Isolation and Characterization of Tributyltin Resistant Mutants of *Escherichia coli*

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Two classes of tributyltin (TBT) resistant, spontaneous mutants of *Escherichia coli* K-12 were isolated, using a cytochrome containing (W1485) and a cytochrome deficient (SASX76) strain. In contrast to the cytochrome sufficient strain, the cytochrome deficient strain was found to be fifty times more sensitive to TBT. The class I mutants, isolated from strain W1485, also showed cross-resistance to triphenyltin (TPT). As compared to its wild type parent, the TBT-resistant mutants exhibited mucoid colony type, aberrant cell morphology and reduced uptake of TPT. Based on these results, it was suggested that the resistance of class I mutants to TBT may be associated with above mentioned alterations.

The class II TBT-resistant mutants were isolated from the cytochrome deficient strain, SASX76. In comparison to class I mutants, these class II mutants were found to have TBT-resistant membrane bound adenosine triphosphatase (ATPase) which may account for their resistance to TBT.

Introduction

Among many other biocides of organometallic category, tributyltin (TBT), an organotin derivative, has been under extensive use in agriculture and industry, for the last three decades [1–4]. TBT is found to be very effective against both, prokaryotes and eukaryotes [2, 4]. Regarding its biochemical mode of action, it is known to catalyze halide/hydroxyl exchange across biomembranes [5–8]; inhibit oxidative and photophosphorylation by interacting with H^+-translocating ATPase [7, 9–12]; and mediates leakage of vital ions [13]. In addition to the processes mentioned above it is also known to inhibit amino acid transport, energy-dependent transhydrogenase and proton translocation in *E. coli* [8, 14–16].

Exclusion, degradation and target site modification are mainly the three different ways by which microorganisms are known to resist the action of antibacterial agents including heavy metals [17, 18].

It is therefore, theoretically possible to isolate mutants resistant to the action of antibacterial agents by virtue of one or more of the above mentioned alterations. In this paper, we report the isolation and properties of TBT-resistant mutants of *E. coli* K-12, showing either reduced uptake of TBT or having a TBT-resistant ATPase, thus suggested to belong to exclusion and target site altered classes of mutants, respectively.

Materials and Methods

Bacterial strains

E. coli K-12 strain W1485 [19] and SASX76 [20] were obtained from Dr. J. R. Guest, University of Sheffield, England, and Dr. A. Sasarman, University of Montreal, Canada, respectively.

Chemicals

Tributyl- and triphenyltin chlorides were purchased from E. Merck (Germany) and Fluka AG, Buche SG (Switzerland), respectively. Amino-levulinic acid (ALA) was obtained from Sigma Chemical Co., St. Louis, Mo. USA.

Media

The composition of minimal salts medium (MSM) was the same as described by Spencer and Guest [21]. Glucose 0.5% (w/v) was used as carbon source, and the pH was adjusted to 6.8. The complex medium (CM), contained in addition to the above, 0.5% (w/v) bacto-peptone and bacto-yeast extract (Difco.). The above media were solidified by the addition of 1.5% (w/v) bacto-agar.

Growth studies

Cells were grown in 30 ml of various media in 100 ml Erlenmeyer flasks fitted with sidearm tubes. As inoculum, 0.4% (v/v) of an overnight culture, grown in the same medium was used. In all cases, flasks were incubated at 37 °C in a thermostated waterbath shaker (Adco, India) under aerobic conditions (180 rev. min⁻¹). Growth was recorded as absorbance (540 nm) against a medium blank with either a Photo-Chem-colorimeter (MK II, India) or Bausch and Lomb Spectronic 20 Spectrophotometer. For transport studies, cells were grown in 200 ml of above media in one liter Erlenmeyer flask, under above conditions. Cytochrome synthesis was induced in E. coli SASX76 by growing the cells in the above medium in the presence of 5 µg/ml of ALA.

Isolation of class I permeability altered mutants resistant to TBT by the disc diffusion method

Nutrient agar petri-plates were flooded with 1.0 ml of an over night broth culture of E. coli W1485 (5×10^9 cells/ml), and the excess liquid was drained off. After 15–20 min, when plates were dried, the filter paper disc soaked in 10 mM ethanolic solution of TBT was placed in center and the plates incubated for 24 h at 37 °C. TBT-resistant colonies, which appeared in the inhibition zone upon further incubation of plates, were then picked and purified twice on the selective plates.

Isolation of class II TBT-resistant ATPase mutants

For the isolation of this kind of mutants, a cytochrome deficient E. coli K-12, strain SASX76 was used. In contrast to cytochrome containing strain W1485, the strain SASX76 was found to be fifty times more sensitive to TBT. The above mutants were isolated by direct selection for TBT resistance, from a non-mutagenized stock of bacterial culture. The first step in mutant isolation consists of treating the bacteria in liquid medium with TBT. For this purpose, 10 ml volume of growth medium lacking ALA, was inoculated with 0.2 ml of stationary phase cells (10^8 cells/ml). As soon as growth commenced, as indicated by an increase in turbidity, TBT (10 µM) was added. After a 60 h incubation period at 37 °C the culture returned to original cell density present at the time of TBT addition. Samples of these cultures were spread on to TBT containing plates which were then incubated at 37 °C. The colonies appearing earliest and largest were circled and tested for resistance to TBT.

Assay of triphenyltin uptake

Since TBT-resistant mutants were also found to be cross-resistant to triphenyltin (TPT), a convenient spectrophotometric method was developed for the transport of TPT. For such studies, cells were harvested and washed twice with 0.05 M Tris-HCl buffer (pH 7.5) at the end of log phase by centrifugation at 10,000 rpm for 10 min. (IEC-25 refrigerated centrifuge, India, 4 °C). Washed cells were resuspended in the same buffer but also containing 10 mM $MgCl_2$ at the desired cell density. Ethanolic solution of TPT was added to cell suspensions to obtain a final concentration of 0.1 mM. At an interval of 0, 10, 20, and 40 min. 5 ml aliquots were removed and the cells were pelleted by centrifugation. The clear supernatant was removed carefully and the concentration of TPT was estimated by measuring its absorbance at 221 nm in a Beckman Double Beam Spectrophotometer (model 35) and was inferred from a standard curve.

Preparation of membrane vesicles

Membrane vesicles were prepared from cells harvested at the end of the exponential phase by the method of Konings and Kaback [22] as modified in [23]. The vesicles were resuspended in 30 mM Tris-HCl buffer (pH 8.0), containing 1 mM $MgCl_2$ and used subsequently for ATPase assay.

Enzyme assay

The ATPase was measured as in [24] and protein was determined by the method of Lowry et al., [25] using crystalline lysozyme as the standard.

One unit of enzyme activity is defined as that amount which catalyses the hydrolysis of 1 nmole of substrate/min. The specific activity is defined as units/mg protein.

Results and Discussion

I. Isolation and properties of altered permeability mutants (class I), resistant to TBT

When filter paper disc, soaked in 10 mM ethanolic solution of TBT was placed in center of plate,

previously seeded with test organism, as a consequence of slow diffusion of TBT in agar, a sharp, circular, inhibition zone appeared around the periphery of the disc, within 24 h of incubation, at 37 °C. Upon further incubation, TBT-resistant colonies appeared in the inhibition zone. These colonies were then picked and purified twice on the selective plates. In this way, ten spontaneous TBT-resistant mutants were isolated and, one of them was examined in some detail with respect to following properties:

1. Growth response of wild type and mutant strains

The mutants isolated by above technique, were screened further for their degree of resistance to TBT, in liquid as well as on solid media. The growth of wild type strain was completely inhibited by 1 mM TBT compared to that of the mutant, which grew normally (Fig. 1). Survival of wild type and mutant strains in the presence of various concentrations of TBT is shown in Fig. 2. At 0.1 mM TBT, the colony forming ability of wild type strain was decreased by 50% but the same concentration had no effect on the mutant strain. At 0.5 mM level, the viability of wild type was lowered by 90% compared to that of the mutant, which was slightly affected. In this way, the strains which grew on
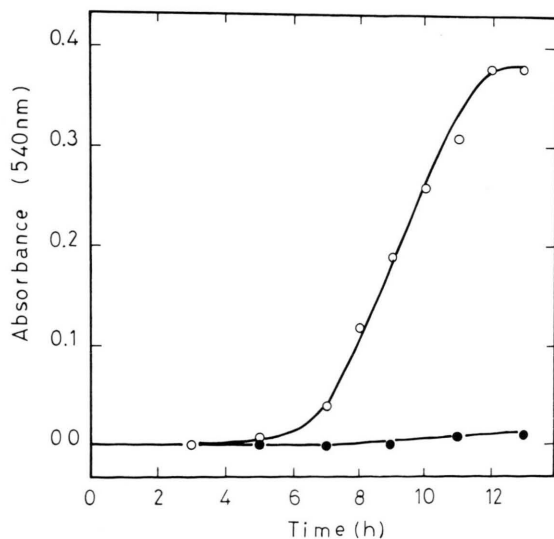

Fig. 1. Effect of 1 mM TBT on the growth of *E. coli* W1485 (●) and its TBT-resistant mutant strain (○) in minimal medium containing glucose (0.5%, w/v) as carbon source.

above media in the presence of 1 mM TBT were considered as TBT-resistant mutants.

While this work was in progress, TBT-resistant mutants of *E. coli* [26] and *Pseudomonas* 244 [27] were isolated from non-polluted and organotin-polluted environments, respectively. These mutants were found to be resistant to 0.147 mM to 0.25 mM TBT.

In contrast to TBT-resistant mutants of *E. coli*, isolated by Ito and Ohnishi [26], our isolate grew normally on agar plates in the presence of glucose, succinate, acetate or malate as carbon sources, which were used to differentiate between the energy coupled versus energy uncoupled mutants in the above study [26]. On the basis of this result the mutants described here belong to the energy uncoupled class. In case of TBT-resistant mutants of *Pseudomonas* [27], no distinctions were made between the above mentioned classes.

2. Colony and cell morphology of mutant strains

As compared to non-mucoid colonies of wild type strain, all TBT-resistant mutants exhibited mucoid colony morphology (results not shown), which may account in part, for their resistance to TBT. It has been reported [28] that the capsulated strain of *Klebsiella aerogenes* was more tolerant to other heavy metals, like Cu^{2+} and Cd^{2+}, compared to its non-capsulated strain. Thus, the resistance or tolerance of mucoid and/or capsulated strains to heavy metals may be due to complexation of such metals by mucoid capsular materials [29].

In addition to colony morphology, the cell morphology was also different in the wild type and mutant strains. For example, the light and electron microscopic studies have revealed that the cells of mutant strains were 5−6 times bigger than the wild type (results not presented). In previous studies [26, 27, 30] no mention was made with respect to colony or cell morphologies of inorganic and organic tin resistant estuarine and non-estuarine bacteria. Aberrant cell morphologies are often observed when bacteria are subjected to heavy metal stresses and has been reported for Ag^{2+} [31], Hg^{2+} [32], and Cd^{2+} [33]. It is quite possible that abnormal cell morphology may be related in some way to the alteration of cell permeability involved partly in bacterial resistance to heavy metals, including TBT.
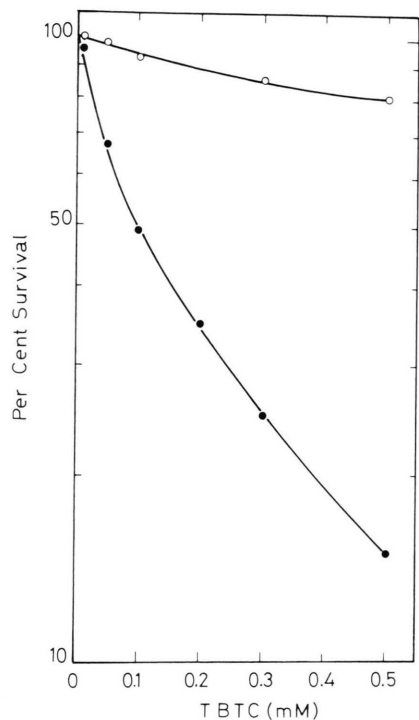
Fig. 2. Effect of various concentrations of TBT on the viability of *E. coli* W1485 (●) and its TBT-resistant mutant (○).
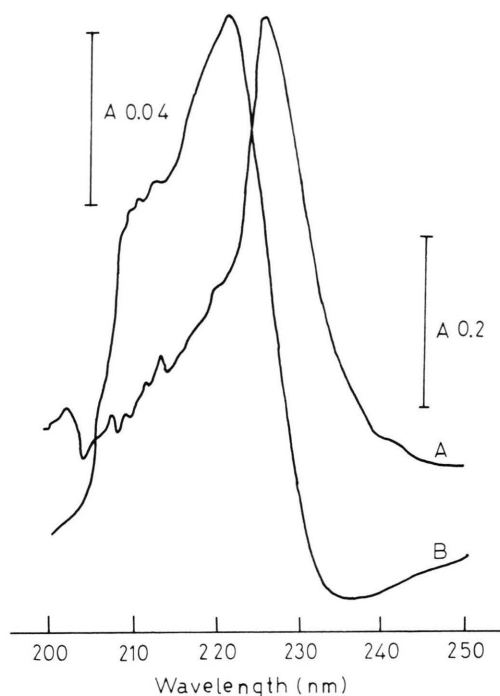

Fig. 3. Absorbance spectra of triphenyltin (TPT) in absolute ethanol (A) and in 0.05 M, Tris-HCl buffer, pH 7.5 (B). The scale at right and left hand sides are for spectrum A and B, respectively.

3. Uptake of triphenyltin in wild type and mutant strains

Microorganisms possess mechanisms by which heavy metal cations can be taken up and accumulated from the environment. There appears to be two main types of metal uptake by organisms. The first involves non-specific binding of the metal to the cell surface, slime layers, extracellular matrices etc., whereas the second mechanism involves metabolism-dependent intracellular accumulation [29]. In order to differentiate between the two classes of permeability mutants, we investigated the transport and/or binding of triphenyltin (TPT) in our TBT-resistant mutant, which is cross-resistant to former compound.

A convenient spectrophotometric method was developed by us to measure the uptake of TPT in whole cells of the test organism. The assay is based on the fact that TPT shows very strong absorption maxima in the UV region. The spectral characteristics of this compound in ethanol and Tris-buffer

(50 mM Tris-HCl, containing 10 mM MgCl₂, pH 7.5) are shown in Fig. 3. There is a strong blue shift (5 nm) in the absorption spectra of TPT in Tris compared to that of ethanol.

The time course of TPT uptake and/or binding by whole cells of W1485 and its mutant strain is shown in Fig. 4. Both, the steady state as well as the initial rate of TPT accumulation was lowered by 40% and 80%, respectively, in mutant strain as compared to W1485. This finding is also consistent with the already reported reduced transport of heavy metals by Co^{2+}-resistant mutants of *Proteus vulgaris* [34] and *A. aerogenes* [35], Cd^{2+} and Cu^{2+}-resistant mutants of *Staphylococcus aureus* [36, 37], and *E. coli*, respectively. Our results could not be directly compared with TBT-resistant mutant of *Pseudomonas* 244 due to lack of comparable metal uptake data for the wild type strain [27]. Consistent with the findings of Yamada *et al.* [39] on tripropyltin chloride accumulation by *E. coli*, the transport of TPT in the present case seems to be mediated by a non-metabolic process, since starved
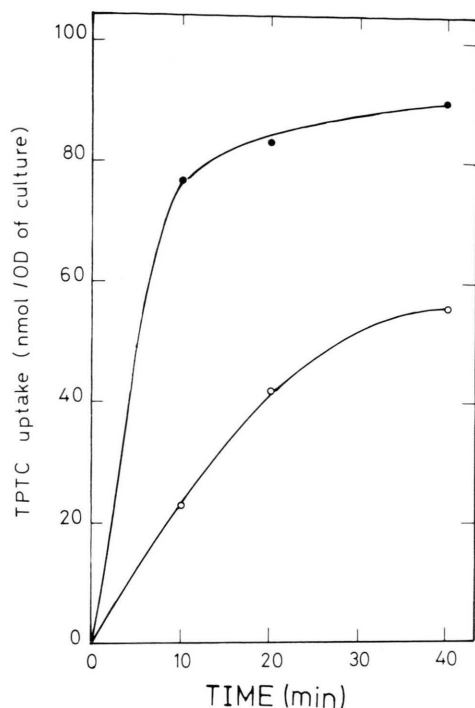
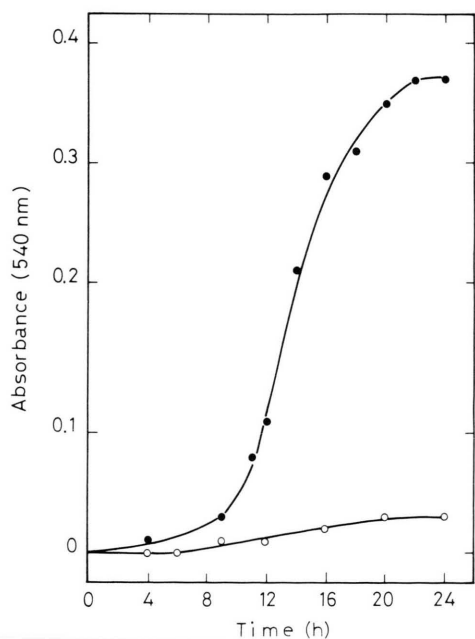Fig. 4. Time course of TPT uptake by whole cells of *E. coli* W1485 (●) and its TBT-resistant mutant (○).

Fig. 5. Effect of 10 μM TBT on the growth of *E. coli* SASX76 (○) and its *tbt-1* mutant (●) in the absence of 5-aminolevulinic acid (ALA).

cells alone, with and without glucose or in the presence of glucose and NaN_3 (a metabolic inhibitor) accumulated same amounts of TPT. Similar results were also obtained in the case of TBT-resistant mutant of *Pseudomonas* [27]. In contrast to *Pseudomonas* [27], the heat killed or boiled cells of wild type *E. coli* did not bind more TPT, and in fact, the uptake was reduced by 80% in such cells compared to unheated control.

Compared to actively growing cells, where the effect of TBT was potentiated by EDTA (Singh and Singh unpublished results), the bound form of TPT by non-growing cells were released when washed with 1 mM EDTA. It seems that TBT and TPT may be sequestered in some manner in the cell envelope, as suggested by Yamada *et al.*, [39, 40] for *E. coli* and tripropyltin. This view is also consistent with inhibitory effect of TBT on amino acid transport in whole cells and on the transhydrogenase activity in everted membrane vesicles of test organism [8, 14–16].

II. Properties of TBT-resistant (class II) ATPase mutants

As shown in Fig. 5, the growth of strain SASX76 (in the absence of ALA) was completely inhibited by 10 μM TBT as compared to *tbt-1* mutant strain, which grew normally under above conditions. Using this kind of selection technique, ten TBT-resistant mutants (*tbt-1* to *tbt-10*) were isolated, and out of these only *tbt-1* was further characterized in this study.

TBT and its derivatives are very potent inhibitors of membane bound ATPase of mitochondria [9] and

Table I. Effect of tributyltin (TBT) on membrane bound ATPase of *E. coli* SASX76 and its TBT-resistant mutant strain, *tbt-1*.

Concentration of TBT	ATPase activity (% control)	
[μM]	SASX76	*tbt-1* mutant
0.0	100	100 [a]
1.0	55	77
2.5	48	74
5.0	36	69
7.5	11	67
10.0	0	64

[a] The 100% control values for SASX76 and *tbt-1* mutant, were 906 and 861 nmol/min/mg protein, respectively.

E. coli [41]. We compared the effect of this agent on the membrane bound ATPase of wild type (SASX76) and its TBT-resistant mutant. Compared to wild type, the membrane bound ATPase in *tbt-1* mutant was resistant to TBT. For example, TBT at a concentration of $10 \, \mu M$ completely inhibited the wild type ATPase, while mutant ATPase was affected only to the extent of 36% (Table I).

Recently, TBT-resistant mutants of a cytochrome containing wild type *E. coli* K-12, isolated by Ito and Ohnishi [26], were found to be cross-resistant to uncouplers and ATPase inhibitors like azide and dicyclohexylcarbodiimide (DCCD). It is interesting to note that our *tbt-1* mutant, isolated from a cytochrome deficient strain SASX76 of *E. coli* K-12, does not show cross-resistance to above compounds. Furthermore, the azide resistant mutants of strain SASX76 isolated by Singh and Bragg (unpublished results) have azide sensitive ATPase. Contrary to the view of Ito and Ohnishi [26], it is evident, from these results that resistance to azide in *E. coli* and other bacteria is not always associated with azide resistant ATPase [42].

It has been reported that TBT and other organotin derivatives affect only the membrane bound ATPase ($F_1 \cdot F_0$, complex) of chloroplast [11], mitochondria [10], and *E. coli* [41, 43] and is without effect on soluble ATPase (F_1). A detailed study on the isolation and characterization of *tbt-1* mutant $F_1 \cdot F_0$ complex will help to resolve the specific site of action of TBT on *E. coli* ATPase; this work is in progress in our laboratory.

In conclusion, the resistance of class I mutants of *E. coli* in this study may be related to production of mucoid colony type, aberrant cell morphology and altered passive permeability. On the other hand, the resistance of class II mutants may be due to presence of a TBT-resistant membrane bound ATPase. However, there may be other mechanisms not mentioned here which may be involved in the resistance of both the classes of mutants to TBT and this possibility requires further investigation.

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