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TRANSPOSON (Tn5)-MEDIATED SUPPRESSIVE INTEGRATION OF ColE1 DERIVATIVES INTO THE CHROMOSOME OF Escherichia coli K12 (dnaA)

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Summary: While integration of ColE1 had not been observed previously by ordinary suppressive integration, a dnaA (Ts) E. coli strain with Tn5 at various sites of the chromosome and ColE1 or its mini-derivative, pAO3, but not pSC101, inserted by the same transposon produced integratively suppressed strains depending on the RecA function. In contrast to Hfr strains made with a stringently controlled plasmid, they contained the plasmid not only in an integrated but in an autonomous state at an amount comparable to the strain containing the plasmid only autonomously. Introduction of a RecA-deficient mutation to the strain with an integrated ColE1 derivative through conjugation failed. This is likely to be due to lethality of such a strain without RecA-dependent excision of the integrated high copy number plasmid or to quantitative deficiency of DNA polymerase I in addition to the recA mutation.

INTRODUCTION

Understanding the mechanism of initiation of DNA replication is one of the most important problem in biology and medicine. Our approach to this problem has been to analyse interactions between two replicons composing a co-integrate. As a specific example of the co-integrate the Hfr strain has been chosen in which an integrated plasmid is regulated differently from that of the chromosome (2, 3). The first choice was made for ColE1 because the replication of this plasmid had been shown not to be affected by chloramphenicol at a concentration inhibitory to initiation of the chromosome replication (4). However, it was subsequently found that ColE1 was not integrated by the ordinary integrative suppression method (1, 2). We turned to use R6K as the replication of this plasmid was also shown not to be affected markedly by the same agent (2) and it is of high copy number exceptionally among the conjugative plasmid but can provoke integrative suppression (2). It was found that the chromosome of an integratively suppressed Hfr strain with R6K replicated in the presence of chloramphenicol at a nonpermissive but not at a permissive temperature (2). This has suggested that the plasmid replication system is active only at a temperature nonpermissive to the chromosome replication system due to the dnaA mutation. A similar conclusion was obtained with the use of a multiphenotypically thermosensitive plasmid, Rts1 (3). However, the Hfr strain with R6K gave only ambi-

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guous results on the effect of the polA mutation and a method to integrate ColE1 into the chromosome was eagerly pursued.

With the progress of knowledge in this field, improvement in constructing a strain with an integrated ColE1 or even its mini-derivative, pAO3 (5), was made as briefly described in the previous paper (6). This communication describes the construction of such an integrated strain with ColE1 or its mini-derivative and a peculiar behavior of the plasmid in such a strain. Nishimura and Hirota made a similar attempt by a different method and presented their results orally. During preparation of this manuscript we read a paper by Yamaguchi and Tomizawa (7) in which a similar strain was constructed by a method somewhat different from ours and the resulting strain was clearly characterized.

MATERIALS AND METHODS

Bacterial strains and plasmids: All strains used are E. coli K12 derivatives, many of which were derived from CRT46 (dnaA46 thy thr leu ilv thi lac mal). YC1214 (mal$^+$ derivative of CRT46) and YC1216 (CRT46 ilv :: Tn5) were described previously (6). YC1261 (CRT46 met :: Tn5) and YC1262 (CRT46 cys :: Tn5) are YC1214 inserted with the transposon at chromosomal sites indicated. The method to introduce a recA mutation from KL16-99 (recA) to derivatives of CRT46 was made by selecting thymine non-requring and kanamycin resistant recombiants as described previously (6). Other strains are shown below or in Results. RSF2124 (ColE1 ::Tn3), pAO3 (mini-ColE1) (5) and pSC101 were given by Drs. Y. Kajiro, A. Oka and E.M. Lederberg, respectively.

Construction of Tn5 - inserted derivatives of RSF2124, pAO3 and pSC101 :To transpose Tn5 onto these plasmids, C600 were transformed to kanamycin resistance by the use of CCC lysates prepared from YC1210 (C600 ilv :: Tn5) (6) with respective plasmids. Then, CCC lysates isolated from these kanamycin resistant transformants of C600 were introduced into YC1214, resulting in strains carrying a Tn5 - inserted plasmid.

Isolation and characterization of thermoresistant (DnaA$^+$) strains : The method was described previously (2, 3, 6, 8).

Detection of autonomous plasmids : Cells were grwon in 250 ml of Penassay broth containing thymine (10 μg per ml) for about 18 hrs at 30°C, harvested by centrifugation and resuspended into 5 ml of 25 % sucrose in 50 mM Tris-Cl (pH 8.1). After addition of 0.05 ml of RNase (RNaseA, Sigma, 5 mg per ml of water) and 0.5 ml of lysozyme (Sigma, 10 mg per ml of 250 mM Tris-Cl (pH 8.1)), the suspension was placed in an ice bath for 5 mins , 2 ml of 250 mM EDTA (ethylenediamine tetraacetate, pH8.1) was added, and then chilled in an ice bath for additional 10 mins. Lysis was accomplished by the addition of 8 ml of "Triton Lysis Mixture" (0.01 % Triton X-100, 62.5 mM EDTA and 50 mM Tris-Cl, pH 8.1) and cleared lysates were obtained by centrifugation. Then, 5 M NaCl and 42 % polyethylene glycol # 6,000 were added to final concentrations of 0.5 M and 10 % , respectively, and stored at 4°C overnight. The precipitate was collected by centrifugation, dissolved in 3 ml of

Table Effect of Tn5 on the formation of thermoresistant ($DnaA^+$) revertants

Expt.	Strain codes	Properties		E.O.P. at 42°C relative to that at 30°C
		Chromosome	Plasmid	
1.	YC1214	-	-	5.5×10^{-7}
	YC1216	ilv::Tn5 a)	-	5.6×10^{-7}
	YC1261	met::Tn5 b)	-	1.2×10^{-6}
	YC1262	cys::Tn5 c)	-	4.7×10^{-7}
	YC1271	-	pMY1111 d)	1.1×10^{-6}
	YC1272	ilv::Tn5	pMY1111	3.2×10^{-4}
	YC1273	met::Tn5	pMY1111	3.0×10^{-4}
	YC1274	cys::Tn5	pMY1111	1.5×10^{-4}
	YC1263	-	pMY1113 e)	1.0×10^{-6}
	YC1264	ilv::Tn5	pMY1113	8.9×10^{-4}
	YC1265	met::Tn5	pMY1113	4.2×10^{-4}
	YC1266	cys::Tn5	pMY1113	7.9×10^{-4}
2.	YC1276	ilv::Tn5	pSC101	5.6×10^{-8}
	YC1277	ilv::Tn5	pMY1115 f)	5.4×10^{-8}
3.	YC1265	met::Tn5	pMY1113	1.7×10^{-3}
	YC1300 g)	met::Tn5	pMY1113	$< 1.4 \times 10^{-7}$

a) Mapped at 83 min. b) Mapped at 84 min. c) Tn5 insertion resulted in cystein requirement but the exact insertion site was not confirmed. d) ColE1::Tn3::Tn5. e) pAO3::Tn5. f) pSC101::Tn5 . g) recA derivative of YC1265.

TEN buffer (0.5 M NaCl, 50 mM EDTA and 50 mM Tris-Cl (pH 8.1)) and added 2.7 g of CsCl together with 0.03 ml of ethidium bromide (20 mg per ml). To separate CCC molecules from other DNA components, ultracentrifugation was done by SW 50.1 rotor (Beckman) at 34,000 rpm for about 40 hrs and the resulting CCC band was directly observed by irradiation with UV-light at 365 nm.

RESULTS

As previously reported (1, 2), ColE1 itself did not increase the reversion frequency of the thermosensitive DnaA phenotype to thermoresistance. A subsequent study with a mini-ColE1 plasmid, pAO3, gave also a negative result. Insertion of a kanamycin resistance transposon, Tn5 , either into any of 3 different sites of the chromosome alone or into the plasmid, RSF2124 or pAO3 alone showed no effect (Table 1, Expt.1).

This is in contrast to the case of R64-11 (Inc Iα) which did not provoke integrative suppression by itself but did it markedly if only the plasmid was inserted by Tn5 (6). In the case of RSF2124 or pAO3, the introduction of Tn5 into both the plasmid and the chromosome resulted in a marked increase in the plating efficiency (Table 1, Expt. 1). This is depending on the RecA proficiency (Table 1, Expt. 3). As 3 different sites of Tn5 insertion on the chromosome gave similar effects, there may be no rigidly specific requirement for the site of transposon insertion. Contrary to ColE1 derivatives, pSC101, did not show a similar effect in the plating efficiency at 42°C even if Tn5 had been inserted into both the plasmid and the host chromosome (Table 1, Expt.2).

Several pure clones were isolated from thermoresistant colonies derived from YC-1264, YC1265 and YC1266 and examined for their stability of thermoresistance. A total of 30 clones gave a plating efficiency at 42°C relative to 30°C of ca. 0.05 to 1.0, indicating that the dnaA (Ts) mutation still exists genotypically but is unstably suppressed phenotypically.

However, contrary to our experience with thermoresistant revertants provoked by stringently controlled plasmids (6) or by an exceptional conjugative but relaxedly controlled plasmid, R6K (2), these revertants provoked by ColE1 derivatives were shown to contain autonomously replicating plasmids as judged by the CsCl-ethidium bromide ultracentrifugation and subsequent transformation by the resulting CCC fraction with the selection by kanamycin resistance that is coded for by Tn5. No difference was observed in the CCC content between DnaA (Ts) parents and their thermoresistant derivatives.

Introduction by transformation of ColE1 :: Tn3 (RSF2124) or pBR322 occurred at a similar rate into both DnaA (Ts) parents with pAO3 :: Tn5 (pMY1113) or ColE1 :: Tn3 :: Tn5 (pMY1111) and their integratively suppressed derivatives. DnaA(Ts) parents thus superinfected by transformation with pBR322 finally lost the resident plasmid, pMY 1111, upon repeated growth in Penassay broth containing tetracycline which should give selective advantage to pBR322 because of incompatibility between two plasmids. On the contrary, their DnaA (Tr) revertants rarely lost the resident plasmid upon similar treatments as judged by colicine E1 production. This most likely indicates that the revertants possess at least one copy of pMY1111 in an integrated state.

An attempt was made to introduce a recA mutation by conjugation into a DnaA (Ts) parent (YC1265) (Table 1, Expt.3) and its thermoresistant revertant, YC1301 by the method described above. While thy$^+$ recombinants of YC1265 jointly inherited the recA mutation at 24 %, apparently no linkage (less than 0.3 % : 0 out of 350) was

shown between thy and recA in the recombinants between KL16-99 (recA) and YC1301. Very rare thy$^+$, recA$^-$ recombinants from YC1301 were shown to be thermosensitive, indicating that the RecA deficiency has been introduced because the integrated plasmid no longer exists and also definitely confirming that YC1301 is genotypically dnaA (Ts).

DISCUSSION

The phenomenon of integrative suppression by a plasmid has so far been observed with only that of low copy number (see the list in reference 6), with only one exception of R6K (2). The representative high copy number plasmid ColE1 could not be used to suppressively integrate into the chromosome (1, 2). This gave us an obstacle in studying the detailed nature of chromosome replication under an integratively suppressed state by a typical PolA-dependent, high copy number plasmid.

In this communication we succeeded in suppressively integrating ColE1 derivatives into the chromosome by introducing a transposon, Tn5, into both the chromosome and the plasmid. It is apparently depending on the RecA function (Table 1, Expt.3). As the RecA-deficient mutation could not be introduced into the resulting DnaA-suppressed strain by ColE1 derivatives, the role of the RecA function in suppressively integrating the plasmid seems to be dual.

Nishimura and Hirota (oral presentation) and Yamaguchi and Tomizawa (7) also succeeded in isolating strains with an integrated ColE1, by the use of that plasmid with an attached DNA fragment derived from the host chromosome. Their results together with ours suggest that the RecA function is required on the one hand for integration by homology dependent recombination. Thus, the inability of ColE1 itself to provoke integrative suppression by the ordinary method is most likely due to the lack of a DNA sequence required for genetic homology.

Another role of the RecA function is presumably related to the finding that the DnaA-suppressed strains contain CCC molecules at an amount comparable to the non-suppressed thermosensitive parent. In a co-integrate, pSC134, that is composed of ColE1 and pSC101, the copy number is high under PolA-proficient condition (9). If there exists a similar regulatory mechanism in the co-integrate composed of ColE1 and the host chromosome under the PolA-proficient condition, it means that the number of chromosome , or at least of its initiation fork increases to almost 20 . This discussion is principally based on the negative repressor theory of Pritchard (10). Such a compulsive increase in the number of chromosome or its initiation fork need not to be supposed if the attachment site

theory is correct (11, 12). The cells with 20 initiation forks of the chromosome replication are likely to be detrimental. One possible mechanism to overcome this is to produce autonomously replicating copies to the extent that the number of chromosomal initiation forks is limited to the physiological level. If the number of autonomously replicating copies is randomly partitioned during cell division as predicted by several investigators (13, 14, 15), then there should be unevenness in the number of autonomous copies. To overcome this, the number of the integrated and/or the autonomous copies should be increased or decreased so as to adjust to the condition to survive. In any case detrimental effects due to these situations seem to require a dynamic shift of plasmid existence between the integrated and the autonomous states. As the integration of ColE1 into the chromosome is likely to have occurred by the RecA-dependent recombination so as to produce a copy of ColE1 flanked by two directed copies of Tn5 , the reverse shift, that is, excision, is also likely to be dependent on the RecA function. Without functional RecA product, this dynamic shift may not occur and hence detrimental effects may result in the death of such cells.

An alternative interpretation for the requirement of the RecA proficiency is that the cells carrying both the integrated as well as the autonomous ColE1 derivatives may be in quantitative insufficiency of DNA polymerase I . This may lead the cells to a state similar to recA polA double mutants which are lethal (16).

A similar but not exactly the same conclusions were obtained by Yamaguchi and Tomizawa (7) by the direct analysis of the role of the polA mutation.

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