The Formation of a Dissociable Plasmid Cointegrate from the *Flac* Factor and the Resident Plasmid of *Salmonella typhimurium* LT2

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SUMMARY

The *Flac* factor showed unstable maintenance in *Salmonella typhimurium* *dnaC* MP10_{LT2}. The properties of a more stable *lac*⁺ derivative (SD-1) are described. SD-1 was *ts* and carried the *fi*⁺ property and the ability to transfer the *lac*⁺ character. It contained a large plasmid of molecular weight about 129×10^6 daltons. The properties of SD-1 and its derivatives suggested that the large plasmid was a cointegrate of *Flac* and the MP10_{LT2} plasmid. *Lac*⁺ transfer was efficient from SD-1 to M799 MP10_{LT2} and one *lac*⁺ exconjugant contained the intact cointegrate. The cointegrate was not successfully transferred to strains lacking MP10_{LT2}. It dissociated into apparently unaltered *Flac* and MP10_{LT2} plasmids, but the deletion of small parts of one or both plasmids during cointegrate formation could not be ruled out. Cointegrate dissociation was more marked in M799 than in SD-1 especially during growth in glucose–Casamino acids minimal medium. In the presence of R1drd19, the cointegrate (like the MP10_{LT2} plasmid) was stably maintained in the *dnaC* strain; maintenance of *Flac* was, however, unstable. It seems likely that replication of the cointegrate was controlled by the MP10_{LT2} plasmid constituent.

INTRODUCTION

During previous work on plasmid replication in *dnaC* and *dnaC*⁺ strains of *Salmonella typhimurium*, it was observed (Rodriguez Lemoine & Rowbury, 1975*b*) that whereas *Flac* was stably maintained in the *dnaC*⁺ strains at all the temperatures tested, maintenance of *Flac* was aberrant at permissive temperatures in the temperature-sensitive *dnaC* mutant. After overnight growth in broth at 32 °C, 90% of organisms were *lac*[−]. Unstable maintenance was dependent on the presence of the resident plasmid as well as on the *dnaC* lesion. Stably *lac*⁺ derivatives can be isolated from this strain and we describe the properties of one such derivative.

The resident plasmid MP10_{LT2} (previously called the LT2 plasmid) is a large stringently controlled plasmid of molecular weight about 60×10^6 daltons (Spratt, 1972; Spratt, Rowbury & Meynell, 1973; Smith *et al.*, 1973). The plasmid, which is apparently non-transferable, is stably maintained, but cured strains (which lack the *fi*⁺ property conferred by it) have been isolated and studied (Smith *et al.*, 1973; Spratt *et al.*, 1973). Macrina & Balbinder (1972) isolated a large plasmid (*FlacS*) from a *F*_{ts114}*lac* derivative of *S. typhimurium*. *FlacS* showed certain properties characteristic of MP10_{LT2} (Macrina & Balbinder, 1973; Rodriguez Lemoine & Rowbury, 1975*a*). Nevertheless, the failure of *FlacS* DNA to hybridize with that of the MP10_{LT2} plasmid led Macrina, Balbinder & Bassel (1973) to conclude that *FlacS* might not be a hybrid of *F*_{ts114}*lac* and MP10_{LT2}. In this paper, we describe

the formation of a large plasmid in *S. typhimurium* IIG *dnaC* *Flac* MP10_{LT2}. This plasmid is apparently a cointegrate of *Flac* and the MP10_{LT2} plasmid, since it conferred properties characteristic of both and dissociated into apparently unaltered *Flac* and MP10_{LT2} plasmids. Transfer of the cointegrate of *Flac* and the MP10_{LT2} plasmid was studied, since it provided a possible means of transferring MP10_{LT2}. Maintenance of the cointegrate was also examined in the presence of another plasmid to ascertain which component governed replication.

METHODS

Bacteria. *Salmonella typhimurium* strains IIG, IIGMP10_{LT2}, IIGMP10_{LT2} *Flac*, IIG *FlacS*, PG154 MP10_{LT2} *Flac*, M827 and M799 MP10_{LT2} have been described previously (Spratt *et al.*, 1973; Rodriguez Lemoine & Rowbury, 1975*b*). Strain M799 *FlacS* was derived from strain M799 MP10_{LT2} by transfer of *FlacS*. *Escherichia coli* strain C600 is a K12 derivative and requires threonine, leucine and thiamin for growth.

Culture media. The minimal medium (MM) used was that of Davis & Mingioli (1950). Citrate was omitted and glucose or lactose (0.2 %) was added as energy source. Required amino acids were added at 20 µg ml⁻¹ (L form), thiamin at 1 µg ml⁻¹ and Difco vitamin-free Casamino acids at 0.1 %. Oxoid nutrient broth no. 2 was used as a rich liquid medium. MM and broth were solidified where necessary with 2 % (w/v) Difco Bacto agar. To detect *lac*⁺ and *lac*⁻ colonies, lactose-deoxycholate (DOC) agar (Meynell & Meynell, 1970) was used.

Viable counts. The number of viable cells in cultures was measured by plating samples (suitably diluted in 0.065 M-phosphate buffer pH 7.4) on nutrient agar or lactose-DOC agar, and counting colonies after 2 days incubation at 25 °C or 30 °C.

Tests for sensitivity to MS2 phage. Two methods were used: (i) 0.1 ml portions of cultures grown overnight in broth were spread on nutrient agar plates and drops of MS2 phage [0.02 ml; 10¹⁰ plaque-forming units (p.f.u.)/ml] were transferred on to marked areas of the plates; (ii) 0.1 ml portions of cultures grown overnight in broth were added to 2.5 ml soft agar (nutrient broth containing 0.8 % Difco Bacto agar and 0.8 % NaCl) and poured on to nutrient agar plates. After setting, drops of MS2 phage (about 0.02 ml; 10¹⁰ p.f.u./ml) were transferred on to marked areas of the plates. After incubation overnight at 30 °C or 37 °C, MS2 sensitive strains showed clear areas where the phage had been placed.

Transfer of *lac*⁺ character by conjugation. Donor strains were grown to late-exponential phase in broth without shaking at the appropriate temperature. Recipient strains were grown to exponential phase in broth with shaking. Recipient cells (0.1 ml of a suitable dilution) were spread on lactose minimal agar plates containing the growth requirements of the recipient. Drops of donor culture [appropriately diluted in 0.85 % (w/v) NaCl] were then transferred on to marked areas of the plates. *Lac*⁺ exconjugants were picked after 72 h at 30 °C or 48 h at 37 °C.

Analysis of plasmid DNA. The growth, labelling and lysis of cells, separation of plasmid DNA on alkaline sucrose gradients, and analysis of gradients were all performed as described previously (Spratt *et al.*, 1973; Rodriguez Lemoine & Rowbury, 1975*a*). The molecular weights of plasmids were estimated from their sedimentation behaviour on alkaline sucrose gradients (Clayton & Vinograd, 1967).

Experiments with *R1drd19*. This R factor was transferred from *Escherichia coli* 153 as described previously (Rodriguez Lemoine & Rowbury, 1975*a*). R⁺ organisms were selected by plating on minimal agar supplemented with the growth requirements of the recipient and with kanamycin (25 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹). Where strains carrying

Fig. 1

Fig. 2

Fig. 1. Analysis of the plasmid content of the stably *lac*⁺ derivative (SD-1) by alkaline sucrose gradient centrifugation. Strains 11G MPIO_{LT2} *Flac* and SD-1 were labelled with [³H]thymidine in lactose-Casamino acids MM. Lysis of the organisms, sedimentation of gradients, gradient analysis and counting procedures were as described in Methods. In all figures, fraction 1 corresponds to the bottom of the gradient tube. (a) SD-1; (b) strain 11G MPIO_{LT2} *Flac*.

Fig. 2. Sedimentation on an alkaline sucrose gradient of the SD-1 plasmid and *FlacS*. SD-1 was grown and labelled with [³H]thymidine as described in Fig. 1. Strain 11G *FlacS* was grown and labelled as described in Fig. 1, but using [¹⁴C]thymidine (4 μ Ci ml⁻¹) instead of [³H]thymidine. The two suspensions were then mixed and lysed together. Lysis, sedimentation and all further analytical procedures were as described in Methods. (a) ¹⁴C counts, i.e. plasmid content of strain 11G *FlacS*; (b) ³H counts, i.e. plasmid content of SD-1.

R1drd19 were tested for the number of R⁺ organisms, plating was on nutrient agar with the same concentrations of kanamycin and chloramphenicol.

RESULTS

A large plasmid in a derivative of Salmonella typhimurium 11G

When *S. typhimurium* 11G *dnaC* MPIO_{LT2} *Flac* (parent) was plated on lactose-deoxycholate agar at 25 °C, many of the organisms formed white colonies with red centres, i.e. *lac*⁺ maintenance was unstable. About 0.5 % of the organisms, however, formed red stably *lac*⁺ colonies. One of these stably *lac*⁺ derivatives (SD-1) was purified and studied.

SD-1 was, like its parent, *ts* and was able to transfer the *lac*⁺ character to strain M799 MPIO_{LT2}. SD-1 was, moreover, resistant to MS2 phage and therefore carried characteristics of both *Flac* (*lac*⁺ character and transfer ability) and the MPIO_{LT2} plasmid (*fi*⁺ character).

The plasmid DNA content of SD-1 was compared with that of its parent by running extracts on alkaline sucrose gradients. The extract prepared from the parental culture showed the two peaks characteristic of the covalently closed circular (CCC) forms of *Flac* (about 1.65 % of the total cellular DNA as CCC plasmid) and the MPIO_{LT2} plasmid (about 1.9 % of the total cellular DNA as CCC plasmid). However, extracts of SD-1 cultures contained substantial amounts (about 3.6 % of the total cellular DNA as CCC plasmid) of a

Fig. 3

Fig. 4

Fig. 3. Analysis of the plasmid content of a *lac*⁻ segregant from SD-1 by alkaline sucrose gradient centrifugation. Organisms of a *lac*⁻ segregant from SD-1 were labelled with [^3H]thymidine in glucose-Casamino acids MM. Strain PG154 MP10_{LT2} *Flac* was labelled with [^{14}C]thymidine in lactose-Casamino acids MM. Cell suspensions were then mixed and lysed together. All subsequent procedures were as described in Methods. (a) ^{14}C counts, i.e. plasmid content of strain PG154 MP10_{LT2} *Flac*; (b) ^3H counts, i.e. plasmid content of the *lac*⁻ segregant from SD-1.

Fig. 4. Analysis of the plasmid content of a *lac*⁺ exconjugant from a cross of SD-1 $\times$ M799 MP10_{LT2} by alkaline sucrose gradient centrifugation. The exconjugant was grown in lactose-Casamino acids MM and labelled with [^3H]thymidine as described in Fig. 1. Sedimentation, analysis of gradients and counting of samples were also performed as in Fig. 1.

large plasmid (Fig. 1), as well as smaller amounts of plasmids which behaved like *Flac* (about 0.35 % of the total cellular DNA) and the MP10_{LT2} plasmid (about 0.36 % of the total cellular DNA).

The large SD-1 plasmid was larger (Fig. 2) than the hybrid *FlacS* factor (molecular weight about 109×10^6 daltons; Macrina *et al.*, 1973) and its molecular weight was estimated to be 128×10^6 to 130×10^6 daltons. It seemed likely that the SD-1 plasmid was a cointegrate of *Flac* and the MP10_{LT2} plasmid, since SD-1 had properties characteristic of both. The molecular weight estimated was somewhat less than that expected for a cointegrate made by fusion of the complete genomes of *Flac* (molecular weight about 80×10^6 daltons) and the MP10_{LT2} plasmid (molecular weight about 60×10^6 daltons). Some DNA might therefore have been deleted in the fusion.

The small amounts of apparently normal *Flac* and MP10_{LT2} plasmids present in SD-1 extracts might have arisen by dissociation of a cointegrate. Dissociation would result in the presence of two types of derivative in SD-1 cultures. Organisms carrying the two constituent plasmids would arise directly; *lac*⁺ maintenance should be unstable in them. From these, *lac*⁻ organisms carrying only the MP10_{LT2} plasmid should arise. When broth-grown cultures of SD-1 were plated on lactose-deoxycholate agar at 25 °C, 5 to 6 % of the organisms formed white colonies with red centres, i.e. maintenance of the *lac*⁺ character was unstable in them (Table 1). Organisms from one of these colonies contained plasmids which behaved like

Fig. 5. Comparison of the derived *Flac* with the parental *Flac* by sedimentation on an alkaline sucrose gradient. Organisms of strain M827 carrying the derived *Flac* (see Table 1) and strain PG154 MP10_{LT2} *Flac* were labelled with [^3H] and [^{14}C]thymidine respectively in lactose-Casamino acids MM. Organisms were lysed together and treated subsequently as in Fig. 2. (a) ^{14}C counts, i.e. plasmid content of PG154 MP10_{LT2} *Flac*; (b) ^3H counts, i.e. plasmid content of M827 derived *Flac*.

Flac and the MP10_{LT2} plasmid on alkaline sucrose gradients, but the large plasmid was absent. A further 2 to 3 % of the organisms from a SD-I culture formed white (*lac*⁻) colonies on lactose-deoxycholate agar. All three of these tested (e.g. Fig. 3) contained only a plasmid which behaved like MP10_{LT2}.

Transfer properties of SD-I

As stated above, SD-I transferred the *lac*⁺ character when incubated with strains such as m799 MP10_{LT2}. However, 5 to 6 % of the organisms in a SD-I culture (those showing unstable *lac*⁺ maintenance) probably carried *Flac* and these might have been responsible for all the *lac*⁺ transfer. Quantitative measurements rule this out. Transfer of the *lac*⁺ character to m799 MP10_{LT2} was somewhat more efficient from SD-I (2.9×10^{-4} *lac*⁺ exconjugants per donor organism) than from 11G MP10_{LT2} *Flac* (2.4×10^{-4} *lac*⁺ exconjugants per donor organism). It follows that organisms carrying the cointegrate were responsible for most of the *lac*⁺ transfer.

Extracts from one *lac*⁺ exconjugant from the cross of SD-I $\times$ m799 MP10_{LT2} contained the plasmid cointegrate (Fig. 4). Dissociation of the cointegrate was more extensive in strain m799 since substantial amounts of plasmids which behaved like *Flac* and MP10_{LT2} were also present (Fig. 4). Growth of the m799 derivative in glucose medium rather than lactose led to even further dissociation (about 1 % of total DNA as CCC form of the SD-I plasmid in glucose-Casamino acids MM, but about 3 % in lactose-Casamino acids MM).

Attempts to transfer the cointegrate into strains lacking MP10_{LT2} were unsuccessful. Very few *lac*⁺ exconjugants arose when SD-I was crossed with M827 and these probably received only *Flac* since 45/45 were MS2 sensitive (i.e. lacked the *fi*⁺ property characteristic of MP10_{LT2} and the cointegrate) and extracts from one contained only a single plasmid which sedimented like *Flac* on alkaline sucrose gradients. Similarly, after a cross of m799 containing the cointegrate with 11G, the three *lac*⁺ exconjugants tested were MS2 sensitive and one of them contained a single plasmid which behaved like *Flac* on alkaline sucrose gradients. When *E. coli* C600 was used as recipient in crosses with SD-I or the m799 derivative carrying the cointegrate, no *lac*⁺ exconjugants were isolated.

Properties of the plasmids deriving from dissociation of the SD-I plasmid

The large plasmid of SD-I evidently arose by fusion of *Flac* and MP10_{LT2}. Its molecular weight, however, appeared to be somewhat less than the sum of the molecular weights of

Table 1. *Plasmid stability in derivatives of Salmonella typhimurium strain 11G*

Flac was transferred into the 11G derivatives from M827 *Flac*. The derived *Flac* originated in an unstable *lac*⁺ derivative of SD-1. From there it was transferred to M827 and thence to the appropriate 11G derivatives. The strain 11G derivative carrying the derived MP10_{LT2} plasmid was a *lac*⁻ segregant from SD-1. Organisms were taken from slopes of supplemented minimal agar plus lactose and grown overnight in broth at 30 °C. Results are expressed as percentages.

Strain	<i>lac</i> ⁻ cells	Unstably <i>lac</i> ⁺ cells	Stably <i>lac</i> ⁺ cells
11G <i>Flac</i>	0.6	NT	NT
11G derived <i>Flac</i>	2.0	NT	NT
SD-1	2.3	6.6	91.1
11G MP10 _{LT2} <i>Flac</i>	80.0	19.7	0.3
11G MP10 _{LT2} derived <i>Flac</i>	71.4	28.4	0.2
11G <i>Flac</i> derived MP10 _{LT2}	78.0	21.7	0.3
11G derived MP10 _{LT2} derived <i>Flac</i>	80.1	19.4	0.5

NT, not tested.

the constituents. It may be that the molecular weight of large plasmids is somewhat underestimated by measurements of sedimentation in alkaline sucrose. It was possible, however, that the SD-1 plasmid lacked parts of one or both constituents, as seems likely for the *FlacS* plasmid. Accordingly we have compared the sizes and properties of the plasmids (derived *Flac* and derived MP10_{LT2}) formed by dissociation of the cointegrate with those of the parental *Flac* and MP10_{LT2} plasmids. The derived *Flac* co-sedimented with the parental plasmid on an alkaline sucrose gradient (Fig. 5) indicating that it was essentially unchanged in size. Transfer of the derived *Flac* (1.1×10^{-2} *lac*⁺ exconjugants per donor organism in a cross of M827 derived *Flac* × 11G MP10_{LT2}) was as efficient as that of the parental *Flac* (1.0×10^{-2} *lac*⁺ exconjugants per donor organism in a cross of M827 *Flac* × 11G MP10_{LT2}) and the derived *Flac* retained sensitivity to repression of its transfer properties by the *fi*⁺ MP10_{LT2} plasmid. The derived *Flac* also behaved like the parental *Flac* in strain 11G MP10_{LT2}. Maintenance of both was unstable and stably *lac*⁺ derivatives arose with approximately equal frequencies (Table 1). In a derivative of strain 11G lacking MP10_{LT2}, both the derived *Flac* and the parental form were stably maintained (Table 1). The derived MP10_{LT2} plasmid co-sedimented with the parental form on an alkaline sucrose gradient (Fig. 3). Furthermore it conferred on strain 11G the *fi*⁺ property characteristic of the parental MP10_{LT2} plasmid. The derived MP10_{LT2} plasmid showed unchanged behaviour in strain 11G *Flac*. Its presence caused unstable maintenance of *Flac* and stably *lac*⁺ derivatives arose with a frequency similar to that observed with 11G *Flac* carrying the parental MP10_{LT2} plasmid (Table 1).

The derived MP10_{LT2} plasmid also retained the characteristic compatibility properties of the parental plasmid. Like the parental plasmid (Rodriguez Lemoine & Rowbury, 1975*a*), it was incompatible with the hybrid *FlacS* as shown by the observation that introduction of *FlacS* into a *lac*⁻ segregant derived from SD-1 caused the loss of MP10_{LT2}.

Control of cointegrate replication; replication in the presence of R1drd19

Unstable maintenance of *Flac* in the *dnaC* strain was not only caused by the MP10_{LT2} plasmid. *Flac* maintenance was also unstable (Rodriguez Lemoine & Rowbury, 1976) in the presence of R factors like R1drd19 (61 % of organisms were *lac*⁻ after overnight growth of *S. typhimurium dnaC Flac* R1drd19 in broth at 30 °C; 100 % were R⁺), whereas the MP10_{LT2} plasmid was stably maintained with R1drd19 (51/51 organisms tested were

MP10_{LT2}⁺ R⁺ after overnight growth of *S. typhimurium* *dnaC* MP10_{LT2} R1drd19 in broth at 30 °C). When R1drd19 was introduced into SD-1, the strain remained stably *lac*⁺ (2.5 % of organisms were *lac*⁻ after overnight growth of SD-1 in broth at 30 °C; 2.7 % were *lac*⁻ after overnight growth of SD-1 R1drd19 in broth at 30 °C). It follows that the cointegrate behaved as though its replication could be under the control of the LT2 plasmid constituent.

DISCUSSION

Flac was not stably maintained in the presence of the MP10_{LT2} plasmid in *S. typhimurium* strain 11G, but stably *lac*⁺ derivatives could arise in a number of ways (Rodriguez Lemoine & Rowbury, 1975*b*). The stably *lac*⁺ character of SD-1 clearly derived from the presence of a single plasmid cointegrate; single plasmids can be stably maintained in strain 11G (Rodriguez Lemoine & Rowbury, 1975*b*; unpublished observations). The following observations support the conclusion that the cointegrate was formed by fusion of *Flac* and the MP10_{LT2} plasmid: (i) the presence of the cointegrate in SD-1 was associated with substantially reduced amounts of *Flac* and the MP10_{LT2} plasmid (Fig. 1); (ii) some organisms in SD-1 cultures carried both *Flac* and the MP10_{LT2} plasmid and the cointegrate was absent from them.

The presence of *Flac* and MP10_{LT2} in extracts from SD-1 cultures (Fig. 1) suggested that the cointegrate was dissociable. The isolation of a derivative containing both *Flac* and MP10_{LT2} from SD-1 reinforced this view. The extent of dissociation was apparently increased in strain M799, especially when organisms were grown with glucose rather than lactose.

The *Flac* and MP10_{LT2} plasmids which derived from the cointegrate were not significantly altered compared to the parental plasmids with respect to molecular weight or other properties tested. This supports the view that the cointegrate contains essentially the complete genomes of the two constituents. In contrast, there was a marked discrepancy between the observed molecular weight of the cointegrate and that expected for a hybrid produced by fusion of the complete genomes of *Flac* and MP10_{LT2}. It may be therefore that the cointegrate lacks a small part of one or both constituents. Studies of the cointegrate by restriction endonuclease 'fingerprinting' or by electron microscopy will be necessary to resolve this problem. If the SD-1 plasmid does carry a deletion then this might be responsible for the failure to transfer the cointegrate to strains lacking MP10_{LT2}.

Transferable cointegrates which have a tendency to dissociate may be useful for transferring otherwise non-transferable plasmids. The MP10_{LT2} plasmid has not so far been transferred as a separate unit but transfer of the SD-1 cointegrate (Fig. 4) may eventually permit establishment of the MP10_{LT2} plasmid in strains lacking it.

Where plasmid hybrids are formed by the fusion of parts or the whole of two constituent plasmids then the hybrid may potentially have two distinct replication systems. Some such hybrids can apparently utilize either replication system, e.g. the replication of the hybrid pSC134 is governed by the ColE1 constituent in the presence of chloramphenicol but by the pSC101 constituent in *polA* mutants (Timmis, Cabello & Cohen, 1974). The SD-1 cointegrate described here clearly contains the replication system of both constituents, since both plasmids derived from the cointegrate were able to replicate in the absence of other plasmids. In the presence of R1drd19, replication of the cointegrate appeared to be under the control of the MP10_{LT2} plasmid constituent in strain 11G since the cointegrate, like this plasmid, was stably maintained. If the cointegrate is under the control of the MP10_{LT2} plasmid constituent in SD-1 itself then we would expect very stable maintenance since the MP10_{LT2}

plasmid and *FlacS* are very rarely lost from strain 11G MP10_{LT2} or strain 11G *FlacS* (Rodriguez Lemoine & Rowbury, 1975*b*). So far we have found no *lac*⁻ segregants of SD-1 which have lost the complete cointegrate; all *lac*⁻ segregants tested carried the MP10_{LT2} plasmid and therefore arose by dissociation of the cointegrate rather than by its loss. We are further examining the replication of the cointegrate to ascertain whether the F factor constituent can govern replication under suitable conditions.

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