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# Degradation of Benzothiophene and Related Compounds by a Soil *Pseudomonas* in an Oil-Aqueous Environment

FERMÍN SAGARDÍA,* JUAN J. RIGAU, AGNES MARTÍNEZ-LAHOZ, FRANCISCO FUENTES, CARMEN LÓPEZ, AND WALDEMAR FLORES

*Department of Microbiology, University of Puerto Rico, School of Medicine, Río Piedras, Puerto Rico 00936,* and the Office of Petroleum Fuels Affairs, Office of the Governor, Commonwealth of Puerto Rico, San Juan, Puerto Rico 00910*

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*Pseudomonas aeruginosa* PRG-1, an isolate from oil-contaminated soil, degrades benzothiophene (BT) and other related compounds in a 5% oil-basal medium system. The organism cannot grow on BT alone; 0.05% yeast extract is a suitable substrate for its growth and for its attack on BT. Although BT is partially toxic to the bacteria, toxicity is reduced when BT is added in this oil system. The oil phase is emulsified by bacterial action during the process. Oxygen uptake studies with washed cell suspensions show increased respiration in the presence of BT. Endogenous respiration is markedly decreased by *p*-hydroxy-mercuribenzoate, whereas respiration due to BT is scarcely affected, suggesting that oxygen is added directly to BT. Results obtained both in direct degradation and in respiration studies indicate that 3-methyl-thiophene is more rapidly and extensively degraded than BT and other related compounds.

The simultaneous presence of high concentrations of sulfur compounds and heavy metals in crude oils poses an acute environmental problem since heavy metals poison the catalysts used in hydrodesulfurization processes. Since both sulfur compounds and heavy metals are concentrated during refinery processing, the problem with residual fuel oils is even more acute. This combined problem is present in the case of Venezuelan oils, which contain 2 to 3% sulfur and 250 μg of Va and Ni per g (H. Drushel, Symp. Heterocompounds, Am. Chem. Soc., Houston, February 1970). The possibility of using microorganisms in such cases for the removal of sulfur compounds has been considered (6). Although organisms capable of degrading some petrosulfur compounds in defined systems have been obtained (6), their application to practical situations has been disappointing. There appear to be at least two main problems in such systems: (i) accessibility of the oil phase components to the bacteria is limited; and (ii) some of the oil components are toxic to the microorganisms. Using benzothiophene (BT) as a model compound, we have isolated a soil *Pseudomonas* that can degrade BT and related compounds in a simple oil-aqueous environment. This system may open a way to an eventual practical solution of this problem.

## MATERIALS AND METHODS

The basal medium used for these studies contained: $KH_2PO_4$, 1 g; $MgCl_2 \cdot 6H_2O$, 1 g; $NH_4Cl$, 5 g; $[(NH_4)_6Mo_7O_{24}]_4 \cdot H_2O$, 5 mg; $MnSO_4$, 5 mg; biotin, 2 μg; and water to 1 liter. The medium was supplemented with 0.05 or 0.1% yeast extract as a growth substrate since the organisms were not able to grow with BT as sole carbon or energy source. Growth was determined in a Coleman spectrophotometer by measuring the optical density at 600 nm.

For the quantitative determination of BT disappearance, cells were grown shaken in 50 ml of medium containing 2.5 ml (5%) of light oil (Johnson's baby oil), with 0.1% BT in 200 to 250-ml Erlenmeyer flasks at 30 C. At suitable intervals, the contents were extracted with 10 ml of *n*-butanol. This extracts the oil and BT into the organic phase. The butanol solution was diluted as needed (approximately 1,000-fold) and its absorbancy or spectrum was determined in a Gilford 2400-S recording spectrophotometer. The oil peaks and yeast extract materials are so diluted by this procedure that they cannot be detected and do not interfere with the measurements of the BT peaks at 225, 255, and 290 nm. We have used the disappearance of the 225-nm peak as a measure of BT disappearance. The amount of BT dissolved in the aqueous phase both in the presence and absence of oil was determined by extracting, after equilibration, 25 ml of the aqueous phase twice with a total of 25 ml of butanol. The absorbance at 225, 255, and 290 nm was measured after dilution, and the concentration of BT

was calculated using the molar absorption coefficient of BT.

Oxygen uptake of washed cell suspensions was measured with a model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co.). In early experiments, we obtained high endogenous and experimental values which more recently we have managed to reduce. These were due to variable characteristics of the membrane system. Cells were grown in the basal medium with 0.1% yeast extract for 48 h. BT was not added, since the cells clumped upon harvesting. Cells were collected by centrifugation at 0 C in a Sorvall RC-2B centrifuge at 10,000 rpm for 10 min, the supernatant was decanted, and the pellet was washed twice by centrifugation and decanted with cold 0.1 M phosphate buffer, pH 7.4. Finally, the cells were resuspended in the same buffer and the optical density was adjusted to 0.60 U at 600 nm with buffer. The bacterial suspension was maintained in ice during the experiment. Oxygen uptake was measured in a mixture of 1.0 ml of the cell suspension and 2.0 ml of the phosphate buffer. The compounds to be studied were added as saturated solutions in the 2.0 ml of phosphate buffer. The control and experimental solutions were thoroughly shaken to saturate them with air before adding the cell suspensions. The reduction in oxygen content of the suspensions were measured at 1-min intervals and were linear with time. The uptake of oxygen was calculated assuming that the original solution contained 5 $\mu$l of oxygen/ml.

## RESULTS

**Isolation and characterization of microorganisms.** Oil-contaminated soil samples from the grounds of Union Carbide Corporation at Guayanilla, Puerto Rico, were obtained. One-gram samples were inoculated into 50 ml of basal media containing 0.1% yeast extract and 0.1% BT. The toxicity of BT to most microorganism was such that a modified enrichment culture was obtained and BT-tolerating bacteria were favored. After several passages in liquid media, the suspension was plated in the same solid medium (with 1.5% agar), but without BT. Two types of colonies were obtained. One was formed by slow-growing gram-positive micrococci, which we have not studied in detail. The other was fluorescent pigmented and was composed of gram-negative rods. This organism was isolated, and its ability to degrade BT was confirmed (see below) and characterized.

The organism is an oxidative, gram-negative rod, producing a green fluorescent pigment. It is motile, utilizes citrate but not urea, possesses lysine decarboxylase activity, and renders glucose and triple sugar iron media alkaline. We have tentatively classified it as *Pseudomonas aeruginosa*, strain PRG-1.

**Nutritional and growth characteristics.** The organism was grown routinely in the basal medium with 0.05 or 0.1% yeast extract and 0.1% BT. Addition of BT directly to the 0.1% yeast medium reduced the maximum growth obtained in 24 h from an optical density of 0.160 to 0.090 and reduced the growth rate from 0.312 to 0.200 per h. The organism could also be grown in an amino acid mixture (0.1% each histidine, glutamic acid, and glutamine in the basal medium) or on 1% glucose with a vitamin supplement (0.1% each folic acid, biotin, riboflavin, and calcium pantothenate).

There was no growth when BT was added directly without other nutrients at 0.1% or lower concentrations and incorporated in an oil layer (see below), in a vapor phase, or in a solid paraffin matrix (2).

**Disappearance of BT from the oil system.** It was difficult to determine quantitatively the amount of BT degraded when the compound was added directly to the aqueous medium, because BT, although having a high boiling point, evaporated rapidly in combination with water. Furthermore, BT dissolved only to the extent of 0.009%; any extra amount added remained suspended. Finally, as mentioned above, BT was partially toxic to the bacteria. We incorporated into the medium 5% light oil (Johnson's light baby oil) into which BT dissolved at a final concentration for the complete system of 0.1% (2.0% in the oil phase). Under these conditions the concentration of BT in the aqueous phase was 0.0013%, there was reduced toxicity to the bacteria (optical density after 24 h of 0.140 in 0.1% yeast medium and a growth rate of 0.263 $h^{-1}$, and the evaporation of BT from the system was slow even after several days of agitation. Care was taken to separate the aqueous and organic phases before taking these measurements. Similar results were obtained in other growth media (unpublished data). Several hours after inoculation of such a system the growing bacteria emulsified the oil and BT gradually disappeared from the oil phase (Table 1). That the removal of BT is not due solely to emulsification of the oil was shown by an experiment in which the bacteria were allowed to grow and emulsify the oil in absence of BT, the system was autoclaved, BT was added to a concentration of 0.1% BT, and shaking was continued for several days. Under these conditions, the concentration of BT in the system remained nearly unchanged. Using amino acids as growth substrates, there was approximately 40% disappearance of BT in 6 days.

**Degradation of other compounds.** Table 2 shows the results obtained when some other BT-related compounds were incubated in the

TABLE 1. *Degradation of BT in the oil-aqueous system by P. aeruginosa PRG-1*[a]

| Time after inoculation days | Control | | Experimental | |
|---|---|---|---|---|
| | BT concn (%) | BT disappearance (%) | BT concn (%) | BT disappearance (%) |
| 0 | 0.101 | | 0.100 | |
| 2 | 0.099 | 2 | 0.074 | 36 |
| 5 | 0.098 | 3 | 0.058 | 42 |

[a] The experimental was inoculated with a drop of a 24-h growth culture in basal medium with 0.05% yeast extract.

TABLE 2. *Degradation of various compounds in the oil-aqueous system by P. aeruginosa PRG-1*[a]

| Compound | λ Maximum used | % Evaporation, controls | % Disappearance due to microorganisms |
|---|---|---|---|
| Bencene | 215 | 7 | 20 |
| Thiolane | 220 | 11 | 48 |
| 2-Me-T | 220 | 16 | 41 |
| 3-Me-T | 230 | 20 | 87 |

[a] Incubation periods were of 4 days in each case. With these compounds there was some evaporation in the controls; the values in the experimental column were corrected for this factor. The actual wavelength used to determine the concentrations of the compounds is given in column 2. 2-Me-T, 2-Methyl-thiophene; 3-Me-T, 3-methyl-thiophene.

oil system with the bacteria in the presence of 0.1% yeast extract. Evaporation of these compounds, which are normally more volatile than BT, was faster. However, after correction for this factor, it is clear that they also disappeared from the system. In particular, the velocity and extent of disappearance of 3-methyl-thiophene was considerably greater than for BT.

**Oxygen uptake studies.** Another approach taken to detect degradation of these compounds was measurement of the oxygen uptake of washed cell suspensions of the bacteria. Preliminary studies showed that the endogenous respiratory rate of cell suspensions was increased in presence of BT. To eliminate the possibility that this could be due to an uncoupling effect of BT, we also studied the effect of *p*-hydroxy-mercuribenzoate on the system.

Results in Table 3 show that in the presence of BT the uptake of oxygen increased considerably over the endogenous rate. *p*-Hydroxy-mercuribenzoate reduced the endogenous rate approximately in half; yet the increase in oxygen uptake in the presence of BT

TABLE 3. *Oxygen uptake of washed cell suspensions of P. aeruginosa PRG-1, and the effects of BT and p-hydroxy-mercuribenzoate*

| System | $O_2$ uptake (nl/min) | Increase caused by BT (nl/min) |
|---|---|---|
| Endogenous | 538 ± 94 | |
| +BT | 1,075 ± 86 | 547 |
| +PMB | 290 ± 22 | |
| PMB + BT | 713 ± 42 | 423 |

[a] Cells were grown in basal medium with 0.1% yeast extract for 48 h, washed with buffer several times, and suspended to give an optical density of 0.60 at 600 nm in buffer. Oxygen uptake was measured in mixtures containing 1 ml of the cell suspension and 2 ml of buffer; the final concentration of BT in the system was 0.006%. The final concentration of *p*-hydroxy-mercuribenzoate (PMB) in the system was $10^{-4}$ M.

under these conditions was about the same as that obtained in the absence of *p*-hydroxy-mercuribenzoate. These results indicate that the enzyme(s) involved in BT degradation were not sulfhydryl enzymes. Furthermore, it would appear that impairment of the respiratory chain (decrease in endogenous oxygen consumption) did not interfere significantly with oxygen uptake due to the metabolism of BT. This may indicate that the uptake of oxygen was due to direct addition to BT.

We have studied the uptake of oxygen in washed cell suspensions in presence of other petrosulfur compounds. Table 4 shows that in the presence of these compounds oxygen uptake increased at least as much as in the case of BT. It may be of some significance that in the case of 3-methyl-thiophene the rate was the highest; as shown in Table 3, degradation of 3-methyl-thiophene is also very rapid in the simulated oil system. The concentrations in the aqueous

TABLE 4. *Oxygen uptake of washed cell suspensions of P. aeruginosa PRG-1 in presence of various compounds*[a]

| Compound | Endogenous rate | Experimental | Difference |
|---|---|---|---|
| BT | 495 ± 36 | 705 ± 64 | +210 |
| Thiophene | 435 ± 48 | 870 ± 105 | +435 |
| 2-Me-T | 405 ± 20 | 630 ± 195 | +225 |
| 3-Me-T | 390 ± 30 | 1,105 ± 205 | +715 |

[a] Cells were grown and treated as described in the legend to Table 3. The compounds were added to the respiratory chamber as saturated solutions in the 2 ml of buffer. 2-Me-T, 2-methyl-thiophene; 3-Me-T, 3-methyl-thiophene.

phase of 2-methyl-thiopene and 3-methyl-thiophene were 0.031 and 0.014%, respectively.

## DISCUSSION

Our results on the degradation of BT by *P. aeruginosa* PRG-1 are consistent with a mechanism whereby oxygen is added directly to the molecule of BT. We are now starting a search for the products of degradation, but an analogy with the degradation of benzene (2), dibenzothiophene (4), and thiophene-2-carboxylate (1) by other *Pseudomonas* suggests that hydroxyl derivatives of one or the other ring are formed; these, being more soluble in water, move into the aqueous phase. The presence and position of a side chain would appear to be of importance since 3-methyl-thiophene is degraded more readily than 2-methyl-thiophene and BT. Although there are differences in the solubilities of these compounds, it should be noticed that the differences in solubilities between 3-methyl-thiophene and BT is relatively small whereas the solubility of 2-methyl-thiophene is the highest of all. Yet the rate of degradation of BT and 2-methyl-thiophene are practically the same and the oxygen uptake rate with 2-methyl-thiophene is the lowest. Thus, it is likely, although not entirely certain, that the position of the side chain is of primary importance.

It is difficult to decide at this stage whether the degradation of BT is due to a co-metabolite phenomenon or whether BT is a nongrowth substrate (3). However, the fact that BT is degraded when the bacteria are grown on a simple amino acid mixture favors the second alternative.

There are clear advantages in degradation experiments in oil-aqueous systems. The main problems expected in such a system are reduced by the fact that the bacteria emulsify the oil, and that the toxic effects of the compounds on the bacteria are reduced. However, it appears that if fuel oil itself is used directly, toxicity effects are severe. Indeed, we have found that although the bacteria will emulsify crude oil, the sulfur content of the oil is not decreased. This simulated oil-aqueous system, however, provides a model for exploring these problems in further detail with a view to their eventual application to natural oils.

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