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# Evidence for a Composite State of an F'*his,gnd* Element and a Cryptic Plasmid in a Derivative of *Salmonella typhimurium* LT2

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A method designed to select mutants constitutive for expression of the histidine operon has been applied to a *Salmonella typhimurium* LT2 strain containing an F'*his,gnd* element and a cryptic plasmid. One of the mutants isolated, strain AA0019, has not only increased levels of histidinol phosphate phosphatase (*hisB*), but also increased levels of gluconate-6-phosphate dehydrogenase (*gnd*). Ultracentrifugation studies of extrachromosomal deoxyribonucleic acid (DNA) isolated from strain AA0019 revealed the presence of a single species of covalently closed circular (CCC) DNA that sedimented more rapidly through neutral and alkaline sucrose gradients than any of its possible plasmid precursors. From neutral sucrose gradients, sedimentation coefficients of 130, 100, and 86*S* were derived, corresponding to the CCC DNA of the large plasmid in strain AA0019, the F'*his,gnd* element and the cryptic LT2 plasmid, respectively. An *Escherichia coli* plasmid-free strain that upon mating had received the large 130S plasmid also contained 86*S* and 100*S* CCC DNA components. A histidine-requiring derivative of strain AA0019 obtained after acridine orange treatment retained the cryptic plasmid DNA. Apparently, the large plasmid in strain AA0019 consists of the F'*his,gnd* element and the cryptic LT2 plasmid of the parental strain.

*Escherichia coli* and *Salmonella typhimurium* cells harboring an F' element appear to have a strict control mechanism that ensures that there are one to two copies of the F' element per host chromosome (10, 19). Similarly, Rownd et al. (34) have shown that there is one copy of an F-like R plasmid per host chromosome in *Serratia marcescens* as well as in *E. coli*. However, when the R factor is transferred into *Proteus mirabilis*, control of R factor replication is relaxed, and the number of R factor copies increases to ten per chromosome. Nordström et al. (28) have isolated mutants of an R factor in *E. coli* having two to four times as many R factor copies per chromosome as the parental strain.

To determine whether a similar alteration in the control of replication of an F' element could be obtained, we applied a technique designed to select for mutants constitutively derepressed for histidine biosynthesis (33) to a strain containing an F'*his,gnd* element. This element, described by Fink and Roth (10), carries the *E. coli* histidine operon and the *gnd* gene, a constitutive gene coding for the enzyme gluconate-6-phosphate dehydrogenase (GND). Although the *gnd* gene is closely linked to the histidine operon, it is unaffected by derepression of that operon (27). Mutants derepressed for histidine biosynthesis can be obtained by selecting clones that are resistant to the histidine analogue 1,2,4-triazole-3-alanine (TRA), a false co-repressor of the operon, and 3-amino-1,2,4-triazole (AT), an inhibitor of one of the histidine biosynthetic enzymes (1, 15, 22). Although TRA does not inhibit wild-type *S. typhimurium* growing in minimal medium, it does inhibit strains having a partial genetic block in the histidine pathway. Such a partial block, in which the histidine operon must be derepressed for growth, can be achieved by growing cells on AT. Since TRA acts as a false co-repressor, it inhibits strains that must be derepressed in order to grow. Therefore, mutants constitutive for histidine biosynthesis are found among cells that are resistant to TRA in the presence of AT. A mutant exhibiting relaxed control for replication of an F'*his,gnd* element would be expected to have increased activity of not only the histidine biosynthetic enzymes, but also GND.

In this paper, we report the isolation of a

TRA-AT-resistant mutant of *S. typhimurium*, designated strain AA0019, having increased levels of both the histidine biosynthetic enzymes and GND. However, DNA ultracentrifugation studies revealed that the higher enzymatic activities are not due to an overproduction of the F'*his,gnd* element. The data presented in this paper suggest that strain AA0019 contains a composite plasmid larger than either of its possible precursors, the F'*his,gnd* element or the LT2 cryptic plasmid present in the parental strain.

## MATERIALS AND METHODS

**Materials.** Reagents and sources were as follows: diethyl sulfate from Aldrich Chemical Co.; ethidium bromide, acridine orange, and triethanolamine hydrochloride from Calbiochem; egg white lysozyme, deoxyadenosine, histidinol phosphate, nicotinamide adenine dinucleotide phosphate (NADP), phenazine methosulfate (PMS), 2-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium (INT), potassium-6-phosphogluconate, 1,2,4-triazole-3-alanine (TRA), and 3-amino-1,2,4-triazole (AT) from Sigma Chemical Co; CsCl (technical grade) from Kawecki Berylco Industries, Inc.; sodium deoxycholate (DOC) from Difco Laboratories; and [*methyl*-$^3$H]thymidine (5 Ci/mmol) and [2-$^{14}$C]thymidine (59 mCi/mmol) from Amersham.

**Bacterial strains.** The characteristics and origins of strains employed in this study are given in Table 1. The *S. typhimurium* strains are derivatives of strain LT2 which contains a cryptic plasmid (8, 37). The *E. coli* strains are derivatives of K-12.

**Media.** Minimal medium E (42) supplemented with 1% (wt/vol) glucose was used unless otherwise indicated. Supplements were added as required at the following concentrations: (i) to solid media: serine, 60 μg/ml; arginine, 30 μg/ml; other L-amino acids, 20 μg/ml; (ii) to liquid media: serine, 480 μg/ml; arginine, 240 μg/ml; other L-amino acids, 20 μg/ml.

**Mutagenesis and isolation of TRA-AT-resistant strains.** Single colonies of strain TR35 were inoculated into 2 ml of minimal medium E supplemented with the required amino acids and incubated overnight at 37 C. The cultures were then mutagenized by adding 0.05 ml of diethyl sulfate for 15 min at 37 C. Samples containing approximately $5 \times 10^7$ cells were spread directly onto minimal agar plates (13 by 55 mm) containing 20 mM AT and 2% glucose (33). Crystals of TRA were placed in the center of the plates, and the plates were incubated for 5 days at 37 C. It has been shown previously that strains containing high levels of the histidine biosynthetic enzymes form wrinkled colonies on media containing 2% glucose (9, 26). Therefore, two wrinkled TRA-AT-resistant colonies within the TRA zone of inhibition were picked from each plate and purified by successively streaking for single colonies on (i) supplemented minimal medium plates, (ii) nutrient agar plates, and (iii) supplemented minimal medium plates. Eighty-six purified TRA-AT-resistant clones were saved for further study.

**Mating procedure.** F-duction experiments were performed by a plate-mating technique (13). The

TABLE 1. *Bacterial strains*

| Strain no. | Genotype[a] | Origin or source |
|---|---|---|
| *S. typhimurium* strains | | |
| LT2 | Wild type | B. N. Ames |
| TR35 | F'*80his,gnd*/Δ*hisDCBHAFIE712 arg-501 ser-821*[b] | The F'*80his,gnd* element is derived from *E. coli* (10, 21) |
| AA0019 | See Results | TRA-AT- resistant mutant derived from strain TR35 |
| AA0020 | See Results | Acridine orange "cured" His$^-$ derivative of strain AA0019 |
| AA0021 | See Results | Spontaneously "cured" His$^-$ derivative of strain AA0019 |
| AA0035 | Δ*hisDCBHAFIE712 arg-501 ser-821* | Acridine orange "cured" His$^-$ derivative of strain TR35 |
| *E. coli* strains | | |
| SB1542 | FS400*his,gnd,rfb*/Δ*his,gnd3157 thr-4 leu-8 proA2 ara$^-$ lacY1 gal-2 str xyl-5 mtl-1 thi-1* (λ)$^-$ | M. J. Voll (43) |
| AA1100 | Δ*his, gnd3157 thr-4 leu-8 proA2 ara$^-$ lacY1 gal-2 str xyl-5 mtl-1 thi-1* (λ)$^-$ | Acridine orange "cured" His$^-$ derivative of strain SB1542 |
| AA1119 | See Results | His$^+$ Gnd$^+$ recombinant from AA0019 × AA1100 |
| AA1135 | F'*80his,gnd*/Δ*his,gnd3157 thr-4 leu-8 proA2 ara$^-$ lacY1 gal-2 str xyl-5 mtl-1 thi-1* (λ)$^-$ | His$^+$ Gnd$^+$ recombinant from TR35 × AA1100 |

[a] Genetic symbols used for *S. typhimurium* strains are those used by Sanderson (35); genetic symbols used for *E. coli* strains are those used by Taylor and Trotter (39).

[b] The symbol Δ indicates a multisite deletion mutation.

resulting recombinants were purified by twice streaking for single colonies on appropriately supplemented minimal medium prior to further study.

**"Curing" experiments.** Merodiploid strains were inoculated into 1 ml of nutrient broth, pH 7.6, containing 50 μg of acridine orange per ml for the *S. typhimurium* strains or 10 μg of acridine orange per ml for the *E. coli* strains (14). The cultures were incubated overnight in the dark at 37 C to allow full growth and then appropriately diluted and plated on nutrient agar. The colonies were then tested by replica plating for loss of plasmid-associated phenotypes.

**Enzyme assays.** For all enzymatic assays cells in exponential growth were collected, washed, and toluene-treated as described by Martin et al. (25). The activity of the enzyme histidinol phosphate phosphatase (specified by the *hisB* gene) was determined by the procedure of Martin et al. (25) with the exception of using 0.02 ml of 50 mM histidinol phosphate instead of 0.01 ml. Gluconate-6-phosphate dehydrogenase was assayed by the technique of Fraenkel and Horecker (11) as modified by Murray and Klopotowski (27).

**Preparation of cleared lysates.** Cells were grown in 30 ml of appropriately supplemented minimal medium E, containing 500 μg of deoxyadenosine per ml and $3.3 \times 10^{-3}$ mCi of [*methyl*-$^3$H]thymidine per ml. At the exponential phase of growth, cells were harvested by centrifugation and washed twice with TES buffer [0.03 M tris(hydroxymethyl)aminomethane(Tris), 0.005 M ethylenediaminetetraacetic acid (EDTA) and 0.05 M NaCl, pH 8.0]. The washed pellet was resuspended in 1 ml of 25% sucrose in 0.05 M Tris-hydrochloride, pH 8.0 (2). Cell lysis, using a Brij 58-sodium deoxycholate mixture, was carried out in an ice bath as described by Clewell and Helinski (3, 4). The crude lysate was centrifuged at 48,000 × *g* for 5 min, and the supernatant fluid was saved for further study.

**Dye-buoyant density equilibrium centrifugation.** Centrifugation was performed in a Beckman L3-50 preparative ultracentrifuge in a type Ti-50 fixed angle rotor at 40,000 rpm at 15 C for 40 to 60 h. Each polyallomer tube contained 7.5 g of CsCl in 5.5 ml of TES buffer, 1.5 ml of 1 mg of ethidium bromide per ml of water, and 1 ml of cleared lysate sample (2). After centrifugation, the bottom of the tube was punctured with a Hoefer gradient fractionator and 20-drop (0.2 to 0.3 ml) fractions were collected. A sample of 50 μliters from each fraction was applied to a 1-inch square of Whatman no. 1 chromatography paper and treated as described below.

**Sucrose density gradients.** The fractions from the ethidium bromide-CsCl density gradients containing covalently closed circular deoxyribonucleic acid (CCC DNA) were pooled and dialyzed at 4 C for 6 h against two changes of a 4-liter volume of 0.1 × SSC (0.015 M NaCl; 0.0015 M sodium citrate, pH 7.4). For neutral velocity gradients, samples of 0.1 to 0.2 ml were layered onto 5-ml 5 to 20% neutral sucrose gradients made up in TES, pH 8.0, and centrifuged at 48,000 rpm for 35 min. For alkaline velocity gradients, samples of 0.1 to 0.2 ml were layered onto 5-ml 5 to 20% alkaline sucrose gradients containing 1.0 M NaCl, 0.01 M EDTA, and 0.3 M NaOH, and centrifuged at 40,000 rpm for 20 min. The centrifugations were performed in an SW50.1 rotor at 15 C in a Beckman L3-50 preparative ultracentrifuge. From both types of gradients, fractions of nine drops each were collected onto 1-inch filter paper squares. The recovery of radioactivity from sucrose gradients was 87 to 100%.

**Counting of radioactivity.** The filter paper squares were washed twice in 5% trichloroacetic acid, ethanol, and ether (2); dried; and counted for radioactivity in a Beckman LS-230 liquid scintillation spectrometer. The $^{14}$C and $^3$H counts in sucrose density gradient profiles have been corrected for spillage of counts from one isotope into the counting window of the other isotope.

**Preparation of [$^{14}$C]LT2 cryptic plasmid DNA and estimation of its molecular weight.** *S. typhimurium* LT2 was grown in 12 ml of minimal medium E containing 500 μg of deoxyadenosine per ml and $3.75 \times 10^{-1}$ μCi of [2-$^{14}$C]thymidine per ml. At the exponential phase of growth, the cells were harvested and washed twice with TES buffer, pH 8.0. Crude cell lysates were prepared by the procedure described by Clewell and Helinski (3, 4), modified by replacing Sarkosyl with 1% sodium dodecyl sulfate in TES. The crude lysate was incubated at 37 C for 10 min, and the DNA was sheared by pipetting 20 times with a 5-ml small-bore pipet. Dye buoyant density equilibrium centrifugation was performed, and the fractions containing CCC DNA were pooled and dialyzed as described above.

The procedure of Willetts and Bastarrachea (44) was used to obtain CCC-DNA from R-factor containing strains.

The R538 plasmid has a molecular weight of $50 \times 10^6$ (5, 40), whereas that of the Sa plasmid is $26 \times 10^6$ (18). By using the empirical formula of Hudson, Clayton, and Vinograd (17), the *S* value of the CCC DNA of each R plasmid was calculated to be 78 and 56*S*, respectively. The *S* value of the CCC DNA of the cryptic LT2 plasmid in neutral sucrose gradients was estimated to be 86*S* by cosedimentation with either differentially labeled R538 CCC DNA or Sa CCC DNA. Upon storage at 4 C (44), the cryptic plasmid CCC DNA sedimenting at 86*S* was converted to an open-circular DNA (OC DNA) sedimenting at approximately 53*S*. The estimated *S* value of the cryptic plasmid CCC DNA corresponds to a molecular weight of approximately $59 \times 10^6$ (17). This value is in good agreement with previously reported values of $60 \times 10^6$ (38) and $58 \times 10^6$ (36) for the molecular weight of the LT2 cryptic plasmid.

## RESULTS

**Isolation of a TRA-AT-resistant mutant with increased levels of histidinol phosphate phosphatase and GND.** Strain TR35 carries the chromosomal deletion Δ*hisDCBHAFIE712* which does not extend into the *gnd* gene. It also harbors the cryptic LT2 plasmid (8, 38) and an *E. coli* F′ factor, F′*80*, carrying the histidine

operon and the *gnd* gene. After diethyl sulfate mutagenesis of strain TR35, 86 TRA-AT resistant, wrinkled colonies were selected and purified. All mutants were assayed for histidinol phosphate phosphatase (*his*B) and GND. In one isolate, strain AA0019, the specific activity of histidinol phosphate phosphatase was increased 2.3-fold and the GND activity was increased 1.5-fold over that of the parental strain TR35 (Table 2). Similar results have been obtained from assays of crude cell extracts. This observation might suggest that there are multiple copies of the F'*his,gnd* element in strain AA0019.

**Analysis of extrachromosomal DNA.** *S. typhimurium*, strain LT2, possesses a cryptic plasmid which can be separated from the chromosomal DNA by the dye-buoyant density equilibrium centrifugation technique. However, this technique does not distinguish between F' strains and strains derived from it that harbor the cryptic LT2 plasmid in addition to the F' factor. The data in Fig. 1, panels A, B, and C, demonstrate that strains LT2, TR35, and AA0019 all contained a denser satellite DNA component corresponding to CCC DNA (32) which formed a band in fractions 13 to 15. The fractions containing the extrachromosomal DNA were pooled, dialyzed, and subjected to sedimentation through (i) 5 to 20% neutral sucrose gradients to determine whether more than one size molecule was present in the plasmid material, (ii) 5 to 20% alkaline sucrose gradients to ascertain whether the plasmids were actually in a closed circular form. The sedimentation profiles obtained are shown in Fig. 2.

TABLE 2. *Enzyme levels of hisB and GND*

| Strain | *hisB* | | GND | |
|---|---|---|---|---|
| | Sp act[a] | Relative sp act[b] | Sp act[a] | Relative sp act[b] |
| LT2 | 2.2 | 1.0 | 6.0 | 1.0 |
| TR35 | 7.2 | 3.3 | 18.0 | 3.0 |
| AA0019 | 16.7 | 7.6 | 28.0 | 4.7 |
| AA0020 | 0.1[c] | 0.05 | 8.0 | 1.3 |
| AA0035 | 0.06[c] | 0.03 | 7.0 | 1.2 |

[a] Enzymatic assays were performed in toluenized cell preparations as described in Materials and Methods. All values are averages of at least five separate experiments performed on separately prepared extracts.

[b] Relative specific activities are expressed relative to the wild-type strain, LT2, which is haploid for the histidine operon and the *gnd* gene.

[c] Deletions of the *hisB* gene show very low enzymatic levels which are presumably due to the presence of a non-specific phosphatase (33).

Figure 2, panel A, demonstrates that the LT2 strain contained a CCC DNA that formed a band at neutral pH with a sedimentation coefficient of 86*S* (fraction 14). This represents the cryptic LT2 plasmid which has been reported by others (8, 37). The heterogeneous band sedimenting at approximately 40*S* represents

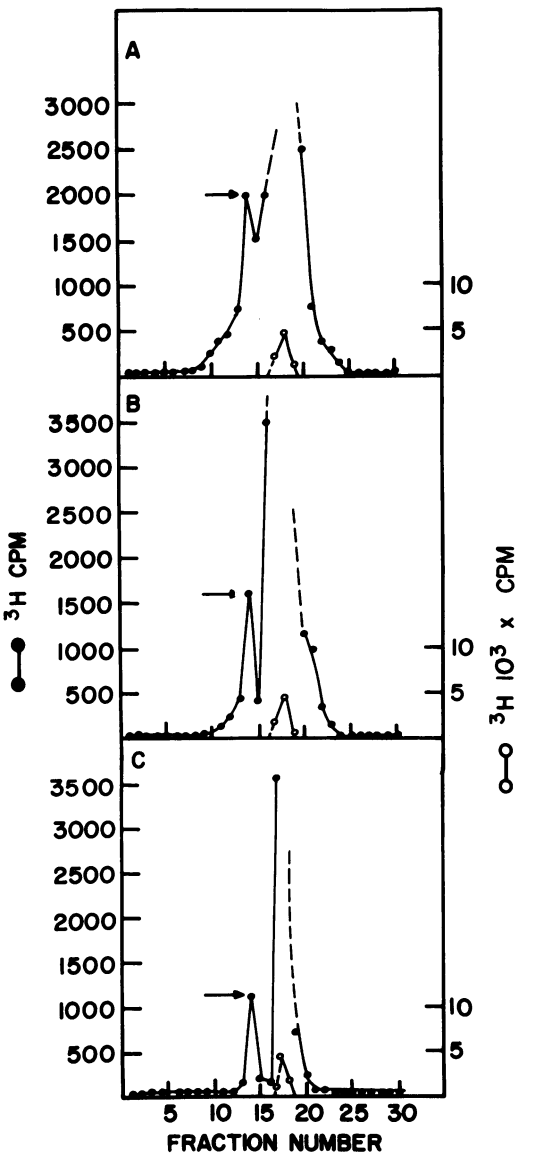


FIG. 1. *Dye-buoyant density centrifugation of $^3$H-labeled cell lysates of S typhimurium. All strains were grown in the presence of [$^3$H]thymidine and [$^3$H]deoxyadenosine. Cleared lysates were centrifuged in a Ti-50 rotor at 40,000 rpm at 15 C for 40 h. The arrows indicate the fractions containing CCC DNA which were pooled for subsequent study. (A) LT2; (B) TR35; (C) AA0019.*

chromosomal DNA (as the chromosomal band obtained in the CsCl dye-buoyant density gradients overlaps the satellite DNA band; Fig. 1, panel A).

The molecular weight of the cryptic plasmid DNA was calculated to be $59 \times 10^6$ (17) which is in good agreement with previously reported values (36, 38). The F'*his,gnd* DNA in strain TR35 sedimented slightly faster (fraction 13) than the cryptic LT2 plasmid DNA which is also present (Fig. 2, panel B). The sedimentation coefficient of the F'*his,gnd* element was estimated to be 100*S*, corresponding to a molecular weight of $79 \times 10^6$ (17). Analysis of the CCC DNA in mutant AA0019 (Fig. 2, panel C) revealed a single component which sedimented 15 to 20% faster than the cryptic plasmid DNA or the F'*his,gnd* element. DNA bands corresponding to these latter two DNA species were no longer present. The sedimentation coeffi-

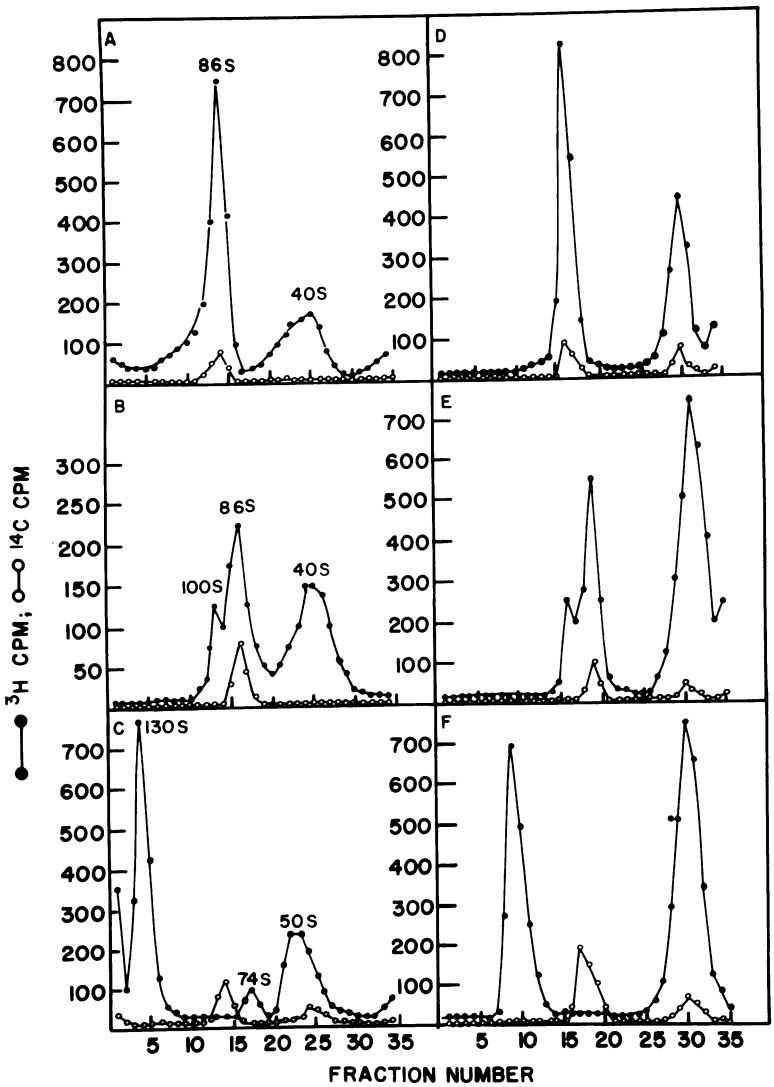


FIG. 2. *Sedimentation analyses of pooled fractions corresponding to [$^3$H]CCC DNA separated by dye-buoyant density centrifugation. The fractions containing [$^3$H]CCC DNA, depicted by arrows in Fig. 1, were pooled and dialyzed. A 0.1- to 0.2-ml sample was layered onto 5-ml 5 to 20% neutral or alkaline sucrose gradients and centrifuged in an SW50.1 rotor at 15 C. Neutral sucrose gradients were sedimented at 48,000 rpm for 35 min. Alkaline sucrose gradients were sedimented at 40,000 rpm for 20 min. (A) LT2 (neutral); (B) TR35 (neutral); (C) AA0019 (neutral); (D) LT2 (alkaline); (E) TR35 (alkaline); (F) AA0019 (alkaline); ●, $^3$H counts per minute; ○, $^{14}$C-labeled CCC cryptic plasmid DNA used to calibrate the sucrose gradients.*

cient of this larger plasmid was estimated to be 130*S*. These data suggest that the fast sedimenting CCC DNA in strain AA0019 represents a composite plasmid resulting from a recombinational event between the F'*his,gnd* element and the cryptic LT2 plasmid.

In Fig. 2 panel C, the DNA component sedimenting at 74*S* (fraction 17) presumably represents the open circular form which results from single strand nicking of the supercoiled 130*S* DNA molecules. The heterogeneous band sedimenting at approximately 50*S* presumably represents further degradation of the open circular molecules to linear forms. Chromosomal DNA cannot account for this band as the satellite DNA band obtained in the CsCl dye-buoyant gradients is well separated from the chromosomal band (Fig. 1, panel C), and under neutral sedimentation conditions chromosomal DNA appears as a broad heterogeneous band sedimenting at approximately 40*S*. Furthermore, storage at 4 C of the dialyzed CCC DNA of strain AA0019 isolated from CsCl-ethidium bromide density gradients resulted in an increase in the size of these slower moving bands (Fig. 2, panel C) and a concomitant decrease in the size of the 130*S* CCC DNA band. These observations on the degradation of CCC DNA upon storage are in accord with those reported by others (7, 41, 44).

To verify that the satellite DNA isolated by CsCl dye-buoyant density gradients is in fact covalently closed, alkaline sucrose gradients were employed. At alkaline pH supercoiled DNA duplexes sediment more rapidly than nicked circular or linear and fragmented DNA molecules. The wild-type LT2 strain revealed only one size of plasmid DNA present (Fig. 2, panel D). In strain TR35 two CCC DNA species were observed: one corresponding to the cryptic plasmid, the other to the F'*his,gnd* element (Fig. 2, panel E). Strain AA0019 contained only one DNA component (Fig. 2, panel F) that sedimented 20 to 30% faster than the DNA components present in the parental strain TR35. These results are in agreement with data obtained from neutral sucrose sedimentation, indicating that strain AA0019 contains a plasmid which is significantly larger in size than either of its possible plasmid precursors. The DNA sedimenting in fractions 25 to 30 (Fig. 2, panels E to D) in the alkaline gradients corresponds to open circular and linear DNA molecules (44).

**Transfer ability of mutant AA0019.** The sex factor F and derivative F' elements promote their own conjugal transfer to recipient cells. The transfer ability of strain AA0019 was determined by qualitative plate matings made with the *E. coli* plasmid-free strain AA1100. In a control experiment, the same recipient was mated with strain TR35 (Table 1). It was established that strains AA0019 and TR35 transferred the *his* and *gnd* loci to recipient cells. The $His^+$ recombinants, purified twice by single colony isolation, were then assayed for histidinol phosphate phosphatase and GND activities. The specific activities obtained from the prepared strains, AA1135 and AA1119, were the same whether they received the F'*his,gnd* element from strain TR35 or strain AA0019. Contrary to our expectation, no higher enzymatic activities were detected in the recombinant strain AA1119.

To determine whether the *E. coli* strains AA1135 and AA1119 have the same plasmid DNA species as their respective *S. typhimurium* parental strains, their CCC DNA was isolated by CsCl dye-buoyant density centrifugation and subsequently analyzed by sedimentation through neutral and alkaline sucrose density gradients. Strain AA1135 contained a single band of CCC DNA (Fig. 3, panels A and B) with a sedimentation coefficient at neutral pH of 100*S*, which is the value obtained for the F'*his,gnd* element when present in *S. typhimurium*. No DNA band corresponding to the cryptic LT2 plasmid was observed in strain AA1135. Furthermore, an acridine orange "cured" $His^-$ derivative of strain AA1135 did not contain any CCC DNA.

In contrast, strain AA1119 contained three species of closed circular DNA molecules with different sedimentation coefficients (Fig. 3, panels C and D). Under neutral sedimentation conditions the faster moving band sedimented at approximately 129*S*. This is the expected value for the larger plasmid of strain AA0019, and thus it was concluded that the plasmid in strain AA0019 does promote its own transfer. The two other circular DNA components (panel C) have sedimentation values of approximately 100 and 86*S* which correspond to the sedimentation coefficients of the F'*his,gnd* element and the cryptic LT2 plasmid, respectively. The band sedimenting at approximately 50*S* (fractions 22 to 26) apparently represents further degradation of the CCC DNA to linear forms, as was observed in strain AA0019. This pattern suggests that the composite AA0019 plasmid when present in the *E. coli* AA1100 host is unstable and is converted to DNA components the size of its apparent precursors.

**Curing of extrachromosomal DNA by acridine orange.** The F' factor is readily eliminated from $F^+$ bacteria by growth in the presence of

acridine orange, thus converting them to F⁻ cells (16). However, not all extrachromosomal elements can be cured by acridine dyes (6, 23). Thus it was of interest to see whether strain AA0019 containing a composite plasmid would lose its extrachromosomal DNA after acridine orange treatment and yield a *S. typhimurium* strain free of plasmid DNA.

Cultures of strains TR35 and AA0019 were treated with acridine orange, and histidine auxotrophs were isolated. Neither of the "cured" strains, AA0035 or AA0020, derived from strains TR35 and AA0019, respectively, were able to promote the transfer of the histidine genes upon conjugation, thus exhibiting a lack of fertility. Enzymatic assays of histidinol phosphate phosphatase and GND in strains AA0020 and AA0035 are shown in Table 2. In both strains, the level of the GND enzyme has decreased to that of wild-type LT2, and there is almost no phosphatase activity present.

To determine whether the "cured" strains contain any extrachromosomal DNA, cleared lysates of strains AA0035 and AA0020 were centrifuged to equilibrium in CsCl-ethidium bromide density gradients, and the CCC DNA was pooled, dialyzed, and further analyzed on neutral sucrose gradients. The sedimentation profiles obtained from the "cured" strains, AA0035 and AA0020 were identical. Both strains contained a single species of CCC DNA that sedimented at approximately 86*S* in neutral sucrose gradients corresponding to the cryptic LT2 plasmid. Similar results were obtained with a spontaneously cured derivative of AA0019 designated strain AA0021 (Table 1).

Acridine orange treatment of *E. coli* strain AA1119 resulted in the loss of ability to grow on histidine. Analysis of the plasmid DNA contained in the His⁻ derivative revealed a single band of CCC DNA which cosedimented with [$^{14}$C]cryptic LT2 plasmid DNA at 86*S*. There-

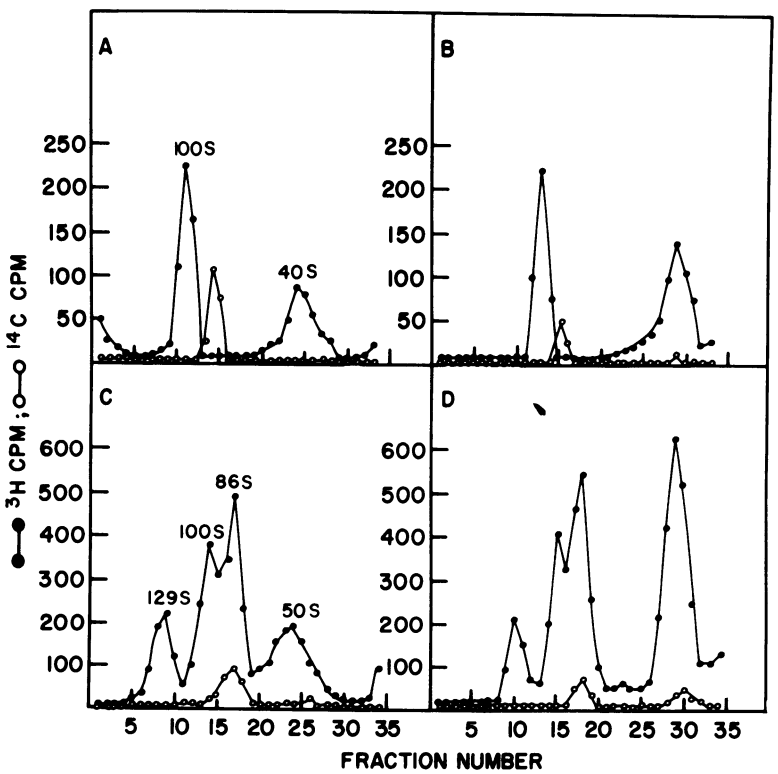


FIG. 3. *Neutral sucrose sedimentation analyses of pycnographically separated plasmid DNA from E. coli strains. Cells were labeled with [$^3$H]thymidine and lysed as described in the Materials and Methods. CCC DNA was isolated by dye-buoyant density equilibrium centrifugation and dialyzed. A 0.1- to 0.2-ml sample was layered onto a 5-ml 5 to 20% neutral or alkaline sucrose gradient and centrifuged as described under Fig. 2. (A) AA1135 (neutral); (B) AA1135 (alkaline); (C) AA1119 (neutral); (D) AA1119 (alkaline); ●, $^3$H counts per minute; O, indicates the position of $^{14}$C-labeled CCC cryptic plasmid DNA used to calibrate the sucrose gradients.*

fore, even though the *S. typhimurium* and *E. coli* strains were cured of the F'*his,gnd* element, the cryptic plasmid was retained.

## DISCUSSION

A mutant resistant to TRA and AT has been isolated by using a selection method devised for isolation of histidine regulatory mutants. This strain, designated AA0019, is derived from *S. typhimurium*, strain TR35, which harbors an F'-*his,gnd* element and a cryptic plasmid. The specific activity of the histidinol phosphate phosphatase in strain AA0019 is increased 2.3-fold over that of the parental strain, while the specific activity of the GND, an enzyme whose structural gene is not affected by derepression of the histidine operon, is increased 1.5-fold. Since the parental strain TR35 is haploid for the *hisB* gene and diploid for the *gnd* gene, these values represent a twofold increase in the expression of both genes. It should be recalled that, although the selection method employed selects for mutants exhibiting increased levels of the histidine biosynthetic enzymes, it does not select for increased activity of the GND enzyme.

Analysis of extrachromosomal DNA from strain AA0019 revealed the presence of a single species of CCC DNA sedimenting under neutral conditions at 130*S*. The F'*his,gnd* element and the cryptic LT2 plasmid found in the parental strain TR35 have an $s_{20,w}$ of 100 and 86*S*, respectively. No bands of plasmid DNA corresponding to these two DNA components were observed in strain AA0019. This suggests that the heavier species of extrachromosomal DNA in strain AA0019 might represent a composite plasmid.

If strain AA0019 were an overproducer of the F'*his,gnd* element, more radioactivity would have been incorporated into the band containing the plasmid DNA in CsCl-ethidium bromide gradients, and no shift in the position of the plasmid DNA band in neutral sucrose gradients would have been expected (28).

Since the large plasmid present in strain AA0019 is an infectious plasmid that promotes the transfer of the *his* and *gnd* genes to recipient cells, genetic material derived from the F'*his, gnd* element which is present in the parental strain, must be involved in its formation. The increased size of the plasmid is probably the result of insertion of DNA into the F'*his,gnd* element. Several possibilities could be considered for the formation of such a larger plasmid: (i) association with the LT2 cryptic plasmid, (ii) duplication of the F'*his,gnd* element, and (iii) insertion of host chromosomal DNA.

Upon mating strain AA0019 with an *E. coli* plasmid free recipient, three species of CCC DNA were recovered in the recombinant strain AA1119. Their neutral sedimentation coefficients, as determined by cosedimentation with $^{14}$C-labeled cryptic LT2 plasmid marker DNA were 129, 100, and 86*S*, corresponding to size of the larger plasmid, the F'*his,gnd* element and the cryptic LT2 plasmid, respectively. It seems likely that in an *E. coli* host the large plasmid is being converted to the smaller plasmid precursors found in the *S. typhimurium* parental strain TR35. Furthermore, strains AA0020 and AA0021 that have lost the ability to promote the transfer of the histidine genes either by acridine orange treatment or spontaneously contained only one species of plasmid DNA that sedimented under neutral conditions at 86*S*, which is the size corresponding to the cryptic LT2 plasmid. An acridine orange-treated derivative of the *E. coli* strain AA1119 also retained the cryptic plasmid DNA.

The $s_{20,w}$ at neutral pH of the large plasmid in strain AA0019 is approximately 130*S*. This is close to the value of 136*S* predicted by using the empirical formula of Hudson et al. (17), if it is assumed that an 86*S* plasmid and a 100*S* plasmid have recombined. If the larger plasmid originated from two F'*his,gnd* elements a value of 146*S* would be expected. Also, we would not expect to recover a plasmid DNA sedimenting at 86*S* in the *E. coli* strain AA1119, as shown in Fig. 3, panel C. Even though this model of an F'*his,gnd* dimer can account for the higher enzymatic activity observed in strain AA0019, we would expect if that were the case that the higher enzymatic activity would be transferred to recipient cells upon mating. However, no increase in the levels of histidinol phosphate phosphatase or GND was detected in the *E. coli* strain AA1119 that has received the large AA0019 plasmid.

At present, our data are consistent with the hypothesis that the larger plasmid in strain AA0019 is the result of an association between the cryptic LT2 plasmid and the F'*his,gnd* element to form a composite plasmid. However, conclusive proof of the DNA components of the large AA0019 plasmid awaits the results of DNA hybridization studies.

Recently, Macrina and Balbinder (23, 24) reported the isolation of a stable plasmid, F'*lacS*, from *S. typhimurium* LT2 strains. F'*lacS*, with an estimated molecular weight of $109 \times 10^6$, appears to be larger than its precursor the F'$_{ts114}$*lac* ($81 \times 10^6$). However, no increase in the levels of the enzyme β-galactosidase was observed in strains carrying F'*lacS* as compared with the parental strain. When trans-

ferred into an *E. coli* plasmid-free recipient, F'*lacS* does not break down into any other DNA components. It was suggested by the authors that F'*lacS* might be the product of a nonreciprocal exchange between the $F'_{ts114}$*lac* and a segment of the LT2 DNA (host chromosome or cryptic plasmid DNA).

Assembly of composite plasmids consisting of heterogenic parental elements has been observed for several R factors (5), the F·ColV·ColB·*trp*·*cys* plasmid (12), and the P1 penicillinase plasmid (30). Novick (29) has termed the recombinational event leading to the cointegration of two independent replicons "associative recombination." The composite product might have special properties resulting from the presence of two operative replication systems. The fusion of the two replicons might affect the rate of transcription of the cointegrate plasmid DNA. This event may account for the higher enzyme levels observed in strain AA0019.

Alternatively, the higher enzymatic levels in strain AA0019 might be the result of multiple events due to the use of the mutagenic agent diethyl sulfate (29). At the present, we cannot offer a conclusive explanation for the increased enzymatic activity of strain AA0019, and why strains that have received the AA0019 plasmid do not show increased enzymatic activity. It is possible that a chromosomal mutation in strain AA0019 is involved. Further experiments to explore this possibility are in progress.

The buoyant density separation of the AA0019 plasmid in ethidium bromide-CsCl gradients indicates that the plasmid is a CCC molecule. This is further supported by the DNA analysis data of alkaline sucrose velocity sedimentation gradients. Preliminary electron microscope studies of the strain AA0019 plasmid DNA appearing in the satellite DNA band of CsCl-ethidium bromide density gradients have revealed the presence of supercoiled DNA molecules larger than either the F'*his*,*gnd* element or the cryptic LT2 plasmid. No catenated forms (20, 31) of the AA0019 plasmid DNA have been detected.

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