HETEROGENEOUS CIRCULAR DNA ELEMENTS IN VEGETATIVE CULTURES OF BACILLUS MEGATERIUM*

By Bruce C. Carlton† and Donald R. Helinski‡

DEPARTMENTS OF BIOLOGY, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT, AND UNIVERSITY OF CALIFORNIA, SAN DIEGO (LA JOLLA)

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Abstract.—DNA preparations from two strains of *Bacillus megaterium*, one which produces megacin and another that is megacin sensitive, were analyzed by dye-buoyant density centrifugation, cesium chloride gradient equilibrium centrifugation in the absence of dye, zone sedimentation in sucrose, and electron microscopy. Both strains were found to contain a substantial proportion of their total DNA as circular, covalently closed, double helical molecules. These DNA elements were heterogeneous in size and appeared slightly less dense than chromosomal DNA. The physiological role and origin of these elements is unknown at present.

The existence of circular and covalently closed, double-stranded DNA elements has been documented recently for a variety of bacterial, viral, and mammalian systems. These elements range in size from *FColVColBtrycys* sex factor-*Col* factor of a molecular weight 107 million[1] isolated from *Proteus mirabilis* to the minute circular DNA elements found in *Micrococcus lysodeikticus*[2] and *E. coli* 15T$^-$ strains.[3] Although the functional significance of certain of these elements such as viral DNA, sex factors, and colicinogenic factors seems clear, for others their biological role is unknown.

Two strains of *Bacillus megaterium* have been examined to determine whether this organism possesses extrachromosomal circular DNA elements that are responsible for megacin production. One of the strains (strain 216) is able to produce two distinct antibiotic proteins, megacins, which can interact with and kill certain other *B. megaterium* strains. The other strain (strain 58) is megacin sensitive. Unexpectedly, it was found that both strains contain circular DNA elements, heterogeneous in size, which comprise a substantial proportion of the total extractable DNA. In this report we present some of the characteristics of these elements.

Materials and Methods.—*Bacterial strains:* *B. megaterium* strains 216 (megacin A and megacin C producer) and 58 (sensitive to megacin B) were obtained from the collection of Dr. I. B. Holland through the cooperation of Dr. D. L. Wulff. The strains were maintained on AK agar (Baltimore Biological Laboratories) sporulation slants. Single-colony isolates were tested for megacin-producing activity by stabbing into (Difco) nutrient agar plates seeded with 5×10^7 cells of an appropriate sensitive tester strain (*B. megaterium* strain C4ma—) in a layer of 4 ml of soft nutrient agar.

Reagents: H^3-thymidine, spec. act. 6.7 c/mM, was purchased from New England Nuclear Corp.; crystalline RNase, lysozyme, and crystalline salmon sperm DNA were products of Worthington Biochemical Co.; phenol was freshly distilled before use and immediately saturated with TES buffer (0.05 *M* Tris-HCl, 0.015 *M* EDTA, 0.05 *M* NaCl, pH 8.0). Ethidium bromide was kindly provided by Boots Pure Drug Co., Ltd., Nottingham, England. Cesium chloride, technical grade, was purchased from Kawecki Chemical

Co., New York, N. Y., and optical grade CsCl was purchased from Harshaw Chemical Co. Sarkosyl NL30 was a product of Geigy Chemical Co.

Media and growth conditions: Cultures were grown overnight from single-colony isolates in Penassay broth (Difco) tubes at 37° and inoculated at a $^1/_{100}$ dilution into flasks containing prewarmed Spizizen's minimal salts medium[4] supplemented with 5 μg/ml L-tryptophan, 0.05% casein acid hydrolysate, 0.01% Bacto-tryptone, 0.5% dextrose, and 250 μg/ml deoxyadenosine. Growth on a rotating water bath shaker at 36° was followed on a Klett-Summerson colorimeter using a 66 filter. When the cells attained a density of 50–60 Klett units, H^3-thymidine was added to a final concentration of 1 mc/liter of culture. Growth was allowed to continue to a Klett value of 150–160 at which time the cells were chilled on ice, sedimented at 13,000 $\times$ g for 10 min at 0°, and washed twice with cold TES buffer. The generation time under these growth conditions was about 75 min.

Preparation of DNA: DNA extraction was performed essentially by the phenol technique described previously.[5] The concentration of the final DNA solution was estimated by the procedure of Burton[6] using crystalline salmon sperm DNA as the reference standard.

Preparative and analytical density gradient equilibrium centrifugation: Preparative density gradient equilibrium centrifugation was carried out on phenol-purified H^3-thymidine-labeled DNA preparations by dissolving the DNA samples in 11.2 ml of CsCl solution (final density of 1.70 gm/cm^3) and centrifuging in a Spinco Ti-50 fixed angle rotor in a Spinco model L-4 or L-2 centrifuge for 48 hr at 46,000 rpm and 15°. Dye-buoyant density equilibrium centrifugation was carried out according to the procedure of Radloff, Bauer, and Vinograd[7] as previously described.[8] Portions of each fraction obtained by collecting 25-drop samples from the bottom of the tubes were assayed for radioactivity as described by Hickson *et al.*[1]

Analytical equilibrium centrifugation in a cesium chloride gradient was performed by the method of Meselson *et al.*[9] as described by DeWitt and Helinski.[10] *Pseudomonas aeruginosa* N^{15} DNA (ρ of 1.742 gm/cc)[11] was used as reference DNA. The photographic plates were scanned on a Joyce-Loebl microdensitometer, and the band positions were calculated with relation to the *Pseudomonas* reference DNA to obtain buoyant density values for the *B. megaterium* DNA fractions.

Alkali denaturation and renaturation: Samples of phenol-purified DNA and DNA purified by cesium chloride-ethidium bromide gradient centrifugation were dissolved in 0.7 *N* NaCl, cooled to 4° in an ice-water bath, and adjusted to 0.11 *N* NaOH by the addition of 1 *N* NaOH. The mixture was gently swirled at 4° for 5 min and then neutralized by the addition of one-half volume of a mixture of 10 parts of 1 *M* Tris-Cl, pH 8.4, and 4 parts of 1 *N* HCl.

Sucrose gradient velocity centrifugation: Samples of DNA were layered on 5 to 20% sucrose gradients and centrifuged in a SW65 swinging-bucket rotor at 50,000 rpm for 90 min, timing from the beginning of acceleration until the time of deceleration. The rotor was allowed to stop without braking and the tubes were punched from the bottom with a hollow needle. Aliquots of 10 drops were collected directly on 1 inch squares of Whatman no. 1 filter paper. After drying under a heat lamp, the samples were counted in 10 ml of a PPO/POPOP/toluene scintillation mixture.

Electron microscopy: DNA samples were prepared for electron microscopy and analyzed as previously described.[5] In order to determine contour lengths of the circular molecules, the negative plates were projected onto sheets of white cardboard, the contours were traced, and the lengths determined with the use of a Keuffel and Esser map tracer calibrated in centimeters. The absolute lengths were calculated from the known magnification which was determined with a calibration grid.

Results.—Density gradient centrifugation with ethidium bromide: DNA isolated from either a megacin-producing strain of *B. megaterium* (216), or a megacin-sensitive strain (58), labeled in the mid-log phase of growth with H^3-thymidine, was centrifuged in a cesium chloride gradient containing ethidium bromide.

As shown in Figure 1, in addition to the main peak of DNA, a second, more dense, peak is observed having a density approximately 0.04 gm/cm^{3} greater than that of the bulk of the DNA. The relative amount of this heavier material under these growth conditions, as estimated from the total isotopic label in the two peaks, varied from about 15 per cent to over 40 per cent of the total DNA, depending on the growth conditions used. Pooling the fractions of the denser peak from the cesium chloride-ethidium bromide gradient and rerunning them in the same type of gradient yielded approximately equal amounts of DNA of the heavy and light buoyant densities, indicating that the more dense DNA material can convert spontaneously to the less dense form. If the same DNA preparations were centrifuged in a cesium chloride gradient without ethidium bromide, the total DNA is found in a single peak.

In two phenol-purified preparations of each of the two strains used, the amount of labeled DNA observed in the denser peak was 14 and 22 per cent for strain 58, and 29 and 22 per cent for strain 216. The relative distribution of label in the denser peak relative to the less dense peak was somewhat lower in the purified preparations than in crude lysates.

Analytical sedimentation equilibrium centrifugation: Analytical density gradient analyses of the total DNA from *B. megaterium* 58, and the isolated two bands of DNA from the preparative cesium chloride-ethidium bromide gradients are shown in Figure 2. The total DNA contained at least two major species of DNA exhibiting buoyant densities of 1.690 gm/cm^{3} and 1.693 gm/cm^{3} (Fig. 2*a*). In contrast, the DNA fractionated by ethidium bromide-CsCl gradient centrifu-

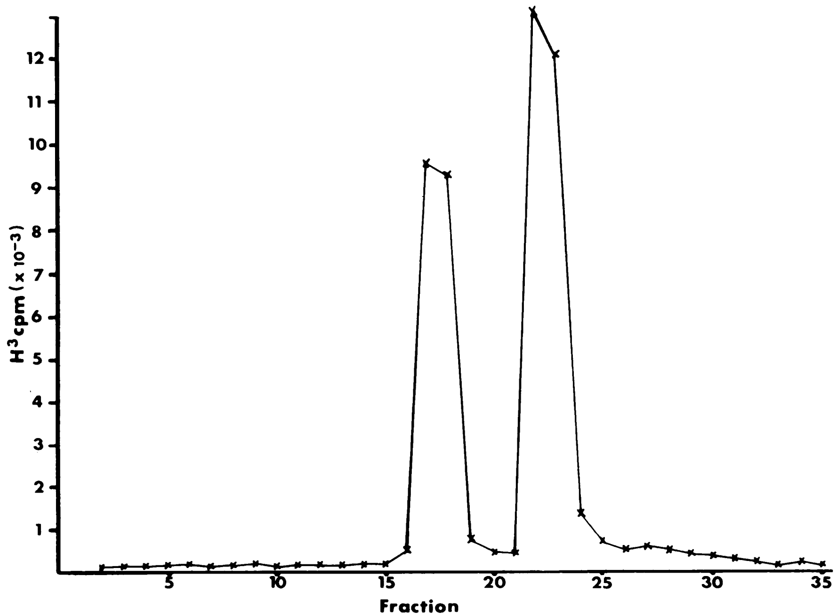

FIG. 1.—DNA elution profiles of preparative cesium chloride density gradients of total phenol-purified DNA from *B. megaterium* 216. Centrifugation was carried out in the presence of ethidium bromide (250 μg/ml).

gation appeared more homogeneous. The denser DNA from this gradient possessed a buoyant density of 1.690 gm/cm³ in a cesium chloride gradient in the absence of the dye, while the less dense band exhibited a buoyant density of 1.693 gm/cm³ (Figs. 2*c* and *e*).

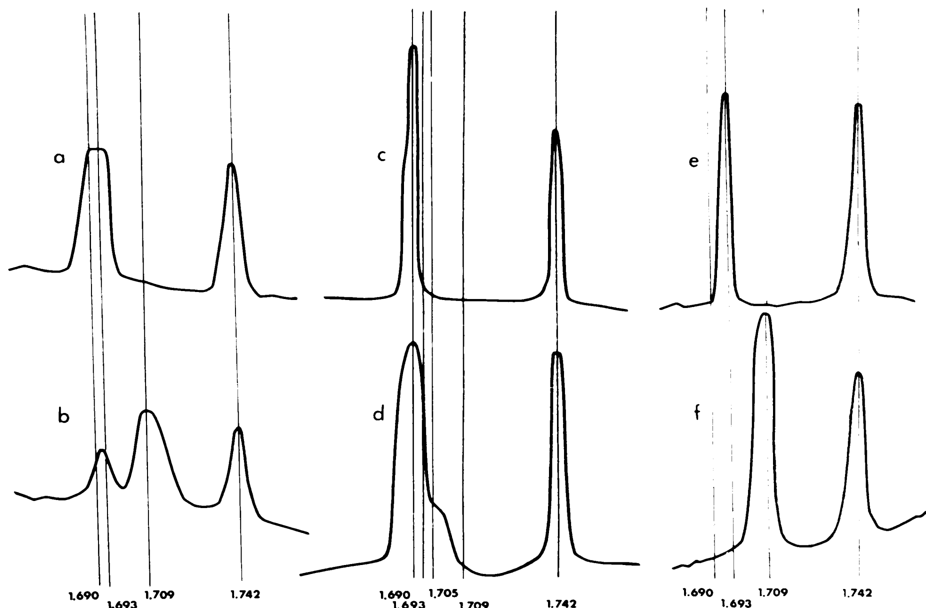

FIG. 2.—Analytical cesium chloride equilibrium density gradient analysis of *B. megaterium* 58 DNA preparations. Depicted here are the microdensitometer tracings of the films taken after 18-hr centrifugation at 44,000 rpm and 25°C.

Total, phenol purified: (*a*), untreated; (*b*) alkali denatured-renatured. Denser band isolated from dye-buoyant density gradient centrifugation; (*c*) untreated; (*d*) denatured-renatured. Isolated lighter band from dye-buoyant density gradient centrifugation; (*e*) untreated; (*f*) denatured-renatured.

Preparations of the samples and conditions of alkali denaturation and renaturation are described in *Materials and Methods*. Each gradient contained approximately 2 μg of sample DNA and 2 μg of N^{15}-*Pseudomonas* reference DNA (1.742 gm/cm³).

Alkali denaturation of the unfractionated DNA yielded some material banding approximately at the density position of native DNA (1.690 gm/cm³), while the rest banded at a buoyant density position corresponding to the denatured form of the unfractionated DNA (Fig. 2*b*). Alkali denaturation of the less-dense fraction of DNA from the dye-buoyant density centrifugation increased the buoyant density of this DNA by approximately 0.016 gm/cc with no native DNA regenerated following neutralization (Fig. 2*f*). The majority of the denser DNA obtained from the dye-buoyant density gradient resisted alkaline denaturation (Fig. 2*d*). Similar results were obtained for the unfractionated and fractionated DNA from strain 216.

Sedimentation velocity centrifugation: The sedimentation profiles of the two DNA fractions obtained by dye-buoyant density centrifugation of the DNA from the two *B. megaterium* strains were determined by centrifugation on neutral

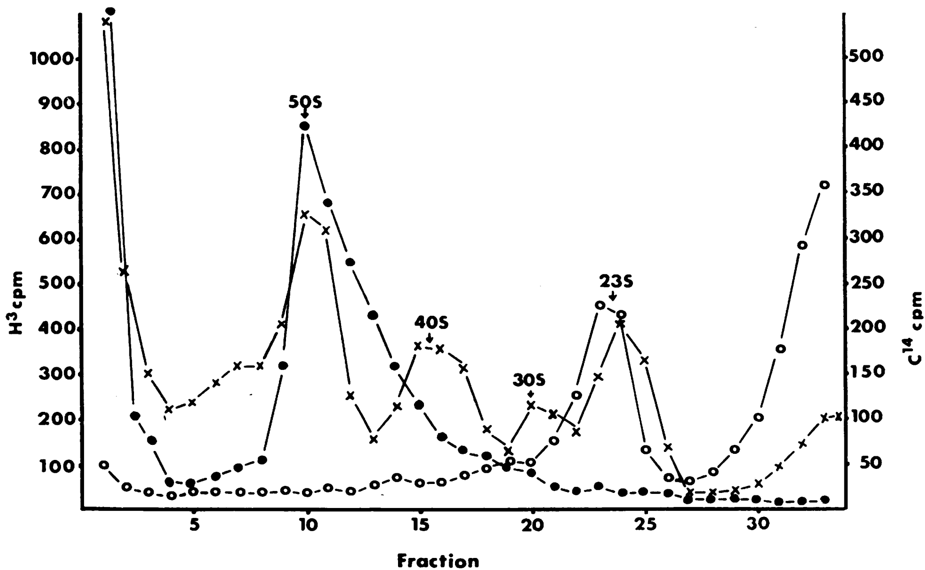

FIG. 3.—Neutral sucrose sedimentation velocity profiles of denser and lighter fractions of *B. megaterium* 58 DNA obtained by dye-buoyant density centrifugation. The denser and less dense fractions were run on different gradients but are presented here superimposed on the same graph. Details of centrifugation are described in the text. x-x-x, denser fraction; ●-●-●, less dense fraction; O-O-O, C^{14}-labeled *E. coli ColE*$_1$ DNA reference marker ($S^0_{20,w}$ of 23).

5 to 20 per cent sucrose gradients (Fig. 3). It is evident from these results that while the less dense DNA material obtained from the preparative CsCl-ethidium bromide gradients sediments with a rather uniform distribution at about 50*S*, the denser band of DNA sediments in a heterogeneous manner with major peaks at 23*S*, 30*S*, 40*S*, and 50*S*. Both fractions of DNA contained some material which sedimented to the bottom of the tube. Attempts to resolve this material by running the gradients for shorter times and at lower speeds were unsuccessful. The presence of broad peaks at each of the indicated *S* values for the more dense DNA material was interpreted as reflecting heterogeneity in the size of the circular DNA and the fact that the material added probably consisted of a mixture of native supercoiled DNA and open circular molecules that were formed during the centrifugation procedures.

Electron microscopy: Examination by electron microscopy of the DNA from both strains fractionated by the ethidium bromide-cesium chloride gradient centrifugation revealed that essentially all of the molecules of the denser fraction were supercoiled or open circular molecules, while the DNA from the less-dense fraction consisted of randomly coiled molecules, most of which had visible broken ends. The denser fraction of DNA contained a variety of different sizes of circular molecules, of which approximately one fourth to one third were open circular (Fig. 4*A*–*E*). The distributions of contour lengths of the open-circular forms are shown in Figure 5. The molecules ranged from about 0.8 μ to 34 μ, with the smaller size classes present in highest amount. The distribution of sizes was similar for both strains examined.

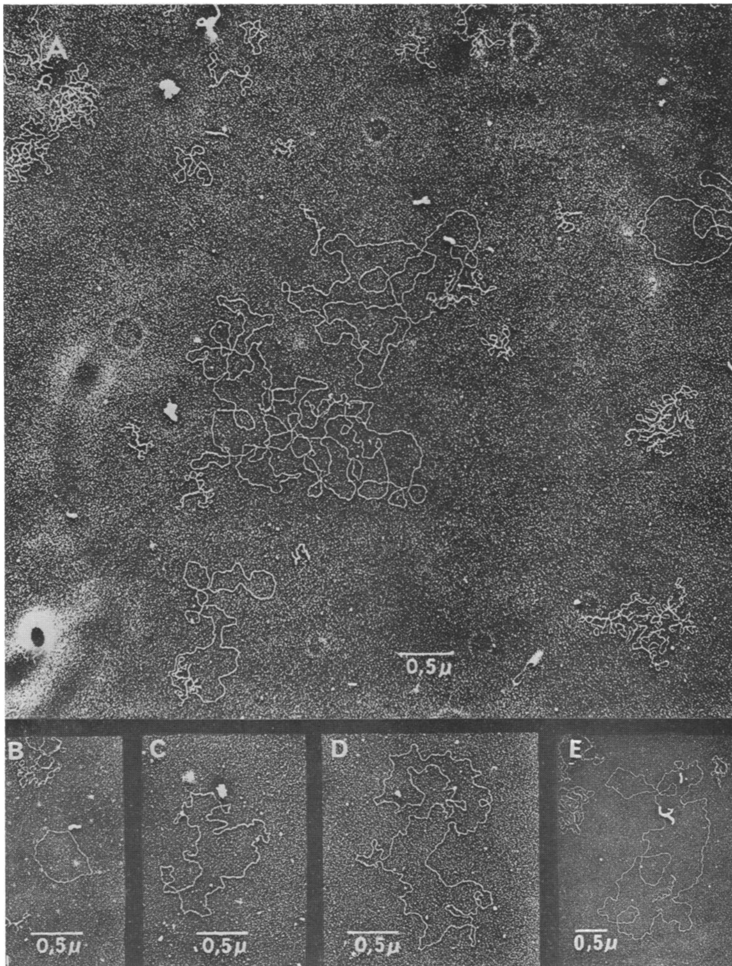

FIG. 4.—Electron micrographs of *B. megaterium* circular DNA molecules. (A) Typical distribution of supercoiled and open-circular molecules present in the denser band of a preparative cesium chloride-ethidium bromide gradient. (*B*), (*C*), (*D*), and (*E*) correspond to open circular molecules of contour lengths 1.9 μ, 7.8 μ, 11.7 μ, and 18.7 μ, respectively.

Discussion.—The finding of a rather considerable portion of the total cellular DNA of certain strains of *Bacillus megaterium*[12] in a form which sediments as a more dense band in a cesium chloride gradient containing the dye ethidium bromide was suggestive of the possible covalently closed circular nature of this material. The results presented here clearly indicate that this DNA is indeed circular, varying in length from approximately 1 μ to more than 30 μ. The total amount of supercoiled DNA in these strains is probably greater than indicated by the procedures used because of the high probability of inducing covalent breaks, particularly in the larger molecules, by the preparative procedure. In fact, when crude lysates were prepared directly in the tube used for the gradient

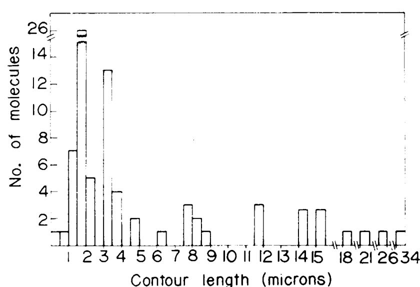

FIG. 5.—Contour length distributions of circular DNA molecules obtained from the denser band fractions after dye-buoyant density centrifugation of DNA preparations from *B. megaterium*.

equilibrium centrifugation by a rapid lysis procedure[13] that involved no pipetting, substantially more DNA was recovered in the supercoiled DNA band.

Although the molecules described here have essentially those properties of circular plasmid and episomal molecules that have been described previously, in some respects there are differences in properties and characteristics which are noteworthy. The analytical equilibrium centrifugation data presented in Figure 2 suggest that the circular material, which is more dense in a cesium chloride-ethidium bromide gradient, consists principally of material of a slightly lower buoyant density than that of the bulk of the chromosomal DNA. While the nonsupercoiled material from the gradient is completely and irreversibly denatured by alkali treatment, the band of supercoiled DNA is able to renature to a considerable extent upon neutralization. The observation that not all of the circular material is consistently renaturable to its native density following neutralization may be due to the fact that the several preparative steps to which the molecules have been subjected have led to covalent bond breaks in a variable portion of the molecules. Alternatively, due to the configurational constraints imposed by their covalent, double-stranded nature, some of the molecules may have resisted renaturation to the completely native configuration, as shown under certain conditions for the replicative form of ϕX174 DNA,[14] thus leading to a form with a buoyant density intermediate between the denatured and native states (see Fig. 2*d*).

The size heterogeneity of the circular DNA demonstrated by neutral sucrose gradient and electron microscopic analyses distinguishes this DNA from previously described bacterial plasmid and episome DNA. While multiple circular forms have recently been demonstrated for colicinogenic factors,[5, 15] mitochondrial DNA,[16] and ϕX174,[17] none of these systems shows the apparent widely dispersed size variability observed in the present studies. In addition, the apparent nonmultiple nature of the size distribution of the *B. megaterium* circular molecules observed here tends to rule out the origin of these forms as a result of nonreciprocal recombination between molecules of a basic unit form[18] or tandem linking of the basic units resulting possibly from errors in the replication mechanism.[15]

The physiological significance of these circular DNA molecules is presently obscure. Since both megacin-producing and megacin-sensitive strains, and even other bacilli, produce the circular material, the production of these elements does not appear to be directly related to megacin production. Furthermore, the

apparent absence of these elements from a number of other spore-forming *Bacillus* species[19] tends to argue against their implication in the specific control of sporulation processes in these organisms. Finally, although many bacilli harbor defective bacteriophages, including perhaps the strains used here, the absence of these circular elements in *B. subtilis* strains known to be carrying at least two such phages and the failure to induce circular elements in these strains by treatment with mitomycin C[19] suggests that the heterogeneous circular molecules are unrelated to this phenomenon. It is important to determine whether each of the different-sized elements observed represents a distinct element which replicates autonomously within a dividing cell, or whether they arise from the cell chromosome by detachment or by differential replication of limited regions of the chromosome. An understanding of the relationship of these elements to each other and to the remainder of the bacterial genome should give some insight to both the physiological role and origin of these elements. Experiments are currently in progress to examine these problems.

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† Visiting Research Associate, University of California, San Diego, 1968.

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