

Mutants Suppressing Novobiocin Hypersensitivity of a *mukB* Null Mutation

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The *mukB* gene is essential for the partitioning of sister chromosomes in *Escherichia coli*. A *mukB* null mutant is hypersensitive to the DNA gyrase inhibitor novobiocin. In this work, we isolated mutants suppressing the novobiocin hypersensitivity of the *mukB* null mutation. All suppressor mutations are localized in or near the *gyrB* gene, and the four tested clones have an amino acid substitution in the DNA gyrase beta subunit. We found that in the *mukB* mutant, the process of sister chromosome segregation is strikingly hypersensitive to novobiocin; however, the effect of novobiocin on growth, which was measured by culture turbidity, is the same as that of the wild-type strain.

The *smtA-mukF-mukE-mukB* operon (22) is located at the 21-min map position of the *Escherichia coli* chromosome. Deletion mutants of each *muk* gene show common phenotypes, such as frequent production of anucleate cells upon cell division, chromosome guillotining (cutting) by septum closure, and temperature-sensitive growth; however, they are nearly normal in chromosome replication, homologous recombination, mutation frequency, and sensitivity to UV irradiation (10, 11, 22; for a review, see reference 4). MukF, MukE, and MukB form a large complex (22). MukB is a member of the SMC superfamily (9). A MukB-green fluorescent protein fusion protein is localized as one, two, or four foci at regular cellular positions in the presence of both MukF and MukE (12). To examine the function of the MukB protein, many suppressor mutants of *mukB* mutations have been analyzed (for a review, see reference 4). Mutations of the *topA* gene encoding topoisomerase I suppress temperature-sensitive growth and anucleate-cell production (16). It has been demonstrated in synchronized cultures that replicated sister chromosomes are associated with one another for substantial times (6, 18). In contrast, results in randomly growing cultures in enriched medium appear to be consistent with the model in which replicated sister copies of *oriC* separate immediately after replication (8). However, it is hard to determine which model is right from the results of such random cultures, because the actual time of initiation of chromosome replication is not clear in this type of experiment.

A *mukB* null mutant is hypersensitive to novobiocin, an inhibitor (20). Deletion mutants of each of the *mukB*, *mukF*, and *mukE* genes and a deletion mutant lacking all three *muk* genes are hypersensitive to novobiocin (14). Weitao et al. (20) showed that a *seqA* null mutation suppressed temperature-sensitive growth, anucleate-cell production, and novobiocin hypersensitivity in the *mukB* null mutation. Inconsistently, Onogi et al. (14) reported that a *seqA* or *dam* null mutation partially suppressed temperature-sensitive growth but failed to suppress

the anucleate-cell production and novobiocin hypersensitivity of these *muk* null mutants.

It is not yet clear what the mechanism of the novobiocin hypersensitivity of these *muk* mutants is. What is the target protein that is hypersensitive to novobiocin in these *muk* null mutants? There are two possibilities. First, DNA gyrase could be the target of a low concentration of novobiocin in *muk* null mutants. Second, an unknown protein could be the target, which should be more sensitive to novobiocin than DNA gyrase and essential for growth only in the defective *muk* background but nonessential in the wild-type *muk* genetic background. In this work, to investigate the identity of the target protein, we isolated novobiocin-resistant suppressor mutants from the *mukB* null mutant strain and characterized them. We found that the beta subunit of DNA gyrase is the target protein of novobiocin in a *mukB* null mutant. We discuss the mechanism of novobiocin hypersensitivity of *mukB* null mutant cells.

MATERIALS AND METHODS

Bacterial strains. YK1100 (*trpC9941*) and AZ5372 (*trpC9941 ΔmukB::kan*) are derivatives of *E. coli* strain W3100, as described previously (14, 22). Bacterial cells were grown in Luria-Bertani (LB) medium at 22°C, which is the permissive temperature for AZ5372. QT186 (strain JC12334 [15]) has the *ma-300::Tn10* marker, which is linked with the *gyrB* gene.

Isolation of mutants that suppress novobiocin hypersensitivity of a *mukB* null mutation. Ten independent single colonies of strain AZ5372 grown on LB agar plates at 22°C were inoculated into LB liquid medium, and the cultures were grown to saturation at 22°C. Fifty microliters of each culture was spread onto an LB agar plate containing 100 or 1,000 μg of novobiocin/ml and incubated at 22°C for 5 days. Confluent growth of cells occurred on the plates containing 100 μg of novobiocin/ml, due to the large number of cells spread on the plates. However, only 10 to 30 novobiocin-resistant colonies appeared on each plate containing 1,000 μg of novobiocin/ml. Single colonies from each plate were picked and purified on LB agar plates at 22°C and named MQ40, MQ41, and MQ43 to MQ50. These purified clones were shown to have the same tryptophan requirement and kanamycin resistance at 22°C as the parental strain, AZ5372.

Sensitivity to DNA gyrase inhibitors. The colony-forming abilities of various strains were analyzed on LB agar plates containing various concentrations of novobiocin or nalidixic acid according to the method of Onogi et al. (14). Novobiocin and nalidixic acid were obtained from Sigma Chemical Co.

Transduction with phage P1vir. Suppressor mutant cells were infected with phage P1vir propagated on QT186 cells. About 500 tetracycline-resistant transductants were obtained on each LB agar plate containing 7.5 μg of tetracycline/ml after incubation at 22°C for 6 days. Fifty of the transductants were

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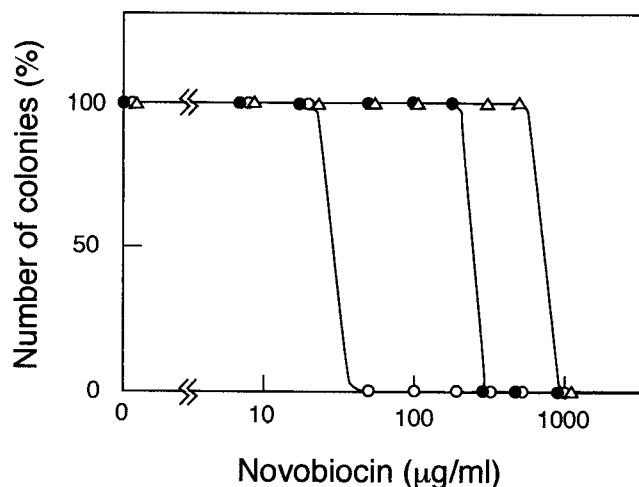


FIG. 1. Novobiocin sensitivities of various strains in colony formation. Solid circles, parental strain YK1100 (W3110 *trpC9941*); open circles, AZ5372 (W3110 *trpC9941 ΔmukB::kan*); open triangles, novobiocin-resistant suppressor mutants that were independently isolated from AZ5372. All 10 suppressor mutants showed the same sensitivity to novobiocin.

isolated from each plate, purified, and analyzed for novobiocin hypersensitivity on LB agar plates containing 300 µg of novobiocin/ml after incubation for 5 days at 22°C.

DNA sequencing. To determine the mutation sites by DNA sequencing, we amplified the 2,580 bp of DNA fragments comprising the *gyrB* gene and its flanking regions from chromosomal DNA samples of four suppressor mutants (MQ40, MQ43, MQ44, and MQ45) and the parental AZ5372 strain by PCR using the forward primer 5'GTGCTGAACACGTTATAGACATGTCGGA CG3' and the reverse primer 5'CAAGATTTTCGTAGGCCTGATAAGCGTA GC3'.

Fluorescence microscopy. Cells were stained with DAPI (4',6-diamino-2-phenylindole) for chromosomal DNA and observed with fluorescence and phase-contrast microscopes according to the method of Hiraga et al. (5).

Survival after irradiation with X rays. Cells grown exponentially in LB medium at 22°C were irradiated by X rays, diluted, spread on LB agar plates, and incubated at 22°C for 5 days. The colonies that appeared were counted.

RESULTS

Isolation and characterization of mutants that suppress novobiocin hypersensitivity of a *mukB* null mutation. Cells

grown at 22°C in LB medium were diluted, spread on LB agar plates containing various concentrations of novobiocin, and incubated at 22°C for 5 days. As shown in Fig. 1, the wild-type strain YK1100 (a *trpC9941* derivative of strain W3110) was able to form colonies in the presence of 200 µg of novobiocin/ml but not at 300 µg/ml. In contrast, the isogenic *mukB* null mutant AZ5372 (W3110 *trpC9941 ΔmukB::kan*) was able to form colonies at 20 µg/ml but not at 50 µg/ml. Thus, the maximum concentrations of novobiocin allowing the survival of >50% of cells were 200 µg/ml in YK1100 and 20 µg/ml in AZ5372. The *mukB* null mutant was 10-fold more sensitive to novobiocin than the wild-type strain, consistent with previous results (14).

To identify the target of novobiocin in the *mukB* null mutant, we isolated 10 novobiocin-resistant mutants in which the novobiocin hypersensitivity of the *mukB* null mutation was suppressed. These suppressor clones were analyzed for novobiocin sensitivity, temperature sensitivity, and anucleate-cell formation. All 10 clones were able to grow at 22°C in the presence of 500 µg of novobiocin/ml but not at 1,000 µg/ml (Fig. 1 and Table 1). Two clones, MQ40 and MQ41, showed partial suppression of temperature sensitivity; colony-forming abilities at 37°C were 6.5×10^{-3} and 1.2×10^{-2} , respectively, compared with that at 22°C. The other eight clones showed the same temperature sensitivity as the parental *mukB* null mutant; colony-forming ability at 37°C was $<2 \times 10^{-6}$ compared with that at 22°C (Table 1). All novobiocin-resistant clones retained the property of frequent production of anucleate cells at 22°C.

Mapping of suppressor mutations. To map the suppressor mutations, we first determined whether they are located in the *gyrB* gene, which encodes the beta subunit of DNA gyrase. The wild-type *gyrB* gene, located at the 83-min map position, can be cotransduced by phage P1vir together with the *tna-300::Tn10* marker of strain QT186, which is located near the *gyrB* gene. Eighty to 95% of tetracycline-resistant transductants in each case exhibited the same degree of novobiocin hypersensitivity as the parent strain, AZ5372. This suggested that when the wild-type *gyrB* gene of the donor QT186 was cotransduced with the *tna-300::Tn10* marker, the transductants became hypersensitive to novobiocin, like the parental AZ5372. This implies

TABLE 1. Properties of 10 mutants suppressing novobiocin hypersensitivity of *mukB* null mutation

Strain	Growth at 22°C with indicated concn of novobiocin (µg/ml) ^a					Colony-forming ability at ^b :		Formation of anucleate cells ^c
	0	20	100	300	500	22°C	37°C	
YK1100	+	+	+	-	-	1	1.3	-
AZ5372	+	+	-	-	-	1	$<2 \times 10^{-6}$	++
MQ40	+	+	+	+	+	1	6.5×10^{-3}	++
MQ41	+	+	+	+	+	1	1.2×10^{-2}	++
MQ43	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ44	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ45	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ46	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ47	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ48	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ49	+	+	+	+	+	1	$<2 \times 10^{-6}$	++
MQ50	+	+	+	+	+	1	$<2 \times 10^{-6}$	++

^a +, growth; -, no growth.

^b Colony-forming ability on LB agar medium at 22°C was defined as 1.

^c -, no anucleate cell; ++, many anucleate cells.

that the suppressor mutations are located in or near the *gyrB* gene. MQ40 and MQ41 showed partial suppression of temperature sensitivity as described above (Table 1). Novobiocin-hypersensitive clones among tetracycline-resistant transductants from MQ40 and MQ41 were unable to form colonies at 37°C on LB agar plates, like the parental AZ5372, implying that the mutation that partially suppresses temperature sensitivity is closely linked to the mutation conferring novobiocin resistance. To avoid the possibility of contamination, all tetracycline-resistant transformants were confirmed to have a tryptophan requirement and kanamycin resistance, like the parental strain, AZ5372.

We sequenced the *gyrB* DNA fragments obtained from MQ40, MQ43, MQ44, MQ45, and AZ5372. We found that in all suppressor mutants tested, the guanosine residue at position 407 (the adenosine residue of the start codon ATG is defined as +1) is changed to thymidine, resulting in the replacement of arginine at position 136 by leucine of the beta subunit of DNA gyrase. The arginine residue at position 136 is conserved in the GyrB proteins of various bacterial species. This arginine residue is one of the key amino acids implicated in novobiocin binding (7; <http://www.sanger.ac.uk/Software/Pfam/index.shtml>). MQ40 showed weak suppression of temperature sensitivity, in contrast to the other sequenced clones (Table 1). We speculate that MQ40 has a second mutation, which affects expression of the altered GyrB protein, outside the sequenced region. The *gyrB* gene is known to be located in an operon, *dnaA-dnaN-recF-gyrB*.

Effect of novobiocin on cell growth and chromosome segregation in *mukB* null mutant cells. To examine why the *mukB* null mutant is hypersensitive to low concentrations of novobiocin, we analyzed the effect of novobiocin on increase of turbidity of cultures in wild-type and *mukB* mutant strains. Cultures growing in LB medium at 22°C were incubated for 6 h in the presence of cephalaxin alone or cephalaxin plus novobiocin, as shown in Fig. 2. Cephalaxin (10 µg/ml) was added to the cultures in order to inhibit cell division to prevent guillotining of the chromosome by septum closure in the *mukB* null mutant (4, 11, 22), because we expected that the abnormal structure of the chromosome in *mukB* mutant cells would be clearly observed in elongated cells incubated in the presence of cephalaxin. Novobiocin was added at concentrations of 1,000 µg/ml in YK1100 and 100 µg/ml in AZ5372. These concentrations were five-fold higher than the maximum concentration of novobiocin allowing the survival of >50% of cells in each strain (Fig. 1). When cephalaxin (10 µg/ml) alone was added, the turbidity of cultures increased exponentially for at least 6 h in both strains, suggesting that DNA and protein syntheses continued normally, although cell division was inhibited by cephalaxin. The doubling times measured by turbidity increase were 155 and 190 min in YK1100 and AZ5372, respectively, under the conditions used. When 1,000 µg of novobiocin/ml was added to the culture of YK1100 together with cephalaxin, the turbidity increased ~1.5-fold for the first 2 h; however, the increase in turbidity was markedly inhibited after that (Fig. 2A). On the other hand, surprisingly, when 100 µg of novobiocin/ml was added to the culture of AZ5372, the turbidity increased exponentially for 6 h without any significant inhibitory effect (Fig. 2B), although the *mukB* mutant was unable to form colonies in the presence of the same concentration of

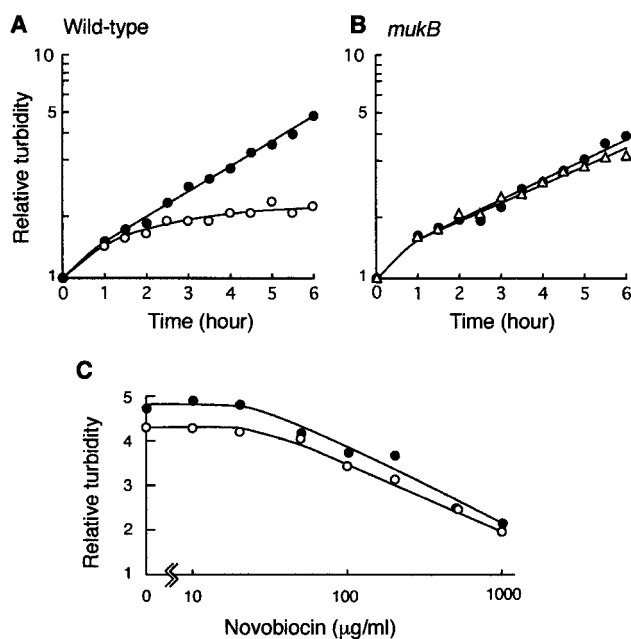


FIG. 2. Effects of novobiocin on growth of cultures in YK1100 and AZ5372. (A) YK1100. Solid circles, cephalaxin (10 µg/ml) alone; open circles, cephalaxin plus 1,000 µg of novobiocin/ml. (B) AZ5372. Solid circles, cephalaxin alone; open triangles, cephalaxin plus 100 µg of novobiocin/ml. (C) Final value of turbidity after incubation for 6 h with or without indicated concentrations of novobiocin. The turbidity at zero time is 1. Solid circles, YK1100; open circles, AZ5372.

novobiocin (Fig. 1). To confirm these results, we analyzed the effects of various concentrations of novobiocin on increase of turbidity after 6 h of incubation in both strains. As shown in Fig. 2C, the effects of novobiocin on growth were the same in both strains, implying that the effects of novobiocin on DNA and protein syntheses were the same in both strains. This indicates that the effect of *muk* mutation on the structure of chromosomal DNA, for example, decompaction or reduced superhelicity of chromosomal DNA (16, 19–21), does not affect DNA and protein syntheses, consistent with expectations. Thus, *mukB* mutant cells lost colony-forming ability in the presence of a low concentration of novobiocin, such as 100 µg/ml, even though DNA and protein syntheses continued at the levels of the wild-type strain. One might ask what the mechanism of novobiocin hypersensitivity is in the *mukB* mutant.

To answer this question, we observed cells by fluorescence microscopy after a 6-h incubation in the presence of cephalaxin and novobiocin. We found a striking effect on the structure of nucleoids in *mukB* mutant cells at 100 µg of novobiocin/ml. The nucleoids were large and frequently formed long twisted strings in elongated filamentous cells of AZ5372, as shown in Fig. 3G, I, J, and K. In contrast, nucleoids were normal in size and arranged at nearly equal distances in elongated filamentous cells in YK1100 at the same concentration of novobiocin (Fig. 3C). These results indicate that segregation of sister chromosomes is strongly inhibited by a low concentration of novobiocin in the *mukB* mutant but not in the wild-type strain.

After incubation with 1,000 µg of novobiocin/ml, YK1100

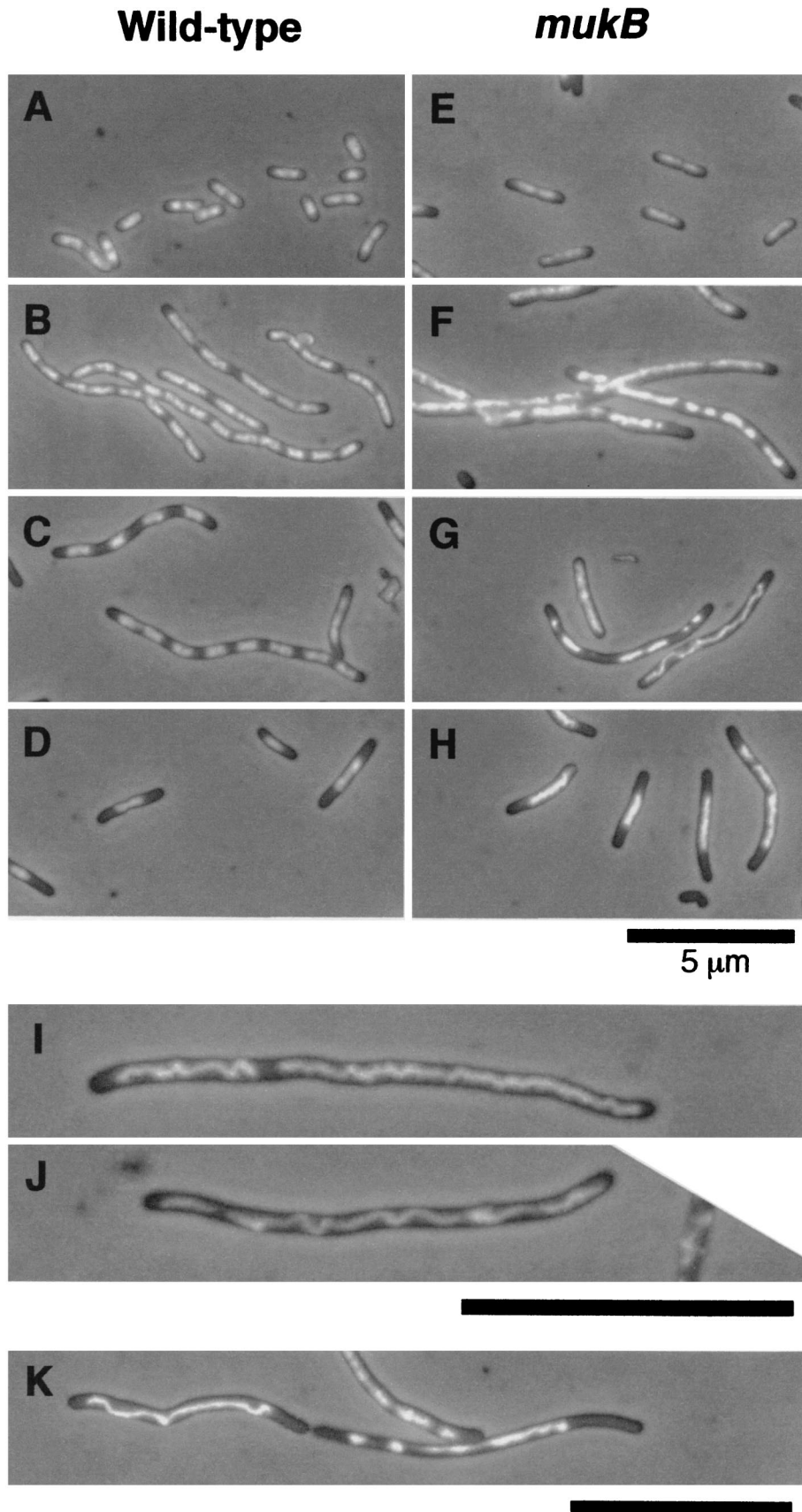


FIG. 3. Merged images of cells stained with DAPI by fluorescence and phase-contrast microscopy. (A to D) YK1100; (E to K) AZ5372. (A and E) Before the addition of antibiotics. (B and F) Cephalexin (10 $\mu\text{g/ml}$) alone for 6 h. (C, G, I, J, and K) Cephalexin (10 $\mu\text{g/ml}$) and novobiocin (100 $\mu\text{g/ml}$) for 6 h. (D and H) Cephalexin (10 $\mu\text{g/ml}$) and novobiocin (1,000 $\mu\text{g/ml}$) for 6 h. The scale bars represent 5 μm .

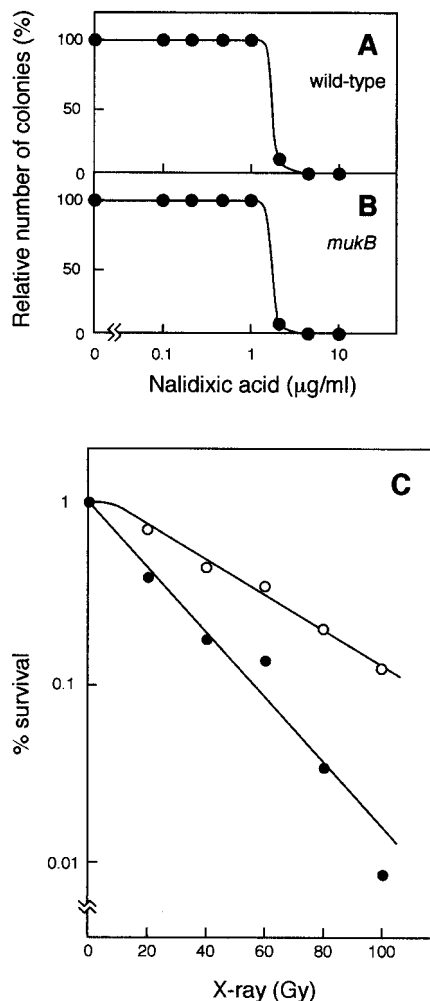


FIG. 4. Effects of nalidixic acid on colony-forming ability (A and B) and survival after X-ray irradiation (C). (A) YK1100; (B) AZ5372; (C) YK1100 (solid circles) and AZ5372 (open circles).

cells were small and had one nucleoid that was localized in the center of the cell or at the constriction site at midcell (Fig. 3D). In a minority of YK1100 cells, two nucleoids existed on both sides, flanking the constriction site. These results suggest strong inhibition of DNA and protein syntheses under these conditions, as expected. In AZ5372 cells incubated with 1,000 μg of novobiocin/ml, nucleoids were frequently larger than those of YK1100 cells (Fig. 3H). The large size of nucleoids in *mukB* mutant cells is presumably a result of a defect in the segregation of sister chromosomes (2, 6; M. Kohiyama, T. Onogi, S. Adachi, and S. Hiraga, unpublished data) before and after the addition of antibiotics.

Sensitivity to nalidixic acid and X-ray irradiation in *mukB* null mutant cells. We analyzed *mukB* null mutant cells for sensitivity to another DNA gyrase inhibitor, nalidixic acid. The maximum concentration of nalidixic acid allowing colony formation was 1 $\mu\text{g/ml}$ in both wild-type and *mukB* null mutant strains (Fig. 4A and B). The *mukB* mutant showed more resistance to X-ray irradiation than the wild-type strain (Fig. 4C). These results are discussed below.

DISCUSSION

The above-mentioned results indicate that the target protein that is hypersensitive to novobiocin in the *mukB* null mutant is DNA gyrase. Novobiocin inhibits DNA gyrase, resulting in a reduction in the superhelicity of circular DNA (1, 3). Another DNA gyrase inhibitor, nalidixic acid, inhibits DNA gyrase and produces double-strand breaks in DNA (17). We have observed no significant difference in sensitivity to nalidixic acid between the wild-type and *mukB* null mutant strains (Fig. 4A and B). This is consistent with our observation that the *mukB* mutant is more resistant to X-ray irradiation than the wild-type strain (Fig. 4C). The resistance to X rays might be due to a larger average number of chromosomes in *mukB* mutant cells (Kohiyama et al., unpublished). X-ray irradiation also induces double-strand breaks in DNA (2). These results indicate that the *mukB* null mutant is hypersusceptible to the reduction in superhelicity of chromosomal DNA but not to double-strand breaks. In this work, we found no significant difference between wild-type and *mukB* mutant strains in the novobiocin concentration that inhibits growth of cultures as measured by turbidity increase (Fig. 2). However, segregation of sister chromosomes is remarkably inhibited in AZ5372, but not in YK1100, by a low concentration (100 $\mu\text{g/ml}$) of novobiocin (Fig. 3). The abnormality in segregation of sister chromosomes should cause lethality (inability to form colonies) in *mukB* mutant cells. A step in the process of sister chromosome segregation might depend strongly on the DNA supercoiling function catalyzed by DNA gyrase in the *mukB* null mutant at the permissive temperature, 22°C. In *mukB* null mutant cells, the abnormal segregation in the presence of a low concentration of novobiocin might cause an irreversible defect, resulting in the loss of colony-forming ability.

In wild-type cells, the replisome complexes associated with diverging replication forks are closely associated with one another at midcell during the early phase of replication; however, they separate and migrate rapidly to one-quarter and three-quarter cell positions during replication. This event causes persistent separation of clockwise and counterclockwise replicating regions of the chromosome (4, 6, 13, 18). Protein-protein linkage between clockwise and counterclockwise replicating regions, e.g., by the hemimethylated DNA binding SeqA protein, might be broken off by the event. Two clusters of the MukFEB complex known to be located at the cell quarter positions (12) might participate in the event of migration of replication forks, because the *mukB* mutant has a defect in the regular subcellular localization of replication forks (Kohiyama et al., unpublished). Flow cytometry and immunofluorescence microscopy revealed that separation of sister chromosomes is delayed in *mukB* null mutant cells (Kohiyama et al., unpublished). This is consistent with frequent production of anucleate cells upon cell division in the mutant. The MukFEB complex might facilitate the resolution of interwound sister chromosomal DNA strands to form two separated nucleoids. Reduced superhelicity of bacterial and plasmid DNAs was observed after proteins in the *mukB* null mutant were removed (16, 19–21). However, no significant difference between the levels of compactness of nascent DNA labeled with 5-bromodeoxyuridine and bulk chromosomal DNA could be detected in the wild-type and *mukB* null mutant strains by im-

munofluorescence microscopy and fluorescence microscopy *in vivo* (Kohiyama et al., unpublished), in contrast to *in vitro* data (19–21). SeqA participates in the compactness of nascent and bulk chromosomal DNA *in vivo*, because decompaction of the chromosome was observed in the *seqA* null mutant but not in the *dam* null mutant *in vivo* (Kohiyama et al., unpublished). Temperature-sensitive growth, but not novobiocin hypersensitivity, of the *mukB* null mutation was suppressed by the *seqA* or *dam* null mutation (14). These results suggest that linkages of SeqA-SeqA interaction between nascent DNA segments in clockwise and counterclockwise replicating regions are probably harmful for cell growth in the genetic background lacking the MukFEB complex above 30°C in rich media. The MukFEB complex would act for separation of the SeqA-SeqA linkages between nascent DNA segments of clockwise and counterclockwise replicating regions. This idea is consistent with the phenomenon that the *seqA* or *dam* null mutations suppress the temperature sensitivity of the *mukB* null mutation.

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