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Molecular structure and translocation of a multiple antibiotic resistance region of a Psychrobacter psychrophilus permafrost strain

Mayya Petrova, Zhosephine Gorlenko & Sofia Mindlin
Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia

Correspondence: Sofia Mindlin, Institute of Molecular Genetics, Russian Academy of Sciences, 2 Kurchatov sq., Moscow 123182, Russia. Tel.: +7 499 196 0015; fax: +7 499 196 0015; e-mail: mindlin@img.ras.ru

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Abstract
A Psychrobacter psychrophilus strain resistant to tetracycline and streptomycin was isolated from a 15 000–35 000-year-old permafrost subsoil sediment sampled from the coast of the Eastern-Siberian Sea. The genes conferring antibiotic resistance were localized on a c. 30-kb pKLH80 plasmid. It was shown that the antibiotic resistance region of this plasmid has a mosaic structure and contains closely linked streptomycin resistance (strA-strB) and tetracycline resistance [tetR-tet(H)] genes, followed by a novel IS element (ISPpy1) belonging to the IS3 family. Both the strA-strB and tetR-tet(H) genes of pKLH80 were highly similar to those found in modern clinical bacterial isolates. It was shown that the ISPpy1 element of pKLH80 can direct translocation of the adjacent antibiotic resistance genes to different target plasmids, either by one-ended transposition or by formation of a composite transposon resulting from the insertion of the ISPpy1 second copy at the other side of the antibiotic resistance region. Thus, our data demonstrate that clinically important antibiotic resistance genes originated long before the introduction of antibiotics into clinical practice and confirm an important role of horizontal gene transfer in the distribution of these genes in natural bacterial populations.

Introduction
According to current views, antibiotic resistance determinants present in clinical isolates of bacteria have evolved from at least two sources. The first one is antibiotic-producing environmental bacteria (streptomycetes) carrying resistance determinants that initially appeared as a mechanism of self-protection. Afterwards, via successive rounds of horizontal transfer, these genes spread among other microbial genera and were integrated into clinical strains of Gram-positive and Gram-negative bacteria (Davies, 1994; Courvalin, 2005; Harbottle et al., 2006). Bacterial genes required for normal metabolism of the bacterial cell (housekeeping structural or regulatory genes) are another possible source of resistance genes, including determinants coding for different efflux systems (Poole, 2005; Piddock, 2006). The spread of multiple drug resistance has been apparently greatly stimulated by the intensive use of antibiotics in clinical practice during the past six decades (Gizman, 2003; Livermore, 2003).

The hypothesis that antibiotic resistance genes of modern clinical bacteria have originated from antibiotic-producing streptomycetes was put forward > 35 years ago (Benveniste & Davies, 1973). In support of this view, bacteria resistant to most commonly prescribed antibiotics such as different aminoglycosides and tetracycline were found among environmental bacterial isolates (Riesenfeld et al., 2004), and some of them were resistant to even more than two antibiotics (Esiobu et al., 2002). Multidrug-resistant bacteria were also found in a large collection of spore-forming bacteria isolated from soil. Most of them were resistant to seven or eight antibiotics of the 21 tested (D’Costa et al., 2006). However, one cannot rule out the possibility that antibiotic resistance determinants found in modern environmental bacteria have in fact been acquired from commensal bacteria or human pathogens by horizontal gene transfer.

We approached the problem by isolating and studying bacterial strains from permafrost sediments formed well before the ‘antibiotic era’ in medicine. Previously, we
described permafrost bacterial strains resistant to mercury compounds, which carried resistance determinants highly similar to the mer-operons of present-day bacteria (Petrova et al., 2002; Kholodii et al., 2003; Mindlin et al., 2005). Recently, we succeeded in isolating permafrost bacterial strains resistant to different antibiotics. We found that about 30% of the isolated strains had been simultaneously resistant to two and more antibiotics of different classes (Mindlin et al., 2008). Furthermore, we demonstrated that in some environmental streptomycin-resistant strains, the strA-strB genes were associated with mobile elements such as plasmids and transposons (Petrova et al., 2008). We have also described a strain of Psychrobacter psychrophilus MR29-12 isolated from a permafrost sample collected at the coast of the Eastern-Siberian Sea (Mindlin et al., 2008), which carried an R plasmid pKLH80 specifying resistance to streptomycin and tetracycline. Although both determinants could be mobilized in mating-out assays (Petrova et al., 2008), their association with transposable elements remained to be clarified. This study was undertaken to examine the molecular structure of the antibiotic resistance genes from the permafrost strain of P. psychrophilus MR29-12 and the structure of the putative mobile element associated with these genes. As a result, we demonstrated that the streptomycin and tetracycline resistance genes of P. psychrophilus MR29-12 were closely related to modern antibiotic resistance genes and were associated with a novel IS element involved in their translocation.

Materials and methods

Bacterial strains and plasmids

The permafrost sampling techniques used for the isolation, storage and transportation of samples, as well as bacterial isolation methods, were as described in Vorobyova et al. (1997) and Petrova et al. (2002). Psychrobacter psychrophilus strain MR29-12 has been described in our previous studies (Mindlin et al., 2008; Petrova et al., 2008). Bacteria and plasmids used in this study are listed in Table 1. Bacteria were grown on Luria–Bertani (LB) agar or in LB broth. Psychrobacter psychrophilus MR29-12 and Acinetobacter calcoaceticus BD413rif were grown at 25°C. Escherichia coli K12 strains were grown at 30 or 37°C. All crosses were incubated at 30°C.

Mobilization of antibiotic resistance determinants in P. psychrophilus MR29-12

To analyze transposition of streptomycin and tetracycline resistance determinants, a mating-out assay with modifications was used (Mindlin et al., 2001). Plasmid pKLH80 (SmR, TcR) was transferred from P. psychrophilus MR29-12 into a laboratory strain of A. calcoaceticus BD413rif (RifR) by conjugation as described in Petrova et al. (2008). A broad-host-range plasmid RP4-5M (KmR) was then introduced by conjugation into A. calcoaceticus BD413rif containing pKLH80. After two serial transfers on selective medium (LB agar plus 25 µg mL⁻¹ kanamycin, 50 µg mL⁻¹ streptomycin and 15 µg mL⁻¹ tetracycline), the Acinetobacter transconjugants were mated with E. coli K-12 JF238 (NalR). SmR·NalR transconjugants, expected to carry RP4-5M harboring transposable SmR determinants (RP4-5M::SmR), were selected on LB plates with 50 µg mL⁻¹ Sm and 20 µg mL⁻¹ Nal and checked for resistance to kanamycin and tetracycline. The genetic linkage between RP4-5M and the streptomycin and tetracycline resistance determinants was verified in crosses with E. coli K-12 C600 rif. The experiments were repeated four times, resulting in four independent isolates of RP4-5M containing antibiotic resistance genes RP4-5M80-I, RP4-5M80-II, RP4-5M80-III and RP4-5M80-IV. The antibiotic resistance genes of RP4-
5M80-I were translocated onto vector plasmid pGEM-7Zf(--). By the conduction method as described previously (Mindlin et al., 2001); the resulting plasmid containing Tn5080 was designated as pGEM-7-80.1. A 4.5-kb fragment of Tn5080 between two NcoI sites was subcloned from RP4-5M80-I into pGEM-5Zf(--), resulting in a pGEM-5-80.3 plasmid (see Table 1).

DNA methodology, sequencing and PCR assays
Basic DNA procedures, including restriction endonuclease digestions, ligations, transformations and agarose gel electrophoreses, were carried out as described (Sambrook et al., 1989). The sequence of Tn5080 was obtained from plasmids pGEM-7-80.1 and pGEM-5-80.3 using Sp6 and T7 standard primers and specific primers corresponding to the Tn5080 sequence. DNA sequencing was performed at the Interinstitute ‘GENOME’ Center (Moscow). Nucleotide and amino acid sequences were analyzed at the website of the National Center for Biotechnology Information via the BLAST network service (Altschul et al., 1997), and using the VECTOR NTI SUITE 9.0.0 (InforMaz Inc., Frederick, MD). The sequence of Tn5080 has been deposited in the EMBL database under accession number AM992204. Positions of the PCR primers are shown in Fig. 1. A combination of primers 1 and 4 was used to detect the left copy of ISPpy1; primers 2 and 5 were used to detect the right copy of ISPpy1. Control experiments demonstrated that these primer pairs can specifically discriminate between the two ISPpy1 copies. Primers 3 and 4 were used to detect the presence of orfX. The copy number of ISPpy1 was estimated by Southern blot hybridization. Probes corresponding to ISPpy1 were obtained by PCR with primers 1 and 2 and radiolabeled as described (Sambrook et al., 1989).

Results and discussion
Characterization of the Tn5080 transposon
To analyze the structure of the antibiotic resistance region of P. psychrophilus MR29-12, we transferred the antibiotic resistance genes from the original pKLH80 plasmid to a broad-host-range plasmid RP4-5M using a mating-out assay. The resulting plasmid was designated RP4-5M80-I (Table 1). Based on this plasmid, we constructed recombinant plasmids carrying the whole or part of the isolated mobile element and determined the sequence of the translocated region using standard sequencing methods (see Materials and methods for details). It was revealed that the mobile element isolated may be considered a typical composite transposon harboring streptomycin and tetracycline resistance genes flanked by two identical copies of a novel IS element (Fig. 1a). The transposon was named Tn5080 (7193 bp in size), while the IS element in Tn5080 was designated as ISPpy1 (IS Psychrobacter psychrophilus 1) (Fig. 1, Table 2).

ISPpy1 displayed features typical for the IS sequences of the IS3 family (Mahillon & Chandler, 1998). In particular, (1) the length of the element was 1275 bp, which is similar to the length of other elements of this family; (2) the length of direct target repeats was 3 bp; (3) the element contained two consecutive ORFs (orfA and orfB), presumably involved in transposition, and (4) the element contained a frameshifting signal between orfA and orfB, necessary for the formation of the OrfAB transposase (Fig. 1a and data not shown). However, it should be noted that the length of the left and right inverted repeats (IRs) in ISPpy1 were 49 and 50 nt, respectively (identity at 34 positions) (Fig. 1b), and that the repeats were terminated with 5'-TA...TA-3', as opposed to typical IS3-family elements characterized by IRs

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** Genetic organization of Tn5080 and ISPpy1. (a) The structure of Tn5080. The location and polarity of the genes are shown by open arrows. orfB and A (orfA), putative transposition genes of ISPpy1; X (orfX), a gene coding for a putative mobilization protein; AY (orfY), the 3'-part of a gene with unknown function. Filled-in arrowheads designate terminal IRs of ISPpy1; the open arrowhead designates the right terminal IR of Tn5393. Open rectangles and rectangles with different shading below the scheme denote regions of different evolutionary origin (for details, see the text). Primers 1–5 used in this work are shown above the map of Tn5080. (b) Terminal IRs of ISPpy1. Sequences of the left (IRl) and right (IRr) terminal repeats are aligned from the 5' end. Identical nucleotides are indicated by vertical bars.
of 20–40 bp and terminated with 5′-TG . . . CA-3′ (Mahillon & Chandler, 1998).

A detailed analysis of the antibiotic resistance region of Tn5080 revealed three different subregions, as followed from their comparison with published bacterial DNA sequences (Figs 1a and 2, Table 2). The leftmost region 1 contained an ORF designated orfX and adjacent sequences showing 63% identity with the nucleotide sequences recently found in the plasmid DNA of several Acinetobacter baumannii strains (see Table 2). The orfX homologues were predicted to encode a putative DNA restriction/modification protein involved in plasmid mobilization.

Region 2 contained the streptomycin resistance genes strA-strB and exactly corresponded to a fragment of the streptomycin-resistance transposon Tn5393 (Chiou & Jones, 1993; Mantegoli & Rossolini, 2005). This transposon and its derivatives are widely distributed among plant-associated bacteria such as Erwinia amylovora, Xanthomonas campestris and Pseudomonas syringae, as well as in many clinical isolates of Gram-negative bacteria (Sundin, 2000). Whereas the transposition module of Tn5393 including the left inverted repeat IR-5393l was completely absent from Tn5080, a 40-bp fragment adjacent to the 5′ end of the strA gene and the right inverted repeat IR-5393r were present at precisely the same positions as in Tn5393 (Fig. 1a). Thus, it is very likely that the strA-strB region in Tn5080 is a relic of Tn5393.

Region 3 contained tetracycline resistance genes tetR–tet(H) and a proximally located fragment of an ORF (orfY). The whole region was essentially identical (with only a single nucleotide substitution) to a fragment of genomic DNA of the Histophilus somni clinical strain 2336 (CP000947). The 446-bp-long orfY fragment corresponded to the 3′-part (nucleotides 73–518) of an ORF found in the H. somni genome and encoding a protein with unknown functions. The 5′-part of the gene was deleted from Tn5393 as a result of a Tn5393 insertion. The tetR-tet(H) genes, highly similar to those of Tn5080, were previously found in plasmids, chromosomes and in transposon Tn5076 in clinical bacterial strains belonging to Pasteurella, Mannheimia, Acinetobacter, Moraxella and Actinobacillus genera involved in a variety of infections in humans and animals (Kehrenberg et al., 1998, 2001; Miranda et al., 2003; Blanco et al., 2006). The 3′-end of the tet(H) gene in region 3 contained a 21-bp-long deletion, which likely resulted from the ISPpy1 insertion. The resulting TetH protein contained 393 amino acids as compared with 400 amino acids in the full-length protein (Table 3). The deletion did not affect the protein function because the tet(H) gene conferred high-level tetracycline resistance both to the original P. psychrophilus isolate and to the A. calcoaceticus and E. coli strains carrying an antibiotic resistance region. It should be mentioned that another shortened version of the TetH protein (392 amino acid residues) was detected previously in plasmid pPAT1 in a single isolate of Pasteurella aerogenes and six isolates of Pasteurella multocida (Table 3) (Kehrenberg & Schwarz, 2000). Other known variants of the tet(H) gene contain single amino acid substitutions in the body of the gene (codons 137 and 252, Table 3).

### Structure of the antibiotic resistance region of pKLH80

The transposon isolation procedure used in our experiments included several transfers of antibiotic resistance determinants between different host bacteria, which might be accompanied by rearrangements of the antibiotic resistance region. Therefore, we carried out additional experiments to further characterize the structure and mechanisms of transfer of the antibiotic resistance region in the original plasmid pKLH80 from P. psychrophilus. We performed three additional independent experiments on the mobilization of the antibiotic resistance determinants from P. psychrophilus. As a result, three new variants of RP4-5M with insertions originating from pKLH80 were obtained and investigated (RP4-5M80-II, III and IV, Table 1). Southern blot hybridization experiments with probes corresponding to ISPpy1 revealed that all three new RP4-5M derivatives and the original pKLH80 plasmid contained a single copy of ISPpy1, as compared with two copies of ISPpy1 in RP4-5M80-I (Fig. 2). A PCR analysis with primer pairs specific to the left and right copies of ISPpy1 in Tn5080 (Fig. 1a) demonstrated that all analyzed plasmids, including pKLH80, contained the

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**Table 2. Molecular analysis of the antibiotic resistance region of Psychrobacter psychrophilus plasmid pKLH80**

<table>
<thead>
<tr>
<th>Region</th>
<th>Coordinates*</th>
<th>G+C (%)</th>
<th>Closest relative</th>
<th>Identity (%)</th>
<th>ACs†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. orfX and adjacent sequences</td>
<td>1276–1788</td>
<td>36</td>
<td>Acinetobacter baumannii str. SDF, plasmid p2ABSDF</td>
<td>63.0†</td>
<td>CU468232</td>
</tr>
<tr>
<td>2. strA-strB and adjacent sequences</td>
<td>1789–3575</td>
<td>56</td>
<td>Alcaligenes faecalis Tn5393d</td>
<td>100†</td>
<td>A6627643</td>
</tr>
<tr>
<td>3. orfY-tetR-tet(H)</td>
<td>3576–5921</td>
<td>41</td>
<td>Histophilus somni 2336</td>
<td>99.9†</td>
<td>CP000947</td>
</tr>
<tr>
<td>ISPpy1</td>
<td>5919–7193</td>
<td>43</td>
<td>Cyanobacte sp. ATCC 51142 linear chromosome</td>
<td>53.1†</td>
<td>CP000807</td>
</tr>
</tbody>
</table>

*The position of the genes in the sequenced region of pKLH80 (AM992204).
†ACs, accession numbers of the closest relative sequences.
†Identity at the nucleotide sequence level.
†Overall identity at the amino acid sequence level (orfA and orfB).
right copy of IS\textit{Ppy1}, whereas the left copy of the element was present only in RP4-5M80-I (data not shown). PCR assays with primers corresponding to different parts of Tn5080 demonstrated that pKLH80 contained the whole antibiotic resistance region as in Tn5080, including the tetracycline and streptomycin resistance genes and \textit{orfX} located at its left side (Fig. 1a and data not shown). Control experiments confirmed the absence of IS\textit{Ppy1} in the original RP4-5M plasmid and in the \textit{A. calcoaceticus} BD413rif strain used in conjugation experiments. Thus, the left copy of IS\textit{Ppy1} in RP4-5M80-I most likely originated from transposition during the procedure of RP4-5M80-I isolation, resulting in the formation of Tn5080.

**Evolutionary origin and modes of translocation of the antibiotic resistance region of pKLH80**

The association of the IS\textit{Ppy1} element with the antibiotic resistance genes in pKLH80 and in all analyzed recombinant plasmids suggests that this element is directly involved in the translocation of the resistance genes. Analysis of the structure of recombinant plasmids demonstrated that IS\textit{Ppy1} can mediate two different pathways of dissemination of antibiotic resistance genes (Fig. 3). First, this element is able to direct translocation of the neighboring antibiotic resistance genes onto different targets, a process known as one-ended transposition (Avila \textit{et al.}, 1988). This reaction was also described for IS911, another member of the IS3-family of insertion sequences (Polard \textit{et al.}, 1994). In addition, one-ended transposition events were detected for the elements of the IS1380 and the IS91 families (Bernales \textit{et al.}, 1999; Poirel \textit{et al.}, 2005). However, because the elements of all three IS families differ in many properties, the mechanism for genomic DNA mobilization might not be common. We are now studying the insertion specificity and the detailed mechanism of IS\textit{Ppy1} transposition. Second, IS\textit{Ppy1} can take part in the \textit{in situ} formation of a putative composite transposon, by insertion of its second copy at the other side of the resistance genes (see Fig. 3). Such a formation of a composite transposon was serendipitously discovered in our experiments during the initial isolation of the antibiotic resistance genes of \textit{P. psychrophilus} MR29-12. Thus, antibiotic resistance regions associated with IS elements similar to the region found in pKLH80 may serve as building blocks for efficient formation of multiple antibiotic resistance transposons, both in nature and in clinical practice.

As shown above, the antibiotic resistance region in pKLH80 plasmid of \textit{P. psychrophilus} has a mosaic structure.

**Table 3. Comparative amino acid sequences of TetH proteins**

<table>
<thead>
<tr>
<th>Origin of the \textit{tet} (H) genes</th>
<th>Amino acid positions in TetH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>137</td>
</tr>
<tr>
<td>\textit{Pasteurella multocida} pVM111</td>
<td>V</td>
</tr>
<tr>
<td>\textit{Pasteurella multocida} pPMT1</td>
<td>A</td>
</tr>
<tr>
<td>\textit{Pasteurella aerogene} pPAT1</td>
<td>A</td>
</tr>
<tr>
<td>\textit{Mannheimia glucosida et al.} pMH1</td>
<td>A</td>
</tr>
<tr>
<td>\textit{Psychrobacter psychrophilus} pKLH80</td>
<td>A</td>
</tr>
</tbody>
</table>

*Combined data from Kehrenberg \textit{et al.} (2001) and this work.

Fig. 2. IS\textit{Ppy1} copy number and structure of the antibiotic resistance regions of pKLH80 and recombinant RP4-5M plasmids. (Left) Southern blot analysis of AvaII-digested plasmid DNA. (Right) Genetic organization of the antibiotic resistance region in RP4-5M80-I (top) and in other recombinant plasmids (bottom). A-AvaII restriction sites. Positions of specific probes used for Southern blot analysis are shown by black lines below IS\textit{Ppy1}. The gene designations are the same as in Fig. 1.
In particular, the streptomycin and tetracycline resistance genes found in this region most likely originated from different sources. The mosaic structure of the antibiotic resistance gene cluster is clearly manifested in the different G+C contents of its different regions (Table 2), which corresponded well to the G+C content values of the well-known bacterial hosts of these genes (Kehrenberg et al., 2001, 2003; Sundin, 2002). Therefore, it may be supposed that the pKLH80 plasmid has descended from an unknown ancestral plasmid by successive insertions of antibiotic resistance genes. Based on sequence analysis, one can suggest the following hypothetical scenario for the formation of the antibiotic resistance region in pKLH80. First, a cluster of the antibiotic resistance genes was formed, probably as a result of two independent events: (1) transposition of Tn5393 into a DNA region containing the tetR-tet(H) genes and (2) insertion of the IS\textsubscript{Ppy1} at the 3’ end of tet(H) (Fig. 3). Then, a one-ended transposition mediated by IS\textsubscript{Ppy1} has likely occurred. The transposition included tetracycline genes and the streptomycin-resistance transposon Tn5393 lacking the transposition module. It is worth noting that a cluster of the same antibiotic resistance genes as in pKLH80 was found recently in the pVM111 plasmid from a clinical strain of \textit{P. multocida} (Kehrenberg et al., 2003). However, in that case, the tetR-tet(H) gene region was inserted into an RSF1010-like plasmid between the sul2 and strA genes, most likely as a result of illegitimate recombination.

The presence in pKLH80 of remnants of Tn5393, a modern clinically important transposon, suggests that this transposon spread among environmental bacterial populations many thousands of years ago. In support of this, we have recently detected the strA-strB genes in association with remnants of the Tn5393 transposition module in several other permafrost bacterial strains (Petrova et al., 2008, and unpublished data). Thus, this transposon likely played an important role in horizontal transfer of the strA-strB streptomycin resistance genes among environmental bacteria.

In conclusion, our data provide the first example, to our knowledge, of a mobile genetic element coding for multiple antibiotic resistance that existed in natural bacterial populations long before the ‘antibiotic era’. This suggests that formation of multiple resistance mobile elements can occur steadily in the environment, thus providing a natural reservoir of antibiotic resistance genes that can be readily transferred into clinical bacteria. The results of our study support the hypothesis that antibiotic resistance genes were spread in natural bacterial populations long before the introduction of antibiotics into clinical practice and confirm an important role of horizontal gene transfer in their distribution.

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