Overproduction of Three Genes Leads to Camphor Resistance and Chromosome Condensation in *Escherichia coli*

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ABSTRACT

We isolated and characterized three genes, *crca*, *cpe* and *crb*, which when present in high copy confer camphor resistance on a cell and suppress mutations in the chromosomal partition gene *mukB*. Both phenotypes require the same genes. Unlike chromosomal camphor resistant mutants, high copy number *crca*, *cpe* and *crb* do not result in an increase in the ploidy of the cells. The *cpe* gene has been previously identified as a cold shock-like protein with homologues in all organisms tested. We also demonstrate that camphor causes the nucleoids to decondense in *vivo* and when the three genes are present in high copy, the chromosomes do not decondense. Our results implicate camphor and *mukB* mutations as interfering with chromosome condensation and high copy *crca*, *cpe* and *crb* as promoting or protecting chromosome folding.

Camphor vapors, which are lethal to many organisms, can be survived by mutants that exhibit an increase in DNA content per cell. Such mutants have been found in a number of bacteria (OGG and ZELLE 1957; WON 1950), including *E. coli* (TRUN and GOTTESMAN 1990, 1991), yeast (BAUCH 1941), and molds (ROPER 1952; SANSOME 1946). While it has been recognized for several decades that camphor dramatically affects cells, its mechanism of action has yet to be elucidated. One possible explanation for the selection of cells with an increased DNA content is that camphor resistance requires one or more genes to be present in multiple copies for the cell to be resistant to camphor. A second possible explanation is that camphor interferes with a specific cellular process and this can be circumvented by increasing the DNA content per cell.

To investigate how camphor impacts on the cell, we have screened a multicopy plasmid library for those plasmids which confer camphor resistance. From this approach, we expect to find either the gene(s) that must be overproduced to result in camphor resistance or gene(s) that indicates what cellular process is affected by camphor. We report here the identification of a chimeric plasmid that contains *E. coli* chromosomal DNA inserted into pBR322 and that confers camphor resistance on a wild-type cell. This new plasmid, pNT2, contains three genes that are required for the camphor resistance phenotype. These same three genes, when present in high copy number, also suppress defects in the chromosomal partitioning gene, *mukB* (YAMANAKA et al. 1994b and this study). We present data that strongly suggest that treatment of cells with camphor causes the chromosome(s) to decondense and that overproduction of the three genes helps to recondense the chromosome(s).

MATERIALS AND METHODS

Strains: The strains used in this study are isogenic derivatives of the *E. coli* K-12 strain, MC4100, except where noted, and are listed in Table 1.

Reagents: Restriction endonucleases and T4 DNA ligase were acquired from New England Biolabs (Beverly, MA) and used according to the manufacturer's protocols. PCR fragments were cloned using the TA Cloning System from Invitrogen (San Diego, CA). Ampicillin (Amp) was used at a final concentration of 50 µg/µl.

Assaying for camphor resistance: Assays for camphor resistance were carried out as previously described (TRUN and GOTTESMAN 1990). Unless otherwise noted, cells were plated on LB media with Amp and grown overnight at 37°.

Isolation and mapping of the camphor resistance-confering plasmid, pNT2: A Sau3A partial digest of chromosomal DNA from MC4100ΔgalΔlon ligated into the BamHI site of pBR322 was obtained from J. TREMPY. Recombinant plasmids were transformed into NT3 and Amp-resistant colonies were isolated on LB + Amp agar at 37°. Colonies were replica plated to LB + Amp agar in the presence of camphor vapors. pNT2 was mapped by probing the Kohara library of lambda phage that represents the entire chromosome of *E. coli* (KOHARA et al. 1987). Briefly, pNT2 was labeled with 32P-dCTP (Amersham, Arlington Heights, IL) using the Random Primers DNA labeling system (Gibco-BRL, MD). The labeled pNT2 was used to probe a filter containing DNA from all of the Kohara phage (Takara, Japan). Hybridization was carried out as previously described (SAMBROOK et al. 1989).

X-ray irradiation: Strains NT555, NT3 and NT3 transformed by pNT2 and pBR322 were grown overnight in aerated minimal media at 30°. Cells were diluted, grown to an

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TABLE 1

<table>
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<th>Strains or plasmid</th>
<th>Genotype</th>
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OD₆₀₀≈0.5 and irradiated in a Gamma-Cell 220 (Atomic Energy of Canada, Ottawa, Canada) at a dose rate of 107 Gy/min. Dosimetry was done using Fricke solution (1 mM FeSO₄·7H₂O, 1 mM NaCl in 0.8 N H₂SO₄) read at 302 nm (FRICKE and BROWNSCOMBE 1933). The temperature increase of the samples during irradiation was less than 5° above ambient temperature. Full electron equilibrium was ensured for all irradiations. Five milliliters of each sample were exposed to X-rays for varying amounts of time corresponding to dosages of 0, 200, 400, 600, 800 and 1000 Gy. The samples were then diluted and plated on LB agar to determine the percent survival of each culture. Survival curves were plotted semilogarithmically, and each curve is the average of three experiments.

Assaying resistance to antibiotics and camphor derivatives: Cultures of NT3 transformed with pNT2, pKH4a, pBR322 and pCRII were grown overnight. Each sample (100 µl) was mixed with 3 ml of molten 0.7% top agar and poured onto LB + Amp plates. Dispense-o-disc antimicrobial susceptibility test disks from Difco (Detroit, MI) were placed on the solidified top agar. The antibiotics tested were Amp (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), lincomycin (2 µg), nalidixic acid (30 µg), rifampin (5 µg) and streptomycin (10 µg). Plates were incubated overnight at 37° and zones of inhibition of growth by each compound were determined. Resistance to the camphor-related compounds, as well as colchicine, benzimidazole, oxysterccycline and isonicotinic acid hydrazide were assayed by placing a small amount (~0.5 g) on the solidified top agar, incubating overnight and examining the plates for zones of growth inhibition. Because the camphor derivatives, camphoric acid, norcamphor, borneol, norborneol and naphthalene are able to vaporize at low temperatures like camphor, they were assayed in the same manner as camphor.

DNA cloning and sequencing: Subclones of pNT2 were constructed as follows: The 700-bp EcoRI fragment from pNT2 was inserted into the multiple cloning site of pMLB1107, yielding pKH3. pESL1, pNT5 and pNT3 were constructed
from pNT2 that was partially digested with EcoRI and then religated, thus deleting various EcoRI segments (see Figure 1). pNT5 lacks the 1.2-kb EcoRI fragment, pNT3 lacks the 700-bp EcoRI fragment, and pESL1 lacks both of these fragments.

The 2.2-kb EcoRI-HindIII fragment from pNT5 was cloned into pBluescript II KS+ (Stratagene, San Diego, CA), yielding pKH1 (see Figure 1). pKH2 was created by deleting the KpnI-HindIII segment from pKH1. pKH3 was created by subcloning the 700-bp EcoRI fragment from pNT2 into the EcoRI site of pBR322.

pKH4a through pKH9a were constructed by PCR amplification (protocol from Invitrogen) of DNA segments from pNT2. Primers roughly 150bp upstream and downstream from the proposed open reading frames (ORFs), crcB, crcE, and crcA were used. The PCR products were ligated into the multiple cloning site of pCRRI. Oligonucleotides used were K1 (5’-CAT TGC CAC CTC CTG TCA-3’), K2 (5’-AGA TAC GTC AGC AAG AAT-3’), K3 (5’-CTT CAA CCG CAC ACT AAA-3’), K4 (5’-GAT GCG TTA TTT TTG ATG TTA-3’), K5 (5’-GCA GAA TTT AGG CCC TTA GGG-3’), and K6 (5’-GCA GAA AAC GAC GCA TCA-3’). pKH4a was constructed from an insert amplified with primers K1 and K6, pKH5a with K1 and K2, pKH6a with K3 and K4, pKH7a with K5 and K6, pKH8a with K1 and K4 and pKH9a with K5 and K6. The genes in pKH4a through pKH9a were cloned so that they are in the same orientation as the lac promoter present in pCRRI. The oligonucleotides used were K1 (5’-GAG TTA TTT TTG ATG TTA-3’) and K6 (5’-GGA GAA AAC GAC GCA TCA-3’).

pKH2 was created by deleting the KpnI-HindIII segment from pKH4b with EcoRV and HindIII sites of pACYC184. pMDG3 was constructed by restricting pKH4b with EcoRV and HindIII sites of pACYC184.

Cloning of cold shock protein genes: Primers approximately 100 bp upstream from the initiation site and downstream of the termination codon of cspA, cspB, cspC, and cspD were synthesized. Each gene was amplified by PCR from NT3 chromosomal DNA. Each amplified PCR product was then ligated into the multiple cloning site of pCRRI. The oligonucleotides used for PCR amplification were 5’-TGG GAA GCC GGC ACA GCT-3’ and 5’-TGG TAG CCA TTG TTT CAT-3’ for cspA, 5’-GCA GTC TGC ATT TCA GGT-3’ and 5’-TGC TAG CCA TTG TTT CAT-3’ for cspB, 5’-AGC GGG TGG TTT TTC GAT-3’ for cspC, and 5’-GGA GAA AAC GAC GCA TCA-3’ for cspD.

Transient camphor assay: Cells were grown overnight in LB at 37°C. The overnight culture was diluted and grown to an OD600 of 0.5. Cells were incubated in the presence or absence of camphor at 37°C for 0, 45 or 90 min. Samples of the cultures (100 μl) were transferred to an Eppendorf tube and incubated for 5 min at room temperature with 5 μl of toluene and 1 μg/ml DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO). Poly-L-lysine (1% stock solution in saline) was placed on a clean microscope slide and incubated at room temperature for 3 min. The slide was then washed with distilled water and air dried, and the sample was placed on the prepared slide. Twenty microliters of 1 μg/ml para-phenylenediamine (free base; Sigma) in 90% glycerol was placed on the slide (to prevent bleaching of the DAPI) and a coverslip was placed over the suspension. Excess moisture was removed by gentle pressure on the coverslip with a tissue and subsequently the coverslip was sealed. Slides were viewed under a X100 Neofluor objective on a Zeiss microscope using both UV and visible light. Photographs were taken on Kodak 400 film using an extended ASA setting and developed using Diafine, according to the manufacturer’s directions.

Prior to using the toluenese and DAPI staining procedure described above, three staining protocols were compared. Wild-type cells, after a minimum of 10 generations in balanced growth, were (a) stained with only DAPI, (b) treated with toluene and DAPI or (c) treated with formaldehyde and DAPI. The cells treated with the formaldehyde exhibited a marked change in cell size and shape compared with treatment (a) and, thus, formaldehyde was eliminated as a general staining method. The cells and their corresponding nucleoids

**Figure 1.** The chromosomal inserts in pNT2 and its derivatives. The relevant subclones, the amount of DNA they contain, their growth properties and their camphor phenotypes are indicated. Asterisk indicates that the camphor phenotype cannot be scored because of an effect on cell growth (see text for details).
treated by either method (a) or (b) looked essentially the same (data not shown) with the major difference being that there was a more even staining of the entire population when the cells were treated with toluene. For this reason, we routinely stain using a brief room temperature treatment with toluene followed by DAPI. In staining cells of many different genotypes, we see a very large difference in nucleoid appearance depending on the strain background and/or the growth conditions. For these reasons, all of the strains to be compared are isogenic, constructed at the same time, and grown for a minimum of 10 generations in balanced growth to eliminate potential artifacts.

To test the effects of camphor on cells in the absence of specific macromolecular synthesis processes, cells were grown to an OD~0.5~ and treated for 90 min with 5-fluorouracil and uracil (40 and 20 μg/ml, respectively, Sigma), 5-fluorouracil (40 μg/ml), rifampicin (20 μg/ml, Sigma), chloramphenicol (50 μg/ml, Sigma), spectinomycin (25 μg/ml, Sigma) or cephalexin (10 μg/ml, Sigma). Cells were then treated for 90 min at 37°C with camphor and the antibiotics present simultaneously. Cells were subsequently processed for microscopy as described above.

For the adaptation to plates, cells were patched to LB + Amp plates and incubated in the presence or absence of camphor for 4, 6 or 8 hr. Cells were scraped from the plate and resuspended in 100 μl of LB. They were stained, viewed and photographed as described above.

RESULTS

Isolation and mapping of a multicopy plasmid conferring camphor resistance: To investigate the mechanism of camphor resistance, we isolated plasmids from a library containing wild-type genome inserts in pBR322, that confer camphor resistance (CmrR) on E. coli. Of 100,000 transformants screened, 28 colonies were camphor-resistant. Twenty-seven carried an identical plasmid, since named pNT2, and one carried an overlapping clone. When pNT2 was used to probe the Kohara library (KOHARA et al. 1987), an ordered lambda library of the entire E. coli chromosome, only phage 168 hybridized with the recombinant plasmid (data not shown). This indicates that pNT2 carries DNA from the 14.2 min region on the E. coli physical map. The restriction map of the insert of pNT2 matches the published map of E. coli at this location (Figure 1) (KOHARA et al. 1987). pNT2 carries 3.9 kb of chromosomal DNA.

Phenotypes of cells containing pNT2: E. coli cells carrying pNT2 exhibit resistance to camphor, but no growth defects when examined at 25°C, 30°C, 37°C, 39°C or 42°C on a variety of rich, indicator (MacConkey) or minimal agar. In direct contrast, the chromosomal CmrR mutant, mbrA4, exhibits sensitivity to growth on rich media or at high temperatures (TRUN and GOTTESMAN 1990). At nonpermissive conditions, mbrA4 mutants become filamentous and continue to increase the amount of their DNA before their subsequent death (TRUN and GOTTESMAN 1990). pNT2 containing cells look similar to wild-type cells at the conditions tested (32°C and 37°C in rich media, data not shown). At this level, the chromosomally encoded camphor resistance and plasmid-encoded resistance do not behave identically.

Determination of DNA content by X-ray irradiation: Because all previously described CmrR mutants are diploid (BAUCH 1941; SANSOME 1946; OGG and ZELLE 1957; TRUN and GOTTESMAN 1990), we determined if pNT2 increases the DNA content of the cell. Cells that contain multiple chromosomes are more resistant to X-ray irradiation because they can use their sister chromosome to repair any double-stranded breaks caused by the X-rays (for review see KUSHNER 1987). Therefore, we can use resistance to X-ray irradiation as a measure of cellular ploidy (LUCKE and SARECHK 1953). NT3 (wild type) and NT555 (mbrA4) served as controls, as their DNA content had previously been determined by flow cytometry (TRUN et al. 1991). As shown in Figure 2, the haploid strain, NT3, is significantly more sensitive to X-rays than the diploid strain, NT555. NT3 carrying pNT2 does not exhibit any difference in X-ray sensitivity compared to NT3 carrying no plasmid or the pBR322 control (Figure 2). Thus, while pNT2 confers camphor resistance, it does not appear to affect the DNA content of the cells.

Resistance to camphor-related compounds and antibiotics: One possible mechanism for camphor resistance by pNT2 is that it alters the cell permeability such that certain compounds, including camphor, are no longer able to enter the cell. We tested this hypothesis by determining the susceptibility of NT5, NT555 (mbrA4) and NT3 carrying either pNT2 or pBR322 to derivatives of camphor and to a variety of antibiotics. There was no difference in resistance to any of the camphor-related compounds (camphoric acid, norcamphor, borneol, norborneol or naphthalene), compounds that affect chromosomal dosage (colchicine,
benzimidazole, oxytetracycline or isonicotinic acid) or antibiotics (Amp, chloramphenicol, erythromycin, kanamycin, lincomycin, nalidixic acid, rifampicin or streptomycin) between any of the cells tested. Each of the camphor derivatives affects cells in the same manner but at a different concentration, due to its vaporization. The sequences from 938 to 2442 had been submitted as cspE (YAMANAKA et al. 1994b). cspE (cold shock protein E) is proposed to be a gene encoding a 69 amino acid (aa) polypeptide. CspE shows 70% identity and 81% similarity to the major E. coli cold shock protein, CspA (GOTTESMAN et al. 1990), as well as high homology to the other E. coli cold shock proteins: CspB (LEE et al. 1994), CspC (LEE et al. 1994) and CspD (GOTTESMAN et al. 1990). There is striking homology between CspE and eukaryotic proteins with cold shock or Y box domains and plant glycine-rich cell wall proteins (Figure 4).

The sequences from 938 to 2442 had been submitted to GenBank as gicA (growth in cold). This ORF is proposed to encode a polypeptide of 186 aa. A third ORF, 100 bp downstream of cspE, is a previously undescribed gene that could encode a protein of 81 aa. A promoter search found the presence of possible promoters immediately upstream of cspE and gicA, though none were found upstream of the ORF (Figure 3). Because the only reference to gicA is as a sequence in GenBank, we have chosen to refer to this ORF as crcA (confers resistance to camphor). By analogy, the previously undescribed ORF downstream of cspE will be referred to as crcB.

By deleting and subcloning various segments from pNT2, six plasmids were constructed: pNT5, pNT3, pESL1, pKH1, pKH2 and pKH3 (Figure 1). Of these, only pNT5 and pKH1 confer camphor resistance. From these data, we can conclude that when cspA, cspE and crcB are present in high copy number (pNT2, pNT5 and pKH1), the plasmids confer camphor resistance. When cells carry cspA and cspE (pNT3 and pESL1) or just crcB (pKH3) in high copy, they are not camphor-resistant. Unexpectedly, when cells contain a plasmid with cspE and crcB but lacking cspA, they exhibit a marked reduction in growth. In cells carrying a plasmid

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\text{Figure 3. — The sequence of the chromosomal insert of pNT2 from the Sph site to the EcorI sites. The three open reading frames are indicated by the shaded boxes. The potential promoters (-10 and -35) in front of cspA and cspE are also indicated by shaded boxes. }
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that affects growth, assaying camphor resistance becomes less accurate because the effects of reduced growth are difficult to differentiate from the effects of camphor. Thus, a clone may reduce cell viability to an extent that its camphor resistance phenotype may be masked. For this reason, if a plasmid severely affects cell growth, we cannot use it to determine which genes are required for the phenotype.

To further investigate the relationship between crcA, cspE and cspB and camphor resistance, PCR-generated fragments containing only the gene(s) in question were cloned (Figure 5). pCRII, a pUC derivative that contains the lac promoter followed by multiple cloning sites and has a higher copy number than pBR322 was used as the recipient of these fragments. When all three genes are present and the lac promoter is reading backwards through the genes (pKH4b), the plasmid confers camphor resistance. If the genes are inverted with respect to the lac promoter (pKH4a), the plasmid markedly affects the growth rate of the cells and camphor resistance cannot be scored. The most probable reason for this difference can be seen when only crcA is cloned downstream of the lac promoter. If the lac promoter is reading in the same direction as crcA (pKH7a), the plasmid again affects the growth of the cells. Whereas, when the lac promoter is reading backwards through cspE (pKH7b), there is no growth defect and the plasmid does not confer camphor resistance. Thus, having the lac promoter reading in the same direction as cspE correlates with the growth defect phenotype and implies that too much CrcA is detrimental to the cell.

When csaB (pKH7b), cspE (pKH6a) or cspB (pKH5a) are present individually in high copy, they do not affect cell growth and do not confer camphor resistance on the cells. If cspE and cspB are present in high copy without csaB (pKH2, pKH8a and pKH8b), the cells exhibit severe growth defects and camphor resistance cannot be scored. The growth defects are orientation-independent, unlike those described above for csaB. If csaB and cspE are present in very high copy without cspB (pKH9a and pKH9b), cells again have a growth defect and camphor resistance cannot be scored. The growth defect is again independent of the orientation of the genes with respect to the lac promoter. Thus, csaB and cspE on
pBR322 grow normally and are camphor sensitive but too much of these gene products affects growth.

From all of the subclones, we can conclude that when all three genes are present, they confer camphor resistance (pNT2, pNT5 and pKH4b). No gene by itself can confer camphor resistance (pKH5a, pKH6a and pKH7b). crA and crpE without crcB do not confer camphor resistance (pESL1 and pNT3). We cannot tell if crpE and crcB can confer camphor resistance because overproduction of these two genes (pKH2, pKH8a or pKH8b) without crA compromises the growth of the cell. While we have not directly tested a clone carrying crA and crpE for camphor resistance, such a clone has been tested for a second phenotype of crA, crpE and crcB in high copy, namely suppression of mukB mutations (see below). In all of the subclones we have tested (see below and Figure 5), if a plasmid confers camphor resistance, it also suppresses mukB mutations. The crA, crpE clone fails to suppress mukB mutations and thus, by analogy, should not confer camphor resistance. We are currently constructing plasmids to test these two combinations of genes for both phenotypes.

**crA, crpE and crcB on different copy number plasmids:**
To determine if the levels of crA, crpE and crcB are important for the camphor resistance phenotype of cells carrying the plasmid, two additional subclones were constructed. The chromosomal insert from pKH4b was cloned into a pSC101 derivative, pTA108 [copy number ~10–14 copies per cell (ARMSTRONG et al. 1984)] and pACYC184 [~20 copies per cell (CHANG and COHEN 1978)]. When the three genes are present on either of these low copy number plasmids, they do not confer camphor resistance on the cell (data not shown). Only when they are present on pBR322 (pNT2) or a pUC derivative (pKH4b) do they confer camphor resistance. Thus, camphor resistance requires that the three genes be present in high copy number.

**crA, crpE and crcB suppression of the phenotypes of mukB mutations:**
The crpE gene was originally identified as an overproduction suppressor of a point mutation in the mukB gene of E. coli (YAMANAKA et al. 1994b). mukB encodes an 180-kD protein that is involved in chromosomal partitioning (NIKI et al. 1991, 1992). mukB106 is a point mutation that affects the ATP binding region of MukB (YAMANAKA et al. 1994a). The ATPase domain is embedded in the NH2 terminal globular domain of the protein (NIKI et al. 1992). Cells carrying mukB106 exhibit a 100-fold increase in DNA-less cells to ~5% of a culture, filamentation of the cells, unfolding of the nucleoids and temperature sensitivity (NIKI et al. 1991). When a high copy number plasmid library was used to identify temperature-resistant suppressors of mukB106, a clone carrying crpE was found (YAMANAKA et al. 1994b). The clone also carried crA and crcB; however, this was not noted in the original report (YAMANAKA et al. 1994b). To show that crpE was involved in the suppression of mukB106, the authors made a 4-bp insertion at the BstEI restriction site (see Figure 1) in the middle of the crpE gene. This insertion abolished the suppression phenotype (YAMANAKA et al. 1994b).

To determine if crA or crcB are involved in the suppression of mukB mutations, the plasmids carrying one, two or all three of the genes were transformed into a mukB deletion strain, a mukB106 strain and a mukB103 strain. The mukB103 mutation resides in the carboxyl terminal globular DNA-binding domain (YAMANAKA et al. 1994a). The mukB deletion is the most severe of the three mutations and strains carrying it will only form colonies at room temperature (NIKI et al. 1991; FUNNEL and GAGNIER 1995). As shown in Figure 5, only plasmids that carry all three genes are able to partially suppress the mukB deletion strain to growth at 30°. The mukB deletion was moved into MC4100 (the parent of NT3), and all of the plasmids behaved as described above. In contrast, the two mukB point mutations have proven intractable to transduction (the original strains are resistant to Plvir) and, therefore, were tested in a different genetic background. In this background, all of the pUC-derived plasmids cause the strains to grow poorly and to exhibit a decrease in cell viability. For this reason, only the pBR322 derivatives were tested for their ability to suppress the point mutations in mukB. Only the plasmid that carries all three genes (pNT2) is capable of suppressing mukB103 and mukB106 (data not shown). From these experiments, we conclude that only plasmids that carry all three genes are capable of suppressing the mukB mutations. In all cases, if a plasmid confers camphor resistance, it also suppresses mukB mutations: These two phenotypes are always present together.

**Overproduction of the other E. coli cold shock protein genes and camphor resistance:**
The screen that identified crpE as an overproduction suppressor of mukB106 also revealed that overproduction of cspC, a related cold shock gene, could suppress mukB106 (YAMANAKA et al. 1994b). Therefore, the four other E. coli cold shock protein genes known to be homologous to crpE were cloned into pCRII and tested in the camphor assay (Table 2). When pKH10 (cspa) was transformed into wild-type E. coli, the cells formed small colonies in the presence of camphor vapors. NT3 carrying pKH11, pKH12, and pKH13 (cspB, cspC, and cspD, respectively) do not exhibit any resistance to camphor. Cells with pKH11 do show reduced growth, whereas none of the other cloned cold shock genes alters cell growth. From these results we can conclude that cspB, cspC or cspD in high copy do not lead to camphor resistance; cspa in high copy leads to partial camphor resistance.

**Transient camphor treatment and cell morphology:**
Given that overproduction of crA, crpE and crcB both suppress mukB mutations and lead to camphor resistance, it is reasonable to investigate if both phenotypes are related. By this argument, the deletion of mukB and
TABLE 2
High copy number cold shock genes and camphor resistance

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gene</th>
<th>Growth*</th>
<th>Camphor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKH10</td>
<td>cspA</td>
<td>++++</td>
<td>R/S</td>
</tr>
<tr>
<td>pKH11</td>
<td>cspB</td>
<td>+++</td>
<td>S</td>
</tr>
<tr>
<td>pKH12</td>
<td>cspC</td>
<td>++++</td>
<td>S</td>
</tr>
<tr>
<td>pKH13</td>
<td>cspD</td>
<td>++++++</td>
<td>S</td>
</tr>
</tbody>
</table>

R, resistant; S, sensitive.

* Growth refers to the ability to form colonies of the same size as a wild-type strain; ++++ is the same as a wild-type strain.

camphor would have the same effect on the cell. From previous studies, two lines of evidence suggest that MukB plays a role in partitioning the chromosome. First, mukB mutant strains exhibit a 100-fold increase in DNA-less cells (NIKI et al. 1991). Microscopy of the mukB deletion-containing cells shows that the DNA, when present, is found throughout the cell and not condensed into a tightly folded nucleoid (NIKI et al. 1991). Overproduction of crcA, cspE, and crcB reverses both the number of DNA-less cells and the nucleoid condensation state (YAMANAKA et al. 1994b). The second line of evidence comes from the structure of MukB. The two globular domains connected by a rod-shaped coiled coil, complete with hinge in the middle of the coiled coil structure (NIKI et al. 1991) is very similar to the structure proposed for the Smc family of eukaryotic proteins (STRUNNIKOV et al. 1993; PETERSON 1994). Included in this family are numerous members that have been shown to be involved in condensing chromosomes at very defined stages in the development of the cells (STRUNNIKOV et al. 1993; CHUANG et al. 1994; HIRANO and MITCHISON 1994). Thus, one function of MukB may be to help condense the chromosome so that it can be partitioned and in the mukB mutants, the chromosome is decondensed. If the two phenotypes seen when the three genes are overproduced are related, it follows then that camphor may function by decondensing the chromosome and overproduction of crcA, cspE and crcB should prevent or overcome this decondensation.

To test this idea, we developed a transient camphor treatment. Cells grown to an OD$_{600}$ ~ 0.5 were exposed to camphor vapors at 37°C in liquid media for 45 or 90 min or on solid media for 4, 6, or 8 hr. The cells were harvested and briefly treated with 3% toluene and 10 μg/ml of DAPI to stain the DNA. After adhering the cells to slides with poly-l-lysine, they were viewed under ×1000 magnification using both visible and UV light sources. As can be seen in Figure 6A, untreated wild-type E. coli contains a tightly condensed nucleoid that does not occupy the entire cell body. When these cells are treated in liquid media with camphor for 90 min, the DNA is no longer tightly condensed and has spread throughout the entire cell (Figure 6B). This could indicate that camphor is decondensing the chromosome or that it is preventing cell division while allowing DNA replication to continue. To determine if blocking DNA replication, transcription, translation or cell division blocks the effect of camphor on cells, we treated the cells with various drugs for 90 min and then exposed them to camphor vapors and examined them as described above. We used 5-fluorouracil and uracil [blocks DNA replication (COHEN et al. 1958)], 5-fluorouracil [blocks DNA replication and transcription (COHEN et al. 1958)], rifampicin [blocks RNA polymerase (McCLURE and Cech 1978)], chloramphenicol [blocks translation (WILLET 1984)], spectinomycin [blocks translation (WILLET 1984)] or cephalaxin [blocks cell division (WILLET 1984)]. In all cases, treatment with the drugs did not prevent the effect of camphor on the DNA in the cells (data not shown). Thus, it is likely that camphor decondenses the chromosome through direct physical contact(s) with either the DNA or some other component of the nucleoid.

When either pBR322 or pNT2 is transformed into the cells and they are exposed to camphor vapors in liquid media, there are slight but not dramatic differences in the appearance of the cells. Because camphor resistance is routinely assayed on agar plates, we patched pNT2- or pBR322-containing cells onto agar plates and exposed the plates to camphor vapors. The cells were then scraped from the plate, resuspended in LB broth and treated with toluene and DAPI (see above). As shown in Figure 7, after 6 hr of camphor treatment, cells carrying pBR322 (Figure 7A) contain decondensed nucleoids that encompass the entire cell. On the other hand, cells containing pNT2- (crcA, cspE and crcB contain mainly tightly condensed nucleoids (Figure 7B). Both populations contain cells that do not stain with DAPI. We do not know if these cells do not contain DNA or they simply do not stain. When the cells to be stained are taken from solid agar, we see this population of unstained cells, regardless of the genotype of the strain being tested. For this reason, we do not believe that the presence or absence of this population impacts on this study. From these results, we conclude that camphor, like the mukB mutations, results in decondensed nucleoids. Overproduction of the three genes, either at least partially recondenses the chromosome or partially blocks the decondensation of the chromosome.

**DISCUSSION**

We describe here the isolation and characterization of pNT2, a pBR322-derived plasmid that contains 3.9 kb of DNA from the 14.2-min region of the E. coli chromosome. pNT2 carries three genes, crcA, cspE and crcB, that are required to confer camphor resistance on cells and to suppress mutations in the chromosomal partition gene, mukB. Using deletion derivatives of pNT2,
we have shown that if a plasmid is missing one or two of the three genes, it fails both to confer camphor resistance and to complement mukB mutations. Thus the same genes are responsible for both phenotypes.

When cells are treated with camphor vapors, their chromosomes decondense and occupy the entire cytoplasm. When the effects of overproduction of creA, cspE and creB are tested, we can show that the nucleoids are no longer fully decondensed in the presence of camphor. Because overproduction of the three genes results in camphor resistance and suppresses mukB mutations, it is likely that mutations in mukB, similar to camphor, lead to chromosome decondensation. This prediction is supported by the evidence that in the absence of mukB the chromosomes appear decondensed (Niki et al. 1991).

Overproduction of creA, cspE and creB, as well as point mutations in specific chromosomal genes (the mbr mu-
E. coli which camphor resistance occurs should allow us to identify mechanisms that lead to camphor resistance. The novel condensing system defined by functional chromosome. The nucleoid and camphor resistance or increasing the amount of DNA per nucleoid (the csp mutants) effectively leads to a more condensed nucleoid and camphor resistance. If this model is correct, studying the ways in which camphor disrupts the condensed nucleoid, presumably in a nonspecific manner, increases (for review see Oberto et al. 1994; Petwijohn 1988), but, again like its eukaryotic counterpart, no one mechanism has been identified that can account for all 2000-fold. This suggests that several different mechanisms act to condense the chromosome to its physiological state. When camphor disrupts the condensed nucleoid, presumably in a nonspecific manner, increasing the amount or efficiency of any one of these mechanisms can result in camphor resistance. Thus, either increasing the amount of DNA per nucleoid (the mbr mutants) effectively leads to a more condensed nucleoid and camphor resistance or increasing the novel condensing system defined by cscA, cspE and cscB leads to a more condensed chromosome and camphor resistance. If this model is correct, studying the ways in which camphor resistance occurs should allow us to define the systems used to maintain a condensed and functional chromosome.

It has been extensively documented (Niki et al. 1991, 1992) that in the absence of mukB, the chromosomes are not correctly partitioned. One potential connection between condensation and partitioning is that condensation may have to occur prior to partitioning. Alternatively, condensation may be one of the reactions that forces the two daughter chromosomes apart after replication has taken place. Further characterization of the three genes should shed light on the connection between chromosome condensation and chromosome partitioning.

As stated above, when cells are treated with camphor, the chromosomes decondense and visibly fill the entire cell. This decondensation is not simply occurring because the cells are dead or dying. When cells are treated with 5-fluorouracil to block DNA replication and transcription, 5-fluorouracil plus uracil to block DNA replication or cephalaxin, which blocks cell division, no difference is seen in nucleoid morphology. In fact, when cells are treated with compounds that interfere with protein synthesis (e.g., chloramphenicol or spectinomycin) the nucleoids actually visibly condense (Kellenberger 1960; Morgan et al. 1967; Danelo-Moore and Higgins 1972). Each of these drugs has dramatic effects on cells, some being bacteriostatic and some bactericidal. Thus, blocking cell growth by a variety of means does not correlate with affecting the state of the nucleoid. This implies that there is some degree of specificity to the effect(s) of camphor. One compound that does affect nucleoid condensation is rifampicin (Danelo-Moore and Higgins 1972). When cells are treated with rifampicin, their nucleoids decondense and they look very similar to camphor-treated cells.

Our data demonstrating the effects of camphor on chromosomal condensation provide an interesting and testable explanation for why there are potentially several mechanisms that lead to camphor resistance. The E. coli chromosome, much like its eukaryotic counterparts, must be condensed approximately 2000-fold to fit inside an E. coli cell. Several genes have been identified that have modest effects on chromosomal condensation (for review see Oberto et al. 1994; Petwijohn 1987), but, again like its eukaryotic counterpart, no one mechanism has been identified that can account for all 2000-fold. This suggests that several different mechanisms act to condense the chromosome to its physiological state. When camphor disrupts the condensed nucleoid, presumably in a nonspecific manner, increasing the amount or efficiency of any one of these mechanisms can result in camphor resistance. Thus, either increasing the amount of DNA per nucleoid (the mbr mutants) effectively leads to a more condensed nucleoid and camphor resistance or increasing the novel condensing system defined by cscA, cspE and cscB leads to a more condensed chromosome and camphor resistance. If this model is correct, studying the ways in which camphor resistance occurs should allow us to define the systems used to maintain a condensed and functional chromosome.
recurring theme is binding to DNA and/or RNA. Given that the nucleoid contains a large amount of DNA and that RNA is one component that holds this DNA together (Worcel and Burgi 1974; Hecht and Pettijohn 1975), the fact that all of the Csp homologues bind to DNA, RNA or both is very intriguing. It is tempting to speculate that CspE, in conjunction with CrcA and CrcB, may affect chromosome condensation by interacting with the DNA in the nucleoid and/or the mRNA that is required to keep a nucleoid from coming apart. Further experimentation will decide if this idea has merit.

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