INACTIVATION OF THE REPLICATION GENES OF A PSC101 DERIVATIVE BY IS1-MEDIATED INTEGRATION OF THE PLASMID PSM1

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The plasmid pSM1 carrying a single copy of IS1 has been shown to integrate into various sites on another plasmid pHS1, a temperature sensitive replication mutant of the tetracycline resistance plasmid pSC101. The resulting cointegrates (named pMZ plasmids) contain two IS1 sequences in a direct orientation, each at a junction of the integration. This paper describes a detailed analysis of such cointegrates and shows that the integration of pSM1 occurs frequently at a region of pHS1, resulting in inactivation of the pHS1 replication system. To do this, we attempted to isolate pHS1 containing IS1 (namely pHS1::IS1) from eight independently isolated cointegrates using the restriction endonuclease PstI, which cleaves at a single site within IS1 and no site within pHS1. After ligation of the PstI digests of a pMZ plasmid at DNA concentrations favorable for recircularization of each fragment in the digests and subsequent transformation of the ligated DNA, tetracycline resistant transformants were selected at 30°. Two cointegrates (pMZ3 and 7) did indeed give rise to the pHS1::IS1 plasmids which showed temperature sensitive DNA replication like pHS1, while the other six cointegrates (pMZ1, 2, 4, 5, 8, and 9) did not. This suggests that the cointegrates pMZ3 and 7 contain a functional pHS1 replication system, while the others contain an inactive system. The inactivation as a result of integration of pSM1 at its IS1 into sites within pHS1 is like translocation of the IS1 sequence itself which has been shown to inactivate various bacterial genes.

We also describe the nucleotide sequence in a pHS1::IS1 (named pMZ71), generated from pMZ7, of the junction region between the pHS1
and IS1 sequences as well as the entire IS1 sequence. pMZ71 would be a useful plasmid with which to study the IS1 sequence genetically and biochemically.

In a previous paper (20) we described a simple genetic system used to isolate cointegrates formed between two plasmids, pSM1 and pHS1, which belong to different incompatibility groups. pHS1, 9.43 kilobase pairs (kb) in length, is a temperature sensitive replication mutant of the tetracycline resistance plasmid pSC101 (4) and replicates at 30° but not at 42°. pSM1, 5.67 kb in length, is a derivative of the resistance plasmid R100 which can replicate at both 30° and 42°. pSM1 is deleted for all resistance genes carried by R100 but retains one copy of the insertion sequence IS1 (14). We have shown that cells which can grow at 42° in the presence of tetracycline can be isolated from cells harboring both pHS1 and pSM1 at a frequency and that these cells contain large cointegrate plasmids homogeneous in size (20). Structural analysis of eight independently isolated cointegrates, named pMZ plasmids, has revealed that the plasmid pSM1, containing IS1, has integrated at IS1 into various sites on the recipient plasmid pHS1. Figure 1 summarizes the sites of integration of pSM1 into pHS1 for the eight pMZ plasmids analyzed. As a result of integration, each cointegrate plasmid has, as its characteristic structure, direct repeats of IS1 at each integration junction as well as a nine base pair (bp) repetition of the target site on pHS1 (20).

Fig. 1. Map of the various integration sites of pSM1 into pHS1 to form pMZ plasmids (see ref. 20).

Numbers are coordinates in kilobase pairs (kb) clockwise from the EcoRI site on the pHS1 sequence. ori, a tentative location of origin of replication (I); Tcr, the tetracycline resistance region.
This paper reports that the integration event mediated by IS1 in pSM1 can sometimes inactivate a gene or genes on the recipient plasmid pHS1. The plasmid genes which we studied are those necessary for autonomous replication, and we have localized their positions to a restricted region of pHS1. This plasmid cointegration system may be similar to that of the integrative suppression of bacterial chromosomal replication by a plasmid, as reported previously (16, 17).

MATERIALS AND METHODS

Bacterium and plasmids. The E. coli K12 strain used was C600 (F⁻ thr leu thi lacY tonA supE). The cointegrate plasmids used are listed in Fig. 1.

Covalently closed circular plasmid DNAs were isolated according to the method described by OHTSUBO et al. (19).

Restriction endonucleases. PstI was purchased from Boehringer Manheim. BstEII, HaeIII, Hhal, HaeII, Hinfl, and HpaII were purchased from Bethesda Research Labs, Inc. BstNI, PvuII and Alul were obtained from New England Bio Labs, Inc. These enzymes were assayed as recommended by the laboratory from which they were obtained.

Gel electrophoresis. The conditions used for agarose and acrylamide gel electrophoresis have been previously described (23).

Ligation of DNA fragments. The plasmid DNA was first cleaved with PstI. The cleaved DNA was then treated twice with an equal volume of phenol saturated with TE buffer [0.01 M Tris-HCl (pH 8.0) and 0.001 M EDTA], washed with ether five times, precipitated by ethanol twice, and resuspended in water. Ligation was performed for 12 hr at 12° in 50 µl of solution containing 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl₂, 10 mM DTT, 0.5 mM ATP, 0.1-1.0 units of T4 DNA ligase (purchased from Bethesda Research Labs, Inc.) and 0.5 µg DNA. The final concentration of the DNA in the ligation solution was determined according to the equations of DUGAIczYK et al. (3), such that the ratio of the effective concentration of one end of one molecule to the other end of the same molecule (j) to the total concentration of DNA ends (i) was equal to or greater than 2, allowing recircularization of each fragment in the PstI digests to occur at the four complementary base pairs at each end. In another experiment, as will be described in RESULTS, the concentration of DNA in the ligation mixture was increased (namely 4 µg of DNA in 50 µl) to allow two or more different DNA fragments to join.

Transformation of E. coli K12. Ligated DNA samples were transformed into C600 using the procedure described essentially by MANDEL and HIGA (11) and KREITSCHMER et al. (8). After transformation, the cells were plated on L-plates containing 5 µg/ml of tetracycline, unless otherwise stated in RESULTS, and incubated overnight at 30°. The tetracycline resistant (Tc⁺) transformants were purified and subjected to the crude lysis method described below.

Crude lysis method. A quick and simple way to visualize and compare the
plasmid DNA in bacterial cells in the crude lysis method. We used the procedure of Machida et al. (9). Forty µl of the supernatant of crude lysate containing plasmid DNA was mixed with 10 µl of solution containing 50% glycerol and 0.025% bromophenol blue, and run in a 0.7% agarose gel.

Test for temperature sensitivity of plasmid replication system. Cells were grown in 5 ml L-broth at 30° until fully turbid. One-tenth of a 1:10⁻⁵ dilution of cells was plated on L-plates containing 0 and 5 µg/ml of tetracycline each of them at both 30° and 42° and incubated overnight. Controls used were cells carrying one of the cointegrates, pMZ2, which is temperature resistant in replication, and cells harboring plasmid pHSI which is temperature sensitive in replication. In a typical experiment, one would count 200–400 colonies on plates containing tetracycline at both 30° and 42° for cells with pMZ2, and 200–400 colonies at 30° but none at 42° for cells with pHSI.

Nucleotide Sequencing. Nucleotide sequence was determined by the method of Maxam and Gilbert (12).

RESULTS

Isolation of pHSI::IS1 from cointegrates, pMZ3 and pMZ7. Figure 2 schematically represents the physical structures of pHSI and pSM1 as well as their cointegrate which contains direct repeats of IS1 at each junction of cointegration. The restriction endonuclease PstI cleaves the pSM1 sequence at four sites, one being within the IS1 sequence present in pSM1 but does not cleave the pHSI sequence (Fig. 2, and ref. 20). PstI, therefore, cleaves each pMZ plasmid at five sites, two of which are within the duplicated IS1 sequences (Fig. 2), thus generating five fragments. The largest fragment contains the entire pHSI sequence plus an IS1 sequence cleaved at its PstI site, whereas the other fragments are identical to the pSM1 fragments (Fig. 3). As shown in Fig. 3, if the pHSI replication system has not been inactivated as a result of integration of pSM1 to give a pMZ plasmid, one can isolate a derivative of pHSI carrying a copy of IS1 (namely pHSI::IS1) from the pMZ plasmid after ligation of the PstI digest and subsequent transformation to select for the tetracycline resistant (Tc+) transformants on a plate containing tetracycline (Tc) at 30°. However, if pHSI replication has been inactivated as a result of integration, the only possible plasmids to be isolated by the same procedure are composite plasmids which contain, in addition to the PstI fragment containing the pHSI and IS1 sequences (see Fig. 3), at least two PstI fragments of pSM1, 1.60 kb and 1.09 kb in length, which are known to be responsible for replication of pSM1 (or R100) (15, 24). The resulting pHSI::IS1 will be temperature sensitive in its replication, while the composite plasmids carrying the pSM1 replication system will be temperature resistant.

We have tried to construct pHSI::IS1 plasmids from two cointegrates, pMZ7 and pMZ3 (see Fig. 1) using PstI. As described in MATERIALS AND METH-
ODS, we ligated *PstI*-digested DNA under low DNA concentration to allow circularization of each fragment in the digest, so that the circularized *PstI* fragment containing the pHS1 and IS1 sequences is efficiently formed. Subsequent transformation and plating on Tc plates containing 5 μg/ml of Tc at 30°C yielded the Tc<sup>r</sup> transformants. Examination of the plasmid DNAs in each transformant by the crude lysis procedure showed that all are equal in size but are slightly larger than pHS1. Figure 4A shows a typical crude lysis analysis of transformants obtained from pMZ7. Temperature sensitivity tests of eight randomly selected colonies obtained from pMZ7 or pMZ3 showed that all the plasmids within were temperature sensitive for replication. Two plasmids, each derived from pMZ7 or pMZ3, were named pMZ7<sub>1</sub> and pMZ3<sub>1</sub>, respectively. Cleavage of pMZ7<sub>1</sub> and pMZ3<sub>1</sub> by *PstI* produced a linear fragment which was larger than pHS1 linear DNA but equal in size to the largest *PstI* fragment of pMZ7 and pMZ3 (see Fig.
Fig. 3. Schematic representation of the in vitro procedure used to isolate pHSI::IS1 and a possible composite plasmid containing the two PstI fragments of pSM1 which are known to be necessary for replication of pSM1.

pMZ plasmid DNA which was digested with PstI and then run on a 0.7% agarose gel is shown in the middle of the figure. The size and gel position of each PstI fragment is shown.

Fig. 4. (A) A 0.7% agarose gel, showing the intact DNA molecules of the control plasmids (lanes a–c) as well as those present in the crude lysates (lanes d–k) of eight Tc\(^r\) transformants from pMZ7 DNA which was digested with PstI followed by ligation.

Lane a, pSM1; lane b, pHSI; lane c, pMZ7. The molecular lengths in kb are given at the positions of the closed circular DNA of these control plasmids. In the crude lysates, the solid arrow indicates the position of the closed circular DNA molecules present in the Tc\(^r\) transformants; Uppermost band is contaminating chromosomal linear DNA which is overlapped with the open circular DNA of the plasmids; Broad bright bands are cellular RNA.

(B) A 0.7% agarose gel of the purified plasmid DNAs digested with restriction enzymes. Lane a shows pH51 DNA digested with EcoRI to generate a linear molecule. Lanes b and c show pMZ7 and its parent pMZ7 DNA digested with PstI, respectively.

(C) A 0.7% agarose gel showing digests of control plasmids (lanes a–c) and various chimera plasmids obtained from pMZ3 (lanes d–f) and pMZ4 (lane g). Lanes a and b show pMZ4 and pMZ3 digested with PstI, respectively; lane c shows pH51 digested with EcoRI; lanes d–g show the PstI digests of pMZ3, pMZ4, pMZ33, and pMZ41, respectively. In pMZ34, the 1.09 kb fragment is double the relative brightness in comparison to that of pMZ32, hence we postulate this chimera to carry two such fragments. With pMZ33, a similar phenomenon is observed for the 1.80 kb fragment, hence the same conclusion.
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4B and C), indicating the structure of these plasmids to be pHS1:: IS1. These results indicate that both cointegrates pMZ7 and pMZ3 contain the entire sequence of pHS1, the replication system of which is still functional and temperature sensitive.

Note that in the experiments described above, we used Tc plates containing 5 µg/ml of tetracycline to select for the Tc' transformants, since efficiency of plating of cells harboring pHS1 and probably pHS1:: IS1 is normal on these plates but is very low on Tc plates containing higher concentrations of tetracycline. In fact, when Tc' transformants from the ligated samples of the PstI digests of pMZ7 or pMZ3 were selected on Tc plates containing 20 µg/ml of tetracycline, we were able to isolate only a very few transformants from pMZ3, and none from pMZ7. Crude lysis of such rare transformants interestingly showed that they contained large plasmids of various sizes, all of which were found to be temperature resistant in replication. PstI digestion of plasmid DNA showed that they contained the largest PstI fragment, which is the pHS1:: IS1 sequence, and several PstI fragments from pSM1, including the 1.60 kb and 1.09 kb fragments (denoted C and E in Fig. 4C) which are necessary for replication of pSM1 (see two such derivatives named pMZ33 and pMZ34, shown in Fig. 4, lanes e and f). These results suggest that the replication system of pSM1, which has 5.5 times higher copy number than pHS1 (20), is selected for under high Tc concentrations and that the dosage of the Tc' gene carried by pHS1:: IS1 is not sufficient under these conditions.

Analysis of cointegrates, pMZ1, 2, 4, 5, 8 and 9. When the ligation procedure under low DNA concentrations was applied to pMZ1, 2, 4, 5, 8 and 9 (Fig. 1), we failed to isolate any pHS1:: IS1 type plasmids similar to pMZ71 and pMZ31 at 30° in the presence of 5 µg/ml of tetracycline. However, using increased DNA concentrations in the ligation mixture to allow the joining of two or more PstI fragments as described in MATERIALS AND METHODS, we obtained Tc' transformants. Temperature sensitivity tests of independently isolated colonies showed them all to be temperature resistant. Crude lysis revealed that all the plasmid DNAs were larger than pMZ71 and pMZ31. PstI digestion of some of these plasmids showed that they contained the largest PstI fragment, containing pHS1:: IS1, plus at least the two fragments of pSM1 necessary for replication. PstI degests of the smallest plasmids obtained from each cointegrate showed the identical gel pattern of these three bands. Figure 4B shows an example of the cleavage analysis of a derivative of pMZ4 (named pMZ41), showing the same pattern as that of pMZ32 which was from pMZ3 and contained the minimal three fragments described in the previous section.

The inability to isolate pHS1:: IS1 from the six cointegrates above clearly indicates that the pHS1 replication system in pMZ1, 2, 4, 5, 8, and 9 is defective and must be rescued by the pSM1 replication system in order to replicate at 30° in the presence of 5 µg/ml of tetracycline.

Structure of pMZ71. We examined the plasmid pMZ71 derived from pMZ7
to see if it contained one copy of IS1 whose sequence is identical to that in pSM1. As described in the previous section pMZ71 is cleaved once by PstI probably at its IS1 since PstI cuts IS1 once at a unique site but does not cut within the pHS1 sequence. We analyzed the nucleotide sequence of IS1 containing the PstI cutting site as well as other regions including the junctions between the IS1 and pHS1 sequences. The nucleotide sequences of IS1 were found to be identical to that of IS1 in pSM1 previously determined by Ohtsubo and Ohtsubo (23) (only a part of IS1 sequences are shown in Fig. 5.). Results of the nucleotide sequence around the junctions between IS1 and pHS1 showed that a nine base pair sequence on pHS1 was duplicated at regions adjacent to the ends of IS1 in a direct orientation as shown in the bottom of Fig. 5. This nine base pair sequence must be that of the original target site on pHS1 which was duplicated during transposition of pSM1 into pHS1 to form the cointegrate pMZ7.
DISCUSSION

We have described *in vitro* construction of pHS1::IS1 from various cointegrates formed between pSM1 and pHS1. We were able to isolate such pHS1::IS1 from pMZ3 and pMZ7. However, attempts to isolate similar plasmids from the cointegrates pMZ1, 2, 4, 5, 8, and 9 failed, indicating that the pHS1 replication apparatus is not active in these plasmids. In the formation of the latter cointegrates, pSM1 most likely integrated into regions of pHS1 whose integrity is essential for autonomous replication. It is interesting that the regions corresponding to the locations of pSM1 integration in these six cointegrates are in a restricted region (see Fig. 2). This region could be a part of the region which has been identified as being required for replication and partitioning of the pSC101 plasmid during cell division (13). As reported in a previous paper (20), nucleotide sequence analysis of a 500 bp region containing the sites of pSM1 integration forming pMZ2, 4 and probably 1 showed it to be rich in A and T base pairs (69%). At present we do not know whether this region is part of a coding region for a protein(s) or part of a junctional site(s) essential for replication of pHS1.

NISHIMURA et al. (17) have described the phenomenon called integrative suppression in which plasmids such as F and R100 can suppress a temperature sensitive replication mutation of *E. coli* K12 by integration into the bacterial chromosome enabling the cells to grow at the restrictive temperature. The integration sometimes occurs within *E. coli* genes and inactivates them, since some of the integratively suppressed cells were found to be auxotrophs (16). Our cointegration system between two plasmid genomes is similar to this integrative suppression, since the plasmid containing IS1 suppresses the temperature sensitive replication mutation present in the second plasmid by integration. The integration also causes the inactivation of a gene(s) in the recipient plasmid. Based on the molecular mechanism in our system, we assume that in the integrative suppression of the *E. coli* replication mutant, F and R100 could integrate into the *E. coli* chromosome by transposition mediated by insertion sequences carried by these plasmids, such as IS1, IS2, IS3, gamma-delta, etc. (2, 5, 6, 22), giving rise to duplication of those sequences at the recombinational junctions. It would be interesting if the integration of F and R100 caused inactivation of some of the *E. coli* genes that are essential for chromosomal replication.

We have also reported that pMZ71 is actually pHS1::IS1 and contains an IS1 sequence identical to that in pSM1 which we have described previously (23). This suggests that in the formation of plasmid cointegrates, *E. coli* chromosomal IS1s, one of which has been shown to have a few base pair differences from the IS1 in pSM1 (7), have not participated in the generation of the IS1 duplication present in these cointegrates. Therefore, this would eliminate one of three possible mechanisms discussed in the previous paper for generation of two copies of IS1 in these cointegrates (see ref. 20).

IS1 in pMZ71 has also been shown to be biologically active in vivo: like that
in pSM1, IS1 in pMZ71 can mediate cointegration between pMZ71 and a second plasmid, CoIE1, which contains no insertion sequences. Concurrently, a duplication of the IS1 sequence at the recombinational junctions in a direct orientation occurs (10, 21). pMZ71 has been a very useful plasmid in studying the genetic aspects of IS1. For example, we have obtained derivatives of pMZ71 carrying mutations inside IS1 by mutagenizing restriction endonuclease cutting sites located exclusively within the IS1 sequence, including the PstI site (19, 21). PMZ71 will also be useful in studying the biochemical aspects of IS1, such as transcription of mRNA and translation of proteins which may be directed by IS1. Note that in all these experiments the plasmid pHSI, having no IS1, can be used as a control for pMZ71.

Finally, since pMZ71 can inactivate a gene(s) by cointegration, it may be generally used for mutagenesis of any other plasmid genomes in vivo.

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