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Tributyltin-resistant marine bacteria: a summary of recent work

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SUMMARY

A tributyltin chloride (TBTCl)-resistant bacterium, *Alteromonas* sp. M-1, was isolated from coastal seawater. This bacterium grew in medium containing 125 μM TBTCl. TBTCl added to the medium was taken up by this bacterium, however, the amount of TBTCl in the cellular fraction was low after the logarithmic phase, suggesting the existence of a TBTCl-efflux system. A genetic library was constructed using plasmid vector pUC 19. Three positive clones were obtained, by which *E. coli* was transformed to TBTCl resistance. Of the three clones, the shortest fragment from *Hin*dIII-library was analyzed. This fragment was 1.8 kb long and contained one complete open reading frame. The predicted amino acid sequence of this open reading frame had a homologous domain to transglycosylases of bacteriophage and *E. coli*. TBTCl-tolerant marine bacteria other than *Alteromonas* sp. M-1 were obtained from natural seawater to which TBTCl was added. DNA–DNA hybridization was performed between the three cloned fragments from *Alteromonas* sp. M-1 and chromosomal DNA of the TBTCl-tolerant bacteria. Some strains hybridized with the fragments and some did not, suggesting that several genes are responsible for TBTCl tolerance.

INTRODUCTION

Triorganotins (TBT) such as tributyltin oxide (TBTO), tributyltin chloride (TBTCl) and triphenyltin chloride (TPTCl) are toxic to both eukaryotes and prokaryotes and are used as industrial biocides in antifouling paints [13,14]. TBT pollution is a serious problem since it is released from fishing boats and nets into marine sediments and degrades slowly [14]. Although the use of TBT is controlled in several European countries, the United States and Japan, it is present in the marine environment.

Although a few researchers have reported degradation of TBT by environmental microorganisms [1], isolation of TBT decomposing bacteria has not been successful so far. In recent years, several groups have found TBT-resistant bacteria [7,9,14,17,20]. Among the reports on TBT-resistant bacteria, *Alteromonas* sp. M-1 [7,18] are the first records of isolation and identification of a TBT-resistant marine bacterium. Moreover, it was found that addition of TBT to natural seawater enriched TBT-tolerant bacteria [5,17]. The purpose of this paper is to summarize our recent work on genetic control of TBT resistance in the marine bacterium *Alteromonas* sp. M-1 and the distribution of homologous genes in TBT-tolerant bacteria enriched by the addition of TBT to seawater.

CHARACTERISTICS OF TBTCl-RESISTANT BACTERIUM, *ALTEROMONAS* sp. M-1

Alteromonas sp. M-1 was isolated from natural seawater obtained from Funka Bay, Hokkaido, Japan. This bacterium was reported as *Vibrio* sp. [7]. However, fermentation in oxidation-fermentation tests was weak compared to the other marine Vibrios. Therefore, a reexamination of taxonomy was performed by sequencing the 16S rRNA. The results indicated that this bacterium should be classified in the genus *Alteromonas* [18]. Strain M-1 is resistant to TBTCl but not to other organometals and metals such as TPTCl, $CdSO_4$ and methyl-Hg. When 125 μM TBTCl was added to the medium, strain M-1 could grow. This concentration is sublethal to sensitive microorganisms.

Uptake of TBTCl by strain M-1 and some other sensitive bacteria revealed that all bacteria tested incorporated TBTCl within 1 h after incubation. Figure 1 shows TBTCl uptake and growth of strain M-1 and one of the sensitive bacteria, *Shewanella putrefaciens*. The sensitive organism did not grow after uptake of TBTCl, which confirmed reports by other researchers [3,21]. However, strain M-1 thrived. Interestingly, TBTCl taken up by strain M-1 decreased with growth. This might be performed by an efflux system(s) of TBTCl.

We have found in strain M-1, that two polypeptides of 12 kDa and 30 kDa were induced in cells cultured with TBTCl [7]. The function of the polypeptides is still not known. However, because these polypeptides could be extracted by 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS), these polypeptides might be associated with the cell membrane. Similar inducible polypeptides were observed in Hg-resistant bacteria [10]. The two polypeptides might be involved in resistance in strain M-1.

GENES RESPONSIBLE FOR TBTCl RESISTANCE IN *ALTEROMONAS* sp. M-1

Extensive studies at the gene level of organomercury resistance have been reported [2,15,16], although the resistance

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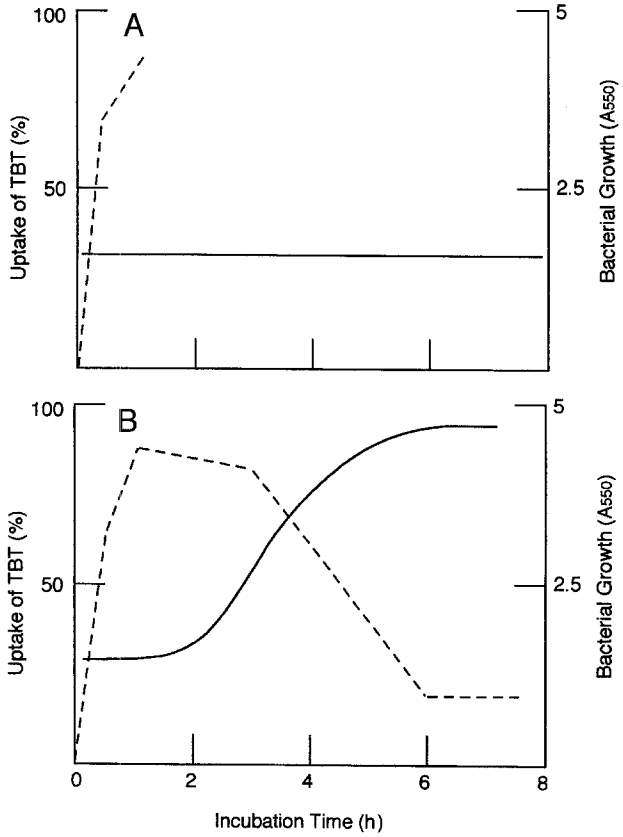

Fig. 1. TBTC1 uptake (dotted line) and growth (solid line) of *Shewanella putrefaciens* (A) and *Alteromonas* sp. M-1 (B). Modified from [7].

mechanism for other organometals has not been clarified so far. The purpose of our study is to reveal the molecular mechanism of TBT resistance in marine bacteria. As a first step of this attempt, cloning of the gene responsible for TBT resistance in *Alteromonas* sp. M-1 was performed.

As the strain M-1 does not have any plasmids, it was considered that the gene(s) responsible for TBTCl resistance are chromosomal. A genetic library of strain M-1 was constructed using plasmid vector pUC 19. *Escherichia coli* JM 109 was transformed with the recombinant plasmids. To determine whether TBT resistance was expressed in *E. coli*, colonies grown on an LB agar plate containing ampicillin were replicated on an LB plate containing 1 mM of TBTCl. Cells from colonies grown on this plate were then inoculated in LB liquid medium containing 100 μM TBTCl. This concentration is sublethal for sensitive bacteria including *E. coli* JM 109. After the two step screening, we obtained three positive clones from *Hin*dIII-library and *Pst*I-library. A positive clone from *Hin*dIII-library designated pTBT 1 possessed a 1.8-kb insert. Since this fragment was the shortest among the three clones, it was sequenced. We reported the whole DNA sequence of the fragment [6]; an open reading frame (ORF) of 324 bp started from base number 990 was found. This ORF encoded 108 amino acids from the initiation codon. However, very recently, two frame shift errors were found within the ORF. From the corrected sequence, a putative protein was constructed which contained 207 amino acids; hydrophobic, neutral and hydrophilic amino acids were 50.5%, 22.1% and 26.9%, respectively. The predicted amino acid sequence from the corrected DNA sequence (Fig. 2) had 44% identity to the *YafG* product of *E. coli*. Koonin and Rudd [11] found that the product of the ORF

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        10         20         30         40         50         60
aagcttgatgttcaaagttgcagtattaggtttaggtgattctagctatgaatttttctg
        70         80         90        100        110        120
caaaccgtaaagactttgaagagcgttaactaaattcaggtgccagacggttatcatcac
       130        140        150        160        170        180
ggcgctgacgtgattatgatgacgaagcagccgacgttggattgaggcgcattaaatgcg
       190        200        210        220        230        240
tttgagccagatttaaaagcccagcaagttgcaactagcggtcaagttgtatcaatgcca
       250        260        270        280        290        300
tttggcgctcctgcagcagccggctagtccagtaccaccaaagccaaaatcccgtttgcc
       310        320        330        340        350        360
ggctagtccagtaccaccaaagccaaaatcccgtttgccggctagagccttaagcttaga
       370        380        390        400        410        420
caggtactggtaaaaaagatgggaaaagaatttattgttcgcgtagagaagttagattgg
       430        440        450        460        470        480
cttgaatcaaatggcaattatgtaaatctacttattgcgctcgaatttcccatttttcgtg
       490        500        510        520        530        540
caaccatgacacaacttataactcagttagagccacaaggattttgccgcattcaccgtt
       550        560        570        580        590        600
ctcatgcggttagattagacgcggtctgaatcaatcacaccattagcaagtggcgagagt
       610        620        630        640        650        660
gaagtaaaacttacaacaggaaaagcacttaacttatcgcgtcgctataaagatcaattt
       670        680        690        700        710        720
aaggaactgttaaattcaactacaagtcattcttaactcacgctatgttaaagtaaccta
       730        740        750        760        770        780
ctttaggttagccgcattcatttttttcggagtagtttagtacgtgacctttaaatcgatt
       790        800        810        820        830        840
tatccgattttgccattagtgttaattttatccggttgtgaaacacacccgaacagccct
       850        860        870        880        890        900
cttcacaacctgatattaaagctaaccaaatattgttgttaaagaacaaaacgaaaatat
       910        920        930        940        950        960
tgagcaaatagctcccttaaggtcctcagaaactcgatgatgtgtggcacgatccgagcc
       970        980        990       1000       1010       1020
gagttacatttgccaatctagccacccagATGTACAACAACGCATTGCATGGTATTTATC
                                    M  Y  N  N  A  L  H  G  I  Y  L
      1030       1040       1050       1060       1070       1080
TCACCCAAATTACATGGATGAAATCAGCGCGTGCTGAGCCTTACCTTTATTATATAGTCA
  T  Q  I  T  W  M  K  S  A  R  A  E  P  Y  L  Y  Y  I  V  T
      1090       1100       1110       1120       1130       1140
CAGAGGTTGAAAAGCGGAACTTACCCATAGAATTAGCATTAATGCCGCTAATTGAAAGTG
  E  V  E  K  R  N  L  P  I  E  L  A  L  M  P  L  I  E  S  D
      1150       1160       1170       1180       1190       1200
ACTTTAACGCCAGTGCCTATTCGCACAAGCATGCATCTGGACTTTGGCAATTAACGCCTG
  F  N  A  S  A  Y  S  H  K  H  A  S  G  L  W  Q  L  T  P  A
      1210       1220       1230       1240       1250       1260
CCATTGCTAAATATTTTAAAGTGCAAATATCCCCTTGGTATGACGGACGTCAGGACGTAA
  I  A  K  Y  F  K  V  Q  I  S  P  W  Y  D  G  R  Q  D  V  I
      1270       1280       1290       1300       1310       1320
TAGACAGTACCCGGGCTGCGTTGAATTTTATGGAATATTTACACAAACGCTTTGATGGTG
  D  S  T  R  A  A  L  N  F  M  E  Y  L  H  K  R  F  D  G  D
      1330       1340       1350       1360       1370       1380
ACTGGTATCACGCTATAGCAGCCTTAAACTTAGGTGAAGGCCGTGTACTTAGAGCAATTA
  W  Y  H  A  I  A  A  L  N  L  G  E  G  R  V  L  R  A  I  S
      1390       1400       1410       1420       1430       1440
GTAATATAAAAAACAAGGCAAACCCACTGATTTTTCAACTTAAAACTGCCCAAACAAACC
  N  I  K  N  K  A  N  P  L  I  F  Q  L  K  T  A  Q  T  N  Q
      1450       1460       1470       1480       1490       1500
AGTCAGTACGTGCCAAAAGGACTAGCTGCGGCACAATTATTAAAAAGCCAAAAAATGCTT
  S  V  R  A  K  R  T  S  C  G  T  I  I  K  K  P  K  N  A  F
      1510       1520       1530       1540       1550       1560
TTCCTGCAATTTTAAACAGCCCAACAATTGCAGTATTGCCTGTTGACTGCGCTGTTATTT
  P  A  I  L  N  S  P  T  I  A  V  L  P  V  D  C  A  V  I  L
      1570       1580       1590       1600       1610       1620
TAGATAACCGAAAGCAATGGCAGCAACTTGAAATCTTTAAACCAATGGTGtgactcgctt
  D  N  R  K  Q  W  Q  Q  L  E  I  F  K  P  M  V  *
      1630       1640       1650       1660       1670       1680
tggcccaggcaatatgatgcgccccacactgtgttccagtgtgaacaaacacaatttaaa
      1690       1700       1710       1720       1730       1740
gacatgctcgctaatcttgattccaatgattatagtcagtggcaacactacagtaaaacg
      1750       1760       1770       1780       1790       1800
tcgtgatagttaagtgttatagcgaaacgctacaaagtaggtattagccaagctcaaagctt
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Fig. 2. Nucleotide sequence of the 1.8-kb *Hin*dIII-fragment from pTBT 1. Predicted amino acids are shown parallel to the nucleotide sequence. This corrected sequence was from Swissprot database with accession number P32820.

had a conserved domain similar to a domain of several transglycosylases as shown in Fig. 3. The ORF product has not been obtained yet. If the product is a murein transglycosylase as mentioned by Koonin and Rudd, and if the enzyme is part of the resistance mechanism of strain M-1, it must be a very interesting one. The transglycosylase might change the transport potential of membrane. When the nucleotide sequences were searched for, sequences presenting the upper position of the ORF showed similarity with some Ca^{2+} transport genes. These results suggest that the gene cloned in pTBT 1 might be a part of a cluster of membrane proteins relating to transportation.

OCCURRENCE OF TBTCl-TOLERANT BACTERIA IN TBT-ADDED SEAWATER

Whether or not contamination by organotins affects the numbers and the flora of resistant organisms is important from the environmental view point. Hallas and Cooney [9] reported that they did not find any significant correlation between tin concentration in the sediment and numbers of tin-resistant organisms. Wuertz et al. [20] also failed to find selection of tin-resistant bacteria in tin-polluted estuarine waters but did not find enrichment in fresh waters. It was suspected that other factors besides the presence of tin influence the selection for tin-resistant bacteria. Their criterion for a TBT-polluted area was more than 200 p.p.b. of TBT in the water or 1.57 nmol g^{-1} of TBT in the sediment. No studies are known that have been performed yet on highly polluted habitats.

An artificially polluted system in glass bottles was made, and the occurrence of TBTCl-tolerant bacteria was surveyed in seawater containing high concentrations of TBTCl. TBTCl-tolerant bacteria were enriched under these experimental conditions [5,17]. Moreover, in these experiments, it was found that the bacteria were tolerant to both TBTCl and $CdSO_4$, or tolerant to both TBTCl and methyl-Hg, although the incidences were low when compared with that of TBT tolerance.

Natural seawater was collected from the sea surface into sterile glass bottles. The bottles of water were treated as follows: a control to which only ethanol (solvent for TBTCl) was added, TBTCl–water to which 40 p.p.m. of TBTCl was added, TBTCl–water to which 40 p.p.m. of TBTCl was added and $CdSO_4$- or methyl-Hg–water to which 40 p.p.m. of $CdSO_4$ or methyl-Hg was added. They were stored at 20 °C in the dark. Viable cells and tolerant bacteria were determined over time in each sample. As shown in Fig. 4(A), only low percentages of TBTCl-tolerant bacteria were found during the incubation period in the control-water. However, in water containing TBTCl, TBTCl-tolerant bacteria gradually increased with increasing incubation time (Fig. 4(B)). This enrichment of TBTCl-tolerant bacteria was reproducible in our experimental system, indicating that enrichment of TBTCl-tolerance can occur in seawater with high contamination. Although the mechanism of the selective enrichment of TBTCl-tolerant bacteria is still not known, there are two possibilities to explain this phenomenon: (1) TBT-sensitive strains may be killed by the presence of high concentrations of TBT, although naturally tolerant strains survive in a seawater environment; (2) sensitive strains might develop resistance to TBT, enabling them to grow.

As mentioned above, our reports [5,17] are the first descriptions of bacteria which were tolerant to both TBT/Cd or TBT/methyl-Hg. Miller et al. (this volume) report that a plasmid coding for chromium resistance conferred increased resist-

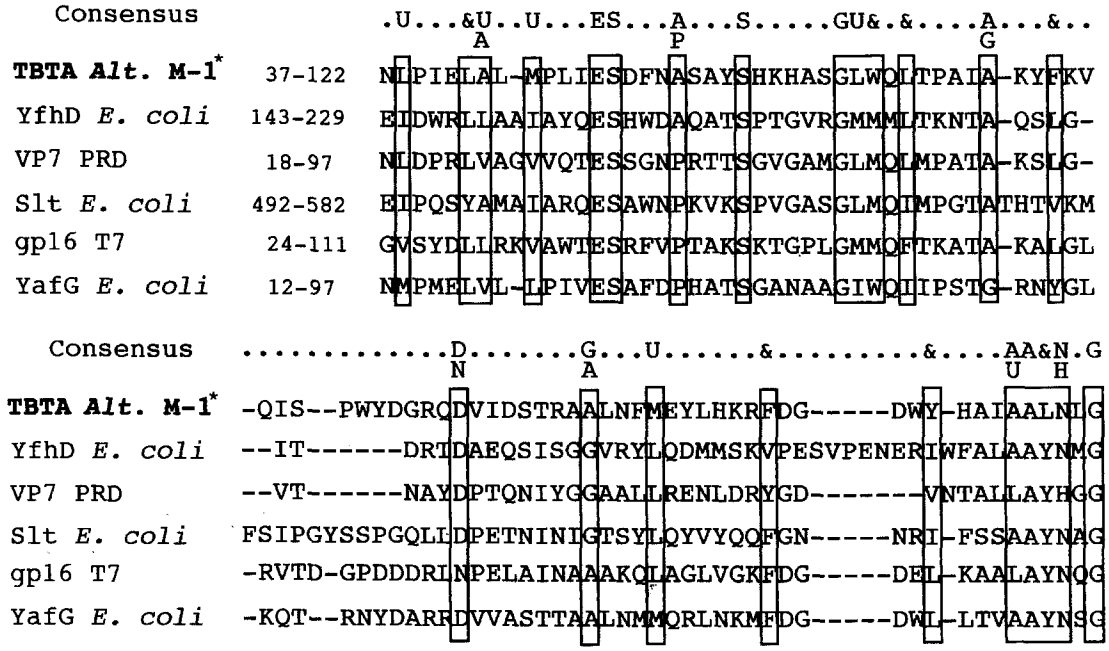

Fig. 3. Multiple alignment of the conserved domains of putative transglycosylases with the ORF in Fig. 2. Compared genes and consensus sequence are from [11], where U is a bulky aliphatic amino acid and "&" is a bulky hydrophobic amino acid. *TBT-resistance associated gene from *Alteromonas* sp. M-1.

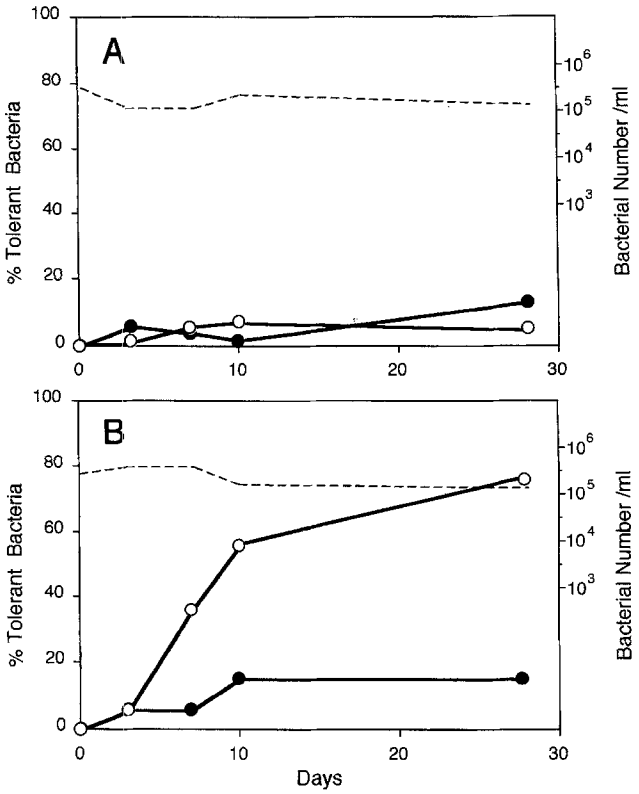

Fig. 4. (A) Time-dependent changes of viable count (dotted line) and occurrence (%) of TBTC1-tolerant (open circle) and methyl-Hg tolerant (closed circle) bacteria in control seawater sample, to which neither TBTC1 nor methyl-Hg was added. (B) Same experiment as A in seawater sample, to which TBTC1 was added (40 p.p.m.; 125 μM). Modified from [5].

ance to TBT when transferred from *Pseudomonas aeruginosa* to a *Beijerinckia* sp. The relationship between Cd or methyl-Hg tolerance and TBTCl tolerance will be further examined.

DISTRIBUTION OF GENE(S) HOMOLOGOUS TO CLONED FRAGMENTS FROM *ALTEROMONAS* sp. M-1

In methyl-Hg resistant bacteria, the gene cruster responsible for the resistance is in the *mer* operon, which is relatively homologous among bacterial species [8,12,19]. Cross-resistant strains to TBT/methyl-Hg obtained in our study were examined to determine whether the strains have a region homologous to the *mer* operon. However, the chromosomal DNA from the cross-resistant strains did not hybridize with a 24-mer oligonucleotide probe, 5′-GCT (C/A)AG (C/G)GC GCA CCA (G/T)GC ATA (C/T)AG-3′ whose sequence was determined from a conserved region of *mer* B (mercury lyase gene) from pDU 1358, pI 258 and the chromosome of *Bacillus* sp. [8,12,19]. This suggests that the methyl-Hg resistant strains isolated from our TBT-addition experiment have a low homology with *mer* B.

A preliminary experiment of DNA–DNA hybridization between cloned fragments from *Alteromonas* sp. M-1 and chromosomal DNA of TBT-tolerant bacteria other than *Alteromonas* sp. M-1 was performed [5]. Probe fragments were a 1.8-kb *Hin*dIII-fragment, a 4.8-kb *Pst*I-fragment and a 6.1-kb *Pst*I-fragment. Hybridization tests were performed for twenty-three TBT-tolerant strains picked up from the experiment shown in Fig. 4(B). Results are summarized in Table 1, showing different hybridization profiles with the three probes. The *Hin*dIII-fragment hybridized with *Alteromonas* sp. M-1, whereas the DNA of the twenty-three strains tested were not detected with this probe. The 4.8-kb *Pst*I-fragment and 6.1-kb *Pst*I-fragment hybridized with nine strains and with fourteen strains, respectively. Among the strains tested, eight strains did not hybridize with any of the three probes. It is interesting that *E. coli* JM 109 DNA was detected with the three probes, despite the fact that it is sensitive to TBT. Gene(s) responsible for TBT resistance in *Alteromonas* sp. M-1 might have other functions in *E. coli*, although we cannot exclude the possibility of random hybridization of the probes. As mentioned above, the conservative domain of transglycosylases was found in *E. coli* and cloned fragment in pTBT 1. This might cause cross hybridization between the three probes and *E. coli* DNA. How-

TABLE 1

Bacterial strains used in the DNA–DNA hybridization study and summary of results. Modified from [5]

No.	Strain #	Hybridize with		
		1.8-kb *Hin*dIII-fr.	4.8-kb *Pst*I-fr.	6.1-kb *Pst*I-fr.
1	T-67	–	++	++
2	T-71	–	+/–	+/–
3	T-73	–	–	+
4	T-76	–	+/–	+/–
5	T-87	–	+	+
6	T-91	–	+	+
7	T-93	–	–	–
8	T-94	–	+/–	+/–
9	T-97	–	–	+/–
10	T-102	–	–	–
11	T-103	–	–	–
12	T-105	–	–	+
13	T-113	–	–	–
14	T-127	–	–	+
15	T-129	–	–	+/–
16	T-130	–	+	++
17	T-138	–	–	–
18	T-139	–	–	–
19	T-141	–	–	–
20	T-149	–	+/–	+
21	C-51[a]	–	++	+
22	C-135[a]	–	–	–
23	C-137[a]	–	–	–
24	JM109[b]	+	++	++
25	M-1[c]	++	++	++
26	S5B[d]	–	–	–

[a] Strains isolated from control-water.
[b] *E. coli* JM109.
[c] *Alteromonas* sp. M-1.
[d] *Alteromonas haloplanktis* S5B.

ever, it can be concluded that more than one gene codes for TBT resistance. TBT resistance in marine bacteria is possibly coded by several genes which have low homology to each other.

CONCLUSION

Although many studies are available on the toxicity of TBT compounds [4], little is known of the biochemistry of resistance to and decomposition of TBT by bacteria. In this paper, we summarize some results obtained in recent studies using marine bacteria. The TBT-resistant bacterium, *Alteromonas* sp. M-1 was isolated and characterized. A TBT-resistant gene was cloned from this bacterium and one of the clones was sequenced. A homology search with other genes revealed that the predicted protein of the ORF had a conservative domain in common with transglycosylases, suggesting that a transglycosylase is involved in TBT resistance.

In addition, an experimental system to obtain TBT-tolerant bacteria was constructed, from which some TBT-tolerant strains and strains cross-tolerant to TBT/Cd and TBT/methyl-Hg were isolated. Between the DNAs of the TBT-tolerant strains isolated and cloned fragments from *Alteromonas* sp. M-1, DNA–DNA hybridization was performed. We found that TBT-resistant bacteria are common in the marine environment, and TBT-resistant organisms can be enriched by the presence of a high concentration of TBT. It is also suggested that more than one gene is responsible for TBT resistance and that there is diversity in the molecular mechanism(s) of TBT resistance.

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