JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 7517–7526
0021-9193/92/237517-10$02.00/0
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Vol. 174, No. 23

# Identification and Characterization of the *smbA* Gene, a Suppressor of the *mukB* Null Mutant of *Escherichia coli*

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**The *mukB* gene encodes a protein involved in chromosome partitioning in *Escherichia coli*. To study the function of this protein, we isolated from the temperature-sensitive *mukB* null mutant and characterized 56 suppressor mutants which could grow at 42°C. Ten of the mutants also showed cold-sensitive growth at 22°C. Using one of the cold-sensitive mutants as host, the wild type of the suppressor gene was cloned. The cloned suppressor gene complemented all of the 56 suppressor mutations. DNA sequencing revealed the presence of an open reading frame of 723 bp which could encode a protein of 25,953 Da. The gene product was indeed detected. The previously undiscovered gene, named *smbA* (suppressor of *mukB*), is located at 4 min on the *E. coli* chromosome, between the *tsf* and *frr* genes. The *smbA* gene is essential for cell proliferation in the range from 22 to 42°C. Cells which lacked the SmbA protein ceased macromolecular synthesis. The *smbA* mutants are sensitive to a detergent, sodium dodecyl sulfate, and they show a novel morphological phenotype under nonpermissive conditions, suggesting a defect in specific membrane sites.**

During the bacterial cell cycle, replicated chromosomal-DNA molecules are spatially separated from each other prior to cell division. How are replicated chromosome molecules partitioned into the two daughter cells in bacteria? A microtubule apparatus for chromosome migration like that in eukaryotic cells has not been found in bacteria. However, nucleoids (folded chromosome masses) migrate rapidly and independently of elongation of cell length in *Bacillus subtilis* (40) and *Escherichia coli* (5, 14, 17), suggesting an unknown cellular apparatus acting for active positioning of nucleoids.

The overall process of chromosome partitioning includes two categories of mechanisms. The first category includes decatenation of replicated chromosomes, resolution of chromosome dimers or oligomers, and other topological events for the separation of daughter chromosomes. The second category includes mechanisms for the active positioning of daughter chromosomes at cell quarter sites before and/or during cell division. In the first category, conditionally lethal mutants showing the $Par^-$ phenotype have been described (18). Cells of these mutants become elongated and have a large nucleoid mass in the center of the cell at the nonpermissive temperature. Some of these cells have a mutation in *gyrA* or *gyrB*, genes which encode the subunits of DNA gyrase (19, 20, 25, 37, 43), or in *parC* or *parE*, genes which encode the subunits of topoisomerase IV (24, 26). These mutants are defective in decatenation of replicated chromosomes and/or in other topological events of replicated daughter chromosomes. Chromosome dimers produced by sister chromatid exchange are resolved by *recA*-independent recombination at a specific chromosome site, *dif* (7, 10, 28, 31).

To analyze the second category of mechanisms, we have developed a strategy to isolate mutants defective in the active positioning of daughter chromosomes to the quarter sites (16). In these mutants, replicated chromosomes are decatenated normally, but they tend to remain close to each other at the position where the chromosome was replicated. Thus, these mutants are able to grow normally except for the production of anucleate cells (chromosomeless cells), which are normal cell length, at a significant frequency (16). Anucleate cells are present as less than 0.03% of the total cell population in wild-type strains (16). We have isolated such anucleate-cell-producing mutants (*muk* mutants) and characterized some of them. The *mukA* mutants have mutations in the *tolC* gene (16). The *tolC* gene codes for an outer membrane protein (34) which is essential for excretion of hemolysin from the cell (46, 47). How the protein encoded by the *mukA* (*tolC*) gene is involved in chromosome positioning is unknown.

A second *muk* gene, *mukB*, is located at 21 min on the chromosome and codes for a newly identified 177-kDa protein (35). The predicted MukB protein consists of five distinct domains: an amino-terminal globular domain containing a consensus sequence for nucleotide binding; two α-helical coiled-coil domains and one globular domain in the central region; and a carboxyl-terminal globular domain which is rich in Cys, Arg, and Lys. The amino-terminal globular domain of the MukB protein was found to be homologous to dynamin D100 (36), which is a microtubule-associated mechanochemical enzyme obtained from rat brain. It was suggested that the MukB protein could be a promising candidate for a force-generating enzyme involved in positioning of the replicated and decatenated daughter chromosomes in *E. coli* (35). A *mukB* null mutant was able to form colonies at 22 but not 42°C (35). When grown at 22°C, this mutant was nearly normal in cell size, but approximately 5% anucleate cells with normal cell sizes were produced in growing cultures. When *mukB* null mutant cells grown at 22°C were transferred to 42°C, cells elongated heterogeneously, and nucleoids were located irregularly in the elongated cells. These observations suggested that the *mukB* gene product is required for chromosome positioning in the temperature range from 22 to 42°C and that the product is not essential for colony formation at 22°C but is essential at 42°C.

As one way of studying the cellular function of MukB protein, we isolated suppressor mutants which grew from the *mukB* null mutant at 42°C. In this paper, we report the

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TABLE 1. Bacterial strains

| Strain | Relevant genotype | Reference, source, or construction |
|---|---|---|
| W3110 | Prototroph | Y. Kohara |
| PB103 | *dadR trpE61 trpA62 tna-5* | 12 |
| GC7528 | Same as PB103 except for Δ*mukB*::*kan* | 35 |
| FS1576 | *recD1009 thi-1 thr-1 leuB6 lacY1 tonA21 supE44* | 41 |
| RK4788 | F⁻ Δ(*argF-lac*)*U169 araD139 recA1 rpsL150 flbB5301 deoC1 thi ptsF25 gyrA219 non metE70* Δ*btuB ompA zcb*::Tn*10* | (=ME8307) A. Nishimura |
| 112(#2) | HfrC *metB proA3 metD88 lac-3 tsx-76 relA1 zaf*::Tn*3* | (=ME8314) A. Nishimura |
| SJ16 | F⁻ *panD2 metB1 relA1 gyrA216* λ$^r$ *zad-220*::Tn*10* | (=ME8385) A. Nishimura |
| AZ5002 | Δ*mukB*::*kan smbA2* | From GC7528 (this study) |
| AZ5009 | Δ*mukB*::*kan smbA9* | From GC7528 (this study) |
| AZ5040 | Δ*mukB*::*kan smbA40* | From GC7528 (this study) |
| AZ5061 | *mukB*$^+$ *zcb*::Tn*10 smbA2* | P1*vir*/RK4788→AZ5002 |
| AZ5120 | *recD1009* (pKX110) | pKX110 DNA→FS1576 |
| AZ5134 | *recD1009* Δ*smbA*::*kan* (pKX110) | DNA[a]→AZ5120 |
| AZ5145 | *dadR trpE61 trpA62 tna-5* (pKX125) | pKX125 DNA→PB103 |
| AZ5149 | Same as AZ5145 except for Δ*smbA*::*kan* | P1*vir*/AZ5134→AZ5145 |

[a] The *Eco*RI DNA fragment of pKX122 contains the disrupted *smbA* gene.

isolation and characterization of a newly identified gene, *smbA*, whose mutations suppress temperature-sensitive growth of the *mukB* null mutant. The *smbA* gene product is essential for cell growth, and cells show a novel morphological phenotype in the absence of the normal function of the SmbA protein.

## MATERIALS AND METHODS

**Bacterial strains and media.** Bacterial strains used and their relevant genotypes are listed in Table 1. L medium and minimal E medium were prepared as described previously (35). When necessary, antibiotics were supplemented at the following final concentrations: ampicillin, 25 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 20 μg/ml; and tetracycline, 15 μg/ml.

**Isolation of temperature-resistant suppressors of the *mukB* null mutant.** Cells of strain GC7528 (Δ*mukB*::*kan*) were grown exponentially in minimal E medium supplemented with glucose (0.5%), Casamino Acids (0.4%), and tryptophan (50 μg/ml) at 22°C. The cells were treated with 1% ethyl methanesulfonate at 22°C for 80 min, diluted with medium, divided into 60 tubes ($10^3$ to $10^4$ viable cells per ml), and incubated overnight at 22°C and then at 42°C for 4 h. Each culture was diluted, plated on an L-agar plate, and incubated overnight at 42°C. Colonies grown at 42°C were tested for growth on L-agar plates containing kanamycin. Fifty-six temperature-resistant suppressor mutants, AZ5001 to AZ5056, were independently isolated.

**Microscopic observation of cells and nucleoids.** Cells grown in L medium were collected, washed with saline, dried on glass slides, fixed with methanol, bound tightly to the slides with poly-L-lysine, and then stained with DAPI (4′,6-diamino-2-phenyl-indole). Cell shape and nucleoids were simultaneously observed by the fluophase combined method of Hiraga et al. as described previously (16).

**Cloning and mapping of cold-sensitive suppressor gene.** A cosmid library of chromosomal DNA extracted from the wild-type strain W3110 was constructed by using the cosmid vector pHSG262 (8) as described previously (16). After in vitro packaging of recombinant cosmid DNA into λ phage particles, the cosmid library was used to infect the cold-sensitive suppressor mutant AZ5061 (*mukB*$^+$ *smbA2*). Both kanamycin- and cold-resistant colonies were isolated at 22°C on L-agar plates containing kanamycin. Recombinant cosmid DNAs were extracted and analyzed, and one of them, pKX19, was used for further study.

The Kohara library of λ transducing phages carrying chromosome segments (27), which covers almost the entire *E. coli* chromosome, was used to map the cloned suppressor gene by DNA hybridization. Each of the 476 recombinant λ DNAs were spotted onto a BA85 nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.). The filters were probed with the recombinant plasmid pKX19 DNA. DNA labeling and detection were carried out with the DIG-ELISA system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer's instructions.

Phage P1*vir* was used for general transduction of chromosomal markers. P1*vir*-mediated transduction was performed as described previously (33).

**DNA cloning and sequencing.** All DNA cloning was carried out essentially by following general procedures (32). DNA fragments for subcloning were purified from agarose gels with GeneClean (Bio 101, Inc., La Jolla, Calif.) according to the manufacturer's instruction. To purify the recombinant plasmid DNAs, QIAGEN (QIAGEN Inc., Chatsworth, Calif.) was used according to the specifications of the manufacturer. For sequencing, DNA fragments were subcloned onto M13mp18 and M13mp19 (49). DNA sequences were determined by the dideoxy-chain termination method (39) using Sequenase (United States Biochemical Corp., Cleveland, Ohio). All restriction enzymes and DNA modification enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Boehringer Mannheim Biochemicals.

**Identification of gene product.** To identify the gene product, plasmid-encoded proteins were analyzed by a prokaryotic DNA-directed translation system (Amersham, Arlington Heights, Ill.) used according to the manufacturer's instructions. Briefly, purified plasmid DNAs, *E. coli* extract, and L-[$^{35}$S]methionine (>1,000 Ci/mmol; Amersham) were incubated at 37°C for 1 h and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (30). The gels were soaked in EN$^3$HANCE (New England Nuclear, Boston, Mass.) and fluorographed.

**Assays for macromolecular synthesis.** The rates of cellular DNA, RNA, and protein synthesis were measured by pulse-labeling with [$^3$H]thymidine, [$^3$H]uridine, and [$^{35}$S]methionine, respectively. At 0, 1, 3, 5, 7, and 10 h after a

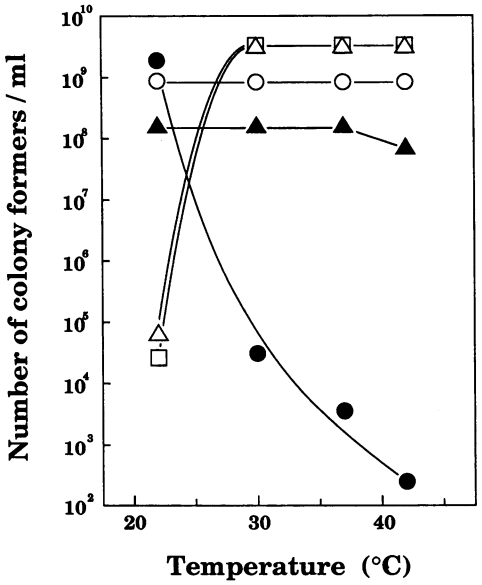


FIG. 1. Colony-forming abilities of mutants at various temperatures. Cultures of mutants were grown in L medium at permissive temperatures to mid-log phase (PB103 at 37°C; GC7528 at 22°C; and AZ5002, AZ5040, and AZ5061 at 42°C). The mutants were plated on L-agar plates and incubated at 22, 30, 37, and 42°C. ○, PB103 (*mukB*$^+$ *smbA*$^+$); ●, GC7528 (Δ*mukB*::*kan smbA*$^+$); △, AZ5002 (Δ*mukB*::*kan smbA2*); ▲, AZ5040 (Δ*mukB*::*kan smbA40*); □, AZ5061 (*mukB*$^+$ *smbA2*).

temperature shift-up to 42°C, 0.4-ml samples from cultures were removed, added to tubes containing the appropriate radiolabeled precursor ([$^3$H]thymidine and [$^3$H]uridine, 2 μCi; [$^{35}$S]methionine, 10 μCi), and incubated at 42°C for 2 min. Fifty microliters of each sample was spotted onto 3MM filter paper (Whatman, Clifton, N.J.). The filters were soaked in ice-cold 5% trichloroacetic acid and rinsed with ice-cold ethanol. Dried filters were counted in an Aloka Liquid Scintillation Counter. [*methyl*-$^3$H]thymidine (25 Ci/mmol), [5,6-$^3$H]uridine (44 Ci/mmol), and [$^{35}$S]methionine (>1,000 Ci/mmol) were purchased from Amersham.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D13334.

## RESULTS

**Isolation of suppressor mutants of the *mukB* null mutant.** Fifty-six extragenic suppressor mutants (AZ5001 to AZ5056), which were able to grow at 42°C, were independently isolated from the *mukB* null mutant GC7528. Ten of them (AZ5001 to AZ5010) were unable to grow at 22°C. We chose to study the suppressor mutant AZ5002, which showed cold sensitivity, first, since this phenotype could be used to clone the wild-type copy of the suppressor gene (named *smbA*, for suppressor of *mukB*) through complementation. Colony-forming abilities of PB103 (*mukB*$^+$), GC7528 (Δ*mukB*::*kan*), and AZ5002 (Δ*mukB*::*kan smbA2*) are shown in Fig. 1. Another class of suppressor mutants (AZ5011 to AZ5056) was also examined for colony-forming abilities. Strain AZ5040 (Δ*mukB*::*kan smbA40*) colony-forming ability was essentially the same as that of the *mukB*$^+$ strain PB103 (Fig. 1).

In order to determine whether the cold sensitivity phenotype shown by AZ5002 resulted from the *smbA2* mutation itself or from the combination of the Δ*mukB*::*kan* and *smbA2* mutations, the wild-type *mukB* gene was cotransduced with *zcb*::Tn*10* into AZ5002 by P1*vir* transduction. The resulting transductant, AZ5061 (*mukB*$^+$ *smbA2*), also retained the cold sensitivity phenotype (Fig. 1). These results indicated that the *smbA2* mutation corrected the temperature sensitivity of the *mukB* null mutant and that this mutation itself showed cold sensitivity, even in the *mukB*$^+$ background.

The *mukB* null mutant can grow at 22°C, and normal-size nucleate and anucleate cells were observed in medium E containing glucose, Casamino Acids, and L-tryptophan (35). Filamentous cells with irregularly distributed nucleoids along the cell were not observed at 22°C but were observed in the temperature range 30 to 42°C in the enriched minimal-salts medium (35). In L medium, anucleate and filamentous cells with nucleoids irregularly distributed along the cell were observed even at 22°C (Fig. 2C). All the *smbA* suppressor mutations we obtained corrected the temperature sensitivity for colony formation of the *mukB* null mutants and partially suppressed filamentation, but they did not prevent anucleate cell production (Fig. 2E, F, G, and H).

The *mukB*$^+$ *smbA2* strain AZ5061 hardly grew at 22°C in L medium and showed a very unique type of morphological phenotype after incubation for 4 h at that temperature: no visible contents in a quarter or a half of the cells at one of the poles or at the center (Fig. 2J and J′). Cells with these translucent segments were also directly observed without fixation under a phase-contrast microscope (data not shown). The results suggest that the septation site is very susceptible to plasmolysis in the *smbA2* mutant at the nonpermissive temperature, so that newborn daughter cells produced by the septation have a weak pole. The same morphological phenotype was observed in the Δ*mukB*::*kan smbA2* strain AZ5002 at the nonpermissive temperature, 22°C (Fig. 2F and F′). The SmbA protein may be involved in stabilization of the septation sites and/or poles of newborn cells.

Cells of the *smbA2* mutant AZ5061 and wild-type strain PB103 were labeled with [$^{35}$S]methionine for 2 h at 42°C in L medium. The labeled cells were washed twice with L medium to remove unincorporated [$^{35}$S]methionine, suspended in L medium, and incubated at 22°C for 5 h. Samples were removed at intervals and centrifuged to remove cells. The radioactivity of the supernatant was measured. The result indicated that labeled cellular components (proteins) in the *smbA2* mutant, as well as in the wild-type strain, did not leak into the medium (less than 5% of the total radioactivity incorporated into cells). When similar labeling experiments with [$^3$H]thymidine instead of [$^{35}$S]methionine were performed, less than 5% of $^3$H-labeled chromosomal DNA leaked into the medium after incubation at 22°C for 5 h in L medium; this was observed in the *smbA2* mutant as well as in the wild-type strain. Thus, it is unlikely that translucent segments are caused by release of cellular components to medium.

The *smbA2* mutant was highly sensitive to a detergent, SDS, at a range of 30 to 42°C. This also suggests that SmbA may be involved in membrane structure and/or function.

**Cloning and mapping of the *smbA* gene.** The *mukB*$^+$ *smbA2* strain AZ5061 was infected with a cosmid library from the wild-type genome, and kanamycin-resistant colonies which could grow at 22°C (Cs$^+$) were isolated. The recombinant plasmids were extracted and used as probes for DNA-DNA hybridization with the recombinant λ DNAs of the Kohara

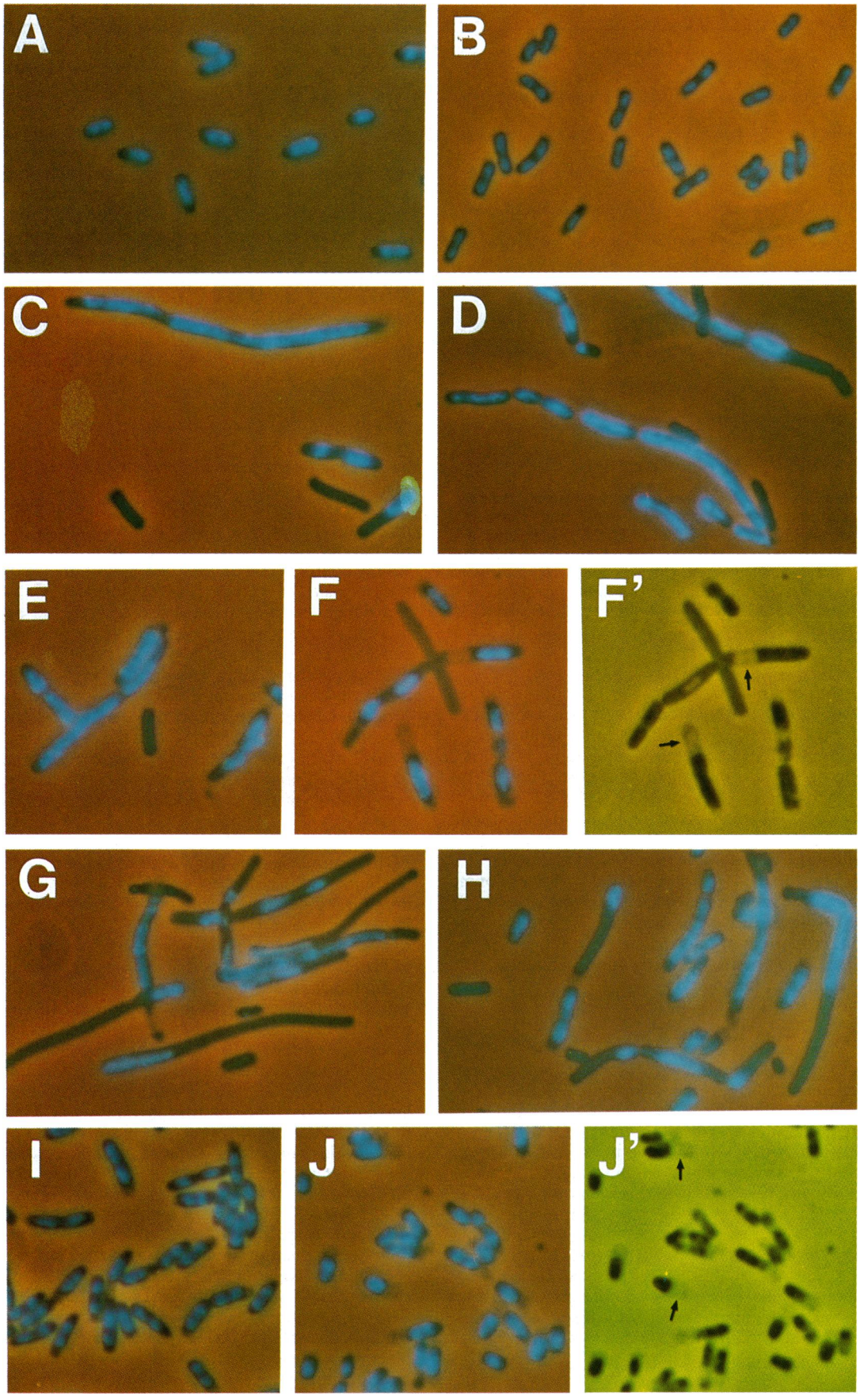
A
B
C
D
E
F
F'
G
H
I
J
J'

FIG. 2. Photographs of cells of strains PB103, GC7528, AZ5002, AZ5040, and AZ5061. Cells grown in L medium were photographed after DAPI staining by Hiraga's fluophase combined method (16). (A and B) Strain PB103 (*mukB*$^+$) was incubated at 22°C (A) and 42°C (B). (C) Strain GC7528 (Δ*mukB*::*kan*) was incubated at 22°C. (D) Strain GC7528 was incubated at the nonpermissive temperature of 42°C for 4 h. (E, G, and I) Strains AZ5002 (Δ*mukB*::*kan smbA2*), AZ5040 (Δ*mukB*::*kan smbA40*), and AZ5061 (*mukB*$^+$ *smbA2*), respectively, were incubated at 42°C. (F, H, and J) Strains AZ5002, AZ5040, and AZ5061, respectively, were incubated at the nonpermissive temperature of 22°C for 4 h. (F′ and J′) Phase-contrast photographs of cells corresponding to F and J, respectively. Arrows indicate the translucent segments.

library. It was found that the *smbA* suppressor gene was located at around 4 min on the *E. coli* chromosome (data not shown). To verify this, P1*vir*-mediated transduction was performed. Phage P1*vir* was grown on strains SJ16 and 112(#2), which contain a Tn*10* (Tet$^r$) at 3.4 min and a Tn*3* (Amp$^r$) at 5.1 min, respectively. Tet$^r$ or Amp$^r$ transductants of AZ5002 were selected at 42°C and subsequently tested for a Cs$^+$ phenotype at 22°C and vice versa. If the *smbA* gene is located around 4 min, a Cs$^+$ phenotype should be cotransduced with the markers used. Cs$^+$ colonies were observed at a frequency of 45% for the Tn*10* marker (3.4 min) and at a frequency of 21% for the Tn*3* marker (5.3 min). These results show that the *smbA* suppressor gene is located at 4 min on the *E. coli* chromosome.

**Subcloning and sequencing of the *smbA* gene.** Restriction mapping revealed that all of the cosmid DNAs which complemented the cold sensitivity of the *smbA2* mutation (AZ5061) contained a common 2.3-kb *Eco*RI segment (data not shown). This fragment was subcloned onto the plasmid vectors pHSG397 and pHSG575 (44). The recombinant plasmids pKX53 and pKX106 were able to complement the Cs$^-$ phenotype of AZ5061 (Fig. 3). Plasmid pKX110 carrying an *Eco*RV DNA fragment 1.4 kb long was also able to complement the phenotype (Fig. 3). Furthermore, when the pKX110 plasmid was introduced into AZ5002 (Δ*mukB*::*kan smbA2*), the transformants were able to grow at 22°C but not at 42°C. These phenotypes are the same as those of GC7528 (Δ*mukB*::*kan*). These results suggest that the *smbA* gene is located within this *Eco*RV DNA segment. Complementation tests using plasmid pKX110 were carried out with all the suppressor mutants. All of the independently isolated suppressor mutations were complemented by pKX110 but not by vector pHSG575. This suggests that all of the suppressor mutations are located within the *smbA* gene. On the other hand, the *mukB* null mutant GC7528 was not complemented with pKX110. This indicates that the overproduction of SmbA protein cannot compensate for lack of MukB function. Growth phenotypes of the various strains are summarized in Table 2.

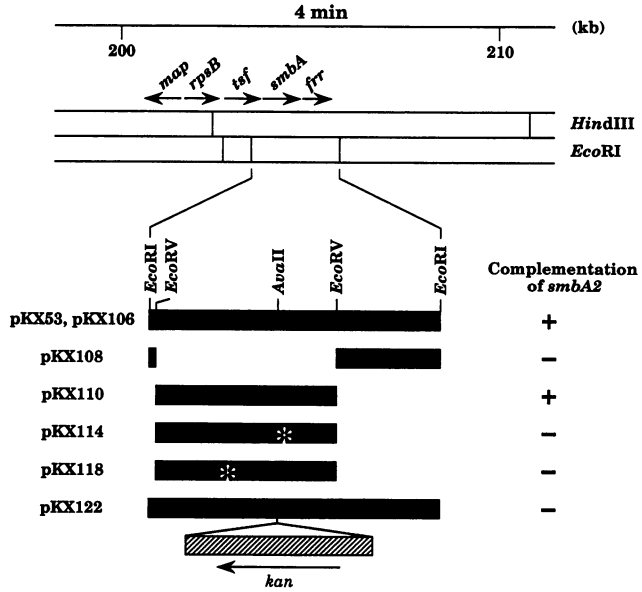


FIG. 3. Cloning of the *smbA* gene. Upper lines represent part of the *E. coli* chromosome restriction map described by Kohara et al. (27). Scales are expressed in kilobase coordinates. Plasmids pKX53 and pKX106 were constructed by cloning the 2.3-kb *Eco*RI DNA fragment of plasmid pKX19 on vectors pHSG397 and pHSG575, respectively. Plasmids pKX108 and pKX110 are derivatives of pKX106. Plasmids pKX114 and pKX118 were constructed by cloning the 1.4-kb *Eco*RV segment from the suppressor mutants AZ5002 and AZ5009, respectively, into vector pHSG575. The white asterisks in the solid bars represent *smbA* mutations. Plasmid pKX122 was constructed by cloning the 1.4-kb *kan* segment of plasmid pACYC177 into plasmid pKX53. Each plasmid was introduced into the cold-sensitive strain AZ5061 (*mukB*$^+$ *smbA2*), and transformants were tested for colony formation at 22°C on L-agar plates. +; complemented, −; not complemented.

The 2.3-kb *Eco*RI DNA segment carried by pKX53 and pKX106 was sequenced. The entire nucleotide sequence and the deduced amino acid sequences of predicted open reading frames (ORFs) are shown in Fig. 4. Nucleotides 1 to 315 correspond to the C-terminal part of the *tsf* gene (2), and nucleotides 1482 to 2036 are identical to the *frr* gene (21). An ORF which could encode a protein of 241 amino acids with a predicted molecular weight ($M_r$) of 25,953 is present from nucleotides 465 to 1187. This ORF is located between the two recognition sites for *Eco*RV (one is at nucleotide 64, and the other is at nucleotide 1491). These results indicate that the ORF might be the *smbA* gene. The *smbA* gene does not appear to be under heat shock regulation or gearbox regulation (45) and does not contain a leader peptide or potential transmembrane-spanning sequences (Fig. 4). The predicted amino acid sequence for the *smbA* gene product was compared with translated GenBank sequences. No remarkable homology was found. Several genes related to the regulation of translation are clustered but not in an operon (Fig. 3). The genes *map-rpsB-tsf-smbA-frr* are ordered clockwise; *map* codes for methionine amino peptidase (4), *rpsB* codes for protein S2 of the 30S ribosome subunit (2), *tsf* codes for

TABLE 2. Summary of colony-forming ability and anucleate-cell production

| Genotype (chromosome/plasmid) | Colony formation at: | | Production of anucleate cells |
|---|---|---|---|
| | 22°C | 42°C | |
| Wild type | + | + | − |
| Δ*mukB*::*kan* | + | − | + |
| Δ*mukB*::*kan*/*smbA*$^+$ | + | − | + |
| Δ*mukB*::*kan smbA2* | − | + | + |
| Δ*mukB*::*kan smbA2*/*smbA*$^+$ | + | − | + |
| *smbA2* | − | + | − |
| *smbA2*/*smbA*$^+$ | + | + | − |

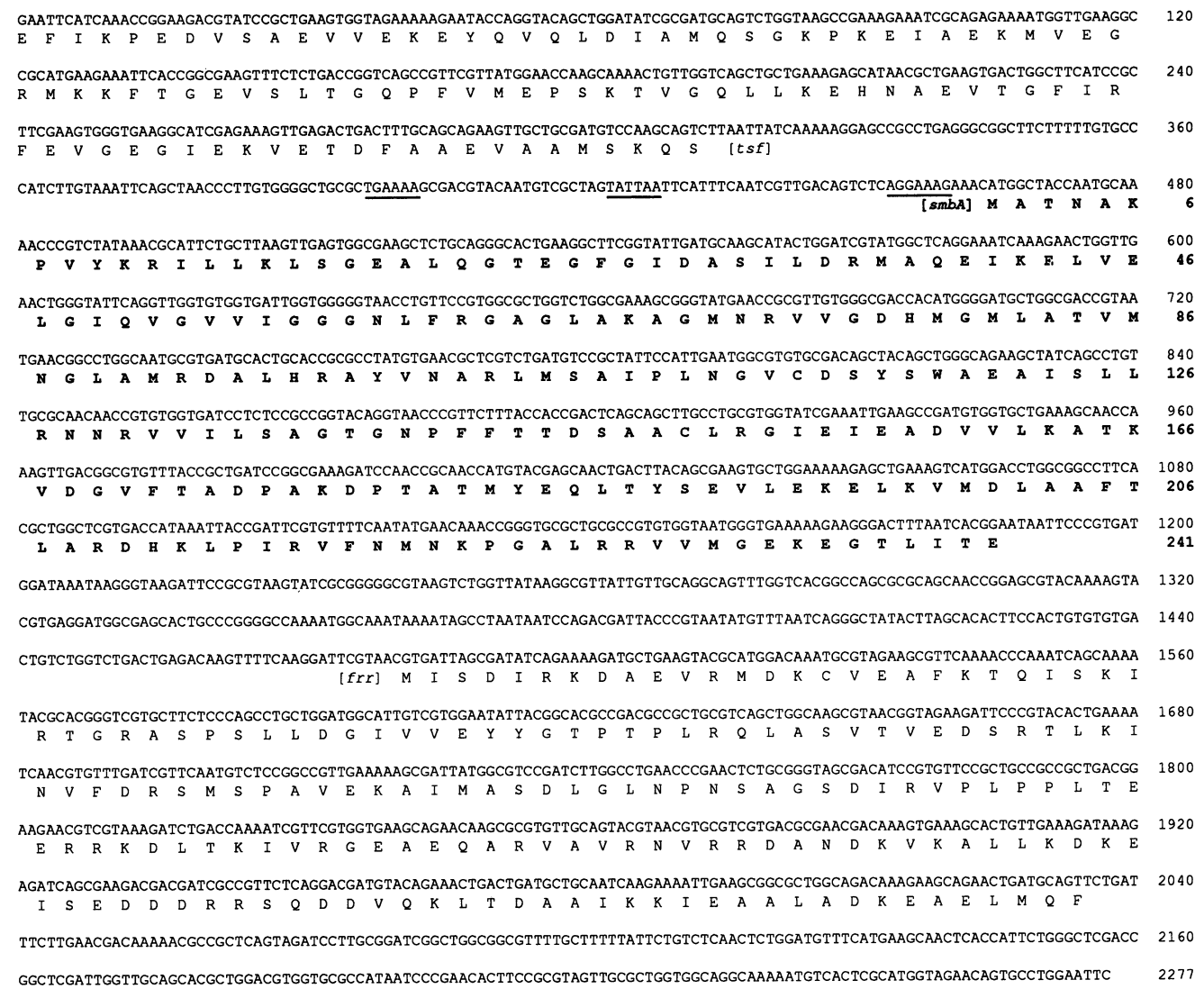


FIG. 4. Nucleotide sequence and predicted amino acid sequence of the 2.3-kb *Eco*RI DNA fragment containing the *smbA* gene. Nucleotides and corresponding amino acids of the SmbA protein are numbered on the right. The putative promoter regions (−35 and −10) and ribosome-binding site of the *smbA* gene are underlined.

protein chain elongation factor EF-Ts (2), and *frr* codes for ribosome-releasing factor (22).

**Identification of the product of the *smbA* gene.** To identify the product of the *smbA* gene, we introduced plasmid pKX53 and vector plasmid pHSG397 into an *E. coli* cell-free transcription-translation system. As shown in Fig. 5A, the recombinant plasmid pKX53 produced two different proteins (28 and 22 kDa) which were not present with vector pHSG397. The 2.3-kb *Eco*RI DNA segment carried by pKX53 encodes SmbA and ribosome-releasing factor (the *frr* gene product) proteins as mentioned above. Shimizu and Kaji (42) reported that the ribosome-releasing factor protein has an apparent $M_r$ of 22,000 on SDS-PAGE. These results indicate that the *smbA* gene is expressed and that its product, SmbA, has an apparent $M_r$ of 28,000 on SDS-PAGE. Furthermore, the 28-kDa product was overexpressed in the cells of PB103 harboring plasmid pKX103. The overexpressed protein could be detected clearly in SDS-PAGE after staining with Coomassie brilliant blue (Fig. 5B). This result is in good agreement with the prediction of the primary sequence (Fig. 4).

**Determination of mutation sites of suppressor mutants.** The 1.4-kb *Eco*RV DNA segments from AZ5002 (*smbA2*) and AZ5009 (*smbA9*) were cloned on plasmid pHSG575 by screening DNA-DNA hybridization using the wild-type *smbA* gene as a probe. The resulting plasmids, pKX114 and pKX118, were isolated (Fig. 3) and sequenced. Both of them have point mutations which lead to the substitution of an amino acid within the *smbA* coding region; Asp-201 was changed to Asn in the case of *smbA2*, and Arg-62 was changed to His in the case of *smbA9*.

The cold sensitivity phenotype of strain AZ5061 (*mukB*$^+$ *smbA2*) is not complemented by plasmid pKX114 carrying the *smbA2* mutation or plasmid pKX118 carrying the *smbA9* mutation (Fig. 3). Thus, overproduction of the altered SmbA proteins encoded by the *smbA2* and *smbA9* genes does not allow the cold-sensitive mutant AZ5061 to grow at 22°C, and

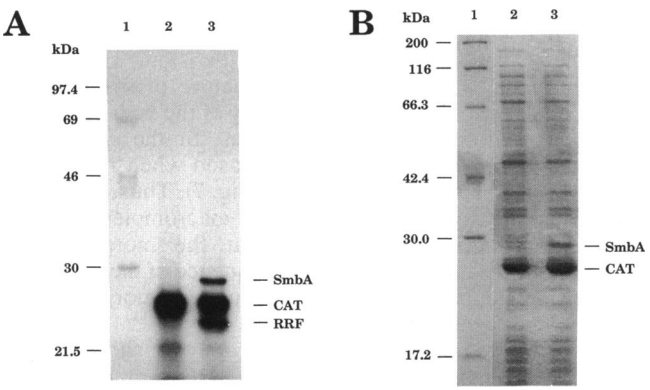


FIG. 5. Identification of the SmbA protein product. (A) In vitro synthesis of protein products made use of a coupled transcription-translation system with pHSG397 (lane 2) and pKX53 (lane 3). Lane 1, molecular weight markers. CAT, chloramphenicol acetyltransferase; RRF, ribosome-releasing factor. (B) Detection of SmbA protein overproduced in pKX103-harboring cells. pKX103 was constructed by cloning the 1.4-kb *Eco*RV segment from pKX53 into vector pHSG399. Lane 1, molecular weight markers; lane 2, PB103 (pHSG399); lane 3, PB103(pKX103). The gel was stained with Coomassie brilliant blue.

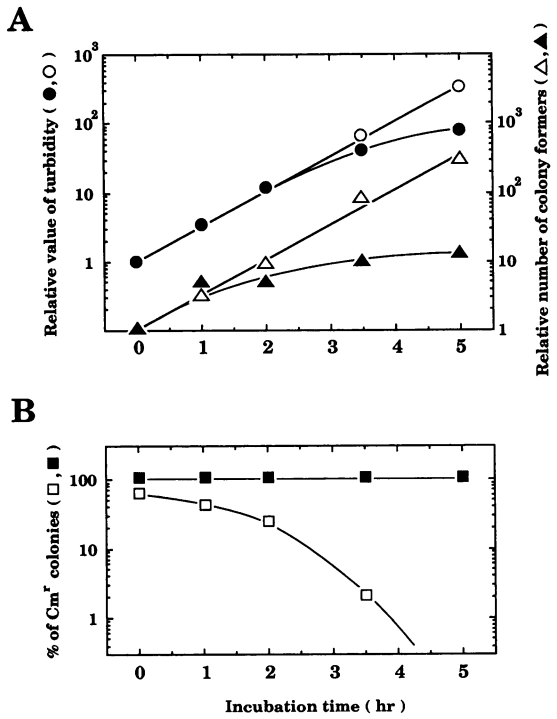


FIG. 6. Growth of cells and stability of plasmid pKX125. The pKX125-harboring *smbA*$^+$ strain AZ5145 (open symbols) and the pKX125-harboring *smbA* null mutant AZ5149 (closed symbols) were grown in L medium at 30°C to early log phase and then transferred to 42°C at zero time. (A) Parts of the cultures were sampled at 0, 1, 2, 3.5, and 5 h, and the turbidity and the number of colony-forming units (at 30°C overnight on L-agar plates) were measured. They are shown relative to amounts at zero time. Circles, relative value of turbidity; triangles, relative number of colony-forming units. (B) Colonies from each sample were streaked on L-agar plates containing 20 μg of chloramphenicol per ml to test for the plasmid. Ratios of the number of chloramphenicol-resistant cells (plasmid-harboring cells) to the total number of cells tested are shown.

these two defective SmbA proteins were not able to complement each other intermolecularly in vivo.

**Construction of *smbA* null mutant in the presence of *smbA*$^+$ plasmid.** To study the cellular function of the SmbA protein, an *smbA* null mutant was constructed. The kanamycin resistance gene (1.4-kb *Hae*II segment) from plasmid pACYC177 (9) was inserted into the coding region of the *smbA* gene at the *Ava*II site (nucleotide 1064 in Fig. 4) of plasmid pKX53. The resulting plasmid, pKX122, was isolated (Fig. 3). The linearized pKX122 DNA fragment containing the disrupted *smbA* gene (Δ*smbA*::*kan*) was introduced into the chromosome of strain AZ5120, which is a *recD* mutant carrying the *smbA*$^+$ plasmid pKX110. *recD* mutants are defective in the activity of endonuclease V but normal in homologous recombination (35a). Kanamycin-resistant transformants carrying the disrupted *smbA* gene on the *E. coli* chromosome were confirmed by Southern hybridization (data not shown). The disrupted *smbA* gene could be transduced with phage P1*vir* into *smbA*$^+$ strain PB103 carrying plasmid pKX110 but could not be transduced into strain PB103 in the absence of pKX110. These results indicated that the *smbA* gene is essential for growth. To confirm this, the *smbA* gene was inserted into plasmid pHSG415, which is a temperature-sensitive replication vector (15). Wild-type strain PB103 carrying the resulting plasmid, pKX125, grew logarithmically after transfer to 42°C (Fig. 6A), and the ratio of cells carrying pKX125 to total colony formers was drastically decreased (Fig. 6B). Plasmid-harboring cells were less than 1% of total cells at 5 h. In contrast, the *smbA* null strain harboring the pKX125 plasmid grew exponentially for the first 1 h after the shift-up of temperature, and thereafter, the rate of increase in the number of colony-forming cells was markedly decreased. The turbidity of the culture increased exponentially for the first 3 h, and then the rate of increase was reduced (Fig. 6A). After the shift-up, no plasmid-free segregant was observed (Fig. 6B), indicating that the *smbA* null mutant cells which lost the *smbA*$^+$ plasmid could not form colonies. Thus, the product of the *smbA* gene plays a critical role in cell proliferation.

To determine whether macromolecular synthesis was affected by the *smbA* null mutation, the uptake of radiolabeled precursors into DNA, RNA, and protein was monitored in the *smbA* null mutant harboring pKX125. After a shift-up of temperature to 42°C, macromolecular synthesis was followed by measuring the rate of incorporation of [$^3$H]thymidine, [$^3$H]uridine, and [$^{35}$S]methionine into trichloroacetic acid-insoluble material. As expected, the incorporation rate of these precursors increased exponentially at least for 10 h at 42°C in the *smbA*$^+$ strain AZ5145 (Fig. 7). In contrast, in the *smbA* null mutant AZ5149, the incorporation rates increased exponentially for about 3 to 5 h, and thereafter the incorporation rates plateaued (Fig. 7). This is consistent with the turbidity of the cultures shown in Fig. 6A.

**Morphological change of cells after loss of *smbA*$^+$ plasmid.** In a pKX125-harboring *smbA*$^+$ strain (AZ5145), no remarkable morphological change was observed after plasmid loss. In contrast, drastic changes were observed in the pKX125-harboring *smbA* null mutant (AZ5149) after plasmid loss: cells with a large translucent segment at a cell pole or in the center of cell were observed after incubation for 5 h at 42°C. These morphological changes are the same as those in *smbA2* cells incubated at 22°C (see Fig. 2J and J'). Widths of AZ5149 cells increased slightly after plasmid loss. No anu-

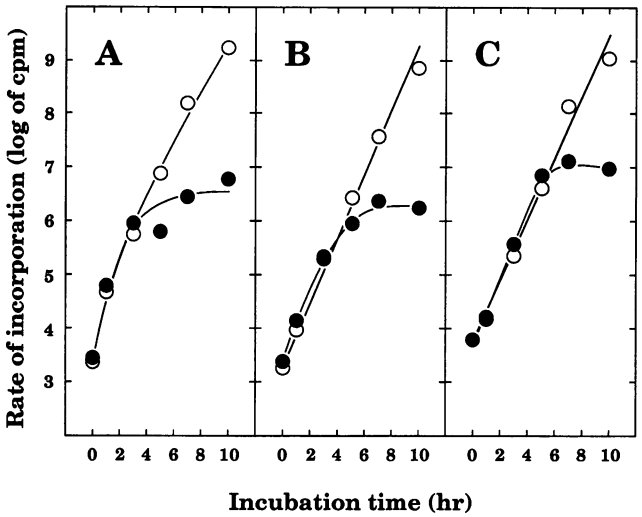


FIG. 7. Effects of macromolecular synthesis in *smbA* disrupted cells. The pKX125-harboring *smbA*$^+$ strain AZ5145 (○) and the pKX125-harboring *smbA* null mutant AZ5149 (●) were grown in L medium at 30°C to early log phase and then transferred to 42°C at zero time. Samples were removed at 0, 1, 3, 5, 7, and 10 h and pulse-labeled with [$^3$H]thymidine (A), [$^3$H]uridine (B), and [$^{35}$S]methionine (C) as described in Materials and Methods.

cleate cells were observed. The morphological changes in the *smbA* null mutant observed after plasmid loss suggest that the SmbA protein might be essential for the stability of the septum-forming site and cell poles.

## DISCUSSION

In the course of studies to determine the cellular function of the MukB protein, we isolated and characterized 56 temperature-resistant suppressor mutants of the *mukB* null mutant. When a plasmid carrying the *smbA*$^+$ gene from the chromosome of the wild-type strain was introduced into these suppressor mutants, all mutants became temperature sensitive. All the suppressor mutations are thus alleles of the *smbA* gene. Although temperature sensitivity for colony formation is suppressed in the mutants, the phenotype of anucleate-cell formation remains. Ten of the 56 suppressor mutants showed cold-sensitive growth. The cold sensitivity phenotype resulted from the suppressor mutation itself; the cold sensitivity phenotype remained even after the *mukB*$^+$ gene was transferred into the Δ*mukB*::*kan smbA2* double mutant (Fig. 1).

A novel morphological phenotype of translucent segments was observed in the *smbA2* point mutant at the nonpermissive temperature of 22°C (Fig. 2J and J′) and the *smbA* null mutant after the loss of the *smbA*$^+$ plasmid. The absence of the normal function of SmbA presumably causes a defect in the septation site and/or cell pole. The defect is followed by plasmolysis of intracellular components from the affected cell segment. Two characteristic findings have been reported concerning the division site and cell poles in *E. coli* (6, 11). One is the functional relationship between the MinC-MinD division inhibitor and the *ftsZ* gene product (6, 13). Their interaction prevents cell division at the polar sites, which leads to minicell formation. The other is the existence of characteristic structures, the periseptal annuli, that connect the inner membrane, murein, and outer membrane (11). The periseptal annuli in the center of each newborn cell determine the division site at the next cell division cycle, and the central periseptal annulus becomes a periseptal annulus of the pole in each new cell after cell division. The SmbA protein might be involved in the structure or synthesis of these cellular sites. The SDS sensitivity of the *smbA2* mutant may be due to the membrane defect. In the *smbA* null mutant, macromolecular synthesis ceased when replication of the *smbA*$^+$ plasmid was inhibited (Fig. 7). Thus, the *smbA* mutations directly or indirectly affect macromolecular synthesis. It is therefore possible that the morphological changes of *smbA* mutants are a consequence of the cessation of macromolecular synthesis even though the morphological changes are observed earlier.

The *mukB106* point mutant is moderately sensitive to SDS at 42°C (48). This suggests that the *mukB106* mutant directly or indirectly affects the cell membrane at 42°C besides causing a defect in the active positioning of daughter chromosomes. The temperature-sensitive growth of the *mukB106* mutant may be due to a defect in cell membrane. The *mukA1* mutant, which shows a typical *muk* phenotype, is also sensitive to SDS (16). The *mukA1* mutant has a mutation in the *tolC* gene, which encodes an outer membrane protein (16). The *tolC* (*mukA*) gene product is required for hemolysin secretion (46). These results suggest that the cell membrane or specific membrane sites are involved in the active positioning of daughter chromosomes. However, there is no certain genetic and/or biological evidence of components in the specific membrane sites so far. All of the 56 *smbA* mutants isolated may correct a structure defect of the putative specific membrane sites caused by the *mukB* null mutation at 42°C. In addition, cold-sensitive *smbA* mutants may be defective in the structure of specific membrane sites at 22°C even in *mukB*$^+$ genetic backgrounds. These cold-sensitive *smbA* mutants and the *smbA* null mutant show the characteristic phenotype of translucent segments in specific cell sites as described above. It is likely that the defects in specific membrane sites, which are caused by an *smbA* mutation, subsequently prevent macromolecular synthesis. However, since the *smbA* suppressor mutations do not correct anucleate-cell production and moderate filamentation of nucleate cells, it remains possible that SmbA is not directly involved in cell division.

Another approach to characterizing the cellular functions of the wild-type genes which are mutated is to screen for multicopy suppressor genes (1, 3, 23, 29). Very recently, we identified a multicopy suppressor gene of *smbA* mutation, named *mssA*, that maps at 21 min, just upstream of the *rpsA* gene on the *E. coli* chromosome (48). It is interesting that overexpression of the *mssA* gene was able to suppress growth defects not only of point mutants but also of the null mutant of the *smbA* gene, which is essential for growth. Since the *mssA* and *rpsA* genes constitute an operon (38), the function of the *mssA* gene product might be related to translation. From studies of the functional relation of the SmbA and MssA proteins, some significant clues about important cellular functions of the SmbA protein should emerge.

## ACKNOWLEDGMENTS

We thank Y. Kohara and A. Nishimura for providing bacterial strains. We also thank Akiko Matsusaka and Chiyome Ichinose for assistance.

This work was supported by Grant-in-Aid for Scientific Research on Priority Areas, Grant-in-Aid for Scientific Research B, and Monbusho International Scientific Research Program for Joint Research to S.H. and by Grant-in-Aid for Encouragement of Young

Scientists to K.Y. from the Ministry of Education, Science and Culture of Japan.

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