

Characterization of the *ssnA* Gene, Which Is Involved in the Decline of Cell Viability at the Beginning of Stationary Phase in *Escherichia coli*

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When grown in rich medium, *Escherichia coli* exhibits a drastic reduction of the number of viable cells at the beginning of stationary phase. The decline of cell viability was retarded by disruption of the *ssnA* gene, which was identified as a gene subject to RpoS-dependent negative regulation. Moreover, *ssnA* expression was induced at the time of decline of cell viability at early stationary phase. The viability decline was augmented in the *rpoS* background, and this augmentation was suppressed by *ssnA* mutation. Cloning of the *ssnA* gene in a multicopy plasmid, pBR322, caused small colony formation and slow growth in liquid medium. Cells harboring the *ssnA* clone showed aberrant morphology that included enlarged and filamentous shapes. The gene product was identified as a 44-kDa soluble protein, but its function could not be deduced by homology searching. From these results, we conclude that *ssnA* is expressed in response to a phase-specific signal(s) and that its expression level is controlled by RpoS, by a mechanism which may contribute to determination of cell number in the stationary phase.

Escherichia coli has a life cycle in which exponential growth occurs in the presence of sufficient nutrients but ceases as nutrients are consumed or as growth-suppressing factors are accumulated; at the same time, cells develop resistance to a variety of environmental stresses (2, 12, 19). In the natural environment, *E. coli* usually lives under starved conditions, coexisting with a large number of other microorganisms, and rarely encounters circumstances which allow it to grow exponentially. Thus, the natural conditions are comparable to those of stationary phase in *in vitro* experiments, in which many specific genes are expected to function. To identify stress-specific genes is thus important for elucidation of the mechanism of maintenance of the bacterial life cycle in nature.

A stationary phase-specific σ^S factor, σ^S , encoded by *rpoS*, has been extensively characterized (7, 9, 12, 23, 24, 26). The factor is believed to be a master regulator in the network of gene expression in stationary phase and to control many genes responding to various physiological stresses (8, 9). When grown in rich medium, *E. coli* exhibits a large decrease in viability, by one to two orders of magnitude, at the beginning of stationary phase, and after that, it retains the same viability for many days (27). To explain this phenomenon, Zambrano and Kolter (26) proposed the GASP (growth advantage in stationary phase) model, in which mutants that acquire a competitive advantage take the place of the population of dying cells. As one of such mutants, *rpoS*^{att}, which was isolated from prolonged culture, was reported to express the GASP phenotype in mixed cultures with the wild type (26, 27). However, the

physiological role and cause of the decline of cell viability are still unknown.

In our previous study, the *E. coli ssn* (subject to RpoS-dependent negative regulation in stationary phase) genes were identified (21). One of the genes, designated *ssnA*, was shown to be specifically induced in stationary phase when cell viability was decreasing and was expressed at a much higher level in the *rpoS* background than in the wild type. Here, we demonstrate that the *ssnA* gene is involved in the decline of cell viability observed at the beginning of stationary phase and that there is a functional relationship between *ssnA* and *rpoS* affecting determination of cell viability. We have begun the molecular analysis by focusing on the bacterial cell death event at the beginning of stationary phase.

MATERIALS AND METHODS

Materials, bacterial strains, and plasmids. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, Mass.). The DNA sequencing kit was from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Other chemicals were analytical grade. *E. coli* strains and plasmids used in this study are shown in Table 1.

Bacterial growth conditions and media. *E. coli* cells were grown with reciprocal shaking (100 times/min) at 37°C in LB (1% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) or M9 minimal medium (13) with 0.2% glucose. For time course experiments, one colony was inoculated into 1.5 ml of medium. After a 12-h preculture, cells were diluted to a turbidity corresponding to an optical density at 600 nm (OD₆₀₀) of 0.1. Thirty microliters of this cell suspension was inoculated into 30 ml of medium in a 100-ml Erlenmeyer flask, and cell growth was then monitored under the conditions indicated in the text.

Colony sizes of different strains were compared on 1.5% agar LB plates after 20 h of incubation. When used, the following drugs were added at these final concentrations: ampicillin, 100 µg/ml; tetracycline, 8 µg/ml; and kanamycin, 50 µg/ml.

Construction of *ssnA* clone and its derivatives. Conventional recombinant DNA techniques (15) were used. The 2.9-kb *PstI* fragment bearing *ssnA* was isolated from the Kohara clone, 6A1 (11), and inserted into the *PstI* site of pBR322 or pACYC177, generating pBRSSNA or pACYCSSNA, respectively. Deletion constructs were made by inserting the 2.3-kb *PstI-NruI* or 0.6-kb *NruI-PstI* fragment from pBRSSNA into the *PstI-ScaI* site in pBR322, generating pBRSSNA-ND or pBRSSNA-NP, respectively. pBRSSNA-MD was generated by deleting a 0.5-kb *MluI* fragment from pBRSSNA. To construct the *ssnA-lacZ* operon fusion, the 0.5-kb *EcoRV* fragment bearing the *ssnA* promoter and the region corresponding to the N-terminal 22 amino acid residues (see Fig. 6A) was subcloned from pBRSSNA into pCB182, generating pSSNALAC.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
<i>E. coli</i> strains		
W3110	IN(<i>rrnD-rrnE</i>) <i>rph-1</i>	K. Mizobuchi
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F⁺ traD36 proAB⁺ lacI^a lacZΔM15</i>	15
MC1000	<i>araD139 Δ(ara-leu)7697 ΔlacX74 galU galK rpsL</i>	5
BL21 (DE3)	<i>F⁺ dcm ompT hsdS(r_B⁻ m_B⁻) gal λDE3(lacI lacUV5-T7 gene 1 ind-1 Sam7 nin-5)</i>	RIKEN DNA Bank
UM122	<i>rpoS::Tn10</i>	A. Ishihama
YU449	W3110 <i>ssnA::kan</i>	This work
YU450	W3110 <i>rpoS::Tn10</i>	This work
YU451	W3110 <i>ssnA::kan rpoS::Tn10</i>	This work
YU381	MC1000 <i>rpoS::Tn10</i>	21
Plasmids		
pBR322	Amp ^r Tet ^r	3
pACYC177	Amp ^r Kan ^r	6
pBRSSNA	pBR322 with the 2.9-kb <i>PstI</i> fragment bearing <i>ssnA</i>	This work
pBRSSNA-ND	pBR322 with the 2.3-kb <i>PstI-NruI</i> fragment from pBRSSNA	This work
pBRSSNA-NP	pBR322 with the 0.6-kb <i>PstI-NruI</i> fragment from pBRSSNA	This work
pBRSSNA-MD	pBRSSNA lacking the 0.5-kb <i>MluI</i> fragment	This work
pACYCSSNA	pACYC177 with the 2.9-kb <i>PstI</i> fragment bearing <i>ssnA</i>	This work
pVEX11	Amp ^r , pBR322 with T7 promoter of ϕ 10 gene and its terminator	20
pVEXSSNA	pVEX11 with the 1.3-kb <i>XbaI-BamHI</i> fragment bearing <i>ssnA</i>	This work
pJQ200mp18	<i>sacB</i> Gen ^r	14
pJQ200SSNA	pJQ200mp18 with the 2.9-kb <i>PstI</i> fragment bearing <i>ssnA</i>	This work
pJQ200SSNA-K	pJQ200SSNA derivative with insertion of <i>kan</i> at the <i>NruI</i> site	This work
pCB182	<i>lacZ galK</i> Amp ^r	17
pSSNALAC	<i>ssnA-lacZ</i> operon fusion in pCB182	This work

Morphological observation. Cells were grown at 37°C in LB medium, and samples taken at the late exponential phase were diluted with 100 mM potassium phosphate (pH 7.0) and immediately incubated with acridine orange or 4',6'-diamidino-2-phenylindole at a final concentration of 30 or 5 μ g/ml, respectively, at room temperature for 3 min. Stained cells were filtered through a polycarbonate membrane and viewed with a UV-1A filter (EX 365/10) for staining with 4',6'-diamidino-2-phenylindole or a B-2A filter (EX450-490) for staining with acridine orange under a Nikon E600 microscope with fluorescence capability (Nikon, Tokyo, Japan). Photomicrographs were taken with a charge-coupled device camera with an exposure time of 10 ms and printed by using a CP710A printer (Mitsubishi, Tokyo, Japan). Reagent solutions and buffers employed in the cell-staining procedures were used after being passed through cellulose acetate filters (pore size, 0.2 μ m).

Mutant construction. An *ssnA*-disrupted mutant of W3110 was constructed as follows. The 2.9-kb *PstI* fragment bearing *ssnA* was inserted into the *PstI* site of pJQ200mp18 (14), generating pJQ200SSNA. The *BglII-BamHI* fragment bearing the kanamycin resistance gene (*kan*) from ColE1::Tn5 (provided by T. Miki) was blunt ended by T4 DNA polymerase treatment (15) and inserted into the *NruI* site in *ssnA* on pJQ200SSNA, to disrupt the *ssnA* open reading frame (ORF). The resultant recombinant, pJQ200SSNA-K, was introduced into W3110 to allow homologous recombination between the *kan*-inserted *ssnA* gene on the plasmid and the *ssnA* gene on the genome, and recombinants were screened on LB plates containing 5% sucrose and kanamycin (15 μ g/ml). Gene disruption was confirmed by Southern blotting by using DNA fragments bearing *ssnA* or *kan* as probes.

From the resultant W3110 *ssnA::kan* (YU449) or UM122 *rpoS::Tn10* (provided by A. Ishihama), W3110 *rpoS::Tn10* (YU450) and W3110 *ssnA::kan rpoS::Tn10* (YU451) were made by P1 transduction (13).

Homology searching. Homology searching was performed by using FASTA and BLAST in the GenBank, DDBJ, EMBL, SWISS-PRO, and NBRF-PIR databases. Comparisons of nucleotide sequences or amino acid sequences were conducted by using GENETYX (Software Development, Tokyo, Japan).

***SsnA* overproduction and cell fractionation.** The 1.3-kb DNA fragment bearing *ssnA* was amplified by PCR by using primers 5'-GGGTCTAGAGGAG GTGTAATCATGTT-3' and 5'-GGAGGATCCCATTTATGCCAGCGCAT-3', which have the *XbaI* and *BamHI* sites, respectively, at their 5' ends, as indicated by underlines. The amplified fragment was digested with *XbaI* and *BamHI* and inserted into the *XbaI-BamHI* site downstream of the T7 promoter in pVEX11. The region encompassing *ssnA* in the recombinant, pVEXSSNA, was sequenced to ensure that no mutations had been introduced during amplification of the DNA fragment. BL21 (DE3) cells harboring pVEXSSNA were grown at 37°C in LB medium containing ampicillin, and after 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to obtain an OD₆₀₀ of approximately 1.0, cultivation was further continued for 4 h. The expressed SsnA protein was separated from other proteins by sodium dodecyl sulfate-12% polyacrylamide gel electrophore-

sis and transferred to a polyvinylidene difluoride membrane. The portion of the membrane containing the protein band was cut out, and the protein was subjected to amino acid sequencing, as described previously (25).

Subcellular fractionation was performed by a modification of the method described previously (25). Cells were harvested from 250-ml cultures by centrifugation at 8,000 \times g for 5 min at 4°C, washed with 0.85% NaCl, and suspended in 10 mM Tris-HCl (pH 7.5). The suspended cells were disrupted by passing twice through a French pressure cell press (16,000 lb/in²). After removal of cell debris by centrifugation at 8,000 \times g for 10 min, the membrane and soluble fractions were separated by centrifugation at 86,000 \times g for 90 min. The resultant membrane fraction was resuspended by homogenization in a volume of 10 mM Tris-HCl (pH 7.5) equivalent to the volume of the soluble fraction.

Primer extension and RT-PCR analysis. *E. coli* total RNA was prepared as described by Aiba et al. (1). For primer extension analysis, 1 μ g of total RNA isolated from MC1000 or YU381 harboring pSSNALAC was subjected to the reverse transcription (RT) reaction using a fluorescein isothiocyanate-labeled primer (5'-CGCCAGGGTTTCCAGTCACGAC-3') as previously described (10). The RT reaction was performed at 60°C with *rTh* DNA polymerase XL (Perkin-Elmer-Cetus Instruments). A standard nucleotide sequence ladder was obtained with the same primer and pSSNALAC DNA as a template. To analyze expression along with cell growth, an RT reaction was carried out at 60°C for 60 min with 0.1 μ g of total RNA from W3110 or YU450 cells and one of the primers used in the *ssnA* amplification, and subsequently PCR consisting of denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 5 min was carried out by using the two primers used for the *ssnA* amplification. The products obtained after PCR were analyzed by 0.9% agarose gel electrophoresis and stained with ethidium bromide. Their relative amounts were compared by measuring band densities after the color of the image taken was reversed by using a model GS-700 Imaging Densitometer (BIO-RAD). Linearity of the amplification was observed up to at least 27 cycles.

β -Galactosidase assay. For analysis of *ssnA* expression during the course of cell growth, MC1000 or YU381 cells harboring pSSNALAC were grown at 37°C in LB containing ampicillin. As the cell growth progressed, samples were taken from the cell culture and β -galactosidase activity was measured according to the procedure described by Miller (13). For determination of activity, the following formula was used (13): Miller units = $1,000 \times (OD_{420} - 1.75 \times OD_{550}) / (t \times v \times OD_{600})$. OD₄₂₀ and OD₅₅₀ were read from the reaction mixture, OD₆₀₀ reflects the cell density just before assay, *t* is the time of reaction expressed in minutes, and *v* is the volume of culture used in the assay expressed in milliliters.

RESULTS

Growth inhibition by the *ssnA* gene cloned in a multicopy plasmid, pBR322. Based on the genomic position of the *ssnA*

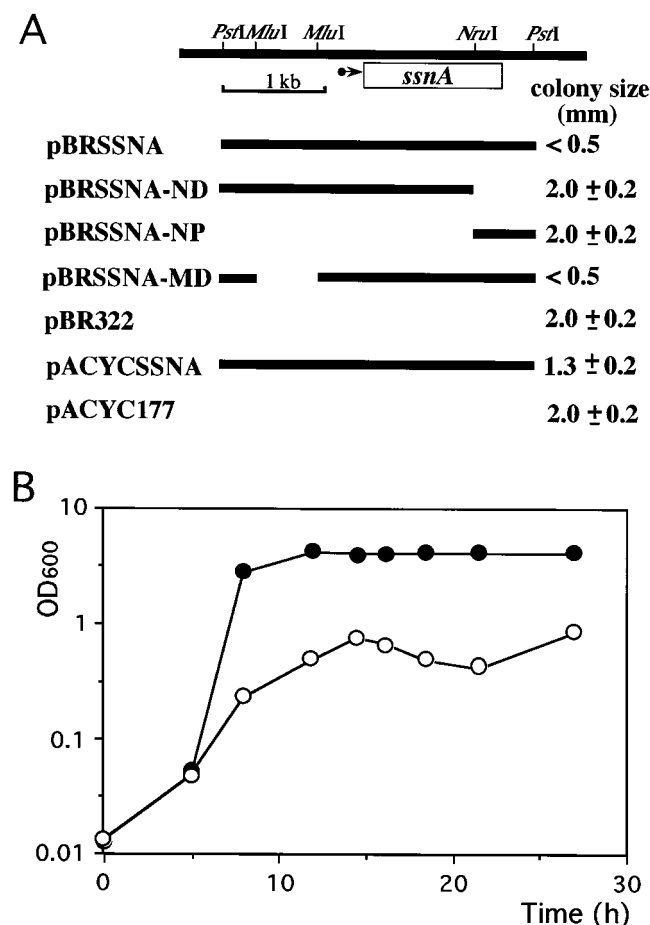


FIG. 1. Effect of the *ssnA* gene on cell growth. *E. coli* W3110 cells harboring pBRSSNA, pBRSSNA-ND, pBRSSNA-NP, pBRSSNA-MD, pBR322 (vector), pACYCSSNA, or pACYC177 (vector) were grown until log phase and plated on LB plates containing tetracycline or kanamycin (A). The mean diameters of the colonies were then compared after 20 h of incubation. The top diagram represents the physical map of the region around *ssnA*, and an arrow indicates the *ssnA* promoter position and transcriptional direction. Black bars represent the DNA fragments inserted into the vectors. (B) W3110 harboring pBR322 (closed circles) or pBRSSNA (open circles) were grown under the conditions described in Materials and Methods, and cell growth was determined by measuring OD₆₀₀.

gene at 65 min in the *E. coli* chromosome (22), the 2.9-kb *PstI* fragment bearing the *ssnA* gene was subcloned from Kohara clone 6A1 into pBR322, generating pBRSSNA. We determined the nucleotide sequence of the fragment and found one ORF, the sequence of which precisely agreed with that of *orf464* in the GenBank database. However, we revised the *orf464* to *orf442* because the N-terminal 9-amino-acid sequence that was determined in this study (see below) corresponds to the 23rd to 31st codons of *orf464*.

Cells harboring pBRSSNA were found to grow slowly on plates, indicating that *ssnA* may have a negative effect on cell growth. To examine whether *ssnA* causes cell growth inhibition, we constructed several deletion-bearing derivatives and tested their effects on cell growth (Fig. 1A). *E. coli* W3110 harboring pBRSSNA or pBRSSNA-MD formed small colonies. In contrast, cells harboring pBRSSNA-ND or pBRSSNA-NP, which lack part of *ssnA*, formed normal-sized colonies. This phenomenon was also observed with other *E. coli* strains, TG1 and MC1000, and with another clone, pACYCSSNA, which is a pACYC177 derivative with a copy number lower

than that of pBR322. Therefore, the formation of small colonies appeared to depend on the presence of the intact *ssnA* gene.

Growth inhibition by *ssnA* was also observed in liquid culture (Fig. 1B). Cells harboring pBRSSNA showed markedly slower growth than cells harboring the vector. In the cultures, insoluble sediments were observed as cell growth progressed, suggesting that the growth inhibition resulted in cell lysis.

Growth-inhibited cells were morphologically analyzed by staining with acridine orange. When wild-type W3110 cells harboring the vector were grown at 37°C until the late exponential phase, they exhibited a rod shape (Fig. 2A and B), whereas most cells harboring pBRSSNA showed enlarged and filamentous shapes (Fig. 2C, D, E, and F). Cell staining with 4',6-diamidino-2-phenylindole showed that the nucleoids formed lines at intervals in the cell filaments (data not shown), suggesting that normal DNA replication occurred in the cells. Lysed cells were also observed in the presence of pBRSSNA. Therefore, increased expression of *ssnA* causes aberrant morphology of the host cell, presumably by hampering some cell function, such as cell division.

Characterization of the *ssnA* mutant. To explore the function of the *ssnA* gene, we constructed an *ssnA*-disrupted strain by inserting the kanamycin resistance gene into the genomic *ssnA* gene of W3110. Consequently, although the *ssnA* mutant, YU449, was not different in colony size and form from the parent strain on LB plates, its cell density and viability were significantly different from those of the parent strain at the beginning of stationary phase (Fig. 3A and B). During the exponential phase, the growth curves of the *ssnA* and wild-type strains were essentially the same in terms of OD and cell viability. Just after cells entered the stationary phase, YU449 showed a cell viability pattern different from that of W3110. As previously reported (27), the number of viable cells of W3110 was observed to increase to about 5×10^9 CFU/ml and then to decrease by more than one order of magnitude, to about 2×10^8 CFU/ml. In contrast, YU449 showed a significant retardation of the decrease in cell viability (Fig. 3B), indicating that the *ssnA* mutation allows the cells to survive longer than the wild type in this phase. After 60 h, the numbers of viable cells of both strains were similarly stable (1×10^8 to 3×10^8 CFU/ml) for several days, and the morphology of both appeared to be essentially the same (data not shown). The OD of YU449 in stationary phase was found to gradually increase and to be higher than that of the wild type. The reason for this may have been reduced lysis of dead cells.

The expression of *ssnA* in stationary phase and its negative control by RpoS. Considering the growth inhibition caused by the increased expression of *ssnA* and the retardation of cell death in the *ssnA* background, the *ssnA* gene seems to be involved in the cell death at early stationary phase. We therefore examined the *ssnA* expression by RT-PCR under the conditions used in the experiments to determine cell viability (Fig. 3C). A 1.3-kb band corresponding to the *ssnA* transcript was detected in cells in stationary phase but not in exponential phase. This indicates that *ssnA* expression is extremely low in exponential phase and increases at the beginning of stationary phase, which appears to be consistent with the time at which cell viability started to decrease. The RT-PCR experiments further revealed that in stationary phase, *ssnA* expression was enhanced more than twofold in the *rpoS* background (Fig. 3C). The stationary phase-specific induction and the enhanced expression in the *rpoS* background were also observed when an *ssnA-lacZ* operon fusion was used (Fig. 4). These results suggest that *ssnA* expression is negatively regulated by RpoS.

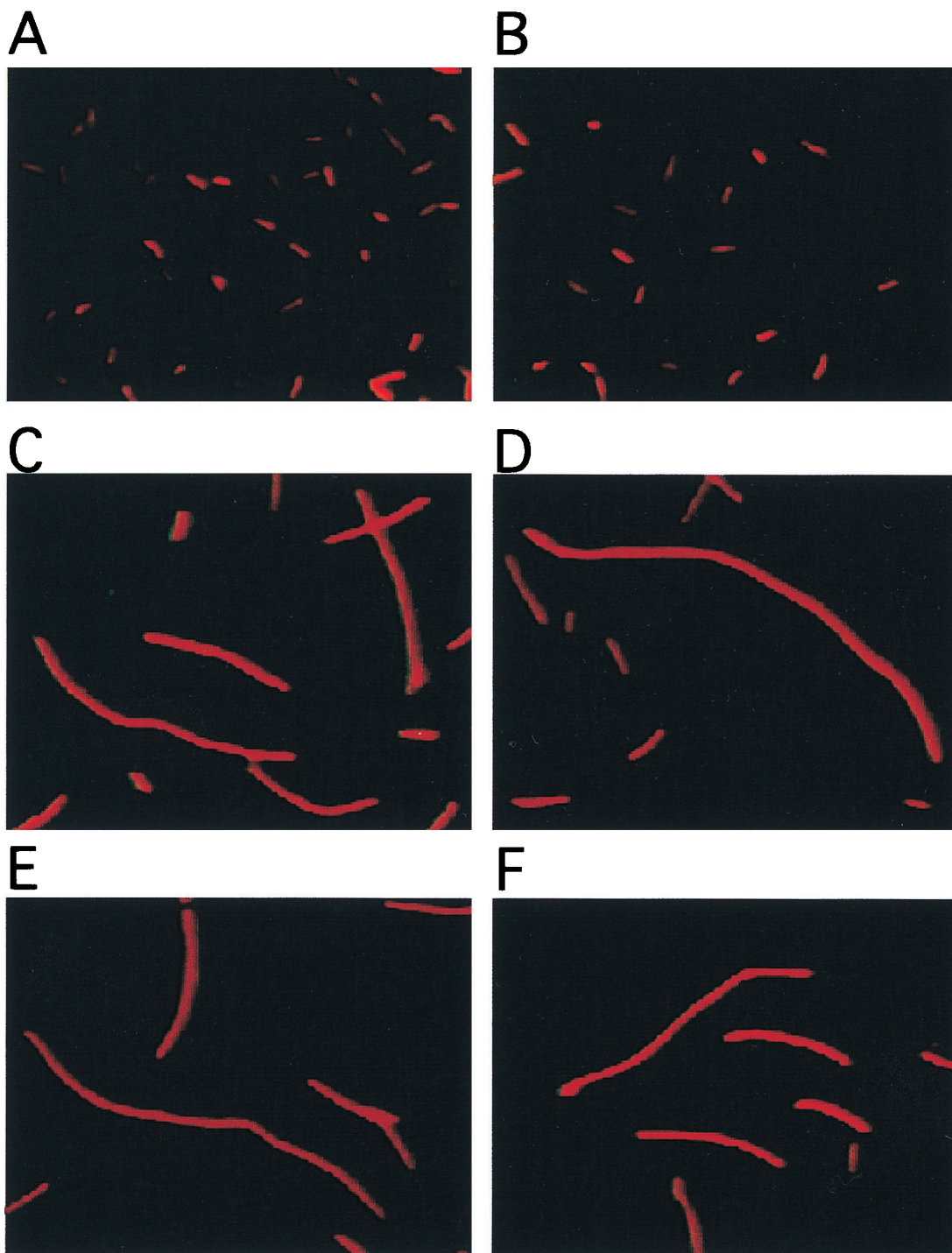


FIG. 2. Morphological analysis carried out by staining with acridine orange. Cells grown for 14 h in LB medium were diluted and stained. W3110 harboring pBR322 (A and B) or pBRSSNA (C, D, E, and F) was analyzed by staining with acridine orange. The scale bar corresponds to 10 μ m.

On the other hand, the *rpoS* mutant reached a lower cell density and its viable cell number decreased more than that of the wild type after cells entered the stationary phase (Fig. 3A and B and Fig. 4B and C). Notably, these effects were ameliorated by the addition of the *ssnA* mutation (as in YU451, an *rpoS ssnA* double mutant, in Fig. 3). Therefore, the further

decrease of viable cells in the *rpoS* background seems to be due to the increased expression of *ssnA* caused by the *rpoS* mutation. These data suggest that *ssnA* is expressed in the early stationary phase under the control of RpoS and that this expressional control may determine cell viability during the subsequent stationary phase.

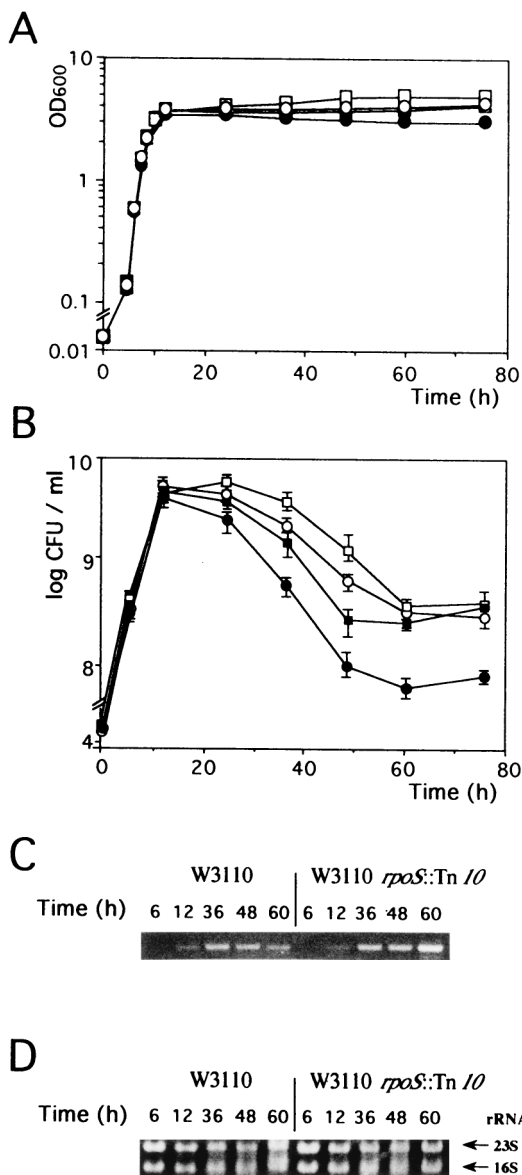


FIG. 3. Cell growth and viability of W3110, YU449 (W3110 *ssnA::kan*), YU450 (W3110 *rpoS::Tn10*), and YU451 (W3110 *ssnA::kan rpoS::Tn10*) and expression of the *ssnA* gene. Cell growth at 37°C in LB medium was monitored by measuring OD₆₀₀ (A). At the same time, the number of viable cells (expressed as CFU per milliliter) was determined by counting the number of colonies formed after 20 h on LB plates (B). In both A and B, closed squares, open squares, closed circles, and open circles represent W3110, YU449, YU450, and YU451, respectively. The solid lines connect average values from three different experiments. The vertical line for each point corresponds to the standard deviation. Total RNA was isolated from W3110 and YU450 (W3110 *rpoS::Tn10*) grown in LB medium at the times indicated, and 0.1 μg of RNA was subjected to RT-PCR (C). The products obtained after a 25-cycle PCR were analyzed by 0.9% agarose gel electrophoresis and stained with ethidium bromide. Portions (5 μg) of the same total RNA samples were electrophoresed in the same gels to compare rRNAs as a control (D).

Formation of small colonies corresponds to the retardation of decline of cell viability in the *ssnA* background. We noticed that small colonies were obtained from liquid cultures at the time that the decline of cell viability occurred at early stationary phase. From 12-h cultures at the late exponential phase, uniform colonies were formed (Fig. 5), whereas, from cultures at 24 to 48 h, when the viability was decreasing, small colonies

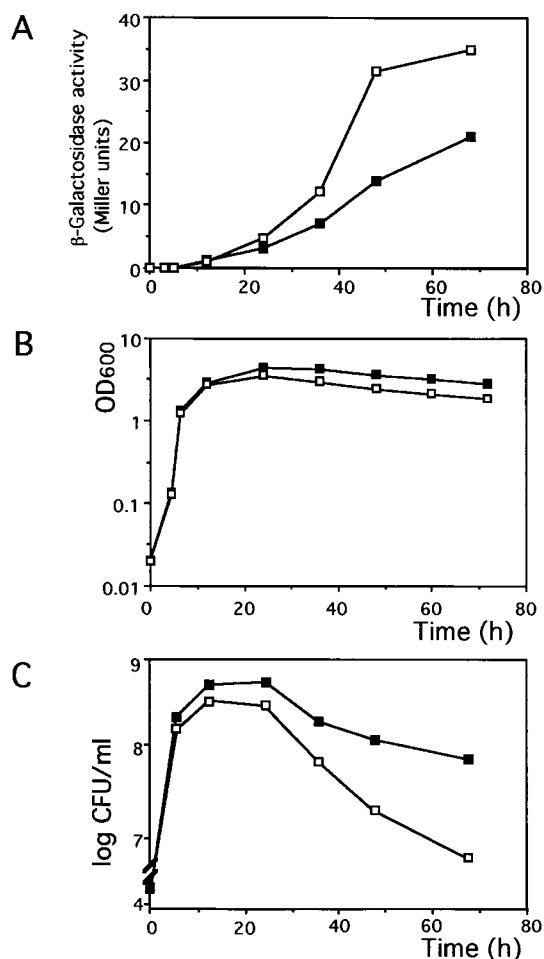


FIG. 4. *ssnA* expression during the course of cell growth. MC1000 (closed squares) or YU381 (MC1000 *rpoS::Tn10*; open squares) cells harboring pSSNA-LAC were grown at 37°C in LB containing ampicillin, and β-galactosidase activity (A), cell density (B), and cell viability (C) were monitored at the times indicated.

were formed in addition to large colonies equivalent in size to those formed at the late exponential phase. A significant difference between the *ssnA* strain and the wild type was observed in this phase. The two strains formed nearly the same numbers of large colonies, but small colonies developed from the 36-h culture of the wild-type strain and from the 36- and 48-h cultures of the *ssnA* strain. Thus, the difference between the two strains in the viable cell number at the beginning of stationary phase may be correlated with the formation of small colonies but not with the formation of large colonies. After the decline of the cell viability (60-h culture), the colonies of the two strains became homogeneous again. Similar numbers of small colonies were formed from 24-h cultures of the *rpoS* strain and from 36- and 48-h cultures of the *rpoS ssnA* strain.

The small size of the colonies may not be caused by mutation, because when cells from the small colonies were subjected to a similar experiment with cultivation in liquid culture, large and small colonies appeared again and the proportion of the two sizes of colonies derived from the small colonies was the same as that derived from the large colonies. The small colonies thus seemed to be formed from cells in liquid cultures during a specific period of stationary phase. From these results, we assume that during such a period, the cells that form small

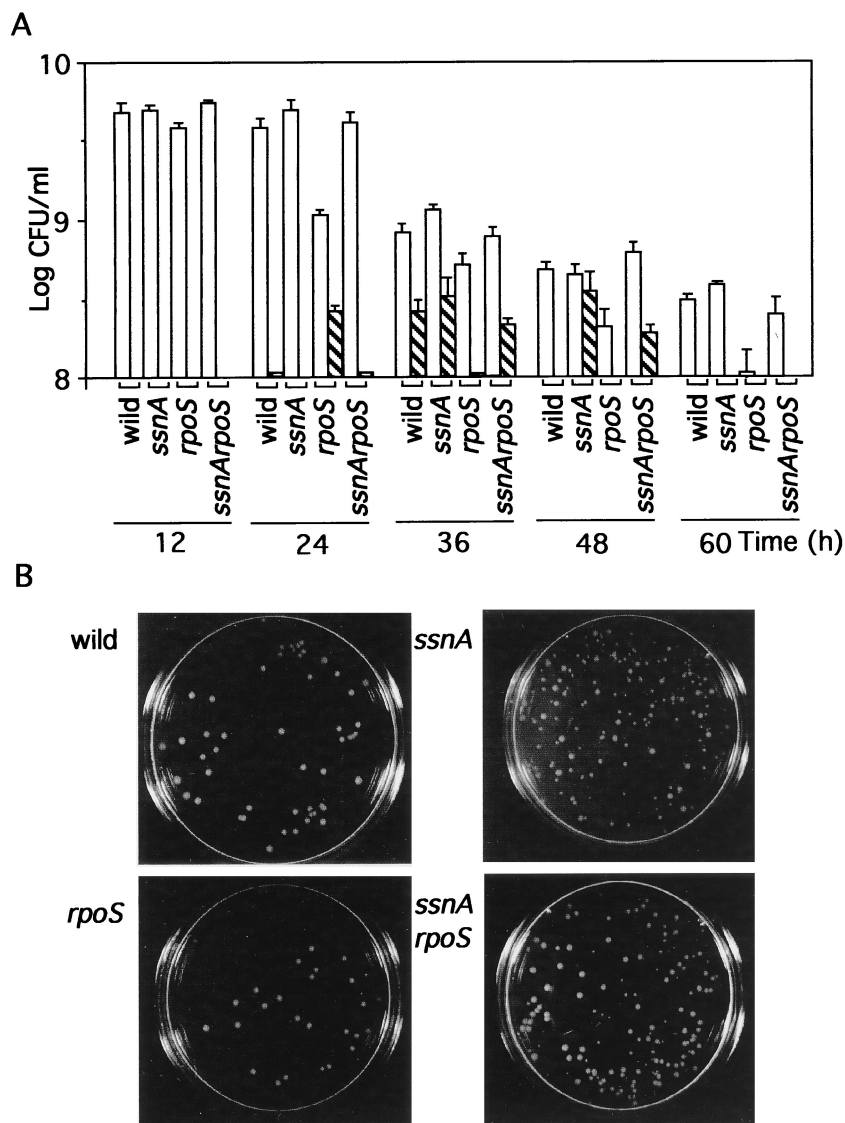


FIG. 5. Small colony formation corresponding to retardation of cell decline in the *ssnA* background. Changes of the number and properties of colonies from W3110, YU449, YU450, and YU451 cells in the liquid culture were examined at the times indicated (A). The numbers of large colonies (with diameters of more than 2 mm) after a 20-h incubation are represented by open columns. Hatched columns represent the number of small colonies (less than 2 mm in diameter). The vertical line for each column corresponds to the standard deviation based on three different experiments. Colonies of each strain obtained from a 48-h culture are shown (B).

colonies may delay the first cell division on plates, and such cells would die if cultivated any longer in liquid medium. In the *ssnA* strain in the 48-h culture (Fig. 5B), many small colonies were formed on plates, whereas in the wild type at the same time, no small colonies were formed. Cells lacking *ssnA* appeared less likely to die compared with the wild type at the early stationary phase. Therefore, it is suggested that *ssnA* reduces the cell viability by enhancing the death of feeble cells at this phase.

The *ssnA* gene and its product. A possible ribosome-binding sequence was found upstream of the initiation codon, ATG, that is 66 bases downstream of the initiation codon of the *orf464* sequence in the GenBank database (Fig. 6A). To test the usage of the initiation codon and to identify the *ssnA* gene product, the 1.3-kb DNA fragment encompassing the region from the ribosome-binding sequence to the stop codon of *ssnA* was cloned under the control of the T7 promoter. SsnA was

then detected as a 44-kDa protein and was found only in the soluble fraction and not in the membrane fraction (Fig. 7). This protein size is roughly equivalent to the mass of 48,841 Da predicted for the amino acid sequence encoded by *orf442*. The sequence of the 9 N-terminal amino acids of the protein was determined, and it completely agreed with the sequence deduced from the nucleotide sequence (data not shown). Therefore, SsnA is suggested to consist of 442 amino acid residues.

The transcriptional start site of *ssnA* was determined by the primer extension method (Fig. 6B). An extension product initiated 211 bases upstream of the translation initiation codon was detected when RNA from cells in stationary phase was analyzed. A band at the same position was also detected in the *rpoS* background (data not shown), whereas no corresponding band could be detected when RNA from cells at exponential phase was analyzed, probably because its level of expression is very low, as described above and previously (21). A sequence

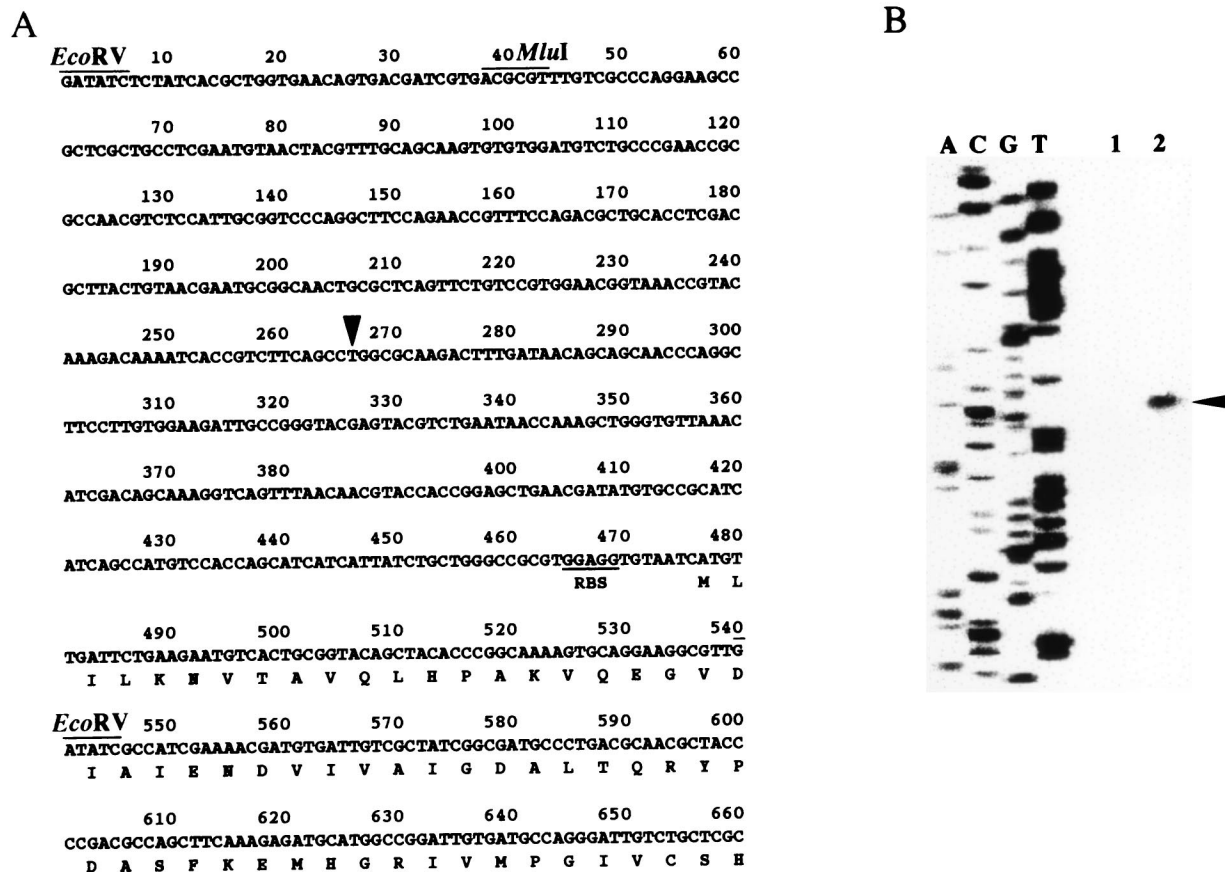


FIG. 6. Nucleotide sequence of the region around the promoter of *ssnA* and its transcription initiation site. (A) The nucleotide sequence of the region around the *ssnA* promoter and the N-terminal amino acid sequence of SsnA are shown. A possible ribosome-binding sequence (RBS) is underlined. The transcriptional start site of *ssnA* is indicated by a triangle. Restriction sites used in this study are indicated above the sequences. (B) Primer extension was performed using a fluorescein isothiocyanate-labeled primer as described in Materials and Methods. Total RNA (1 μ g) from MC1000 harboring pSSNALAC in exponential phase (6-h culture) (lane 1) or in stationary phase (48-h culture) (lane 2) was used. The sequencing ladder (lanes A, C, G, and T) was obtained by using pSSNALAC DNA as a template and the same primer. An arrowhead indicates the extended band.

(CGTACA-15 bases-TCTTCA) weakly similar to the canonical σ^{70} -dependent promoter start sequence was found upstream of this putative transcriptional start site. There is a possible ρ -independent terminator (CCCTCTTCCC—GGGAAGAGGGTTAAATAA) 10 bases downstream of the stop codon of *ssnA*.

Homology searching revealed that SsnA showed weak identity (23.6%) in a region of almost its full length (415 amino acid residues) to the *N*-ethylamine chlorohydrolase homologue in *Methanococcus jannaschii* (accession no. D64492), of which the cellular function has not yet been clarified (4). *N*-Ethylamine chlorohydrolase (TrzA; accession no. L16534) in *Rhodococcus corallinus* has been characterized (18) but is much less similar to SsnA (23.5% identity in a region of 226 amino acid residues) than to the homologue in *M. jannaschii*. Thus, homology searches did not allow us to predict the function of SsnA.

DISCUSSION

When grown in rich medium, *E. coli* exhibits a large decrease in viability at the beginning of stationary phase, and after that, the viable cell number is maintained for many days (26, 27). This process occurs with experimental reproducibility, which means that a decline in cell viability certainly starts and stops at a definite time and/or cell number. When the cells after the decline of cell viability were reinoculated into new medium, a

similar decline in cell viability was observed (data not shown). Therefore, the decline of viability may be due to a cellular mechanism(s) specific for some phase in the life cycle of the organism.

The *ssnA* gene characterized here was found to be specifically expressed as the cell viability was decreasing in the early stationary phase. A mutant lacking the *ssnA* gene showed a delay of the decrease in viability compared with the wild type, although no significant difference was observed in the exponential phase (Fig. 3). Therefore, *ssnA* appears to promote a unique cell death event in the early stationary phase. The *ssnA* gene, however, may be dispensable for the decline of cell viability because it eventually occurs even in *ssnA* mutants.

Although the enzymatic function of SsnA is still unknown, cells harboring the *ssnA* gene on a multicopy plasmid exhibited a filamentous shape, which is one of the characteristic phenotypes of cell division-defective mutants, and sometimes exhibited swollen or burst shapes (Fig. 2). The viable cell number (expressed in CFU per milliliter) was about 100-fold lower than the total cell number scored directly by microscopic observation (data not shown). Therefore, the enhanced production of SsnA might make the host cell defective in cell division and thus nonviable. The *ssnA* gene on the genome is thus assumed to have a negative effect on cell division, although it is unclear whether SsnA directly affects the division machinery.

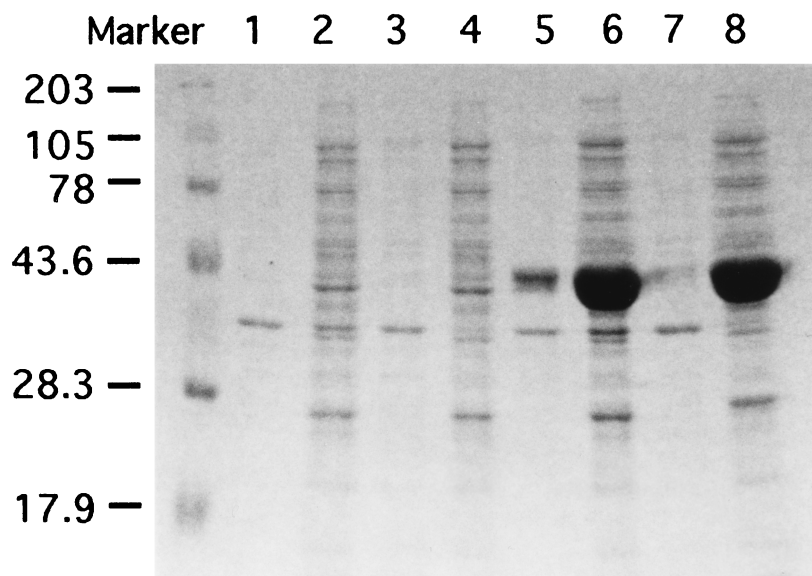


FIG. 7. The subcellular localization of the *ssnA* product. Cells harboring pVEX11 (lanes 1 to 4) or pVEXSSNA (lanes 5 to 8) were grown at 37°C to an OD₆₀₀ of approximately 1.0 and then grown for 4 additional h in the presence of 1 mM IPTG. Subcellular fractions were obtained by French pressure cell press and ultracentrifugation, as described in Materials and Methods, and the fractions were subjected to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, followed by staining with Coomassie brilliant blue. Lanes 1 and 5, unbroken cells recovered by low-speed centrifugation after treatment with a French press; lanes 2 and 6, cell extracts recovered after treatment with a French press; lanes 3 and 7, membrane fractions recovered after ultracentrifugation; lanes 4 and 8, soluble fractions recovered after ultracentrifugation. Samples used in all lanes were from comparable aliquots of the cell cultures. Sizes of the prestained molecular weight markers were as follows: myosin, 203,000; phosphorylase *b*, 105,000; bovine serum albumin, 78,000; ovalbumin, 43,600; carbonic anhydrase, 28,250; β -lactoglobulin, 17,900.

Interestingly, the *ssnA* mutant showed slightly higher cell density than the wild type at stationary phase (Fig. 3A), which might be explained by such a defect in inhibition of cell division and the evidence of retardation of the decline in cell viability in the *ssnA* mutant, we surmise that *ssnA* may be necessary for determining the viable cell number in stationary phase in the nutrient medium used.

A clue for elucidating the physiological function of the *ssnA* gene may lie in the properties of colonies formed from cells in the early stationary phase when the cell viability was decreasing (Fig. 5). The retardation of the decline in the viable cell number in the *ssnA* background seems to be correlated with the appearance of small colonies, which are assumed to be formed from feeble cells that are about to die but to maintain a small degree of viability in the liquid culture at that phase. The *ssnA* gene may reduce the viability of such feeble cells, and thereby the cell population may be promptly adjusted to the level that exists under stationary phase conditions.

Another interesting finding revealed in this study was the relationship between the *ssnA* and *rpoS* genes. The *ssnA* expression that is induced in stationary phase was found to be negatively regulated by *rpoS* (Fig. 3 and 4). The augmented decrease in viable cell number in the *rpoS* background may thus be due to the increased expression of *ssnA*, which was confirmed by measurement of the *ssnA* mRNA by RT-PCR (Fig. 3C). Therefore, *rpoS* may repress the excessive expression of *ssnA*, thereby controlling the number of viable cells in stationary phase. Although the molecular mechanism of the *rpoS*-dependent repression has not been determined, control by *rpoS* may prevent *ssnA* from overexerting its cellular function.

A drastic change in the number of viable cells in early stationary phase is observed in rich medium but not in minimal medium (27). *ssnA* expression in early stationary phase in minimal medium was very low, like that in exponential phase (data not shown), suggesting that an unknown inducer(s) of *ssnA*

expression may be produced in early stationary phase in cells grown in rich medium. Cells in stationary phase may control the viable cell number in their own population, which would be crucial for withstanding nutrient-limited or stressful conditions or for smooth transition of the population from exponential to stationary phase.

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