On the Origin of the Chloramphenicol Resistance Transposon Tn9

By SHIGERU IIDA

Department of Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70,
CH-4056 Basel, Switzerland

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The widely studied chloramphenicol resistance (Cm^r) transposon Tn9 came from phage P1Cm0. This phage, however, had acquired its Cm^r 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^r) 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^r 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 & Mitsuhashi (1964) as a plaque-forming, specialized Cm^r 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^r transposons (Arber *et al.*, 1978; Iida & Arber, 1980; Iida *et al.*, 1980; Iida *et al.*, 1981a). Furthermore, *de novo* genesis of IS1-flanked Cm^r transposons (e.g. Tn2651) with structures similar to that of Tn9 was demonstrated using a Cm^r 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^r) 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).

Bacteria	Comments	Source/Reference
ML4	<i>E. coli</i> O-26 (pSM14)	From H. Hashimoto (Kondo <i>et al.</i> , 1962)
ML40	<i>E. coli</i> K12 (F) (pSM14) (λ)	From H. Hashimoto (Kondo <i>et al.</i> , 1962)
WA921	<i>E. coli</i> K12 (F ⁻) <i>thr leu met thi hsd_K</i>	Wood (1966)
R Plasmids		
R100-1	Cm ^r Sm ^r Su ^r Hg ^r Tc ^r Tra ⁺ IncFII	Egawa & Hirota (1962)
R1- <i>drd</i> 19	Cm ^r Sm ^r Su ^r Ap ^r Km ^r Tra ⁺ IncFII	Meynell & Datta (1967)
pSM14	Cm ^r Tc ^r Tra ⁺ IncFII	Kondo <i>et al.</i> (1962)
pSH1158	Ap ^r Cm ^r , pBR327 derivative	This study
Phages		
P1- <i>cts</i> 225	Carrying IS1	Scott (1968); Iida <i>et al.</i> (1978)
P1-15 <i>cts</i> 225	P1-15 hybrid 2, carrying no IS1	Iida <i>et al.</i> (1980)
P1Cm0	Carrying one copy of Tn9	Kondo & Mitsuhashi (1964); Iida & Arber (1979)
λ r14::IS1	Used as IS1 probe	Hirsch <i>et al.</i> (1972)
λ r32::IS2	Used as IS2 probe	Hirsch <i>et al.</i> (1972)

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 made lysogenic for P1-15*cts*225. The resulting lysate served to infect strain WA921 at a multiplicity of about 0.03 plaque-forming phages per cell. Cm^r transductants appeared with a frequency of about 2×10^{-6} per plaque forming phage. In each experiment, 30 Cm^r transductants were screened for Cm^r and Tc^s phenotypes and for the ability to produce high frequency Cm^r transducing lysates. About half of the Cm^r transductants usually fulfilled these criteria. Thus, the frequency of formation of P1-15Cm 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 *Hind*III and *Bgl*II and the Cm^r fragment was cloned into the *Hind*III-*Bam*HI 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 sulphonamide (Su) (Kondo *et al.*, 1962). Only the Cm^r and Tc^r 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^r and Tc^r markers were transduced with bacteriophage P1 to an F⁺ strain of *E. coli* K12 and Cm^r Tc^r transductants, which were able to transfer both the Cm^r and Tc^r markers further into a recipient by conjugation, were isolated (Kondo *et al.*, 1962). Strain ML40 is such a Cm^r Tc^r transductant (H. Hashimoto, personal communication). The Cm^rTc^r plasmids from ML4 and ML40 are now shown to be incompatible with the R plasmid R1-*drd*19 (data not presented), indicating that pSM14 belongs to the incompatibility group FII. Plasmid pSM14 also confers fusidic acid resistance.

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 λ r32::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 IS1a 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 λ r14::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 λ r14::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^r* 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 pSH158 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 λ cam1 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 λ cam1 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 P1Cm0. 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 32 P-labelled *Ar14*::IS1; 8, pSM14 from ML4; 9 and 10 P1Cm0; 11-13, three independent P1-15::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 P1Cm0 and P1-15::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; *PstI*-Cm, 1.9 kb; *PstI*-a, 46.7 kb; *PstI*-Cm, 1.9 kb (Alton & Vapnek, 1979; Iida, 1980).

Fig. 2. (a) Restriction cleavage map of the R plasmid pSM14. The circular map is arbitrarily drawn linearly from IS1a. The fragments are labelled alphabetically in the order of their electrophoretic mobility in agarose, except for the *Pst*I fragments X, Y and Z which contain the IS1 sequences. The indicated locations of IS1, IS10, Tn9, Tn10, Tra and Rep are based on a comparison with the map of R100-1 (Iida, 1980). The shadowed box between a *Hind*III site and IS1b indicates the DNA segment about 1.5 kb longer than the corresponding part of R100-1. The bracket under the restriction map represents the *Bgl*II-*Hind*III fragment cloned into pBR327 to yield pSH1158.

(b) Restriction cleavage map of the *Bgl*II-*Hind*III fragment containing Tn9. The map is drawn in the same way as (a). The arrow from one *Tth*1111 site indicates the strategy used to sequence the end of the IS1a.

(c) DNA sequence of the end of IS1a. The DNA sequence of the end of IS1a from the *Tth*1111 site (at position 712 of the IS1, 56 bp from the end) is identical to that of IS1b of R100-1 (Ohtsubo & Ohtsubo 1978). Only the 14 bp sequence at the end of the IS1a sequence and the 2 bp sequence next to the IS1 are shown. T in parenthesis at 12 bp from the end (at position 757) represents the G to T transversion found in the IS1 variant derived from P1 or from Tn9 on *λcam*1 (Galas *et al.*, 1980; Iida *et al.*, 1981b).

In order to examine this hypothesis, this end of Tn9 on pSM14 was sequenced. The Tn9-containing *Hind*III-*Bgl*II 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 *λcam*1 (Fig. 2c; Ohtsubo & Ohtsubo, 1978; Alton & Vapnek, 1979; Galas *et al.*, 1980). This result is consistent with the idea that the terminal sequence of Tn9 on *λcam*1 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 *Cm*^r transposon Tn9 and the *Tc*^r 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 *Cm*^r or *Tc*^r 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 Tn10 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 P1Cm phages studied carry Tn9 within the residential IS1 element of P1. The arrowheads below the P1-15 map indicate independent insertion sites of Tn10. The known restriction cleavage maps for Tn10 (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 Tn10 into the P1-15 genome were estimated as 1×10^{-6} and 4×10^{-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^r 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 *Bam*HI, *Bgl*II or *Hind*III, the cleavage sites for which on P1-15 DNA are known (Arber *et al.*, 1980; Iida *et al.*, 1981c).

For Tn10 transposition, four P1-15Tc phages from two independent experiments were chosen and their genome structures were analysed. Three of these were P1-15 : Tn10 and their Tn10 integration sites were mapped using the enzymes *Bam*HI, *Bgl*II, *Eco*RI and *Hind*III (Fig. 3). The characteristic snap-back structure of Tn10 was observed on the P1-15Tc DNA under the electron microscope (J. Meyer & S. Iida, unpublished).

One of the P1-15Tc phages studied carries the DNA segment including Tn10 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 6×10^{-5} per plaque-forming P1 phage. Four P1Cm

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 λ DNA yields $\lambda::$ Tn9 such as λ cam1 (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 conjugative, incompatibility group FII plasmid pSM14 coding for Cm^r and Tc^r 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. I 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 conjugative plasmid as well as a prophage. Therefore, the conjugative transfer of the Cm^r 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 $\lambda::$ 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 $\lambda::$ Tn9, called λ cam1, 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.

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