

## NOTE

# Fluorescence Microscopy of Condensed DNA Conformations of Bacterial Cells

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Cellular DNA in prokaryotes is organized in nucleic acid-protein self-assemblies referred to as the nucleoid. The physical forces responsible for its stability inside the poor solvent properties of the cytoplasm and their functional implications are not understood. Studies on the organisation and functioning of the cytosol of cells largely rely on experimental protocols performed in highly dilute solutions using biochemically purified molecules, which is not a reliable substitute for the situation existing *in vivo*. Our current research interest is focused on the characterization of biological and physical forces determining the compaction and phase separation of DNA in *Escherichia coli* cytoplasm. We have emphasized the effect of excluded volume in solutions with high macromolecular concentrations (macromolecular crowding) upon self-association patterns of reactions. The prokaryotic cytosol was simulated by addition of inert polymer polyethylene glycol (PEG) (average molecular weight 20000), as an agent which afterwards facilitates the self-association of macromolecules. Fluorescence microscopy was used for direct visualization of nucleoids in intact cells, after staining with DAPI (4',6-diamidino-2-phenylindole dihydrochloride). Addition of the crowding agent PEG 20,000, in increasing concentrations generated progressively enhanced nucleoid compaction, the effect being stronger in the presence of 0.2 M NaCl and 5 mM MgCl<sub>2</sub>. Under these conditions, the nucleoids were compacted to volumes of around 2  $\mu\text{m}^3$  or comparable sizes with that of living cells.

**Key words:** *Escherichia coli*, DNA condensation, macromolecular crowding, phase separation, fluorescence microscopy.

Nucleic acid compaction in small spatially restricted compartments and how it governs the gene expression level has always been regarded as a crucial meeting point between biophysical chemistry and microbiology. Of particular interest is bacterial genomic DNA, the structure-function relationships of which are still being presented differently by various research groups. The *E. coli* chromosome, also referred to as the nucleoid (reviewed by Pettijohn, 1996), is a polyelectrolyte with a molar mass of around  $2.8 \times 10^9$ , which is packed, or condensed inside the inner volume of the bacterial cell. Low resolution of the optical microscopy led researchers to rely on electron microscopy, which helped to unravel further details of nucleoid structure. However, fixation artifacts during sample preparation of the latter method are well-known (Bendich, 2001). As a result, the explanation of how the overall

morphology and structural transitions of the nucleoid determine metabolic activity is still under debate. The statement of electron microscopists (Pettijohn, 1996), that the nucleoid region is restricted to only a ribosome-free region, is open to doubt because of the belief that the DNA in growing bacterial cells is in a dynamic state, part of which exceeds the bulk of the nucleoid, to be further transcribed in the cytoplasm. The proposal of phase separation of macromolecular assemblages within cytoplasm (Pogliaro, 2000), appears to be an alternative cytoplasmic organization to the historically very attractive model of specific association of nucleoid to bacterial membrane (Funnell, 1996). However, there is no direct proof of the existence of phase separation within cytoplasm. Even if it exists, the question of whether it is inevitable remains speculative. More detailed studies of this level of cellular organization are difficult to perform because the latter is below the resolution limit of conventional microscopy. Despite these obstacles, chemical approaches suggest that

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phase separation may occur in bacteria, supported also by theoretical considerations (Odijk, 1998). To understand the nature of this phase separation within the cytoplasm, the work presented herein concentrates on weak interactions, coupled with macromolecular crowding, thus establishing the non-membrane bound cytoplasmic organization of DNA. The aim was to study certain bio-inorganic aspects of either intramolecular (collapse) or intermolecular condensation (aggregation) of DNA to form conformations of a certain size. Divalent cation-induced compaction of isolated *E. coli* nucleoids is presented, in the context of recent direct evidence of cation effects on the structural integrity of chromosomes (Strick *et al.*, 2001). An attempt is made to relate these ion effects on formation of optimum size of DNA condensates. In principle, direct observation with a fluorescent microscope gives information on nucleoid conformation. It is shown here that microscopic images correlate with well-established physicochemical relationships between compaction state, cation concentration and cation valency, thus indicating an electrostatic mechanism of *in vivo* DNA compaction. Understanding of nucleic acid compaction is important for non-viral gene delivery vector design. Methodological limitations of studies on bacterial DNA compaction determine its isolation as a crucial point for development of further assays, depending on the final objective. Most of the applied procedures for isolation of the bacterial nucleoids have employed harsh chemical treatments after initial disruption of cells. Alternatively, invasive physical treatments to isolate nucleoids were applied. The present work is an attempt to consider polymer-induced aqueous phase separation as a subcellular model for the liquid phase of *E. coli* cytoplasm, applying direct visualization of intact bacterial nucleoids, as well as their release patterns after gentle osmotic lysis.

## Materials and Methods

### Bacterial strain and growth conditions

A laboratory stock of *E. coli* strain PJ4271 was cultivated at 37°C with shaking in 300mOsm glucose minimal medium, containing ampicillin (200 µg/ml). The osmolarities of the growth media and osmotic shock solutions were determined by vapor pressure osmometer and further necessary adjustments were done with 2 M NaCl. A few colonies from agar plates were inoculated into 3 ml fresh pre-warmed glucose minimal medium. The use of Casamino acids was avoided, because of their property of creating high background fluorescence for microscopic observation (Shellman and Pettijohn, 1991). Growth was followed by turbidity measurements with the spectrophotometer at 600 nm. At an optical density of 0.3, which corresponds to the early exponential phase, the culture was diluted 150 times and poured into fresh media. The

growth of the strain was continued at 37°C in a thermostatically controlled water bath with a constant shaking speed of 250 r.p.m. Cell doubling time under these growth conditions was 60 min. Exponential phase cells were used in all experiments, since the gross morphology of the nucleoids is growth phase-dependent (Azam *et al.*, 1999). A coulter counter (Coulter Electronics Ltd., UK) was used to characterize the bacterial metabolic state, as described (Kubitschek and Friske, 1986). Achievement of constant average cell mass was used as a criteria for reaching the steady state. Bacterial growth was documented by measuring the optical density (OD<sub>600</sub>) of the turbid suspension and following the changes of cell concentration for a number of generations. The kinetics of reaching constancy of average cell mass distributions was followed by plotting OD×10<sup>9</sup> cells/ml vs time, until a horizontal line was reached.

### Nucleoid isolation

10 ml (or approximately final density of 2.6×10<sup>9</sup> cells/ml) of exponentially growing cells was centrifuged at 5 000 × g (at 0°C). The obtained cell pellet was resuspended in 1 ml of lysis buffer, containing 0.4 µg/ml egg lysozyme (E. C.3.2.1.17), 10×PBS; pH=7.2, 0.25 M EDTA, 0.8 M sucrose. The turbid suspension was vortexed briefly and the spheroplast suspension was left for 5 min at room temperature and re-chilled. Osmotic shock was performed by 1:100 dilution of spheroplasts in water. Besides avoiding possible artifacts of chemical and physical methods, the osmotic stress technique is suitable for evaluation of thermodynamic parameters as a function of the applied osmotic stress, as described previously for *E. coli* plasmids (Reich *et al.*, 1995). Since fluorescent dye was used, because it only very slightly affects cell growth (Shellman and Pettijohn, 1991), nucleoids were visualized by adding 1µg/ml of DAPI (4',6-diamidino-2-phenylindole dihydrochloride) during either bacterial growth, or after rupturing spheroplasts prior to microscopic examination.

### DNA compaction assay

The macromolecularly crowded *E. coli* cytoplasm (Johansson *et al.*, 2000) was modelled by adding increasing amounts of volume-occupying polymer polyethylene glycol (PEG) from 2% up to 14% (vol/vol) of known osmotic pressures, dissolved in PBS or PBS+5 mM MgCl<sub>2</sub>, where specified. The water-salt solutions of PEG were prepared by dissolving the required amounts of crystalline PEG 20,000 in NaCl containing a solution of the required concentration. Under these conditions, PEG is mostly excluded from the DNA phase (Lorman *et al.*, 2001). The estimated pressure created by PEG 20,000 is 5.6×10<sup>4</sup>–1.6×10<sup>6</sup> dyn/cm<sup>2</sup> (see <http://aqueous.labs.brocku.ca/data/> and Raspaud *et al.*, 2000). The relation between the osmotic pressure created by PEG molecules and the density of the stressed DNA phase is well established (Strey *et al.*, 2000). After equil-

ibration at room temperature (26°C-28°C) the PEG solutions were used without further treatment.

### **Fluorescence microscopy and image analysis**

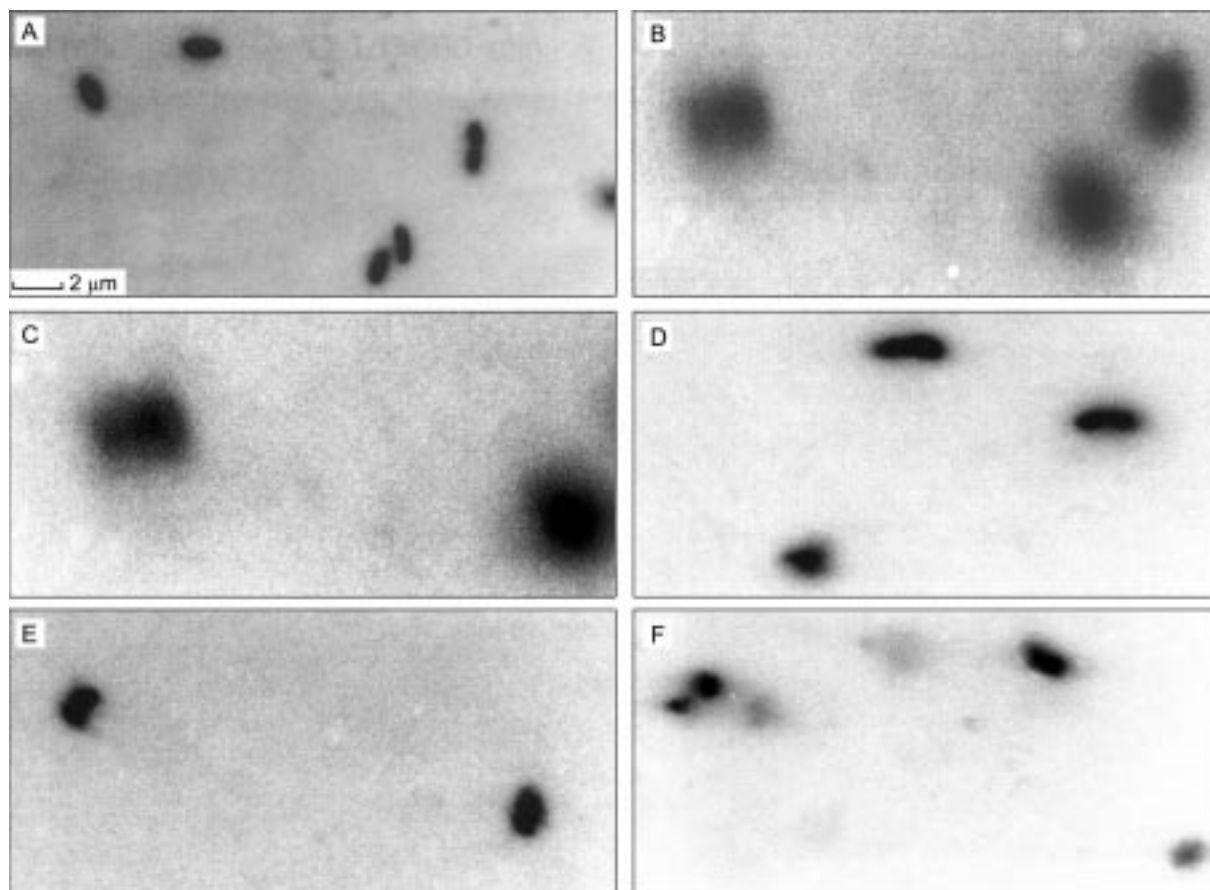
Microscopy object slides were flamed and kept in humidified Petri dishes. Under these conditions most of the non-permeabilized cells do not bind (Shellman and Pettijohn, 1991). A 4 µl sample of cells was poured on object slides pre-treated with 80 µl of a cooled layer of 1% agarose in water. The agarose was dissolved by boiling in a microwave oven and kept warm. Low melting point, low-gelling point (hydroxymethylated) agarose preparations from FMC BioProducts (Rockland, ME) were used. An ice-cold cover glass was placed on the specimen, which afterwards was kept cold until gelation finished. Chilled slides were warmed to room temperature during microscopic observation. However, in this procedure, first the agarose was melted and spread on the glass surface and beneath the coverglass of the object slides, after which the agarose solidifies, thus creating certain shear force-induced degradation of the sample (see Bendich, 2001). Therefore, parallel experiments were run with a nail polisher for sealing the coverslip, instead of agar. This treatment stops the movement of the nucleoids for further quantification. The cells and, subsequently the spheroplasts, were visualized with an Olympus BH-2 fluorescence microscope equipped with 100x oil-immersion objective of the Princeton<sup>TM</sup> charge-coupled device (CCD) camera. Fluorescence images were obtained by a fluorescence (DAPI) filter of the microscope, after irradiation of the fluorochrome with an Hg-lamp. Since this treatment rapidly kills the cells, images were taken following a very short exposure time of a couple of seconds. The images of the released nucleoids after osmotic shock were further digitalized by using computerized image quantification software Object Image<sup>TM</sup> (<http://simon.bio.uva.nl/object-image.html>). Determination of the number of nucleoids per cell was done manually employing the length measurement capabilities of the software package. The quantified images were transferred to Adobe Photoshop 5.0<sup>TM</sup> or Canvas<sup>TM</sup> and printed on a high quality dye printer.

## **Results and Discussion**

Inside the living bacterial cells, the nucleoid can be visualized by light microscopy, when cells are placed in an adjusted medium, so that differences in the refractive index of the cytoplasm compared to that of the nuclear body could be used. *E. coli* nucleoids are observed with fluorescence microscopy, or alternatively by cryofixation and freeze-substitution modes of electron microscopy. Fluorescence microscopy offers an advantage over autoradiography and electron microscopy, since observation of DNA during structural perturbations is possible (Ben-

dich, 2001). Much better images are obtained, however, by employment of confocal laser scanning microscopy (our unpublished data), which permits working in a non-invasive manner by optical sectioning, thus dissecting the three-dimensional cell architecture.

In the present study, quantitative fluorescence microscopy was used to visualize nucleoids in intact cells following gentle osmotic rupture of the bacterial spheroplasts. The final aim was to use the isolated nucleoids for various physicochemical DNA condensation assays, trying to relate the observed patterns to certain biological functions of prokaryotic genome compaction. Traditional assumptions of laboratory biochemistry, valid for dilute aqueous solutions, cannot be adapted directly to intact cell cytoplasm. The living bacterial cell interior is characterized by limited reaction space, where volume exclusion (macromolecular crowding) of ions and biopolymers behave in highly heterogeneous and a non-ideal manner (Kuthan, 2001; Ellis, 2001). A molecular crowding reagent, PEG 20,000 was used to define the consequences of perturbing the macromolecular interactions within the *E. coli* cytosol. This reagent is preferred compared with a wide variety of readily available macromolecular crowding agents (Harris, 1992). Polyethylene glycol(s) are easily soluble both in organic media and in water. In addition, their commercial availability in a variety of molecular weights, uncharged molecular structure and low viscosity, as well as well-understood physicochemical properties, in terms of known osmotic pressure values, makes them widely employed reagents. The concept of macromolecular crowding is based on the thermodynamically non-ideal behaviour of proteins (Minton, 2001), which is expressed by their virial coefficients (Behlke and Ristau, 2000). The origin of osmotic stress, created by different solutes is a result of the size exclusion effect. The concentration of proteins in *E. coli* cytosol is involved in volume exclusion, a property which is replaced here by the excluded volume effect of PEG 20,000 molecules, thus relating macromolecular crowding to regulation of cell volume. Therefore, the so-created osmotic pressure is sensed in a manner apparently different from the well-known hydrodynamic perspectives. The physical and biological forces responsible for DNA compaction within living bacterial cells were approached by employing an osmotic shock procedure, avoiding the destructive physical disruption of bacteria as well as ionic and non-ionic detergents in the lysis step. The latter was commonly used throughout the nucleoid isolation protocols until recently and gave rise to sample preparation artifacts. Moreover, nucleic acid-protein dissociations were limited by omitting detergents and performing the lysis at low ionic strength. Parallel experiments were carried out under high ionic strength conditions for comparison of patterns of nucleoid release from osmotically ruptured *E. coli* spheroplasts (data not shown). The EDTA-lysozyme-sucrose procedure used

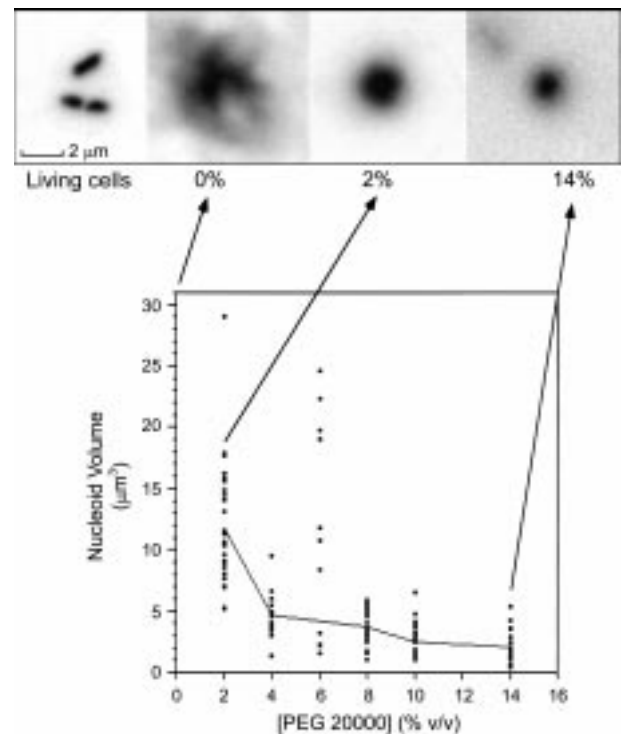


**Fig. 1.** Fluorescence microscopy (DAPI filter) of nucleoids in intact bacterial cells (A) and released following osmotic shock (B through F). Early stationary phase cells of *E. coli* PJ4271 were diluted 150-fold in fresh pre-warmed glucose minimal medium, and stained with DAPI for following the cell morphology, as described in Materials and Methods. Total number of cells per volume and the average cell volumes were measured in 100  $\mu$ l sample holder, with a Coulter Counter, equipped with 30  $\mu$ m orifice. The specimens are adsorbed on glass surface. (B): Osmotically released nucleoid in water; (C): Bacterial nucleoid in water and 0.2 mol/l NaCl. Concentrations of crowding agent used are: (D): 2% PEG; (E): 5% PEG and (F): 10% PEG, respectively. Free-moving nucleoids, prepared in agar layer of 30  $\mu$ m thickness possess volumes of about 12  $\mu$ m<sup>3</sup>, the nucleoids being collapsed upon attachment to the glass surface. The color of fluorescence images is inverted. The reproducibility of measurements is  $\pm 0.05$   $\mu$ m (96% confidence limits).

here brings about roughly 80% efficiency in obtaining spheroplasts. Changes of morphology of cells during various growth periods were followed by the phase-contrast mode of the light microscope before proceeding further with fluorescence observations. Exponentially grown bacterial cells (Fig. 1A) were subjected to osmotic lysis, to release the cytoplasmic constituents. The nucleoids were visualized with DAPI, followed by the compaction assay, as described in Materials and Methods. Fig. 1B shows the osmotic shock-induced release pattern of nucleoids in water. DNA compaction is a well-known salt-dependent reaction, as shown in Fig. 1C, where the effect of salt addition to water is shown. Both Fig. 1B and 1C show results of experiments performed in the absence of osmotic stress. DNA in water-salt solution (Fig. 1C) behaves as a highly rigid wormlike coil with a little tendency for interaction between distantly located portions of the duplex DNA chain, without any substantial tertiary

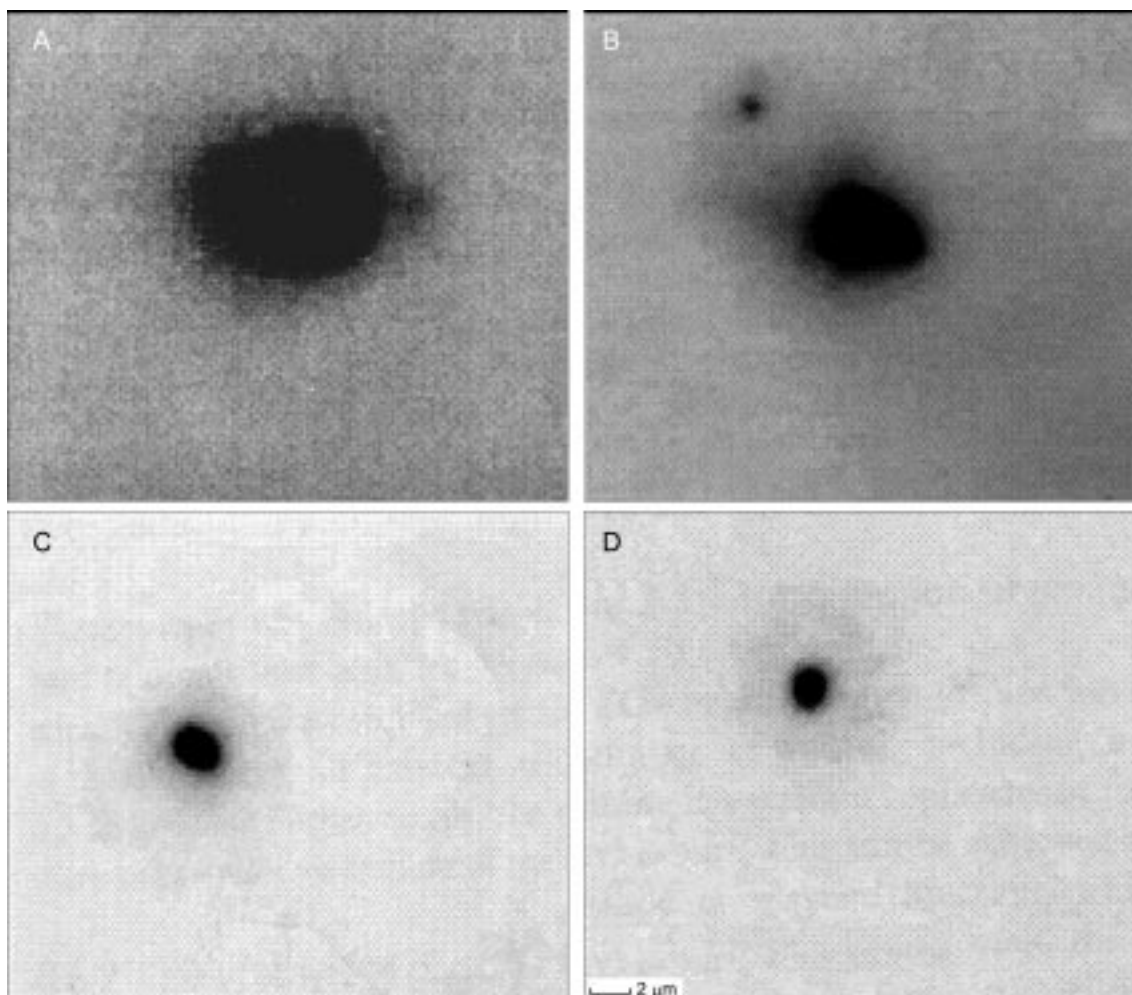
structure. Wide conformational changes are observed, however, after addition of certain polymers, such as polyethylene glycol, also used in this study (Fig. 1D-F). Compaction is an intramolecular event, in which PEG competes with DNA for water molecules. This is essentially a steric effect, by which reactions proceed with changes of the amount of sequestered water in spacially inaccessible cavities. The addition of PEG to a water-salt solution of DNA gives rise to a decrease in rigidity of duplex DNA thus leading to a conformational transition of the latter, referred to as compaction. The PEG-collapsed DNA state is caused mainly by repulsive DNA-polymer interactions, in which state the salt neutralizes repulsive DNA-DNA effects (Bloomfield *et al.*, 2000). The phenomenon of DNA compaction is determined by excluded-volume effects. The effect of this volume exclusion is the creation of depletion forces, which brings together the solute-excluding regions. Such a solution

separates into two non-mixable phases and the DNA state presented here is one of these separate phases. DNA molecule forms individually compacted states. The volume exclusion effect is shown in Figs. 1D through 1F, where the created high osmotic pressures remove the sequestered water from the exposed surfaces. For brevity, only some of the varying PEG concentrations are shown. More informative is Fig. 2, which shows the influence of more PEG concentrations on nucleoid size. Each dot represents a separate experiment. The enhancement of compaction of nucleoids with increasing the concentration of the crowding agent PEG 20,000 is shown. The main property of a crowding exclusion is dependence of the excluded volume on solute size, rather than on chemical nature and thus solute-solute interactions are neglected. Hence, the Gibbs-Duhem approach is applied in terms of included water (Parsegian *et al.*, 2000; Rand *et al.*, 2000). Therefore, due to its dependence on solute size, our approach employed PEG polymer, instead of other available crowding agents, due to its well-understood physicochemical mode of action. This  $\Psi$  form of DNA can be envisaged as a very primitive model of native compaction processes, e.g. bacteriophage head self-assembly, bacterial nucleoid phase separation, or condensed eukaryotic chromatin. The observed sedimentation behaviour of phages, prokaryotic nucleic acids, and finally the condensed chromatin found in metaphase chromosomes, shares similarities with this form of DNA, demonstrating its value as a reliable model of all these condensation processes. Electron microscopy confirmed previously this resemblance (Evdokimov *et al.*, 1972). In addition, diffusion coefficients ( $D$ ), as measured by dynamic light scattering for condensates observed in solution are of comparable sizes with those determined by electron microscopy (Hoppert and Meyer, 1999a,b; Bloomfield *et al.*, 2000). Evolution only selected other macromolecules to achieve the final aim of DNA folding in a more accurate and ordered way, than that seen with  $\Psi$  DNA, namely with participation of histones and protamines. In conclusion, DNA observed *in vivo* as liquid crystal arrays showed that the amount of osmotic stress needed for compaction and ordering into the liquid-crystalline state is in the same order for DNA *in vitro* (Strey *et al.*, 1998; Hansen *et al.*, 2001; Lorman *et al.*, 2001). More importantly, the PEG-induced DNA structure is very similar to crystals formed by many other polymers, particularly DNA-lipid self-assemblies, or stress proteins in *E. coli*, implying that nature employs similar thermodynamic packing strategies in different species. In general, the phenomenon of DNA collapse is treated as coil to globule transition. Cellular DNA is imagined as a globule, but this state remains to be confirmed by direct visualization in intact cells. On the other hand, one can claim that the biological implication of this physical state is used to bring distant sequence elements into close proximity. This strategy has been used by phages, plasmids, and



**Fig. 2.** Change in nucleoid volume as a function of the concentration of macromolecular crowding agent. The wide range of measured nucleoid volume values is due to different kinetics of their osmotic release and subsequent collapse on the cover glass. Photo bleaching was reduced to a minimum, as described in Materials and Methods. Since the induced compaction takes place in polymeric solvent, most of the added polymer is excluded from the DNA globule. Nucleoid volumes were quantified with Object-Image™ software. The line represents a curve fit of the determined nucleoid sizes and shows their trend to decrease as the concentration of PEG 20 000 increases. Arrows depict representative images of osmotic stress-free (0%) and two cases (2% and 14%) of polymer-induced compaction and phase separation of *E. coli* nucleoids, respectively.

transposons to facilitate the formation of site-specific DNA-protein interactions, to accommodate recombination and initiation of replication, as well as in transcription. The compacted DNA phases presented in the current study can serve as evidence for the globular state of DNA and its phase separation within the bacterial cytoplasm. As far as we know, no experimental protocol exists for analysing DNA phases which are close to equilibrium. Katritch *et al.* (2000) designed the interesting Metropolis-Monte Carlo modelling, which considers the chromatin fiber at thermodynamic equilibrium. The bead used in this and similar approaches for force-extension analysis somehow perturbs the DNA molecule. Moreover, extension of DNA molecules is limited for approaching with fluorescence microscopy, due to resolution limits of the latter. Our approach allows undisturbed spreading and certain temporary collapse of the osmotically released nucleoids on the glass surface, in order to achieve near equilibrium



**Fig. 3.** Nucleoid compaction in the presence of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  ions. The color of fluorescence images is inverted. (A): nucleoid in water; (B): nucleoid in 0.2 M NaCl; (C): nucleoid in 10% PEG 20,000 and 0.2 M NaCl and (D): nucleoid in 10% PEG 20,000, 0.2 M NaCl and 5 mM  $\text{MgCl}_2$ .

conditions. This partially extended DNA conformation falls within the resolution limits of the fluorescence microscope, which can be used for digitizing the dynamics of DNA molecules. Their compaction, achieved afterwards, is a function of the concentration of the added macromolecular crowding agent (Figs. 1D-F. and Fig. 2). These figures show results of fluorescence microscopy of cells grown in 300 mOsm minimal medium, for comparative reasons, to test the already experimentally determined cytoplasmic water activities of *E. coli* as a function of osmolality of cell growth and its significance in regulating periplasmic volumes at moderate levels of osmolality (Cayley *et al.*, 2000). Working with cells grown on other media of different osmolalities, as well as treated with transcription/translation inhibitors evaluating the concept of V. Norris transertion model (Norris and Madsen, 1995) becomes a necessity for this reason. Fig. 3 shows the final part of the *E. coli* nucleoid compaction assay in the presence of  $\text{Na}^+$  and  $\text{Mg}^{2+}$ . The images were obtained with DAPI staining using thin-layer preparations

of specimens on agar or directly on glass surfaces. Our current intention is to employ thick-layer preparations using scanning two-photon absorption microscopy. Comparison with Fig. 1 already creates a good impression for much tighter DNA compaction under excess salt conditions. In *E. coli*, where proteins are zwitterionic, the nucleic acids are the major polyelectrolytes. Therefore, other agents (e.g. various cations) should neutralize the rest of the charges of these polyanions. Particularly,  $\text{Na}^+$  and  $\text{Mg}^{2+}$  are naturally abundant and thus are essential solvation parts of nucleic acids. DNA transitions are therefore highly dependent on activities and charges of cations in the surroundings. Since  $\text{Mg}^{2+}$  is an essential stabilizing element of nucleic acid structure and a detrimental factor of chromosome integrity (Strick *et al.*, 2001) *in vivo*  $\text{Mg}^{2+}$ -induced compaction of nucleoids is of particular interest. Nucleic acid-ligand binding of this sort and its effects on condensation reactions are explained by G.S. Manning's polyelectrolyte theory. However, this approach ignores the role of size exclusion effects. Hence, the cur-

rent study gives an opportunity to compare different approaches, at least theoretically. In terms of biophysical chemistry of cellular electrolytes, Fig. 3 depicts experiments with two different counterions of different valencies. This system more closely approximates real systems and is described by Manning's two-variable counterion condensation theory, by the Poisson-Boltzmann approach, and by the Monte Carlo self-consistent field method. Previous cryo-electron microscopic studies on circular DNA provided evidence that twist, writhe, effective diameter and overall shape of supercoiled DNA changes due to cations' effects. Here,  $\text{MgCl}_2$  diminishes the effective diameter of DNA within the nucleoid. Thus, both  $\text{Na}^+$  and  $\text{Mg}^{2+}$  ions act via long-range electrostatic effects on persistence length and effective diameter, with a specific effect on the mean linking number.  $\text{Mg}^{2+}$  competes with  $\text{Na}^+$  for binding to DNA, since almost no additive effects on the duplex stability is known for these two ions, divalent cations being much more effective in stabilizing duplex structure. Steric exclusion is the source of the preferential hydration in water-PEG systems (see Fig. 1 and Fig. 2). PEG 20,000 is excluded nonspecifically from native globular proteins, while  $\text{Mg}^{2+}$  raises the surface tension of water (Fig. 3). The resultant extremely crowded surroundings would have promoted DNA self-assembly events, resulting in highly compacted and thermodynamically stable DNA condensates. Addition of 5 mM  $\text{MgCl}_2$  has both practical and functional reasons. Added below this concentration,  $\text{Mg}^{2+}$  would be removed by the EDTA in the lysis buffer, while above this range would exert adverse effects in a temperature-dependent manner. The biological importance of this result is seen from the fact that during DNA compaction into aggregates, the local DNA concentration enhances the catenated state. It seems that 5 mM is the critical concentration of  $\text{Mg}^{2+}$  in combination with competing  $\text{Na}^+$ . In addition, 5 mM  $\text{Mg}^{2+}$  is the critical range for intracellular phase transitions and phase separation processes in bacteria, by means of structural sequestration. Thus, free  $\text{Mg}^{2+}$  concentration decreases upon starvation, both intra- and extracellularly (A. Minsky, personal communication). An important functional implication is that salt effects influence the ability of cells for osmotic regulation, thus determining the presence of evolutionary selected inorganic cations in nucleoid fiber. The latter fact is important, since the  $\text{Mg}^{2+}$  served as a primitive enzyme during the RNA-based protocell formation. Molecular evolution afterwards substituted the thermodynamic affinities of DNA for counterions, replacing the inorganic, and selecting more efficient proteinaceous biomolecules, such as DNA-bending proteins besides other enzymatic agents. It was proposed recently (Odijk, 1998), that DNA-DNA interactions occur in a self-excluded volume, much less than the calculated value for DNA-protein depletion energy, due to crowding of cytoplasmic proteins, as the cross virial coefficient is

high. Hence, phase separation between nucleoid DNA and cytoplasm occurs, which forms the basis for microcompartmentation and metabolic channeling. This osmotic compaction of the nucleoid into a globular liquid crystalline state was monitored in the study presented here experimentally at near to equilibrium conditions, employing the macromolecular crowding agent PEG 20,000. The volume of the nucleoids achieved after the size exclusion effect is comparable to those in living cells (compare Fig. 3D with Fig. 1A). The bacterial nucleoid, however, cannot be envisaged as a static structure. Having considered the current debate concerning the structure of *E. coli* nucleoid, unravelling the molecular details responsible for prokaryotic genome compaction and its influence on regulation of gene expression remains a major objective for molecular biology. How condensed fibers are produced, why are they so precisely packed and finally, why they unfold widely reaching the border of the membrane to facilitate transcription and translation are questions awaiting answers (Norris and Madsen, 1995; Funnell, 1996).

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