On the Origin of the Chloramphenicol Resistance Transposon Tn9

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(Received 11 August 1982; revised 25 September 1982)

The widely studied chloramphenicol resistance (Cm') transposon Tn9 came from phage P1 Cm0. This phage, however, had acquired its Cm' marker from the R plasmid pSM14. The analysis of the physical structure of pSM14 has now revealed that this plasmid already carried Tn9 and also the tetracycline resistance transposon Tn10. Physical and functional studies indicated that Tn9 of pSM14, although capable of transposition, probably translocated to the P1 genome by reciprocal recombination processes.

INTRODUCTION

The chloramphenicol resistance (Cm') transposon Tn9 is one of the most extensively studied transposons. It is 2638 bp long and includes two flanking directly repeated IS1 elements (MacHattie & Jackowski, 1977; Alton & Vapnek, 1979). The gene cat for Cm' is carried in the 1102 bp central DNA segment and codes for chloramphenicol acetyltransferase which is also responsible for resistance to fusidic acid (Alton & Vapnek, 1979; Shaw et al., 1979; Völker et al., 1982). Transposition of Tn9 was first demonstrated from bacteriophage P1Cm0 (originally called P1CM) to phage λ (Gottesman & Rosner, 1975). Transposition experiments were later carried out using either P1Cm0 or λ::Tn9 as donor genomes (Bukhari & Froshauer, 1978; Johnsrud et al., 1978; Galas et al., 1980).

Phage P1Cm0 was originally isolated by Kondo & Mitsushashi (1964) as a plaque-forming, specialized Cm' transducing phage from a P1 lysate prepared from cells harbouring the R plasmid pSM14 (originally named R14). It was thus assumed that transposition of Tn9 from pSM14 into the P1 genome produced P1Cm0 (Campbell et al., 1977). However, subsequent physical analysis of the P1 and P1Cm0 phage genomes revealed that P1 DNA contains an IS1 as a natural constituent, the location of which coincides with that of Tn9 on P1Cm0 DNA (De Bruijn & Bukhari, 1978; Iida et al., 1978; Iida & Arber, 1979). Transposition was thus not necessarily involved in the formation of P1Cm0, which might rather have resulted from restructuring processes including reciprocal recombination between IS1 elements and IS1-mediated deletion formation. These processes were shown to have been involved in the formation of a family of IS1-mediated Cm' transposons (Arber et al., 1978; Iida & Arber, 1980; Iida et al., 1980; Iida et al., 1981 a). Furthermore, de novo genesis of IS1-flanked Cm' transposons (e.g. Tn2651) with structures similar to that of Tn9 was demonstrated using a Cm' segment on a plasmid containing no IS1 sequence, and the resident IS1 of phage P1 (Iida et al., 1980).

These observations prompted me to suspect that pSM14 might not necessarily carry Tn9 and that Tn9 might have its origin in the derivation of the P1Cm0 genome. Experimental evidence shows that this hypothesis is not correct and that Tn9 is already carried on pSM14 from which it is able to transpose as a unit. These results indicate that P1Cm0 was formed by two successive recombinations between IS1 sequences of P1 and of pSM14. Indeed, P1Cm, the genome structure of which is identical to that of P1Cm0, were repeatedly isolated from cells carrying both P1 and pSM14. The structural analysis also revealed that pSM14 is a relative of the R plasmid R100 (or NR1) and carries the tetracycline resistance (Tc') transposon Tn10 and IS2.
Table 1. Properties of bacteria, bacteriophages and drug resistance plasmids

Nomenclature is according to Bachmann & Low (1980), Campbell et al. (1977) and Novick et al. (1976).

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<tr>
<th>Bacteria</th>
<th>Comments</th>
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<tr>
<td>ML4</td>
<td><em>E. coli</em> O-26 (pSM14)</td>
<td>From H. Hashimoto (Kondo et al., 1962)</td>
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<tr>
<td>ML40</td>
<td><em>E. coli</em> K12 (F) (pSM14) (2)</td>
<td>From H. Hashimoto (Kondo et al., 1962)</td>
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<tr>
<td>WA921</td>
<td><em>E. coli</em> K12 (F') thr leu met thi hsdK</td>
<td>Wood (1966)</td>
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**R Plasmids**

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<tr>
<td>R100-1</td>
<td>Cm'Sr'Su' Hg'Tc' Tra' IncFII</td>
<td>Egawa &amp; Hirota (1962)</td>
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<tr>
<td>pl1-dr19</td>
<td>Cm'Sr'Su' Ap' Km' Tra' IncFII</td>
<td>Meynell &amp; Datta (1967)</td>
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<td>pSM14</td>
<td>Cm'Tc'Tra' IncFII</td>
<td>Kondo et al. (1962)</td>
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<tr>
<td>pSH1158</td>
<td>Ap'Cm', pBR327 derivative</td>
<td>This study</td>
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**Phages**

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<td>Plets225</td>
<td>Carrying IS1</td>
<td>Scott (1968); Iida et al. (1978)</td>
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<tr>
<td>P1-15ets225</td>
<td>P1-15 hybrid 2, carrying no IS1</td>
<td>Iida et al. (1980)</td>
</tr>
<tr>
<td>P1cm0</td>
<td>Carrying one copy of Tn9</td>
<td>Kondo &amp; Mitsuhashi (1964); Iida &amp; Arber (1979)</td>
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<tr>
<td>A'14::IS1</td>
<td>Used as IS1 probe</td>
<td>Hirsch et al. (1972)</td>
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<tr>
<td>A'32::IS2</td>
<td>Used as IS2 probe</td>
<td>Hirsch et al. (1972)</td>
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**METHODS**

*Microbial strains and media.* The *Escherichia coli* strains, plasmids and bacteriophages used are listed in Table 1. Growth media were as described previously (Iida & Arber, 1977; Iida, 1980).

*Transduction and isolation of P1-15::Tn9 phages.* The general procedures for transduction and isolation of plaque-forming specialized P1 phage derivatives were those described by Iida & Arber (1977). For the isolation and determination of the frequency of formation of P1-15::Tn9 phages, phage P1-15 was prepared by heat induction of strain ML40 lysogenic for P1-15ets225. The resulting lysate served to infect strain WA921 at a multiplicity of about 0.03 plaque-forming phages per cell. Cmr transductants appeared with a frequency of about 2 x 10^-6 per plaque forming phage. In each experiment, 30 Cm' transductants were screened for Cm' and Tc' phenotypes and for the ability to produce high frequency Cmr transducing lysates. About half of the Cmr transductants usually fulfilled these criteria. Thus, the frequency of formation of P1-15 Cm phages can be estimated as 10^-6 per plaque-forming phage. The genome structure of P1-15::Tn9 was confirmed by restriction cleavage analysis (Iida & Arber, 1980). P1-15::Tn10 and P1Cm phages were isolated similarly.

*Physical analysis of pSM14.* Isolation of plasmids, construction of restriction cleavage maps and Southern hybridization of restriction fragments were performed as described before (Southern, 1975; Iida, 1980; Iida & Arber, 1980). Restriction enzymes were purchased from Boehringer (Mannheim, F.R.G.) or from New England Biolabs (Massachusetts, U.S.A.).

*Construction of the pBR327 derivative pSH1158.* Plasmid pSM14 was digested with HindIII and BglII and the Cm' fragment was cloned into the HindIII-BamHI region of the plasmid pBR327 (Soberon et al., 1980).

*DNA sequencing.* This was done by the method of Maxam & Gilbert (1977).

**RESULTS**

*Origin of the R plasmid pSM14*

The R plasmid pSM14, originally called R14, was isolated by K. Ochiai from *Shigella flexneri* 3b N-1, a strain conferring resistance to Cm, tetracycline (Tc) and sulphamamide (Su) (Kondo et al., 1962). Only the Cm' and Tc' characters were shown to transfer to *S. flexneri* 3a 87 and then to *E. coli* O-26 and *E. coli* K12 (Kondo et al., 1962). Strain ML4 is such an *E. coli* O-26 (pSM14) transconjugant (H. Hashimoto, personal communication). From an *E. coli* K12 (pSM14) transconjugant, the Cm' and Tc' markers were transduced with bacteriophage P1 to an F' strain of *E. coli* K12 and Cm' Tc' transductants, which were able to transfer both the Cm' and Tc' markers further into a recipient by conjugation, were isolated (Kondo et al., 1962). Strain ML40 is such a Cm' Tc' transductant (H. Hashimoto, personal communication). The Cm'Tc' plasmids from ML4 and ML40 are now shown to be incompatible with the R plasmid R1-dr19 (data not presented), indicating that pSM14 belongs to the incompatibility group FII. Plasmid pSM14 also confers fusidic acid resistance.
The origin of Tn9

Restriction cleavage analysis of pSM14

Plasmids isolated from strains ML4 and ML40 were cleaved with restriction endonucleases and the cleavage patterns were compared with those of R100-1 and F (Ohtsubo & Ohtsubo, 1977; Iida, 1980). As Fig. 1 shows, ML4 yielded a pure plasmid preparation of pSM14, while the pattern of restriction fragments of plasmids obtained from ML40 points to a mixture of pSM14 and the Inc FI plasmid F. The relative intensity of the fragments produced from pSM14 and F suggests that the copy number of pSM14 in strain ML40 is approximately 2–3 times higher than that of F. The pSM14 plasmids from ML4 and ML40 yielded identical cleavage patterns, indicating that no genetic rearrangement had taken place during conjugation and transduction. Therefore, the physical structure of pSM14 in these strains is likely to be the same as that in the original S. flexneri strain.

The restriction cleavage maps of pSM14 (Fig. 2a) were constructed by comparing the cleavage patterns of pSM14 with those of R100-1, for which restriction cleavage maps exist (Iida, 1980). From the analysis illustrated in Fig. 1, slots 1–3, it was concluded that EcoRI fragments derived from the RTF part of R100-1 are identical to those from pSM14 except for the R100-1 fragments EcoRI-c and EcoRI-h. The pSM14 fragment EcoRI-B corresponds to R100-1 fragment EcoRI-c, but it carries a 1-3 kb insertion which contains one additional HindIII site. Cleavage at this HindIII site produced pSM14 fragments HindIII-A and HindIII-C which correspond to the R100-1 fragment HindIII-a (Fig. 1, slots 5 and 6). This 1-3 kb insertion on pSM14 was tentatively assigned as insertion sequence IS2, since two fragments from EcoRI-B produced by EcoRI-HindIII double digestion hybridized with the 32P-labelled IS2 probe λr32::IS2 (data not shown). IS2 is 1327 bp long and carries one HindIII site 871 bp from one end (Ghosal et al., 1979). The location of IS2 in Fig. 2 is based on this HindIII site and it includes an ambiguity of about 0.4 kb depending on the orientation of the IS2 insertion. An IS2 is also carried in the homologous region of the tra genes of plasmid R6, a relative of R100-1 (Hu et al., 1975; Timmis et al., 1978).

Most of the R100-1 fragments originating from the r-determinant part are absent in pSM14. As Fig. 1 (slots 1–3) indicates, the pSM14 fragment EcoRI-A corresponds to the R100-1 EcoRI-a fragment which includes ISla and the N-terminal part of the cat gene (Lane & Chandler, 1977; Arber et al., 1978; Miki et al., 1978; Marcoli et al., 1980). In R100-1, IS1b is contained in the fragment EcoRI-h, while in pSM14, the fragment EcoRI-G as well as the fragment EcoRI-A hybridized with the 32P-labelled IS1 probe λr14::IS1 (data not shown). Instead of the approximately 22 kb multiple drug resistance r-determinant of R100-1, pSM14 carries a DNA segment of about 4 kb containing the cat gene and two copies of IS1.

To examine further if pSM14 carries the IS1-flanked Tn9, pSM14 was cleaved with PstI and the resulting fragments were hybridized with 32P-labelled λr14::IS1 DNA (Fig. 1, slots 7–9). Since IS1 carries a unique PstI site, a Tn9-containing plasmid should produce at least three PstI fragments hybridizing with the 32P-labelled IS1 probe. Of these three fragments, one (the PstI fragment Z) has the same size as the internal PstI fragment of Tn9, indicating that pSM14 indeed carries the 2-6 kb Cm' transposon Tn9 (Fig. 2). One of the other two hybridizable fragments, PstI-Y, is the same size as the IS1a-containing fragment produced from R100-1, while the fragment PstI-X is about 1.5 kb longer than the corresponding fragment from R100-1. This additional 1.5 kb DNA segment was localized within the region common to HindIII-B and PstI-X fragments (Fig. 2a). This assignment was further confirmed by the analysis of the plasmid pSH1158 which carried the Tn9 containing HindIII-BglII segment of pSM14 (Fig. 2b).

DNA sequence of one end of Tn9 on pSM14

It has been reported that the IS1a of Tn9 transposed from λcam1 is an IS1 variant and that its DNA sequence has a G to T transversion at position 757, 12 bp from the end of the element (Galas et al., 1980). However, the end sequence of this variant IS1 was thought to originate from the IS1 of phage P1, because the same sequence alteration was found in another IS1 originating from P1 (Iida et al., 1981b) and because the Tn9 on λcam1 was derived from P1Cm0 (Gottesman & Rosner, 1975).
Fig. 1. Patterns of restriction cleavage and hybridization with a radioactive IS1 probe of DNA from R100-1, pSM14, F and PlCm0. Electrophoresis was performed in 0.9% agarose. Slots 1-4, EcoRI fragments: 1, R100-1; 2, pSM14 from ML4; 3, pSM14 and F from ML40; 4, F. Slots 5 and 6, HindIII fragments: 5, R100-1; 6, pSM14 from ML4. Slots 7-13, PstI fragments: 7, pSM14 from ML4 hybridized with 32P-labelled Ar14::IS1; 8, pSM14 from ML4; 9 and 10 PlCm0; 11-13, three independent Pl-15::Tn9 isolates.

Capital letters refer to pSM14 fragments corresponding to the labelling in Fig. 2. Small letters refer to R100-1 fragments in alphabetical order according to the electrophoretic mobility in agarose gels except x (Iida, 1980). In slots 1–3, the fragments EcoRI-c and EcoRI-B migrate close to other fragments EcoRI-d and EcoRI-C, respectively. The pSM14 fragments labelled X, Y and Z in slot 7 contained IS1 sequences. An arrow with x marks the position of a PstI fragment produced from the Rep-IS1b region of R100-1 (Fig. 2; Hänni et al., 1982). The fragments containing the cat gene on PlCm0 and Pl-15::Tn9 genomes are marked with Cm. The size of the fragments are: EcoRI-a, 20.5 kb; EcoRI-c, 11.0 kb; EcoRI-h, 4.85 kb; HindIII-a, 46.7 kb; PstI-Cm, 1.9 kb (Alton & Vapnek, 1979; Iida, 1980).
The origin of Tn9

In order to examine this hypothesis, this end of Tn9 on pSM14 was sequenced. The Tn9-containing HindIII-BglII fragment of pSM14 was cloned into pBR327. Its restriction sites are shown in Fig. 2(b). The DNA sequence at the end of IS1a on Tn9 in pSM14 was found to be identical to that of IS1b in R100-1 and of IS1b in Tn9 and to be different from that of IS1a on Tn9 which originated from λcam1 (Galas et al., 1980; Iida et al., 1981b). This result is consistent with the idea that the terminal sequence of Tn9 on λcam1 is derived from that of the resident IS1 or P1 (Iida et al., 1981b).

Transposition of Tn9 and Tn10 into the genome of phage P1-15

The restriction enzyme cleavage patterns are consistent with pSM14 being a relative of the R plasmid R100 (or NR1) and carrying the Cmr transposon Tn9 and the Tcr transposon Tn10. In order to demonstrate that these transposons on pSM14 could transpose normally, plaque-forming derivatives of phage P1-15 showing specialized transduction for either Cmr or Tcr were isolated as described in Methods. The transposition of Tn9 and Tn10 into the P1-15 genome was verified by analysing the DNA of P1-15Cm and P1-15Tc phages with restriction enzymes.
Fig. 3. Sites of insertion of Tn9 and Tnl0 on the P1 and P1-15 genomes. Physical maps of P1 and P1-15 are according to Iida et al. (1981c). Short bars above the lines indicate EcoRI restriction sites and the numbers identify the EcoRI fragments. Phage P1 carries an IS1 element (large box) which contains the unique PstI site of P1 DNA. P1-15 carries neither IS1 nor a PstI site and its genome is about 1 kb shorter than the P1 genome. The horizontal arrow at the left end indicates the origin and orientation of DNA packaging. The small boxes in the C-loop represent the 0.6 kb inverted repeats flanking the invertible DNA segments. Res, Mod and cl indicate the locations of genes for restriction, modification and repressor, respectively. Vertical arrows above the map point to independent Tn9 insertion sites. All P1 Cm phages studied carry Tn9 within the residential IS1 element of P1. The arrowheads below the P1-15 map indicate independent insertion sites of Tnl0. The known restriction cleavage maps for Tnl0 (Jorgensen et al., 1979) and for P1-15 DNA (Arber et al., 1980) served in the determination of these sites.

Transposition frequencies of Tn9 and Tnl0 into the P1-15 genome were estimated as $1 \times 10^{-6}$ and $4 \times 10^{-5}$ per plaque forming P1-15 phage, respectively.

In four independent Tn9 transposition experiments, between 10 and 60% of the P1-15Cm prophages carried by Cm' transductants produced no EcoP15 restriction, i.e. they had a Res(P15)$^-$ phenotype. This suggests, in accordance with earlier findings (Meyer et al., 1980), that the Res Mod region of the P1-15 serves as a good target for the transposing Tn9 element. In each of the four experiments, one P1-15Cm Res(P15)$^+$ and one P1-15Cm Res(P1)$^-$ phage was chosen for restriction cleavage analysis. All P1-15Cm phage genomes had acquired a DNA segment of about 2-6 kb and the short PstI fragments derived from these phage DNAs were identical to that from P1Cm0 (Fig. 1, slots 10-13), confirming the P1-15 :: Tn9 structure of these phages. Since Tn9 carries a single EcoRI site in the cat gene and its two unique PstI sites within the IS1 elements, a comparison of the EcoRI cleavage patterns with those of EcoRI–PstI double digests revealed the locations of the Tn9 insertions (Fig. 3). Finer mapping was done by double digestion with PstI and BamHI, BglII or HindIII, the cleavage sites for which on P1-15 DNA are known (Arber et al., 1980; Iida et al., 1981c).

For Tnl0 transposition, four P1-15Tc phages from two independent experiments were chosen and their genome structures were analysed. Three of these were P1-15 :: Tnl0 and their Tnl0 integration sites were mapped using the enzymes BamHI, BglII, EcoRI and HindIII (Fig. 3). The characteristic snap-back structure of Tnl0 was observed on the P1-15Tc DNA under the electron microscope (J. Meyer & S. Iida, unpublished).

One of the P1-15Tc phages studied carried the DNA segment including Tnl0 of pSM14 delimited by IS1a and IS10R within the left part of P1-15 EcoRI-2 fragment (data not shown; see also Fig. 2a). One may speculate that this P1-15Tc phage was formed by IS1a-mediated transpositional cointegration and subsequent IS10R-mediated deletion formation (Iida & Arber, 1980; Hänni et al., 1982).

Isolation of P1Cm phages identical to P1Cm0

We have also isolated plaque-forming P1Cm phages in the same way as P1-15Cm. The estimated frequency of their formation was $6 \times 10^{-5}$ per plaque-forming P1 phage. Four P1Cm
The origin of Tn9

Fig. 4. A putative pathway for the formation of P1Cm0 from P1 and pSM14. Reciprocal recombination between IS1 elements is proposed to first form a P1-pSM14 cointegrate and in a second step the P1Cm0 genome. Transposition of the Tn9 from P1Cm0 to λDNA yields λ::Tn9 such as λlam1 (Gottesman & Rosner, 1975). The filled boxes represent IS1.

Res(P1)+ phages from two independent experiments were analysed. They all carried Tn9 at the site of the residential IS1 of P1 DNA (Fig. 3), exactly as is known for P1Cm0. Therefore, P1Cm phages, the genome structure of which is identical to that of P1Cm0 previously isolated by Kondo & Mitsuhashi (1964), are repeatedly produced when P1 phage is grown in rec+ cells harbouring pSM14.

DISCUSSION

We have shown that the conjugal, incompatibility group FII plasmid pSM14 coding for Cm' and Tc' carries Tn9 and Tn10. The physical structure of pSM14 is similar to that of the well-studied R plasmid R100 (or NR1). Both R plasmids were originally isolated from S. flexneri in Japan (Kondo et al., 1962; Rownd et al., 1966).

Kondo & Mitsuhashi (1964) isolated a plaque-forming specialized transducing phage P1Cm0 in experiments involving transduction and conjugation. Phage P1Cm0 carries Tn9 at the resident IS1 of P1. Therefore propose that P1Cm0 must have been formed by two successive reciprocal recombinations between IS1 elements (Fig. 4). Like the cointegrate P1-R100-20 (Iida, 1980), P1-pSM14 should be a conjugal plasmid as well as a prophage. Therefore, the conjugal transfer of the Cm' marker with the phage P1 genome during the isolation of P1Cm0 (Kondo & Mitsuhashi, 1964) could be reinterpreted as the result of transmission of a P1-pSM14 cointegrate. Because of its large size the genome of P1-pSM14 could not be packaged intact into a single P1 virion. In contrast, the smaller P1Cm0 segregant genome resulting from reciprocal recombination between two IS1 elements can be packaged as a unit into P1 particles. Thus these form plaques, and this property allows for their easy isolation (Iida, 1980; Iida & Arber, 1980).

Translocation of Tn9 from pSM14 to P1 was reproducible. Its frequency by processes of reciprocal recombination with the IS1 of P1 is about 50-fold higher than that of the transposition of Tn9 from pSM14 to the genome of P1-15, which is largely homologous to P1 DNA, but devoid of an IS1 element. This reciprocal recombination between IS1 sequences depends mainly upon the host recA function (Iida & Arber, 1980; Iida et al., 1981a; Iida et al., 1982).

If the proposed scheme is correct, both ends of Tn9 on P1Cm0, and therefore also of Tn9 on λ::Tn9 (Fig. 4), could have been derived from the ends of the resident IS1 element carried on P1 DNA. Sequencing data supported this view and revealed the same base alteration at one end of the IS1 element derived from λ::Tn9, called λcam1, and at the same end of an IS1 which had been transposed from P1 DNA to a small plasmid pBR325 (Galas et al., 1980; Iida et al., 1981b). Furthermore, this alteration was not found at the corresponding end of IS1a on the original Tn9 in pSM14 (Fig. 2c).

It is very likely that pSM14 carried Tn9 already when it was isolated from S. flexneri. The true origin of Tn9 remains unknown. Tn9 could have been produced from a longer r-determinant by
IS1-mediated deletion or it could have been generated by two successive integrations of IS1 elements on either side of the cat gene.

I wish to thank Werner Arber for his encouragement, support and advice in writing the manuscript, Solveig Schrickel and Rosmarie Hiestand for their excellent technical assistance, Hajime Hashimoto for providing strains, Jürg Meyer and Katsutoshi Mise for discussions, and Thomas A. Bickle for reading the manuscript. This work was supported by grant no. 3.479.79 from the Swiss National Science Foundation.

REFERENCES


