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Characterization of *Escherichia coli* nucleoids released by osmotic shock

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ABSTRACT

Nucleoids were isolated by osmotic shock from *Escherichia coli* spheroplasts at relatively low salt concentrations and in the absence of detergents. Sucrose-protected cells, made osmotically sensitive by growth in the presence of ampicillin or by digestion with low lysozyme concentrations (50–5 μg/ml), were shocked by 100-fold dilution of the sucrose buffer. Liberated nucleoids stained with 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI), the dimeric cyanine dye TOTO-1, or fluorescent DNA-binding protein appeared as cloud-like structures, in the absence of phase contrast. Because UV-irradiation disrupted the DAPI-stained nucleoids within 5–10 s, they were imaged by time-lapse microscopy with exposure times less than 2 s. The volume of nucleoids isolated from ampicillin- or low-lysozyme spheroplasts and minimally exposed to UV (<2 s) was on average ~42 μm^3. Lysozyme at concentrations above 1 μg/ml in the lysate compacted the nucleoids. Treatment with protease E or K (20–200 μg/ml) and sodium dodecyl sulfate (SDS; 0.001–0.01%) caused a twofold volume increase and showed a granular nucleoid at the earliest UV-exposure; the expansion could be reversed with 50 μM ethidium bromide, but not with chloroquine. While DNase (1 μg/ml) caused a rapid disruption of the nucleoids, RNase (0.1–400 μg/ml) had no effect. DAPI-stained nucleoids treated with protease, SDS or DNase consisted of granular substructures at the earliest exposure similar to UV-disrupted nucleoids obtained after prolonged (>4 s) UV irradiation. We interpret the measured volume in terms of a physical model of the nucleoid viewed as a branched DNA supercoil crosslinked by adhering proteins into a homogeneous network.

1. Introduction

The bacterial nucleoid has been studied extensively both *in situ* and as an isolated structure. Electron microscope observations of thin sections showed a meshwork of aggregated DNA fibers in well-delineated or dispersed regions in which the DNA must have suffered structural changes due to fixation and dehydration (for reviews see Robinow and Kellenberger, 1994; Woldringh and Nanninga, 1985). Additional information was obtained from isolated nucleoids prepared by the so-called cytochrome-*c*-spreading technique, showing for the first time supercoiled, branched DNA loops radiating out of a "spider-like" core (Delius and Worcel, 1974; Kavenoff and Bowen, 1976; Meijer et al., 1976). These structures have frequently been interpreted as resulting from protein and RNA cross-links and have led to the so-called "rosette" model for a folded chromosome (see for review Toro and Shapiro, 2010). The folds or domains are considered to represent independent supercoiled loops, but the nature of possible crosslinks that form the supercoiling barriers and that determine the size of isolated nucleoids has remained obscure.

When analyzing the various procedures historically used to release the bacterial nucleoid, we may discern two agents that seem to have been important in determining its size and structure: (i) the presence of detergents and (ii) the concentration of lysozyme in protocols without detergent. Since the introduction of the first protocol by Stonington and Pettijohn (1971), detergents (non-ionic Brij-58 and anionic deoxycholate) at a high salt concentration (1 M NaCl) or spermidine have been used in most studies (Worcel and Burgi, 1972, 1974; Kornberg et al., 1974; Drlica and Worcel, 1975; Meijer et al., 1976; Materman and van Gool, 1978; Murphy and Zimmerman, 2000; Foley et al., 2010). Depending on the lysis temperature, the detergent-salt method produced membrane-attached (at 10 °C) or membrane-free (at 25 °C) "particles" as analyzed by sedimentation through sucrose gradients (Worcel and Burgi, 1974) or by "visualization" with the electron microscope (Delius and Worcel, 1974). The results supported the interpretation that the released nucleoids were intact and supercoiled as

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addition of ethidium bromide caused them to relax at a concentration of 2 μg/ml or re-compact at higher concentrations (>4 μg/ml; Worcel and Burgi, 1972). However, the hydrodynamic properties determined from sedimentation in sucrose gradients showed large variations (Hecht et al., 1977; Odijk, 2002). In part, these could be caused by entanglement with envelope fragments (Meijer et al., 1976; Materman and van Gool, 1978). The theoretical analysis (Odijk, 2002) of the sedimentation data of nucleoids obtained by the detergent method (Hecht et al., 1977) proves that their dimensions are typically of the order of 0.5 μm (see also Hecht et al., 1975). This is much smaller than the nucleoids obtained by the osmotic shock method (Cunha et al., 2001b; this work). The implication is that we have to be careful in interpreting the response to enzymatic treatments in the respective isolation methods.

Nucleoids released by the detergent method were found to be sensitive to treatment with RNase (Worcel and Burgi, 1972; Pettijohn and Hecht, 1973) and proteases (Drlica and Worcel, 1975) possibly indicating the existence of crosslinks. Moreover, detergents can be expected to dissociate proteins from the DNA and they could therefore influence subsequent structural transitions upon enzyme treatments. To circumvent such problems, Sloof et al. (1983) introduced an osmotic shock procedure without detergents using protoplasts of *Bacillus licheniformis*. In a previous study (Cunha et al. (2001a) we compared this method with the lysis with detergents using the same *Escherichia coli* spheroplast suspension. We observed that both procedures resulted in comparable sizes of the isolated nucleoids visualized by fluorescence microscopy, in spite of the large difference in salt concentrations (1 M NaCl in the detergent method *versus* 10 mM NaCl in the osmotic shock method).

In subsequent studies (Cunha et al., 2001b) we emphasized the osmotic shock method and we determined the compaction of isolated nucleoids by polyethyleneglycol (PEG) and measured the dynamics of fluorescently labeled DNA regions in the isolated structures (Cunha et al., 2005). We found that when released by osmotic shock, the nucleoids expanded like a spring into cloud-like structures with an average volume of about 30 μm^3. Romantsov et al. (2007) obtained nucleoids by osmotic shock in the shape of rounded, delineated structures with a volume of about 18 μm^3. These nucleoid volumes are well below the theoretical value of *un-crosslinked* nucleoids expanded by excluded volume effects and estimated to be about ten times larger (Cunha et al., 2001b). What causes this variation in size of isolated nucleoids and what prevents their full expansion?

As lysozyme is known to be a basic protein (Kuehner et al., 1999) it could well bind to DNA causing nucleoid compaction. In this work we have therefore refined previous protocols and we have prepared spheroplasts either in the absence of lysozyme, though by growing cells in the presence of ampicillin, or by using much lower concentrations of lysozyme (as low as 0.5–0.05 μg/ml in the lysate). The goal of nucleoid isolation is to prepare nucleoids that are expanded versions of their counterparts *in vivo*. Ideally, they should not be perturbed by the compounds used in the isolation protocol. We hope that our new protocols are a further step in realizing this ideal.

The present nucleoids obtained by osmotic shock and stained with DAPI, appear to expand faster than previously described (Cunha et al., 2001a,b). We have therefore measured their sizes from images taken by time-lapse microscopy within 2 s of UV-exposure. The cloudlike structures appear to be larger (42 μm^3) and to fall apart fast during UV-irradiation as a network of granules with what appears to be Brownian motion superposed. A similar expansion of granular substructures was observed after treatment with protease, sodium dodecyl sulfate (SDS) or DNase. In contrast to studies using the detergent method (Worcel and Burgi, 1972), RNase appeared to have no effect. We show that our current method of preparing and imaging low-lysozyme or lysozyme-free nucleoids is particularly suitable for establishing their dimensions. These sizes will be discussed in terms of a physical model in which the nucleoid is considered to be a branched DNA supercoil crosslinked by adhering proteins into a homogeneous network (see Fig. 5 and Appendix B for a description of this model).

2. Materials and methods

2.1. Strains and growth conditions

Strains *E. coli* K-12 MC4100 (laboratory strain LMC500) and *E. coli* 500/pSACT11 were grown at 28 °C in glucose minimal medium containing 6.33 g of $K_2HPO_4.3H_2O$, 2.95 g of KH_2PO_4, 1.05 g of $(NH_4)_2SO_4$, 0.10 g of $MgSO_4.7H_2O$, 0.28 mg of $FeSO_4.7H_2O$, 7.1 mg of $Ca(NO_3)_2.4H_2O$, 4 mg of thiamine, 4 g of glucose and 50 mg of lysine, per liter pH 7.0 at 28 °C (doubling time at 28 °C was about 85 min). NaCl was added to adjust the osmolarity of the medium to 300 mosM (Micro-Osmometer, Advanced Instruments). Cell growth was monitored at 450 nm with a spectrophotometer. Exponential growth was maintained by periodical dilutions of the culture. For *E. coli* LMC500, we estimate the number of chromosome equivalents per nucleoid to be about 1.58 (see e.g. Huls et al., 1999). *E. coli* 500/pSACT11, grown in the presence of 100 μg/ml ampicillin, was used so as to visualize nucleoids with the fluorescent fusion protein HupA-mRFP (red fluorescent protein) after induction with 0.3 mM IPTG. Strain *E. coli* K-12 FH2973/pFHC2973 (obtained from F. Hansen, Biocentrum DTU, Denmark. See Nielsen et al., 2006) was grown in the same medium with 0.5% glycerol as carbon source and supplemented with 100 μg/ml ampicillin (doubling time at 28 °C was about 120 min). When 0.58 M sucrose was added to *E. coli* LMC500 growing in glucose-minimal medium (see below) the doubling time at 28 °C increased to ~120 min. When indicated (as TY) the glucose minimal medium was supplemented with 10 g of bactotryptone, 5 g of yeast extract, 5 g of NaCl and 15 mmol NaOH per liter giving a doubling time at 28 °C of about 60 min.

2.2. Plasmid construction

A HupA-mRFP protein fusion was constructed using standard laboratory techniques (Miller, 1992). mRFP from pGEM-XbBg-mRFPsv (Alexeeva et al., 2010) was used to create a mRFP fusion to the C-terminus of HupA, amplified from chromosomal *E. coli* MC4100 DNA. See Supplementary data (S2.1) for details.

2.3. Chemicals

DAPI was obtained from Sigma and was prepared by dissolving 500 μg/ml in distilled water with the help of sonication. TOTO-1 (dimeric cyanine dye) and FM4-64 were obtained from Molecular Probes (Invitrogen), sodium dodecyl sulfate from Koch-Light Laboratories and glutaraldehyde from BDH (UK). RNase A was purchased from Sigma, bovine serum albumin (BSA) and protease E from Calbiochem, protease K from Invitrogen, pancreatic Dnase I from Roche Diagnostics.

2.4. Preparation of lysozyme-spheroplasts

Except for the application of a cold-shock (4 °C) to the cells before lysozyme addition, the protocol was that described essentially by Cunha et al. (2005). In short: 2 ml of *E. coli* cells grown in glucose minimal medium (Gmin) to an OD_{450} of 0.2 (~5×10^7 cells per ml) were centrifuged in an Eppendorf centrifuge for 5 min at 10,000 rpm. The cell pellets were resuspended in 475 μl of