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 (tcpC941) and AZ5372 (tcpC941 ΔmukB::kan) are derivatives of E. coli strain W3110, 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 trp-306::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 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
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’GTGCAGAACATGTTAAGACATGTGCAGA 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 × 10⁻⁹ 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::Tn 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth at 22°C with indicated concn of novobiocin (μg/ml)</th>
<th>Colony-forming ability at 22°C</th>
<th>Formation of anucleate cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  20  100  300  500</td>
<td></td>
<td>22°C  37°C</td>
</tr>
<tr>
<td>YK1100</td>
<td>+  +  +  +  +</td>
<td>1 1.3</td>
<td>+ +</td>
</tr>
<tr>
<td>AZ5372</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ40</td>
<td>+  +  +  +  +</td>
<td>1 6.5 × 10⁻³</td>
<td>++</td>
</tr>
<tr>
<td>MQ41</td>
<td>+  +  +  +  +</td>
<td>1 1.2 × 10⁻²</td>
<td>++</td>
</tr>
<tr>
<td>MQ43</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ44</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ45</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ46</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ47</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ48</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ49</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
<tr>
<td>MQ50</td>
<td>+  +  +  +  +</td>
<td>1 &lt;2 × 10⁻⁶</td>
<td>++</td>
</tr>
</tbody>
</table>

* +, growth; −, no growth.

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

++ −, 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 temper-
ature sensitivity as described above (Table 1). Novobiocin-
gene. MQ40 and MQ41 showed partial suppression of temper-
 gyrB
that the suppressor mutations are located in or near the

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 posi-
tion 407 (the adenosine residue of the start codon ATG is
defined as +1) is changed to thymidine, resulting in the re-
placement 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 tem-
perature 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 segre-
gation 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 cephalexin alone or cephalexin plus
novobiocin, as shown in Fig. 2. Cephalexin (10 μg/ml) was added to
the cultures in order to inhibit cell division to prevent guillo-
tining 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
cephalexin. Novobiocin was added at concentrations of 1,000
μg/ml in YK1100 and 100 μg/ml in AZ5372. These concentra-
tions were five-fold higher than the maximum concentration of
novobiocin allowing the survival of >50% of cells in each strain
(Fig. 1). When cephalexin (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 con-
tinued normally, although cell division was inhibited by ceph-
alexin. 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 cephalexin, 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 novobi-
ociocin/ml was added to the culture of AZ5372, the turbidity
increased exponentially for 6 h without any significant inhibi-
tory effect (Fig. 2B), although the mukB mutant was unable to
form colonies in the presence of the same concentration of
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 mu-
tant.

To answer this question, we observed cells by fluorescence
microscopy after a 6-h incubation in the presence of cephalexin
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 filamen-
tous cells in YK1100 at the same concentration of novobiocin
(Fig. 3C). These results indicate that segregation of sister chro-
mosomes is strongly inhibited by a low concentration of novo-
biocin 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 μg/ml) alone for 6 h. (C, G, I, J, and K) Cephalexin (10 μg/ml) and novobiocin (100 μg/ml) for 6 h. (D and H) Cephalexin (10 μg/ml) and novobiocin (1,000 μg/ml) for 6 h. The scale bars represent 5 μm.
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 µg/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 µg/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-

**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).
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 above 30 °C and would act for separation of the SeqA-SeqA linkages above 30 °C and would be likely harmful for cell growth in the genetic background lacking the mukB gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA 97:1671–1676.


