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Unfolding of the Bacterial Nucleoid Both In Vivo and In Vitro as a Result of Exposure to Camphor

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Both prokaryotic and eukaryotic cells are sensitive to killing by camphor; however, the mechanism by which camphor kills has not been elucidated. We report here that camphor unfolds the nucleoid of *Escherichia coli* and that unfolding does not require DNA replication, translation, or cell division. We show that exposure of isolated nucleoids to camphor results in unfolding of the chromosome.

Camphor vapors are lethal to many organisms (1, 11, 13). To date, there are two manners by which cells can become resistant to camphor treatment. First, both prokaryotic and eukaryotic cells that have doubled their DNA content per cell are resistant (1, 15). Second, *Escherichia coli* cells which increase the condensation of their chromosome are resistant (7).

While investigating this second mode of resistance, we examined by microscopy the effect on cells of treatment with camphor. In untreated cells, the nucleoid occupies approximately a third of the cytoplasm, whereas in treated cells, the nucleoid occupies the entire cytoplasm (7). This expansion of the nucleoid could be the result of decondensation of the nucleoid or overreplication of the chromosome, or it could be an indirect consequence of the inhibition of some other cellular process.

To investigate the nature of the nucleoid expansion in the presence of camphor, we pretreated cells separately with inhibitors of each of the macromolecular synthesis processes, followed by treatment with camphor. To determine the concentration of inhibitor and the time of treatment that are effective, cells actively growing in Luria-Bertani broth were treated with the inhibitor and the number of viable cells was measured over time (Fig. 1). Cells were treated with 5-fluorouracil (400 μg/ml) and uracil (200 μg/ml) (Fig. 1A) to inhibit DNA replication (2), with cephalexin (50 μg/ml) (Fig. 1A) to inhibit cell division (16), with rifampin (20 μg/ml) (Fig. 1B) to inhibit RNA synthesis (10), with chloramphenicol (50 μg/ml) (Fig. 1B) to inhibit protein synthesis (16), or with 5-fluorouracil (400 μg/ml) (Fig. 1C) to inhibit DNA replication. For all inhibitors, a 60-min treatment at the stated concentration proved effective in inhibiting cell growth (Fig. 1).

To determine the effect of inhibiting each macromolecular synthesis process on the action of camphor, separate 5-ml cultures of cells were treated with each inhibitor for 60 min, 0.5 g of camphor was added to the cells, and incubation was continued for 90 min at 37°C. During the camphor treatment, the inhibitors were present at the indicated concentrations. The cells were then stained with the DNA-specific fluorescent dye DAPI (4,6-diamidino-2-phenylindole) (1 μg/ml; Sigma, St. Louis, Mo.) and placed on poly-L-lysine-treated slides (7). They were subsequently viewed under both UV and visible light sources with a 100× Neofluor objective on a Zeiss phase-contrast microscope and were photographed as previously described (15).

Inhibiting DNA replication, DNA replication and transcription, protein synthesis, or cell division did not prevent camphor from causing the nucleoids to expand (Fig. 2B, C, E, and F, respectively). Rifampin by itself caused the nucleoids to decondense, and camphor did not alter this reaction (12) (Fig. 2D). From this experiment, it is not possible to determine the effect of inhibiting RNA synthesis on nucleoid expansion by camphor. As can be seen (Fig. 2E), treatment of cells with

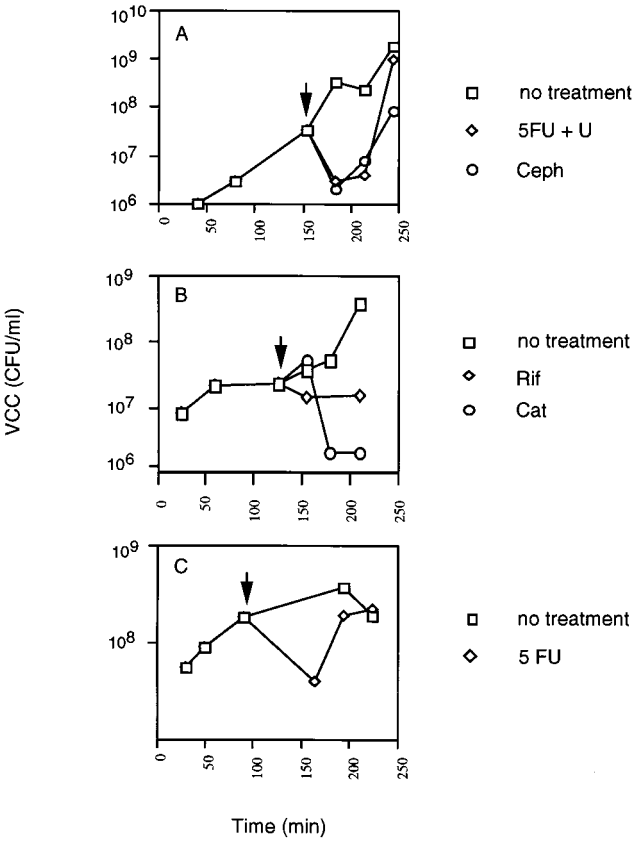

FIG. 1. Viable cell counts (VCC) of NT3 after treatment with various inhibitors. The arrows indicate the time of addition of each compound. Ceph, cephalexin; 5FU + U, 5-fluorouracil plus uracil; Cat, chloramphenicol; Rif, rifampin. See the text for details.

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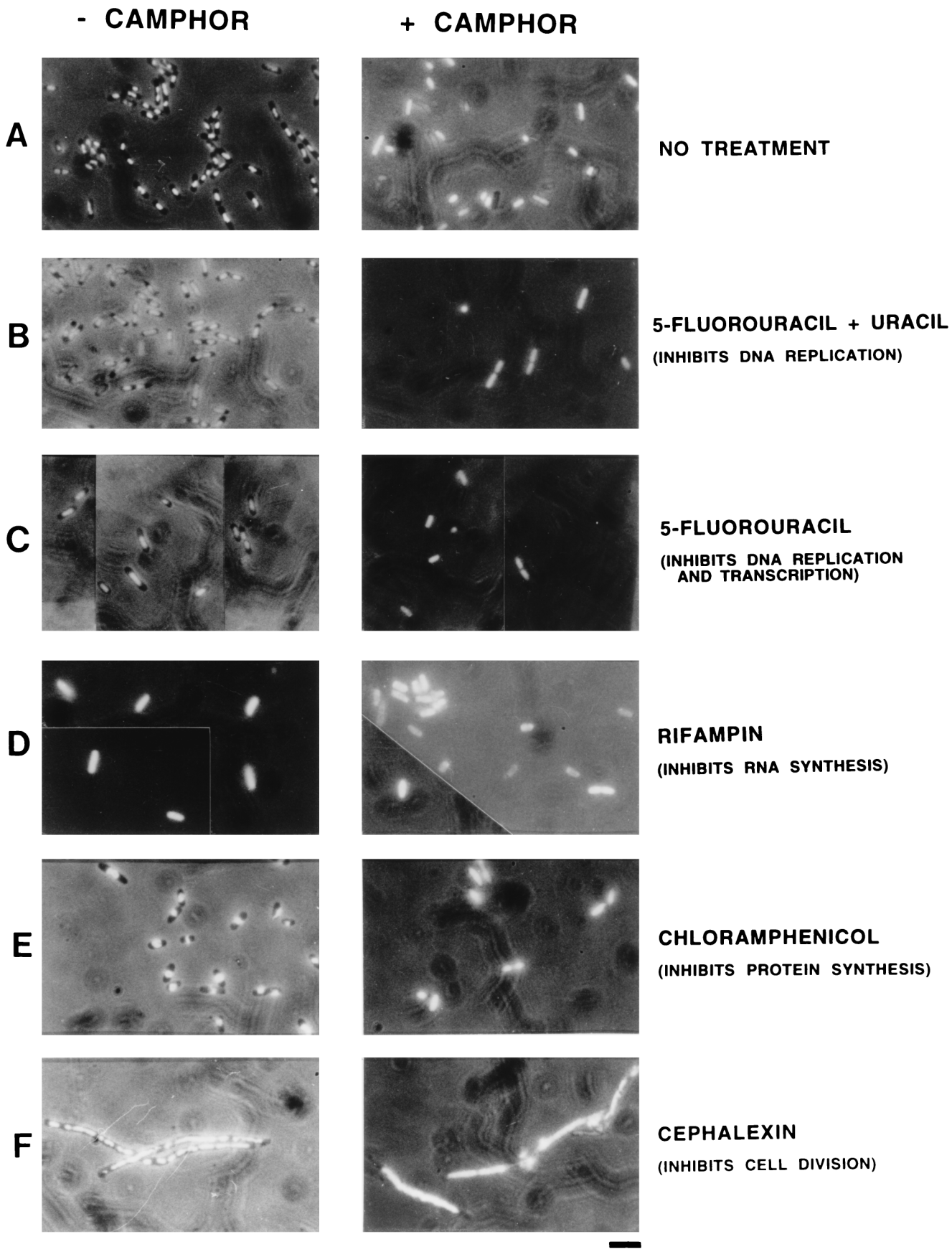

FIG. 2. DAPI-stained NT3 cells viewed by fluorescence microscopy before (column 1) and after (column 2) camphor treatment. The inhibitor used in each row is listed at the right. See the text for details. Bar, 2 μm.

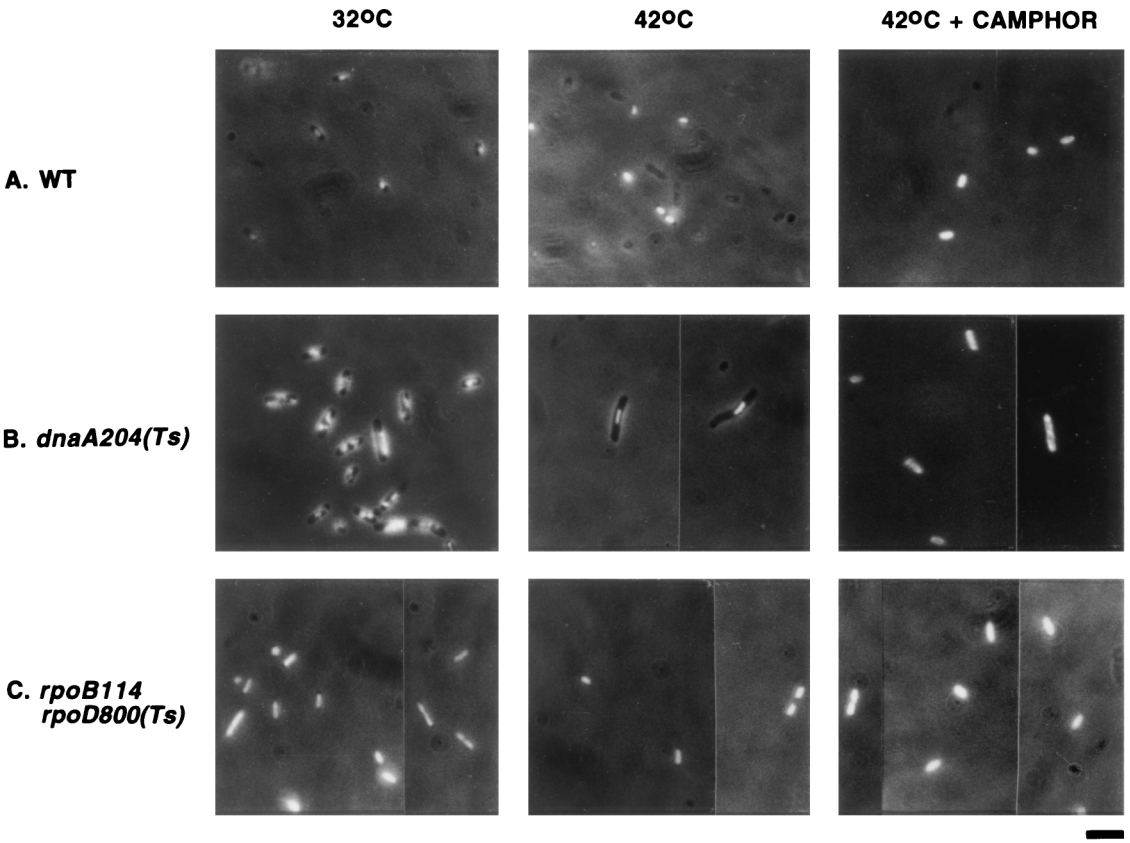

FIG. 3. Effects of temperature shifts on wild-type (WT) and mutant cells with and without camphor treatment. (A) NT3 cells grown at 32°C (column 1), shifted to 42°C for 1 h (column 2), or shifted to 42°C for 1 h and then treated with camphor at 42°C for 90 min (column 3). (B) The same experiment with an isogenic strain carrying *dnaA204*(*Ts*). (C) The same experiment with the *rpoB114 rpoD800*(*Ts*) double mutant. Bar, 2 μm.

chloramphenicol actually causes the nucleoid to condense more than in untreated cells to form a characteristic doughnut-shaped structure (8). This ultracondensed nucleoid is still subject to disruption by camphor. Treatment of cells with cephalexin (Fig. 2F) causes the cells to form filaments (6) and the nucleoids not to be as uniformly shaped as those of wild-type cells, but the nucleoids are still decondensed by camphor. From these data, transcription, protein synthesis, and cell division are not required for unfolding of the nucleoid by camphor.

The data from the 5-fluorouracil-plus-uracil treatment (Fig. 2B) demonstrate that camphor cannot be leading to nucleoid expansion via DNA overreplication, since expansion occurs even when DNA replication is blocked (Fig. 2B). To independently verify that DNA replication is not required for camphor decondensation, we carried out a temperature shift experiment with a temperature-sensitive mutant that is defective in initiation of DNA synthesis [*dnaA204*(*Ts*)] (5). Cells were grown at 30°C, shifted to 42°C for 60 min to block new rounds of DNA replication from initiating, and subsequently treated with camphor for 90 min at 42°C to maintain the block of initiation. As expected, the DNA initiation mutant exhibited a decrease in the DNA-to-mass ratio after the shift to the nonpermissive temperature (Fig. 3B, column 2) (5), and this DNA could be decondensed by camphor (Fig. 3B, column 3). Because 5-fluorouracil plus uracil interferes with DNA synthesis and *dnaA204*(*Ts*) interferes with the initiation of DNA replication, we conclude that DNA replication is not required for decondensation of the nucleoids by camphor.

Because treatment with rifampin decondenses the nucleoid by itself (12), we decided to use an alternative method to determine the need for RNA synthesis in decondensation by camphor. Beginning with an *rpoB114 rpoD800*(*Ts*) double mutant (18) that is temperature sensitive for RNA synthesis due to mutations in the β subunit (*rpoB*) and σ subunit (*rpoD*) of RNA polymerase, we first raised the temperature to 42°C for 60 min to inhibit RNA synthesis. Subsequently, we treated the cells with camphor for 90 min at 42°C and processed them as for the inhibitor studies. As shown in Fig. 3C, the temperature-sensitive RNA polymerase mutant has decondensed nucleoids at all temperatures, which makes it impossible to use this assay to determine the effect of inhibiting RNA synthesis on decondensation by camphor.

The data presented above indicate that camphor decondenses the nucleoid independently of new DNA replication, protein synthesis, or cell division. No conclusion regarding the requirement for RNA synthesis can be drawn. This suggests that camphor may be interacting directly with the DNA in the nucleoid or with a preexisting protein or RNA component of the nucleoid to mediate unfolding. If this is true, then camphor should be able to decondense isolated nucleoids in vitro. To test this idea, we isolated nucleoids and exposed them to camphor.

Three types of preparations of nucleoids from *E. coli* can be isolated. In the classic preparation (14, 17), the nucleoids are isolated in 1 M NaCl. If the procedure is carried out at 25°C, then membrane-free nucleoids (type I) are predominant, whereas if the procedure is carried out at 10°C, membrane-

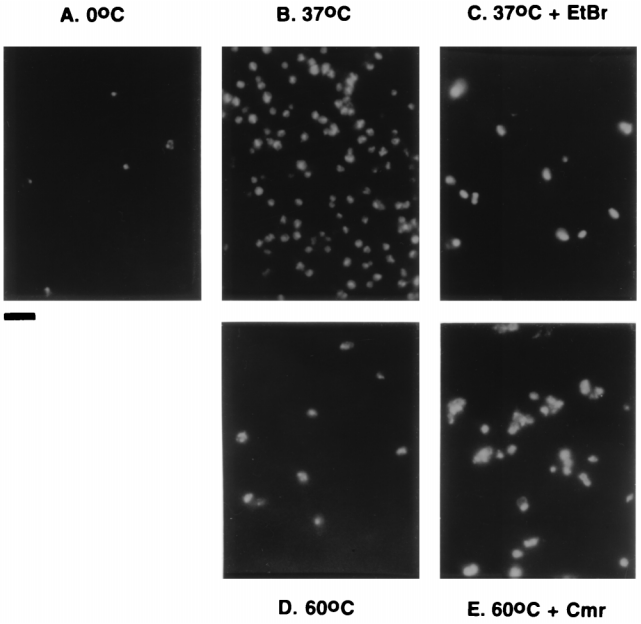

FIG. 4. Effects of camphor on type III nucleoids in vitro. (A) Nucleoids isolated from NT3 cells as previously described (9); (B) the same nucleoids incubated at 37°C for 90 min; (C) the nucleoids incubated with 1 mg of ethidium bromide (EtBr)/ml at 37°C for 90 min; (D) the nucleoids incubated at 60°C for 90 min; (E) the nucleoids incubated at 60°C with camphor (Cmr) for 90 min. Bar, 2 μm.

attached nucleoids (type II) are predominant (17). Nucleoids can also be isolated by using 10 mM spermidine to stabilize the nucleoids instead of 100 mM NaCl (type III) (9). This is reported to result in somewhat more physiologically relevant nucleoids that are more stable than type I or type II nucleoids (4).

We isolated type III nucleoids and treated them with camphor at different temperatures (0, 37, 50, and 60°C) for 30, 45, 60, 75, or 90 min. Camphor is active as a vapor; using approximately 0.5 g per 1.5-ml tube ensures saturation by the vapors, and increasing the temperature increases the amount of vaporization. The nucleoids were then stained with DAPI at room temperature and visualized by microscopy by the same procedure as for whole cells (see above). The most effective time for observing a visible change in the nucleoids was after 90 min of camphor treatment, exactly the same as in whole cells; after shorter times, smaller changes were observed (data not shown).

Figure 4 shows the results of decondensing type III nucleoids and Fig. 5 shows the analysis of these results. As can be seen, type III nucleoids held at 0°C are very compact (Fig. 4A and 5A). As the temperature was increased to either 37°C (Fig. 4B and 5B) or 60°C (Fig. 4D and 5D), the nucleoids appeared larger and bound approximately threefold more DAPI. Nucleoid staining intensity was calculated by measuring the intensity of 15 nucleoids from each preparation with an Eagle-Eye II (Stratagene, La Jolla, Calif.) and averaging the values. When the nucleoids were treated at 60°C with camphor (Fig. 4E and 5E), they expanded further and their ability to bind DAPI further increased approximately threefold over the nucleoids held at 60°C without camphor. As a positive control for expansion, the nucleoids were also exposed to 1 mg of ethidium bromide/ml (3) at 37°C (Fig. 4C and 5C) and exhibited an additional approximately threefold increase in intensity over nucleoids held at 37°C without ethidium bromide. Figure 5A, B, D, and E also demonstrate that nucleoids held at 0, 37, or 60°C or treated at 60°C with camphor, respectively, exhibit a relatively narrow intensity distribution. Nucleoids treated at 37°C with ethidium bromide show a broad intensity distribution, although the majority (14 of 15) have a higher staining intensity than nucleoids held at 37°C without ethidium bromide treatment (Fig. 5C). All attempts to isolate type I or type II nucleoids from our strains have met with limited success. While unhelpful, this result was not completely unexpected as the original technique was somewhat strain specific (17).

The results presented here indicate that camphor decondenses the bacterial nucleoid in the absence of DNA replication, translation, or cell division. Interfering with RNA synthesis by two different means affects nucleoid decondensation independently of camphor. Camphor is capable of decondensing the nucleoid in vitro and increases the nucleoid's ability to bind DAPI by a factor of about three. It is not possible from these studies to determine the exact amount of decondensation caused by camphor because the relationship between decondensation and detection of DAPI binding is influenced by many factors and will most likely not be linear. Determining the degree of decondensation after camphor treatment must await further experiments using type I and II nucleoids. However, the data clearly indicate that camphor has a dramatic effect on the nucleoid both in vivo and in vitro. The fact that camphor unfolds the chromosome in vitro indicates that it must be interacting with a preexisting structure on the folded

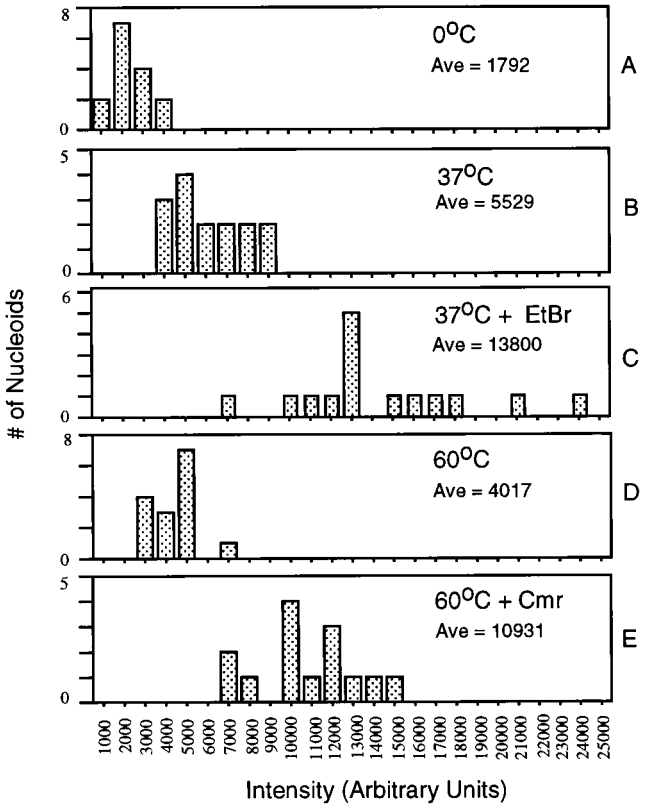

FIG. 5. Staining intensity distribution of type III nucleoids under each of the indicated conditions. Photographs of the nucleoids were scanned with an Eagle-Eye II, and the intensity of 15 nucleoids from each preparation was measured. The number of nucleoids at each intensity is plotted against the different intensities obtained. Nucleoids listed at an intensity of 1,000 have a value between 0 and 1,000, those shown at 2,000 have a value between 1,001 and 2,000, etc. Ave, average; EtBr, ethidium bromide; Cmr, camphor.

chromosome. This could be either the DNA itself or a protein(s) or RNA(s) needed to keep the nucleoid compacted. The fact that camphor has an effect on both prokaryotic and eukaryotic cells suggests that the component(s) it interacts with is present in many different cell types.

From studies on chromosomally encoded camphor resistance mutants, one way that cells can become resistant to camphor is to double the amount of DNA per cell (1, 11, 15). Why doubling the amount of DNA in the cell leads to camphor resistance is unclear. It is possible that the extra DNA affects the packing of the DNA in the nucleoid, making it harder for the camphor to unfold it. This could be the case if camphor interacts with the DNA. Alternatively, it is possible that the extra DNA induces the cell to make more of the components that condense the chromosome, leading to camphor resistance. This could be the case if camphor interacts with a component of the condensing machinery. Studying the effects of camphor on susceptible cells and our camphor-resistant mutants should shed light on the component(s) affected by camphor and allow us to begin to dissect how the bacterial chromosome is folded to fit inside the cell.

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