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] 1×10^{-5} M, 0.1% acetone in buffer. [b] 5×10^{-5} M, 0.1% acetone in buffer. [c] 5.75×10^{5} cells in 5 ml 10 mM Tris-Cl, pH 7.4. [d] 1.3×10^{6} cells in 5 ml 10 mM potassium phosphate, pH6.1. [e] 6×10^{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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2 To whom reprint request should be addressed.
3 Tateishi, M., Suzuki, S., and Shimizu, H., J. biol. Chem. *253* (1978) 8854.
4 Bakke, J.E., and Price, C.E., J. envir. Sci. Health *B14* (1979) 427.
5 Begum, S., Scheunert, I., Haque, A., Klein, W., and Korte, F., Pesticide Biochem. Physiol. *11* (1979) 189.
6 Koss, G., Koransky, W., and Steinbach, K., Archs Toxic. *42* (1979) 19.
7 Stillwell, W.G., Bouwsma, O.J., Thenot, J.-P., Horning, M.G., Griffin, G.W., Ishikawa, K., and Takaku, M., Res. Commun. Chem. Path. Pharmac. *20* (1979) 509.
8 Lamoureux, G.L., and Rusness, D.G., ACS Symp. Ser. *158* (1981) 133.
9 Renner, G., Xenobiotica *11* (1981) 435.
10 Lau, E.P., Niswander, L., Watson, D., and Fall, R.R. Chemosphere *9* (1980) 565.
11 Murphy, S.E., Drotar, A.M., and Fall, R., Chemosphere *11* (1982) 33.
12 Drotar, A.M., Ph. D. Thesis, University of Colorado, 1982.
13 Ogrydziak, D., Bassel, J., Contopoulou, R., and Mortimer, R., Molec. gen. Genet. *163* (1978) 229.
14 Weisiger, R.A., Pinkus, L.M., and Jakoby, W.B., Biochem. Pharmac. *29* (1980) 2885.
15 Alexander, M., Science *211* (1981) 132.
16 Bergman, A., Brandt, I., Darnerud, P.O., and Wachmeister, C.A., Xenobiotica *12* (1982) 1.

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

A.P. Singh[1] and K. Singh

Laboratory of Bioenergetics, Department of Botany, Banaras Hindu University, Varanasi-221005 (India), 23 February 1984

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

also found to prevent growth, solute transport, proton translocation, biosynthesis of macromolecules and energy-dependent transhydrogenase[11-18].

In a previous study[19] we have shown that the inhibitory effect of tributyltin (TBT) on the growth of *E. coli* was modulated by various abiotic factors such as pH, chloride, sulfate, phosphate, and Tris ions; presence and absence of peptone, glucose, succinate and the reducing agents. In this report, the role of cytochromes on the susceptibility of *E. coli* SASX76 to TBT has been examined.

Materials and methods. All chemicals were of reagent grade purity. Tributyltin chloride was purchased from E. Merck (Federal Republic of Germany) and 5-aminolevulinic acid (ALA) was obtained from Sigma Chemical Co., St Louis, USA.

Escherichia coli SASX76 (formerly SHSP18)[20] (F^-, hem A^-, met^-, trp^-, lac^-, str^-) and *E. coli* W1485[21] were generous gifts of Drs A. Sasarman (University of Montreal, Canada) and J. R. Guest (University of Sheffield, England), respectively.

Bacteria were grown aerobically or anaerobically at 37 °C in 100-ml culture flasks containing 20 ml of a minimal-salts-glucose medium[22], supplemented with 0.5% (w/v) bacto-peptone and 0.1% (w/v) bacto-yeast extract (Difco), pH 6.8. Cytochrome synthesis was induced in *E. coli* SASX76 by growing the bacterium in the above medium in the presence of 25 µg/ml of 5-aminolevulinic acid (ALA) as described elsewhere[23-25]. Growth was recorded as absorbance (540 nm) against a medium blank with either a Photo-Chem. colorimeter (MK II, India) or a Bausch and Lomb Spectronic[20] spectrophotometer as described previously[18].

Results and discussion. As reported elsewhere[23-25], *E. coli* SASX76 does not form cytochromes unless the growth medium is supplemented with ALA. The role of cytochromes in the sensitivity of *E. coli* to TBT was examined by growing the cells of strain SASX76 in the presence and absence of ALA. The effects of different concentrations of TBT (added either at the time of inoculum addition or during the exponential phase of growth) on the growth of cytochrome-containing and cytochrome-deficient cells are shown in figures 1 and 2. In the absence of TBT, the latter cells exhibited a longer growth lag and a 5 to 6 times lower cell yield in comparison to the former cells (compare figs 1A and 1B or figs 2A and 2B). In the presence of increasing concentrations of TBT, the growth lag in both cytochrome-sufficient and cytochrome-deficient cells was progressively increased when TBT was added at the time of ionoculum addition (cf figs 1A and 1B). In contrast to the former cells, the growth of the latter cells was extremely sensitive to TBT, when this compound was added either at the time of inoculum addition or during the exponential phase of growth (figs 1B and 2B). For example, 10 µM TBT completely blocked the growth of cytochrome-deficient cells but had very little or no effect on cytochrome-sufficient cells. More than 200 µM TBT was required for full inhibition of the growth of the latter cells (figs 1A and 2A). On the basis of these results it was concluded that the cytochrome-deficient cells were at least 20 times more sensitive to TBT as compared to cytochrome-containing cells. The differential sensitivity of these two types of cells to TBT tends to suggest that cytochromes play a positive

Effect of tributyltin on various energy-linked reactions of *Escherichia coli*. The data presented here are computed mainly from our previous studies

Energy-linked reactions	Concentrations causing 50% inhibition of maximal activity (nmoles/mg protein)	References
1) Dissipation of ΔpH	0.15	12, 14
2) ATPase activities		
a) ATP hydrolase	2.5 (1.2)*	18 (26)
b) ATP synthase	> 2.5? (8.6)*	(26)
3) Oxidation of substrates (NADH, succinate, D-lactate)	> 50.0	12, 15
4) Glycolysis (intracellular ATP pools)	> 50.0	14
5) Solute transport (amino acids)		
a) At pH 6.6	3.3	14
b) At pH 7.5	> 50.0	14
6) Energy-linked transhydrogenase	8.0	15
7) Synthesis of macromolecules		
a) Proteins	35.3 µM**	17
b) DNA and RNA	70.6 µM**	17
8) Growth of		
a) cytochrome-sufficient cells	100.0 µM	This study
b) cytochrome-deficient cells	5.0 µM	This study

* Numbers in the parenthesis are values for mitochondrial ATPase complex. ** These values refer to tripropyltin chloride.

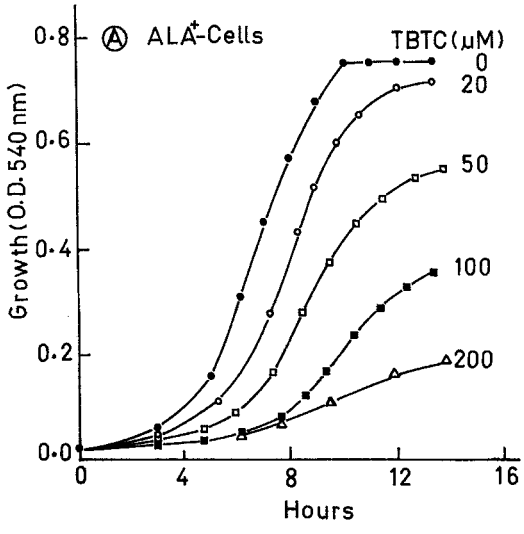

Figure 1A. Effect of different concentrations of tributyltin chloride (TBTC) on the growth of cytochrome-containing (ALA^+) cells of *Escherichia coli* SASX76. For induction of cytochrome synthesis, the culture medium was supplemented with 25 µg/ml of 5-aminolevulinic acid (ALA). TBTC was added at the time of inoculum addition.

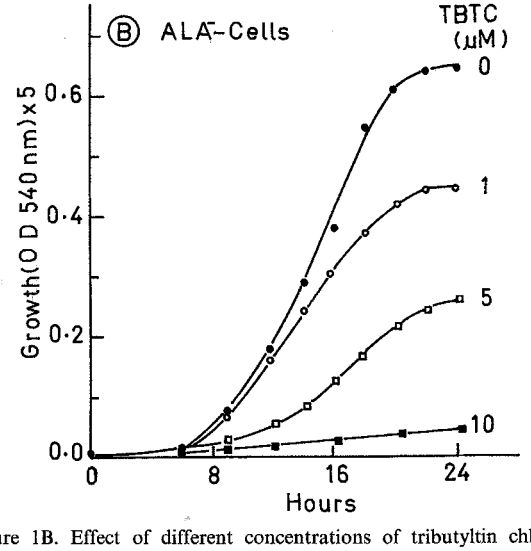

Figure 1B. Effect of different concentrations of tributyltin chloride (TBTC) (added at the time of inoculum addition) on the growth of cytochrome-deficient (ALA^-) cells of *E. coli* SASX76.

role in reducing the toxicity of TBT to *E. coli*. The exact mechanism(s) involved in the resistance of cytochrome-sufficient cells of *E. coli* to TBT, as opposed to cytochrome-deficient cells, is not clear; however, to explain the role of cytochromes in the above processes the following possibilities must be considered.

1) The observed effect of cytochromes in TBT resistance of *E. coli* may be related to a differential mode of energy generation in the presence and absence of cytochrome by cells of this bacterium. As shown previously[25], in the absence of cytochromes, *E. coli* SASX76 generates ATP via glycolysis and via phosphoroclastic cleavage (under anaerobic conditions) of pyruvate. Under the above conditions this bacterium produces energy for cellular functions in the form of a proton electrochemical gradient ($\Delta\bar{\mu}H^+$) which constitutes ΔpH and $\Delta\Psi$, from ATP hydrolysis catalized by ATPase. TBT is known to abolish only the ΔpH component of $\Delta\bar{\mu}H^+$ by exchange of OH^- for Cl^-, and does not affect $\Delta\Psi$; hence, only ΔpH driven processes are affected under these conditions. However, at higher concentrations of TBT (table) in addition to ΔpH, formation of $\Delta\Psi$ is also prevented by inhibition of ATPase enzyme.

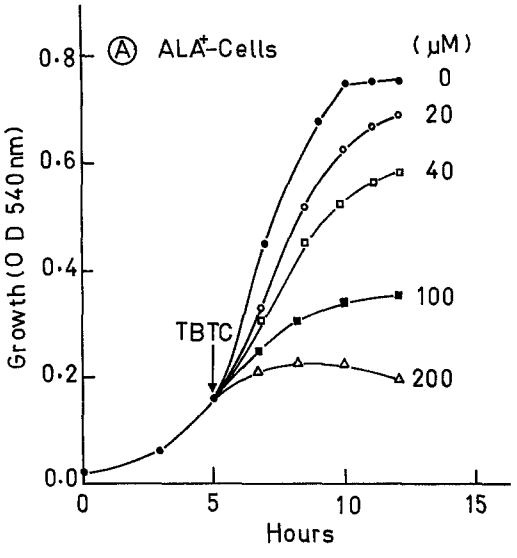

Figure 2A. Effect of different concentrations of TBTC (added during exponential phase, at the arrow) on the growth of cytochrome-containing (ALA^+) cells of *E. coli* SASX76. The synthesis of cytochromes was induced as described in the legend of figure 1A.

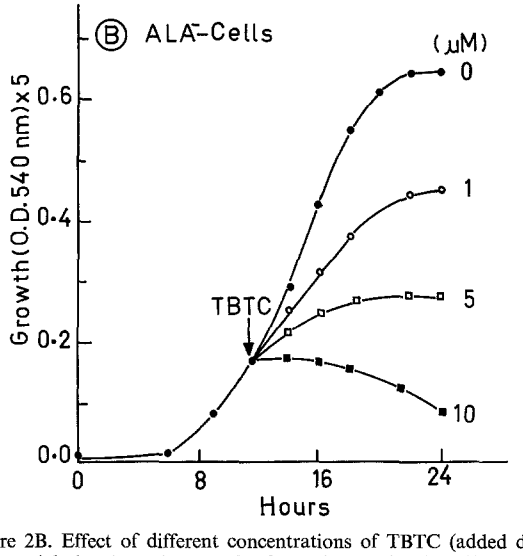

Figure 2B. Effect of different concentrations of TBTC (added during exponential phase) on the growth of cytochrome-deficient (ALA^-) cells of *E. coli* SASX76.

In contrast to cytochrome-deficient cells, the formation of $\Delta\Psi$ via substrate oxidation is insensitive to TBT in cytochrome-containing cells[12]. As in the former cells, in the latter cells the supply of ATP is maintained via glycolysis which is also resistant to TBT[13]. AT 5 μM of TBT, which completely inhibited $\Delta\bar{\mu}H^+$ formation and ATPase activity, the intracellular ATP levels were unaltered[13]. Even at 50 μM TBT, intracellular ATP levels (produced via glycolysis) dropped by 20–25% only[13]. If a situation like that in mitochondria[26] prevails in *E. coli* with respect to the differential sensitivity of ATP synthetic and ATP hydrolytic reactions of the ATPase complex to triorganotins, including TBT, ATP will also be made available from oxidative phosphorylation. However, this possibility requires a direct demonstration of a differential effect of TBT on the above reactions in *E. coli*.

On the basis of these considerations it is apparent that the energy levels in the form of ATP and $\Delta\bar{\mu}H^+$ will be higher in cytochrome-containing cells in comparison to cytochrome-deficient cells. Thus in the presence of TBT the latter cells will be subjected to greater stress. In the deficient cells, as compared to the sufficient the demand for energy utilizing or consuming reactions to support cellular function overrides the energy-producing reactions.

2) In addition to the above noted effect of cytochromes, it has been observed that the pH of the culture media is also influenced by the presence and the absence of cytochromes in an indirect way. In the absence of cytochromes the pH of the culture medium during cell growth decreased from pH 6.8 to pH 5.5 or less, whereas in the presence of cytochrome it increased from pH 6.8 to pH 7.8. As reported previously[11,19], the growth of *E. coli* is more sensitive to TBT at acid pH than at basic pH. This is related to the fact that at pH 5.5, the major component of $\Delta\bar{\mu}H^+$ in *E. coli* cells was found to be ΔpH, but at pH 7.5 it was $\Delta\Psi$[27,28]. As pointed out before, TBT affects only ΔpH but not $\Delta\Psi$; this may account in part for the hypersensitivity of cytochrome-deficient cells to TBT compared to their cytochrome-containing counterparts.

3) In addition to the above factors, it is quite possible that the differential sensitivity of cytochrome-sufficient and cytochrome-deficient cells to TBT could be due to the presence of ALA itself in the culture medium of the former cells. However, this view is contradicted by the fact that TBT produced the same effect on other cytochrome-containing cells of *E. coli* W1485 which do not require ALA for cytochrome synthesis.

In conclusion, on the basis of the above results it is proposed here that the hypersensitivity of cytochrome deficient cells of *E. coli* to TBT is useful in the isolation of a mutant ATPase which is resistant to this compound[18]. Based on these findings, we anticipate that TBT-resistant mutant ATPase could easily be isolated from cytochrome-deficient anaerobic (fermentative bacteria) as opposed to their aerobic cytochrome-containing counterparts.

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1 To whom all correspondence should be addressed (Until June 85: Department of Biological Sciences, Brock University, St Catharines, Ontario, L2S 3A1, Canada.)

2 Piver, W.T., Envir. Hlth Perspect. *4* (1973) 61.
3 Smith, P., and Smith, L., Chem. Brit. *11* (1975) 208.