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Cryptic Plasmids in a Minicell-Producing Strain of *Salmonella typhimurium*

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A minicell-producing strain of *Salmonella typhimurium* contains two cryptic plasmids. One has a molecular weight of 2.6×10^6 to 2.8×10^6, is present in multiple copies per cell, and segregates into minicells. The other has a molecular weight of 130×10^6, is present in few copies per cell, and probably does not segregate into minicells.

A variety of bacterial strains have been shown to harbor circular plasmid deoxyribonucleic acid (DNA) of unknown function (cryptic DNA). Cryptic DNAs, having molecular weights as indicated in parenthesis, have been obtained from *Escherichia coli* 15(1.5×10^6, 63 $\times 10^6$, and 104×10^6) (7, 12), *Salmonella pullorum* (1.47×10^6, and 55×10^6) (15, 18), *Salmonella typhimurium* LT2(62×10^6) (10), *Shigella paradysenteriae* (3.4×10^6 and 1.0×10^6) (13), *Shigella dysenteriae* (10^6, 1.3×10^6, 2.6×10^6, 3.8×10^6, 20×10^6 and 24×10^6) (21), *Micrococcus lysodeikticus* (0.88×10^6) (16), and *Bacillus megaterium* (1.96×10^6 to 58.8×10^6) (4).

We report here on two cryptic circular DNA species present in a *S. typhimurium* strain (χ-1313), which is an abnormal cell division mutant isolated by W. L. Tankersley (Master's thesis, University of Tennessee, 1970), that buds off minicells, similar to those formed by the *Escherichia coli* K-12 strain described by Adler et al. (1). Minicells, which are easily separated from the cells that produce them, lack chromosomal DNA but may contain extrachromosomal DNA (20) and therefore are useful for the isolation and characterization of such extrachromosomal elements.

Overnight growth of the *Salmonella* minicell-producing strain χ1313 in the presence of tritiated thymidine ([^{3}H]dThd) produced minicells containing about eight times more acid-insoluble radioactivity than minicells from the F$^-$ *E. coli* minicell-producing strain χ925. About 0.3% of the total radioactivity in the culture was in the minicells. Analysis (3) of this radioactivity labeled material from *Salmonella* minicells by cosedimentation with M13 phage DNA (28*S*) on neutral sucrose gradients revealed the presence of a DNA species with a sedimentation coefficient of 19.7*S* (Fig. 1).

Ethidium bromide-CsCl density gradient centrifugation (19) of [^{3}H]dThd-labeled DNA extracted from partially purified strain χ1313 minicells (i.e., containing 1 to 2% contaminating cells) is presented in Fig. 2. The material in fractions 18 to 25 was pooled and examined in the electron microscope. Two sizes of circular DNA molecules were observed. One had an average contour length of 1.34 μm (Fig. 3 and Table 1) and was the predominant species (range of 47:1 to 187:1 ratios), and the other had an average contour length of 66.3 μm (Fig. 3 and Table 1). The smaller molecule was shown to sediment with an *S* value of 19.7 in neutral sucrose gradients (Fig. 1). This *S* value corresponds to a molecular weight of 2.8×10^6 (2) which is in good agreement with the molecular weight of 2.6×10^6 calculated from contour lengths (Table 1). The large molecule found in the ethidium bromide-CsCl density gradients either sediments to the bottom of the centrifuge tube in the neutral sucrose gradients or is not segregated into minicells. To decide between the above alternatives, DNA from partially purified *Salmonella* cells (from minicells on sucrose gradients) and from purified *Salmonella* minicells was analyzed on 5 to 20% (wt/vol) alkaline sucrose gradients. Covalently closed circular DNA molecules of an appropriate size will sediment far ahead of linear

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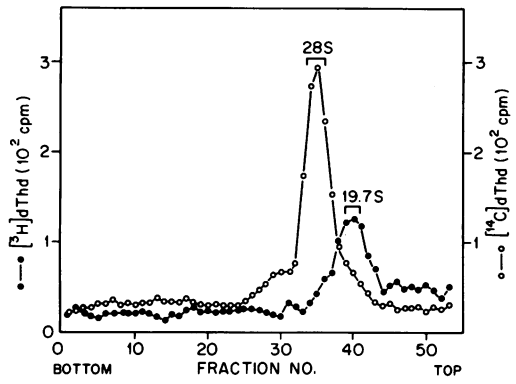

FIG. 1. *Neutral sucrose gradient centrifugation of [³H]dThd-labeled DNA from S. typhimurium minicells. The minicell-producing parent was inoculated 1:100 into 30 ml of a minimal salts solution (8) supplemented with Casamino Acids (1.5%), adenosine (200 μg/ml), and 20 μCi of [³H]dThd/ml. The culture was incubated at 37 C on a rotary shaker for approximately 12 h. Minicells were purified by two successive centrifugations on 35 ml, 5 to 20% (wt/vol) linear sucrose gradients (20). Purified minicells were suspended in 0.3 ml of a chilled buffer consisting of 0.05 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 800 μg of lysozyme/ml, 0.02 M ethylenediaminetetraacetic acid (EDTA), and 0.01 M KCN. After 15 min at 4 C, the minicells were lysed by adding 100 μliters of 2% sodium dodecyl sulfate. Minicell lysates were directly layered on a 3.6-ml linear sucrose gradient (pH 7.4) of 5 to 20% sucrose (wt/vol) containing 0.7 M NaCl and 0.01 M EDTA. Gradients were centrifuged at 30,000 rpm at 21 C for 130 min in the SW-56 rotor in a Beckman model L3-50 ultracentrifuge. Four-drop fractions were collected on paper strips (5), washed in 10% trichloroacetic acid and 95% ethanol, air dried, and counted. [¹⁴C]dThd-labeled M13 DNA (28S), ○; [³H]dThd-labeled DNA from S. typhimurium minicells, ●.*

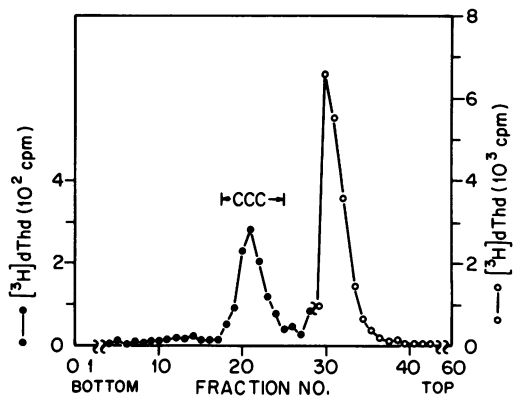

FIG. 2. *Ethidium bromide-CsCl density gradient centrifugation of [³H]dThd-labeled DNA in partially purified minicells. The minicell-producing parent was labeled and grown as described in Fig. 1, except 5 μCi of [³H]dThd/ml was used in a total volume of 60 ml of culture. After ~12 h of growth, the minicells were purified on one 35-ml, 5 to 20% (wt/vol) linear sucrose gradient per 20 ml of culture, and lysed according to the method of Clewell and Helinski (6). Partially purified minicells (containing about 1% contaminating cells) at an A_{620nm} of 0.8 were suspended in 1 ml of 25% sucrose in 0.05 M Tris at pH 8.0. Lysozyme in TES (0.003 M Na_2EDTA, 0.05 M NaCl) was added to a final concentration of 1 mg/ml. After 5 min at 25 C, predigested Pronase in TES was added to a final concentration of 1 mg/ml. After another 5 min, lysis was accomplished by adding sarkosyl to a final concentration of 1%. Ethidium bromide was then added to the lysate to a final concentration of 400 μg/ml. This solution was then mixed by inversion, 7.21 g of CsCl was added, and the refractive index was adjusted to give a median ρ = 1.550. The ethidium bromide-CsCl solution was poured into polyallomer tubes and centrifuged at 44,000 rpm (15 C) for ~60 h in a 50-Ti rotor in a Beckman model L3-50 ultracentrifuge. Gradients were collected from the bottom by inserting a hollow needle through the gradient and pumping the gradient out with a proportionating pump. Fractions were collected at 30-s intervals into plastic well dishes, and 25-μliter samples were taken from each raction for analysis of radioactivity. CCC, covalently closed circular DNA.*

chromosomal fragments in an alkaline sucrose gradient (11). Figure 4 demonstrates that 3% of the cellular DNA is fast-sedimenting (fractions 4 to 91, whereas this DNA species is not detectable from the minicell preparation. We conclude that the larger molecule is present in very few copies per chromosome and does not segregate into minicells at measurable frequencies (less than 1 large molecule for every 3,000 small molecules in minicells). The small plasmid often segregates into minicells which each contain about 10 such molecules. Since minicells have about one-tenth the volume of cells, we infer that there are about 100 small plasmids per cell.

The *Salmonella* strain χ1313 and *S. typhimurium* LT2 are sensitive to the same antibiotics. However, χ1313 is lysogenic for three phages that infect *S. typhimurium* LT2. We are continuing our investigations to ascertain if either plasmid represents a prophage genome.

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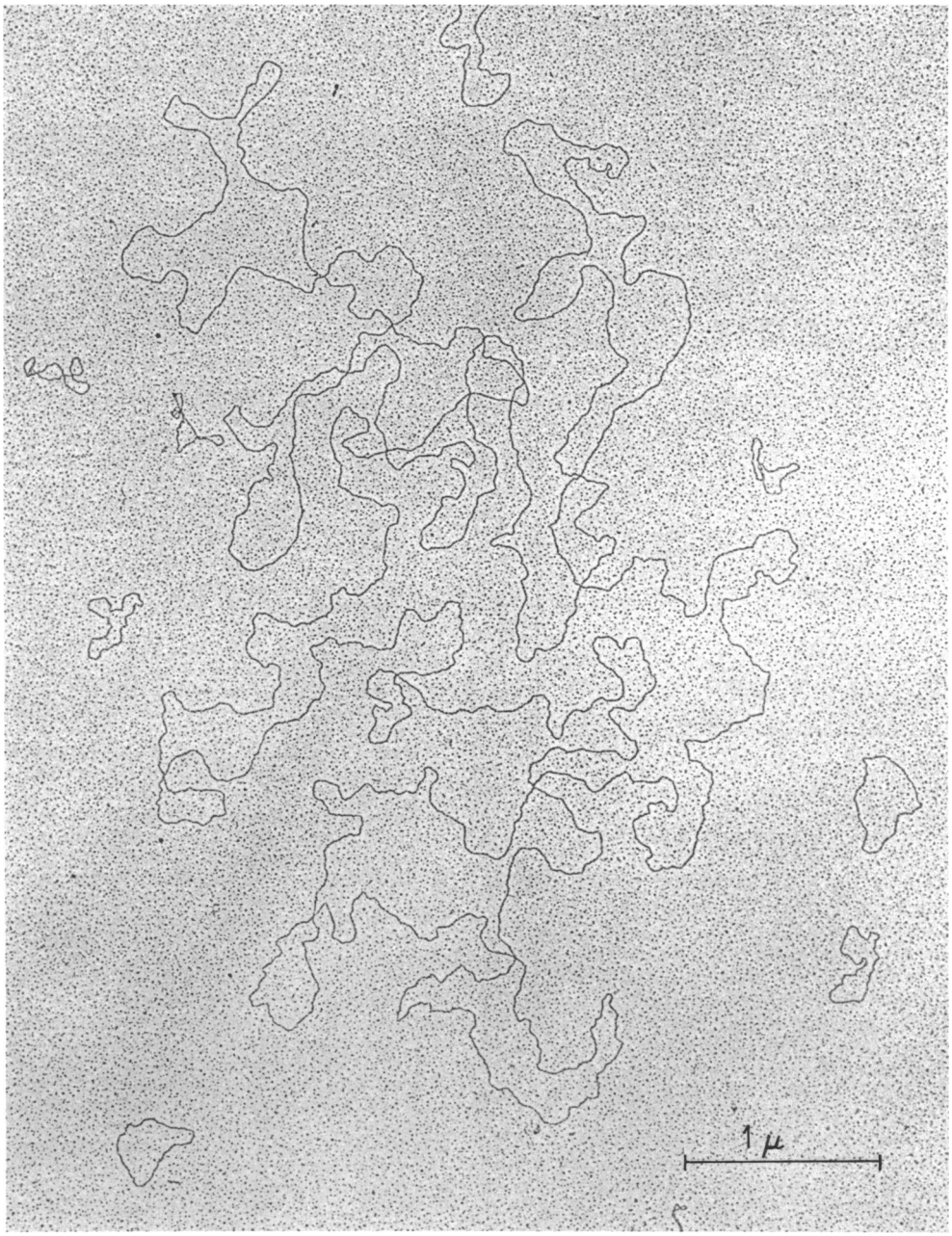

FIG. 3. *Electron micrograph of several small (~1.34 μm) and one large (~66.3 μm) circular DNAs isolated from partially purified Salmonella minicells on ethidium bromide-CsCl density gradients as described in the legend to Fig. 2. Material from fractions 18 to 25 was pooled (Fig. 2). The ethidium bromide was extracted with isopropanol, and the DNA was dialyzed against 0.50 M ammonium acetate buffer containing 0.001 M EDTA and adjusted to a pH of 6.0 with acetic acid. A 0.1-ml sample of dialyzed DNA was mixed with 0.001 ml of 1% cytochrome c and spread onto a 0.25 M ammonium acetate (pH 7.5) hypophase by using the protein film technique (14). Samples were picked up on parlodion-covered grids, stained with uranyl acetate (9), and shadowed with platinum. Electron micrographs were made with a Siemens Elmiskop I electron microscope, calibrated with a diffraction grating replica (Fullman 54, 864 lines/inch).*

TABLE 1. *Mean contour lengths and estimated molecular weights of the two circular DNA molecules present in the Salmonella typhimurium minicell producer (χ1313)*[a]

No. of molecules measured	Avg length ± SD (μm)	Estimated mol wt
5	66.3 ± .45	130×10^6
50	1.34 ± .10	2.6×10^6

[a] Molecular weights were calculated by assuming a mass of 1.96×10^6 daltons/μm (17). Contour lengths were taken by projecting electron micrograph negatives on a screen and tracing on paper. Lengths were determined from tracings with a curvimeter.

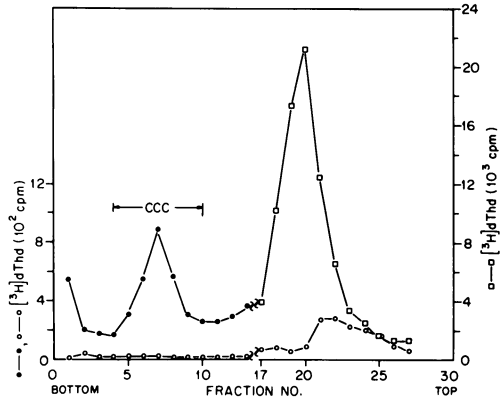

FIG. 4. *Alkaline sucrose gradient centrifugation of [³H]dThd-labeled DNA from Salmonella cells and purified minicells. Salmonella minicells were labeled and purified as described in Fig. 1. The cells were taken from the pellet of the first sucrose gradient during the minicell purification procedure. One-half milliliter of minicells, at an A_{620nm} of 0.2, and 25 μliters of cells were each suspended in washing buffer (0.01 M $KHPO_4$, 0.001 M $MgSO_4$, and 0.001 M $CaCl_2$ [pH 7.0]) and then in 200 μliters of lysis buffer (0.05 M EDTA, and 0.02 M Tris [pH 9.1]) (11). Alkaline sodium dodecyl sulfate (100 μliters of 1% solution) was added to this suspension to bring about lysis. The sample was sheared for 30 s on a Vortex mixer operating at top speed, and 250-μliter samples were layered on top of 3.6 ml of 5 to 20% (wt/vol) linear alkaline sucrose gradients. Gradients were centrifuged at 30,000 rpm for 40 min in a SW-56 rotor in a Beckman model L3-50 ultracentrifuge. Eight-drop fractions were collected on paper strips (5), washed in 10% trichloroacetic acid and 95% ethanol, air dried, and counted. S. typhimurium minicells, ○; S. typhimurium cells, ● and □; CCC, covalently closed circular DNA.*

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