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Environmental Impact of Tributyltin-Resistant Marine Bacteria in the Indigenous Microbial Population of Tributyltin-Polluted Surface Sediments

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We compared the TBT-resistant ability of resting cells prepared from isolates that formed colonies on nutrient agar plates containing 100 μM tributyltin (TBT) chloride, such as *Photobacterium* sp. TKY1, *Halomonas* sp. TKY2, and *Photobacterium* sp. NGY1, with those from taxonomically similar type strains. *Photobacterium* sp. TKY1 showed the highest ability among those three isolates. The number of surviving *Photobacterium* sp. TKY1 cells was hardly decreased after 1 h of exposure to 100 μM TBTCI, regardless of the number of resting cells in the range from $10^{9.4}$ to $10^{4.2}$ CFU mL⁻¹. In such an experimental condition, the maximum number of TBT molecules available to associate with a single cell was estimated to be approximately $6.0 \times 10^{11.8}$. Resting cells prepared from type strains *Photobacterium ganghwense* JCM 12487^T and *P. halotolerans* LMG 22194^T, which have 16S rDNA sequences highly homologous with those of *Photobacterium* sp. TKY1, showed sensitivity to TBT, indicating that TBT-resistant marine bacterial species are not closely related in spite of their taxonomic similarity. We also estimated the impact of TBT-resistant bacterial species to indigenous microbial populations of TBT-polluted surface sediments. The number of surviving TBT-sensitive *Vibrio natriegens* ATCC 14048^T cells, $10^{6.2 \pm 0.3}$ CFU mL⁻¹, was reduced to $10^{4.4 \pm 0.4}$ CFU mL⁻¹ when TBT-resistant *Photobacterium* sp. TKY1 cells, $10^{9.1 \pm 0.2}$ CFU mL⁻¹, coexisted with $10^{9.4 \pm 0.2}$ CFU mL⁻¹ of *V. natriegens* ATCC 14048^T cells in the presence of 100 μM TBTCI. These results indicate that the toxicity of TBT to TBT-sensitive marine bacterial populations might be enhanced when a TBT-resistant marine bacterial species inhabits TBT-polluted surface sediments.

Key words : TBT-resistant marine bacteria / Busy ports / TBT-polluted surface sediments / Survivability.

INTRODUCTION

The usage of organotin compounds had expanded worldwide because of their broad applications in industry and agriculture as catalysts, polyvinyl chloride stabilizers, biocides for fungi, bacteria, and insects, and wood preservatives (Hoch, 2001). In particular, tributyltin (TBT) oxide, TBT chloride (TBTCI), and triphenyltin (TPhT) chloride were used to prevent biofouling on ship hulls since the 1970s, resulting in extensive pollution by these organotin compounds in the marine envi-

ronment (Sudaryanto et al., 2002; Murai et al., 2005; Furdek et al., 2012). Pollution by organotin compounds has already spread to the Antarctic Ocean. In sediments close to the channels of Antarctica, high concentrations of organotin compounds, up to $2,290 \mu\text{g Sn kg}^{-1}$ sediment, were detected (Negri et al., 2004). Therefore, marine pollution by TBT originating from antifouling paints is still a great concern (Antizar-Ladislao, 2008).

Bioaccumulation of organotin compounds causes chronic toxicity in living things (Ebdon et al., 1989; Ueno et al., 2004; Harino et al., 2007). The compounds also act as endocrine disrupters (Oberdörster et al., 1998; Horiguchi et al., 2002). In addition to their toxicity, mutagenic effects on the *Bacillus subtilis* Rec⁺ or Rec⁻

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strain (Hamasaki et al., 1992), *Salmonella* Typhimurium (Hamasaki et al., 1993) and the marine worm (Hagger et al., 2002) have been confirmed. The International Maritime Organization (IMO) has since prohibited the use of such organotin compounds as antifouling biocides through the International Convention on the Control of Harmful Anti-fouling Systems on Ships (IMO, 2008). While the use of TBT must be avoided, Lewis et al. (2004) emphasized the importance of preventing biofouling on ship hulls to prevent the invasion of non-indigenous marine organisms.

Tributyltin-resistant, or TBT degrading, bacteria have been isolated from marine environments. A TBT-resistant marine *Alteromonas* sp., growing in the presence of 125 μ M TBTCI, was isolated from coastal seawater (Fukagawa et al., 1992). A protein from the cloned genes deduced to be responsible for TBT resistance was found to be related to Na^+/H^+ antiporters and various Ca^{2+} transporters (Fukagawa and Suzuki, 1993). A multidrug efflux pump was shown to be essential to TBT resistance in *Pseudomonas stutzeri* (Jude et al., 2004). Adsorption of TBT molecules is an important function of cell walls for *Pseudoalteromonas* sp. to prevent the inflow of molecules into the cytoplasm (Kubota et al., 2004; Mimura et al., 2008a; Mimura et al., 2008b). Recently, a TBT-degrading *Aeromonas veronii*, which uses TBT as a carbon source, was isolated from an estuarine environment (Cruz et al., 2007). It seems that the strain could be applied to repair to TBT-polluted sediments.

Organotin compounds are stable and have existed mainly in sediments, rather than in seawater, for prolonged periods (Dowson et al., 1993; Hashimoto, 1998; Hoch, 2001; Saeki et al., 2007; Antizar-Ladislao, 2008), indicating that the frequency of mutations related to TBT resistance might occur in indigenous marine bacteria in sediments (Wuertz et al., 1991; Suzuki et al., 1992; Chen and White, 2004) and that sublethal concentrations of TBT in such sediments give considerable damage to the bacterial ecosystems. Therefore, in this study, we compared survivability of an isolate, from sediments of busy ports such as Tokyo Bay and Ise Bay, with taxonomically similar type strains in the presence of TBT to know whether or not the tolerance is in the genus level. In addition, the impact of an indigenous TBT-resistant bacterium was estimated for microbial populations in TBT-polluted surface sediments.

MATERIALS AND METHODS

Isolation of TBT-resistant marine bacteria

Sampling of surface sediments was carried out in 2008 using a Van Dorn water sampler (5026, Rigosha

Co., Ltd., Tokyo, Japan) on board a training ship when anchored at the quarantine anchorages in Tokyo Bay (35.58 N, 139.85 E) and Ise Bay (34.97 N, 136.78 E), Japan. The depth of the water at the sampling sites was 14 to 15 m, and the seawater temperature was 20 to 24°C. Each surface sediment sample was poured into an autoclaved bottle with a screw cap. The samples thus obtained were stored on board up to 7 days at 4°C. When the ship called at a port, samples kept in a cool box were sent to the laboratory.

One hundred μ L of the sample was taken after vigorous shaking and spread onto a nutrient agar plate containing 5.0 g Bacto Peptone (Difco, Sparks, MD, USA), 1.0 g yeast extract (Difco, Sparks, MD, USA), 15 g agar in seawater (liter^{-1}), and 100 μ M TBTCI as a final concentration. TBTCI was added to the medium after it was autoclaved. After the sample was spread onto an agar plate, it was incubated for 5 days at 25°C, and a single colony formed on the plate was picked up with an autoclaved toothpick and streaked onto a new agar plate. Three TBT-resistant species thus obtained were used for identification and experiments.

Identification of isolates

Identification of the isolates was carried out commercially on the basis of partial 16S rDNA sequences. Briefly, a partial 16S rDNA fragment from the V1 to V3 region (about 500 bp) was amplified by PCR and sequenced with a MicroSeq[®] 500 16S rDNA kit (Applied Biosystems, Foster City, CA, USA). The sequencing data obtained were used for homology analysis using the database, Apron DB-BA Version 4.0 (TechnoSuruga Laboratory, Shizuoka, Japan). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Partial 16S rDNA sequences of *Photobacterium* sp. TKY1, *Halomonas* sp. TKY2, and *Photobacterium* sp. NGY1 were deposited in the DDBJ/GenBank/EMBL under the accession numbers of AB501122, AB501213, and AB511030, respectively.

Enumeration of colony-forming cells after exposure to TBT

Resting cells prepared from the isolate and two type strains most like it based on partial rDNA sequences were examined. Cells grown until the early stationary phase of growth were harvested by centrifugation (10,000 $\times g$, 5 min), washed twice with 50 mM potassium-phosphate (K-Pi) buffer, pH 7.8, containing 0.5 M NaCl, and resuspended in the same volume with the buffer (1 mL). We varied the initial number of resting cells (mL^{-1}) by serial dilution prior to the addition of TBTCI at a final concentration of 100 μ M. After exposure to TBT for 1 h, the cell suspension was diluted

serially and spread onto a nutrient agar plate. After incubation for 4 days at 25°C, colonies on the plate were enumerated.

We used a nutrient agar plate containing 5.0 g Bacto Peptone, 1.0 g yeast extract, 20 g sucrose, 0.04 g bromothymol blue, and 15 g agar in seawater (liter⁻¹) to enumerate each number of surviving cells such as *V. natriegens* ATCC 14048^T as well as *Photobacterium* sp. TKY1 in their mixture in the presence and absence of 100 µM TBTCl. We could distinguish colonies of *V. natriegens* ATCC 14048^T from those of *Photobacterium* sp. TKY1 because the former strain grew faster than the latter on the agar plate, and sucrose metabolites from *V. natriegens* ATCC 14048^T acidified the medium pH, with changes in the color of a colony to yellow-orange.

Measurement of cell-associated TBT

Photobacterium sp. TKY1 cells were pre-incubated for 1.5 days at 25°C in the medium containing 5.0 g Bacto Peptone, 1.0 g yeast extract, and 29.2 g NaCl in 50 mM K-Pi buffer (liter⁻¹). After pre-incubated cells (30 µL) were inoculated into the medium (30 mL), incubation was carried out for 1.5 days at 25°C. Cells in the broth (10 mL) were harvested with centrifugation (5 min, 10,000×g), washed twice with 50 mM K-Pi buffer, pH 7.5, containing 0.5 M NaCl, and resuspended in the buffer (10 mL). Cells were also grown in the presence of 100 µM TBTCl for 4 days at 25°C.

As for the cells grown in the absence of TBTCl, 30 mM TBTCl dissolved in ethanol was added to the cell suspension in a glass test tube (diameter: 20 mm; length: 180 mm, AGC Techno Glass Co., Ltd., Shizuoka, Japan) to give a final concentration of 100 µM. Then the glass test tube was vigorously shaken for 1 min and left for 1 h. After centrifugation, supernatant was pipetted out and added to a different glass tube with a screw cap. The centrifuge tube was briefly centrifuged again, and then remaining supernatant at the bottom of the tube was removed carefully by pipetting. A cell pellet thus obtained was resuspended in 50 mM K-Pi buffer, pH 7.5, and the volume was adjusted up to 10 mL with the buffer. As for cells grown in the presence of 100 µM TBTCl, the samples prepared from the supernatant and the cell pellet were obtained using the same procedure described above. The sample was also obtained from the growth medium alone after adding 100 µM TBTCl as a final concentration. The glass test tube containing growth medium (10 mL) and 100 µM TBTCl was vigorously shaken for 1 min, and then an aliquot of the mixture (1 mL) was pipetted out and kept in a glass tube with a screw cap.

The samples of the supernatant as well as the growth medium alone, in which TBTCl was added to give a final

concentration of 100 µM, were measured with a HPLC (Waters 2695, Milford, MA, USA) equipped with an electrospray tandem mass spectrometer (Waters Quattro Micro, Milford, MA, USA) (HPLC/MS/MS) without pretreatment. Pretreatment was carried out to measure cell-associated TBT. Cell suspensions (100 µL) were pipetted out and added to acetonitrile (9 mL). After sonication for 5 min, the sample was centrifuged. The supernatant thus obtained was adjusted to 10 mL with acetonitrile.

The mixture of 0.1% formic acid (60 mL) with acetonitrile (40 mL) was used as the moving phase of the liquid chromatographic column (2.0 mm inner diameter x 150 mm length), in which TSKgel Octadecyl-2PW (5-µm mean particle size) was filled. The mixture's flow rate was 0.2 mL/min. The injection volume of the sample was adjusted to 10 µL. A stable isotope, TBT-d27Cl, was used as a surrogate, in which hydrogen was replaced with deuterium. The method of selected reaction monitoring was employed to quantify TBT in the sample.

Observation of morphological changes in isolates by scanning electron microscopy

Resting cells suspended in 50 mM K-Pi buffer, pH 7.5, that contained 0.5 M NaCl were prepared from *Photobacterium* sp. TKY1 cells grown in the medium described previously for 4 days at 25°C in the absence or presence of 100 µM TBTCl. Resting cells were also prepared from cells grown in the absence of TBTCl for 1.5 days at 25°C, and exposed to TBTCl at a final concentration of 100 µM for 1 h.

Resting cells prepared from *V. natriegens* ATCC 14048^T reaching the early stationary phase of growth were mixed with those obtained from *Photobacterium* sp. TKY1 cells, and then the cell mixture was exposed to TBT at a final concentration of 100 µM for 1 h.

Cells examined were fixed with 1% (vol/vol) glutaraldehyde, as a final concentration, for 1 h at 4°C, dehydrated once for 1 h in 50, 70, 90, 95, and 100% ethanol, and suspended in 100% *t*-butyl alcohol. After being freeze-dried, they were pasted on a carbon tape and coated with Pd-Pb particles under vacuum. Samples thus obtained were observed with a scanning electron microscope (XL30 CP, FEI Company, Eindhoven, the Netherlands).

Purchase of chemicals

Tributyltin chloride, of greater than 95% purity, was purchased from Wako Pure Chemical Industries, Ltd. (Wako, Japan). Other chemicals used were of high grade.

RESULTS AND DISCUSSION

Phylogenetic similarity of each isolate with type strains

The homologous percentage of the type strain to each isolate was more than 98.5%, based on partial 16S rDNA sequences, except for *P. halotolerans* LMG 22194^T (94.2%) and *P. damsela* ATCC 33539^T (95.2%) (Table 1). So far, *Photobacterium* sp. TKY1, *Halomonas* sp. TKY2, and *Photobacterium* sp. NGY1 were very close to *P. ganghwense* JCM 12487^T, *H. venusta* DSM 4743^T, and *P. rosenbergii* LMG 22228^T, respectively.

Tributyltin-resistant ability of resting cells

We examined changes in the TBT-resistant ability of each isolate as well as those of its type strains to a constant number of TBT molecules, $6.0 \times 10^{19} \text{ mL}^{-1}$ when the number of resting cells varied up to 10^9 CFU mL^{-1} (Fig. 1). The number of cell-associated TBT molecules with a single cell increases in response to the reduction of the number of resting cells in the experiment. As a result, the number of resting cells of *Photobacterium* sp. TKY1 was hardly reduced even when the initial number of resting cells varied from $10^{9.4}$ to $10^{4.2} \text{ CFU mL}^{-1}$, at which the number of surviving cells was $10^{3.8} \text{ CFU mL}^{-1}$ (Fig. 1A). In such experimental conditions, the number of cell-associated TBT molecules increased by

TABLE 1. List of two type strains with high similarities to the 16S rDNA sequences of each isolate

Strain isolated	Type strains ^a	Origin of the type strain	References
<i>Photobacterium</i> sp. TKY1	<i>Photobacterium ganghwense</i> JCM 12487 ^T (99.4%) ^b	Seawater	Park et al. (2006)
	<i>Photobacterium halotolerans</i> LMG 22194 ^T (94.2%)	Saline lake	Rivas et al. (2006)
<i>Halomonas</i> sp. TKY2	<i>Halomonas venusta</i> DSM 4743 ^T (99.6%)	Seawater	Baumann et al. (1972)
	<i>Halomonas alkaliphila</i> DSM 16354 ^T (99.6%)	Salt pool	Romano et al. (2006)
<i>Photobacterium</i> sp. NGY1	<i>Photobacterium rosenbergii</i> LMG 22228 ^T (98.5%)	Bleached coral	Thompson et al. (2005)
	<i>Photobacterium damsela</i> ATCC 33539 ^T (95.2%)	Damselfish skin ulcers	Smith et al. (1991)

^aWe selected two kinds of type strains, which showed high first and secondary similarities to the 16S rDNA sequences of each isolate, based on the database of Apron DB-BA Version 4.0 (TechnoSuruga Laboratory Co., Ltd.).

^bThe homologous percentage between the isolate and each type strain is shown in parentheses. The type strain listed in the upper line made a cluster with the isolate on the phylogenetic tree.

FIG. 1. Changes in the survivability of the isolate and the taxonomically similar type strains in relation to the given numbers of resting cells exposed to TBT

Surviving cells were counted by the colony-counting method after exposure to $100 \mu\text{M}$ of TBT for 1 h. The number of cell-associated TBT molecules varied with the number of resting cells exposed to TBT. Changes in the number of surviving cells of *Photobacterium* sp. TKY1 (●), *P. ganghwense* JCM 12487^T (△), and *P. halotolerans* LMG 22194^T (□) are shown in relation to given numbers of resting cells (Fig. 1A). The results obtained from *Halomonas* sp. TKY2 (●), *H. venusta* DSM 4743^T (△), and *H. alkaliphila* DSM 16354^T (□) cells are shown in Fig. 1B. For *Photobacterium* sp. NGY1 (●), *P. rosenbergii* LMG 22228^T (△), and *P. damsela* ATCC 33539^T (□), the number of surviving cells is shown in Fig. 1C. The dotted line in each figure means perfect survivability, regardless of the initial number of resting cells.

5-orders of magnitude, indicating that the isolate was highly resistant to TBT. When resting cells of $10^{9.2}$ CFU mL⁻¹ were exposed to TBT for 1 h, surviving cells of *P. ganghwense* JCM 12487^T were reduced to $10^{8.5}$ CFU mL⁻¹. Survivability, however, drastically reduced from $10^{5.3}$ CFU mL⁻¹ to zero when the number of resting cells varied from $10^{7.9}$ to $10^{7.0}$ CFU mL⁻¹. *P. halotolerans* LMG 22194^T also showed a result similar to that of *P. ganghwense* JCM 12487^T. The number of surviving cells was $10^{6.3}$ CFU mL⁻¹ when resting cells of $10^{9.2}$ CFU mL⁻¹ were exposed to such a number of TBT molecules (mL⁻¹). The number of surviving cells was reduced to $10^{1.8}$ CFU mL⁻¹ when the resting cells of $10^{7.0}$ CFU mL⁻¹ were exposed to the same concentration of TBT.

The sensitivity of *Halomonas* sp. TKY2 and *H. venusta* DSM 4743^T to TBT were enhanced in relation to the reduction of the initial number of resting cells (Fig.1B). When the resting cells of $10^{7.0}$ CFU mL⁻¹ were examined, the surviving cells of *Halomonas* sp. TKY2 and *H. venusta* DSM 4743^T were reduced by 3- and 2-orders of magnitude, respectively, whereas the reduction of surviving cells prepared from each strain was repressed by 0.6-order of magnitude to the initial number of resting cells, $10^{8.9}$ CFU mL⁻¹. For *Halomonas* sp. TKY2 and *H. venusta* DSM 4743^T cells, no surviving cells were observed when the number of resting cells was less than $10^{6.0}$ CFU mL⁻¹. Of the three *Halomonas* species examined thus far, *H. alkaliphila* DSM 16354^T cells showed the highest survivability. The number of surviving cells hardly decreased when the number of resting cells varied from $10^{9.2}$ to $10^{5.1}$ CFU mL⁻¹. The reduction of surviving cells was on 0.3-order of magnitude, even when the resting cells, such as $10^{5.1}$ CFU mL⁻¹, were exposed to TBT. *H. alkaliphila* DSM 16354^T was isolated from the water of a salt pool in Italy (Romano et al., 2006). It might be plausible to think that the highly resistant ability is not caused by prolonged exposure to sublethal TBT concentrations, but fundamentally possessed by the strain. Further study of the resistance of *H. alkaliphila* DSM 16354^T to TBT is necessary.

Changes in the survivability of a given number of resting cells of *Photobacterium* sp. NGY1, *P. rosenbergii* LMG 22228^T, and *P. damsela* ATCC 33539^T were examined (Fig.1C). The number of surviving cells of *Photobacterium* sp. NGY1 drastically decreased to $10^{4.2}$ CFU mL⁻¹ when the initial number of $10^{9.3}$ CFU mL⁻¹ was exposed to TBT for 1 h. *P. rosenbergii* LMG 22228^T showed relatively higher survivability as compared to *Photobacterium* sp. NGY1. The numbers of surviving cells were $10^{6.8}$ and $10^{2.0}$ CFU mL⁻¹, respectively, when resting cells of $10^{8.7}$ and $10^{6.8}$ CFU mL⁻¹ were exposed to TBT for 1 h. Resting cells of *P. damsela* ATCC 33539^T showed similar sensitivity to *P. rosenbergii* LMG 22228^T.

The isolates, *Photobacterium* sp. TKY1, *Halomonas*

sp. TKY2, and *Photobacterium* sp. NGY1, formed colonies on the nutrient agar plate containing 100 μ M TBT within 5 days of incubation at 25°C (see MATERIALS AND METHODS). The sensitivity to TBT of *Halomonas* sp. TKY2 and *Photobacterium* sp. NGY1, however, was enhanced with the reduction of the initial number of resting cells in the presence of 100 μ M TBT, indicating that some kinds of amino acids might be essential for the survival of the strains, i.e., syntheses of stress proteins for coping with the toxicity of TBT (Nováková et al., 2009). Resting cells prepared from the isolate, *Photobacterium* sp. TKY1, and the type strain, *H. alkaliphila* DSM 16354^T, showed high survivability to TBT, indicating that they might have constitutively expressed transporters such as the multidrug efflux pump, TbtABM, in the membrane found in *P. stutzeri* (Jude et al., 2004).

Capacity of adsorption of TBT molecules as an indicator of tolerance to TBT

Cell-associated TBT molecules were measured with a HPLC/MS/MS. As shown in Table 2, growing *Photobacterium* sp. TKY1 cells as well as resting cells, $10^{9.4}$ CFU mL⁻¹, adsorbed TBT. These results indicate that the isolate could grow in the nutrient liquid medium to cope with the adsorption of TBT molecules by the growing cells. The cell wall of the isolate might play an important role to prevent the interaction of TBT molecules with the cell membrane. The concentration of TBT recovered from the medium alone was found to be 65.0 nmol mL⁻¹. The concentration was lower than the adjusted concentration of 100 nmol mL⁻¹. It seemed that some of the TBT molecules were not recovered due to their adhesion onto the inside of the glass test tube and chips used for pipetting.

We have pointed out previously that the cell wall of a marine *Pseudoalteromonas* sp. plays an important role in preventing the inflow of TBT molecules into the cytoplasm across the membrane (Kubota et al., 2004; Mimura et al., 2008a; Mimura et al., 2008b). The isolate, *Photobacterium* sp. TKY1, could adsorb TBT molecules, approximately $3.5 \times 10^{6.6}$ (a single cell⁻¹), when resting cells were examined. The order of the value was consistent with that obtained from TBT-resistant marine *Pseudoalteromonas* sp. cells examined in the presence of 100 μ M TBTCl (Mimura et al., 2008b). Those results indicate the importance of the cell wall's function and capacity to adsorb TBT molecules on the surface in relation to the resistance of the species to TBT toxicity.

Estimation of the impact of a TBT-resistant bacterium on the indigenous microbial population

We estimated the impact of a TBT-resistant marine bacterium on an indigenous microbial population in

TABLE 2. Adsorption of TBT molecules by *Photobacterium* sp. TKY1 cells

	TBT in supernatant (nmol mL ⁻¹)	Cell-associated TBT ^d (nmol (mg cell protein) ⁻¹)
Cells grown in the presence of TBTCl ^a	7.2	187.4
Resting cells ^b	7.6	142.5
Medium alone ^c	65.0	—

^a Cells were grown in the presence of 100 µM TBTCl for 4 days at 25°C. After their harvest, TBT in the supernatant as well as the cell pellet were measured.

^b Resting cells were prepared from the cells grown in the absence of TBTCl for 1.5 days at 25°C. After being harvested and washed twice, cells were suspended in 50 mM K-Pi buffer, pH 7.5, containing 0.5 M NaCl. TBT concentrations in supernatant and cell pellets were measured after the addition of 100 µM TBTCl, as the final concentration, for 1 h.

^c TBTCl, as the final concentration of 100 µM, was added into the medium. After vigorous shaking, TBT in the sample was measured.

^d Cell proteins of resting cells grown in the absence or presence of TBTCl were 0.29 mg mL⁻¹ and 0.42 mg mL⁻¹, respectively.

TABLE 3. Changes in survivability of TBT-sensitive *Vibrio natriegens* ATCC 14048^T cells when TBT-resistant *Photobacterium* sp. TKY1 cells coexist in the presence of TBTCl

Strains and each number of resting cells (CFU mL ⁻¹)	Surviving cells in the presence of TBTCl (100 µM) ^a	
	<i>Photobacterium</i> sp. TKY1 (CFU mL ⁻¹)	<i>Vibrio natriegens</i> ATCC14048 ^T (CFU mL ⁻¹)
<i>Photobacterium</i> sp. TKY1 (10 ^{9.1±0.2}) and <i>Vibrio natriegens</i> ATCC 14048 ^T (10 ^{9.4±0.2}) ^b	10 ^{9.1±0.2}	10 ^{4.4±0.4}
<i>Vibrio natriegens</i> ATCC 14048 ^T alone (10 ^{9.4±0.2})	—	10 ^{6.2±0.3}

^a Experiments were carried out three times independently, and the data are shown as the averaged value ± standard deviation.

^b No reduction of colony-forming *V. natriegens* 14048^T cells was observed after being mixed with *Photobacterium* sp. TKY1 cells for 1 h in the absence of TBTCl.

surface sediments polluted with a sublethal level of TBT (Table 3). When TBT-sensitive *V. natriegens* ATCC 14048^T cells, 10^{9.4±0.2} CFU mL⁻¹, were exposed for 1 h to 100 µM TBTCl, as a final concentration, the number of surviving cells was reduced to 10^{6.2±0.3} CFU mL⁻¹. When resting cells of *V. natriegens* ATCC 14048^T were mixed with those of TBT-resistant *Photobacterium* sp. TKY1, the number of surviving cells of *V. natriegens* ATCC 14048^T was found to be 10^{4.4±0.4} CFU mL⁻¹, the value of which decreased 2-orders of magnitude as compared with that when *V. natriegens* ATCC 14048^T was examined alone. This result indicates that a coexistent TBT-resistant marine bacterium stimulates TBT toxicity in TBT-sensitive microbial populations in surface sediments polluted with sublethal TBT concentrations. In order to maintain the microbial ecosystem, TBT contamination should be prevented, especially in the surface sediments of busy ports.

Morphological observation of *Photobacterium* sp. TKY1 and *V. natriegens* ATCC 14048^T cells in the presence of TBT

Obvious morphological changes were not observed when *Photobacterium* sp. TKY1 cells were grown in the presence of 100 µM TBTCl (Fig.2B) as well as after the addition of TBTCl to the resting cells (Fig.2D). Morphological changes of TBT-sensitive *V. natriegens* ATCC 14048^T cells mixed with those of *Photobacterium* sp. TKY1 were observed in the presence of TBTCl (Fig. 2F). After exposure to TBT, ruptured or wrinkled *V. natriegens* ATCC 14048^T cells were observed, while *Photobacterium* sp. TKY1 cells in the mixture retained their normal cell morphology. Since the cells were suspended in the presence of 0.5 M NaCl, such changes in morphology seem to be closely related to the disruption of osmoregulation by the cation transport across the membrane (Fukagawa and Suzuki, 1993).

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FIG. 2. Morphological changes of the isolate, *Photobacterium* sp. TKY1, *Vibrio natriegens* ATCC14048^T, and their mixture in the presence of TBTCl with a scanning electron microscope

Morphological changes were observed with a scanning electron microscope with the following experimental conditions: *Photobacterium* sp. TKY1 cells were grown in the absence (Fig. 2A) or presence (Fig. 2B) of 100 μ M TBTCl as the final concentration; Resting cells (Fig. 2C) of *Photobacterium* sp. TKY1 were exposed to the same concentration of TBTCl for 1 h (Fig. 2D); A mixture of resting cells of *Photobacterium* sp. TKY1 and those of *V. natriegens* ATCC14048^T (Fig. 2E) was exposed to the same concentration of TBTCl as described above for 1 h (Fig. 2F).

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