Transformation of Tetralin by Whole Cells of Pseudomonas stutzeri AS39*

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Summary. A bacterium capable of growing on tetralin (1,2,3,4-tetrahydronaphthalene) as the sole source of carbon and energy has been isolated from soil of a coal dump. It has been identified as Pseudomonas stutzeri. The organism converts tetralin to 1-tetralol and 1-tetralone. The generation time is 50 h with tetralin, and 1.2 h with salicylate, respectively, as carbon source. Under conditions where the cells grow on tetralin the highest yield of 1-tetralol and 1-tetralone so far obtained is about 3% of the tetralin-input. The oxidation of an alicyclic ring structure by an arene-degrading bacterium is being discussed.

Introduction

Microbial degradation of various classes of hydrocarbons has been intensively investigated, as shown by biochemical, genetical, and ecological studies (Watkinson 1978). Very little is known, however, about interaction of microorganisms and the frequently used solvent tetralin, a bicyclic molecule combining both aromatic and alicyclic structures. With increasing importance of optimal use of raw materials many biotechnological approaches on hydrocarbon degradation emerged (Harrison et al. 1980). We investigated the bacterial degradation of tetralin, because some of its oxidation products e.g., 1-tetralol and 1-tetralone are of great importance as pharmaceutical agents and as base chemicals for the production of drugs and insecticides (Christova and Dantschev 1978; Collin 1979; Ivanov et al. 1978; Welch et al. 1977). Alkanes and aromatic hydrocarbons (arenes) serve as sole sources for carbon and energy for a large variety of bacteria (Hopper 1978; Ratledge 1978). Cycloalkanes, however, are relatively persistent against microbial attack (Trudgill 1978). So far, no detailed report on the microbial metabolism of tetralin has been given. In few publications dealing with microbial degradation of hydrocarbons tetralin was only casually taken into account. Tetralin does not support growth of Alcaligenes sp. 559 (Tsuchii et al. 1977) and it does not stimulate endogeneous respiration by cells of Corynebacterium (Ladd 1956). Tetralin induces the enzymes required for the conversion of p-xylol to 2,5-dimethylmuconic acid in cells of Nocardia salmonicolor A-100 (Hosler and Eltz 1969), and Nocardia corallina V-49 produces 4-phenyl-(2'-hydroxy)-butyric acid as a result of alicyclic ring cleavage (Jamison et al. 1970). Gana-pathy et al. (1966) found low yields of 1-tetralol and 1-tetralone as products of tetralin transformation by Aspergillus niger.

We used whole cells of newly isolated bacterial strains during our investigations of tetralin transformation. This approach guarantees stability of oxygenases and applicability in microbial technology (Drozd 1980). Conventional tests for substrate utilization showed that none of 41 newly isolated strains grew on tetralin. Tetralin was converted, however, when we used the technique of co-oxidation, which has been reviewed by Perry (1979). Subsequently we detected growth of P. stutzeri, strain AS39 on tetralin vapour. In this paper we describe the bacterial production of 1-tetralol and 1-tetralone by arene-adapted cells of P. stutzeri AS39, a result that we did not await. It shows a deviation from predicted pathways which has been reported by Chapman (1979) for arylhydrocarbon oxygenases in Pseudomonas.

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Methods

Bacterial Strains. *Pseudomonas stutzeri*, strain AS39 was isolated from a coal dump overlayed by partly recultivated soil near Herten, Germany. Other strains which were tested for their capability of tetralin degradation were isolated from different samples of polluted soils, mud and waters.

Culture Media. For growth in liquid culture the mineral salts medium as described by Dorn et al. (1974) was used. Solid media were prepared by addition of Bacto agar (1.5%) to the liquid medium. The same medium was used for isolation and enrichment of bacteria. For supplementation of media yeast extract (0.05%) or a vitamins solution was used (Schlegel 1974).

Strain Isolation. For direct isolation of bacteria samples of soil (1-3 g) or water (2-4 ml), collected from different biotops, were suspended in 50 mM neutral phosphate buffer (10-30 ml), plated on minimal agar, and grown with the selective carbon source as described under culture conditions. Equally, bacteria were isolated by classical enrichment techniques using liquid media (30 ml) instead of agar. For enrichment of bacteria in a dessicator samples of soil or water (total volume 200 ml) were mixed and supplemented with liquid tetralin (50 ml), which was pipetted into the middle of the mixture. Every second day fresh air was introduced into the dessicator. Tetralin was added again when it's characteristic odour diminished.

Culture Conditions. Cultures were incubated at 30°C. Small cultures grew in shaking flasks (100 and 500 ml) containing 10-100 ml of medium, aerated on a gyratory shaker at 170 rpm; quantities up to 200 ml grew in cylindrical bubbling columns (250 mm by 30 mm), and cultures up to 750 ml grew in 1-l fermentors (Schütt, Götingen, Germany), which were equipped with a selfconstructed mechanical defoamer (US patent no. 4,310,437). Air was introduced at a rate of 0.1-0.3 l/min and the culture was magnetically stirred at 800 rpm. Air flow was adjusted by flow controls (Model 9844 Brooks Instruments, Venzendal, The Netherlands), and flow was indicated by flow meters (Rota, Wehr, Germany). Openings of shaking flasks and fermentors were sealed by teflon lined screwcaps to prevent loss of volatile substrates, when necessary. Non-hydrocarbon substrates were added from stock solutions to give 5 mM final concentrations in solid media. Solid hydrocarbons were placed into the inverted cup of a petry dish. Fermentor cultures were supplied with hydrocarbon vapour by the air flow as described by Schreiber and Winkler (manuscript in preparation). Liquid cultures in shaking flasks were supplied with hydrocarbon vapours from centre-well reservoirs. Total cell counts were obtained microscopically and viable cell titers by plating diluted samples on nutrient broth agar and incubation for about 2 days.

Substrate Utilization and Strain Identification. Tests for substrate utilization were made on minimal agar supplemented with the test compound as described under culture conditions. Growth on test plates was compared with growth on blank plates, in order to detect unspecific growth. Biochemical identification of strains was carried out with commercial sets of test tubes (Oxi/ferm, Hoffman-LaRoche, Grenzach-Wyhlen, Germany).

Chemical Analysis. The amount of tetralin and naphthalene vapour consumed by cells growing in liquid cultures was determined as UV-absorbance (OD280) after periodically trapping with adsorption cartridges (Sep-Pak-C18, Waters, Königstein, Germany) as described by Schreiber and Winkler (manuscript in preparation).

For estimation of metabolites 20 ml of the culture supernatant were examined gaschromatographically after adsorption of the organic components by the reversed-phase of cartridges [Sep-Pak-C18 (see above)]. Products of tetralin oxidation were removed from adsorbent by 0.5 ml of methanol and 1.5 ml of acetonitrile. 1-tetralol and 1-tetralone were identified and quantitatively determined by retention times and co-chromatography of standard chemicals. Gaschromatographic conditions were: Gaschromatograph Model HP5710 (Hewlett Packard, Avondale, U.S.A.), 6' stainless steel column, 3% Carbowax 20M on Chromosorb, gas-flow rates: N2 : H2 : air = 40 : 30 : 360 (ml/min, each), temperatures of detector and flame-ionisation detector: 250°C; the column oven was programmed from 120°C, initial temperature being held for 3 min, to 180°C at a rate of 6°C/min. The detection limit of 1-tetralol and 1-tetralone was 0.05 mg/l of culture medium.

Chemicals. Chemicals were of analytical-grade quality and were purchased from Merck, Germany. Tetralin (99%) was a gift from Dr. K. Handrick, Bergbau-Forschung GmbH, Essen, Germany. Trace amounts of oxidation products (1-tetralol and 1-tetralone, 0.2% each) were not removable, neither by distillation nor by chromatography on silica. Special chemicals were purchased from: Aldrich Europe, Beere, Belgium (1-tetralol); Baker, Groß-Gerau, Germany (n-hexane, n-heptane); Fluka, Buchs, Switzerland (3- and 4-hydroxybenzoïc acid, 3-methylbenzoïc acid, n-octane, 2-tetralone, 5-tetralol, 6-tetralol, tetralin-2-carbonic acid); Sigma, Munich, Germany (5-hydroxy-1-tetralone).

Results

Isolation and Characterization

Samples of soil, mud, and water from 32 different places in West Germany were screened for tetralin-utilizing bacteria. Although growth occured in several mixed cultures containing tetralin as the sole source of carbon and energy, none of 41 isolated strains utilized tetralin in pure culture, when tested on minimal agar with tetralin vapour, or in liquid cultures with liquid tetralin. The present strain (No AS39) was isolated from a mixture of soil and coal originating from a coal dump. The bacterium is a gram-negative, oxidase-positive, motile rod, which was identified as *Pseudomonas stutzeri*. Tests for substrate utilization revealed that the strain utilizes for example naphthalene, protocatechuate, 4-hydroxybenzoiac, salicylate, toluate, benzoate, succinate, and glucose as growth substrates; only in liquid culture this strain grows with tetralin vapour. Strain AS39 was not capable of growing on adipate, pimelate, n-hexane, n-heptane, n-octane, cyclohexane, gentisate, phenol, cyclohexanol, and cyclohexanone.

Transformation of Tetralin

1-tetralol and 1-tetralone were recovered from various cell-free culture media as products of bacterial tetralin transformation (Figs. 1-3). Resting cells of