Freshwater Bacteria Can Methylate Selenium through the Thiopurine Methyltransferase Pathway

Lionel Ranjard,† Sylvie Nazaret, and Benoit Cournoyer*

Research Group on Opportunistic Pathogens and Environment, UMR CNRS UCBL 5557 Ecologie Microbienne (Center for Microbial Ecology), Université Claude Bernard-Lyon 1, 69622 Villeurbanne Cedex, France

Received 6 September 2002/Accepted 15 April 2003

Involvement of the bacterial thiopurine methyltransferase (bTPMT) in natural selenium methylation by freshwater was investigated. A freshwater environment that had no known selenium contamination but exhibited reproducible emission of dimethyl selenide (DMSe) or dimethyl diselenide (DMDSe) when it was supplemented with an organic form of selenium [(methyl)selenocysteine] or an inorganic form of selenium (sodium selenite) was used. The distribution of the bTPMT gene (tpm) in the microflora was studied. Freshwater bacteria growing on 10 μM sodium selenite and 10 μM sodium selenate were isolated, and 4.5 and 10% of the strains, respectively, were shown by colony blot hybridization to hybridize with a Pseudomonas syringae tpm DNA probe. Ribotyping showed that these strains are closely related. The complete tpm sequence of one of the strains, designated Hsa.28, was obtained and analyzed. Its closest phyletic neighbor was found to be the Pseudomonas anguilliseptica tpm sequence. The Hsa.28 strain grown with sodium selenite or (methyl)selenocysteine produced significant amounts of DMSe and DMDSe. The Hsa.28 tpm gene was isolated by genomic DNA library screening and sequencing. BLASTP comparisons of the deduced Hsa.28 bTPMT sequence with P. syringae, Pseudomonas aeruginosa, Vibrio cholerae, rat, and human thiopurine methyltransferase sequences revealed that the levels of similarity were 52 to 71%. PCR-generated Escherichia coli subclones containing the Hsa.28 tpm open reading frame were constructed. E. coli cells harboring the constructs and grown with sodium selenite or (methyl)selenocysteine produced significant levels of DMSe and DMDSe, confirming that the gene plays a role in selenium methylation. The effect of strain Hsa.28 population levels on freshwater DMSe and DMDSe emission was investigated. An increase in the size of the Hsa.28 population was found to enhance significantly the emission of methyl selenides by freshwater samples supplemented with sodium selenite or (methyl)selenocysteine. These data suggest that bTPMT can play a role in natural freshwater selenium methylation processes.

Selenium (Se) is a metalloid which is essential for most living organisms. This element is found in several enzymes (e.g., glutathione peroxidases) either as a cofactor or in the form of seleno-amino acids (18). However, it can become toxic at high doses (14). Regular intake of about 850 μg of selenium per day by humans can lead to selenosis (hair and nail brittleness and loss) and other symptoms, such as a garlic breath odor and nervous system abnormalities. Pollution by this element can lead to selenosis (hair and nail brittleness and loss) and other symptoms, such as a garlic breath odor and nervous system abnormalities. Pollution by this element is one type of VI metal hazard pollution (3). In the environment, Se can be released through the weathering of selenium-containing minerals (6) and into the atmosphere through microbial assimilation into amino acids and proteins and are mobilized in the atmosphere through biological methylation (7, 12). It is noteworthy that in remote areas atmospheric concentrations of Se are far greater than the concentrations predicted to result from anthropogenic sources, suggesting that natural biological methylation is a key process involved in the flux of selenium in the atmosphere (17, 27).

Spontaneous selenium methylation can be observed in biologically active aqueous sewage sludges and soil extracts. In most environments, it is also observed after addition of inorganic and organic selenium compounds, suggesting that a broadly distributed process(es) is involved (4, 8, 15, 20, 27). Bacteria have been identified as the predominant Se-methylating organisms (7, 11). The Se-methylating bacteria have been found to mainly belong to two bacterial groups, the Proteobacteria (Enterobacter cloacae, Aeromonas sp., Pseudomonas sp., Rhodospirillum, Rhodococcus) and the Cytophagaes (Flavobacterium sp.) (4, 7, 9). Bacterial exposure to oxyanions (selenate \( \text{SeO}_4^{2-} \) or selenite \( \text{SeO}_3^{2-} \)) or organic forms of selenium (selenocystine, selenocysteine, selenomethionine) was found to generate dimethyl selenide (DMSe) and, at a lesser extent, dimethyl diselenide (DMDSe) (7, 25, 27). Selenium biomethylation leads to detoxification and removal of Se from contaminated sites. The volatile forms, DMSe and DMDSe, are 500 to 700 times less toxic than other derivatives (14), and this prevents Se from entering the food chain (1).

The genetics and enzymology of selenium methylation are far from being fully understood. So far, only Cournoyer et al. (5) have characterized a tellurite-selenite resistance genetic
determinant, designated \( tpm \), that was found to be involved in this methylation process. This gene, isolated from *Pseudomonas syringae* pv. *sisi* (a pea pathogen), encodes a bacterial thiol-purine methyltransferase (bTPMT) which was shown to catalyze the S-adenosyl methylation of thiopeptide heterocyclic sulfhydryl compounds (5). The \( tpm \) determinant, subcloned in *Escherichia coli*, conferred resistance to tellurite and the ability to grow at high concentrations of sodium bismuthenate. We recently determined the role of bTPMT in Se methylation by performing gas chromatography (GC)-mass spectrometry (MS) analyses. *E. coli* cultures harboring various \( tpm \) constructs and grown with selenite, selenate, and (methyl)selenocysteine were shown to produce DMSe and DMDSe (25). The presence of these selenides was directly related to expression of the bTPMT (25).

In this study, the likelihood of the presence of bTPMT-driven selenium methylation in natural environments was investigated. An aquifer which had no known selenium contamination but exhibited reproducible emission of methylated Se derivatives after it was spiked with selenite or (methyl)selenocysteine was used to investigate this possibility. Bacterial heterotrophs growing on selenate and selenite were isolated, and the distribution of the \( tpm \) gene in these strains was investigated. The diversity and functionality of the freshwater \( tpm \) sequences identified, as well as the phylogenetic affiliations of the \( tpm \)-harboring bacteria, were studied. The contribution of \( tpm \)-harboring bacteria to the emission of methylated Se derivatives by this freshwater aquifer was analyzed.

### MATERIALS AND METHODS

**Freshwater sample and bacterial counting.** Samples were obtained from an aquifer in Les Vosges, France. The samples had a pH of 7 and a total selenium content of less than 2 ppb.

Homogeneous freshwater samples were serially diluted with a sterile saline solution (0.08% NaCl), and 100-μl portions of the appropriate dilutions were plated on tryptic soy agar (TSA) (Difco) that was diluted 10-fold (TSA/10). Plate counts (in CFU) of total viable heterotrophs were determined. Selenite- and selenate-grown bacteria were isolated on TSA/10 supplemented with 10 μM Na₂SeO₃ (sodium selenite; Sigma, St. Quentin Fallavier, France) or 10 μM Na₂SeO₄ (sodium selenate; Sigma, St. Quentin Fallavier, France) or with 30 mM sodium selenite or 30 mM sodium selenate. The plates were incubated for 4 days at 28°C. Only plates containing 10 to 100 colonies were counted. All experiments were performed in triplicate.

**Genomic DNA extraction, PCR-amplified \( tpm \) probe, DNA blotting, and DNA hybridization.** Bacterial genomic DNAs were extracted and purified by using Quick genomic tips (Qiagen, Courtabœuf, France). A 600-bp PCR fragment, designated the \( tpm \) probe, of the *P. syringae* pv. *sisi* \( tpm \) gene was amplified by using primers 314 and 310 of Cournoyer et al. (5) as described by these authors. The \( tpm \) probe was labeled with \( [32P]dCTP \) by using a random priming kit and the recommendations of the supplier (Roche, Meylan, France).

Bacterial colony blots were prepared as described by Sambrook et al. (28). Briefly, the bacterial strains were grown on sterile nylon membrane filters (*GeneScreen Plus*; DuPont de Nemours, Les Ulis, France) layerad on top of Luria-Bertani (LB) agar. After incubation for 24 h at 28°C, the membranes were removed and treated by the alkaline lysis procedure. Southern blotting was performed as described by Sambrook et al. (28).

DNA blot hybridization with the \( tpm \) probe and washing were performed at 65°C as recommended by the *GeneScreen* membrane supplier. Hybridization signals were detected and analyzed by a Bio-Rad GS-525 molecular imager and the Molecular Analyst software (Bio-Rad, Paris, France) and by autoradiography.

**Genomic DNA library, \( tpm \) subcloning, and DNA sequencing.** A cosmogenic DNA library was constructed as described by Baure and Collmer (2). The genomic DNA was partially digested with SmaI and fractionated by a sucrose gradient centrifugation procedure (28). DNA fragments that were 30 to 50 kb long were purified and cloned in the pCPP47 BamH1 site. Cosmid packaging was carried out by using a Gigapack Gold packaging kit (*Stratagene*, La Jolla, Calif.). Transduction was performed by using *E. coli* XL1-blue MR cells obtained from Stratagene as recommended by the supplier. Library clones were selected on LB agar containing tetracycline (10 μg/mL). Positive clones were screened by \( tpm \) colony blot hybridization (see above), \( tpm \)-hybridizing library clones were extracted by the Maxiprep procedure (*Qiagen*) and were digested with various restriction enzymes, including NotI, EcoRI, PstI, XhoI, and XmnI, by following the manufacturer's recommendations. Restriction profiles were visualized by using a 0.8% agarose gel, and \( tpm \)-hybridizing restriction fragments were identified (see below), excised (using a Qiaex kit [Qiagen]), ligated into restricted pBlueScript SK+- (Stratagene) with the T4 DNA ligase (GIBCO BRL, Cergy Pontoise, France), and transformed into subcloning-efficient *E. coli* DH5α cells (GIBCO BRL). Library subclone plasmid DNAs were extracted by the Qiaamp prep procedure (*Qiagen*) and sequenced with the T3 and T7 primers. All DNA sequencing were performed by the Act Gene-Euro Sequence Gene Service (Grenoble, France) by using an ABI377 sequencer and an ABI PRISM dye terminator cycle sequencing Ready Reaction kit with AmpliTaq DNA polymerase (*Perkin-Elmer*, Paris, France). The library subclone DNA sequences were used to design primers for gene walking with one of the library clones by successive DNA sequencing. DNA sequencing was performed on both strands.

Two PCR-generated subclones, one by using the Expand High Fidelity system (Roche) according to the manufacturer's instructions to obtain the PCR fragments. Denaturation was performed at 95°C for 1.5 min, and elongation was performed at 72°C for 1 min. Primers PHF3 (CAGCAGCCCTGTGCCG; positions 1 to 17 of accession no. AY259120) and PHR3 GCCGGATCAAGATGCCAG; positions 1951 to 1932 of accession no. AY259120) were used for 1 min at an annealing temperature of 53°C to generate the pF31 PCR insert. The \( tpm \)-like ORF was amplified by using primers PHF4 (CGGCTGGACCTCTTGGGCC; positions 571 to 589 of accession no. AY259120) and PHR4 (CGGCTGATGACAGGACCCAG; positions 1338 to 1358 of accession no. AY259120) at an annealing temperature of 55°C for 1 min. The PCR fragments were ligated into the pGEM-T Easy vector by using pGEMT Easy vector system (Promega) and were transformed into subcloning-efficient *E. coli* DH5α. The positions and orientations of the inserts were verified by sequencing. These inserts were then subcloned into pBlueScript SK–.

**Ribosomal DNA analyses.** The intergenic spacer between *rrs* and *rfl* was PCRamplified by using primers and conditions described by Ranjard et al. (24). PCR products were restricted with *AatII* and *XbaI* and visualized on a 2% agarose gel. The *rrs* gene of one of the *tpm*-hybridizing strains was PCR-amplified and sequenced by using conditions and primers described by Viallard et al. (30).

**Selenium methylation assay.** Flasks (150 ml) containing 50 ml of LB medium (22) were inoculated with the bacterial strains and incubated for 48 h at 28°C. The 10 ppm of sodium selenite or 10 ppm of (methyl)selenocysteine (Sigma) was added, and the flasks were sealed and incubated for an additional 3 days at 28°C. The headspace gases of the flasks (containing the bacterial broth media) were analyzed by a GC-MS method to estimate the production of DMSe and DMDSe (25). These experiments were carried out with a Hewlett-Packard 6890 gas chromatograph coupled to a model 5973 mass spectrometer. The chromatography column was a Supelco VOCOL column (60 m by 0.32 mm i.d., film thickness 0.25 μm). A milliliter of headspace gas was injected in each analysis. The split ratio was 0.1:1, and the flow rate was 200 μl per min. The gas saver was set at 20 ml per min for 2 min. The carrier gas was helium (obtained from Air Liquide) and was used at a flow rate of 1.7 ml per min in the column and at a flow rate of 4.2 ml per min in the injector. The gas pressure was 180 kPa. The temperature of the injector was 210°C. The elution conditions were 2 min at 30°C, followed by an increase in the temperature from 30 to 210°C at a rate of 5°C per min. The retention times of DMSe and DMDSe were estimated by using gases purchased from Sigma-Aldrich (St. Louis, Mo.). The detection limits were about 50 ng of DMSe per liter and 10 ng of DMDSe per liter.

The methylation activities of the freshwater samples were measured by using the modified procedure of Chau et al. (4). Flasks (150 ml) containing 50 ml portions of freshwater samples, supplemented or not supplemented with nutrient broth (0.5%), glucose (0.1%), and sodium selenite or (methyl)selenocysteine (Sigma) (10 ppm), were sealed and incubated for 1 week at 25°C. The headspace gases from the flasks were analyzed as described above, except for 100-fold to 10,000-fold dilutions of the headspace gases. 10³ to 10² cells of a freshwater *tpm*-harboring strain per ml were added along with sodium selenite or (methyl)selenocysteine.

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Methylation phenotypes of E. coli or DMDSe detected; nd, not determined. The positions and orientations of orfA and selenium precursor, DMDSe were detected with and without nutrient broth and glucose were required. When (methyl)selenocysteine was used as the methyl donor, addition of sodium selenite (but nutrient broth and glucose were omitted) produced methylated derivatives of selenium after addition of sodium selenate. The values are means ± standard deviations of the means. All samples were analyzed at least twice, and all analyses were performed at least three times.

### Results and Discussion

**Bacterial selenium methylation by freshwater.** A freshwater environment exhibiting reproductive emission of DMSe and DMDSe after it was spiked with various selenium substrates was used to investigate the role of bTPMT-expressing bacteria in this process. This freshwater environment was found to produce methylated derivatives of selenium after addition of inorganic and organic Se precursors (Table 1). DMSe was detected in the headspace gases of the freshwater supplemented with sodium selenite (but nutrient broth and glucose were required). When (methyl)selencysteine was used as the selenium precursor, DMDSe was detected with and without addition of nutrient broth and glucose, and DMDSe was detected only after addition of nutrient broth and glucose (Table 1). In the absence of supplemental selenium, no volatile selenium derivatives were detected. These observations confirmed previous reports suggesting that most environments have the ability to biologically transform selenium derivatives into DMSe and DMDSe (4, 8, 15, 27). Emission of DMDSe could not be reproducibly detected after addition of sodium selenite to water samples supplemented with nutrient broth and glucose, and the levels of DMDSe were always less than the DMSe levels (data not shown); however, DMDSe emission was greater than DMSe emission (below detectable levels) in samples that were not supplemented with nutrient broth and glucose but were spiked with (methyl)selencysteine. Thus, it appears that a distinct microbial flora or processes can be favored depending on the carbon and nitrogen sources available, creating variations in the ratio of methylated selenium species produced by an environment.

**Occurrence of tpm in freshwater bacteria.** Bacteria growing on 10 μM and 30 mM sodium selenite or on 10 μM and 30 mM sodium selenate were isolated from the freshwater environment described above. About 2.3 × 10^4 and 1.7 × 10^5 CFU · ml⁻¹ were obtained with 10 μM sodium selenate and 10 μM sodium selenite, respectively, representing 7.9 and 5.9% of the viable bacterial heterotrophs (2.9 × 10^6 CFU · ml⁻¹), respectively. Around 1.4% of the viable bacterial heterotrophs (corresponding to 4 × 10^5 cells · ml⁻¹) were able to grow on media supplemented with 30 mM selenite. Bacterial strains were not obtained on media supplemented with 30 mM sodium selenite. These data confirmed that even in the absence of Se contamination (the freshwater studied contained less than 2 ppb of total selenium), Se oxyanion-resistant bacteria could be isolated. This is not surprising because the ability to detoxify or grow on heavy metals or toxic metalloids seems to be widely distributed and not necessarily linked to recent contamination by the elements, as previously observed for selenium (3) and for mercury (26).

Colonies of a representative set (around 50 strains) of the bacterial heterotrophs isolated on sodium selenite and sodium selenate was performed, and the strains were screened by DNA hybridization by using a PCR-amplified tpm probe from P. syringae pv pisi strain 203 total DNA. Ten percent (5 of 48 strains) and 4.5% (2 of 45 strains) of the strains growing on media containing 10 μM sodium selenite and 10 μM sodium selenate, respectively, gave positive hybridization signals.
but none of the 37 strains obtained in the presence of 30 mM sodium selenate gave positive hybridization signals. Our freshwater environment thus harbored tpm-hybridizing sequences that could play a role in the global selenium methylation activity. However, only a small portion of the bacterial heterotrophs selected seemed to harbor tpm-hybridizing sequences. It is noteworthy that about one-half of our freshwater strains growing on TSA containing 10 μM selenite produced red intracellular deposits indicative of accumulation of amorphous elemental selenium, but red colonies were not observed on TSA containing 10 μM or 30 mM selenate. These observations confirm that reduction can play a role in the adaptation of natural bacterial communities to selenite and that selenate-grown bacterial cells rarely produce elemental selenium (3, 21).

Diversity and phylogenetic affiliation of tpm-hybridizing bacteria. The seven tpm-hybridizing bacteria were typed by using the length and sequence polymorphism of the intergenic spacer between the rrs and rrl tRNA genes. This approach was previously shown to be reliable for typing bacterial strains at the species and genus levels (16). No difference between tpm-hybridizing strains was observed. All of these strains had intergenic spacers that were about the same size (about 620 bp) and similar restriction patterns (data not shown). The entire rrs sequence of one of these strains, designated Hsa.28, was determined (accession no. AY259120). BLAST analysis of this sequence suggested that the most closely related rrs sequences in the GenBank database are sequences from members of the γ subclass of the Proteobacteria affiliated with the genus Pseudomonas. A phylogenetic analysis of the Hsa.28 rrs gene sequence and 50 other rrs sequences from representative Pseudomonas species was performed. The neighbor-joining tree inferred from this data set showed that the Hsa.28 rrs sequence groups significantly with Pseudomonas anguilliseptica sequences (supported by 94% of the bootstrap replicates) (data not shown). It has been well established that rrs molecular phylogeny can be used to classify bacterial species. Thus, we concluded from this analysis that the Hsa.28 strain is a Pseudomonas strain closely related to P. anguilliseptica. However, the average percentage of divergence (which is more than 1%) with P. anguilliseptica sequences suggests that strain Hsa.28 belongs to a different species. This should be investigated further by performing total DNA-DNA hybridization.

Genetic characterization of a freshwater bacterial tpm sequence. The tpm determinant of Pseudomonas strain Hsa.28 was isolated by constructing and screening a genomic DNA library of this strain. tpm-positive clones were identified by colony hybridization by using a PCR-amplified tpm probe (see Materials and Methods). Two hybridizing cosmId clones were detected and designated pHLN.1 and pHLN.2. Only pHLN.1 was kept and used for further study. DNA blot analysis of this cosmid clone harbored an approximately 800-bp hybridizing PstI fragment, which was subcloned (the construct was designated pA1) (Fig. 1) and sequenced. DNA sequence analysis of the insert (794 bp) confirmed that the library cosmid clone harbored a tpm gene sequence. BLASTX analysis revealed 51% identity and 70% similarity between the deduced amino acid sequence encoded by the P. syringae tpm gene (positions 26 to 217; accession no. L49178) and the deduced amino acid sequence encoded by the P. syringae tpm gene (positions 26 to 217; accession no. L49178) and the deduced amino acid sequence encoded by the P. syringae tpm gene (positions 26 to 217; accession no. L49178) and the deduced amino acid sequence encoded by the P. syringae tpm gene (positions 26 to 217; accession no. L49178) and the deduced amino acid sequence encoded by the P. syringae tpm gene (positions 26 to 217; accession no. L49178).
The Hsa.28 sequence encoded by the PstI fragment. The PstI sequence was used to design sequencing primers and perform additional sequencing of the Hsa.28 tpm sequence (and the flanking region) with the pH1N.1 library clone. A 1,794-bp region was completely sequenced (accession no. AY259120) and analyzed. Testcode, frameplot, and BLASTX analyses of this sequence allowed us to detect three highly probable coding regions: (i) orfA from position 498 to position < 1, (ii) tpm from position 641 to position 1297, and (iii) htx from position > 1,794 to position 1,365. The deduced amino acid sequences encoded by the coding regions exhibited 78% similarity from position 2 to position 156 of a 256-amino-acid hypothetical protein (designated OrfA; accession no. AAG06227 and related COG 3384), and 69% similarity from position 81 to position 287 of HtpX (a 293-amino-acid heat shock protein; accession no. BAB35962), and 52% similarity from position 24 to position 237 of accessions no. AAD17293), and 52% similarity from position 27 to position 208 of accession no. AAB71627), respectively. The positions and orientations of orfA, htx, and tpm-like determinants are shown in Fig. 1. htx was previously found next to tpm in P. syringae and P. aeruginosa genomes. Such a close and repetitive association of tpm and htx could indicate that htx is involved in bacterial adaptation to metalloids. A multiple alignment of all bTPMTs and human TPMT revealed the conserved nature of some amino acid residues in this family of methyltransferases (Fig. 2). In this multiple alignment, the Bordetella pertussis bTPMT sequence was deduced from the promoter region of a virulence gene, and it does not appear to be functional. Its genetic drift explains the higher level of divergence observed with the other sequences. The Pseudomonas stutzeri tpm sequence was deduced from a partial coding sequence. A PLoCG domain, from position 46 to position 50, is conserved in bacteria and humans. TPMT appears to be one of the few proteins which can be used to compare phylogenetic trends in eukaryotes and prokaryotes. This phylogenetic proximity suggests that bacteria could be used to investigate TPMT-driven methylation of various drugs, including the well-known thiopurines (10). TPMT is a major human detoxifying enzyme (29).

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Medium</th>
<th>Conc of DMSe (10^3 AU · ml⁻¹)</th>
<th>Conc of DMDSe (10^3 AU · ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas strain Hsa. 28</td>
<td>LB</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>LB + sodium selenite</td>
<td>1,263 ± 687</td>
<td>748 ± 397</td>
</tr>
<tr>
<td></td>
<td>LB + (methyl)selenocysteine</td>
<td>3,109 ± 708</td>
<td>BDL</td>
</tr>
<tr>
<td>E. coli harboring pHLN.1d</td>
<td>LB</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>LB + sodium selenite</td>
<td>BDL</td>
<td>55 ± 15</td>
</tr>
<tr>
<td></td>
<td>LB + (methyl)selenocysteine</td>
<td>250 ± 110</td>
<td>84 ± 80</td>
</tr>
<tr>
<td>E. coli harboring pF31d</td>
<td>LB</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>LB + sodium selenite</td>
<td>1,065 ± 476</td>
<td>246 ± 69</td>
</tr>
<tr>
<td></td>
<td>LB + (methyl)selenocysteine</td>
<td>2,043 ± 338</td>
<td>35 ± 30</td>
</tr>
<tr>
<td>E. coli harboring PQC1d</td>
<td>LB</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>LB + sodium selenite</td>
<td>307 ± 118</td>
<td>64 ± 21</td>
</tr>
<tr>
<td></td>
<td>LB + (methyl)selenocysteine</td>
<td>4,284 ± 1,344</td>
<td>245 ± 30</td>
</tr>
<tr>
<td>E. coli harboring pSK−</td>
<td>LB</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>LB + sodium selenite</td>
<td>342 ± 239</td>
<td>368 ± 149</td>
</tr>
</tbody>
</table>

* E. coli cells were grown in LB broth (24) with either 10 ppm of sodium selenite or 10 ppm of (methyl)selenocysteine. See the text for a description of the GC-MS method used to estimate DMSe and DMDSe production.

** Data are expressed as GC-MS spectrum peak areas of (in arbitrary units [AU]) of DMSe or DMDSe per milliliter of injected headspace gases. The values are means ± standard deviations of the means. All samples were analyzed at least twice, and all analyses were performed at least three times.

b BDL, below detectable level or absent. The detection limit was between 10 and 50 ng · liter⁻¹.

d Details concerning plasmid construction are shown in Fig. 1.

### Table 3. DMSe and DMDSe produced by freshwater samples supplemented with sodium selenite or (methyl)selenocysteine

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Pseudomonas strain Hsa.28 density (CFU · ml⁻¹)</th>
<th>Conc of DMSe (10^3 AU · ml⁻¹)</th>
<th>Conc of DMDSe (10^3 AU · ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium selenite</td>
<td>10⁵</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>BDL</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>45 ± 45</td>
<td>BDL</td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>240 ± 129</td>
<td>BDL</td>
</tr>
<tr>
<td>(Methyl)selenocysteine</td>
<td>10⁵</td>
<td>932 ± 158</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁴</td>
<td>2,991 ± 301</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10³</td>
<td>2,556 ± 1,800</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10²</td>
<td>1,259 ± 318</td>
<td></td>
</tr>
</tbody>
</table>

* Freshwater samples were incubated with either 10 ppm of sodium selenite or 10 ppm of (methyl)selenocysteine. See the text for a description of the GC-MS method used to estimate DMSe and DMDSe emission.

** Number of Hsa.28 CFU per milliliter of freshwater.

a Data are expressed as GC-MS spectrum peak areas (in arbitrary units [AU]) of DMSe or DMDSe per milliliter of injected headspace gas. The values are means ± standard deviations of the means. All samples were analyzed at least twice, and all analyses were performed at least three times.

b BDL, below detectable level or absent. The detection limit was between 10 and 50 ng · liter⁻¹.
Selenium methylation by freshwater bTPMT. In a previous study it was shown that E. coli cells expressing tpm of P. syringae methylate selenite, selenate, and (methyl)selenocysteine into DMSe and DMDSe (25). In the present study, significant levels of DMSe and DMDSe were detected in the headspace gases of flasks containing Hsa.28 LB broth media which were supplemented with sodium selenite or (methyl)selenocysteine (Table 2). These results demonstrated that freshwater bacteria (which likely express bTPMT) are able to methylate selenium in organic and inorganic forms. To show that the tpm determinant is involved in the Hsa.28 selenium methylation process, two PCR-generated subclones, pF31 (containing the pHLN.1 sequenced region) and pQC1 (containing the tpm ORF), were constructed (Fig. 1 shows the details of construction). E. coli DH5α harboring the vector pSK—was included as a negative control in all these experiments. The headspace gases of cultures of E. coli strains harboring these constructs or pHLN.1 (cosmid) were then analyzed by GC-MS after growth with sodium selenite and (methyl)selenocysteine supplements. E. coli cells harboring pHLN.1 produced significant levels of DMDSe, but DMSe could not be detected. pHLN.1 has a long insert that is about 30 to 40 kb long. It is thus possible that other gene products of pHLN.1 interfered with the emission of DMSe. E. coli cells harboring pF31 and pQC1 produced significant levels of DMSe and DMDSe when they were grown on sodium selenite and significant levels of only DMSe (P < 0.05) when they were grown on (methyl)selenocysteine (as opposed to negative control) (Table 2). The levels of DMSe emitted were always higher (P < 0.05) in the presence of (methyl)selenocysteine than in the presence of sodium selenite. DMDSe was always detected but only at significant levels (compared to the control levels) with tpm-harboring strains grown with sodium selenite (Table 2). These results are in line with those obtained when the P. syringae tpm sequence was used (25).

The involvement of tpm-harboring Pseudomonas strain Hsa.28 in the global selenium methylation activity of a freshwater environment was investigated. The effects of the size of the Hsa.28 population in freshwater supplemented with sodium selenite or (methyl)selenocysteine on the production of DMSe and DMDSe were estimated. Production of DMSe from sodium selenite was increased by reinoculation of 10^6 Hsa.28 CFU · ml⁻¹ and seemed to increase significantly with larger populations (P < 0.05) (Table 3). Addition of (methyl)selenocysteine was found to increase the production of DMSe and/or DMDSe after inoculation of 100 Hsa.28 CFU · ml⁻¹ (P < 0.05) (Table 3). The amount of DMSe produced seemed to be correlated with the number of inoculated cells (P < 0.05), whereas the amount of DMDSe produced remained constant (P > 0.05). These results showed that enrichment of Pseudomonas strain Hsa.28 can increase selenium methylation in freshwater.

In conclusion, this is the first study in which it was shown that bTPMT was found in a natural freshwater selenium-methylating environment. The presence of the enzyme in this environment supports the hypothesis that it is involved in the natural selenium methylation processes. Analyses of the distribution of tpm in other water and soil selenium-methylating environments are in progress. These analyses should provide further insight into the ecological role of the bTPMT detoxifying enzyme.

ACKNOWLEDGMENTS

We thank the Pôle Expertise Eau of Danone France for performing the GC-MS analyses.

This work was supported in part by the CNRS (France).

REFERENCES


