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Integration of R Plasmid Rts1 to the *gal* Region of the *Escherichia coli* Chromosome

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An R plasmid Rts1 was integrated into the *gal* region of the chromosome of *Escherichia coli* XA-7012 (*galE*) strain by the directed transposition technique. The integration of the Rts1 genome was confirmed mainly by conjugation studies and also by transduction experiments using phage P1. As a result, it was found that the integrated genome contained genes responsible for kanamycin resistance, conjugal transferability, and for autonomous replication. As reported previously, Rts1 is temperature sensitive in replication and inhibits the growth of the host at nonpermissive temperature. However, although a plasmid derived from the integrated Rts1 genome still demonstrates temperature sensitivity upon transfer and high level of kanamycin resistance, this plasmid no longer displays temperature sensitivity in replication and the inhibitory effect on the host. These results indicate that the temperature sensitivity of replication of Rts1 and its inhibitory effect on the host cell are due to the presence of a gene or gene cluster on the Rts1 genome and that the gene(s) is clearly discriminated from the one responsible for the temperature sensitivity of transfer.

Until recently there had been no report on the integration of R plasmid into the host chromosome except one in which the chloramphenicol resistance of an R plasmid *NR1* alone translocated on the chromosome (7). However, the integrative suppression method using *dnaA* temperature-sensitive mutant of *Escherichia coli* has been developed (10), and as a result F-like R plasmids can be integrated into the host chromosome (9, 11, 17). By the integrative suppression method, the integration of plasmid might occur anywhere on the chromosome. Ippen et al. devised a directed transposition technique employing *galE* strain of *E. coli* as the host and succeeded in constructing strains in which F*lac* were integrated into the *gal* region of the chromosome (6). This technique is based on the phenomenon that although a *galE* strain is unable to grow on galactose-containing medium, it can grow on the medium if the *gal* operon is inactivated by insertion of some other fragment of deoxyribonucleic acid (DNA) in the region. Therefore, by adding galactose to the medium, we can select directly for mutations in the *gal* region, and can expect to isolate some bacteria that have the integrated plasmid in the region.

In this communication we report on the isolation of an *E. coli* strain in which an R plasmid Rts1 was integrated into the *gal* region of the chromosome by the directed transposition technique. As reported previously, Rts1 makes the growth of the host cell temperature sensitive (13). Although the inhibitory effect of Rts1 was studied extensively by DiJoseph et al. (3), its mechanism still remains uncertain. In the present studies we have isolated a plasmid which is derived from the integrated genome of Rts1. It was found that this plasmid has lost the temperature sensitivity in replication as well as the inhibitory effect on the host. We also discuss the detrimental effect of Rts1 by comparing the properties of the bacteria carrying Rts1 with those carrying the plasmid derived from the integrated Rts1.

MATERIALS AND METHODS

Bacterial strains and R plasmid. The bacterial strains used are listed in Table 1. An R plasmid Rts1 confers resistance to kanamycin (Km), and its replication is temperature sensitive (14, 15). The Rts1-carrying XA-7012 (XA-7012/Rts1) was constructed by bacterial matings between CSH-2/Rts1 and XA-7012, in which conjugation was carried out at 30 C. Strain CSM-2 is a mutant induced from CSH-2 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Media. Penassay broth (PAB, Difco) was used for growth of bacteria. When used as solid media, agar (no. 1, Oxoid) was added to PAB at a concentration of

TABLE 1. *Bacterial strains used*

Strain	Genotype	Source
Escherichia coli K-12		
XA-7012	F⁻ Δ*lac galE*	J. R. Beckwith
WD-7001	F⁻ *gltA* Sm^r	J. R. Beckwith
W677	F⁻ *lac leu thr thi*	
CSH-2	F⁻ *met*	
CSM-2	F⁻ *met glt*	CSH-2, by NTG[a] treatment
JC-1569	F⁻ *recA1 gal leu his* Sm^r	H. Uchida

[a] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

1.0%. When bacterial growth was assayed in the presence of sodium dodecyl sulfate (SDS, Wako Chemical Co., Tokyo), SDS was added to PAB at a concentration of 0.03%. The pH of the SDS-containing medium was adjusted to 6.8. M9 (1) agar medium supplemented with appropriate amount of sugars, amino acids, and drugs was used for selection of transconjugants and transductants.

Transduction by P1 phage. The recipient strain WD-7001 was grown in L broth (8) to a concentration of 4×10^8 cells per ml and concentrated to 2×10^9 cells per ml by centrifugation. Then, the cells were infected with the phage P1kc grown on strain XR27, one of the clones of XA-7012 that have integrated R*ts*1 into the *gal* region. The multiplicity of infection was 0.3, and adsorption was made for 30 min at 30 C. Transductants were selected on M9 agar plate containing 100 μg of streptomycin (Sm) per ml. For selecting Km^r transductants, 30 μg of Km per ml was added to the above selective plate with or without 20 μg of glutamate per ml.

Bacterial growth. Bacterial growth was monitered by turbidity measurements at 560 nm with a spectrophotometer (Coleman, Junior II).

Isolation of DNA and density gradient centrifugation in CsCl gradient. These procedures were carried out as described previously (12, 14).

RESULTS

Isolation of Gal^r Km^r strains. XA-7012/R*ts*1 was grown in PAB at 30 C into the stationary phase. After 5 ml of the culture was centrifuged, the cells were resuspended in 0.5 ml of fresh PAB and spread onto M9 minimal agar plates containing 0.1% glycerol, 0.2% galactose, and 25 μg of Km per ml. Fifty-seven colonies were formed on the plate after 3 days of incubation at 42 C. The reason for employing the high temperature of incubation was as follows: since R*ts*1 is temperature sensitive in replication and inhibits the growth of the host at 42 C (13), cultivation of the R*ts*1$^+$ cell at the nonpermissive temperature in the presence of Km would facilitate selection of the cell having integrated R*ts*1 into the chromosome. The ensuing colonies were purified on galactose-Km agar plate at 42 C. Finally, seven clones were isolated as stable Gal^r Km^r mutants. One of these strains, XR27, was used for further studies.

Characterization of XR27. Because XR27 was Gal^r Km^r, it was presumed that R*ts*1 or at least the resistance determinant of R*ts*1 was inserted into the *gal* region of the chromosome. To determine whether the sex factor of R*ts*1 was integrated and to decide the direction of the insertion, conjugation and transduction studies were performed.

In matings between XR27 and WD-7001 at 30 C for 90 min, *gltA* was transferred with a frequency of 10^{-5}, but the Km resistance gene, *kan*, was rarely transferred (Table 2). The transfer of *gltA* was clearly temperature sensitive. When recombinants were selected on M9-Km-Sm agar plate intending to obtain *gltA*$^+$ *kan*$^+$ WD-7001, only a few colonies developed on the plate. This *gltA*$^+$ *kan*$^+$ WD-7001 strain also had Hfr properties as described below. As also shown in Table 2, in the matings with W677 Sm^r, *leu* of XR27 was transferred more frequently than *thi* gene. Although 14% of these *leu*$^+$ recombinants were found to be *thr*$^+$ *thi*$^+$, none of them was Km resistant. From the conjugation studies it was concluded that XR27

TABLE 2. *Recombination frequency of kan and chromosomal genes of XR27*[a]

Donor	Recipient	Temp of mating (C)	Recombination frequency of:				
			kan	*gltA*	*gltA kan*	*leu*	*thi*
XR27	WD-7001	30	1×10^{-7}	3×10^{-5}	3×10^{-8}		
		37	$<10^{-8}$	2×10^{-7}	$<10^{-8}$		
XR27	W677 Sm^r	30	$<10^{-8}$			8×10^{-7}	1×10^{-7}
		37	$<10^{-8}$			6×10^{-8}	$<10^{-8}$
XA-7012/R*ts*1	WD-7001	30	5×10^{-2}	$<10^{-8}$	$<10^{-8}$		
XA-7012/R*ts*1	W677 Sm^r	30	4×10^{-2}			$<10^{-8}$	

[a] Matings were carried out for 90 min at either 30 or 37 C. XR27 is a strain derived from strain XA-7012 in which R*ts*1 was integrated into the chromosome.

was an Hfr strain and that the chromosome was transferred in the order *O gltA--leu thr thi---kan*. Therefore, the main component of the sex factor of Rts1 should be located between *gltA* and *kan*.

Insertion of *kan* of the Rts1 into the *gal* region of the chromosome was also confirmed by transduction experiments. The *gltA* gene of XR27 was transduced to WD-7001 by phage P1 at a frequency of 6×10^{-6} per adsorbed phage (Table 3). Among the 130 *gltA*$^+$ transductants tested, 84 were *kan*$^+$. The *gltA*$^+$ *kan*$^+$ cotransductants were also obtained directly by selecting the transductants on M9-Km-Sm plate. Transferability of *kan* of these transductants was examined by bacterial matings with CSH-2 Nal^r, but *kan*$^+$ transconjugants were not obtained. These experiments suggest that the transduced segment including *kan* does not contain the entire genome of the sex factor of Rts1, although *gltA* and *kan* were cotransducible.

Temperature sensitivity of XR27. As reported previously Rts1 affects the growth of the host cell at 42 C (13). This phenomenon was also observed in XA-7012/Rts1. Furthermore, the temperature sensitivity of the Rts1$^+$ cell became remarkable if 0.03% SDS was added to the culture (Fig. 1). In the case of XR27, however, the growth was not inhibited at 43 C, even in the presence of SDS. This loss of the inhibitory effect can not simply be ascribed to the integrated state of Rts1, since it is presently unknown whether the integrated genome of Rts1 still harbors a postulated gene(s) responsible for the inhibitory effect. This problem will be discussed in a later section.

Km-resistance level of XR27. Km resistance of Rts1 is due to phosphorylation of the drug (unpublished data). Minimal inhibitory concentration of Km with XR27 was over 400 μg per ml, which was the same value obtained with XA-7012/Rts1. This means that the Km resistance is readily expressed wherever Rts1 exists in the host cell of *E. coli*.

TABLE 3. *Transduction studies of XR27 by phage P1*[a]

Selective marker	Transduction frequency	Transfer frequency of *kan* of transductants[b]
gltA$^+$	6×10^{-6}	NT
gltA$^+$ *kan*$^+$	4×10^{-6}	$<10^{-8}$
kan$^+$	5×10^{-7}	$<10^{-8}$

[a] Phage P1 grown on the strain XR27 was mixed with the cells of WD-7001. The infected cells were selected for the transductants with the markers indicated.

[b] The transferability was determined by examining conjugal transfer of *kan* of each 10 *kan*$^+$ transductants from the groups except that selected for only *gltA*. CSH-2 Nal^r was used as the recipient of the bacterial matings. NT, Not tested.

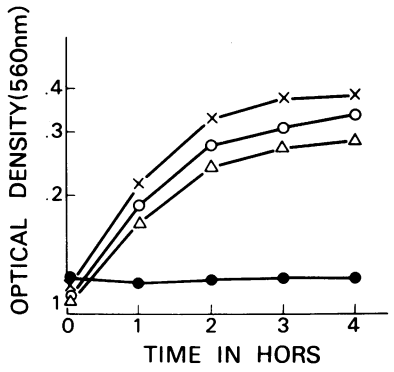

FIG. 1. *Growth of XA-7012 strains in the presence of SDS at 43 C. Exponential cultures of XA-7012, XA-7012/Rts1, XR27, and XA-7012/pTW2 grown at 30 C were diluted into fresh PAB containing 0.03% of SDS. Cell density was adjusted to approximately 1 × 10^8 per ml, respectively. Then the cells were grown at 43 C with gentle shaking. Growth was monitored by measuring turbidity using a spectrophotometer (Coleman, Junior II). Symbols: ○, XA-7012; ●, XA-7012/Rts1; ×, XR27; △, XA-7012/pTW2.*

Successive isolation of an Hfr strain and derivation of a mutant from the integrated Rts1 genome. When XR27 was mated with WD-7001, a small number of *gltA*$^+$ *kan*$^+$ recombinants were obtained as described above. One of these strains, TW100, was further studied, and it was found that *gltA* and *met* genes were transferred to the recipient CSM-2 (Table 4). In the matings with W677 Nal^r, *leu*$^+$ recombinants were obtained more frequently than *thi*$^+$ ones, and these *leu*$^+$ recombinants did not carry *kan*. These findings indicate that TW100 is an essentially the same type of Hfr strain as XR27. The reason for the higher transfer frequency of *kan* of TW100 than that of XR27 is unknown, but it could be due to the presence of a cell fraction harboring *kan*$^+$ plasmid in the population of TW100.

The second type of *gltA*$^+$ *kan*$^+$ WD-7001 was constructed by transferring *gltA* and *kan* genes from TW100 to CSH-2 and finally to WD-7001. One of the recombinants thus obtained, TW200, transferred its Km resistance to the recipient CSM-2 with high frequency, but *gltA* was transferred with 10^{-5} times lower frequency than *kan*, as opposed to the case of TW100 (Table 4). TW200 could also transfer its Km resistance to a *recA* strain JC1569, and the

TABLE 4. *Recombination frequency of kan and chromosomal genes of TW100 and TW200*[a]

Donor[b]	Recipient	Temp of mating (C)	Recombination frequency of:			
			kan	*gltA*	*gltA met*	*leu*
TW100	CSM-2	30	7×10^{-7}	5×10^{-6}	2×10^{-6}	
		37	$<10^{-8}$	2×10^{-7}	6×10^{-8}	
TW100	W677 Nal^r	30	2×10^{-6}			2×10^{-5}
TW100	JC-1569	30	$<10^{-8}$	$<10^{-8}$		
TW200	CSM-2	30	$>10^{-2}$	7×10^{-7}	3×10^{-7}	
		37	2×10^{-4}	$<10^{-8}$	$<10^{-8}$	
TW200	W677 Nal^r	30	$>10^{-2}$			$<10^{-8}$
TW200	JC-1569	30	$>10^{-2}$	$<10^{-8}$		
WD-7001/R*ts*1	CSM-2	30	$>10^{-2}$	$<10^{-8}$	$<10^{-8}$	

[a] The period of mating was 4 h when CSM-2 was used as the recipient, since recombinant was rarely obtained if the mating was terminated within a shorter period. Other matings were interrupted at 2 h.

[b] TW100 is a strain derived from WD-7001 in which R*ts*1 was integrated into the chromosome. TW200 is a strain derived from WD-7001 in which the mutant R*ts*1 plasmid pTW2 was harbored as a plasmid.

resistance in JC1569 was stably transmitted to the progeny, whereas Km resistance of TW100 was not transferred to JC1569. Furthermore, as shown in Fig. 2, an analysis of DNA of TW200 by CsCl density gradient centrifugation revealed the presence of plasmid DNA, whose density was almost the same as that of R*ts*1 DNA (5). In TW100, however, plasmid DNA was not observed.

These findings would suggest that in TW200 a large part of the R*ts*1 genome exists as a plasmid combining a small but unknown fragment of the chromosome, whereas *gltA* is integrated into the chromosome. It may be assumed that owing to the homology of this plasmid DNA with the chromosome, particularly with the gal region, it could easily recombine with the chromosome at the time of mating. As shown below, this plasmid was revealed to have lost various properties of R*ts*1 and designated pTW2.

Temperature sensitivities of the cell carrying pTW2 and pTW2 itself. WD-7001 was very useful to employ as the host to examine the curing of R*ts*1, since R*ts*1 was easily eliminated from this host at nonpermissive temperature. However, as mentioned above, the *gltA* gene in TW200 is considered to be integrated into the chromosome, which means the chromosome of TW200 is not identical to that of WD-7001. Therefore, it was assumed inappropriate to use TW200 as the host cell of pTW2. For this reason, WD-7001/pTW2 was newly constructed by transferring the plasmid from TW200 to CSH-2 Nal^r and then back to WD-7001. The WD-7001/pTW2, named TW300, was used for further studies on the temperature sensitivities of the host and the mutant plasmid pTW2 itself.

Midexponential cultures of TW300 and WD-7001/R*ts*1 grown at 30 C were diluted into fresh PAB, and each was divided into two portions and grown either at 30 or 43 C. At intervals, samples were withdrawn from each of the four cultures and viable cell counts were performed. At the same time, elimination of these plasmids was scored by examining Km resistance of at least 100 colonies in each sample. Doubling time was calculated from the increase of viable cells during the first 2 h from the shift, since elimination of R*ts*1 or pTW2 was rarely observed in these earlier periods of incubation at nonpermissive temperature.

As shown in Fig. 3, the doubling times of TW300 and WD-7001/R*ts*1 at 43 C were 45 and 90 min, respectively. At 30 C however, no difference was observed between them.

To examine the effect of pTW2 on the host cell growth at nonpermissive temperature, SDS sensitivity of the pTW2 carrying cell was tested, since as described above remarkable SDS sensitivity was observed when the cells harbored R*ts*1 as a plasmid. For this purpose XA-7012/pTW2 was constructed by mating between TW200 and XA-7012, and its growth was examined at 43 C in the PAB containing 0.03% SDS. As shown in Fig. 1, the growth of XA/pTW2 was not affected by the presence of pTW2, whereas XA-7012/R*ts*1 did not grow in the medium.

These findings suggest that R*ts*1 loses the

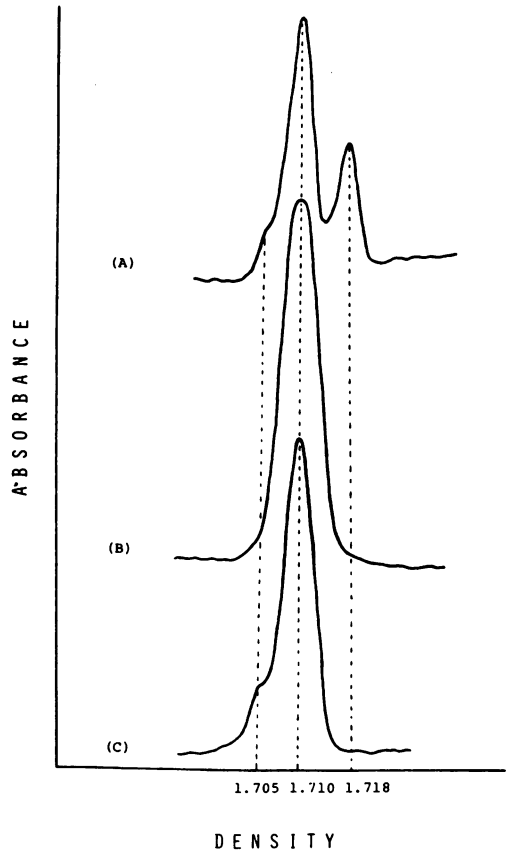

FIG. 2. *Density profiles of DNA prepared from exponential cultures of E. coli strains harboring pTW2 or Rts1. (A) DNA prepared from TW200. Serratia marcescens DNA (density of 1.718 g/cm³) was added as a reference. (B) DNA prepared from TW100. (C) DNA prepared from CSH-2/Rts1.*

detrimental properites against the host during the process at either integration into XA-7012 chromosome forming XR27 or detachment from the integrated chromosome forming the mutant plasmid pTW2.

Rts1 was easily eliminated from the host at 43 C, and the R⁻ fraction in the population reached over 70% within 4 h after the temperature shift. In contrast, at 30 C Rts1 was highly stable (data not shown). Contrary to Rts1, pTW2 was not efficiently eliminated at nonpermissive temperature. Although a small number of pTW2⁻ cells were present in the culture grown at 43 C, they did not increase in number during the incubation period. In addition to this, almost the same number of pTW2⁻ cells were observed in the culture grown at 30 C. These results indicated that replication of pTW2 was no longer temperature sensitive.

DISCUSSION

An R plasmid Rts1 was integrated into the *gal* region of *E. coli* chromosome by the directed transposition technique. It seems that this technique is very useful for isolating cells that have integrated other bacterial or plasmid genes into its chromosome, as discussed by Ippen et al. (6). This technique permits plasmid DNA to integrate strictly to the *gal* region of chromosome. This is quite different from the case employing the integrative suppression method (11, 17), by which the integration of plasmid occurs at a site among many possible regions of the chromosome. It had been reported that integrative suppression is made possible only by F and F-like plasmids (9). Although Rts1 belongs to the incompatibility group T of R plasmids (2), which is not an F-like plasmid, Rts1 can construct a stable recombinant with an F-like R plasmid *NR1* (16). Therefore, it is supposed that the Rts1 DNA would share homologous portion with F in their nucleotide sequences.

As shown in the conjugation studies with

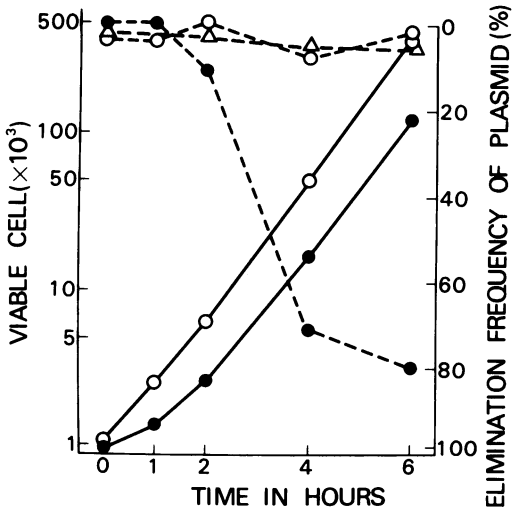

FIG. 3. *Growth of WD-7001 harboring pTW2 or Rts1 and elimination of the plasmid from the host at 43 C (elimination of pTW2 at 30 C is also shown). Exponential cultures of TW300 and WD-7001/Rts1 grown at 30 C were diluted into fresh PAB, adjusting the cell density to approximately 1 × 10⁸ per ml, respectively. Then the cells were grown at either 43 or 30 C with gentle shaking. Samples were withdrawn at intervals and spread onto PAB agar plate. The resultant colonies were used for viable cell counting and for scoring elimination frequency of the plasmid by examining the Km resistance of at least 100 cells in each sample. Symbols: ———, viable cell; – – – –, elimination; ○, TW300 at 43 C; △, TW300 at 30 C; ●, WD-7001/Rts1 at 43 C.*

XR27, the R*tsl* integrated into the chromosome maintained conjugal transferability. On the other hand, the transduced segment derived from the integrated R*tsl* genome did not contain the entire sex factor of R*tsl*. These contradictory findings would be reconciled by assuming that circular R*tsl* molecule was broken at midportion of the sex factor upon insertion into the chromosome, and as a result the sex factor genome was separated to both sides of the resistance determinant. Since the R*tsl* DNA appears to be almost the same as the phage P1 DNA in size (preliminary observation), the frequency of transducing such a separated sex factor as a single unit might be extremely low.

As shown in the studies on TW100 and TW200 strains, once a plasmid is integrated into the chromosome, and a state of Hfr is established, it is rather easy to isolate successively Hfr and/or mutant of the plasmid by transferring the integrated genome to the other strain. In these instances the integration of plasmid tends to occur at the same region of the chromosome as where it had been inserted in the parent strain.

DiJoseph et al. (3) studied the growth inhibitory effect of R*tsl* on its host cell and found that the effect was caused by a thermosensitive product mediated by R*tsl*. They suggested that this product was present at the permissive temperature and underwent a temperature-induced alteration resulting in a lethal effect on the host cell. As revealed in the present studies, pTW2 has lost the detrimental effect on the host, and at the same time it no longer shows temperature sensitivity in replication. These factors raise the possibility that the gene of R*tsl* responsible for the temperature sensitivity in replication and for the inhibitory effect on the host cell growth is the same; in other words, the gene product which inhibits the replication of R*tsl* itself can also affect the growth of the host at nonpermissive temperature. Recently it has been reported that the R*tsl* molecule in *E. coli* does not convert to a covalently closed circular form at nonpermissive temperature (4), suggesting that the gene product mentioned above might induce the formation of the covalently closed circular molecule of R*tsl* at permissive temperature.

It is considered that the isolation of this temperature insensitive plasmid pTW2 from R*tsl* would indicate the presence of an additional gene on the R*tsl* genome inducing the temperature sensitivity in replication besides a normal replication gene.

We are now attempting to isolate recombinants between λ phage and R*tsl*, since the integrated site of R*tsl* on the host chromosome is adjacent to the *att*λ locus.

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