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## Microbial methylation of benzenethiols and release of methylthiobenzenes

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*Summary.* Three phylogenetically diverse microorganisms methylated several different chloro- and nitro-substituted benzenethiols to yield the corresponding methylthiobenzenes. These products were identified by gas chromatography–mass spectrometry. In several cases large percentages of the methylthio products were released by intact cells into the medium, suggesting that microbial methylation of xenobiotic thiols may be a significant biotransformation in many ecosystems.
*Key words.* Methylthiobenzenes; benzenethiols; xenobiotics; ecosystems.

In recent years it has been recognized in animal and plant systems that many xenobiotics, including drugs and pesticides, are converted to sulfur containing metabolites[3–9], some of which are free thiols or their disulfide derivatives. We have recently discovered that many phylogenetically distinct microorganisms contain the enzyme glutathione-S-transferase that catalyzes formation of thioether conjugates between glutathione and a variety of electrophiles[10]. In one of these organisms, *Tetrahymena thermophila,* metabolism of the fungicide pentachloronitrobenzene is initiated by glutathione-S-transferase and a major excreted metabolite is pentachloromethylthiobenzene[11]. Excretion of the thioanisole follows methylation of pentachlorobenzenethiol by a S-adenosylmethionine-dependent thiol methyltransferase, an enzyme we have purified and characterized from the above organism, from the yeast *Saccharomycopsis lipolytica,* and from the green alga *Euglena gracilis*[12]. Given the nonspecific substrate specificities of the thiol methyltransferases in these organisms[12], it is possible that many methylthio metabolites are released into the environment as a result of microbial transformation of xenobiotic thiols.

During these studies we wondered whether intact microbial cells could take up exogenous thiols and methylate them via the thiol methyltransferase system. To study this possibility whole cells of *E. gracilis, T. thermophila* and *S. lipolytica* were incubated with various aromatic thiols bearing chloro- or nitro-substitutions. These thiols were chosen because they are good substrates for the thiol methyltransferase as measured in vitro[12], and because of the ease of detection of methylated chloro- and nitro-metabolites using gas chromatography coupled to electron capture detection.

Cells were grown as previously described[10,11], harvested in exponential growth by gentle centrifugation, and washed and suspended in 10 mM Tris-Cl, pH 7.4, for *T. thermophila* and *S. lipolytica* or 10 mM potassium phosphate, pH 6.1, for *E. gracilis*. The cell concentrations were determined by hemacytometer counts. Reaction mixtures contained cells in a volume of 5 ml. Aromatic thiols were prepared or obtained commercially as described elsewhere[12]. Thiols were dissolved in acetone and added to the incubation mixtures at concentrations of $1–5 \times 10^{-5}$ M (0.1% v/v final acetone concentration), and the mixtures shaken gently for 3 h at 25 °C. At the end of the incubation cells were pelleted in a clinical centrifuge and resuspended in fresh buffer. Both the resuspended cells and reaction medium were extracted with 5 ml hexane (pesticide grade, Burdick and Jackson Laboratories, Muskegan, MI) twice after adding 50 µl of 10 N NaOH; for determinations of total product the centrifugation step was omitted. The hexane layer was analyzed for the methylthio products by gas chromatography on a 1.8 m column packed with SP2250 on 100/120 Supelcoport (Supelco Inc., Bellefont, PA). The oven temperature was 180 °C, the detector temperature was 250 °C and the injection port temperature was 200 °C. A nitrogen flow rate of 25 ml/min was used. Control reaction mixtures without added thiol or with boiled cells added were treated identically. Quantitation of methylthio products was accomplished by measuring peak areas, compared to peak areas of authentic methylthio derivatives prepared as described elsewhere[12].

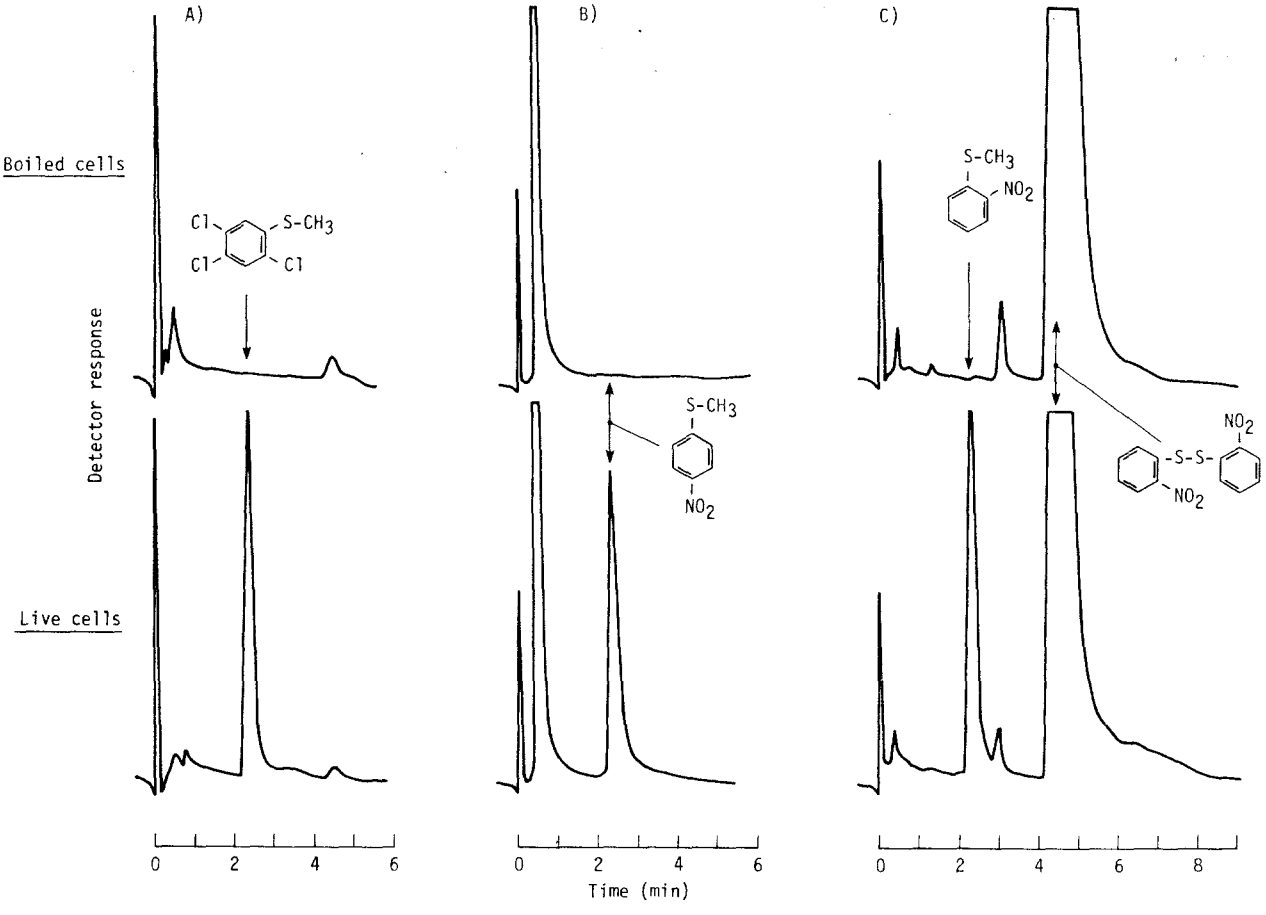
The production of methylthiobenzene derivatives of three substituted benzenethiols by whole cell incubations with *T. thermophila* is shown in the figure. The major product in each case (compared to boiled cell controls) had a retention time identical to the authentic methylthio derivative run at the same time. The amount of each methylthiobenzene recovered was dependent on the length of incubation, the number of cells present

and the amount of substituted benzenethiol added (data not shown). Identical results were obtained in incubations with *E. gracilis* and *S. lipolytica*, although the methylthio product yield varied (as described below).

In order to confirm the identity of the metabolite peaks shown in the figure, gas chromatography–mass spectrometry was used. Large scale incubations (50 ml) with the following thiols, *p*-nitrobenzenethiol, *o*-nitrobenzenethiol and 2,4,5-trichlorobenzenethiol, were carried out with *T. thermophila* as described in the legend to the figure. After extraction with hexane, and evaporation of the hexane layer, samples were analyzed on a Hewlett-Packard model 5982A gas chromatograph–mass spectrometer (Hewlett-Packard Co., Palo Alto, CA). A capillary column was used: fused silica, 30 m × 0.32 mm i.d. with a DB-5 1-μm film thickness (J and W Scientific, Rancho Cordova, CA), connected directly to the ion source. The instrument was operated with a 70 eV ionization voltage, a source temperature of 150 °C and temperature programming from 20 to 300 °C. Retention times and mass fragmentation data for the unknowns were compared to authentic standards run at the same time. The presumptive methylthiobenzenes produced by *T. thermophila* (figure) were shown unambiguously to be: *p*-nitromethylthiobenzene, from *p*-nitrobenzenethiol, *o*-nitrobenzenethiol, and 2,4,5-trichloromethylthiobenzene from 2,4,5-trichlorobenzenethiol. In addition, in separate experiments *p*-chloromethylthiobenzene was produced from *p*-chlorobenzenethiol, and pentachloromethylthiobenzene was produced from pentachlorobenzenethiol.

It was also of interest to determine if the methylthio products were accumulated intracellularly or if they were excreted into the incubation medium. For these experiments we analyzed all three cell types, using gas chromatographic identification of the methylthio products in each case. After incubating cells for specified times with substituted benzenethiols, whole cells were removed from the incubation medium and both medium and cells were extracted and analyzed separately. The time course of production of total methylthio products, and percentage of these products excreted into the medium was measured for each cell type and the results are summarized in the table.

The rates of production of the methylthio products differed significantly between organisms. In the case of pentachloromethylthiobenzene *T. thermophila* retained 43% of the product after 3 h incubation and released 57% the remainder into the medium. *E. gracilis* excreted about 81% and *S. lipolytica* only about 18% in this same period of time. Using other thiols revealed a more complex pattern of appearance of different methylthio products as summarized in the table. In *T. thermophila* approximately 50% of four different methylthio products was excreted in each case despite the fact that the rate of production varied approximately 20-fold. In *E. gracilis* the percent of product excreted varied from 52% to 91%, and in *S. lipolytica* the excretion ranged from 13% to 72%. Since the mechanism of excretion is unknown, the reason for these differences is unclear. Different results might be obtained with cells incubated in growth medium instead of buffer or cells harvested at different growth stages.




Gas chromatography of hexane extractable metabolites of substituted benzenethiols produced by *T. thermophila*. Cells, boiled or live, were incubated with the indicated concentration of benzenethiols for 3 h in 5.0 ml reaction mixtures. The complete incubation mixture was made basic with the addition of 50 μl 10 N NaOH to minimize the subsequent extraction of the thiol, and then extracted with hexane. The hexane layer was analyzed by gas chromatography. A: $1 \times 10^{-5}$ M, 2,4,5-trichlorobenzenethiol; B: $5 \times 10^{-5}$ M, *p*-nitrobenzenethiol; C: $4 \times 10^{-5}$ M, *o*-nitrobenzenethiol.

Whole cell production and release of methylthiobenzenes after exposure to benzenethiols for 3 h

| Substrate | *T. thermophila*[c] | | | *E. gracilis*[d] | | | *S. lipolytica*[e] | | |
|---|---|---|---|---|---|---|---|---|---|
| | nmoles total | nmoles/ mg cells (dry wt) | % in media | nmoles total | nmoles/ mg cells (dry wt) | % in media | nmoles total | nmoles mg cells (dry wt) | % in media |
| Pentachlorobenzenethiol | 2.68 | 5.8 | 57 | 0.51 | 1.0 | 81 | 1.15 | 1.2 | 18 |
| 2,4,5-Trichlorobenzenethiol[a] | 1.6 | 2.5 | 57 | 0.47 | 0.9 | 52 | 1.05 | 1.2 | 39 |
| *p*-Nitrobenzenethiols[b] | 29.0 | 62.5 | 52 | 15.0 | 28.6 | 90 | 3.80 | 3.7 | 72 |
| *o*-Nitrobenzenethiol[b] | 12.8 | 27.6 | 53 | 10.4 | 19.9 | 91 | 7.72 | 8.0 | 68 |

[a] $1 \times 10^{-5}$ M, 0.1% acetone in buffer. [b] $5 \times 10^{-5}$ M, 0.1% acetone in buffer. [c] $5.75 \times 10^{5}$ cells in 5 ml 10 mM Tris-Cl, pH 7.4. [d] $1.3 \times 10^{6}$ cells in 5 ml 10 mM potassium phosphate, pH6.1. [e] $6 \times 10^{7}$ cells in 5 ml 10 mM Tris-Cl, pH7.4.

In the experiments shown in the table greater than 80% of all three cell types were still viable after the 3-h incubation. Viable cells were measured by counting the number of cells still swimming in the cases of *T. thermophila* and *E. gracilis*, and for *S. lipolytica* by counting colonies after plating on YMS agar plates[13]. These results suggest that release of products from dead cells is probably not a major factor in the appearance of methylthiobenzenes in the medium.

These studies establish that methylthiobenzenes, produced by microbial methylation of benzenethiols, accumulate in readily detectable amounts outside the cell. Since the methylthio derivatives are generally less toxic than the parent thiol[14], this biotransformation could be beneficial to the ecosystem as a whole. However, if these methylthio products can be further processed by the same or different organisms toxification could result. For instance, soil organisms are known to transform the sulfide containing insecticide, aldicarb, into its sulfoxide and sulfone, products with toxic effects[15]. A similar toxification scheme is seen in rats that produce a methylsulfone derivative of some chlorinated biphenyls[16]. The toxicological significance and extent of microbial methylation of thiols in natural environments is unknown, although methylthio derivatives of pesticides have been seen by others as reviewed by Renner[9]. Further work on these reactions in natural mixed populations must be done before any environmental impact can be assessed.

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## Differential sensitivity to tributyltin of cytochrome-containing and cytochrome-deficient cells of *Escherichia coli* SASX76

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*Summary.* The effect of tributyltin (TBT) chloride on the growth of cytochrome-deficient and cytochrome-containing cells of *Escherichia coli* SASX76 was examined. The former cells were found to be at least 20 times more sensitive to TBT. It is proposed that the differential sensitivity of these two cell types to the biocide, TBT, may be due to a different mode of energy generation by cytochrome-deficient and cytochrome-sufficient cells. In addition to the energy state, the pH change caused by the presence and absence of cytochromes which occurred during growth also resulted in a differential sensitivity of these cells.
*Key words. Escherichia coli;* cytochrome deficiency; tributyltin; differential sensitivity.

Organotin compounds including tributyltin (TBT) have considerable commercial importance as biocides, antifouling agents, plastic stabilizers, and catalysts[2–4]. TBT is found to be most effective against both the prokaryotes and the eukaryotes[5–7]. Regarding its biochemical mode of action it is known to catalyze halide/hydroxyl exchange across biomembranes[8]; inhibits oxidative and photophosphorylation by interacting with proton translocating ATPase[9]; and mediates leakage of vital ions[10]. In *Escherichia coli*, in addition to the above-mentioned processes, TBT and other triorganotin compounds were