Metabolism of Naphthalenesulfonic Acids by Pseudomonas sp. TA-2

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A 2-naphthylamine-1-sulfonate (tobias acid)-degrading Pseudomonas strain was isolated from soil. This organism degraded 1-naphthalenesulfonate and 2-naphthol-1-sulfonate as well as tobias acid. When the cells grown on a nutrient medium were incubated with tobias acid, 1-naphthalenesulfonate, and 2-naphthol-1-sulfonate, salicylate was temporarily accumulated in the culture medium. A salicylate-degrading enzyme and a gentisate-degrading enzyme were induced by salicylate and gentisate, respectively. On the other hand, the enzymes which degrade sulfonated naphthalenes to salicylate were constitutive. Ammonia, sulfate, and a small amount of sulfite were detected in the course of degradation of tobias acid. 2-Naphthol-8-sulfonate or 1-naphthol-5-sulfonate was converted into 2,4-dihydroxybenzoate or 2,6-dihydroxybenzoate respectively. Both cis-1,2-dihydroxy-1,2-dihydro-3-naphthalenesulfonate and sulfate were formed when the cells were incubated with 2-naphthalenesulfonate. It is suggested that tobias acid was degraded by 1,2-dioxygenation in the initial process of the degradation pathway.

The degradation pathways of naphthalene by microorganisms have been well studied. According to Gibson, cis-1,2-dihydroxy-1,2-dihyronaphthalene is the primary product of naphthalene catabolism.1,2) This compound is rearomatized through a cis-dihydriodiol; dehydrogenase yielding 1,2-dihydroxynaphthalene,3) and 1,2-dihydroxynaphthalene was further metabolized via salicylate.

On the other hand, sulfonated naphthalene was a recalcitrant compound, and the C-S bond of the sulfonate group is thermodynamically stable. Some reports have described the degradation of naphthalenesulfonate. 1- and 2-naphthalenesulfonate were degraded by Pseudomonas sp.4) Amino and hydroxynaphthalenesulfonate were degraded by bacterial communities.5) Disulfonated naphthalene was also degraded by Moraxella sp. and Pseudomonas sp.6,7)

It was suggested by Knackmuss et al. that these compound were desulfonated spontaneously after 1,2-dioxygenation in naphthalene, and further degraded via the known catabolic sequence of naphthalene. Previous work from our laboratory has shown that 2-naphthylamine-1-sulfonate (tobias acid) was degraded by Pseudomonas sp. strain TA-1 via salicylate and gentisate.8) However, the degradation pathway for amino naphthalenesulfonate by a bacterium is not well understood. In addition, the mode of elimination of the sulfur and nitrogen from the naphthalene skeleton is still ambiguous. We has isolated from soil another Pseudomonas sp., TA-2, which had different characteristics from TA-1.8) In
this paper, we describe the characteristics of tobiac acid degradation mechanisms in *Pseudomonas* sp. TA-2.

### Materials and Methods

**Media and growth conditions.** For the cultivation of naphthalene-derivative-assimilating bacteria, a nutrient medium or a mineral salt medium was used. The mineral medium contained per liter: KH₂PO₄ (0.7 g), MgSO₄·7H₂O (0.7 g), NH₄NO₃ (2 g), CaCl₂ (10 mg), FeCl₃·6H₂O (4 mg), ZnCl₂ (4 mg), and MnCl₂·4H₂O (2 mg). The mineral salt medium was supplemented with an appropriate naphthalene derivative as a carbon source. The nutrient medium contained, per liter: peptone (5 g), meat extract (5 g), and NaCl (5 g). Cells were grown in 500-ml shaking flasks in 80 ml of the mineral salt medium containing a naphthalene derivative, or nutrient medium. The flask were incubated with shaking at 27°C.

To examine the degradation pathway for naphthalene derivatives, an incubation medium was used, containing, per liter: K₂HPO₄ (2 g), KH₂PO₄ (2 g), MgSO₄·7H₂O (0.5 g), and a naphthalene derivative (0.4 mmol). In the measurement of sulfate, MgSO₄·7H₂O was removed from the incubation medium. Cells at the late logarithmic growth phase grown in the nutrient medium or the mineral salt medium containing tobiac acid were harvested by centrifugation, washed twice with 0.2% potassium phosphate (pH 7.0) and then resuspended in the same buffer. The washed cells were then added to 100-ml shaking flasks containing 20 ml of the incubation medium to make O.D. 0.5 at 660 nm then incubated at 30°C with shaking.

**Isolation and Identification of bacteria.** A bacterial strain that grew on tobiac acid as a sole source of carbon and nitrogen were obtained from soil, as reported previously.  

The isolated bacteria were taxonomically characterized by routine methods.  

**Analytical methods.** Two-milliliter samples of the incubation medium were taken out at intervals during the incubation, and the cells were removed by centrifugation. The supernatants were used for measurement of tobiac acid, other related compounds, sulfate, and sulfite. Tobiac acid and related compounds were measured with a reverse-phase high performance liquid chromatograph (HPLC) equipped with an ultraviolet detector (wavelength, 220 nm). Separation was achieved on a Polyygel C₁₈ column (4 × 50 mm) acetonitrile-water containing 10 mm potassium dihydrogen phosphate and 10 mm tetra-n-butylammonium hydrogen sulfate, the flow rate 1 ml per minute.

The method used for measurement of sulfate in the incubation medium was a modification of the method of Bertolacini and Barrey.  

Sulfite in the incubation medium was measured by the method of Grant.  

The infrared (IR) absorption spectrum of the intermediate was measured with a SIMAZU spectrophotometer using KBr tablets.

The nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ¹H and 22.4 MHz for ¹³C in DMSO-d₆ and D₂O on JEOL JNM GSX-500 and JEOL JNM FX-90Q instruments with TMS and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal references, respectively.

**Mass spectrometry** was measured with a JOEL JMS-DX303H spectrometer at 70 eV.

Ultraviolet spectra were measured in water by a SIMAZU UV-240 spectrophotometer.

**SEM analysis** was done with a JEOL JTD 2000.

The growth of cultures was monitored by measurement of the optical density at 660 nm.

**Conversion of 2-naphthalenesulfonate (2NS) and 1-naphthalene-sulfonate (1NS) into dead end products, and purification of the compounds from the incubation medium.** Twenty five grams of the washed cells were resuspended in 21 of 25 mm phosphate buffer (pH 7.0) containing 2NS or 1NS (0.04%). After incubation at 27°C for 1 day, the cells were removed by centrifugation and the incubation medium was concentrated by evaporation. The concentrated medium were separated by thin layer chromatography (2 x 50 mm) acetonitrile-water containing 2 mm potassium dihydrogen phosphate and 2 mm tetra-n-butylammonium hydrogen sulfate, the flow rate 1 ml per minute.

**Conversion of 2-naphthol-8-sulfonate (2N8S) and 1-naphthol-5-sulfonate (1N5S) into dead end products, and purification of the compounds from the incubation medium.** Fifty grams of the washed cells were resuspended in 4 l of phosphate buffer (pH 7.0) containing 2N8S (0.04%). After this was incubated at 27°C for 1 day,
the cells were removed by centrifugation from the medium.
The incubation medium was concentrated and dried by a
rotary evaporator. The residue was dissolved in 200 ml
of methanol and then insoluble material were removed
by centrifugation. The product was separated by paper
chromatography (Toyo No. 50) with the upper layer of
solution: butanol, acetate, water (4, 1, 5). The band of the
product, that fluoresced a dark blue color under ultraviolet
(UV) light, was extracted with water. Insoluble materials
were removed by centrifugation. The solution was con-
centrated and dried by a rotary evaporator. White crystals
were obtained. To remove contaminating acetate, the
residue was washed with 3 ml of methanol.

Results

Relative rates of degradation of various com-
 pounds

Besides tobias acid, 1-naphthalenesulfo-
 nate (1NS) and 2-naphthol-1-sulfonate (2NS1S)
were degraded by the cells grown on tobias
acid (Table I). The cells had low activities
with 1N5S, 1-naphthol-8-sulfonate, 2N8S,
and 2NS, and new metabolites were detected
in the culture medium by HPLC analysis.
Since the amount of these products did not
decrease after the substrates disappeared in
the medium, they seemed to be dead end products.
The other sulfonated naphthalenes were not
degraded by the cells. Gentijsonate, protocat-
echuate, and salicylate were degraded by the
cells. Especially gentisolate showed a faster
degradation rate than that of tobias acid or
salicylate. The other dihydroxybenzoates were
not degraded. Catechol was converted into
yellow dead end product.

Metabolism of tobias acid

When the washed cells grown on tobias acid
were incubated with tobias acid, they degraded
tobias acid at a constant rate (Fig. 1). The
sulfate produced was equimolecular to the
amount of tobias acid degradation. Since the
cells could oxidize sulfite into sulfate (Fig. 1),
a small amount of sulfite was temporarily
accumulated during the degradation of tobias
acid, and when tobias acid had been degraded

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degradation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphthylamine-1-sulfonate (tobias acid)</td>
<td>100</td>
</tr>
<tr>
<td>2-Naphthylamine-6-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1-Naphthylamine-6-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1-Naphthalenesulfonate</td>
<td>101</td>
</tr>
<tr>
<td>2-Naphthalenesulfonate</td>
<td>5.3°</td>
</tr>
<tr>
<td>1-Naphthol-2-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1-Naphthol-3-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1-Naphthol-4-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1-Naphthol-5-sulfonate</td>
<td>46.8°</td>
</tr>
<tr>
<td>1-Naphthol-8-sulfonate</td>
<td>47.2°</td>
</tr>
<tr>
<td>2-Naphthol-1-sulfonate</td>
<td>58.5</td>
</tr>
<tr>
<td>2-Naphthol-6-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>2-Naphthol-7-sulfonate</td>
<td>0</td>
</tr>
<tr>
<td>2-Naphthol-8-sulfonate</td>
<td>15.9°</td>
</tr>
<tr>
<td>1,5-Naphthaldenedisulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1,6-Naphthaldenedisulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1,7-Naphthaldenedisulfonate</td>
<td>0</td>
</tr>
<tr>
<td>2,6-Naphthaldenedisulfonate</td>
<td>0</td>
</tr>
<tr>
<td>1,7-Naphthalenediol</td>
<td>0</td>
</tr>
<tr>
<td>2,3-Naphthalenediol</td>
<td>0</td>
</tr>
<tr>
<td>2,7-Naphthalenediol</td>
<td>0</td>
</tr>
<tr>
<td>Salicylate</td>
<td>38.1</td>
</tr>
<tr>
<td>Catechol</td>
<td>26.4°</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoate</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2,4-Dihydroxybenzoate</td>
<td>0</td>
</tr>
<tr>
<td>2,5-Dihydroxybenzoate (gentisolate)</td>
<td>160</td>
</tr>
<tr>
<td>2,6-Dihydroxybenzoate</td>
<td>0</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoate (protocatechuate)</td>
<td>43.2</td>
</tr>
<tr>
<td>3,5-Dihydroxybenzoate</td>
<td>0</td>
</tr>
</tbody>
</table>

* Accumulated dead end products in the culture
medium.

Fig. 1. Course of Tobias Acid Degradation by Washed
Cells of Strain TA-2 Grown on Tobias Acid.

Strain TA-2 grown on tobias acid was in the incubation
medium containing tobias acid at 30°C with shaking.
Tobias acid (●), sulfate (■), sulfite (□), and ammonia (▲)
were measured every hr. The cells were incubated with sulfite (○).
completely, sulfite disappeared in the culture medium. The amount of the accumulated ammonia was equal to half the amount of the degradation of tobias acid and when tobias acid had disappeared, the production of ammonia stopped, but did not decrease.

Catabolism of 1-sulfonated naphthalenes, salicylate and gentisate by the cells cultured on the nutrient medium and on tobias acid medium

The cells grown on the nutrient medium degraded tobias acid, 1NS, and 2N1S without an induction phase (Fig. 2A, B, C). Salicylate was temporarily accumulated in the culture medium and disappeared after the substrates disappeared in the culture medium.

The addition of chloramphenicol (CM) had no influence on the initial degradation rate of tobias acid by the cells grown on the nutrient medium. When CM was added to the medium, accumulated salicylate did not decrease, and the amount of substrate degradation and that of salicylate formation were approximately stoichiometrically equal.

The cells grown on nutrient medium assimilated salicylate and gentisate lower than the cells grown on tobias acid. The rate of both salicylate and gentisate degradation by the cells grown on nutrient medium increased during the degradation (Fig. 2D, E). The addition of CM prevented the cells grown on the nutrient medium from increasing the degradation rate of the salicylate or gentisate. But small amounts of degradation of these substrates were detected.

On the other hand, the cells grown on tobias acid rapidly degraded salicylate and gentisate without the time lag, and the addition of CM did not influence the degradation rate of salicylate or gentisate by the cells grown on tobias acid.

Conversion of 2N8S, 1N5S, and 2NS into dead end products, and purification and identification of these compounds

The dead end products from 2N8S and 1N5S were purified and identified. The procedure of the purification was described under Materials and Methods. With regard to the UV spectrum, the mass spectrum, and the IR spectrum, the purified products had the same properties as those of authentic 2,4-dihydroxybenzoate and 2,6-dihydroxybenzoate respectively.

The cells also formed a dead end product from 2NS. This compound was also purified and its chemical structure was identified. The procedure of the purification was described under Materials and Methods. The purified...
compound could be hardly dissolved in organic solvents such as ether, and acetone. Therefore, it seemed to have large polarity. This compound was stable in neutral conditions but it was degraded instantly in acidic conditions.

The structure of this compound was identified as 1,2-dihydroxy-1,2-dihydro-3-naphthalenesulfonate (DDNS). Physical evidence for the identity of the compound was provided by $^1$H NMR and $^{13}$C NMR spectroscopy. $^1$H-Decoupled- $^{13}$C NMR spectrum data at 22.4 MHz in D$_2$O are described as follows: 139.99 (s), 136.74 (s), 130.94 (d), 130.67 (d), 130.18 (s), 129.26 (d), 128.29 (d), 125.36 (d), 71.35 (d), 65.93 (d): multiplicity observed in $^{13}$C offresonance decoupling experiment. $^1$H NMR assignments of this compound were done using conventional NOE and COSY experiments (Table II). The $J_{1-4}$ coupling constant of 4.0 Hz for the carbinol hydrogens in the $^1$H NMR spectrum was consistent with a stereochemistry in cis-1,2-dihydroxy-1,2-dihydronaphthalene. $^{14}$

Further, the IR spectrum suggested the existence of a hydroxy group ($\nu$OH: 3360 cm$^{-1}$) and sulfonate group ($\nu$ as SO$_3^-$: 1180 cm$^{-1}$, $\nu$ s SO$_3^-$: 1060 cm$^{-1}$) and the existence of sulfur and potassium in this compound was confirmed by SEM analysis. The formula corresponded to C$_{10}$H$_9$O$_5$SK by elemental analysis. Found: C, 42.47; H, 3.33%. Calcd. for C$_{10}$H$_9$O$_5$SK: C, 42.83; H, 3.21%.

Table II. $^1$H NMR ASSIGNMENT OF DDNS IN DMSO-$d_6$ (500 MHz)

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical shift (ppm)</th>
<th>Description</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.38</td>
<td>1H (dd, $J_{1-4}$=4.0 Hz, $J_{1-2}$=3.7 Hz)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.20</td>
<td>1H (d, OH, $J_{2-1}$=3.7 Hz)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.23</td>
<td>1H (d, OH, $J_{3-4}$=6.4 Hz)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.54</td>
<td>1H (dd, $J_{4-1}$=4.0 Hz, $J_{4-3}$=6.4 Hz)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.86</td>
<td>1H (s, olefinic)</td>
<td></td>
</tr>
<tr>
<td>6, 7</td>
<td>7.16-7.23</td>
<td>2H (m, aromatic)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>7.24-7.31</td>
<td>1H (m, aromatic)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>7.50</td>
<td>1H (d, aromatic, $J_{9-8}$=7.6 Hz)</td>
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</tr>
</tbody>
</table>

Table III. MUTUAL INHIBITION OF TOBIAS ACID, 1-NAPHTHALenesulfonate and 2-NAPHTHALenesulfonate ON THE DEGRADATION OF SUBSTRATE BY THE CELLS GROWN ON TOBIAS ACID

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Degradation$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobias acid</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1NS (0.43 mM)$^b$</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>2NS (4.3 mM)$^b$</td>
<td>101</td>
</tr>
<tr>
<td>1NS</td>
<td>None</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Tobias acid (0.41 mM)$^b$</td>
<td>54</td>
</tr>
<tr>
<td>2NS</td>
<td>None</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Tobias acid (0.44 mM)$^b$</td>
<td>7.4</td>
</tr>
</tbody>
</table>

$^a$ Initial degradation rate for 1 hr.  
$^b$ Initial concentration.

Fig. 3. Course of 2-Naphthalenesulfonate Degradation by Washed Cells Grown on Tobias Acid.  
2NS (---), cis-1,2-dihydroxy-1,2-dihydro-3-naphthalenesulfonate (-□-) and sulfate (-○-) were measured every hr.
Figure 3 shows the course of 2NS degradation by the washed cells grown on tobias acid. Both sulfate and DDNS were accumulated in the incubation medium. The amounts of sulfate and DDNS were nearly equal and the sum of the amount of sulfate and DDNS was about 70% of that of the 2NS degradation.

Inhibition of Tobias acid, 1NS, 2NS degradation by the other naphthalenesulfonate

Table III shows the degradation of tobias acid, 1NS, or 2NS in the presence of one of the other naphthalenesulfonates by the cells grown on tobias acid. Tobias acid degradation was inhibited by 1NS but not 2NS. On the other hand, tobias acid was also an inhibitor of the degradation of 2NS or 1NS.

Discussion

In our previous paper, we reported the study of the degradation pathway of tobias acid by Pseudomonas strain TA-1. As mentioned in this paper, the other Pseudomonas strain TA-2 also assimilated tobias acid as a sole source of carbon and nitrogen. The cells grown on tobias acid degraded salicylate and gentisate, and tobias acid. Under the degradation of tobias acid, 1NS, and 2N1S by the cells grown on the nutrient medium, salicylate was transiently accumulated in the culture fluid. 2N8S and 1N5S were converted to 2,4-dihydroxybenzoate and 2,6-dihydroxybenzoate, respectively. These compounds were accumulated in the culture medium as dead end products, for they were not degraded. This strain probably cleaved the naphthalene ring to which sulfo substituent group bonded. It is presumed from these results that strain TA-2 degrades tobias acid via the same pathway as that of TA-1, that is to say, the pathway of tobias acid → salicylate → gentisate (Fig. 4).

In this bacterium, the enzymes for salicylate degradation and gentisate degradation were induced by the salicylate and gentisate respectively. On the other hand, the enzymes in the degradation pathway from naphthalenesulfonate to salicylate was constitutive, because the bacterium grown on the nutrient medium degraded tobias acid, 1NS and 2NS, as well as the bacterium grown on the tobias acid medium, and the addition of CM hardly influenced the initial degradation rate of tobias

![Fig. 4. Proposed Degradation Pathway of Naphthalenesulfonates by Strain TA-2.](image-url)

Letters are compound designations as follows: a, tobias acid; b, salicylate; c, gentisate; d, 2-naphthol-8-sulfonate or 1-naphthol-5-sulfonate; e, 2,4- or 2,6-dihydroxybenzoic Acid; f, 2-naphthalenesulfonate; g, cis-1,2-dihydroxy-1,2-dihydro-3-naphthalenesulfonate.
Metabolism of Naphthalenesulfonic Acids

Acid and other two naphthalenesulfonates by the cells grown on the nutrient medium. On the other hand, the TA-1 cells grown on peptone-glucose couldn't degrade tobias acid without an induction period. It is thought that these enzymes in TA-1 were inducible. Sulfite was transiently detected in a small amount in the culture medium in the course of degradation of tobias acid. Since the cells had high oxidase activity of sulfite, it was assumed that the elimination of sulfur from the naphthalene skeleton was occurred as sulfite and then sulfite was immediately oxidized to sulfate.

Knackmuss et al. obtained 1,2-dihydroxy-1,2-dihydro-naphthalene-2-carboxylate by co-oxidation of 2-naphalenecarboxylate using the 2NS degrading strains BN6 and A3. They describe how these organisms have a naphthalene dioxygenase with very low substrate specificity but high regioselectivity and if acidic groups exist in the 1 or 2 position, the highly selective model of 1,2-dioxygenation of naphthalene occurs. On the other hand, when the TA-2 cells, which could assimilate 1NS, 2N1S, and tobias acid, were incubated with 2NS as the only substrate, 2NS was converted to DDNS. In addition, sulfate was produced from 2NS simultaneously.

These results indicate that the strain metabolized 2NS through two pathways. Since tobias acid inhibited 2NS and 1NS degradation, it is presumed that 2NS was degraded first by the same enzyme as the first enzyme of tobias acid degradation pathway. However, the tobias acid degradation was not inhibited by 2NS. 2NS seemed hardly to influence tobias acid degradation, for tobias acid is highly in affinity for the first enzyme of tobias acid degradation than 2NS.

Figure 4 summarizes the proposed degradation pathway of the various naphthalenesulfonates. 1-Sulfonated naphthalenes such as tobias acid, 1NS, 2N1S, 2N8S, and 1N5S seem to be oxygenated at the 1,2-position at first and dihydrodiols are produced. When the substrate is 1-sulfonated naphthalene, this first degradation enzyme probably has a disposition to low substrate specificity but high regioselectivity. It is, however, assumed that 1,2- or 3,4-dioxygenation occurred equally on 2NS. Therefore, in this strain highly selective 1,2-dioxygenation of naphthalene may not necessarily occur. In the former case, the 1,2-position is oxygenated, and the dihydroxy-dihydro-naphthalenesulfonate seems to be further degraded to salicylate after desulfonation. In the latter case, DDNS accumulates in the culture medium as a dead end product, because the cells converted from 2NS to DDNS, and it was not degraded by the cells.

Whether dihydrodiols are desulfonated and degraded spontaneously or enzymatically is not known.

References