Translocation of transposition-deficient (Tn<sup>d</sup>PKLH2-like) transposons in the natural environment: mechanistic insights from the study of adjacent DNA sequences

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A family of plasmid-borne DNA fragments of different length, apparently inherited from an ancient plasmid, has been identified in the world population of environmental Acinetobacter strains. These fragments, named PPFs (parental plasmid DNA fragments), were ≥99.8% identical to each other in the common regions, and contained in their central region a variant of an aberrant mercury-resistance transposon (Tn<sup>d</sup>PKLH2) that has lost its transposition genes. As a rule, recombinogenic elements were found at the breakpoints of identity between the different PPFs. Of these recombinogenic elements, a newly identified IS6 family element, a transposon, or a resolvase gene interrupted one end of the PPFs. At the opposite end, the breakpoint of some PPFs was mapped to the recombination point within, in each case, a different variant of a res site (RS2), whilst in other PPFs, this end was eroded by insertion of a newly identified IS6 family element. On the basis of DNA sequence data, possible mechanisms of translocation of defective Tn<sup>d</sup>PKLH2-like elements via recombination events implicating the nearby res (resolution) site and IS element are proposed.

INTRODUCTION

Mercury-resistance (mer) operons are well recognized for their propensity to spread worldwide in environmental bacterial populations (Hobman & Brown, 1997; Osborn et al., 1997; Yurieva et al., 1997; Nakamura & Silver, 1994; Bogdanova et al., 2001; Barkay et al., 2003). A considerable body of data has been accumulated about two circumstances contributing to the widespread distribution of Hg(II) detoxification systems among Gram-negative bacteria.

These are (i) the frequent location of mer operons on plasmids and (ii) their association with transposons (Ogawa et al., 1984; Khesin & Karasyova, 1984; Griffin et al., 1987; Jobling et al., 1988; Hobman et al., 1994; Hobman & Brown, 1997; Kholodii et al., 1993, 1995, 1997, 2000, 2002; Pearson et al., 1996; Reniero et al., 1998; Kalyaeva et al., 2001; Mindlin et al., 1986, 2001). Amongst the best-characterized and most widely distributed mer operons are those present in class II transposons. Examples of such transposons belong to the Tn5053/Tn402 (Hobman et al., 1994; Kholodii et al., 1995; Reniero et al., 1998) and Tn3 families (Grinsted et al., 1990; Kholodii et al., 1997, 2000, 2002; Liebert et al., 1999; Mindlin et al., 2001) in Gram-negative bacteria, whilst in Gram-positive bacteria only Tn3 family mercury-resistance transposons have as yet been identified (Huang et al., 1999; Bogdanova et al., 2001). The Tn3 and Tn5053/Tn402 family transposons are characterized respectively by 38–47 bp or 25 bp inverted repeat termini, which together with the transposition genes (respectively tnp or tni) form the transposition module, within which the mer module and other additional DNAs may be inserted.

A considerable fraction of Tn3 family mercury-resistance transposons found in environmental bacteria appeared to be transposition-deficient (Pearson et al., 1996; Yurieva...
et al., 1997; Hobman et al., 1994; Martinez et al., 2001; Mindlin et al., 2001; Kholodii et al., 2002). The inability of at least some of these elements to transpose has not apparently prevented their further spread, since nearly identical copies of certain defective transposons have been found on different plasmids (pKLH272 and pKLH247) in bacteria from different taxa, which were isolated from geographically separated populations (Yurieva et al., 1997; G. Kholodii, unpublished). If this apparent widespread dissemination of elements has not been associated with real transposition events, what mechanisms could ensure the translocation of defective transposons? To our knowledge, no single study on this question is found in the literature. One of the defective transposons we were interested in was a derivative of a Tn21 subgroup (Tn3 family) mercury-resistance transposon, which was found in plasmid pKLH2 from an Acinetobacter strain (Kholodii et al., 1993; Osbourn et al., 1995). This transposon, named here Tn^dPKLH2, was found to be interrupted at an incomplete hybrid res site (RS1) consisting of only one subsite, resL. From these data, it has been proposed that the participation of an aberrant resolution event, resulting in the loss of accessory resolution subsites (resII and resIII) of the original RS1 site, and abutted transposition genes (tnpR, A), occurred during the evolution of Tn^dPKLH2 (Kholodii et al., 1993).

Previously, short DNA sequences, identical or nearly identical to those of Tn^dPKLH2, have been found in a number of Acinetobacter strains from geographically remote areas (Lomovskaya & Nikiforov, 1988; Pearson et al., 1996; Kholodii, 2001), raising the possibility that Tn^dPKLH2-like elements are widespread. Where tested, these sequences were found to be located on plasmids (Mindlin et al., 1986; S. Mindlin & Zh. Gorlenko, unpublished). In two plasmids, pKLH204 and 205, the termini of the presumptive Tn^dPKLH2-like elements were sequenced and found to be identical to the termini of Tn^dPKLH2; the location of these elements on different plasmids was inferred from differences in the flanking DNAs (Kholodii, 2001). From these data, Tn^dPKLH2-like elements appeared to be a good model for epidemiological studies, and elucidation of the mechanisms of transposition of defective transposons. One of the 'less obvious' mechanisms for transposition was tested previously (Kholodii, 2001). In that work, a powerful co-integrative function of typical DNA resolution systems such as cinH-RS2 (identified in pKLH2, 204 and 205 near the mer locus) and tnpR-res (from Tn1721) was identified. These systems (especially cinH-RS2) were able to act on heterogeneous res sites with a range of identity from 35 to 100 %. What was inferred from the data obtained (Kholodii, 2001) is that movement of DNA fragments by typical resolvases is possible. In the present work, to understand the modes of inter-replicon movement of defective transposons, extended epidemiological and molecular studies on environmental Acinetobacter Hg^R loci were undertaken. The results obtained have allowed us to propose additional mechanisms for translocation of DNA fragments.

**METHODS**

**Media and cell growth.** Bacteria were propagated at 26–30 °C in Luria–Bertani broth (LB), Adams minimal medium (0·1 % NH₄Cl, 0·15 % KH₂PO₄, 0·35 % NaH₂PO₄, 0·01 % MgSO₄·7H₂O, pH 7·2) and on solid media containing 20 g Bacto agar l⁻¹. When required, antimicrobial agents were added to the media at the following final concentrations (μg ml⁻¹): ampicillin (Ap) 100, chloramphenicol (Cm) 20, HgCl₂ (Hg) 5 in minimal-medium plates and 5–20 in LB plates, gentamicin (Gm) 5, nalidixic acid (Nal) 50, rifampicin (Rif) 40, streptomycin (Str) 100, tetracycline (Tc) 15.

**Genetically modified bacterial strains, plasmids and matings.** The strains and plasmids used are listed in Table 1. Intra- and inter-species matings between Acinetobacter sp. and Escherichia coli strains were performed on LB plates as described previously (Kholodii et al., 1995).

**Isolation of environmental Acinetobacter strains.** Both mercury-resistant (Hg^R) and mercury-sensitive (Hg^S) Acinetobacter strains were isolated during this study. Part of the Acinetobacter sp. collection consisted of isolates from mercury mines in different regions of the former Soviet Union: Khaidarkan (Kyrgyzstan, Central Asia), Sakhalin (Northern Caucasus), Nikitovka (Eastern Ukraine), and Bot’shoi Shayan (Transcarpathians). Additional strains in the collection were isolated from soil and water samples from the USA (Bethesda, East Lansing) and the Russian Federation (the Kamchatka Peninsula, the Kurils, the Chuckchee Peninsula, the Kolyma-Indigirka lowland permafrost grounds, and European Russia). However, these sampling regions (except the Kamchatka Peninsula) were not associated with any mercury deposits. Several of the Hg^R isolates have been described previously (Khesin & Karasova, 1984; Mindlin et al., 1986; Bogdanova et al., 1988; Petrova et al., 2002).

The permafrost strains were isolated from ice core samples removed from a geological layer (the Edoma suite) in the Arctic (152–153 °E, 69·5–70·5 °N; the Khomsus Yuryakh river region, Kolyma-Indigirka lowland, North-Eastern Siberia). In this region, the late Pleistocene layer—the so-called ‘Icy Complex’ or Edoma suite—is represented by syngenetically frozen sediments with ice veins, which indicates that they have not thawed since first frozen. On the basis of 14C studies of the Kolyma-Indigirka lowland permafrost grounds (Sher & Plakh, 1988) and the stratigraphic characterization of the sediment cores recovered from drill-holes 6X-Yu and 4/89-34E (from which the Edoma samples used in this work originated), the age of permafrost bacteria within these samples has been estimated to be between 15 000 and 40 000 years old. The drilling/sampling techniques, storage/transportation of permafrost samples, and the precautions against and tests for exogenous contamination were similar to those described previously (Vorobyova et al., 1997; Shi et al., 1997; Petrova et al., 2002).

Hg^R and Hg^S bacterial strains were isolated on LB plates without any drug and on LB plates supplemented with HgCl₂, respectively, or by using an enrichment culture method (Mindlin et al., 1986). The strains were identified according to morphological, cultural and biochemical characteristics of the genus Acinetobacter (Junji, 1978), and the results of genetic transformation of strain BD413 ilv (performed as described previously: Mindlin et al., 1990), which is extremely specific for bacteria from the genus Acinetobacter (Junji, 1978).

**Screening for active Hg^R transposons resident in environmental Acinetobacter strains.** To test for transposition of Hg^R determinants, a broad-host-range plasmid (RP1) was introduced into Acinetobacter isolates by mating with E. coli K-12 HB101(RP1). Hg^R Acinetobacter transconjugants containing RP1 were propagated for 90–120 generations on selective LB plates and mated with E. coli K-12 HB101. Hg^R Str^R transconjugants expected to carry an RP1-containing transposable Hg^R determinant (RP1::Hg^R) were selected.
Plasmids were identified in crude cell extracts according to the method of Eckhardt (1978). Plasmid size was assessed by using known plasmids as size standards, and by restriction enzyme analysis of isolated DNA. Plasmid DNA was tested for the presence of the characteristic pKLH2 fragment in Southern hybridization using transconjugant total DNA and the probe described above.

### DNA manipulations and analysis

Standard DNA manipulations were performed as described by Sambrook et al. (1989). Sequencing was performed manually on both strands of DNA using the dideoxy chain-termination method (Sanger et al., 1977). Computer analysis of nucleotide and deduced amino acid sequences was performed using the VOSTORG (Zharkikh et al., 1991) and BLAST (Altschul et al., 1997) programs. Multiple alignment and subsequent phylogenetic analysis were performed using CLUSTAL W (Higgins et al., 1992) and MEGA2 (Kumar et al., 2001), respectively.

### RESULTS

#### Screening of the Acinetobacter collection

The ‘worldwide geography’ of the collection consisting of HgR and HgS Acinetobacter isolates is given in Methods. Most of the HgR isolates examined carried HgR determinants on large plasmids (60–100 kb) and only a few of them, on smaller plasmids (~18 kb). In addition to the HgR plasmid, all isolates carried plasmid(s) not associated with the HgR phenotype, which were >1.5 kb in size (data not shown).

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**Table 1. Laboratory strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Vector</th>
<th>Description</th>
<th>Resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acinetobacter calcoaceticus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BD413 ilv</td>
<td>ilv</td>
<td></td>
<td>None</td>
<td>Juni (1978)</td>
</tr>
<tr>
<td><strong>Escherichia coli K-12</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM83</td>
<td>F' thi ara Δ(lac–pro) rpsL 80lacZM15, a host plasmid constructs</td>
<td>StrR</td>
<td>Vieira &amp; Messing (1982)</td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>F' thi-1 proA2 leu-2 recA13 hisD20 rpsL20</td>
<td>StrR</td>
<td>Sambrook et al. (1989)</td>
<td></td>
</tr>
<tr>
<td>UB5201 RifB</td>
<td>F' pro met recA56 gyrA rpoB</td>
<td>RifB</td>
<td>Kholodii et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>JF238</td>
<td>F' gyrA91</td>
<td></td>
<td>NaR</td>
<td>Kholodii et al. (1995)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cloning vector</td>
<td>CmR, TcR</td>
<td>Chang &amp; Cohen (1978)</td>
<td></td>
</tr>
<tr>
<td>pKLH2.41</td>
<td>pACYC184 Contains the mer operon of pKLH2</td>
<td>CmR, HgR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH208.5</td>
<td>pACYC184 Contains the mer operon of pKLH208</td>
<td>CmR, HgR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH201.L1</td>
<td>pUC19 Contains the 1·3 kb BglII–EcoRI fragment bearing IS1007 (from pKLH201)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH201.5</td>
<td>pUC19 Contains the 5·6 kb EcoRI fragment bearing IS1008 (from pKLH201)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH204.27</td>
<td>pUC19 Contains the 1·1 kb EcoRI–XbaI fragment bearing IS1007.1 (from pKLH204)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH203.14</td>
<td>pUC19 Contains the 1·7 kb HindIII–EcoRI fragment bearing IS1006 (from pKLH203)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH272.R1</td>
<td>pUC19 Contains the 3·2 kb EcoRI–HindIII fragment bearing IS26 (from pKLH272; AC Y08992)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>pKLH5036.98</td>
<td>pUC19 Contains the trpR.A genes of Tn5036 (Yurieva et al., 1997)</td>
<td>ApR</td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>RP1</td>
<td>IncP group plasmid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRP1.2</td>
<td>RP4 derivative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pOX38gen</td>
<td>F factor lacking all mobile elements and carrying a gentamicin-resistance gene</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

on LB plates with 4–6 μg HgCl₂ ml⁻¹ and 100 μg streptomycin ml⁻¹. Genetic linkage between RP1 and the mercury-resistance determinant was verified in crosses with E. coli strain K-12 JF238, and further analysis was performed if genetic linkage was found. The transposition frequencies were assessed by dividing the fraction of RP1::HgR transconjugants by the RP1 transfer rate.

**Plasmid size estimation and localization of HgR determinants.** Plasmids were identified in crude cell extracts according to the method of Eckhardt (1978). Plasmid size was assessed by using known plasmids as size standards, and by restriction enzyme analysis of isolated DNA. Plasmid DNA was tested for the presence of HgR determinants by Southern blotting with a 32P-labelled 0·512 kb EcoRI–HindIII fragment from the mer operon of pKLH2 (Fig. 1, top). The presence of an HgR determinant in a conjugative plasmid was determined from transmission of mercury resistance in crosses with a Str⁻ derivative of BD413 ilv, followed by identification of the characteristic pKLH2 fragment in Southern hybridization using transconjugant total DNA and the probe described above.
Southern hybridization showed that the majority of the Hg$^R$ strains contained a 2.7 kb EcoRI fragment of a mer operon characteristic of Tn$^d$PKLH2 (Table 2, columns 3 and 4). In the transposition assay, which was similar to those applied to bacteria from genera other than Acinetobacter (Kholodii et al., 1993, 1997, 2000, 2002; Mindlin et al., 2001), no active Hg$^R$ transposons were identified in the collection (Table 2, last two columns), although some of the strains carried Tn21-related trpA sequences (column 5). Several strains, including KHW14 detailed below, were suspected to contain an active Hg$^R$ transposon, as each of them was found in the transposition assay (described in Methods) to produce transconjugants with genetic linkage between the target plasmid RP1 and the Hg$^R$ determinant. Detailed molecular and genetic analyses showed, however, that genetic linkage was caused by formation of a fusion between RP1 and the full Hg$^R$-containing plasmid. The mechanism of fusion formation was not related to transposition of an Hg$^R$ determinant, but was probably due to IS element activity (data not shown). Most of the Hg$^R$, but not the Hg$^S$, isolates hybridized with an IS26 probe (Table 2, column 6), suggesting physical linkage between the Hg$^R$ determinants and IS elements. To confirm this linkage, Hg$^R$ plasmids from arbitrarily chosen strains which contained the 2.7 kb EcoRI fragment were taken for further study. Of these, four strains (KHW14, TC108, NC13-1 and BW3; Table 3) hybridized with the IS26 probe, and one (ED23-35) did not. Additionally, Hg$^R$ plasmids that had previously been partially sequenced were studied. These were the reference plasmid pKLH2 (Kholodii et al., 1993; Kholodii, 2001), which was subjected to further sequencing 3’ to the merR gene and 5’ to the cinH gene; and pKLH204 and pKLH205, previously sequenced in the regions responsible for the function of the CinH recombinase (Kholodii, 2001).

**Association of the 2.7 kb EcoRI mer fragment with Tn$^d$PKLH2-like elements**

All plasmids sequenced across the mer region contained a Tn$^d$PKLH2-like element, which we named Tn$^d$PKLH201, Tn$^d$PKLH202, etc. (according to the designations of corresponding plasmids pKLH201, pKLH202, etc.; Table 3). Each Tn$^d$PKLH2-like element was flanked by a 38 bp terminal inverted repeat (TIR) 3’ to the merR gene, and the hybrid res site, RS1, overlapping with the 3’ end of urf-2Y (Fig. 1). A number of specific base substitutions that were identified during sequencing differentiated Tn$^d$PKLH2-like elements from Tn$^d$PKLH2, and each other (Fig. 1 and legend). Unlike the other elements, Tn$^d$PKLH205 and 208 had changes of a recombinogenic nature, i.e. they were genetic mosaics as they contained distinct divergent regions, each being clearly indicative of a single-event allelic exchange by homologous recombination. In Tn$^d$PKLH205, the divergent region (γ) was small, 334 bp, lay in the last third of the merA gene, and was identical to the corresponding region of Tn501 [GenBank accession no. (AC) Z00027]. In Tn$^d$PKLH208, the divergent region was large: a region of 2424 bp started at the 5’ end of merA and ended at the centre of merD. The
Table 2. Incidence of defective HgR transposons and IS26-relatives amongst environmental Acinetobacter isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source</th>
<th>Hybridizing strains*</th>
<th>total</th>
<th>containing:</th>
<th>Mobility of HgR determinants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mer operon type</td>
<td>tnpA</td>
<td>‘IS26’</td>
<td>RP1-accepting strains/total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7 kb† (TnPKLH2-like)</td>
<td>Others‡</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>HgR</td>
<td>Mercury mine</td>
<td>21/24</td>
<td>1/24</td>
<td>17/24</td>
<td>14/24</td>
</tr>
<tr>
<td></td>
<td>Soil</td>
<td>12/17</td>
<td>0/17</td>
<td>12/17</td>
<td>11/17</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>9/10</td>
<td>3/10</td>
<td>7/10</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td>Permafrost</td>
<td>7/7</td>
<td>NT</td>
<td>5/7</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>49/58 (84%)</td>
<td>4/51 (7%)</td>
<td>41/58 (71%)</td>
<td>38/58 (66%)</td>
</tr>
<tr>
<td>HgS</td>
<td>Soil</td>
<td>NT</td>
<td>4/25</td>
<td>0/17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>NT</td>
<td>1/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Permafrost</td>
<td>NT</td>
<td>0/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5/36 (14%)</td>
<td>0/17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NT, Not tested.

*In the case of the mer operon sequences, total DNA from each strain digested with EcoRI was characterized (hybridized to a specific probe), whereas the tnp- and IS26-related sequences were characterized by colony hybridization. DNA hybridization probes were as follows: the 0.512 kb EcoRI–HindIII fragment from pKLH2 (containing the merR gene; Fig. 1, top) directed against the mer operon; the 1.24 kb EcoRI fragment from Tn21 (from pVS982) directed against the transposase gene (tnpA); and the 0.298 bp PstI–XhoII fragment from an IS26 variant found near the mer operon from pMJ501 (G. Kholodii, unpublished), which was directed against the IS elements.

†2.7 kb† refers to the mer operons containing the 2.7 kb EcoRI fragment that was identified. For the location of this fragment, see Fig. 1, top. ‘Others’ refers to the other mer operons, differing from each other by the size of the EcoRI fragment that was identified (3.7; 3.8; 4.0; 7.8; 8.0; and 9–10 kb).

‡Frequency of transposition of HgR determinants was <10⁻⁸ (see Methods for details).

left 456 bp of this region (δ) contained three mismatches compared to the equivalent DNA sequence from pDU1358 (AC M24940), whereas the right part (ω) was occupied by a mer sequence belonging to a previously unrecognized type, 89–91% identical to the equivalent sequence of Tn5053 (Kholodii et al., 1995; AC L40585), pPB (Reniero et al., 1998; AC U80214) and Tn501 (AC Z00027). It contained an extra 689 bp between the merA and merD genes (shown as a flag in Fig. 1), within which a merB gene was identified. The predicted 208 aa MerB protein from pKLH208 was 59% identical to the organomercurial lyase enzymes from pDU1358 (Griffin et al., 1987; AC M15049).

Table 3. List of Acinetobacter strains carrying the HgR plasmids studied in detail

<table>
<thead>
<tr>
<th>Acinetobacter strain</th>
<th>Origin</th>
<th>TnPKLH2-like transposon</th>
<th>Plasmid characteristics</th>
<th>Size of fragment cloned (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. calcoaceticus KHP18</td>
<td>Kyrgyzstan, mercury mine (Kholodii et al., 1993)</td>
<td>TnPKLH2</td>
<td>pKLH2</td>
<td>60</td>
</tr>
<tr>
<td>A. calcoaceticus KHW14</td>
<td>Kyrgyzstan, mercury mine (Mindlin et al., 1986)</td>
<td>TnPKLH2</td>
<td>pKLH201</td>
<td>60</td>
</tr>
<tr>
<td>A. lwoffii TC108</td>
<td>Carpathians, mercury mine</td>
<td>TnPKLH2</td>
<td>pKLH202</td>
<td>100</td>
</tr>
<tr>
<td>A. junii NC13-1</td>
<td>Caucasus, mine</td>
<td>TnPKLH2</td>
<td>pKLH203</td>
<td>60</td>
</tr>
<tr>
<td>Acinetobacter sp. LS56-7</td>
<td>Moscow region, soil (Kholodii, 2001)</td>
<td>TnPKLH2</td>
<td>pKLH204</td>
<td>60</td>
</tr>
<tr>
<td>Acinetobacter sp. ED45-25</td>
<td>Permafrost, Kolyma-Indigirka lowland (Kholodii, 2001)</td>
<td>TnPKLH2</td>
<td>pKLH205</td>
<td>60</td>
</tr>
<tr>
<td>Acinetobacter sp. BW3</td>
<td>Bethesda, MD, USA</td>
<td>TnPKLH2</td>
<td>pKLH207</td>
<td>18</td>
</tr>
<tr>
<td>Acinetobacter sp. ED23-35</td>
<td>Permafrost, Kolyma-Indigirka lowland</td>
<td>TnPKLH2</td>
<td>pKLH208</td>
<td>60</td>
</tr>
</tbody>
</table>

*Tra⁺, narrow-host-range conjugative plasmids; Tra⁻, non-conjugative plasmids.

†The region of pKLH202 downstream of the merR gene (Fig. 3) was sequenced from a PCR product.
and R831b (Ogawa et al., 1984; AC U77087), and was found to be functional (see below).

**Determination of functional activity of the merB gene from pKLH208**

pKLH208.5 and pKLH2.41, containing respectively the mer operons from pKLH208 and pKLH2 (cloned in pACYC184), and pACYC184 were used to test the resistance of E. coli strain K-12 JM83 to an organomercurial drug, phenylmercuric acetate (PMA). PMA resistance, associated in E. coli with MerB activity (Ogawa et al., 1984; Hobman & Brown, 1997), was determined by bacterial culture titration on LB plates containing different concentrations of the drug. Inhibitory concentrations of PMA were determined according to complete growth inhibition. JM83(pKLH208.5), which was resistant to PMA, grew at 7.5 μg PMA ml⁻¹, whereas the susceptible strains, JM83(pKLH2.41) and JM83(pACYC184), grew at 2.5 μg PMA ml⁻¹. Each experiment was repeated four times.

**Sequences surrounding the TndPKLH2-like elements**

The adjacent regions, sequenced from the cloned fragments (Table 3, last column), contained a large number of recombinogenic elements (20 IS elements, 15 resolution system determinants, and two transposons from the Tn21 subgroup), five putative cation efflux system genes, and other determinants. DNA sequence data described below, and restriction enzyme analysis of unsequenced regions of the cloned fragments (data not shown), demonstrated that, except for the common regions clearly inherited from the last common ancestor of these plasmids (named parental plasmid DNA fragments, PPFs), the remainder of the DNAs varied greatly in molecular structure. These data were clearly indicative of the location of PPFs identified on different plasmids.

**Characterization of plasmids bearing the cinH-RS2 system.** The high sequence identity between the plasmids (> 99.8 %), shown by the hatched bars in Fig. 2(a), continued in regions flanking the TndPKLH2-like elements in pKLH2, 204, 205 and 208. On the right (Fig. 2a), the resolution site RS1 of these plasmids was followed by nearly identical 1451 bp regions, containing a 5‘-truncated IS17-related element (IS1011.D1), and the cinH-RS2 DNA resolution system. The identity was interrupted at the centre of the resolution site RS2 crossover subsite, resI (Fig. 2b), suggesting involvement of independent site-specific recombination in the generation of each particular breakpoint. On the left (Fig. 2a), the identity was interrupted either because of the insertion of an IS26-related element (IS1007.1) in pKLH204, a transposon (Tn5049) in pKLH208, or an unknown recombination event in pKLH205. The exact breakpoint of identity with pKLH208 was not determined, but fine restriction analysis showed that pKLH2 did not contain Tn5049 5‘ to orf900, suggesting that Tn5049 or an ancestor of it had interrupted the original (common) DNA sequence in pKLH208. In the partially sequenced Tn5049, the 38 bp TIR and adjacent resolution system (tnpR-RS3) were most similar to those of Tn501 (AC Z00027), showing respectively 79 % and 93 % identity at the DNA level.

**Characterization of plasmids that have lost the cinH-RS2 system.** DNA sequencing of the remainder of the plasmids showed that IS26-related elements (IS1007, IS1006, IS1006.1 and IS1008) might be the major factors that had caused truncation of the PPFs on either side (Fig. 3). pKLH201, 202, 203 and 207 contained a short (38, 5, 62 and 5 bp, respectively) region of additional identity to pKLH2 at the left flank (shown, in each case, by a hatched bar adjoining TIR of the aberrant transposon; Fig. 3) and a shortened or lost region of additional identity, if compared to pKLH2, 204, 205 and 208 (Fig. 2a), at the right flank (Fig. 3). Unlike the other plasmids, pKLH202 and 207 contained newly acquired common flanking regions (NACRs) that had substituted for the primary (PPFs) flanks. On the left flank, pKLH202 and 207 shared 100 % identity in the corresponding regions (marked with a ‘NACR’ box in Fig. 3). Here a 234 bp relic (tnpRD1) of a resolvase gene closely related to cinH (72 % identical nucleotides) was identified at the breakpoint of the PPFs. On the right flank, the newly acquired regions were characterized by 12 mismatches over 1895 bp. They started within the right half of RS1 and ended after the sequence of an IS17 copy began. These data suggested that pKLH202 and 207 evolved via an intermediate common ancestor. An intermediate common ancestor might also be suggested for pKLH204 and 201, as they contained IS1007 or its one-base variant IS1007.1 inserted at the same site (Figs 2a and 3).

**Tn5047: a way for recreation of a two-ended transposable element**

In pKLH201, IS1008 inserted into IS1011.D1 interrupted the common (PPF) fragment at the right flank (Fig. 3). This IS element was followed by a one-ended, apparently recombinant, Tn3 family transposon named Tn5048. It contained (from left to right) a relic of a tnpR gene (tnpR.D2), a divergently oriented complete tnpA gene, and a 38 bp TIR. The predicted TnpA protein clustered with transposases from Tn21 subgroup elements (up to 74 % identity), whereas the predicted truncated TnpR polypeptide (TnpR.D2) was distantly related to resolvases from Tn21 subgroup elements (≤ 20 % identity) but was much more similar (69 % identity) to the corresponding region of TnpR encoded by Tn5403 from Klebsiella pneumoniae (Rinkel et al., 1994; AC X75779). The spacer region between the tnpRD2 and tnpA genes of Tn5048 was too short (94 bp) to accommodate a regular res site (occupying 101–130 bp; Sherratt, 1989) and showed low identity (46 %) to the corresponding (93 bp) spacer within Tn5403. The tnpRD2–tnpA spacer regions from Tn5048 and Tn5403 did not contain recognizable res subites I, II or III. We
noted that two one-ended Tn21-subgroup transposons, Tn5047 and Tn5048, formed a new mercury-resistance transposon carrying IS1008. The TIRs that flanked this structure had only four mismatches. A transposition assay similar to that described previously (Kholodii et al., 1995) demonstrated that the chimeric 9207 bp element, named Tn5047, was able to transpose into pRP1.2 when the Tn5036 tnpA gene was supplied in trans with pKLH5036.98. The transposition frequency of Tn5047 was $2.5 \times 10^{-5}$ per pRP1.2 transfer. In accordance with the sequence data, no Tn5047-mediated resolution activity was detected.

**DNA insertion sequences: molecular characterization**

Most of the IS elements flanking the Tn5047 variant mer operons (Figs 2a and 3) were highly similar to either the 820 bp IS26 from Proteus mirabilis (Mollet et al., 1983, 1985) belonging to the IS6 family (Mahillon & Chandler, 1998), or the 1040 bp IS17 recently found in Acinetobacter haemolyticus BM2714 (AC U95013). Nine out of a total of twenty IS elements identified in this study were truncated. IS1011.D1 and IS17 shared 82% identical nucleotides. In both elements, the longest ORF encoded a polypeptide that
exhibited the highest similarity (60% identical residues) to ORFL1, a putative transposase from *Janthinobacterium* sp. J3 (AC AB095952), and was 39% identical to the transposase of a well-characterized element, IS\textsubscript{903} (Weinert et al., 1983; Mahillon & Chandler, 1998). The predicted transposases from elements IS\textsubscript{1011}.D1 and IS\textsubscript{17} were characterized by a catalytic motif, D(73)D(67)E, closely related to the IS\textsubscript{903} element's transposase motif, D(71)D(67)E (Polard & Chandler, 1995; Mahillon & Chandler, 1998). In accordance with these data, phylogenetic analysis (Fig. 4a, bottom) placed IS\textsubscript{1011}.D1 and IS\textsubscript{17} into the IS\textsubscript{903} group that is part of the IS\textsubscript{5} family (Mahillon & Chandler, 1998).

Despite the widespread distribution of IS\textsubscript{26} relatives amongst bacteria of different taxa (determined by database searches), until now these have not been found in the genus *Acinetobacter*. As seen in Figs 2(a) and 3, these elements (IS\textsubscript{1006}–1010) were found in five of the eight plasmids studied. Full-sized IS elements that we have identified were either 819 or 820 bp. IS\textsubscript{1006} and IS\textsubscript{1007} were one-base variants of IS\textsubscript{1006} and IS\textsubscript{1007}, respectively, and IS\textsubscript{1006}.D1 and IS\textsubscript{1006}.D2 were truncated copies of IS\textsubscript{1006}. IS\textsubscript{1008}, IS\textsubscript{1009} and IS\textsubscript{1010} were recombinant, each containing sequences characteristic of two or more IS\textsubscript{26} relatives (Fig. 4b). The *Acinetobacter* IS elements displayed characteristics typical of the IS\textsubscript{6} family (Mahillon & Chandler, 1998), namely, their 15 bp TIRs began with a conserved 5' dinucleotide GG; and a single ORF preceded by the potential −35, −10 and RBS sites encoding a putative transposase displaying a distinct DDE motif, D(59)D(34)E, the same as in the IS\textsubscript{26} ORF. DNA sequence identity to IS\textsubscript{26} varied from 71% to 75%, and was at least 13% less than that shared by any two *Acinetobacter* IS elements identified in this work. A phylogenetic study has placed the *Acinetobacter* IS elements into a new *Acinetobacter* subgroup of DNA insertion sequences in the IS\textsubscript{6} family, named the ACI subgroup (Fig. 4a, top); within this group the recently identified IS\textsubscript{Our1} (apparently originating from an *Acinetobacter* strain: Mammeri et al., 2003) was present.

In the *Acinetobacter* plasmids studied, no target site duplication characteristic of transposition events of IS\textsubscript{26} and other IS elements (Iida et al., 1984; Mahillon & Chandler, 1998) was found in the insertion site of either of the individual ACI subgroup IS elements or potential transposons bracketed by these IS elements. DNA insertion sequences from other families (IS\textsubscript{1}, IS\textsubscript{3} and IS\textsubscript{21}) were also identified (Figs 2a and 3; see also the table published as supplementary data with the online version of this paper at http://mic.sgmjournals.org).
**IS26 relatives: a functional study**

Since the *Acinetobacter* IS elements were closely related to IS26, a functional study was performed on them similar to that described for IS26 (Iida *et al.*, 1984), which is believed to transpose via a replicative mechanism. pUC19 (Ap<sup>R</sup>) derivatives containing a cloned copy of IS1006 (pKLH203.L14), IS1007 (pKLH201.L11), IS1008 (pKLH204.27), IS1009 (pKLH201.5) or an IS26 variant (pKLH272.R1) were examined for IS-mediated fusion with the conjugative plasmid pOX38gen (Gm<sup>R</sup>). After each pKLH plasmid was co-transformed with pOX38gen into *E. coli* K-12 HB101 (recA<sup>+</sup>), Gm<sup>R</sup> Ap<sup>R</sup> clones were selected, replated three or four times on selective LB agar, mated with JF238 (recA<sup>+</sup> Nal<sup>R</sup>), and the frequencies of mobilization of the pUC19 derivatives measured by the ratio of Ap<sup>R</sup> Gm<sup>R</sup> Nal<sup>R</sup> transconjugants. In all but one case these were less than the lowest detectable (2·5 × 10<sup>−9</sup>). With pKLH201.5, the frequency was 40–1000 times higher. After the genetic linkage between the Ap<sup>R</sup> and Gm<sup>R</sup> markers was established, four JF238 Ap<sup>R</sup> Gm<sup>R</sup> Nal<sup>R</sup> recA<sup>+</sup> transconjugant clones of independent origin were replated three or four times – to allow dissociation of the cointegrates which were expected to contain the direct copies of IS1008 at the junctions of pOX38gen and pKLH201.5 – and then mated with a recA recipient, UB5201 Rif<sup>R</sup>. In these matings, ≤1 % of Gm<sup>R</sup> Rif<sup>R</sup> transconjugants were Ap<sup>R</sup>. Southern hybridization and terminal sequencing showed that these Ap<sup>R</sup> segregants carried, in each case, a pOX38gen::IS1008, probably resulting from a homologous recombination-mediated resolution event between the direct copies of IS1008 in the cointegrate. Where tested, a target site duplication of 8 bp flanking IS1008, was identified. These data were similar to those obtained in the case of IS26 (Iida *et al.*, 1982, 1984).

**Time elapsed from the last common ancestor of the PPFs**

The isolation of Tn<sup>4</sup>PKLH2-type transposons and corresponding PPFs from the permafrost samples, and the divergence observed between them (Fig. 1, pKLH205 and 208; and data not shown), provided evidence that these entities evolved before they were frozen (15 000–40 000 years ago). Given that the mean evolutionary synonymous distance (*D<sub>s</sub>* and the rate of synonymous site evolution (*μ*)) are available, the divergence time elapsed since the last common ancestor of these entities may be computed by dividing *D<sub>s</sub>* by *μ* (Rich *et al.*, 1998; Achtman *et al.*, 1999). *D<sub>s</sub>* was computed from the polymorphisms observed within the contemporary Tn<sup>4</sup>PKLH2-like transposons (from pKLH2, 201–204, 207; Fig. 1) with the help of several models embedded in the MEGA2 software program (Kumar *et al.*, 2001). These models (Nei–Gojobori, modified Nei–Gojobori, Li–Wu–Luo, Pamilo–Bianchi–Li, and Kumar’s) described elsewhere (Nei & Kumar, 2000), produced close estimates of *D<sub>s</sub>* ranging from 0·00141 ± 0·00072 to 0·00181 ± 0·00101. A number of *μ* estimates were available: for the genus *Pseudomonas*, 2·3 × 10<sup>−9</sup> mutations per
synonymous site per year (Ochman et al., 1999); for mammals, the same value or similar (Kumar & Subramanian, 2002); for E. coli/Salmonella enterica, 4.5 x 10^{-9} (Ochman & Wilson, 1987); and for other entities (Ochman et al., 1999). The similar μ values observed amongst disparate life forms such as bacteria and mammals further confirm the data of Kumar & Subramanian (2002) that neither the generation time, nor the particular features of the organism’s physiology, nor the population size, provide factors determining μ. One more inference from these data seems to be that μ values for chromosomal and plasmid genes can hardly differ significantly. Taking this consideration into account, and using the μ value from the genus Pseudomonas [as, of bacteria for which a μ estimate was available (Ochman et al., 1999), Acinetobacter spp. were most closely related to Pseudomonas spp. (Olsen et al., 1994)], we have calculated the age of the hypothetical plasmid that was the source of the Acinetobacter PPFs identified in this study as being between 300 000 and 830 000 years.

**DISCUSSION**

In this study, we have found that nearly identical defective transposons located on various plasmids are widespread among HgR Acinetobacter strains from natural microbiocenoses from dispersed global locations. The high frequency of occurrence of Tn^{dPKLH2}-like transposons (84%) was associated with low frequencies of occurrence of the Tn21-related tnp genes (7%) and transposition-proficient HgR transposons (<3%) in environmental Acinetobacter isolates. In contrast, 35–52% of environmental Gram-negative strains from other taxa (Enterobacteriaceae), which were isolated concomitantly with the Acinetobacter strains screened in this work, carried an active HgR transposon (Mindlin et al., 2001).

Why is the inactive transposon widespread and how has it appeared in various plasmids? The fact that the transposon is often located on a transmissible plasmid (Table 3) answers the first of the questions. To answer the second question, we have sequenced the regions flanking Tn^{dPKLH2}-like transposons in different plasmids. These regions appeared to contain multiple tracks of previous recombination events, > 36 over ~70 kb sequenced. Such events included: independent breakpoints of PPFs in RS2 (Fig. 2b); insertions/deletions of different DNA elements and regions adjacent to them (Figs 2a and 3); and homologous recombination events in mer operons and IS elements, producing genetic mosaics (Figs 1 and 4b). Some older tracks, e.g. insertion of ‘IS3’ and IS17.D1 ancestors (Fig. 2, pKLH204), were covered by later recombinations, so the whole list and succession of events cannot be reconstructed accurately. However, it is clear that certain events in the evolution of the Acinetobacter mer loci occurred before the formation of the Arctic permafrost. These events included: (i) recombination at the res site of the active transposon to produce the hybrid site RS1; (ii) recombination events underlying the formation of both the IS1011.D1-RS2 conglomerate and versions of RS2 in pKLH205 and 208 (Fig. 2); (iii) recombination events that formed the left flank in pKLH205 and 208; and (iv) allelic exchanges that resulted in the mosaic mer loci (Fig. 1).

An unexpected finding in this study was the high sequence identity between different Acinetobacter plasmids in the regions that flanked the Tn^{dPKLH2}-like elements. The length of additional identity varied from 5 to 1451 bp (Figs 2a and 3, hatched bars) and, as a rule, this identity was interrupted by a mobile element (IS26- or Tn21-related), or ended at the recombination point of the cinH-RS2 DNA resolution system (Fig. 2b). The very closely related and apparently transferable HgR units we have identified and named PPFs, which were flanked by recombinogenic elements, do not fit into any known categories of bacterial mobile elements, such as pathogenicity islands (Hacker & Kaper, 2000), introns (Martínez-Abarca & Toro, 2000; Dai & Zimmerly, 2002), class II transposons (Sherratt, 1989; Grinsted et al., 1990; Craig, 1996) (even including the possibility for a one-ended transposition mechanism: Mötsch et al., 1985), IS elements (Mahillon & Chandler, 1998; Mendiola et al., 1994) and gene cassettes (Recchia & Hall, 1995; Hall & Collis, 1998).

The most reasonable view from the data we obtained is that PPFs are relics of an ancient (300 000–800 000 years old) plasmid that has passed through numerous rounds of fusions with other plasmids followed by deletions (resolutions) stabilizing the resulting novel HgR plasmids. The hypothesis for the stabilizing events stems from the necessity to consider the cinH-RS2 DNA resolution system as a factor implicated in transposition of Tn^{dPKLH2}-like elements, taking into account that its co-integrative and dissociative functions (and those of other typical resolution systems) are reversible (Bliska et al., 1991; Kholodii, 2001). In support of this hypothesis, it seems to be no accident that multiple events of damage to the resolution determinants were seen in the plasmids we studied. These events included: the ‘insertion’ of an IS1011.D1, substituting for res subsites II and III of the ancestral RS1 site (Figs 1 and 2a, top); the impingement of IS1006.1 elements into RS1 in pKLH201 and 207 (shown by the vertical wavy lines in Figs 1 and 3); deletions of the tnpR genes seen with the tnp.RD1 and tnp.RD2 relics (Fig. 3, pKLH202, 207 and 204); and, in the case of pKLH201, 204, 202 and 207, partial deletions of the 44 bp element (shown by the diamond in Figs 2a and 3), the most probable cis-acting factor stimulating the formation of replicon fusions by CinH (Kholodii, 2001).

In contrast to true transposition, which is related to acquisition of a DNA segment, the models we propose, which take into account and explain why no target duplications associated with mobile elements were identified, are related to redistribution of genetic material between replicons. Thus based on the co-integrative capacity of
typical resolvases (Kholodii, 2001), transposition of the pKLH208 fragment, delimited by RS3 and RS2 (Fig. 2a), may be proposed by the mechanism described previously (Kholodii, 2001), where both reactions producing transposition are resolvase-mediated. For the origin of pKLH208, insertion of a class II transposon (Tn5049 ancestor) into the common parental plasmid is sufficient to give ‘pKLH208’. Transposition by typical resolvases is also applicable for the movement of the pKLH2 PPF, if the second res site exists in the unsequenced region. Other scenarios, applicable for the movement of the pKLH204 PPF (Fig. 5a) and the formation of pKLH205 (Fig. 5b), differ from the mechanism discussed above. In these proposed mechanisms, the cointegrate could dissociate either by intramolecular duplicative transposition initiated by a relative of IS26 (possibly similar to the mechanism detailed in the IS1 system: Turlan & Chandler, 1995), or by illegitimate recombination, which is suggested at the left flank of pKLH205. Considering the co-integrative activity of the IS26-related element as an initiating event, the succession of reactions shown in Fig. 5(c), which is the reverse of that shown in Fig. 5(a), is very feasible for the formation of pKLH204. The co-integrative function is also characteristic of other IS elements, but is best expressed in IS26 (Mahillon & Chandler, 1998; Iida et al., 1984). Our transposition models, explaining the origin of pKLH204 and 202/207 (Fig. 5c, d), are based on this feature of transposition, as well as the fact that in the case of IS26, the cointegrates, which are believed to form by a replicative mechanism, show considerable stability, i.e. these are not resolved for a long time by homologous recombination to give the transposition products (Iida et al., 1982). This latter feature allows rearrangement of these cointegrates to be produced by other recombination events. Apparently, precisely because of the deferred resolution and therefore the possibility of non-transposition type resolutions, i.e. those not involving homologous recombination between the two IS elements, the target site duplication produced by IS26 (Iida et al., 1984) is rarely detectable near IS26 elements (database search results). Since the data we have obtained in this work for the IS26-related elements from the genus Acinetobacter [which form a new (ACI) subgroup (Fig. 4a, top)] demonstrate or suggest all of the above properties characteristic of IS26, this gives a basis for the models proposed. The fact that an IS26 variant and most ACI subgroup elements tested in this work have not exhibited mobility might be explained by a particular nucleic acid environment, because there is evidence that the transposition activity of IS26 is significantly increased when the element is placed downstream of a strong promoter (K. Vögél, cited by Mahillon & Chandler, 1998).

In principle, translocation of a TndPKLH2-like element or any other DNA fragment may occur via two or three recombination events, any of which could be stimulated by any recombination system. For example, taking into account the recombinant structure of IS1010 and IS1008 (Fig. 4b), one can suppose that the formation/resolution

Fig. 5. Proposed transposition mechanisms. Large circles show different replicons; mer, HgR locus of the PPFs; X, RS2 or its variant; X’, a res site compatible with RS2; XX’, a recombinant res site; IS, an IS26 relative. (a) Translocation by resolvase followed by IS element-mediated resolution. (b) Translocation by resolvase followed by illegitimate recombination (IL). (c) Translocation by IS transposition followed by resolvase action. (d) Translocation by IS transposition followed by illegitimate recombination. (e) Translocation by homologous recombination (Rec) between IS elements. The asterisk marks IS1007. Co-integrative reaction yields IS1008 as a product; and dissociative reaction yields IS1010 as a product.
of a cointegrate in the evolution of pKLH201 occurred by homologous recombination (Fig. 5e). Of course, we do not rule out the translocation of PPFs (Tn\textsuperscript{d}PKLH2-like elements) by true transposition, either within putative compound (class I) transposons seen in plasmids pKLH201 and 203 (Fig. 3) or within the newly arisen (two-ended) class II transposon like Tn5047. However, as there were no active transposons amongst the Acinetobacter isolates (Table 3, last two columns) and no target site duplications in pKLH201 and 203, no conclusion can be made that these were major events. The models proposed (Fig. 5) imply that mobility is characteristic of DNA segments located not only between two ISs or two TIRs, or two res sites, but also between an IS (or another recombinogenic structure) and a resolution/attachment site.

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REFERENCES


of an aberrant mercury resistance transposable element from an environmental Acinetobacter strain. *Plasmid* 30, 303–308.


