MOLECULAR CHARACTERIZATION OF A STABLE *Flac* PLASMID

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SUMMARY

FlacS is a thermostable extrachromosomal element isolated in *Salmonella typhimurium* which is altered in its replication as compared to its precursor $F_{ts}114$ *lac*. Sedimentation of both these plasmids in alkaline sucrose gradients has indicated a difference in their sizes. Contour length measurements of open circular plasmid DNA molecules photographed in the electron microscope have revealed the estimated molecular weight of $F_{ts}114$*lac* to be 81 X 10^6 daltons while that of FlacS is 109 X 10^6 daltons. FlacS may carry a segment of *S. typhimurium* chromosomal or cryptic plasmid DNA.

INTRODUCTION

The replication of bacterial extrachromosomal elements (plasmids) is an ill-defined process. In hopes of providing insight into this process, we have studied a novel plasmid, designated FlacS, which is altered in one or more of its replicative functions, as compared to its precursor, $F_{ts}114$*lac* (F_{ts}*lac*;1,2). FlacS was isolated as a spontaneous mutant in a strain of *S. typhimurium* which carried F_{ts}*lac* (1). F_{ts}*lac* is lost from cells grown at 42 C due to a block in some stage of its replication (3). FlacS, however, shows no thermolability at this temperature and, in addition, is spontaneously lost at frequencies which are 20-100 less than the wild-type Flac (F42; precursor of F_{ts}*lac*). FlacS is also able to resist selective elimination (curing) by acridine orange, ethidium bromide, and sodium dodecyl sulfate although

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these agents promote the rapid curing of F_{ts}lac and F42 (1). FlacS donor pili appear altered based on their very inefficient adsorption of RNA donor-specific bacteriophages (2). Furthermore, FlacS is not able to inhibit efficiently the growth of the female-specific bacteriophage T7 while F_{ts}lac and F42 do mediate such inhibition.

In this report we present evidence indicating that the FlacS plasmid molecule is substantially larger in size than F_{ts}lac.

MATERIALS AND METHODS

Bacterial strains and media. The following F^- strains (and their isogenic F' derivatives) of Escherichia coli K-12 and Salmonella typhimurium LT-2 were used. E. coli X5097 was obtained from J. R. Beckwith and its genotype is: proB-lac$_{X111}$ str^r mal thi. S. typhimurium trpC109 was from the collection of M. Demerec. The F_{ts}lac plasmid was isolated by Cuzin and Jacob (3) as a thermosensitive replication-defective mutant of Flac (F42). FlacS was isolated as a spontaneously occurring thermostable mutant of F_{ts}lac carried by S. typhimurium trpC109 (1). Conjugal transfer of plasmids was accomplished as previously described (1). Penassay broth (Difco) was used exclusively in these studies.

Radioisotopes. Thymidine-methyl-^{3}H (20 Ci/mmole) and thymidine-methyl-^{14}C (20-30 mCi/mmole) were from the New England Nuclear Corporation.

Alkaline sucrose gradient analysis of cell lysates. Early log-phase cells were labelled for 90 min. with ^{3}H-thymidine (15 μCi/ml) and/or ^{14}C-thymidine (2.5 μCi/ml) in Penassay broth containing 250 μgm/ml deoxyadenosine at 37 C (32 C for F_{ts}lac-containing strains). Sheared sarkosyl (Geigy Chem. Co.) lysates of labelled E. coli cells were prepared according to Bazaral and Helinski (4). The Brij-58 (ICI America, Inc., Wilmington, Del.) lysis method as described by Kline and Helinski (5) was used in the preparation of S. typhimurium sheared lysates. Either 0.1 or 0.2 ml of a sheared lysate was layered onto a 5-20% linear alkaline sucrose gradient (6). Centrifugation conditions are described in the legends to Figures 1 and 2. Gradient fractionation and measurement of radioactivity have been described (2).

TABLE 1. Mean contour lengths of open circular F_{ts}*lac* and *Flac*S molecules and their estimated molecular weights[a]

Plasmid	n[b]	Contour length ± SD (μm)	Estimated molecular weight (daltons)
F_{ts}*lac*	7	44.8 ± 1.6	81 X 10^6
*Flac*S	12	60.6 ± 2.2	109 X 10^6

[a]Photographic enlargements of open circular plasmid molecules were measured with a map measuring device. Molecular weights were computed assuming a mass per unit length relationship of 1.8 X 10^6 daltons/μm for a water hypophase (15,16).

[b]Number of molecules measured.

Isolation and electron microscopic examination of plasmid DNA. Covalently closed circular (CCC) F_{ts}*lac* and *Flac*S plasmid DNA was isolated from *E. coli* X5097 according to the method of Palchaudhuri et al. (7). X-irradiation was used to convert CCC DNA to the open circular form. DNA was prepared for electron microscopy by a modification (8) of the method described by Kleinschmidt (9) employing a water hypophase and was observed using a Siemens Elmiskrop 1A electron microscope.

RESULTS AND DISCUSSION

Alkaline sucrose gradient analysis of ^{3}H-labelled cell lysates of *E. coli* was previously used to establish that *Flac*S existed as an autonomously replicating plasmid and was not present in multiple copies per cell (2). Such studies enabled us to rule out chromosomal integration and an increased replication rate (resulting in multiple *Flac*S copies) as reasons for the unique stability of *Flac*S. In these experiments, the fast-moving peak corresponding to *Flac*S CCC DNA was always observed to move further than the F_{ts}*lac* (or F42) peak in identical gradients. We have confirmed this observation by examining the sedimentation of differentially radioactively-labelled F_{ts}*lac* (^{3}H) and *Flac*S (^{3}H-^{14}C) CCC DNA through alkaline sucrose gradients. Figure 1 reveals

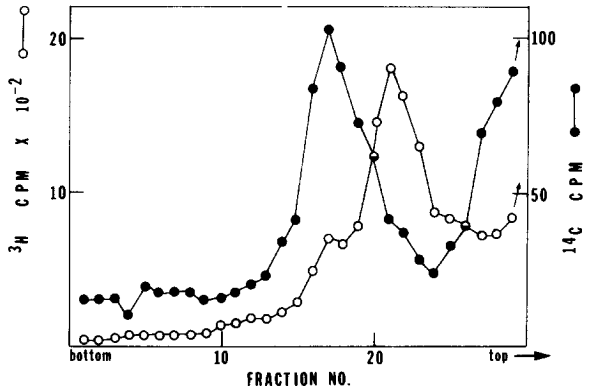

FIGURE 1. Alkaline sucrose gradient analysis of ^{14}C-^{3}H-labelled cell lysates of E. coli X5097. ^{3}H-labelled cells containing F$_{ts}$lac and ^{14}C-^{3}H-labelled cells containing FlacS were mixed (1:1), lysed, and DNA sheared as described in MATERIALS AND METHODS. A 0.2 ml sample of the sheared lysate was layered onto a 5-20% alkaline sucrose gradient and centrifuged at 20 C for 20 minutes at 114,000 X g in a Beckman L2-65B Preparative Ultracentrifuge using a SW50L rotor. Only the first 29 fractions of a 48 fraction gradient are shown. The ^{3}H and ^{14}C chromosomal peaks occur at the top of the gradient and reach maximum values of 197,937 cpm and 14,012 cpm respectively at fraction 46. There is an ^{3}H shoulder under the ^{14}C peak since the FlacS-harboring strain was simultaneously pulsed with ^{3}H- and ^{14}C-thymidine. Recovery of ^{3}H and ^{14}C counts was greater than 90%.

that the ^{14}C peak corresponding to FlacS CCC DNA shows roughly a 25% increase in sedimentation velocity as compared to F$_{ts}$lac (or F42; data not shown). Thus, a size difference between these plasmids seemed apparent.

F$_{ts}$lac and FlacS plasmid CCC DNA was isolated as described in MATERIALS AND METHODS and electron micrographs of open circular plasmid molecules were obtained. Measurements of their respective contour lengths were made and converted to molecular weight values (Table 1). These data show that FlacS carries approximately 28 X 10^6 daltons more DNA than F$_{ts}$lac. The increase in molecular weight is consistent with the alkaline sucrose gradient data (see Fig. 1). Since FlacS was first isolated in S. typhimurium, this observation has led us to the hypothesis that FlacS was generated by the addition of S. typhimurium DNA to F$_{ts}$lac. Such a sizable increase ($\sim$34% of F$_{ts}$lac molecule) makes it reasonable to assume this insertion plays a role in FlacS behavior. These possibilities are discussed below.

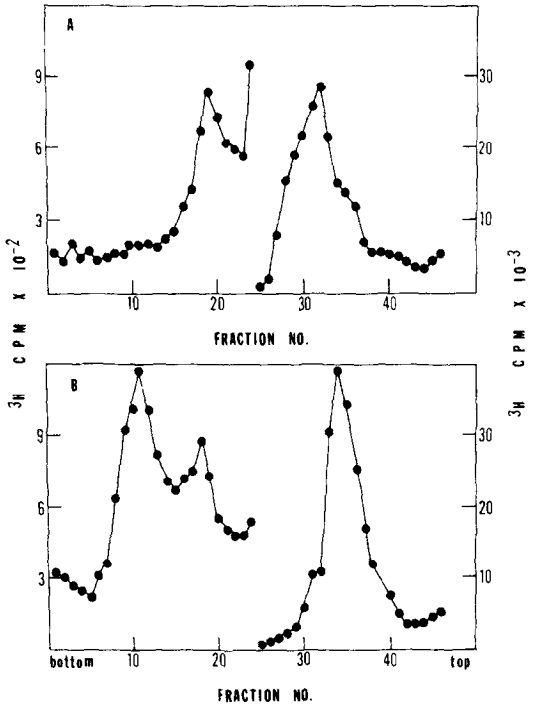

FIGURE 2. Alkaline sucrose gradient analysis of ^{3}H-labelled cell lysates of S. typhimurium trpC109. F$^-$ and FlacS-harboring cells were labelled with ^{3}H-thymidine and lysed as described in MATERIALS AND METHODS. Samples of 0.1 ml were layered onto 5-20% alkaline sucrose gradients and centrifuged at 20 C for 25 minutes at 114,000 X g in a Beckman L2-65B Preparative Ultracentrifuge using an SW50.1 rotor. A: S. typhimurium trpC109 F$^-$ gradient profile. B: S. typhimurium trpC109/FlacS gradient profile. The peak in panel A corresponding to the fast-moving component (fractions 16 - 22) is 1.6% of the peak corresponding to the host chromosome (fractions 25 - 46). The peaks in panel B corresponding to the fast-moving components (fractions 7 - 14 and fractions 16 - 20) represent 2.8% and 1.6% respectively of the peak corresponding to the host chromosome (fractions 25 - 45). Recovery of ^{3}H counts in both instances was greater than 90%. Note that there is a change of scale between fractions 24 and 25.

S. typhimurium LT-2 is known to carry a plasmid (molecular weight = 62 X 10^6 daltons) of unknown function (10,11). If the above hypothesis is correct, the insertion carried by FlacS may be of chromosomal or cryptic plasmid origin. We have verified the presence of this cryptic plasmid in S. typhimurium trpC109. It may be seen as a fast-moving peak on an alkaline sucrose gradient in Figure 2 (panel A, fractions 16-22). Similar analysis of S. typhimurium trpC109/FlacS (Figure 2, panel B) reveals peaks corresponding to CCC DNA of FlacS (fractions 7-14) as well as the cryptic plasmid (fractions

16-20) indicating that these two plasmids can stably coexist within the same cell (i.e., are compatible). F_{ts}lac and F42 compatibility with this cryptic plasmid has also been demonstrated using alkaline sucrose gradient analysis (not shown).

Using the alkaline sucrose gradient profiles, the amount of FlacS and cryptic plasmid CCC DNA may be computed relative to the total host chromosomal DNA (see Figure 2 legend). Assuming a molecular weight value of 62 X 10^6 daltons for the cryptic plasmid (10,11) and 109 X 10^6 daltons for FlacS (Table 1), we calculate that both these plasmids are present to the extent of approximately one copy per chromosomal equivalent. These estimations are based on a molecular weight value of 3.2 X 10^9 daltons for the S. typhimurium chromosome (12). A similar result was previously reported for the number of copies of FlacS (and F_{ts}lac) per E. coli chromosomal equivalent (2). We thus conclude that FlacS stability is not due to the presence of multiple plasmid copies in cells of E. coli or S. typhimurium.

Molecular hybridization studies between DNA extracted from S. typhimurium trpC109 (F^-) and FlacS or F_{ts}lac DNA (prepared from E. coli X5097) have not allowed us to detect any S. typhimurium DNA carried by FlacS. These negative data, however, are not sufficient to rule out our hypothesis. Cryptic plasmid-FlacS hybridization studies as well as heteroduplex mapping (13) of FlacS and F_{ts}lac with the cryptic plasmid should be carried out in order to elucidate the nature of the additional DNA carried by FlacS.

Irrespective of its origin (i.e., insertion of host DNA or duplication of plasmid DNA) the role of this additional DNA in the stability of the FlacS remains unclear. In the context of the replicon model (14), perhaps it serves as a new and highly efficient initiator site or provides for an optimal plasmid-membrane association which facilitates FlacS replication. The genetic alteration(s) resulting in the male-specific phage insensitivity and lack of inhibition of T7 associated with FlacS may or may not have been coincidental with the acquisition of the additional DNA.

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