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Macromolecular crowding can account for RNase-sensitive constraint of bacterial nucleoid structure

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ABSTRACT

The shape and compaction of the bacterial nucleoid may affect the accessibility of genetic material to the transcriptional machinery in natural and synthetic systems. To investigate this phenomenon, the nature and contribution of RNA and protein to the compaction of nucleoids that had been gently released from *Escherichia coli* cells were investigated using fluorescent and transmission electron microscopy. We propose that the removal of RNA from the bacterial nucleoid affects nucleoid compaction by altering the branching density and molecular weight of the nucleoid. We show that a common detergent in nucleoid preparations, Brij 58, plays a previously unrecognized role as a macromolecular crowding agent. RNA-free nucleoids adopt a compact structure similar in size to exponential-phase nucleoids when the concentration of Brij 58 is increased, consistent with our hypothesis. We present evidence that control and protein-free nucleoids behave similarly in solutions containing a macromolecular crowding agent. These results show that the contribution to DNA compaction by nucleoid-associated proteins is small when compared to macromolecular crowding effects.

1. Introduction

In vivo, the bacterial nucleoid is compacted over one thousand fold during the log-growth phase [1], however the majority of the bacterial chromosome exists in a transcriptionally accessible state [2,3]. Given recent advances toward building synthetic genomes *de novo*, we aim to understand whether chromosomal compaction results from physical properties of the biomolecules involved, or if specific molecular mechanisms enable this accessibility and will therefore must be incorporated into synthetic genome design.

Several physical forces contribute to bacterial nucleoid compaction. Chromosomal DNA is negatively supercoiled through the action of several topoisomerases, and cellular polyamines such as spermidine and spermine are thought to participate in DNA compaction by partially neutralizing the charge of the DNA backbone [4]. *In vitro*, physiological levels of polyamines compact DNA several fold by inducing a first order random coil to globule transition for naked DNA chains [5–8]. Cytoplasmic macromolecular crowding also plays a role in chromosomal compaction. The concentration of macromolecules (proteins and RNA) in bacterial cytoplasm is approximately 340 mg/mL, producing significant compaction forces due to excluded volume effects [1,9–11]. Compaction of DNA by macromolecular crowding has been observed *in vitro* as a model for *in vivo* processes [7,12,13], but the extent of its contribution *in vivo* is still ambiguous.

In addition to physical forces, the nucleoid may be compacted by biochemical forces, namely RNA and nucleoid-associated proteins (NAPs). An RNA-based DNA compaction force has been observed for isolated nucleoids [1,14–16]; yet the nature of an RNA constraint remains controversial. The removal of RNA from nucleoid preparations causes nucleoid instability and decompaction regardless of whether the RNA is removed *in vivo* (rifampicin-treated cells) or following nucleoid release (RNase-treated nucleoids). However, the RNA molecule(s) responsible for nucleoid stabilization has not been identified. Similarly, several NAPs exist in bacteria, many of which can compact DNA *in vitro* [17]. However this function has been called into question. NAP-mediated DNA compaction *in vitro* requires protein levels that are several times higher than intracellular levels. In addition, genomic deletion of specific NAPs does not appear to alter global DNA structure [18]. Zimmerman has argued that NAPs may not compact DNA *in vivo*, since removing NAPs by washing has little effect on the size of isolated nucleoids [12].

Given recent advances toward the design and synthesis of a synthetic genome [19–23], we wanted to determine whether specific proteins or RNA molecules are required to maintain

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functional and accessible genomes in a simplified bacterial cell. We hypothesized that nucleoid compaction is mediated mainly by physical forces, specifically macromolecular crowding effects, and that specific RNA molecules and proteins are not necessary to achieve overall chromosomal compaction in bacteria. Here, we present evidence that previously observed RNA-based DNA compaction forces result from changes to the physical structure of the nucleoid, and that these changes may affect interactions between the nucleoid and surrounding polymers. Specifically, we present evidence that Brij 58, a detergent used extensively in *Escherichia coli* nucleoid preparations, also serves an unrecognized role as a macromolecular crowding agent in these preparations. The removal of RNA from the nucleoid either *in vivo* or following cell lysis resulted in reduced Brij 58-dependent nucleoid compaction. In addition, we present evidence that the contribution of nucleoid-associated proteins to nucleoid compaction is small when compared to macromolecular crowding effects, as native and protein-free nucleoids behaved similarly with respect to changes in macromolecular crowding.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Escherichia coli B/r A (ATCC 12407) was grown in C-medium (17.2 mM dibasic potassium phosphate, 11.0 mM monobasic potassium phosphate, 9.5 mM ammonium sulfate, 0.41 mM magnesium sulfate, 0.17 mM sodium chloride, 3.6 μM ferrous sulfate, 1.0 μM EDTA) containing 0.1% glucose [24] at 37 °C, shaking at 350 rpm to OD_{600} ~0.4. The doubling time for these cultures was 44 min. Where indicated, chloramphenicol or rifampicin was added to a final concentration of 30 μg/mL or 40 μg/mL, respectively, and cultures were incubated at 37 °C, 350 rpm for 30 min. Cells were harvested by centrifugation at 14,000*g*, 4 °C for 10 min.

2.2. Nucleoid preparations for fluorescence microscopy

Nucleoids for fluorescent microscopy were isolated using a modified version of the low-salt procedure developed by Murphy and Zimmerman [10]. Briefly, harvested cell pellets were resuspended in 250 μL Solution A (20% sucrose, 10 mM Tris–HCl, pH 8.1, 0.1 M sodium chloride) containing a 1/200 dilution of Picogreen (Invitrogen Co., Carlsbad, CA) and mixed briefly. After 2 min, 50 μL of Solution B (4 mg lysozyme per mL in 120 mM Tris–HCl, pH 8.1, 50 mM EDTA) were added and suspensions were held at room temperature for 1 min. Ten microliters of the suspension was diluted 10-fold into Solution D (7.1% sucrose, 14 mM Tris–HCl, pH 8.1, 36 mM sodium chloride, 10 mM EDTA, 5 mM spermidine, 5 mg Brij 58/mL, 0.22% sodium deoxycholate). The concentrations of Brij 58 and spermidine were varied as indicated. Cells were incubated at room temperature for >20 min to allow nucleoid release. The final nucleoid solution contained the same concentration of all components except Picogreen as the previously described low-salt procedure. Aliquots of each nucleoid suspension were viewed with an Olympus BX-51 fluorescent microscope and imaged using a Cooke SensiCam CCD camera. The images were analyzed with ImageJ version 1.38x software (NIH, Bethesda MD). The contrast of the images was adjusted to the same level and the area of each nucleoid was measured three times using the freehand selection tool. The average area was taken to be the nucleoid area.

2.3. Fragmented E. coli chromosomal DNA preparations

Escherichia coli B/r cell suspensions (10 mL, OD_{600} ~0.4) were centrifuged for 10 min at 14,000*g*, 4 °C. DNA was isolated from cell pellets using the FastDNA® Kit and the FastPrep® Instrument (Qbiogene, Inc., CA). The size of the fragmented DNA ranged from 5 to 6.5 kb as measured by agarose gel electrophoresis (data not shown). Fragmented DNA was diluted to 2.5 μg/mL in solutions containing a 1/200 dilution of Picogreen with Brij 58 and sodium chloride or spermidine. DNA compaction was observed by fluorescent microscopy as previously described.

2.4. Nucleoid isolation for electron microscopy

Nucleoids were isolated from harvested *E. coli* B/r A cells using a high- or low-salt procedure. The 'high-salt procedure' developed by Stonington and Pettijohn was employed with the following modifications [2]. Harvested cell pellets were resuspended in 250 μL Solution A and mixed briefly. After 2 min, 50 μL of Solution B was added and the samples were inverted to mix. After 1 min, 250 μL of Solution C (1% Brij 58, 0.4% sodium deoxycholate, 10 mM EDTA, pH 8.0, 2 M sodium chloride) was added and the samples were incubated at room temperature for 10–20 min, until the suspensions began to clear. Nucleoid suspensions were layered onto 10–30% sucrose continuous gradients in 10 mM Tris–HCl, pH 8.1, 1 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and centrifuged at 28,000*g*, 4 °C for 45 min in a Beckman SW41 rotor. Acceleration and deceleration were set to one. The 'low-salt procedure' was based on Kornberg et al.'s modification of the Stonington and Pettijohn procedure, where the 2 M NaCl in Solution C was replaced with 10 mM spermidine [25]. In addition, cell suspensions were incubated at 37 °C for 5 min to allow cell lysis. Nucleoid suspensions from low-salt preparations were loaded onto 15–30% sucrose continuous gradients in 20 mM sodium diethylmalonate, pH 7.1, 5 mM magnesium chloride, 1 mM β-mercaptoethanol. Gradients were centrifuged at 3000*g*, 4 °C for 35 min in a Beckman SW41 rotor. Acceleration and deceleration were set to one.

Gradients from both high- and low-salt preparations were fractionated into 0.3 mL aliquots. Picogreen was added to a portion of each fraction to a final dilution of 1/400, and the DNA concentration of each fraction was determined according to manufacturer's recommendations (Invitrogen Co., Carlsbad, CA).

2.5. Electron microscopy of isolated nucleoids

Nucleoids were attached to Butvar-coated nickel grids that were freshly coated with carbon by glow-discharge as described by Postow et al. [26]. Aliquots (8 μL) from the peak fraction were adsorbed to the grid for 2 min. Grids were rinsed for consecutive 1 min intervals in 0.1 M ammonium acetate, 0.01 M ammonium acetate, and 2% uranyl acetate. Grids were viewed using a Tecnai T12 transmission electron microscope (FEI Co., Hillsboro, Oregon), and imaged with an SIS Megaview III CCD camera. Images were analyzed with ImageJ software. The condensed area of each nucleoid was measured three times using the freehand selection tool, and averaged. Membrane fragments and single loops extending from the central body of the nucleoid were excluded.

3. Results

3.1. Brij 58 serves as macromolecular crowding agent in nucleoid preparations

Escherichia coli nucleoids were prepared using a low-salt procedure in which *E. coli* cells are sequentially exposed to a sucrose/sodium chloride solution to disrupt the outer membrane, lysozyme to degrade the peptidoglycan layer, and finally a stabilizing solution containing Brij 58, sodium deoxycholate, EDTA, and spermidine (final concentrations 1%, 0.4%, 10 mM and 10 mM, respec-