Horizontal spread of *mer* operons among Gram-positive bacteria in natural environments

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Horizontal dissemination of the genes responsible for resistance to toxic pollutants may play a key role in the adaptation of bacterial populations to environmental contaminants. However, the frequency and extent of gene dissemination in natural environments is not known. A natural horizontal spread of two distinct mercury resistance (*mer*) operon variants, which occurred amongst diverse *Bacillus* and related species over wide geographical areas, is reported. One *mer* variant encodes a mercuric reductase with a single N-terminal domain, whilst the other encodes a reductase with a duplicated N-terminal domain. The strains containing the former *mer* operon types are sensitive to organomercurials, and are most common in the terrestrial mercury-resistant *Bacillus* populations studied in this work. The strains containing the latter operon types are resistant to organomercurials, and dominate in a Minamata Bay mercury-resistant *Bacillus* population, previously described in the literature. At least three distinct transposons (related to a class II vancomycin-resistance transposon, Tn1546, from a clinical *Enterococcus* strain) and conjugative plasmids are implicated as mediators of the spread of these *mer* operons.

Keywords: *mer* operon, horizontal gene transfer, *Bacillus*, *Enterococcus*, Tn1546, class II transposons

INTRODUCTION

Mercury resistance (Hg^{R}) in both clinical and environmental isolates of bacteria has been widely reported (Nakahara *et al.*, 1977; Gilbert & Summers, 1988; Olson *et al.*, 1989; for reviews see Robinson & Tuovinen, 1984; Gadd, 1990). Mercury resistance operons (*mer*) have been characterized (Misra, 1992; Hobman & Brown, 1997). Because the nucleotide

sequences of at least 11 *mer* operons from different bacteria are known (see reviews by Summers, 1986; Silver & Phung, 1996; Hobman & Brown, 1997; Osborn *et al.*, 1997), Hg^{R} provides an excellent model system for studying horizontal gene transfer in the environment. Narrow-spectrum *mer* operons confer resistance to inorganic mercury salts: they encode a mercury-responsive regulatory protein (MerR), one or more mercury-transport proteins, and the enzyme mercuric reductase (MR). MR (encoded by the *merA* gene) reduces divalent mercury (Hg^{II}) to the relatively non-toxic metallic mercury (Hg^0) in an NADPH-dependent reaction. Broad-spectrum *mer* operons confer resistance to organomercurials (for example, phenylmercuric acetate, PMA) as well as inorganic mercury (Brown *et al.*, 1989) and contain an additional gene (*merB*) encoding the enzyme organomercurial lyase, which catalyses the cleavage of the C–Hg bond of many organomercurials (Begley *et al.*, 1986).

Abbreviations: MR, mercuric reductase; PMA, phenylmercuric acetate.

The EMBL accession numbers for the nucleotide sequences of *mer* operons and their flanking regions reported in this paper are: pKLH3, X99457 and Y08064; pKLH301, Y09027, Y10104 and Y10105; pKLH302, Y09024; pKLH304, Y09907; *B. cereus* RC607 *mer* operon flanking region, Y08065. The EMBL accession numbers for the nucleotide sequences of the internal regions of the 16S rRNA genes are: *Exiguobacterium* TC38-2b, Y15051; *Bacillus sphaericus* FA8-2, Y15045; *B. cereus* TA32-5, Y15050; *B. cereus* RC607, Y15048; *B. cereus* CH70-2, Y15046; *B. cereus* VKM684, Y15053.

With a few possible exceptions (Shiratori *et al.*, 1989; Kholodii *et al.*, 1993b, 1997), *mer* operons from Gram-negative bacteria have been found on plasmids. There is little evidence for plasmid-borne *mer* operons in Gram-positive bacteria isolated from the environment (Belliveau & Trevors, 1990) and several papers report the apparent chromosomal location of such *mer* operons (Witte *et al.*, 1986; Mahler *et al.*, 1986; Wang *et al.*, 1987; Nakamura & Silver, 1994). In Gram-negative bacteria, *mer* operons have often been found on transposons, many of which are class II (Tn3 family) transposons of the Tn21 sub-group (for a review see Grinsted *et al.*, 1990) or the recently discovered transposons Tn5041 (Kholodii *et al.*, 1997) and Tn5053 (Hobman *et al.*, 1994; Kholodii *et al.*, 1993b, 1995). The association of *mer* operons with transposons has not been reported for Gram-positive bacteria, though Siemieniak *et al.* (1990) have reported the nucleotide sequence of a Tn3-related class II transposon, Tn4556, from *Streptomyces fradiae*. This transposon contained ORFs that had a low shared identity (30–40%) with bacterial mercuric reductases, but did not confer Hg^R on the host organism.

Recent studies demonstrate considerable diversity in Gram-negative bacterial *mer* operons at the DNA sequence level (Kholodii *et al.*, 1993a, 1995; Osborn *et al.*, 1995, 1996; Yurieva *et al.*, 1997; Liebert *et al.*, 1997), but at the same time there is evidence of a wide geographical distribution of the same sequences. The geographical spread of *mer* operons has been accompanied by horizontal transfer between different bacterial genera (Yurieva *et al.*, 1997). Diversity and dissemination of *mer* operons in Gram-positive bacteria has been less thoroughly studied (Hobman & Brown, 1997; Osborn *et al.*, 1997). Three different *mer* operons have been sequenced from Gram-positive bacteria: *Staphylococcus aureus* plasmid pI258 (Laddaga *et al.*, 1987), *Bacillus* sp. RC607 (Wang *et al.*, 1989), and *Streptomyces lividans* 1326 (Sedlmeier & Altenbuchner, 1992). All of the *mer* operons contained mercuric ion transport genes showing little similarity to Gram-negative transport genes, and *merA* genes homologous to Gram-negative *merA* genes. The *Streptomyces* MR lacked the N-terminal domain characteristic of MRs from Gram-negative bacteria. This domain (homologous to the Hg²⁺-binding protein MerP and dispensable for *in vitro* enzyme activity) is thought to have functional significance in natural environments (Silver & Phung, 1996). The staphylococcal MR contained a single N-terminal domain, whilst the *Bacillus* RC607 MR contained a longer duplicated domain. Using immunological methods, we have previously identified at least five distinct subtypes of MRs in *Bacillus* and related strains isolated from terrestrial samples from different parts of the former Soviet Union (Bogdanova *et al.*, 1988). Trypsin cleavage experiments demonstrated that only three strains contained MRs with a long N-terminal domain while the remaining 22 strains tested contained MRs with the short N-terminal domain (Bogdanova & Mindlin, 1989, 1991; E. Bogdanova & S. Mindlin,

unpublished results). Nakamura & Silver (1994) reported surprisingly low diversity of *mer* operons among 78 mercury-resistant *Bacillus* strains isolated from sediments from Minamata Bay, Japan, the site of a severe human methylmercury poisoning epidemic. All of these strains contained apparently chromosomally located broad spectrum *mer* operons encoding MRs with the duplicated N-terminal domain. Most of the strains contained *mer* operons showing restriction maps that were identical, or closely related, to that of the *Bacillus* sp. RC607 *mer* operon isolated from Boston Harbor, USA.

Here we confirm by DNA sequence analysis the considerable diversity of *mer* operons in terrestrial *Bacillus* strains suggested by our earlier immunological studies. We demonstrate that wide geographical spread and horizontal transfer of distinct *mer* operon variants has occurred amongst *Bacillus* species in the natural environment. We report DNA sequences characteristic of class II transposons at the ends of the *mer* operons studied. This is the first clear indication of the involvement of class II transposons in dissemination of mercury resistance in Gram-positive bacteria.

METHODS

Bacterial strains and plasmids. The strains used in this study are shown in Table 1. Most of these are from our collection of Hg^R low G+C content Gram-positive bacteria (mainly *Bacillus* spp.), which originally included 14 terrestrial strains from the Kamchatka peninsula, three strains from the Kuril islands, one strain from Central Asia, one strain from the Moscow region and six strains from Carpathia (Ukraine) (Bogdanova *et al.*, 1989; Bogdanova & Mindlin, 1991). The strains were collected with the aim that each geographical locale should represent a maximum species diversity, and strains which were identical by morphological and physiological criteria were therefore not duplicated. The strains were collected between 1985 and 1987. The freshly isolated strains were at that time preserved for long term storage using two methods: by freeze drying and under a layer of mineral oil. Stock cultures were maintained at 4–8 °C on Luria-Bertani (LB) agar with 5–7 µg HgCl₂ ml⁻¹. In mating experiments, we used the Hg^S derivative of strain TC38-2b (Bogdanova & Mindlin, 1989). In this work we constructed its spontaneous mutant, VKM382-2 (Rif^R Str^R), which was resistant to 30 µg rifampicin ml⁻¹ and 100 µg streptomycin ml⁻¹ on LB agar. The cloning vectors pACYC184, pUC19, pTZ18 and pTZ19 were used. The *mer* operon recombinant clones (Table 2) were obtained by standard methods (Sambrook *et al.*, 1989) using LB agar plates supplemented with the appropriate selective agents at the following concentrations (µg ml⁻¹): ampicillin, 200; tetracycline, 20; chloramphenicol, 50; HgCl₂, 10.

Bacteria were identified both according to the taxonomic scheme of Bergey's Manual (Claus & Berkely, 1986) and by determination of the nucleotide sequence of region 343–709 of the 16S rRNA gene. The 16S rRNA gene was amplified from genomic DNA by PCR with the forward primer BCF1, described by Cano *et al.* (1994), and the reverse primer 5' ACATCTCTACGCATTTCACC 3' (this work) and cloned into a plasmid vector using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. The 16S rRNA fragment was sequenced in both directions from the vector primers SP6 and T7. The FA8-2 and TC47-5 16S rRNA gene

Table 1. Characteristics of strains and *mer* operons

Strain	Source*	Hybridization with probes†			Location of <i>mer</i> operon	Resistance to PMA‡	Mercuric reductase	
		pKLH3.2 <i>merR</i> , ORFs	pKLH3.3 <i>merA</i>	pKLH3.4 <i>merB</i>			N-terminal domain§	Immunological subtype
1 <i>Bacillus sphaericus</i> FA8-2	1	+(9.5 kb)	+(9.5 kb)	—	pKLH301	0.1 (5)	Short	II-1
2 <i>B. cereus</i> TA32-5	1	+(~15 kb)	+(~15 kb)	—	pKLH302	<0.1–0.1 (7)	Short	II-1
3 <i>B. cereus</i> CH70-2	2	+(~15 kb)	+(~15 kb)	—	pKLH303	0.1 (2)	Short	II-1
4 Unidentified TA32-12	1	+(7 and 8 kb)	+(7 and 8 kb)	—	Chromosome	<0.1–0.1 (2)	Short	II-1
5 <i>B. licheniformis</i> FA6-12	1	+(3.2 kb)	+(3.2 kb)	—	Chromosome	0.1 (3)	Short	II-2
6 <i>Exiguobacterium</i> sp. TC38-2b	3	+(1.3 kb)	+(16.8 kb)	+(16.8 kb)	pKLH3	0.25–1 (5)	Long	II-3
7 <i>B. cereus</i> RC607	4	+(1.3 kb)	+(6.8 kb)	+(6.8 kb)	Chromosome¶	0.5–>5 (7)	Long	II-3
8 <i>B. cereus</i> VKM684 (ATCC 10702)	6	+(1.3 kb)	+(6.5 kb)	+(6.5 kb)	Chromosome	0.5–1 (3)	Long	II-3
9 <i>B. megaterium</i> MK64-1	5	+(1.3 kb)	+(4.6 kb)	—	pKLH304	1–1.5 (3)	Long	NT
10 ' <i>B. macroides</i> ' TC47-5	3	+(2 kb)	+(2 and 3.8 kb)	—	pKLH305	0.25–1 (3)	Long	NT
11 Unidentified TC22-9	3	+(3 kb)	+(3 and 5 kb)	—	Plasmid	0.1–0.25 (2)	Short	II-4
12 Unidentified FA11-3	1	±	±	—	Plasmid	NT	Short	II-7

NT, Not tested.

* Source indicates the area of isolation: 1, the Kamchatka Peninsula; 2, Central Asia (mouse intestine); 3, Carpathia (Ukraine); 4, Boston Harbor (USA); 5, Kuril Islands; 6, not known (Bogdanova *et al.*, 1992).

† Location of *mer* operons was determined by Southern hybridization of plasmid DNA preparations with the *mer* DNA probes as described in Methods. When visible restriction fragments showed hybridization with the probes we concluded that the *mer* operon was located in a plasmid. In the absence of hybridization of plasmid DNA we considered the *mer* operon as chromosomal. +, Hybridization of *Eco*RI fragments(s) with the probes described in Table 2 and shown in Figs 1 and 3(b); —, no hybridization with probe; ±, weak hybridization with probe.

‡ Resistance to PMA was determined as described in Methods. Number of experiments is shown in parentheses. Strains 6–10 show broad-spectrum resistance, others show narrow-spectrum resistance. Strain 12 was not tested because it does not grown on LB agar.

§ Bogdanova & Mindlin (1989, 1991).

|| Numbering of immunological subtypes from II-1 to II-7 reflects decreasing immunological similarity to MR from *B. sphaericus* FA8-2. *Staphylococcus aureus* MR represents the subtype II-7 (Bogdanova *et al.*, 1988).

¶ Wang *et al.* (1989).

Table 2. Recombinant plasmids

Plasmid	Phenotype	Description
pYW33*	Hg ^R	12.8 kb <i>Hind</i> III fragment from RC607 in pUC9 (Fig. 3b)
pKLH301.1†	Hg ^S	9.5 kb <i>Eco</i> RI fragment from pKLH301 in pACYC184 (Fig. 3a)
pKLH302.1†	Hg ^S	5.5 kb <i>Hind</i> III fragment from pKLH302 in pACYC184 (Fig. 3a)
pKLH302.2	Hg ^S	2.8 kb <i>Bgl</i> II fragment from pKLH302.1 in pTZ19 (Fig. 1)
pKLH6.1‡	Hg ^R	3.2 kb <i>Eco</i> RI fragment from FA6-12 chromosome in pACYC184
pKLH3.1‡	Hg ^R	12.5 kb <i>Hind</i> III fragment from pKLH3 into pACYC184 (Fig. 3b)
pKLH3.2	Hg ^S	1.3 kb <i>Eco</i> RI fragment from pKLH3.1 in pUC19 (Fig. 3b)
pKLH3.3	Hg ^S	2.1 kb <i>Eco</i> RI/ <i>Bgl</i> II fragment from pKLH3.1 in pUC19 (Fig. 1)
pKLH3.4	Hg ^S	1.1 kb <i>Nco</i> I fragment from pKLH3.1 in pACYC184 (Fig. 3b)
pKLH3.5§	Hg ^S	16.8 kb <i>Eco</i> RI fragment from pKLH3 in pACYC184
pKLH304.1‡	Hg ^R	8.0 kb <i>Hind</i> III fragment from pKLH304 in pACYC184 (Fig. 3b)
pKLH304.2§	Hg ^S	4.6 kb <i>Eco</i> RI fragment from pKLH304 in pACYC184
pKLH305.1‡	Hg ^R	20 kb <i>Hind</i> III fragment from pKLH305 in pACYC184
pKLH305.2	Hg ^R	Self-ligation of 5.8 kb <i>Nco</i> I fragment from pKLH305.1

* Obtained from I. Mahler, Brandeis University, Waltham, MA, USA.

† Selected by hybridization with the probes pKLH3.2 and pKLH3.3.

‡ Selected by resistance to 10 µg HgCl₂ ml⁻¹ on LB agar.

§ Selected by resistance to HgCl₂ in the presence of plasmid pYW22 (obtained from I. Mahler) containing the mercury transport genes (Wang *et al.*, 1987).

were sequenced directly from the PCR fragments. The DNA sequences were compared with those in the Ribosomal Database Project (RDP) (Maidak *et al.*, 1994) and analysed by

using the SIMILARITY RANK and ALIGN SEQUENCE programs in RDP, and with those of the NCBI non-redundant database by using the BLAST program (Altschul *et al.*, 1990). To construct

the dendrograms, sequence alignments were carried out using the VOSTORG package (Zharkikh *et al.*, 1991). Genetic distances were estimated, bootstrap analysis conducted, and dendrograms constructed by TREECON for Windows version 1.1 (Van de Peer & De Wachter, 1994).

Mating conditions. Donor (the original TC38-2b, Hg^R Rif^S Str^S) and recipient (VKM382-2, Hg^S Rif^R Str^R) cultures were grown separately in LB broth (Sambrook *et al.*, 1989) with the appropriate selection (3 µg HgCl₂ ml⁻¹ or 10 µg rifampicin ml⁻¹ and 20 µg streptomycin ml⁻¹, respectively) overnight at 30 °C with shaking. The cultures were diluted 1:10 in fresh LB broth and incubated for a further 6–8 h. After this additional growth, the donor and recipient cultures were mixed in a 1:1 ratio; 10 µl aliquots of each mixture were spread over a 1 cm² area on LB agar and incubated for 16–18 h at 30 °C. The mixed growth was scraped off the plate, resuspended, diluted and plated on selective LB agar to determine the number of c.f.u. of the donor (5 µg HgCl₂ ml⁻¹), recipient (30 µg rifampicin ml⁻¹ and 100 µg streptomycin ml⁻¹), and transconjugants (5 µg HgCl₂ ml⁻¹, 30 µg rifampicin ml⁻¹ and 100 µg streptomycin ml⁻¹). The plates were incubated for 24–30 h at 30 °C. All tested transconjugants which were resistant to both HgCl₂ and antibiotics contained a plasmid with the same *EcoRI* restriction pattern as pKLH3 (data not shown). The pKLH3 transfer frequencies varied from 1.5 × 10⁻⁵ to 2.5 × 10⁻⁶ per recipient cell. The frequencies of conversion to Hg^R in recipient cultures and to antibiotic resistance in donor cultures grown separately on LB agar were less than 1 × 10⁻⁸.

Determination of narrow- versus broad-spectrum resistance type. Overnight cultures of bacteria grown at 30 °C in LB broth containing 1 µg HgCl₂ ml⁻¹ were supplemented with 4 vols LB broth containing 0.5 µg HgCl₂ ml⁻¹. After 1.5 h incubation bacterial suspensions were diluted to OD₆₀₀ 0.1 with LB broth containing 0.5 µg HgCl₂ ml⁻¹; 5 µl aliquots were dropped on LB agar plates containing varying concentrations of HgCl₂ (5–80 µg ml⁻¹) or PMA (0.1–5.0 µg ml⁻¹), and grown for 24 h at 30 °C. The highest concentrations of HgCl₂ and PMA that did not show inhibition of bacterial growth were recorded.

Plasmid and total DNA preparation. Plasmid DNA from the strains of environmental origin was isolated by alkaline lysis (Birnboim & Doly, 1979) with modifications. The best yields were obtained when it was possible to grow cells in a poor medium and to lyse them quickly at the lowest possible temperature. Accordingly, strains FA8-2, TC38-2b, MK64-1 and TC47-5 were grown in LB broth; the remaining strains were grown in M9 medium containing aminopeptide and glucose (Bogdanova & Mindlin, 1991). Both media contained 1 µg HgCl₂ ml⁻¹. Cell pellets from 10–20 ml overnight cultures were washed with lysis buffer (50 mM glucose, 25 mM Tris/HCl, pH 8.0, 20 mM EDTA) and incubated in the same buffer containing 2 mg lysozyme ml⁻¹ for 10–20 min at 37 °C (strains TA32-5, CH70-2, RC607 and VKM684), for 15 min at 25 °C (strain MK64-1), or for 2–30 min at 0–4 °C (all other strains) with subsequent addition of NaOH/SDS. Immediately after isolation DNA was deproteinized with phenol/chloroform according to standard procedures (Sambrook *et al.*, 1989). To isolate total DNA, bacterial lysis was carried out in 10 mM Tris/HCl (pH 8.0), 50 mM EDTA containing 2 mg lysozyme ml⁻¹ for 1–3 h at 37 °C (strains TA32-5, CH70-2, RC607 and VKM684) or for 15–30 min at 30 °C (all other strains). The cell suspensions were then rapidly homogenized in EDTA/SDS solution (final concentrations of 0.1 M and 5%, w/v, respectively) and mixed with an equal volume of phenol containing 0.1% hydroxyquinoline, and saturated

with 0.1 M EDTA, 2% SDS and 0.5 M NaClO₄. NaClO₄ (5 M solution) was added to 0.5 M, the mixture was shaken for 30 min and 0.1 vol. chloroform was added. After three or four phenol extractions DNA was precipitated by the addition of 1 vol. ethanol, resuspended, and treated with ribonuclease and proteinase according to standard procedures (Sambrook *et al.*, 1989). Recombinant plasmid DNA from *E. coli* was prepared by a standard alkaline lysis procedure (Birnboim & Doly, 1979). Restriction enzyme digests were carried out by standard procedures (Sambrook *et al.*, 1989). The size of the plasmids was determined as a sum of the molecular masses of their *EcoRI* fragments.

Southern blot hybridization. DNA probes prepared by restriction enzyme digestion of the recombinant plasmids were excised from low-melting-point 0.7% agarose Tris/borate/EDTA (TBE) gels and labelled with [³²P]dATP or dCTP by nick-translation (Sambrook *et al.*, 1989). Total and plasmid DNA preparations were digested with the appropriate restriction enzymes and fractionated by electrophoresis on 1% agarose TBE gels. The DNA was transferred overnight onto BA membrane filters (Schleicher & Schuell). The filters were prehybridized and then hybridized with the labelled probes for 16 h at 65 °C in a solution containing 4 × SSC, 0.1% SDS, 5 × Denhardt's reagent and 0.1 mg denatured, fragmented calf spleen DNA ml⁻¹. Filters were washed in 10 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA and 0.2% SDS, at room temperature, and exposed to X-ray film at -70 °C for 1–7 d.

DNA sequencing. DNA was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) either using an automated DNA sequencer (Applied Biosystems model 370A) with fluorescently labelled dNTP and *TaqI* polymerase, or manually using ³²P-labelled dATP and the Sequenase 2.0 DNA sequencing kit (Amersham) in accordance with the manufacturer's instructions. DNA fragments were cloned into plasmids pTZ18 or pTZ19 for sequencing and M13 forward (5' TGAAAACGACGGCCAGT 3') and reverse (5' CAG-GAAACAGCTATGAC 3') oligonucleotides (Biotech) were used as sequencing primers. Alignment of DNA sequences was performed using VOSTORG (Zharkikh *et al.*, 1991) and BLAST (Altschul *et al.*, 1990) programs.

RESULTS AND DISCUSSION

Expression and location of *mer* operons of terrestrial *Bacillus* strains

In this work, we studied ten strains from our collection. These included all of the strains containing MRs with long N-terminal domains, and seven strains containing MRs with short N-terminal domains, representing all immunological subtypes (Table 1). We failed to detect statistically significant differences in HgCl₂ resistance levels within the collection studied, although *in vitro* testing may not be sufficiently sensitive to detect differences that are only evident under environmental conditions. However, the strains containing MRs with short N-terminal domains were PMA sensitive, whilst those containing MRs with long N-terminal domains were PMA resistant, i.e. displayed broad-spectrum resistance. Only one of the broad-spectrum resistant strains contained a *merB* gene detectable by hybridization with the *merB* gene of the RC607 *mer* (Table 1). This indicates that different *merB* genes exist in *Bacillus*

strains, as was recently shown for the *merB* genes of Gram-negative bacteria (Reniero *et al.*, 1995).

Nine strains contained *mer* operons that effectively hybridized with DNA probes to *merR*, the transport genes, or the *merA* gene of the TC38-2b *mer* operon (Table 1). One strain (FA11-3) carried a *mer* operon that showed weak hybridization with these probes. All strains listed in Table 1 except TA32-12 were found to contain plasmids. DNA hybridization experiments demonstrated that the *mer* operons resided on plasmids in most of the strains (Table 1). For TC38-2b, TC47-5 and FA8-2, this conclusion was confirmed in Southern hybridization experiments with Hg^S derivatives of these strains (Bogdanova & Mindlin, 1991) that had lost HgCl₂ resistance as a result of plasmid elimination. Plasmid preparations from these Hg^S derivatives did not hybridize with the *mer* probes.

A mercury-resistance plasmid, pKLH3, found in *Exiguobacterium* sp. TC38-2b, was transferred by conjugation from TC38-2b to its plasmidless Hg^S derivative. This suggests that pKLH3 and other plasmids listed in Table 1 could be involved in the horizontal transfer of *mer* operons. Surprisingly, no plasmids were found in the Minamata Bay collection (Nakamura & Silver, 1994). It is unclear whether this is due to possible technical problems of plasmid isolation from certain *Bacillus* strains or reflects a real local variation characteristic of a particular habitat.

Three *mer* operons encoding MRs with the long N-terminal domains (from *Exiguobacterium* sp. TC38-2b, *Bacillus megaterium* MK64-1 and '*B. macroides*' TC47-5) and three *mer* operons encoding MRs with the short N-terminal domain, representing the most common immunological subtypes (from *B. sphaericus* FA8-2, *B. cereus* TA32-5 and *B. licheniformis* FA6-12), were cloned as described in Methods. In *E. coli*, the cloned *mer* operons from TC38-2b and FA6-12 expressed MR activity constitutively, while the TC47-5 operon showed HgCl₂-inducible MR activity (data not shown); all three *mer* operons conferred mercury resistance to *E. coli*. The *mer* operons from strains FA8-2 and TA32-5 did not express mercury resistance and MR activity in *E. coli*, but all of the *mer* operons studied showed inducible MR activity in their original host strains (data not shown). The cause of the differences in the expression of the *mer* operons is under investigation.

Restriction mapping suggests a high diversity of *mer* operons of terrestrial *Bacillus* strains, and long-distance geographical spread of at least two *mer* operon variants

Five distinct variants were identified among the cloned *mer* operons, by Southern blot mapping with 12 restriction endonucleases (Fig. 1). The *mer* operon variant found in TC38-2b was indistinguishable from the *mer* operon of RC607. Identical *mer* operons were found in TA32-5 and FA8-2. Plasmid DNA Southern blot mapping using seven restriction endonucleases revealed no differences between the CH70-2, TA32-5

and FA8-2 *mer* operons and between the VKM684, TC38-2b and RC607 (Fig. 1). The *mer* operons from FA6-12, MK64-1 and TC47-5 were different from each other and from the TC38-2b/RC607/VKM684 and TA32-5/FA8-2/CH70-2 operons. We failed to construct a definitive *mer* operon map for strain TA32-12, since this apparently contained two *mer* operons. A rudimentary map of TC22-9 suggested that it contained a unique variant *mer* operon distinct from those shown in Fig. 1. The *mer* operon from FA11-3 showed poor hybridization with the R607 *mer* and obviously represented a separate variant. Thus, at least seven distinct variants of *mer* operons can be recognized at the DNA sequence level among the ten strains studied. The DNA sequence classification of *mer* operon variants was in agreement with the immunological typing of MRs (Table 1). It remains to be elucidated whether the observed diversity of the *merA* and *merB* gene structures and variations in the regulation of *mer* operon expression detected in this paper reflect environmental selection.

Formally, the same number of restriction map classes of *mer* operons was recognized in the Minamata Bay collection and in our collection. On closer inspection, the *mer* operons of the Minamata Bay collection are actually less diverse than the *mer* operons from our collection. Indeed, the differences within classes I–IV and V–VI identified by Nakamura & Silver (1994) are minor ones and mainly depend on the presence or absence of a single Bgl/II site. This may not be a real difference, but may be a reflection of the protection of Bgl/II sites, possibly by methylation, in certain *Bacillus* strains, as was noted in our work for strains FA8-2 and TA32-12 (see Fig. 1 legend).

Comparison of our collection with the Minamata Bay collection suggests that local populations may differ considerably in the prevalence of various *mer* operon variants. Thus, the narrow-spectrum *mer* operons encoding MRs with a single N-terminal domain are major variants in our collection, but are absent or very rare in the Minamata Bay population. The broad-spectrum *mer* operons of the RC607 type, encoding MRs with a duplicated N-terminal domain, flourish in Minamata Bay sediments (Nakamura & Silver, 1994), but seem to be minor components of the terrestrial Hg^R *Bacillus* populations so far analysed (Bogdanova & Mindlin, 1989, 1991; this paper). It would be interesting to learn whether this is a peculiarity or a general property of marine sediment populations.

Only one *mer* operon variant was common to both our terrestrial strain collection and the Minamata Bay marine sediment collection. This variant, whose presence is reported in strain VKM684 (this paper) was isolated from a non-documented locale and habitat (Dornbush & Pelcak, 1948), in strains from the USA (Wang *et al.*, 1989), Japan (Nakamura & Silver, 1994), Ukraine (this paper) and Great Britain (Osborn *et al.*, 1997), and is apparently distributed worldwide. We hypothesize that the *mer* variant found in TA32-5, FA8-2 and CH70-2 is also disseminated all over the

Fig. 1. Restriction endonuclease maps of *mer* operons. The restriction maps were constructed by Southern blot hybridization using the clones described in Table 2 and their subclones (E. Bogdanova, unpublished data) using pKLH3.2 (Fig. 3b) and pKLH3.3 (Fig. 1) as probes. Horizontal arrows indicate the location and orientation of the *mer* genes verified by DNA sequencing; dashed lines indicate the approximate location of the *mer* genes. *merR* denotes the gene encoding the regulatory protein MerR; *merA* denotes the gene encoding a mercuric reductase (MR); the functional roles of *orf2*, *orf3* and *orf4* are discussed elsewhere (Wang *et al.*, 1989). The cleavage sites are shown by vertical bars. Open boxes indicate the sequenced portions of the FA8-2 operon. The *Bgl*II restriction sites shown in this figure were not detected in plasmid or total DNAs from the original FA8-2 strain (and from TA32-12) using several methods of DNA purification. However, the *Bgl*II restriction site was detected by restriction (and sequencing) after cloning the *mer* operon from FA8-2 in *E. coli* in pKLH301.1. The presence of *Bgl*II and *Hpa*I sites in the CH70-2 strain *mer* operon has not been studied. The *mer* operon from the VKM684 strain was identical to the RC607 operon with all of the enzymes studied: *Eco*RI, *Bgl*II, *Stu*I, *Sph*I and *Nco*I.

world since we were able to detect it in two geographically separated terrestrial habitats (Kamchatka and Central Asia) in a survey of only ten strains. Further studies are required to outline the geographical distribution of other *mer* operon variants described in this work.

***mer* operons representing the same restriction map variant show no DNA sequence divergence, while different variants show 20 % DNA sequence divergence**

We have determined the complete nucleotide sequence of two *mer* operon variants. Sequence analysis confirmed that these *mer* operons had the same relative gene organization as that of the RC607 *mer*. Inspection of the sequences at the beginning of the *merA* genes confirmed our earlier suggestion, based on limited proteolysis data, that the MR of TC38-2b had a duplicated N-terminal domain, while TA32-5 had a single such domain. The *mer* operon of *Exiguobacterium* sp. TC38-2b (from Carpathia, Ukraine) was virtually identical to that of

Bacillus cereus RC607 from Boston Harbor, USA (nine nucleotide substitutions over 4.2 kb of sequenced DNA, i.e. 0.2 % difference), while that of TA32-5 showed a 20 % difference from RC607. We determined the DNA sequence of 1.8 kb of the *mer* operon of FA8-2 (Fig. 1) and found that it was identical to the sequence of the *mer* operon of TA32-5.

16S rRNA sequence characterization of mercury-resistant strains suggests horizontal transfer of two *mer* operon variants

Finding virtually identical *mer* operons in bacteria isolated from different parts of the world may indicate either wide dissemination of a particular strain – a high rate of strain migration in natural populations of certain *Bacillus* species has been demonstrated by Roberts & Cohan (1995) – or wide horizontal dissemination of the *mer* operon between distinct strains. To resolve this question we sequenced the 16S rRNA genes of the strains carrying the *mer* operons shown in Fig. 1. The 16S rRNA gene region 343–709 sequence of strain TC38-

Fig. 2. Neighbour-joining distance dendrograms of the 16S rRNA gene fragment 343–709, showing the relationship between the strains from our collection (a) and the *merA* gene from the same strains (b). The accession numbers for the *merA* genes are: *B. cereus* RC607, M22708; *Exiguobacterium* sp. TC38-2b, X99457; *B. cereus* TA32-5, Y09024; *B. sphaericus* FA8-2, Y10104 and Y10105. *Staphylococcus aureus* was used as the formal outgroup strain. The 16S rRNA and *merA* (p1258) accession numbers are D83353 and L29436, respectively. Genetic distance was estimated by the Jukes–Cantor algorithm. The distance scale indicates the number of nucleotide substitutions per site that have actually occurred since divergence of each pair of sequences. The distance between any two sequences is the sum of the lengths of the horizontal branches between the two; (a) and (b) are drawn to different scales, as indicated. Bootstrap percentages (100 replicates) are shown to the left of the node being considered. EMBL accession numbers for 16S RNA sequences nt 343–709 are: *Exiguobacterium* TC38-2b, Y15051; *Bacillus cereus* RC607, Y15048; *B. cereus* TA32-5, Y15050; and *B. sphaericus* FA8-2, Y15045.

2b showed 99.7% identity with that of *Exiguobacterium* sp. (GenBank accession number X86064) and 98.5% identity with that of *E. acetylicum* (GenBank accession number D55730). These identification data were in agreement with the fatty acid analysis of strain TC38-2b that was kindly conducted by Dr K. J. Purdy (Natural Environment Research Council Institute of Virology and Environment Microbiology, Oxford, UK). The nucleotide sequences of the 343–709 16S rRNA gene region from strains RC607, TA32-5 and VKM684 were identical to that of a reference *B. cereus* strain (GenBank accession number D16266), while the nucleotide sequence of the corresponding fragment from strain CH70-2 showed 99.7% identity with that of the reference *B. cereus* strain. This difference may be an

artifact of selecting one of the multiple *rrn* sequences which are present in *B. cereus* (Johansen *et al.* 1996). So, strains RC607, TA32-5, VKM684 and CH70-2 fell into a group of very closely related species, which include *B. cereus*, *B. thuringiensis*, *B. anthracis* and *B. mycoides* (Ash *et al.*, 1991). Data on bacterial cell mobility, haemolysis, and colony morphology suggested that all four strains were *B. cereus*. Strains RC607, TA32-5 and CH70-2 were not identical, since they differed in their products of repetitive extragenic palindromic sequence PCR (de Bruijn, 1992). Strain FA8-2 was identified as *B. sphaericus* (99.1% identity with the 16S rRNA gene fragment of a reference *B. sphaericus* strain; GenBank accession number D16280). The 16S rRNA of RC607, and that of TC38-2b differed at 10% of nucleotide positions. Using a calibration of the molecular clock for 16S rRNA (Ochman & Wilson, 1987) we estimate that these strains diverged approximately 500 million years ago. The small difference between the sequences of the *mer* operons found in strains RC607 and TC38-2b (<0.2%) suggests that the time elapsed after their divergence is much smaller than that for 16S rRNA. Accordingly, we conclude that one or both of these strains, and possibly VKM684, acquired their *mer* operons relatively recently due to horizontal transfer (Fig. 2). This conclusion is graphically confirmed by the comparison of phylogenetic trees for the 16S and *merA* genes. Fig. 2(a, b) shows that the phylogenies of the 16S rRNA and *merA* genes are not congruent. Horizontal *mer* operon transfer is also evident for strains FA8-2 and TA32-5, which show 9% nucleotide differences between the 16S RNA genes, and no difference in their *mer* operons (Fig. 2a, b). TA32-5 and CH70-2 show 0.3% difference between their 16S rRNA sequences, so we can not exclude vertical inheritance of the *mer* operons between these strains. The source of *mer* operons in the transfer studied is unknown: the G+C content of *mer* operons of RC607 and TC38-2b (39 mol%); TA32-5 and FA8-2 (40 mol%) is in the range of the chromosome G+C content for the *Bacillus* strains: RC607 (33 mol%), TC38-2b (44 mol%), TA32-5 (30 mol%) and FA8-2 (35 mol%). In the Minamata Bay collection, the RC607 *mer* operon variant was found in strains phenotypically identified as *B. subtilis*, *B. firmus*, *B. lentus* and *B.adius*. It is possible that the RC607 *mer* operon variant experienced horizontal transfer amongst these species. However, definitive testing of this hypothesis requires sequencing of the *mer* operons and 16S RNA genes of these organisms, and the construction and comparison of evolutionary trees based on these data.

DNA sequencing downstream of *mer* operons suggests involvement of class II transposons in dissemination of mercury resistance in Gram-positive bacteria

In looking for the possible involvement of transposons in translocations of *mer* operons from plasmids to chromosome and between plasmids, we characterized the regions flanking the *mer* operons. Southern hybridization restriction mapping and partial DNA sequencing

Fig. 3. Restriction endonuclease maps of regions flanking *mer* operons; (a) and (b) are drawn to different scales, as indicated. For TA32-5, FA8-2, RC607, TC38-2b and MK64-1 strains, the restriction maps were constructed by Southern hybridization of cloned fragments described in Table 2 with pKLH3.2, pKLH3.3, pKLH3.4 and pKLH302.2 as probes. *R*, *orf* and *A* denote the *merR*, *orf2-orf3-orf4* and *merA* genes, respectively. For CH70-2, the plasmid DNA was probed with pKLH302.2 using the restriction enzymes marked by filled circles. Filled squares mark restriction sites also present in the Minamata Bay strains (Nakamura & Silver, 1994). The boxes downstream of the *mer* operons represent DNA fragments following the transposon IRs. The sequences surrounding these IRs are shown in Fig. 4(a, b).

demonstrated that the high degree of similarity between TA32-5 and FA8-2 on the upstream side of the *mer* operons, continued up to the leftmost *Hind*III site shown in Fig. 3(a). The exact position where homology was lost was not determined. The identity of the TC32-5 and FA8-2 DNA sequences downstream of *merA* continued for 220 bp to the end of an element (109 bp) showing 85% identity with the transposase-distal end of a class II (Tn3 family) vancomycin-resistance transposon Tn1546 from a clinical *Enterococcus* strain (Fig. 4a). For TA32-5 and CH70-2, restriction mapping demonstrated that high similarity or identity continued much further beyond the end of the putative transposon (Fig. 3a). This suggests that the transposons found in these strains are located in related plasmids.

Southern hybridization restriction mapping demonstrated that the high degree of similarity between RC607 and TC38-2b on the upstream side of the *mer* operons continued beyond the leftmost *Hind*III site shown in Fig. 3(b). The exact position where the homology finished was not determined. On the downstream side, both strains contained identical *merB* genes separated from the end of *merA* by a 1.8 kb region with an undefined

function (Fig. 3b). This region was not compared by DNA sequencing but was probably identical in the two strains. The identity continued for 635 bp downstream of the *merB* gene and finished precisely at the end of a 38 bp element showing up to 60% identity with IRs of class II transposons (Fig. 4b). This element was preceded by a 162 bp region encoding a peptide showing homology with the C-terminal end of class II transposases (Fig. 5). Sequence analysis demonstrated that the putative transposon found in strains RC607 and TC38-2b was more distantly related to Tn1546 than the putative transposon found in TA32-5 (Fig. 4b and Fig. 4a). However, the putative transposon found in RC607 and TC38-2b was slightly more closely related to a Tn1546-Tn5044 branch of class II transposons (Yurieva & Nikiforov, 1996; Kholodii *et al.*, 1997), containing transposons from Gram-positive bacteria, than to the Tn21 branch containing transposons from Gram-negative bacteria. Southern hybridization restriction mapping demonstrated the absence of homology between downstream *mer* operon regions from RC607 and VKM684.

From mapping data for RC607 and numerous Minamata

Fig. 4. (a) Alignment of the regions downstream of *merA* from *Bacillus cereus* TA32-5 and *B. sphaericus* FA8-2 strains, with the *Enterococcus faecium* transposon Tn1546 transposase-distal end. The numbering is from the first nucleotide after the *merA* gene. A dot indicates a base identical to that in the *B. cereus* TA32-5 sequence; a gap is indicated by a dash. The 38 bp transposon IR is overlined. The EMBL database accession numbers for these sequences are: TA32-5, Y09024; FA82, Y10105; Tn1546, M97297. (b) Alignment of the regions downstream of the *merB* gene from the *B. cereus* RC607 chromosome and an *Exiguobacterium* sp. TC38-2b plasmid (pKLH3) with the region downstream of the *merA* gene from *B. megaterium* MK64-1 plasmid (pKLH304). The terminal IRs of the putative transposons from RC607/TC38-2b, MK64-1, and TA32-5/FA8-2 are aligned with IRs of class II transposons. Numbering is from the first nucleotide after the *merB* gene, except in the MK64-1 sequence where it is from that after the *merA* gene. A dot indicates a base identical to that of the *B. cereus* sequence. No differences were detected in the DNA sequence of the first 635 bp after the end of *merB* gene, between the RC607 chromosome and *Exiguobacterium* sp. TC38-2b plasmid, pKLH3. In our hands, the sequence of RC607 chromosome differed from the published data (Wang *et al.*, 1989) by twelve single-base extensions of homonucleotide tracks in the 100 bp following the *merB* gene. The amino acid sequence of the putative transposase fragment of the RC607/TC38-2b transposon is shown above the nucleotide sequence. The sequences of IRs of Tn1546 from *Enterococcus faecium*, Tn4430 from *B. thuringiensis*, Tn21 from *Shigella flexneri* and Tn3 from *E. coli* are from Arthur *et al.* (1993). The accession numbers for other sequences are: RC607, Y08065; pKLH3, Y08064; pKLH304, Y09907; pKLH302, Y09024.

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RC607      GLKKIGQLSTNISLREDLLKHISPLGWEHINFLGEYTFDMKKVASLSLRPLIQ
TC38-2b    .....
Tn1546     TTAVEYKKR.G-.FN.....H.M.....L.....H.NSE..V..N.....KLS
Tn4439     EKAVEELKRG-EP.....MPYAW.....K.EGLHDTGGQM.....RIKEPFYS
Tn21       ERAAHALRGNGHAUDD.S..QYL.....LT.D.LWRSSAKIGAGKF.....RPLQPA
Tn3        QAALDHLRAQGET.NDEDIARL...CHG...M..H.S.TLAELVTGKH.....KEASEAENVA

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Fig. 5. Sequence similarity between the deduced amino acid sequences of the transposase fragment of the putative transposon of RC607/TC38-2b strains and of the class II transposases. The alignment of transposases at their COOH end is from Yurieva & Nikiforov (1996).

Bay strains (Nakamura & Silver, 1994), we conclude that the high similarity or identity between these strains continues further beyond the end of the putative transposon (Fig. 3b). This suggests that the suspected horizontal transfer of *mer* operons between the replicons of these strains (see the section above on 16S rRNA sequence) involved a larger element than the transposon found in the RC607 strain. It would be interesting to explore the nature of this element by examining the extent of similarity between different Minamata Bay strains on both sides of the *mer* operons.

Inspection of the sequence downstream of the *merA* gene of MK64-1 demonstrated that it bore no homology to the sequence downstream of the *merA* gene of RC607, but showed high similarity to the transposon element found downstream of the *merB* gene in RC607 and TC38-2b (Fig. 4b). This suggests that MK64-1 contains a transposon (closely related to the transposon from RC607) from which a *merB* gene-containing fragment may have been deleted. This may explain why the MK64-1 DNA did not hybridize with the *merB* gene of RC607 (Table 1).

The identification of at least three different class II transposon sequences adjacent to the *mer* operons described above, suggests that this family of transposons was involved in dissemination of mercury resistance not only in Gram-negative bacteria but also in Gram-positive bacteria. It remains to be elucidated whether the putative transposons reported in this paper contain a complete set of transposition genes or are defective, as has often been found in Gram-negative bacteria (Yurieva *et al.*, 1997).

Conclusions and perspectives

The principal result of this study is that a geographical and horizontal dissemination of *mer* operons in low G+C content Gram-positive bacteria in the natural environment has occurred, mirroring the worldwide dissemination of *mer* operons in Gram-negative bacteria (Osborn *et al.*, 1997; Yurieva *et al.*, 1997). Class II (Tn3-like) transposons were apparently involved in this process. As in the case of Gram-negative bacteria (Yurieva *et al.*, 1997), the spread of *mer* operons reported in this paper was detected in a survey of a very small collection of strains. This suggests that promiscuous clones of *mer* operons constitute a considerable, if not the major, fraction of the worldwide population of *mer*

operons in culturable environmental isolates of bacteria. This conclusion does not eliminate the existence of strictly local clones of *mer* operons, but suggests that detection of these would require the large-scale screening of culture collections from all over the world.

The timing and the driving force of the spread of the *mer* operons remains an open question. One possibility is that the observed dissemination occurred because of increased selective pressure caused by recent industrial pollution. Another possibility is that the spread of *mer* operons occurs periodically and occurs over longer time spans, as has been postulated for the periodic replacement of chromosomal alleles in *E. coli* populations (Milkman & McKane, 1995). That would mean that the worldwide distributions that we detect now are neither the first nor the last pandemics in the history of *mer* operons. A promising way to investigate this issue further is to compare *mer* operons from palaeobacteria, which have been preserved in permafrost grounds for more than 30 000 years (Gilichinsky *et al.*, 1992), with presentday bacteria, and to compare the *mer* gene distribution amongst Gram-positive bacteria isolated from sites with different levels of mercury contamination.

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