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Electrorelease of *Escherichia coli* Nucleoids

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ABSTRACT. Bacterial chromosome is assembled and folded into one or several nucleoids, depending on the metabolic status of the cell. Development of reliable nucleoid isolation protocols has always been an objective for researchers. A rapid and reproducible procedure for isolation of *E. coli* nucleoids is described here, while the cell envelope is maintained. Membrane dispersions and vesicles were prepared by lysozyme–EDTA treatment with subsequent rupture of the spheroplasts by electric field. Under these conditions the yield of electroreleased nucleoids was around 90 %. The extent of DNA-envelope contacts was determined by light microscopy employing phase contrast and fluorescence modes.

Despite the fact that bacterial chromosomes are appreciated sometimes also as prokaryotic counterparts of eukaryotic chromatin and nucleosomes (Sharpe and Errington 1999), the *E. coli* chromosome is a unique nucleic acid-protein machinery highly compacted in the cell. For a long time, the understanding of the structure-function relationships of the *E. coli* nucleoids relied on data coming from pioneering electron microscopy work (for review *see* Robinow and Kellenberger 1994) and sedimentation analysis (Worcel and Burgy 1972). The former experimental approach visualized the nucleoids as a large dense and nonviscous ribosome-free mass occupying the center of *E. coli* cytoplasm, the remaining space being filled with polyribosomes. However, the published pictures of the nucleoid structures varied largely depending on the preparative methods, thus rendering the bacterial nucleoid as a structure without defined boundaries. In addition, different methodologies used to visualize the nucleoids resulted in either membrane-bound (Pontalier and Worcel 1976), or membrane-free (Kavenoff and Bowen 1976) structures depending on experimental conditions applied. These two structures were distinguished afterwards according to their different sedimentation rates. Most of the laboratory procedures applied for the purification and further characterization of the bacterial nucleoids either used physical disruption or mild lysis with various detergents, which resulted in DNA breakage or in appearance of viscous DNA particles, reflecting the unfolding of the chromosome, mostly due to charge repulsion of phosphate groups in the DNA (Hirschbein and Guillen 1982). Thus, depending on the purification method used nucleoid components differed from one another.

Despite its immature status and poorly understood molecular mechanisms, the use of electric fields on biological cells, organelles and tissues is nowadays widely applied in cell biophysics and biomedicine (Frey 1994). The impulse for this has originated from the practical need to achieve stable and nondestructive transformation of bacteria, yeasts, animal and plant cells with further potential in the fields of genetic engineering, nonviral gene transfer and therapy, as well as in agrobiotechnology. In this context, the objective is to achieve direct transfer of nucleic acids (DNA and RNA), proteins and other important pharmaceuticals and biomacromolecules by means of electroporation into various recipient cells, the main advantage of this approach being that cell suspensions are manipulated in chemically intact maner (Szostková *et al.* 1999). The strength of the applied electric field and the duration of the pulse(s) are adjusted according to the cell size and type, density of the cell suspension, medium composition, *etc.* (Tsong 1990; Neumann 1992; Sowers 1992). Interestingly, electric field effects were applied mostly to permeabilize cell membranes for various genetic and pharmacological purposes (Orlowski and Mir 1993), whereas their use in electrorelease of cellular components with their subsequent characterization was not studied in detail until now. Having considered the above drawbacks of the experimental procedures applied so far for the isolation of bacterial nucleoids (*see also* Bendich 2001), the latter possibility opens new avenues for isolating and further characterizing them as a model system for studying the *in vivo* properties of bacterial DNA.

Membrane permeability has a natural bioelectrochemical origin (Hong 1994), both in terms of regulation of gene expression (Matzke and Matzke 1991) and cell signaling (Olivotto *et al.* 1996). Electrical

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fields induce either irreversible or reversible effects on bilayer permeability. The former case involves modification of lipid asymmetric structures and proteins within it, leading to disruption of cytoplasmic constituents. When electrical parameters and certain medium characteristics, such as temperature and ionic strength, are suitably chosen, reversible and nondestructive cell membrane permeabilization is achieved, with viable self-assembling of the electro-broken cellular constituents (Zimmermann and Neil 1996). Since the importance of lipid–nucleic acid associations are well established in terms of various models for the origin of protocells (Deamer 1997; Süleymanoğlu 1999), evolution of prokaryotes (Smith and Szathmary 1995), metabolic activities (Hellingwerf and Palmen 1996; Funnell 1996), transcriptional activity of prokaryotic chromosomes (Gorke and Rak 2001), as well as in the field of development of novel tools of non-viral gene therapy vectors, obtaining of membrane-bound nucleoids is emphasized. Molecular details of these self-assemblies and their functional implications are not understood. This work describes the electric-field-induced release of envelope-bound *E. coli* nucleoids, which can be used further as models of relevant *in vivo* macromolecular assemblages.

MATERIALS AND METHODS

Chemicals and sources. Low-melting point agarose, egg white lysozyme (EC 3.2.1.17), NaCl, EDTA were purchased from *Sigma* (USA) 4′,6-diamidino-2-phenylindole (DAPI) and FM 4-64 were obtained from *Molecular Probes* (USA). Glycerol and formaldehyde were purchased from *Aldrich*. All other chemicals used were of highest analytical grade. Unless otherwise stated, all experiments were performed at room temperature (26–28 °C).

Bacterial strain and growth conditions. Glycerol stock of *E. coli* strain MC1000 (kindly provided by M. Wery, *Laboratoire de Physiologie Bacterienne, IBPC,* Paris, France), was grown under vigorous shaking at 30 °C in glucose minimal medium. Growth was followed turbidimetrically at 450 nm. A single colony of agar plates was inoculated into 5 mL fresh glucose minimal medium, supplemented with ampicillin, pH = 7.2; osmotic pressure 300 mmol/L (determined osmometrically). During early stationary phase the culture was diluted appropriately into prewarmed medium and incubated at 30 °C with shaking at 3.3 Hz in a rotary shaker (*New Brunswick* G-25D) in a-500 mL Erlenmeyer flask for better aeration. The culture was diluted several times to reach exponential phase. Determination of the total number of cells was done with Coulter Counter (*Coulter Electronics*, UK) in a-30 μm orifice and in a 100-μL volume. Cells with constant average cell mass were used as the criterion for steady-state culture. Since bacterial cell size and DNA (Akerlund *et al.* 1995; Gasol *et al.* 1999), as well as protein content (Azam *et al.* 1999) is growth-phase-dependent, it was important to use steady-state cultures.

Electrolysis procedure. Ten mL of the exponential-phase cells were centrifuged (83 Hz, 2 min) in 10 Eppendorf tubes. The pellets were collected into a single Eppendorf tube and kept on ice, into which 200 μL lysozyme buffer (10 g/L lysozyme, 10× phosphate buffer saline (PBS), pH = 7.2; 0.25 mol/L EDTA, 1 mol/L sucrose) was added. The resulting turbid mixture was left for 5 min at room temperature and rechilled. Spheroplast suspension was subjected to electromanipulation as follows. One-mm cuvettes were kept at −20 °C and put on ice before use. *E. coli* pulser (*Bio-Rad Laboratories*, USA) current was adjusted to 1.8 kV/cm. One-mL 10 % ice-cold glycerol was added into each cuvette, along with 50 μL of spheroplast suspension with careful mixing. A single pulse was used for electrical breakdown of the spheroplasts. Both DAPI and FM 4-64 stains were added afterwards.

Light microscopy and image analysis. Both DAPI and FM 4-64 were added to spheroplast suspension at final concentration of 1 g/L (Pogliano *et al.* 1999). Glass cover slips were placed immediately onto 10 μL sample and sealed with nail polish, to prevent the movement of electroreleased nucleoids. These slides were left for 10 min and examined by phase contrast, fluorescence, or combined phase contrast–fluorescence modes of *Olympus* BH-2 fluorescence microscope, equipped with Princeton CCD camera, using 100× SPlan PL phase contrast objective. The microscopy samples can be used during the next 1–2 d without any substantial damage of the patterns of the electroreleased *E. coli* nucleoids in terms of their subsequent quantification.

RESULTS AND DISCUSSION

Isolation of *E. coli* nucleoids has always been approached by research groups. Despite the fact that even nowadays different viewpoints concerning the term “isolated nucleoids” are shared, a great success has been achieved with regard to reproducibility of isolation procedures. The difficulty arises from the fact that

the isolated nucleoid size, morphology and molecular properties do not necessarily correspond to those existing *in vivo*. The latter is still controversially documented by various research groups, due to methodological limitations, such as cryofixation artifacts of electron microscopy protocols, as well as those created by the use of various detergents in the lysis step. In addition, the nucleoids decompact after lysis of the bacterial cells and thus the visualized structures are far from being reliable substitutes of those in living *E. coli* cells (Murphy and Zimmerman 2001). Hence, to approach closely the living liquid and crowded state of bacterial cytoplasm, there is a need to visualize the nucleoids directly both in growing cells and upon their lysis, while avoiding the use of ionic and nonionic detergents and destructive microscopy artifacts. One alternative of the use of chemicals is the application of physical forces, which permit work in a controllable chemical-free way. The wide use of electric fields in biotechnology is mainly to permeabilize the membrane of the host cell for introduction of various macromolecules with commercial and therapeutic benefit, and even organelles, but the use of electric fields for isolation of biomolecules of interest from the crowded cytoplasm is only rudimentary.

Fig. 1A is a phase-contrast image of viable *E. coli* cells. The nucleoid was visualized here within living cells as a body of undefined edges by using phase contrast and DAPI fluorescence images (Fig. 1B). In these exponential-phase cells DNA appears to occupy most of the cell volume, being irregular in shape and with edges reaching the membrane, visualized here with FM 4-64 staining (Fig. 1C). FM 4-64 is an envelope-impermeant dye, which binds to the outer leaflet of lipid bilayers (Pogliano *et al.* 1999). To determine the extent of phospholipid–DNA associations, exponential-phase cultures of *E. coli* MC1000 strain were stained with both FM 4-64 and DAPI, either during cultivation of bacteria or immediately before microscopic examination. Both FM 4-64 and DAPI do not interfere with growth rates of cells and can be used for their staining at different points of their growth curve for microscopic observation. Fig. 1C shows the staining pattern of FM 4-64 on living bacterial cells. Their membranes appear as intensely stained mass surrounding dark cytoplasm, which is occupied by nucleoid, as seen by DAPI image in Fig. 1B. Thus, combination of DAPI and FM 4-64 images brings out a membrane–nucleoid association pattern which is also a valid model for living cells of *E. coli* (Funnell 1996). Phase-contrast and fluorescence-microscopic images of the spheroplasts are shown in Figs 1D–F. The DNA content within the spheroplasts (Fig. 1E) has a much greater intensity than nucleoids in living *E. coli* cells (Fig. 1B), which is an indication of their compaction within a smaller volume of vesicles. Some aggregation of spheroplast on the right-hand side (Fig. 1E, F) is due to the presence of inorganic bivalent cations. The intensity of the FM 4-64 signal of spheroplast suspension (Fig. 1F) more or less matches that of living exponential phase *E. coli* MC1000 cells. The results are in agreement with previous observations of Pogliano *et al.* (1999). The morphology of the nucleoids changes upon electric breakdown of the spheroplasts (Fig. 1G). Electrolysis effects are seen here in the disappearance of the nucleoids from the phase-contrast mode of the microscope. The released DNA somehow decompacts (as described by Murphy and Zimmerman 2001); as indicated by the decreased intensity of the DAPI signal. In addition, nucleoids collapse on the surface of the microscopic glass slides. The amount of DNA released from different individual spheroplasts also varies, indicating probably a certain degree of electric-field-induced fusion of several aggregated *E. coli* vesicles prior to release of the nucleic acid content.

Nucleoids in lysozyme-induced spheroplasts were inaccessible to nucleases and other agents disrupting DNA–membrane contacts, as well as to SDS treatments. All these agents disaggregate these complexes (Fig. 1G, H), upon electrolysis (*data not shown*). Spheroplats swell due to the action of the colloid osmotic pressure of the cytoplasmic macromolecules, because of either dielectric breakage or electroosmosis with subsequent hydrodynamic entrance of water and inorganic ions into the vesicles (Tsong 1990; Deuticke and Schwister 1992). The size of the induced electropores also increases steadily, leading to colloid overswelling and finally to rupture of the vesicle membranes and lysis after their critical inner volume has been exceeded. Apparently, membrane blebs are created at this stage and larger broken lipid pieces may be released from the spheroplasts (Tsong 1990). Such a Donnan-osmotic cytolysis (Glaser 2001) involves an electro-induced effect on both membrane proteins and lipids (Deuticke and Schwister 1992; Akinlaja and Sachs 1998). Since FM 4-64 cannot distinguish between various lipid domains and stains with equal efficiency the whole bilayer structure, the observed fluorescence intensities of different membrane regions would indicate their number and topology (Pogliano *et al.* 1999). It is interesting to note the much greater intensity of the ghosts (Fig. 1H), as compared with the unlysed *E. coli* cells (Fig. 1C), with an obvious effect of delayed lipid asymmetry of the original cell envelope. *E. coli* spheroplasts were obtained by lysozyme–EDTA–sucrose procedure (Taketo and Kuno 1969). Phase contrast (Fig. 1D), DAPI fluorescence (Fig. 1E) and FM 4-64 (Fig. 1F) show the match of all three microscopic images of the same spheroplasts, while the intensity of the DAPI signal is much higher than FM 4-64. Membrane dye shows a staining pattern of the ghost surrounding the nucleoid. These spheroplasts are very clearly defined also by phase-contrast microscopy (Fig. 1D), which is in agreement with recent observations (Zimmerman and Murphy 2001). The

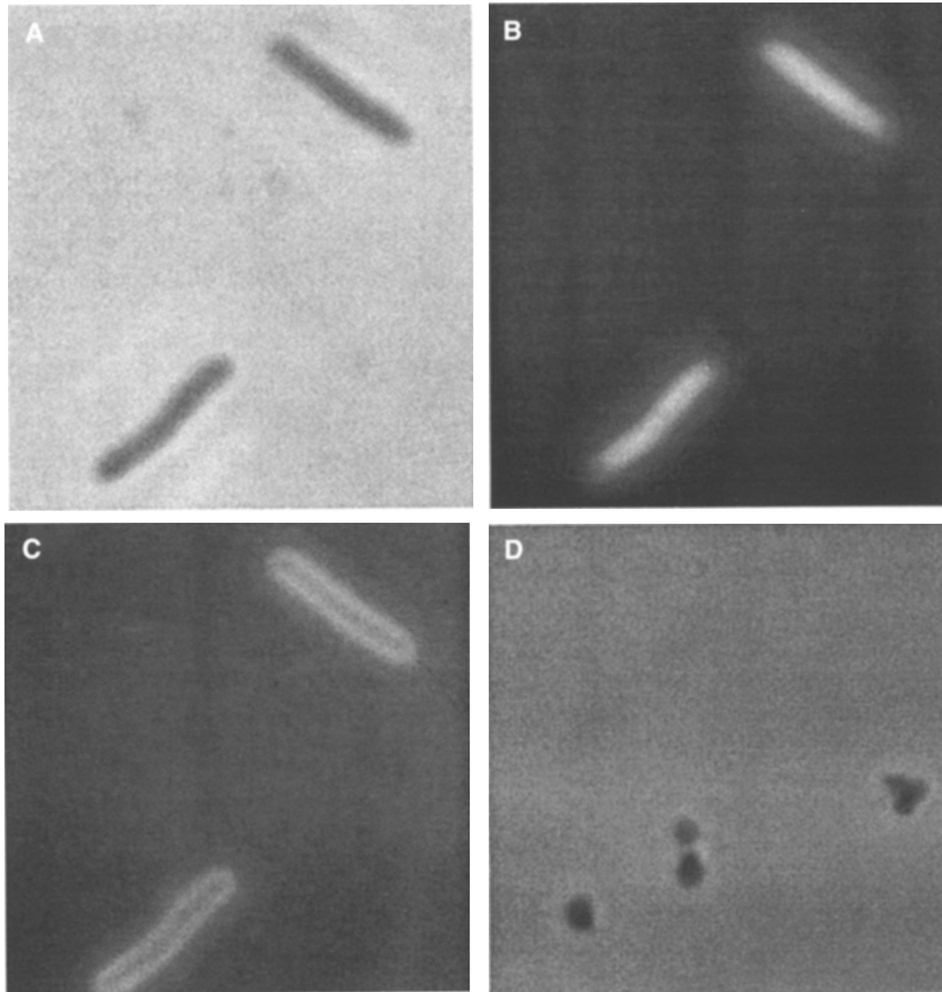
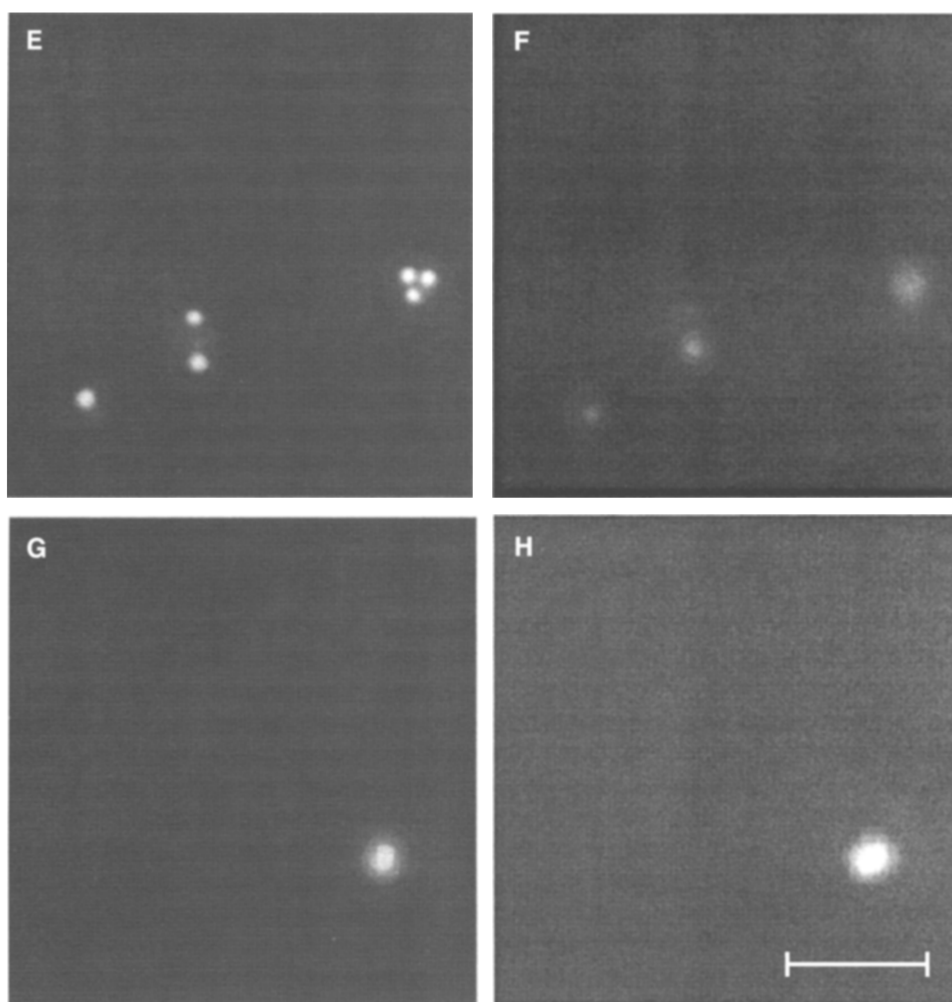


Fig. 1. Light microscopy of *E. coli* cells and electroreleased nucleoids from EDTA–lysozyme spheroplasts. Exponential-phase *E. coli* MC1000 cells are shown as phase contrast (**A**), DAPI- (**B**) and FM 4-64-stained (**C**) images; spheroplasts were obtained by classical EDTA–lysozyme procedure (*see Materials and Methods*); **D** – phase contrast, **E** – DAPI-treated, **F** – FM 4-64-stained *E. coli* vesicles; electric-field-induced shrinkage of the spheroplasts with resulting envelope-bound nucleoids is shown as DAPI (**G**) signal for nucleoids and as FM 4-64 (**H**) pattern for broken membrane dispersions, surrounding the DNA; *bar* – 5 μm.

amount of lysozyme to be used depends on the cell type. A low lysozyme amount was used here to prevent its well-known effect on nonspecific association between bacterial membranes and DNA (Silberstein and Inouye 1974). Lysozyme acts as *N*-acetylmuramidase by degrading the bacterial polysaccharide chains to disaccharide fragments. Complete breakdown of the bacterial cell is prevented by doing lysis in a weak hypertonic or isotonic solution of sucrose. Under these conditions, spheroplasts highly sensitive to osmotic manipulations are formed (Fig. 1D–F), which can be ruptured only if placed in a hypotonic medium leaving then remnants of plasma membranes. Spheroplasts are structures in which permeability is increased but cellular metabolism is not altered (Hagenmaier *et al.* 1997). The release of nucleoids from these lysozyme–EDTA spheroplasts was achieved by applying electric fields (Fig. 1G, H), instead of osmotic shock, to avoid the drawbacks of chemical reagents, such as salt effects (Baumgarten and Feher 1995). Application of an electric pulse to living *E. coli* cells does not lead to their breakdown (*data not shown*), because of the strength and rigidity of the peptidoglycan layer of the envelope. Under these circumstances the sequence of bacterial responses following a single square wave pulse of the electropulsator is orientation of the bacterium with the long axis in the direction of the external field and, subsequently, electro-induced permeabilization of the envelope, in which the lipid component is affected (Neumann 1992; Eynard *et al.* 1998). However, this permeabilization is not sufficient for the release of cytoplasmic contents. In order to obtain functional cells with weakened peptidoglycan layer of the bacterial envelope, lysozyme–EDTA spheroplasts were prepared, as described above (Fig. 1D–F). Electric pulses can now be applied to rupture the spheroplasts, thus releasing nucleoids. Physico-chemical parameters, such as osmotic pressure, ionic strength of the conducting media, *etc.* influence the induced permeability of the spheroplasts (Orlowski and Mir 1993). In this



context, it is important to avoid high fields, which give rise to partial strand denaturation of DNA and formation of DNA clots, where viscous medium is used to avoid the osmotic shock. Obviously, the pulse duration is a critical parameter in such cases (Eynard *et al.* 1998). Living cell membranes possess natural dielectric properties. Upon overloading with an external electric field creating a potential exceeding their dielectric strength, the membrane resistance to permeation breaks down (Tsong 1990). Therefore, following this approach, the possibility exists to achieve controlled electrorelease of bacterial nucleoids (Fig. 1G, H), as well as other cytosolic products. However, structural information of the described nucleoids must be related to their functional implications for living bacterial cells and the information obtained and models built in this context remain to be tested.

Besides the broad range of applications of the isolated nucleoids, mentioned by Hirschbein and Guillen (1982) and Zimmerman and Murphy (2001), it is of particular interest to employ them as biochemical and biophysical models of self-assembling nucleic acid–lipid particles with respect to their *in vivo* compaction and condensation properties, and to study how the folding of the bacterial chromosome fiber within the macromolecularly crowded *E. coli* cytoplasm is related to physico-chemical and bio-inorganic regulation of prokaryotic gene expression.

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