of view, in that they show that observed lag phases need not always be related to microbially mediated reactions in which induction of enzymes and bacterial growth are often rate determining.

In summary, the results of the various experiments described above suggest that the exudate components responsible for mediation of the reduction of nitroaromatic compounds are species that react very much like the quinone model compounds (Table 4). This suggests that the two quinones cinnaquinone and dicinnaquinone (Fig. 9, structures D and E, respectively), identified as major exudate

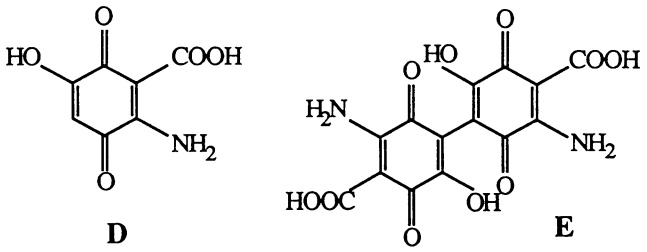

FIG. 9. Structures of the quinones cinnaquinone (D) and dicinnaquinone (E).

components of cultures of strain Tü 2484 by Korff (9), were probably responsible for this mediating activity.

When assuming that these two quinones are the major electron transfer mediators in the exudate and that they were present at a total concentration of 50 mg of C per liter (1), their reactivity can be compared to that of dissolved organic material derived from various natural waters and to that of the model mediators. Such a comparison shows that, as electron transfer mediators, these exudate components are more than 2 orders in magnitude more reactive than dissolved organic matter from groundwaters and from streams that drain bog areas (5) and are of an order similar to that of the model mediators (15). It would be interesting to evaluate to what extent such microbial exudates contribute to the electron transfer mediators present in natural systems.

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