Bacterial Degradation of Styrene Involving a Novel Flavin Adenine Dinucleotide-Dependent Styrene Monoxygenase

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By using styrene as the sole source of carbon and energy in concentrations of 10 to 500 μM, 14 strains of aerobic bacteria and two strains of fungi were isolated from various soil and water samples. In cell extracts of 11 of the bacterial isolates, a novel flavin adenine dinucleotide-requiring styrene monoxygenase activity that oxidized styrene to styrene oxide (phenyl oxirane) was detected. In one bacterial strain (SS), styrene metabolism was studied in more detail. In addition to styrene monoxygenase, cell extracts from strain SS contained styrene oxide isomerase and phenylacetaldehyde dehydrogenase activities. A pathway for styrene degradation via styrene oxide and phenylacetaldehyde to phenylacetic acid is proposed.

Styrene is used in large quantities by the chemical industry, mainly as a starting material for synthetic polymers such as polystyrene and styrene-butadiene rubber. It is also used as a solvent in the polymer processing industry and consequently is present in many industrial effluents. Airborne emissions of styrene often cause problems, even at low concentrations (less than 1 volume per million volumes [vpm]), due to the malodorous properties of the compound. Removal of styrene from industrial waste gases could be accomplished by using styrene-degrading bacteria as biocatalysts. In view of this potential application, it is important to know the metabolic fate of the styrene that is transformed by the biocatalysts in order to prevent the possible accumulation of more toxic styrene degradation products, e.g., styrene oxide.

Mammalian metabolism of styrene has been studied quite extensively in view of the intensive industrial use of styrene and its possible toxic and carcinogenic properties (19). The first step in the major pathway of mammalian styrene metabolism is the oxidation to styrene oxide.

Knowledge concerning the microbial metabolism of styrene is very scarce. The first attempt to isolate styrene-degrading microorganisms from more than 100 soil samples was unsuccessful (15). Subsequently, Sielicki et al. (18) described a styrene-utilizing mixed culture, and the isolation of pure cultures degrading styrene was first reported by Shirai and Hisatsuka (17), who isolated 31 strains. One strain, designated Pseudomonas sp., was studied in more detail. On the basis of the detection of small amounts of styrene oxide when cells were incubated in the presence of styrene, it was proposed that this Pseudomonas strain, styrene is degraded via styrene oxide (16).

Biotransformation of styrene to styrene oxide by whole cells has been described previously (6, 9). By using Methylotrophus trichosporium OB3b cells, styrene oxide was the only product detected when this methanotroph was incubated with styrene (9). The oxidation is probably a result of the broad specificity of the methane monoxygenase present in these cells. Nocardia corallina B-276, which contains a monoxygenase acting on a wide range of 1-alkenes, also forms styrene oxide from styrene (6). Oxidation of styrene to styrene oxide by cell extracts has been reported for Methylococcus capsulatus (Bath) (4) and the propane-utilizing Brevibacterium sp. strain CRL 56 (10). Styrene oxidation by cell extracts of styrene-grown microorganisms has, to our knowledge, not been reported in the literature.

Recently, we described a Xanthobacter species isolated on styrene that contained novel styrene oxide isomerase activity which isomerized styrene oxide to phenylacetaldehyde (8). Styrene transformation by whole cells of this strain was oxygen dependent, but we were not successful in resolving the nature of the oxidation product. We therefore set out to isolate other styrene-degrading organisms. As we anticipated that styrene would be toxic at higher concentrations, three different isolation methods were used in which relatively low concentrations of styrene were employed. All three methods resulted in various new isolates. In addition to two fungal isolates, 14 bacterial isolates, which appeared to be morphologically different, were selected. In the new bacterial isolates, we investigated the transformation of styrene. In most strains, we detected a novel flavin adenine dinucleotide (FAD)-requiring styrene monoxygenase (SMO) activity which formed styrene oxide. The SMO and styrene metabolism in one strain were studied in more detail, and a degradative pathway for styrene is proposed.

**MATERIALS AND METHODS**

**Isolation of styrene utilizers.** Styrene-degrading microorganisms were enriched either by adding 5 μl of styrene directly to an Erlenmeyer flask containing 50 ml of mineral salts medium with inoculum or by adding 25 μl of styrene to a test tube with 5 ml of dibutyl phthalate which was placed in a similar Erlenmeyer flask with 50 ml of mineral salts medium and inoculum. The mineral salts medium has been described previously (8), and various local soil and water samples were used as inoculum. The Erlenmeyer flasks (300 ml) were fitted with Teflon Mininert valves (Precision Sampling) to prevent styrene evaporation. Flasks were incubated at 30°C on a shaker. After growth was observed, 0.1 ml of serial dilutions was plated onto agar plates with mineral salts medium. Plates were incubated in a desiccator containing a flask with 2% (vol/vol) styrene in dibutyl phthalate. The third method used to isolate styrene-degrading microorganisms consisted of directly plating dilutions of samples without prior enrichment onto agar plates with mineral salts medium and incubating these plates in a desiccator in which styrene was supplied via the gas phase as described above. Colonies that developed on the agar plates with styrene as the carbon

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source were isolated and checked for purity by plating on yeast extract-glucose agar plates. The fungi were isolated from enrichment cultures containing 0.5 mM styrene in mineral salts medium which had been adjusted to pH 4.5 by the addition of hydrochloric acid.

**Growth conditions.** Strains were subcultured once a month and grown at 30°C on mineral salts medium agar slopes in a desiccator with an Erlenmeyer flask containing 2% (vol/vol) styrene in dibutyl phthalate. After 1 week, the agar slants were removed from the desiccator and stored at room temperature. Growth experiments were performed by using the mineral salts medium described previously (8), with the carbon sources (Table 1) added aseptically at a concentration of 0.01% (wt/vol) after sterilization of the mineral salts medium. Cultures were incubated at 30°C on a shaker (8).

Culture doubling times with styrene as the growth substrate were determined by monitoring the absorbance increase at 600 nm with a Vitatron UPS photometer of cultures growing in a fermentor with a working volume of 1 liter at 30°C. Styrene was supplied via the gas phase by passing 10% (20 ml/min) of the total air flow (200 ml/min) into the fermentor through a bubble column containing styrene which was kept at 20°C. Styrene-grown cells for the preparation of cell extracts of the strains S1, S3, S4, S5, S6, S8, S9, and S14 were grown as batch cultures in the fermentor under the same conditions as described above. Styrene-grown cells for the preparation of cell extracts of strains S7, S10, S11, and S12 were grown in 5-liter Erlenmeyer flasks as previously described for *Xanthobacter* strain 124X (8).

Cells were harvested, concentrated, and washed with potassium phosphate buffer by centrifugation (7) and were used directly to prepare cell extracts. Cell extracts were prepared by ultrasonication, and dialysis of extracts was performed with a Sephadex G-25 column with 50 mM potassium phosphate buffer, pH 7.0 (7).

**Experiments with whole cells.** Oxygen uptake experiments with washed cells were performed with dilute suspensions of freshly harvested, washed cells (8). The incubation of strain S5 cells with styrene was performed as described previously for *Xanthobacter* strain 124X (8).

**Enzyme assays.** All assays were performed at 30°C by using extracts from freshly harvested cells. Activities are expressed in nanomoles of product formed (NADH or NADPH) or substrate consumed (styrene or O$_2$) min$^{-1}$ mg of protein$^{-1}$.

SMO activity was measured by determining styrene consumption in the headspace of 30-ml vials fitted with Teflon Mininert valves preventing styrene evaporation. The reaction mixture consisted of cell extract (usually 5 to 10 mg of protein per 0.5 ml), 0.2 ml of a solution containing 5 mM NADH or NADPH, and 0.1 mM FAD in water and potassium phosphate buffer (50 mM, pH 7.0) to a total volume of 2.0 ml. The vial was placed in a shaking water bath, and after 2 min the reaction was started by the addition of 0.1 ml of phosphate buffer saturated with styrene. The temperature optimum of SMO was determined with the standard assay at different temperatures. The pH optimum was determined at 30°C with 50 mM potassium phosphate buffer at the desired pH.

Styrene oxide isomerase, 2-phenylethanol dehydrogenase (NAD(P)$^+$ dependent), and phenylacetaldelyde dehydrogenase (NAD(P)$^+$ dependent) were assayed spectrophotometrically as described previously (8). Phenazine methosulfate (PMS)-dependent 2-phenylethanol dehydrogenase and phenylacetaldelyde dehydrogenase were assayed by determining oxygen consumption rates as previously described (8).

**Determination of partition coefficient.** The partition coefficient of styrene between air and mineral salts medium (Henry coefficient) was determined by measurement of the styrene content in the gas phase in a series of Erlenmeyer flasks fitted with Teflon Mininert valves. The Erlenmeyer flasks all contained the same amount of styrene and varying ratios of air/mineral salts medium. After equilibration at 30°C, samples of the gas phase were analyzed, and the partition coefficient was calculated for all pairs of Erlenmeyer flasks by using the peak area determined by the integrator of the gas chromatograph in combination with the volumes of the gas and liquid phases. The dibutyl phthalate-mineral salts medium partition coefficient was determined in a similar way by varying the amount of dibutyl phthalate added to Erlenmeyer flasks containing a fixed amount of mineral salts medium and styrene.

**Analytical methods.** Protein was determined by the method of Lowry et al. (13) by using bovine serum albumin as the standard. Spectrophotometric assays were performed on a Perkin-Elmer 550A spectrophotometer. Oxygen uptake experiments were carried out by using a biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). Styrene was determined by gas chromatographic analysis of headspace samples (8). Reverse-phase high-pressure liquid chromatographic analysis of styrene oxide, 2-phenylethanol, phenylacetaldelyde, and phenylacetic acid was performed as previously described (8).

**Chemicals.** Aromatic compounds were obtained from Janssen Chimica, Beere, Belgium, except for styrene oxide, benzene, and mandelic acid, which were from E. Merck AG, Darmstadt, Federal Republic of Germany. PMS and styrene glycol were from EGA Chemie, Steinheim, Federal Republic of Germany. Biochemicals were from Boehringer GmbH, Mannheim, Federal Republic of Germany, and Sephadex G-25 was from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

**RESULTS AND DISCUSSION**

**Isolation of styrene-degrading bacteria.** The three different methods described in Materials and Methods were all successfully used to isolate styrene-degrading microorganisms from various soil and water samples. In earlier experiments (15), 101 soil samples were tested but no styrene-degrading microorganisms with styrene as the carbon source at a concentration of 2% were isolated. This concentration is well above the solubility of styrene in water of 1.5 mM at 25°C (3), and it is therefore not surprising that no organisms were isolated under these conditions. Recently, however,
Pseudomonas putida IH-2000 was isolated in the presence of 30% (vol/vol) toluene (12). This solvent-resistant strain also grew on a complex medium in the presence of high concentrations of styrene, thus illustrating that high styrene concentrations are not always growth inhibiting.

From our results, it appeared that it is possible to isolate styrene-degrading organisms very readily, provided low substrate concentrations are used. Styrene-degrading microorganisms are apparently very common in nature. This is not surprising when it is realized that styrene is also produced in natural ecosystems (17). Styrene concentrations in the aqueous phase of the closed cultures were calculated by using an air-mineral salts medium partition coefficient (Henry coefficient) of 0.2 and a dibutyl phthalate-mineral salts medium partition coefficient of 2,000. Enrichment cultures set up with dibutyl phthalate as a substrate reservoir (resulting in a styrene concentration in the water phase of about 11 μM) as well as enrichment cultures with 0.01% (vol/vol) styrene added directly to the mineral salts medium (resulting in a styrene concentration of about 0.5 mM in the water phase after separation and equilibration of styrene between the air and liquid phases) resulted in styrene-dependent growth with all inocula used. It was also possible to routinely obtain colonies of styrene-utilizing organisms by directly plating soil and water samples on mineral salts medium plates which were incubated in an atmosphere in equilibrium with 2% (vol/vol) styrene in dibutyl phthalate, resulting in a concentration of about 0.09 mM styrene in the water phase of the agar plates.

Although dibutyl phthalate itself can support microbial growth when added directly to the growth medium, transfer of dibutyl phthalate via the gas phase, as would be the case in our enrichment flasks, is apparently too slow to allow substantial microbial growth. The vapor pressure for dibutyl phthalate is about 0.01 Pa at 25°C (11), whereas the vapor pressure for styrene at the concentration used in the enrichment cultures was 2.2 Pa (22 vpm), illustrating why styrene transfer was much higher than dibutyl phthalate transfer. The solubility of dibutyl phthalate in water at 20°C is about 10 μM (11), which is almost the same as the equilibrium concentration of styrene in the aqueous phase under enrichment conditions. Apparently, the mass transfer resistance in the gas phase is rate limiting for dibutyl phthalate transfer to the aqueous phase. An organic solvent as a reservoir for toxic compounds in enrichment experiments offers the advantage that it is possible to achieve relatively high biomass concentrations at low substrate concentrations in the water phase without having to continuously monitor and adjust the substrate concentration.

By using the three different methods, many different isolates were obtained. Fourteen bacterial isolates that appeared to be morphologically different after growth on agar slants were selected for further study. They were designated strains S1 to S14. Organisms with a macroscopic morphology similar to that of strain S1 were present in all enrichment cultures. Strains S6, S8, S9, and S12 were gram negative and motile. All other bacterial isolates were gram positive. Further identification of the isolated bacteria was not attempted. Two styrene-degrading fungi were also isolated, but not further studied.

**Growth experiments.** The doubling times with styrene as the growth substrate of the strains isolated directly from agar plates (S3 and S4, 5.6 h; S5, 3.4 h; S6, 4.4 h; and S13, 9 h) did not differ significantly from the values obtained for the strains which were isolated from the enrichment cultures (S8, 2.5 h; S9, 3.9 h; and S14, 7.5 h). We did not determine the doubling time of strain S12 as we did not succeed in growing it in the fermentor. All the new isolates grew faster with styrene as the growth substrate than did the previously described Xanthobacter strain 124X (S8), which has a doubling time of 19 h.

To further characterize the new isolates, growth experiments were performed with various aromatic compounds as the sole source of carbon and energy (Table 1). Apart from styrene, all strains utilized styrene oxide and 2-phenylethanol as the growth substrate. Strain S3 was the only strain that could utilize all aromatic compounds tested as the growth substrate. This strain also utilized the polycyclic aromatic compounds anthracene, phenanthrene, and pyrene as the sole sources of carbon and energy.

**Oxidation of potential intermediates of styrene degradation.** Respiration experiments using suspensions of styrene-grown cells were performed to get an indication of possible intermediates of styrene metabolism. The addition of styrene oxide and phenylacetaldehyde resulted in an increase in the rate of oxygen consumption with all strains. Phenylacetic acid was oxidized by all strains except for strain S3. This strain, however, was the only one which oxidized acetophenone and ethylbenzene. Styrene glycol was not oxidized by any of the strains. The above results indicate that, with the exception of perhaps strain S3, which may attack styrene on the aromatic moiety of the molecule, all strains could degrade styrene via styrene oxide, phenylacetaldehyde, and phenylacetic acid.

With all strains tested, styrene degradation by suspensions of styrene-grown cells was inhibited under an atmosphere of nitrogen gas but could be restored after the addition of oxygen. This indicated that styrene is initially attacked by an oxygenase-type enzyme, possibly resulting in styrene oxide formation. To provide evidence that styrene is oxidized to styrene oxide, experiments using cell extracts were performed.

**Styrene degradation in cell extracts.** Styrene degradation in crude extracts was tested in all strains except S2 and S13. In the presence of NADH, styrene degradation was detected in all crude extracts tested with the exception of extracts from strains S3 and S14 and Xanthobacter strain 124X. In some cases, activity was also present in the absence of exogenously added NADH, possibly because sufficient NAD or NADH was already present in these extracts. With crude extracts from strain S14, styrene degradation was observed only if NADH was replaced by NADPH. With extracts from strains S1, S3, S8, S9, S10, S12, and S14, it was shown that styrene degradation by cell extracts was also dependent on the presence of molecular oxygen. To further assess the dependency of the reaction on the presence of NADH or NADPH, extracts of a number of strains were dialyzed. By using these dialyzed extracts, styrene consumption was no longer detected. Apparently, besides both NADH (or NADPH) and molecular oxygen, another low-molecular-weight component is required for enzymic activity.

By adding FAD to the assay mixture, styrene degradation activity of dialyzed extracts was restored. In this manner, it was possible to detect NADH: O2- and FAD-dependent styrene oxygenase activity in dialyzed extracts prepared from styrene-grown cells of strains S1, S4, S5, S6, S8, S9,
from the pH and product formation of phenylacetic acid (Fig. 1). Incubation plates.

S10, S11, and S12. Styrene oxygenase in strain S14 differed from the activity in the other strains in that, besides FAD, it had an absolute requirement for NADPH instead of NADH. NADPH could not replace NADH in the styrene oxygenase assay of extracts derived from the other strains.

**Styrene degradation in strain S5.** It was decided to examine styrene degradation in more detail in strain S5. This nonmotile, gram-positive organism formed pink colonies on agar plates. Incubation of a suspension of washed, styrene-grown cells with styrene resulted in the transient accumulation of phenylacetic acid (Fig. 1). We also attempted to detect product formation in crude extracts. To determine the optimal conditions for styrene degradation in crude extracts, the pH and temperature optima of the assay for styrene oxidation were determined (Fig. 2). The optima were close to the conditions already used in the assay for SMO activity. Incubation of dialyzed cell extracts with styrene in the presence of NADH, FAD, and molecular oxygen revealed styrene-dependent accumulation of three aromatic compounds. On the basis of retention time and UV spectrum upon high-pressure liquid chromatography analysis of the reaction mixture, phenylacetaldehyde, 2-phenylethanol, and phenylacetic acid were identified. No styrene oxide (phenyl oxirane) or any hydroxy styrenes, which would result from chemical rearrangement of arene oxides formed as a result of epoxidation of the aromatic ring or as a result of direct hydroxylation, could be detected. That styrene oxide was the initial oxidation product of styrene is, however, in agreement with the formation of the detected compounds. Crude extracts of styrene-grown cells contained a high styrene oxide isomerase activity (8), which transforms any styrene oxide produced to phenylacetaldehyde. Crude extracts also contained NADH-dependent 2-phenylethanol dehydrogenase and NADP+-dependent phenylacetaldehyde dehydrogenase (Table 2). These three enzymic activities would result in the transformation of any styrene oxide formed from styrene into the three aromatic compounds detected. Besides an NADH-dependent phenylacetaldehyde dehydrogenase, NADP+- and PM-dependent phenylacetaldehyde dehydrogenase activities were also present in cell extracts from styrene-grown cells (Table 2).

The above results suggest a degradative pathway of styrene involving an initial epoxidation to styrene oxide, which is subsequently isomerized to phenylacetaldehyde and oxidized to phenylacetic acid. The proposed pathway for styrene degradation in strain S5 is shown in Fig. 3 and is based on simultaneous adaptation experiments, the transient accumulation of phenylacetic acid (Fig. 1), and the presence of the required enzymic activities (Table 2). The involvement of phenylacetic acid in bacterial styrene metabolism has been proposed previously (2, 18), but evidence concerning the involvement of styrene oxide as an intermediate in bacterial styrene metabolism is not available. Shirai and Hisatsuka (16) detected styrene oxide in the culture broth of a *Pseudomonas* species degrading styrene and isolated 2-phenylethanol from whole cell incubations with both styrene and styrene oxide. Those authors suggested that styrene was transformed by a monooxygenase to styrene oxide, which was then reduced to 2-phenylethanol. Considering our results, it would seem possible that the reduction of styrene oxide to 2-phenylethanol as observed by Shirai and Hisatsuka (16) proceeds in two steps involving phenylacetaldehyde as an intermediate.

The further metabolism of phenylacetic acid was not investigated, although several potential intermediates of phenylacetic acid metabolism were tested as growth sub-

**FIG. 1.** Transient accumulation of phenylacetic acid (O) from styrene (●) by washed cells of strain S5 (1 mg of protein per ml).

**FIG. 2.** Specific activities of SMO in crude extracts of strain S5 as a function of pH (A) and temperature (B).

**FIG. 3.** Proposed pathway of styrene metabolism in strain S5.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sp act (nmol min⁻¹ mg of protein⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>SMO</td>
<td>7</td>
</tr>
<tr>
<td>Styrene oxide isomerase</td>
<td>395</td>
</tr>
<tr>
<td>Phenylacetaldehyde dehydrogenase</td>
<td>132</td>
</tr>
<tr>
<td>PMS dependent</td>
<td>162</td>
</tr>
<tr>
<td>NAD+ dependent</td>
<td>25</td>
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<tr>
<td>2-Phenylethanol dehydrogenase</td>
<td>0</td>
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<tr>
<td>PMS dependent</td>
<td>0.5</td>
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<tr>
<td>NADP+ dependent</td>
<td>0.3</td>
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TABLE 2. Enzyme activities in cell extracts of styrene-grown strain S5

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*HC-CH₂*
strates. Mandelic acid and 4-hydroxyphenylacetic acid did not support growth of strain S5, whereas 2- and 3-hydroxyphenylacetic acid did.

The oxidation of styrene to styrene oxide has been demonstrated previously by using crude cell extracts from the propane-utilizing Brevibacterium sp. strain CRL 56 (10) and the methylotroph M. capsulatus (Bath) (4). The SMO activity present in styrene-degrading bacteria differs from these monoxygenases and alkene monoxygenase (5) in its dependence on FAD for enzymic activity. The methylene and alkenic monoxygenase activities can be easily determined by analyzing the rate of 1,2-epoxypropane formation from propene. Whole cells of strain S5 containing SMO activity did not oxidize propene, further indicating that SMO differs from the bacterial monoxygenases described by others. The requirement of FAD for SMO activity indicates the involvement of a flavoprotein in styrene oxidation. Most known bacterial flavin-dependent monoxygenases only perform hydroxylation reactions on the ring of substituted aromatic compounds (14). To our knowledge, there are no flavin-dependent monoxygenases that epoxidize alkenes (20). SMO could thus appear to be a flavoprotein with a novel catalytic activity.

The previously described Xanthobacter strain 124X (8) and strain S3 were the only strains in which no SMO activity was detected. Styrene metabolism in these strains possibly proceeds via an initial oxidation of the aromatic nucleus. An oxidation of this type has previously been proposed for a styrene-degrading Nocardia species in which styrene was apparently degraded via a dihydrodiol intermediate with the side chain still intact (1).

Although styrene oxide is probably an intermediate in the styrene degradation pathway of most organisms, we could not detect the accumulation of this toxic compound in growing cultures or in suspensions of washed cells incubated with styrene. Apparently, styrene oxide isomerase activities are sufficiently high to prevent such an accumulation. On the basis of these observations, it would seem that application of styrene-degrading bacteria in processes for the biological treatment of waste gases containing styrene will not result in the accumulation of the more toxic styrene oxide.

LITERATURE CITED