APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1981, p. 39-43
0099-2240/81/070039-05$02.00/0
Vol. 42, No. 1

# Enrichment and Isolation of Naphthalenesulfonic Acid-Utilizing Pseudomonads

C. BRILON,[1] W. BECKMANN,† M. HELLWIG,[2] AND H.-J. KNACKMUSS[3]‡*

*Institut für Mikrobiologie der Universität, Göttingen[1]; Gesellschaft für Strahlen- und Umweltforschung, Munich[2]; and Lehrstuhl für Chemische Mikrobiologie der Universität-Gesamthochschule, Wuppertal,[3] Federal Republic of Germany*

Received 12 December 1980/Accepted 7 April 1981

Naphthalenesulfonate-degrading bacteria were obtained by continuous enrichment from a naphthalene-degrading population from sewage. In addition to naphthalene, *Pseudomonas* sp. A3 can utilize 2-naphthalenesulfonate (2NS) and *Pseudomonas* sp. C22 can utilize both 1-naphthalenesulfonate (1NS) and 2NS as sole carbon sources. In a mixture of 1NS and 2NS, the former substrate is utilized by strain C22 only after complete consumption of 2NS. During exponential growth, approximately 10% of the organic carbon of naphthalenesulfonates is temporarily excreted. These unidentified metabolites can readily be used by other bacteria, which, by supplying strain C22 with vitamins, allow optimal growth in stable mixed cultures. The degradative capability of *Pseudomonas* sp. A3 for 2NS was irreversibly lost under nonselective growth conditions and could be transferred from the wild type to a distinguishable cured strain of the wild type.

Naphthylsulfonates are important products of industrial chemical processes. Their sodium salts are used as solubilizers. The alkylnaphthalenesulfonates belong to the surface active compounds and are applied as wetting agents and emulsifiers. In addition, naphthylsulfonates serve as building blocks for the large-scale synthesis of azo dyes with naphthol- and naphthylaminesulfonates as intermediates. In conventional sewage plants naphthalenes carrying $SO_3H$ groups as substituents resist biodegradation or are incompletely degraded. As a consequence, 7 to 15% of the organic pollution of the Rhine water is caused by sulfonated naphthalenes and its secondary products (6).

In principle, aromatic compounds carrying a sulfonic acid group as a xenobiotic structural element can be totally degraded and utilized by bacteria. Several studies (see reference 3) describing the bacterial degradation of benezene- and alkylbenzenesulfonates encouraged us to investigate whether naphthalenesulfonate-degrading bacteria could be enriched and isolated.

## MATERIALS AND METHODS

**Continuous enrichment.** A chemostat was used as described by Hartmann et al. (4) for continuous enrichment. Air loaded with variable concentrations of naphthalene was introduced at a rate of 1 liter/min through a G2 porous glass filter plate, which covered the entire bottom of the chemostat vessel. The naphthalene concentration in the incoming air was regulated by admixing defined amounts of naphthalene-saturated air. This was generated by passing air through a column (50 by 250 mm) which was filled by a mixture of powdered naphthalene and glass beads. The dilution rate of the chemostat was adjusted to 0.03 $h^{-1}$.

**Culture conditions.** For cultivation in continuous and batch culture a mineral medium was used, containing per liter: 12 g of $Na_2HPO_4 \cdot 2H_2O$, 2 g of $KH_2PO_4$, 0.5 g of $NH_4NO_3$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 50 mg of $Ca(NO_3)_2 \cdot 4H_2O$, 7.5 mg of $FeCl_2 \cdot 4H_2O$, and 0.1 ml of a trace element solution according to Pfennig and Lippert (7). When sulfate concentrations were determined during growth with naphthylsulfonates, the trace element solution had the following composition: 50 mg of $ZnCl_2$, 30 mg of $MnCl_2 \cdot 4H_2O$, 300 mg of $H_3BO_3$, 200 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, 30 mg of $Na_2MoO_4 \cdot 2H_2O$ in 1 liter. The mineral salts medium was supplemented with appropriate carbon sources from concentrated separately autoclaved stock solutions.

Small quantities of cells were grown in 500-ml fluted Erlenmeyer flasks containing 50 ml of medium. The flasks were incubated at 28°C on a rotary shaker at 150 rpm. A magnetically stirred fermentor (Multigen F 2000 from New Brunswick, Edison, N.J.) was used for 1-liter cultures. Growth was monitored by measuring the turbidity at 546 nm.

Solid media were prepared by addition of 2% Ionagar no. 2 (Oxoid) to the mineral medium with appropriate carbon sources. When naphthalene was used as a carbon source, mineral agar plates were incubated upside down with a few crystals of naphthalene in the lid of the petri dish.

**Analytical methods.** Concentrations of naphthyl-

† Present address: Gewerbeaufsichtsamt, D-2210 Itzehoe, Federal Republic of Germany.

‡ Present address: Institut für Mikrobiologie der Universität, D-3400 Göttingen, Federal Republic of Germany.

sulfonates in the culture fluid were determined by reverse-phase high-pressure liquid chromatography. Experimental details including sample preparation are described in the accompanying paper (2).

For the determination of sulfate ion concentration in the culture fluid, the method of Bertolacini and Barney (1) was modified. The culture fluid (2 ml) was diluted with distilled water to a final volume of 30 ml. Ethanol (50 ml) was added, and the solution was acidified with citric acid (1 M) to pH 4. After admixture of barium chloranilate (300 mg), the sample was diluted again with distilled water to a final volume of 100 ml and shaken for 10 min. Undissolved particles were removed by centrifugation. Photometric measurements were carried out at 530 nm using glass cuvettes of 20-mm path length. A sulfate-free probe of mineral medium was treated correspondingly and used as a standard. The calibration curve was established by using standard solutions of sodium sulfate ($10^{-6}$ to $4 \times 10^{-5}$ M).

The sulfite concentration in the culture fluid was measured by the method of Johnston et al. (5).

**Chemicals.** Pure (87%) naphthalenesulfonic acids were generously supplied by Bayer AG, Leverkusen, Germany. Technical-grade quality of 1-naphthalenesulfonic acid (1NS), which contained approximately 30% 2-naphthalenesulfonic acid (2NS) as the major impurity, cycloheximide, and 6,6'-dinitro-3,3'-dithiobenzoic acid were purchased from Fluka, Buchs, Switzerland. Nutrient broth and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Mitomycin C was supplied by Sigma Chemical Co., St. Louis, Mo. All other chemicals used for mineral salts media and buffer solution were of analytical grade quality and were purchased from Merck, Darmstadt, Germany.

## RESULTS AND DISCUSSION

**Isolation of strains.** The naphthylsulfonate-degrading bacteria used in this and the accompanying study (2) were obtained by continuous enrichment from a naphthalene-degrading population originating from activated sludge samples of the Göttingen sewage plant. The chemostat was initially fed with naphthalene vapor as carbon source. By use of several pulses of cycloheximide (10 mg/liter), growth of eucaryotic cells was suppressed and the culture was stabilized within 14 days. Stepwise increasing amounts of 2NS (up to 2 g/liter) were added to the mineral medium in the reservoir over a period of 8 weeks. Simultaneously the concentration of naphthalene in the vapor was gradually decreased. Constant growth with 2NS as the only carbon source was obtained after 5 months of continuous enrichment. At that time the steady-state concentration of 2NS in the continuous culture had dropped below 0.01 mM. When plated on mineral agar the majority of the cells in the chemostat could grow on naphthalene. However, only a few colonies were able to utilize both naphthalene and 2NS as sole carbon sources. One of these, strain A3, was selected and purified by replating on nutrient broth and 2NS mineral agar.

The 2NS-utilizing continuous culture could further be adapted to the utilization of 1NS. When 2NS in the mineral salts medium was replaced by 10 mM 1NS from Fluka, which contained about 30% 2NS as an impurity (this mixture is designated as 1NS-Fluka below), both isomeric naphthalenesulfonates were completely removed from the culture fluid within 4 further months of continuous enrichment. No major change in the population density ($E_{546}$ of ca. 1) was observed even when pure 1NS (Bayer) was used as the sole carbon source. However, on 1NS mineral agar plates, no single colonies could be obtained from the population of the chemostat. On agar plates with 10 mM 1NS-Fluka plus 1 mM succinate, 32 isolates were selected and transferred to roll tubes containing mineral salts medium supplemented with the same carbon sources. High-pressure liquid chromatography analysis revealed some turnover of 2NS as well as of 1NS with four of these isolates. One of these isolates with the highest activity for the sulfonates was used for further investigations. When this isolate was plated on succinate mineral agar, three different strains were distinguished by their colony forms. Strain C22 exhibited small, round, and clear colonies, whereas strain R22 gave large and irregularly formed rough colonies. The colonies of the third form, strain T22, appeared round and turbid. None of the single strains could grow on 1NS-Fluka mineral medium. However, growth was observed when the three isolates were recombined.

Strain A3 and strain C22 exhibited most of the phenotypic characters of the species *Pseudomonas testosteroni*, which are listed in the diagnostic key from Stanier et al. (8). Since the escorting strains R22 and T22 were not involved in naphthalenesulfonic acid degradation (see below), they were not identified.

**Naphthalenesulfonic acids as growth substrates.** Both *Pseudomonas* strains A3 and C22 utilized 2NS as the sole source of carbon and energy. When strain C22 was grown in pure culture it required some vitamin $B_{12}$ (see below). To avoid acidification during growth with the sulfonic acids, the buffer concentration in the medium was 80 mM. Under these conditions the doubling time was found to be 4 h for strain A3 and 7.5 h for strain C22. Sulfite accumulation in the growth medium with a maximum concentration in the late exponential growth phase followed by a strong decrease in the stationary phase was found with both organisms (Fig. 1). All of the organic sulfur was finally converted to sulfate. During growth of *Pseudomonas* sp. C22

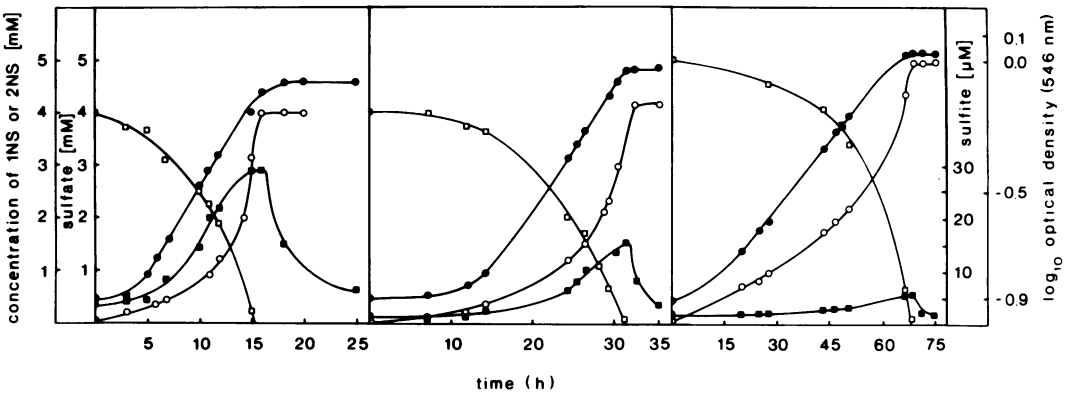


FIG. 1. *Utilization of 2NS by strain A3 (left) and by strain C22 (middle) and of 1NS by strain C22 (right). The organisms were grown at 30°C in a 2-liter fermentor containing 1 liter of mineral salts medium (pH 7.5). The initial substrate concentrations were 4 mM for 2NS and 5 mM for 1NS. For strain C22 the medium was supplemented with vitamin $B_{12}$ (0.2 mg/liter). Samples were taken at intervals for turbidity measurements, quantification of 2NS and 1NS by high-pressure liquid chromatography, and determination of sulfate and sulfite. Symbols: □, 1NS or 2NS; ●, turbidity, ■, sulfite; ○, sulfate.*

with 1NS, a considerably longer doubling time ($d_t$ = 26 h) with this substrate was observed. The maximum concentration of sulfite from 1 NS in the growth medium at the end of the exponential growth phase never exceeded 6 μM.

**Growth of strain C22 with a mixture of 1NS and 2NS.** Strain C22 grew readily with 1NS-Fluka as the sole carbon source provided that the escorting strains R22 and T22 were present. Plating on 1NS-Fluka-succinate agar demonstrated that the ratio of viable cells of the three strains C22, R22, and T22 was almost constant (10:1:1) during the entire course of exponential growth. Optimum growth rates were found with the mixed culture of the three strains. None of the single strains were able to grow on mineral salts medium with naphthalenesulfonic acid as the sole carbon source. Some growth could be obtained with strain C22 when the 1NS-Fluka mineral salts medium was supplemented with yeast extract and vitamin $B_{12}$. However, growth was always inferior to the mixed culture of the three strains (Table 1).

No catabolic activity for naphthylsulfonates could be measured by high-pressure liquid chromatography when the strains R22 and T22 were grown on 10 mM succinate in the presence of naphthalenesulfonic acids.

The two escorting strains R22 and T22 could grow on culture filtrates taken from an exponentially grown culture of *P. testosteroni* C22 when the latter organism was cultivated with naphthylsulfonates in the presence of vitamin $B_{12}$. The dry weight yield of biomass indicated that about 10% of the carbon source was excreted into the growth medium during exponential growth. However, culture filtrates from strain C22, taken from the stationary growth phase, did not support significant growth of strain R22 or T22 (Fig. 2).

TABLE 1. *Growth of strains R22, T22, and C22 in pure and mixed culture with 1NS-Fluka as a carbon source*[a]

| Pure or mixed cultures | Turbidity after 52 h; mineral salts medium | | | |
|---|---|---|---|---|
| | Without supplement | Supplemented with: | | |
| | | Vitamin $B_{12}$ | Yeast extract | Vitamin $B_{12}$ + yeast extract |
| Strain R + C + T | 0.4 | 0.41 | 0.52 | 0.51 |
| Strain R + C | 0.22 | 0.26 | 0.33 | 0.34 |
| Strain T + C | 0.23 | 0.25 | 0.33 | 0.33 |
| Strain R + T | 0.0 | 0.0 | 0.0 | 0.0 |
| Strain C | 0.0 | 0.22 | 0.21 | 0.46 |
| Strain R | 0.0 | 0.0 | 0.0 | 0.0 |
| Strain T | 0.0 | 0.0 | 0.0 | 0.0 |

[a] Mineral medium contained 5 mM 1NS-Fluka (7:3 mixture of 1NS and 2NS), yeast extract (0.05%), and vitamin $B_{12}$ (0.2 mg/liter). The cultures (10 ml in 50-ml Erlenmeyer flasks) were incubated at 30°C. Samples were taken after 3 days, when approximately one-third of maximum growth yield was attained. Growth was observed turbidimetrically at 546 nm. Turbidity values were corrected by those obtained from control cultures without naphthylsulfonates.

The culture of *Pseudomonas* sp. C22 can readily be contaminated even under the selective growth conditions conferred by naphthalenesulfonic acids as the sole carbon source. Apparently the unindentified compounds, which are transiently excreted during exponential growth, can

be readily utilized by other bacteria. These could probably replace the escorting strains R22 and T22 to satisfy the vitamin $B_{12}$ requirement of strain C22.

A characteristic growth curve was observed with 1NS-Fluka (Fig. 3), i.e., a mixture of the isomeric sulfonic acids. Sulfite accumulation in the medium exhibited two maxima corresponding to maximum turnover of the two growth substrates. This growth curve was not significantly changed when *Pseudomonas* sp. C22 was grown in mixed culture with strain R22 and T22 without vitamin $B_{12}$. A slightly shorter doubling time was observed under these conditions. Although relatively small amounts of sulfite are excreted into the medium, it appears to be the primary metabolite of the sulfonic acid groups. Oxidation of sulfite to sulfate is only slightly accelerated by naphthalenesulfonic acid-grown cells. Turnover of 1NS is strongly inhibited by 2NS (Fig. 3), so that 1NS is not utilized until all 2NS has been consumed by the growing culture. However, the existence of a biphasic growth curve could not be substantiated. Cells taken from the growth phase of 2NS utilization were fully induced for 1NS oxidation. In agreement with this, data in the accompanying paper show that degradation of both 1NS and 2NS by *Pseudomonas* sp. C22 follows the same catabolic route where both substrates compete for the same initial enzyme.

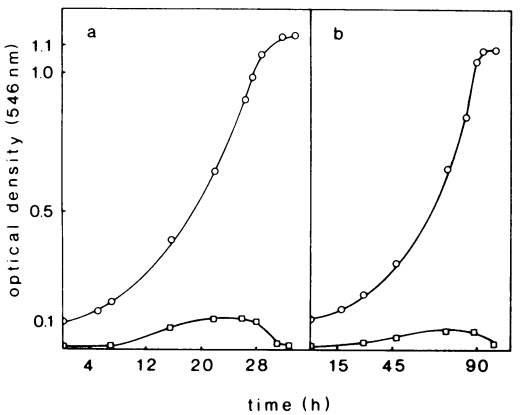


FIG. 2. *Growth of Pseudomonas sp. C22 with 2NS (a) and 1NS (b) as sole carbon sources. The organism was grown in mineral salts medium in a 2-liter fermentor as described in Fig. 1. The initial concentrations of the naphthalenesulfonic acids were 5 mM each. Samples (3 ml) were taken at intervals, cells were removed by sterile filtration, and the fluid was transferred to roll tubes containing 2 ml of mineral salts without carbon source. After being inoculated with a cell suspension of strain T22 (1 ml, $E_{546}$ = 0.1) the tubes were incubated at 30°C by use of a roller drum. Increase of turbidity resulting from growth at the expense of excreted metabolites in the samples was measured after 48 h. Symbols: ○, growth of strain C22; □, increase in optical density at 546 nm of strain T22 48 h after inoculation.*

**Loss and transmissibility of degradative capability.** When *Pseudomonas* sp. A3 was subcultured on nonselective agar plates, an irreversible loss of degradative capability for 2-naphthalenesulfonate was noticed. When cultivated in nutrient broth at 30°C, approximately 50% of the cells had lost the ability to utilize 2NS after 10 generations of growth. When mitomycin C (22 μg/ml) was incorporated into the growth medium, the extent of curing increased

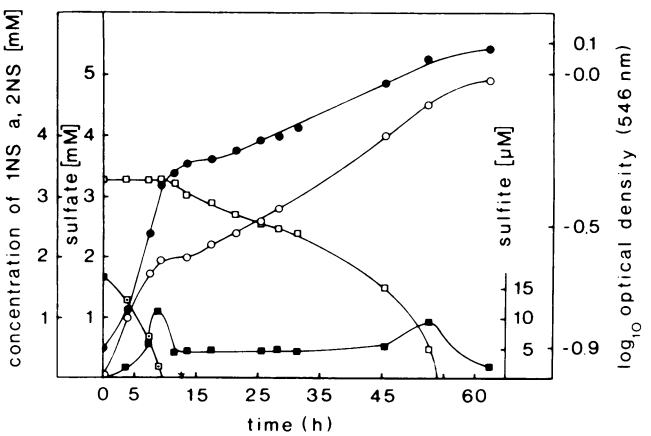


FIG. 3. *Growth of P. testosteroni strain C22 with 1NS-Fluka (7:3 mixture of 1NS and 2NS) as sole carbon source. Mineral salts medium contained 5 mM naphthalenesulfonic acids (1NS + 2NS) and vitamin $B_{12}$ (0.2 mg/liter). Samples were taken at intervals for turbidity measurements, analysis of the growth substrates by high-pressure liquid chromatography, and determination of sulfate and sulfite. Symbols: □, 1NS; ⊡, 2NS; ●, turbidity; ○, $SO_4^{-2}$; ■, $HSO_3^-$.*

to 80%. Colonies which had lost the ability to grow on 2NS were also unable to utilize naphthalene. However, all individual colonies tested still retained the ability to utilize salicylate. No reversion to the wild-type degradative ability was noticed when $5 \times 10^9$ cells of a cured clone of strain A3 were plated on 2NS mineral agar.

The ability to degrade naphthalene or 2-naphthalenesulfonate can be transferred from the wild type to a distinguishable cured strain of the wild type. The donor and a streptomycin-resistant recipient culture were grown overnight on nutrient agar plates. Cells were suspended in phosphate buffer (pH 7.4). One milliliter of each suspension containing $10^8$ cells was filtered through a 0.2-$\mu$m membrane filter (Millipore Corp.) which was then incubated for 48 h on the surface of nutrient agar plates at 30°C. Growth was suspended in 2 ml of phosphate buffer. The suspension and serial dilutions of it were spread onto the selection plates (2NS mineral medium containing 500 $\mu$g of streptomycin per ml) for transconjugant colonies to grow. Donor and recipient cultures were plated onto the selection plates as controls. The frequency of transfer calculated as the number of transconjugants per donor cell was found to be $0.6 \times 10^{-6}$ to $1.0 \times 10^{-6}$.

ACKNOWLEDGMENT

Part of this study was supported by a grant from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Bertolacini, R. J., and J. E. Barney.** 1957. Colorimetric determination of sulfate with barium chloranilate. Anal. Chem. **29**:281–283.
2. **Brilon, C., W. Beckmann, and H.-J. Knackmuss.** 1981. Catabolism of naphthalenesulfonic acids by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22. Appl. Environ. Microbiol. **42**:44–45.
3. **Endo, K., H. Kondo, and M. Ishimoto.** 1977. Degradation of benzenesulfonate to sulfite in bacterial extract. J. Biochem. **82**:1397–1403.
4. **Hartmann, J., W. Reineke, and H.-J. Knackmuss.** 1979. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a pseudomonad. Appl. Environ. Microbiol. **37**:421–428.
5. **Johnston, J. B., R. Murray, and R. B. Cain.** 1975. Microbial metabolism of aryl-sulfonates. A reassessment of colorimetric methods for the determination of sulfite and their use in measuring desulfonation of aryl- and alkylbenzenesulfonates. Antonie van Leeuwenhoek. J. Microbiol. Serol. **41**:493–511.
6. **Malle, K. G.** 1978. Wie schmutzig ist der Rhein? Chem. Unserer Zeit **12**:111–122.
7. **Pfennig, N., and K. D. Lippert.** 1966. Über das Vitamin $B_{12}$-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:245–256.
8. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic Pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**:159–271.