SHORT COMMUNICATIONS

Instability of the *Flac*⁺ Factor in a *dnaC* Mutant of *Salmonella typhimurium*

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INTRODUCTION

The *dnaC* gene of *Escherichia coli* maps at 89 min on the genetic map and its product is involved in the initiation of chromosome replication (Gross, 1972). It also plays a role in plasmid replication since, for example, the *Flac*⁺ factor fails to replicate when the *dnaC* gene is inactivated (e.g. at restrictive temperature in a temperature-sensitive *dnaC* mutant; Zeuthen & Pato, 1971). *Salmonella typhimurium* strain 11G is a temperature-sensitive strain which forms colonies at up to about 35 °C but not at higher temperatures. It is mutated in the *dnaC* gene or its equivalent since the lesion, like *dnaC* of *E. coli*, maps close to the *leu* genes and the strain is altered in the initiation of chromosome replication at 38 °C (Spratt & Rowbury, 1971). Strain 11G also fails to replicate the resident LT2 plasmid and the *Flac*⁺ factor at 38 °C (Spratt, 1972).

Thus in both *E. coli* and *S. typhimurium*, inactivation of the *dnaC* gene product prevents both initiation of chromosome replication and the replication of plasmids. Here we show that under certain conditions the *Flac*⁺ factor, but not the LT2 plasmid or the hybrid *Flac*⁺S, shows an instability in strain 11G at permissive temperature which is apparently related to the presence of the mutated *dnaC* gene.

METHODS

Bacteria. *Salmonella typhimurium* strains 11G LT2⁺, M827 LT2⁻, PG154 LT2⁺, PG154 LT2⁺ *Flac*⁺ and M799 LT2⁺ have been described previously (Spratt & Rowbury, 1971; Spratt, Rowbury & Meynell, 1973). The isolation of strain 11G LT2⁻ *Flac*⁺S by introduction of the *Flac*⁺S factor into 11G LT2⁺ has also been described (Rodriguez Lemoine & Rowbury, 1975). Strain 11G LT2⁻ was isolated from a rare white *lac*⁻ colony produced on deoxycholate-lactose agar (see below) by 11G LT2⁻ *Flac*⁺S. Strain 11G LT2⁺ ts⁺ was isolated by plating 10⁹ cells of 11G LT2⁺ on nutrient agar and picking one of the large revertant colonies which grew at 37 °C.

Culture media. The minimal medium (MM) used was that of Davis & Mingioli (1950) lacking citrate and with glucose or lactose (0.2 %) as energy source. Required amino acids were added at 20 µg/ml (L form). Oxoid nutrient broth No. 2 was used throughout as a rich liquid medium. Both MM and broth were solidified where necessary with 2 % Difco Bacto agar.

To detect *lac*⁺ and *lac*⁻ colonies, lactose-deoxycholate (DOC) agar (Meynell & Meynell, 1970) was used.

Measurement of total cell number and viable count. The total number of cells in cultures was measured on samples diluted in Isoton diluent (Coulter Electronics Ltd, Dunstable, Bedfordshire) with a Coulter model ZB1 electronic particle counter (30 µm orifice, aperture current 1, amplification 2, lower threshold 2.5). The number of viable cells in cultures was

measured by plating samples (suitably diluted in 0.065 M-phosphate buffer pH 7.4) on lactose-DOC agar and counting colonies after 2 days' incubation at 25 °C.

Tests for sensitivity of strains to MS2 phage. In some experiments *Flac*⁺ strains were tested for MS2 sensitivity. Two methods were used: (i) portions (0.1 ml) of overnight broth-grown cultures were spread on to nutrient agar plates and drops of MS2 phage (about 0.02 ml; 10¹⁰ p.f.u./ml) looped on to areas of the plates; (ii) portions (0.1 ml) of overnight broth-grown cultures were added to 2.5 ml soft agar (nutrient broth containing 0.8 % Difco Bacto agar and 0.8 % NaCl). The soft agars were poured on to nutrient agar plates and, after setting, drops of MS2 phage (about 0.02 ml; 10¹⁰ p.f.u./ml) were looped on to areas of the plates. In both types of test MS2-sensitive strains showed clear areas where the phage drops had been placed.

Transfer of Flac⁺ by conjugation. *Flac*⁺ donor strains were grown to late-exponential phase in broth without shaking at the appropriate temperature. Recipient strains were grown with shaking in broth and used in exponential phase. Recipient cells (0.1 ml of a suitable dilution) were spread on lactose minimal agar plates containing the requirements of the recipient and drops of donor culture (tenfold diluted in 0.85 % w/v, NaCl) were looped on to areas of the plates. Exconjugants were picked after 72 h at 30 °C (strain 11G and derivatives) or 48 h at 37 °C (other recipients).

Separation of plasmid DNA. The growth and labelling of cells, lysis, separation of plasmid on alkaline sucrose gradients and analysis of gradients were all performed as described previously (Spratt *et al.* 1973; Rodriguez Lemoine & Rowbury, 1975).

RESULTS AND DISCUSSION

To test for stability of *Flac*⁺ we introduced it into strain 11G LT2⁺ (this is the normal 11G strain carrying the resident LT2 plasmid) and its parent, strain PG154 LT2⁺ ts⁺, and into another *dnaC*⁺ strain, M799 LT2⁺. Organisms from all three *Flac*⁺ strains were then grown overnight in broth at 25 °C and tested for *lac*⁻ cells by plating on lactose-deoxycholate agar at 25 °C. The *Flac*⁺ plasmid was stable in PG154 LT2⁺ and in M799 LT2⁺ (< 1 % *lac*⁻ cells) but highly unstable in 11G LT2⁺ (about 39 % *lac*⁻ cells). There were also virtually no *lac*⁻ cells present after overnight growth of PG154 LT2⁺ *Flac*⁺ and M799 LT2⁺ *Flac*⁺ at 37 °C (see Table 1). Strain 11G LT2⁺ *Flac*⁺ will not grow at 37 °C but after overnight growth at 32 °C almost all organisms had lost the *Flac*⁺ (about 91 % *lac*⁻ cells). Thus not only was the *Flac*⁺ unstable in the *dnaC* mutant at permissive temperature but the tendency to segregate *lac*⁻ cells increased with increasing temperature.

The temperature-dependent loss of *Flac*⁺ shown in liquid medium by strain 11G LT2⁺ *Flac*⁺ also occurred on solid medium. Organisms plated on lactose-DOC agar at 25 °C formed red (*lac*⁺) colonies with thin white (*lac*⁻) areas encircling them. Colonies formed at 32 °C were white with small red centres. Under these conditions colonies containing substantial numbers of *lac*⁻ cells arose from initially *Flac*⁺ cells. It seems likely therefore that many of the *lac*⁻ cells present after overnight growth of strain 11G LT2⁺ *Flac*⁺ in broth at 32 °C (Table 1) were formed in the same way, i.e. from the *Flac*⁺ cells present in the initial inoculum and not predominantly from the few *lac*⁻ cells initially present.

Of the *lac*⁺ cells remaining after overnight growth of strain 11G LT2⁺ *Flac*⁺ at 25 or 32 °C, 99 % still showed unstable maintenance of *Flac*⁺. When restreaked on lactose-DOC agar at 25 °C they produced red (*lac*⁺) colonies with white (*lac*⁻) areas around them.

It is assumed above that replication of *Flac*⁺ was aberrant in strain 11G LT2⁺ *Flac*⁺ at 32 °C and that *lac*⁻ cells arose from *Flac*⁺ ones. This interpretation is supported by the

Table 1. *Effect of temperature on Flac⁺ stability and viability of strains of S. typhimurium*

The *Flac⁺* derivatives were prepared as described in Methods. The donor strains were (i) PG154 LT2⁺ *Flac⁺* for transfer of *Flac⁺* to M799 LT2⁺ and M827 LT2⁻ and (ii) M827 LT2⁻ *Flac⁺* for transfer of *Flac⁺* to the 11G derivatives. The *Flac⁺* derivatives were taken from slopes of minimal agar and lactose and grown overnight in broth at the stated temperature. Strain 11G LT2⁺ was taken from a nutrient agar slope and grown in broth at the stated temperature. For viable counts and counts of *lac⁺* and *lac⁻* organisms, suitable dilutions from the initial inocula and/or the overnight cultures were plated on lactose-DOC agar and the plates incubated at 25 °C. Total cell counts were made with the Coulter counter as described in Methods.

Strain	Percentage of <i>lac</i> ⁻ cells				Percentage of viable cells after overnight growth at 32 °C
	In initial inoculum	After overnight growth at stated temperature			
		25 °C	32 °C	37 °C	
11G LT2 ⁺ <i>Flac</i> ⁺	5.8	38.8	90.7	—	73.0
11G LT2 ⁺	N.T.	N.T.	N.T.	N.T.	68.5
11G LT2 ⁻ <i>Flac</i> ⁺	0.0	0.6	0.6	—	60.0
PG154 LT2 ⁺ <i>Flac</i> ⁺	0.0	0.9	N.T.	0.9	N.T.
M799 LT2 ⁺ <i>Flac</i> ⁺	0.8	0.7	N.T.	0.7	N.T.
11G LT2 ⁻ <i>Flac</i> ⁺ S	0.0	0.0	0.0	—	N.T.
11G LT2 ⁺ <i>Flac</i> ⁺ ts ⁺	1.3	1.7	1.0	4.3	N.T.

N.T., not tested.

behaviour of the strain on solid medium. Another possibility however is that after growth at 32 °C, *Flac⁺* organisms showed plating sensitivity and were unable to form colonies on solid medium. To test this, organisms were grown overnight at 32 °C and total cell counts (made with the Coulter counter) were compared with viable counts on lactose-DOC agar. For strain 11G LT2⁺ *Flac⁺*, 73 % of organisms were viable after overnight growth at 32 °C (Table 1). Loss of viability, however, was apparently unrelated to either the presence or the instability of *Flac⁺*, since strain 11G LT2⁺ (which does not carry *Flac⁺*) and strain 11G LT2⁻ *Flac⁺* (which showed stable maintenance of *Flac⁺*; Table 1) both had a reduced viability (after overnight growth at 32 °C) similar to that of strain 11G LT2⁺ *Flac⁺* (Table 1).

Not all plasmids show instability in strain 11G. The resident LT2 plasmid was rarely lost during growth at 25 or 30 °C (data not shown) and the hybrid *Flac⁺*S factor, which appears to contain parts of the LT2 plasmid and can replace it (Rodriguez Lemoine & Rowbury, 1975), was maintained stably in 11G at both 25 and 32 °C (Table 1). Instability of *Flac⁺* appears from the above to be associated with the *dnaC* lesion. To throw further light on this relationship we have tested the stability of *Flac⁺* in a ts⁺ revertant of strain 11G LT2⁺. Table 1 shows that one such ts⁺ revertant showed almost stable maintenance of *Flac⁺*. The percentage of *lac⁻* cells after growth at 37 °C was somewhat higher than for PG154 LT2⁺ *Flac⁺*, but although a large colony revertant was picked, this strain still showed slower growth at 37 °C than the parent and therefore may still have a *dnaC* product with slightly reduced activity at 37 °C. The greater stability of *Flac⁺* in this revertant strain was not due to loss of the LT2 plasmid (see below), since examination of its DNA on alkaline sucrose gradients showed that both *Flac⁺* and the LT2 plasmid were present. Thus all the above suggests that instability of *Flac⁺* is related to inactivation of the *dnaC* product.

As stated above, the LT2 plasmid and *Flac⁺*S are stable in strain 11G at 25 and 32 °C. The LT2 plasmid apparently remains stable at 32 °C in the presence of *Flac⁺*, since after over-

night growth of 11G LT2⁺ *Flac*⁺ at 32 °C all 11 of the *lac*⁺ clones tested were resistant to MS2 phage (i.e. had the *fi*⁺ property shown to be characteristic of LT2⁺ strains by Smith *et al.* 1973). Also, as stated above, 99 % of these *lac*⁺ clones showed active segregation of *lac*⁻ cells; such segregation is indicative of the continued presence of the LT2 plasmid with *Flac*⁺. In the absence of the LT2 plasmid, *Flac*⁺ is stable (Table 1, strain 11G LT2⁻ *Flac*⁺).

The instability of *Flac*⁺ in 11G LT2⁺ might indicate some major difference in replication mechanism. Another possibility is that strain 11G can stably maintain only a single plasmid even at permissive temperature; where two normally compatible plasmids are present then the resident LT2 (or its derivative *Flac*⁺S) is replicated normally while replication of the second is aberrant. To test this possibility we have introduced *Flac*⁺ into a strain of 11G which carries no autonomous LT2 plasmid (it is probably cured, since it lacks the *fi*⁺ property). The resultant strain (11G LT2⁻ *Flac*⁺, Table 1) showed stable maintenance of *Flac*⁺ at both 25 and 32 °C. It appears, therefore, that strain 11G can maintain a single plasmid (LT2 or *Flac*⁺S or *Flac*⁺) at permissive temperature but that when *Flac*⁺ is present in addition to the LT2 plasmid then the replication of the latter is favoured and *Flac*⁺ tends to be lost.

The reason for the unstable behaviour of *Flac*⁺ in strain 11G is at present conjectural. The *dnaC* gene product probably plays an enzymic role in the initiation process for both chromosome and plasmid, and when the level of active *dnaC* product is limited then there is competition for it and the 'foreign' *Flac*⁺ plasmid evidently fails to compete effectively for it. We are at present testing whether other plasmids show unstable behaviour in 11G LT2⁺.

Although *Flac*⁺ was unstable in 11G LT2⁺, 0.5 % of the *ts lac*⁺ clones were stably *lac*⁺. These still carried the *fi*⁺ property characteristic of the LT2 plasmid. In one strain the presence of both *Flac*⁺ and the LT2 plasmid was confirmed by examining its DNA on alkaline sucrose gradients. The nature of the change in this strain is at present under investigation. Stable *lac*⁺ behaviour of 11G derivatives would also be expected if the LT2 plasmid were lost or if *Flac*⁺ recombined with the LT2 plasmid, forming a hybrid. Such a hybrid plasmid would probably confer *fi*⁺ properties. Recombination of this kind would be analogous to that involved in the formation of *Flac*⁺S from Fts114*lac*⁺ and the LT2 plasmid (Macrina & Balbinder, 1972). Studies of further stable *lac*⁺ derivatives of 11G are in progress to ascertain whether any of them contain hybrid plasmids or lack the LT2 plasmid.

The instability of *Flac*⁺ in strain 11G LT2⁺ may offer a method of easily isolating *dnaC* mutants. If *dnaC* mutants in general (or all initiation mutants) show poor ability to maintain *Flac*⁺ when the LT2 plasmid is present, then after mutagenesis of LT2⁺ *Flac*⁺ strains it may be possible to detect temperature-sensitive *dnaC* mutants (or initiation mutants in general) as those which show unstable *Flac*⁺ maintenance at 25 °C (i.e. red colonies with white sectors or areas on lactose-DOC agar) associated with failure to grow at 37 °C. This is at present under investigation.

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