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DIALOG

Ring-Like Nucleoids and DNA Repair through Error-Free Nonhomologous End Joining in *Deinococcus radiodurans*

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Shortly after its isolation from canned ground meat in 1956, the bacterium *Micrococcus radiodurans*, later reclassified as *Deinococcus radiodurans*, was found to be capable of withstanding radioactive irradiation in doses that are 2 to 3 orders of magnitude higher than those most other organisms can cope with. Following exposure to these irradiation doses as well as to other assaults, such as extreme desiccation, that damage chromatin, each of the genome copies in *D. radiodurans* is shattered into 150 to 200 fragments (1, 4). The ability to reconstitute the whole genome from multiple fragments in an error-free process is remarkable because homologous recombination pathways of DNA repair cannot occur in the absence of an intact chromosome that acts as a template. The mystery surrounding the resilience of *D. radiodurans* was intensified following the complete sequencing of its genome, which failed to clarify the genetic elements that promote accurate DNA mending of hundreds of fragments. Specifically, the annotated sequence implied that most of the typical complements of DNA repair proteins are present in *D. radiodurans* (2, 29).

In 2003, we reported our high-resolution electron microscopy results, which indicated that chromosomes in *D. radiodurans* cells adopt highly condensed ring-like organizations (14). We proposed that within this particularly stable mode of tight DNA packaging, DNA ends generated by double-strand breaks are kept in close spatial proximity, allowing for accurate DNA repair through nonhomologous end joining (NHEJ) (6, 14, 18). This proposal was supported by our observation that, under particular growth conditions that sustain normal proliferation of *D. radiodurans* cells but completely abolish radioresistance, the ring-like shape of the genome is no longer detected, being instead replaced by a dispersed, irregular conformation (14).

In their recent article, Eltsov and Dubochet used cryoelectron microscopy of vitreous sections (CEMOVIS) to examine the structure of *D. radiodurans* (5). By using this method, the authors found that the nucleoids of exponentially growing cells appear to adopt a diffuse shape, whereas in stationary-phase cells, some local order was detected. Eltsov and Dubochet argue that dense toroidal DNA packaging probably does not exist in *D. radiodurans*, in contrast to our previous results. On the basis of these observations, it was concluded that the structure of the nucleoid in *D. radiodurans* does not directly contribute to DNA repair in this bacterium.

We do not agree with the interpretation of the experimental data presented in the Eltsov and Dubochet article, nor do we accept the final conclusion. Specifically, we claim that nucleoids in *D. radiodurans* as well as those in other highly resistant bacterial species do adopt unique conformations and that these conformations represent a sine qua non factor of DNA repair by directly promoting the efficiency and accuracy of NHEJ processes in these organisms. In the following sections, we analyze the experimental data reported in the Eltsov and Dubochet article (5) and juxtapose these data with results derived from other structural techniques. We then proceed to examine the interpretation of the data in light of recently reported findings that directly pertain to the structural basis of DNA repair in *D. radiodurans*.

STRUCTURAL DATA FOR THE *D. RADIODURANS* GENOME

"Pros and cons" of electron microscopy techniques used to analyze chromatin conformations. The most challenging aspect of high-resolution electron microscopy studies of biological samples is associated with the accurate preservation of native features. Conventional methods, in which chemical fixatives such as aldehydes and/or osmium compounds are used, were shown to produce extensive artifacts, which are particularly severe in studies of bacterial chromatin organization (26). These artifacts originated from, mainly, the propensity of chemical fixatives to effect a reorganization of bacterial nucleoids into tightly packed aggregates. In order to minimize the shortcomings associated with chemical fixatives, cryofixation techniques were introduced, whereby biological specimens are vitrified by fast cooling under high-pressure conditions that prevent the formation of ice crystals. In cryofreeze-substitution (CFS) methods, the vitrified samples are substituted at a low temperature with an organic solvent, embedded in resins, sectioned, and stained. In contrast, the technique of CEMOVIS used by Eltsov and Dubochet enables electron microscopy studies of fully hydrated specimens. In this method, the unsubstituted, unstained, and frozen samples are cut into thin sections that are inspected at low temperatures in a cryoelectron microscope (5).

While CEMOVIS has been shown to be appropriate for visualizing cell envelopes and extracellular matrixes, we claim that its suitability and reliability in detecting nonmembranous

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intracellular structures are inherently limited due to the following reasons.

(i) Contrast and density differences. Density differences in biological specimens are usually small, and this is particularly so when the structure of bacterial chromatin is investigated because DNA contrast is intrinsically low (11, 26). In CFS techniques, contrast is enhanced by heavy metal ion staining. Because frozen specimens cannot be stained, density differences in samples prepared by the CEMOVIS procedure are exceedingly small, even when large defocus values are used.

Information on the location and morphology of chromatin in electron microscopy of bacterial specimens is regularly derived from cytoplasmic regions from which ribosomes (whose contrast is higher than chromatin and hence more easily detected) are excluded (11, 26). In this regard, the CEMOVIS technique presents several severe problems. In the resulting unstained specimens, even the densities revealed by ribosomes remain very low, making it difficult to distinguish between regions that contain ribosomes and those from which ribosomes have been excluded. This problem is further aggravated by the extensive cutting artifacts that are associated with the CEMOVIS technique, including knife marks and crevasses (5). As a result of these artifacts, the distinction between DNA-containing regions that are characterized by "real" low contrast due to ribosome exclusion and regions whose low contrast originates from cutting artifacts is rendered ambiguous (for details, see Fig. 1A to C in reference 5). In contrast, in stained samples obtained via CFS techniques, the borderline between DNA-containing and ribosome-containing regions is usually clear-cut. In addition, in samples prepared by CFS (but not by CEMOVIS), chromatin can be specifically labeled with DNA-staining reagents, such as osmium-ammine, which provide direct data on its localization and morphology.

(ii) Axial distortions. A particularly severe sample distortion characteristic of the CEMOVIS technique is a substantial compression of the specimen that occurs in the cutting direction, which leads to the shortening of one axis relative to the other by more than 25% (for details, see Fig. 1C in reference 5). The consequences of this distortion are detrimental when structures such as toroids are considered, since the compression of a ring along one axis may prevent its classification as a ring. This is particularly true when the inner diameter of the ring is small relative to its thickness, as indeed is the case for DNA toroids in *D. radiodurans*. Consequently, in many cases, toroidal structures will erroneously be scored as rod-like in samples prepared by CEMOVIS.

Statistical considerations. Following both CFS and CEMOVIS preservation techniques, samples are randomly cut into thin (60- to 80-nm) sections for transmission electron microscopy. This considered and when the diameter (~300 nm) and thickness (~120 nm) of DNA toroids in *D. radiodurans* cells are taken into account, only those toroids whose main axis is positioned in angles between −15° and +15° relative to the *x* and *y* axes of the section will be scored as such. Since in transmission electron microscopy of thin sections only the projection of structures embedded within these sections is derived, ring-like conformations whose main axes are positioned at higher angles will be scored as unstructured or as rod-like morphologies. Consequently, if all genomes in *D. radiodurans* adopt a ring-like conformation, only one out of six (30°/180°) will be scored as such. In our studies, more than 3,000 cells were inspected for each growth condition, leading to the conclusion that more than 90% of the genomes in stationary-state cells adopt a toroidal structure, whereas in vegetative cells, toroids appear in 70 to 75% of the bacteria. No data are provided on statistical analyses in the Eltsov and Dubochet article (5).

We claim that the unfavorable probability of detecting ring-like DNA structures in thin sections, combined with the low contrast and the severe cutting and compression artifacts that innately characterize the CEMOVIS technique, renders a correct assessment of nucleoid morphologies by this technique unlikely. The fact that, on a few occasions, ring-like structures could still be detected in both exponentially growing and stationary-phase cells following CEMOVIS (5) attests to the abundance of such structures in *D. radiodurans* cells.

Dehydration artifacts. Eltsov and Dubochet claim that the CEMOVIS procedure is superior to CSF because the latter fixation technique involves dehydration by organic solvents, which might lead to artifacts associated with DNA aggregation. This claim is groundless, as numerous studies unambiguously indicated that chemically induced DNA aggregation does not occur during CFS (for details, see reference 13 and references cited therein). In fact, it has been shown that the immobilization of bacterial chromatin already occurs following fast freezing and that this immobilization effectively resists subsequent DNA aggregation (13).

The assertion that a ring-like structure represents the native conformation of the genome in *D. radiodurans* as well as in several other radioresistant members of the *Deinococcaceae* family is supported by numerous observations that indicate that, following CFS fixation, chromatin in logarithmically growing *Escherichia coli*, *Bacillus subtilis*, *Myxococcus xanthus*, and many other bacteria reveals a fully dispersed, apparently disordered morphology (7, 8, 26). Even if freeze-substitution does induce DNA aggregation (which, as discussed above, it has specifically been shown not to), it is hard to envisage why this process would lead to DNA toroids in *D. radiodurans* cells, whereas in all other bacteria, it does not. Most significantly, our studies performed on *D. radiodurans* cells following their exposure to irradiation or to an excess of Mn(II) ions demonstrated that, under these conditions, the tight ring-like structure of the chromatin is no longer present (14). The fact that these cells were also immobilized by CFS clearly argues against the possibility that the ring-like genome conformation represents a technique-dependent artifact.

Granules. In their studies, Eltsov and Dubochet observed one or several large electron-dense granules in exponentially growing *D. radiodurans* cells that were not detected in stationary or long-stationary cells (5). On the basis of these observations, they raised the possibility that since such granules are lost during sample preservation through the CFS technique, thus creating a hole, dispersed nucleoids were misinterpreted as rings. This suggestion is refuted by three facts. First, in our CFS preparations, the remains of granules could indeed be detected on a few occasions (less than 10% of the cells) as small, poorly preserved spots. However, in most cases, these granules were located outside of the DNA ring-like structure. Second, in all cases where ring-like DNA structures were observed, an island of ribosome-rich cytoplasm was detected in

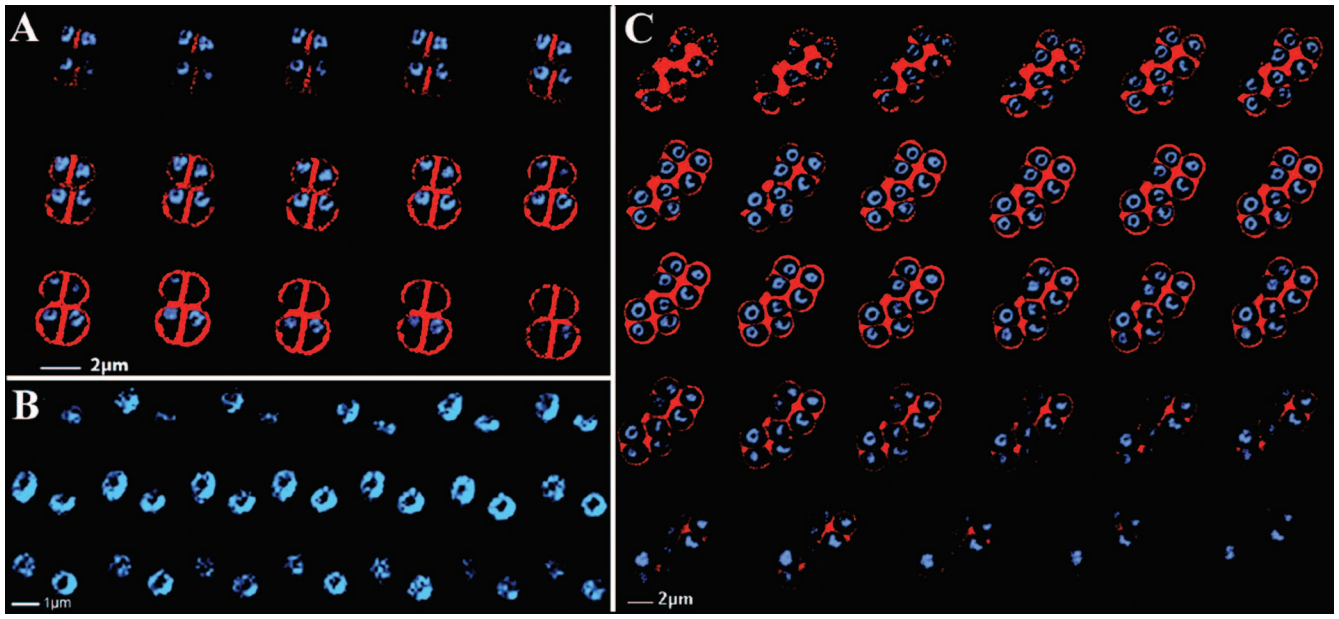

FIG. 1. (A) Optical sections of a *D. radiodurans* tetrad. (B) Optical sections of nucleoids in a pair of *D. proteolyticus* cells (only the DAPI [4′,6′-diamidino-2-phenylindole]-stained DNA is shown in the panel). (C) Optical sections of a pair of *Deinococcus murrayi* tetrads. Images were taken at 100-nm intervals. In all images, the DNA (blue) is stained with DAPI and the lipid membrane (red) is stained with FM-4-64. (Adapted from reference 30 with permission.)

the center (14). Third, and most significant, is the observation that following either the CEMOVIS or the CFS technique, granules could never be detected in stationary-phase *D. radiodurans* cells. Yet, ring-like DNA structures were found to be most abundant during this phase (14).

Fluorescence studies. Nucleoid morphologies in several radioresistant members of the family *Deinococcaceae* were recently evaluated by using epifluorescence and deconvolution techniques (Fig. 1). The genomes of most of these species revealed flat or somewhat tilted ring-like morphologies (30), which were proposed to originate from DNA spooling around a proteinaceous core. While we claim that such a core is not required (and indeed is not detected in electron microscopy studies) in light of the large intrinsic propensity of DNA molecules to adopt a toroidal conformation, the presence of tightly packed and well-defined DNA structures is unambiguously demonstrated by the fluorescence studies. Notably, by using the same technique, it was shown that *E. coli* cells exhibit fully dispersed nucleoid morphologies (30), again indicating that toroidal chromatin conformations in *D. radiodurans* and in other radioresistant bacteria are genuine and unique. Curiously, Eltsov and Dubochet ignore these observations but speculate that nucleoid segregation, which they observe in long-stationary-phase cells, reduces damage caused by free radicals generated by irradiation. In raising this notion, they disregard numerous observations that indicate that the unique radioresistance exhibited by *D. radiodurans* derives from the capacity of this organism to repair, rather than protect, its DNA complement (1, 2, 17, 20, 21).

While most species belonging to the *Deinococcaceae* family reveal ring-like nucleoid conformations, some members do not, instead exhibiting highly condensed DNA structures (30). On the basis of this observation, it was proposed that the crucial factor that promotes DNA repair in radioresistant bacteria is not the actual nucleoid morphology but rather DNA tight packaging (3, 18, 30). We concur with this assertion but note that DNA toroids correspond to one of the most frequent modes of DNA condensation and that restricted diffusion of DNA termini, which we proposed to represent the crucial factor in promoting high-fidelity DNA repair in *D. radiodurans*, is a general feature of tightly packed DNA conformations (18).

DNA PACKAGING AND REPAIR IN *D. RADIODURANS*

On the basis of their cryoelectron microscopy studies of vitreous sections, Eltsov and Dubochet argue that "the arrangement of the nucleoid does not play a key role in the radioresistance of *D. radiodurans*" and that "the unusual efficiency of DNA repair in *D. radiodurans* is more likely to have a physiological than a structural basis" (5). We disagree with these conclusions and claim not only that the technique used by the authors is inadequate but also that they neglect to consider important observations that contradict their reasoning.

The concept of DNA condensation and restricted diffusion. Our principal argument is that the phenomenal abilities of *D. radiodurans* and other highly resistant species cannot be interpreted solely in terms of physiological pathways because such pathways, effective as they might be, are intrinsically incapable of solving the informational problem associated with the repair of multiple double-strand DNA breaks in the absence of an intact template. This premise is supported by biochemical studies as well as by complete-genome analysis, which demonstrated that *D. radiodurans* possesses a typical bacterial complement of DNA repair enzymes that are generally similar to those found in other nonresistant bacteria (15, 17, 29).

Our morphological studies indicated that the genome in *D. radiodurans* adopts a ring-like structure (14). On the basis of this observation, which is now supported by electron microscopy and fluorescence studies of *D. radiodurans* and other members of the *D. radiodurans* family (6, 30), we proposed that the tight packaging renders DNA rings into a matrix in which DNA linear continuity is physically preserved even following numerous breaks. Within this matrix, free DNA termini generated by irradiation or other DNA-damaging factors are kept firmly together due to restricted molecular diffusion, allowing for rapid and efficient DNA repair through template-independent NHEJ of DNA fragments (6, 14, 18). Notably, while NHEJ is usually considered an error-prone repair pathway, the physical continuity of DNA fragments in tightly packed DNA conformations, such as those found in *D. radiodurans*, renders this mechanism into an error-free mechanism. The notion that structural factors, such as condensed rings, play a key role in promoting DNA repair in radioresistant species has been subsequently extended to include other DNA conformations whose common denominator is a condensed DNA organization (3, 6, 18, 30). It should be emphasized here that in order to enable accurate repair of double-strand DNA breaks through NHEJ, the conformation of the nucleoid does not need to be very tight or highly ordered, as is the conformation encountered in DNA toroids obtained in vitro. Specifically, all DNA conformations in which lateral motion (i.e., motion perpendicular to the main axis of the DNA molecule) is restricted due to relatively tight packaging would promote accurate ligation of DNA ends, even if axial motility (motion along the main DNA axis) is unconstrained.

Liquid crystalline phases of DNA. Eltsov and Dubochet interpret their observations obtained from exponentially growing *D. radiodurans* cells as implying a diffuse, unstructured nucleoid morphology. The considerations presented above indicate that data on the morphologies of bacterial nucleoids obtained by CEMOVIS are equivocal due to severe cutting and compression artifacts, low contrast, and an inability to use contrast-enhancing reagents or specific DNA stains that characterize this particular technique. When these drawbacks are combined with the intrinsically low statistical probability of observing DNA toroids in thin electron microscopy sections, the detection of ring-like structures when the CEMOVIS technique is used becomes highly unlikely, even if such intracellular structures are abundantly present.

Data derived from long-stationary phase were taken to imply that, in this late phase, the nucleoid in these cells adopts a cholesteric arrangement, which is stabilized by Mn(II) ions and leads to some segregation of the chromatin (5). The authors further claim that, since this arrangement occurs in only aging cells, it cannot significantly contribute to the enhanced radioresistance that is already exhibited by early-stationary-state *D. radiodurans* cells. In support of the notion that the tight liquid crystalline organization is irrelevant to DNA repair, the authors refer to two observations: the notion that DNA ends remain mobile in condensed DNA phases (12) and the fact that dinoflagellattes, whose genomes are normally in the form of cholesteric liquid crystals, are not radioresistant.

DNA segments within condensed cholesteric DNA phases are indeed mobile. The mobility is, however, mainly confined to the long axis of the DNA molecules but drastically limited in directions perpendicular to this axis. Thus, and as explained above, the physical continuity of DNA fragments and hence the close proximity of the termini of these fragments are preserved in tightly packed liquid crystalline phases, axial motility notwithstanding. Indeed, in vitro studies of DNA annealing and ligation processes conducted under conditions that promote DNA packaging into toroids or other tightly packed structures demonstrated that, within these structures, annealing and ligation are dramatically enhanced relative to their rates and efficiencies in dispersed DNA (12, 32).

The case of dinoflagellates is particularly informative. The following points should be considered. (i) While most dinoflagellate chromatin adopts a tight cholesteric organization, a small but significant part always remains dispersed in the nucleoplasm. This decondensed fraction, in the form of extrachromosomal loops that continually move out from and back to the chromosomal body, corresponds to the transcriptionally active fraction (16). Thus, at any given time, all transcribed genes in dinoflagellates are not condensed and are hence susceptible to DNA-damaging agents. The situation in *D. radiodurans* is fundamentally different. Our electron microscopy data indicated that, in exponentially growing *D. radiodurans* cells, several of the multiple copies of their chromosomes are tightly and completely packed in a ring-like form, whereas the remaining copies are dispersed (14). We proposed that the packed copies promote DNA repair and radioresistance through NHEJ, while the dispersed copies allow for physiological activity (6, 18). In contrast, we find that practically all copies of the genome in stationary-state *D. radiodurans* cells adopt tight toroidal structures, thus accounting for the enhanced resistance of cells in this phase relative to that exhibited by exponentially growing cells (14). (ii) While we propose that structural elements play a key role in DNA repair in *D. radiodurans* and other members of the *Deinococcaceae* family, the contribution of specific enzymatic pathways cannot be ignored. In the absence of such particular activities (e.g., in dinoflagellates), no resistance to irradiation or desiccation may evolve, genome packaging notwithstanding.

Until recently, it was assumed that NHEJ repair of double-strand DNA breaks is restricted to eukaryotic cells. However, several recent studies identified and characterized a functionally homologous bacterial NHEJ repair apparatus (23, 27, 28) and direct evidence of the presence of an active NHEJ pathway in mycobacteria has been reported (10). Remarkably, the radiation-induced protein PprA identified in *D. radiodurans* was shown to bind to DNA ends and to stimulate DNA end joining catalyzed by ATP- and NAD-dependent DNA ligases (22). The authors proposed that PprA plays a critical role in NHEJ repair in *D. radiodurans*. This suggestion is supported by the observation that an ATP-dependent DNA ligase, in addition to an NAD-dependent ligase, is expressed in *D. radiodurans* (29). The ATP-dependent ligase is induced by irradiation, whereas the typical NAD-dependent ligase is down-regulated, implying that the ATP-dependent ligase might be involved in postirradiation repair in *D. radiodurans*. The small size (22 kDa) of *D. radiodurans* ATP-dependent ligase, which sets it apart from the typically much larger DNA ligases, is likely to facilitate access of the enzyme to double-strand breaks within the tightly packed DNA conformation. As discussed above, the physical

proximity of DNA fragments within tightly packed DNA conformations renders this repair pathway error free.

A tightly packed ring-like chromatin conformation was shown to occur during the early steps of spore formation in *B. subtilis* (24) and was found to persist in dormant spores (9) as well as germinating spores (25). These observations are significant because DNA repair through homologous recombination cannot occur in germinating spores that regularly carry only one copy of their genomes. In light of the fact that an NHEJ repair apparatus has been identified in *B. subtilis* as well as in other spore-forming bacteria (28), it is tempting to speculate that the substantial resistance of spores to conditions that effect double-strand DNA breaks, such as desiccation and ionizing irradiation, might be promoted by NHEJ repair processes occurring during germination within their tight and ordered genomes.

In 1983, Zimmerman and Pheiffer suggested that within the highly crowded intracellular environment, which promotes DNA condensation, blunt-end DNA ligation is likely to represent a widely occurring and highly effective process in vivo (31). The considerations presented in this article vindicate this insight and underline the crucial contribution of structural determinants to DNA repair under extreme conditions (19).

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