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Occurrence of Tributyltin-Tolerant Bacteria in Tributyltin- or Cadmium-Containing Seawater

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Tributyltin chloride (TBTCl)-tolerant bacteria accounted for 90% of the flora in natural seawater to which TBTCl was added. These tolerant bacteria were insensitive to 250 nmol of TBTCl per disc, and all were *Vibrio* species. Total counts of viable bacteria did not decrease upon storage of the TBTCl-treated seawater, indicating that enrichment of tolerant strains took place. Addition of $CdSO_4$ to seawater resulted in the occurrence of TBTCl-tolerant bacteria as well as Cd-tolerant bacteria, suggesting some correlation of Cd tolerance and TBTCl tolerance.

Triorganotin compounds, such as tributyltin oxide, tributyltin chloride (TBTCl), and triphenyltin chloride, can be used as antifouling agents in marine paints (8) because they are toxic for plankton or benthic organisms. These compounds are toxic not only for eukaryotes, but also for bacteria (12). It has been reported that gram-negative bacteria are inhibited by tributyltin compounds (TBTs) (9).

A few researchers have, however, reported evidence for the presence of organotin-resistant microorganisms (9). Hallas and Cooney (5) reported that organotin-resistant organisms form colonies on agar containing 15 ppm of Sn as dimethyltin chloride, however, no significant correlation was found between Sn concentrations and the numbers of Sn-resistant microorganisms in sediments. Barug (1) reported that *Pseudomonas aeruginosa* degraded tributyltin oxide when the compound was present at concentration of 2.5 ppm. However, there is no evidence as yet for enrichment of triorganotin-tolerant bacteria. We have isolated many highly tolerant bacteria after addition of TBTCl to natural seawater. Moreover, as is reported here, most Cd-tolerant bacteria are also tolerant to TBTCl.

Samples of natural seawater were collected from the sea surface in Hakodate Bay, south Hokkaido, Japan, in July 1990 in sterile glass bottles (1-liter volume). Each sample of seawater was divided into three aliquots as follows: a control sample, to which nothing was added; a TBTCl sample, to which 40 ppm of TBTCl (as TBTCl) was added; and a Cd sample, to which 40 ppm of $CdSO_4$ (as $CdSO_4$) was added. All water samples were stored aerobically at 20°C in the dark. Viable cells were counted at 0, 1, 2, and 4 weeks by plating on ZoBell 2216E medium (13) unless otherwise noted. Plates were incubated at 25°C, and 30 colonies were randomly isolated from each plate and tested for TBTCl and $CdSO_4$ tolerance by using a disc method as follows. The isolated strains were propagated in ZoBell 2216E broth. The resulting cell suspensions harvested at late log phase were spread onto a plate, after which a disc pad, 8 mm in diameter, containing an appropriate amount of TBTCl or $CdSO_4$ was placed on the cells. A sensitive strain showed a circular zone of inhibition around the disc, while a tolerant strain did not. Any strain having an inhibition circle less than 0.5 mm from the edge of the pad was identified as tolerant. As a standard tolerant strain, *Alteromonas* sp. strain M-1 (formerly reported as *Vibrio* sp. strain M-1) (4) was used, and *Alteromonas haloplanktis* S5B was used as the negative control.

In the water sample in the control group, viable cell numbers decreased gradually from 10^4 to 10^3 cells per ml by week 4 (Fig. 1). No bacteria tolerant to either TBTCl or $CdSO_4$ were detected throughout the storage period.

However, in the water sample in the TBTCl-treated group, over 90% of the viable cells were tolerant to TBTCl (25 nmol per disc) within 1 week (data not shown). Even when 250 nmol per disc was used, 93% of the viable cells were tolerant as of 2 weeks (Fig. 2). In addition, 26% of the cells were tolerant to Cd at 2 weeks. The total viable cell numbers did not decrease throughout the storage period of 4 weeks, unlike the decrease in total cell numbers that occurred upon storage of untreated seawater, which suggests that TBTCl-tolerant bacteria had dramatically increased.

Many reports of metal-resistant bacteria have been made (10, 11). Organomercury-resistant organisms (2, 7) are espe-

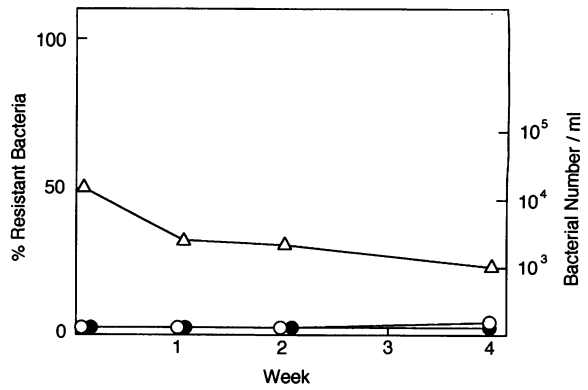

FIG. 1. Time-dependent changes of viable count (△) and occurrence (%) of TBTCl-tolerant (○) and Cd-tolerant (●) bacteria in seawater samples in the control group, to which neither TBTCl nor $CdSO_4$ was added. The assays for tolerance were performed with discs containing 25 nmol of each compound per disc.

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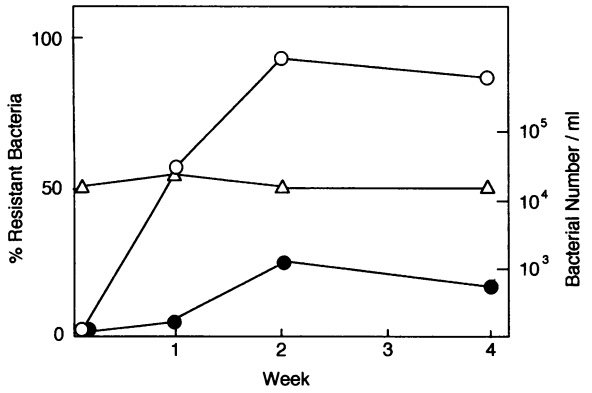

FIG. 2. Time-dependent changes of viable count (△) and occurrence (%) of TBTCl-tolerant (○) and Cd-tolerant (●) bacteria in TBTCl (40 ppm)-treated seawater. The assays for tolerance were performed with discs containing 250 nmol of TBTCl per disc or 25 nmoles of $CdSO_4$ per disc.

cially well known. An organomercury resistance gene is known to be conveyed in a plasmid (7). We used two methods (3, 6) to check for the presence of an extrachromosomal plasmid in our TBTCl-tolerant bacteria. None of the tolerant bacteria had a plasmid, which suggests that the gene involved in the tolerance is coded in chromosomal DNA.

In the Cd-treated group of water sample (Fig. 3), 63% of bacteria were Cd tolerant after 1 week, a proportion which was maintained until almost 4 weeks. In addition, TBTCl-tolerant bacteria were observed at 1 week and the proportion increased to 50% by 2 weeks.

Cross-tolerance in the strains isolated is summarized in Tables 1 and 2. As indicated in Table 1, most of the TBTCl-tolerant strains were not tolerant to Cd, although several exceptions were observed. On the other hand, most Cd-tolerant strains isolated from the Cd-treated group after 2 weeks were cross-tolerant to TBTCl (Table 2). These results suggest that TBT tolerance might be independent from Cd tolerance. The addition of Cd to seawater did not prevent a decrease in total cell numbers, however, since the viable count decreased to 10^3 cells per ml after 4 weeks in the Cd group, a phenomenon which was similar to that

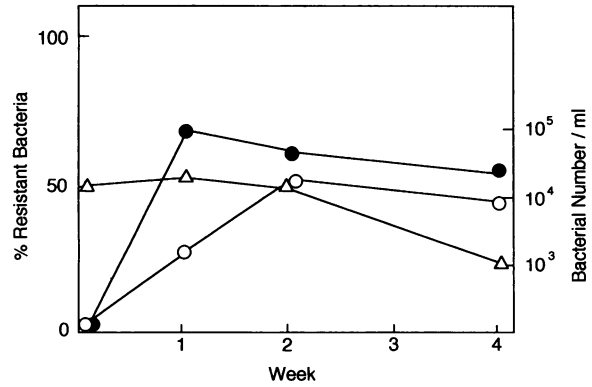

FIG. 3. Time-dependent changes of viable count (△) and occurrence (%) of TBTCl-tolerant (○) and Cd-tolerant (●) bacteria in $CdSO_4$ (40 ppm)-treated seawater. The assays were performed with discs containing 25 nmol of TBTCl or $CdSO_4$ per disc.

TABLE 1. Cross-tolerance for TBTCl and Cd of the strains isolated from TBTCl-treated group

Time tested and strain	Tolerance[a] for: TBT	Cd	Time tested and strain	Tolerance[a] for: TBT	Cd
7 days			T-16″	++	−
T-1′	++	−	T-17″	++	−
T-2′	++	−	T-18″	++	−
T-3′	++	−	T-19″	++	−
T-4′	++	−	T-20″	++	−
T-5′	+	−	T-21″	++	−
T-6′	++	−	T-22″	++	−
T-7′	++	−	T-23″	++	−
T-8′	++	−	T-24″	++	−
T-9′	+	+	T-25″	++	−
T-10′	+	−	T-26″	++	−
T-11′	+−	−	T-27″	++	−
T-12′	−	−	T-28″	++	+
T-13′	+	−	T-29″	++	−
T-14′	−	−	T-30″	++	+
T-15′	++	−	28 days		
T-16′	+	−	T-1‴	++	−
T-17′	+	−	T-2‴	++	−
T-18′	+	−	T-3‴	++	−
T-19′	++	−	T-4‴	++	−
T-20′	++	−	T-5‴	++	−
T-21′	+	−	T-6‴	++	−
T-22′	++	−	T-7‴	++	−
T-23′	++	−	T-8‴	++	+
T-24′	++	−	T-9‴	++	−
T-25′	++	−	T-10‴	++	+
T-26′	+	−	T-11‴	++	+
T-27′	++	−	T-12‴	++	−
T-28′	+	−	T-13‴	++	−
T-29′	+	−	T-14‴	NG[b]	
T-30′	−	+	T-15‴	++	−
14 days			T-16‴	++	−
T-1″	++	−	T-17‴	++	−
T-2″	++	−	T-18‴	++	−
T-3″	++	−	T-19‴	++	−
T-4″	+	−	T-20‴	++	−
T-5″	++	−	T-21‴	++	−
T-6″	++	−	T-22‴	++	−
T-7″	++	+	T-23‴	++	−
T-8″	++	+	T-24‴	++	+
T-9″	++	−	T-25‴	++	−
T-10″	+	−	T-26‴	−	−
T-11″	++	+	T-27‴	−	−
T-12″	++	+	T-28‴	−	−
T-13″	++	+	T-29‴	NG	
T-14″	++	−	T-30‴	−	−
T-15″	++	+			

[a] Criteria of tolerance for TBTCl: ++, tolerant of 250 nmol of TBTCl per disc; +, tolerant of 25 nmol of TBTCl per disc. Criteria of tolerance for Cd: +, tolerant of 10 nmol of $CdSO_4$ per disc.

[b] NG, no growth in tolerance test.

which was observed in the water sample in the control group. The mechanism of Cd tolerance in the strains described here is not known at present, although the mechanism for Cd resistance are known in other bacteria (11). The relationship between Cd tolerance and TBTCl tolerance will be further examined.

A total of 68 highly tolerant strains for TBTCl were isolated from the water treated with TBTCl. These were preliminarily characterized and all of them were identified as *Vibrio* species on the basis of the following characteristics: they were gram-negative rods and were motility positive,

TABLE 2. Cross-tolerance for TBTCl and Cd of the strains isolated from Cd-treated group

Time tested and strain	Tolerance[a] for:		Time tested and strain	Tolerance[a] for:	
	TBT	Cd		TBT	Cd
7 days			C-16″	−	−
C-1′	+	+	C-17″	−	−
C-2′	+	+	C-18″	−	−
C-3′	+	+	C-19″	−	−
C-4′	+	+	C-20″	−	−
C-5′	−	+	C-21″	−	−
C-6′	+	+	C-22″	+	+
C-7′	−	−	C-23″	+	+
C-8′	−	+	C-24″	−	−
C-9′	+	+	C-25″	+	+
C-10′	+	+	C-26″	−	−
C-11′	−	+	C-27″	−	−
C-12′	−	−	C-28″	+	−
C-13′	−	+	C-29″	+	−
C-14′	−	+	C-30″	++	+
C-15′	−	+	28 days		
C-16′	−	+	C-1‴	+	+
C-17′	+	+	C-2‴	−	−
C-18′	−	+	C-3‴	++	+
C-19′	−	+	C-4‴	++	+
C-20′	−	+	C-5‴	−	+
C-21′	−	−	C-6‴	++	+
C-22′	−	−	C-7‴	−	−
C-23′	−	−	C-8‴	++	+
C-24′	−	−	C-9‴	−	+
C-25′	−	−	C-10‴	−	−
C-26′	−	−	C-11‴	+	+
C-27′	−	−	C-12‴	−	−
C-28′	−	+	C-13‴	−	−
C-29′	−	−	C-14‴	−	−
C-30′	−	−	C-15‴	−	+
14 days			C-16‴	−	+
C-1″	−	+	C-17‴	++	−
C-2″	+	+	C-18‴	+	+
C-3″	+	+	C-19‴	++	+
C-4″	+	+	C-20‴	−	−
C-5″	−	−	C-21‴	+	+
C-6″	+	+	C-22‴	+	+
C-7″	+	+	C-23‴	−	−
C-8″	+	+	C-24‴	−	−
C-9″	−	+	C-25‴	−	−
C-10″	+	+	C-26‴	−	−
C-11″	−	−	C-27‴	−	−
C-12″	+	+	C-28‴	+	+
C-13″	+	+	C-29‴	+	−
C-14″	−	+	C-30‴	+	+
C-15″	−	+			

[a] Criteria of tolerance for TBTCl: ++, tolerant of 250 nmol of TBTCl per disc; +, tolerant of 25 nmol of TBTCl per disc. Criteria of tolerance for Cd: +, tolerant of 10 nmol of $CdSO_4$ per disc.

oxidation-fermentation test fermentative, oxidase positive, and gas production negative. We have already isolated another TBTCl-tolerant bacterium, *Alteromonas* sp. strain M-1 (formerly reported as *Vibrio* sp. strain M-1) (4). Studies of the mechanism by which tolerance occurs and the tolerance itself are now being undertaken.

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