The yhhP Gene Encoding a Small Ubiquitous Protein Is Fundamental for Normal Cell Growth of Escherichia coli

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Received 18 December 1997/Accepted 20 February 1998

H-NS is a major constituent of the Escherichia coli nucleoid, whereas $\sigma^S$ is a stress-induced sigma factor. An hns null mutation affects the cellular content of $\sigma^S$ in such a way that a remarkable accumulation of $\sigma^S$ is observed in the logarithmic growth phase, which results in enhanced expression of a number of $\sigma^S$-dependent genes, including the katE gene. We isolated an extragenic mutation that affects the expression of the katE-lacZ fusion gene in the $\Delta$hns background. The relevant gene was identified as yhhP, which encodes a small polypeptide of 81 amino acids. Lesion of this gene seemed to affect the stability of $\sigma^S$. A deletion analysis of yhhP revealed that this small protein plays a fundamental role in the general physiology of E. coli. The yhhP-deficient cell is not capable of growing in standard laboratory rich medium (i.e., Luria broth), resulting in the formation of filamentous cells. Homologs of this intriguing protein occur in a wide variety of bacterial species, including archaean species.

H-NS is a major constituent of the Escherichia coli nucleoid (1), whereas $\sigma^S$ is a stress-induced sigma factor (10, 12). The former influences the transcription of a number of apparently unlinked genes on the chromosome (6, 19), whereas the latter modulates the transcription of a certain subset of genes whose expression is enhanced at the stationary phase (5, 13). Although intensive studies of these two proteins have been done separately, it is known that there is a clear link between their cellular functions. We and others previously showed that a mutational lesion of the hns gene (i.e., the hns::neo allele) affects the cellular content of $\sigma^S$ in such a way that a remarkable accumulation of $\sigma^S$ is observed in the logarithmic growth phase (2, 18). This accumulation was shown to be the result of an elevated translational efficiency of $\rho\sigma^S$ mRNA and also of an increased stability of newly synthesized $\sigma^S$ in the $\Delta$hns cells. Consequently, such an event results in enhanced expression of a number of $\sigma^S$-dependent genes, including the katE gene, which encodes a stationary-phase-specific catalase. However, the mechanism by which H-NS controls the cellular content of $\sigma^S$ has been the subject of debate (2, 18). To gain insight into the underlying molecular mechanism, we attempted to search for chromosomal mutations that affect (or decrease) the expression of $\sigma^S$. A deletion analysis of yhhP revealed that this small protein plays a fundamental role in the general physiology of E. coli. The yhhP-deficient cell is not capable of growing in standard laboratory rich medium (i.e., Luria broth), resulting in the formation of filamentous cells. Homologs of this intriguing protein occur in a wide variety of bacterial species, including archaean species.

Isolation of mutants that affect $\alpha^S$ production in an hns deletion mutant. As shown in Fig. 1A, a mutational lesion of the hns gene (i.e., the hns::neo allele) affects the cellular content of $\sigma^S$ in such a way that a great accumulation of $\sigma^S$ is observed in the logarithmic growth phase (GY41, wild type; GY43, $\Delta$hns). Consequently, remarkably enhanced expression of katE-lacZ (or $\beta$-galactosidase) in GY43 was strictly $\sigma^S$ dependent, although the katE-lacZ fusion gene was not significantly expressed in the wild-type strain grown to the logarithmic growth phase in M9 glucose medium (Fig. 1B). In this study, we attempted to search for chromosomal mutations that affect (or decrease) the expression of katE-lacZ in the $\Delta$hns background. GY43 carrying both the hns and katE-lacZ alleles gave dark blue colonies on M9 glucose agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside). The cells were mutagenized with 2% ethyl methane sulfonate (20% viability) and then spread on the selection plate. After an extensive screening, 36 candidates showing white or pale-blue color were isolated, each of which seemed to express a lower level of $\beta$-galactosidase activity. Among them, 15 were found to be due to mutations in the katE-lacZ fusion gene itself while 16 had mutations that mapped in the $\rho\sigma^S$ gene itself. The remaining five candidates were assumed to have extragenic mutations that somehow affect the H-NS-$\sigma^S$-katE regulatory circuit. Levels of $\sigma^S$ in these mutants were directly measured by means of immunoblotting with a $\sigma^S$ antiserum (Fig. 1A), whereas levels of katE-lacZ expression were measured by monitoring $\beta$-galactosidase activities (Fig. 1B). Only one mutant (type 1) exhibited a lower level of $\sigma^S$ (about 50%) than that of the parental strain, while the other four mutants (type 2) had more or less the same level of $\sigma^S$ as the parental strain (Fig. 1A). However, all of them exhibited decreased levels of $\beta$-galactosidase activity (40 to 60% of that of the parental strain) (Fig. 1B). We then focused our attention on the type 1 mutant. Although its observed phenotype is not striking, its putative mutation was assumed to affect the content of $\sigma^S$ in the $\Delta$hns background, thereby resulting in a reduction of katE expression.

Identification of the mutant gene. To transfer this putative mutation into a fresh genetic background and to map its position on the chromosome, a rough mapping was first carried out by means of F-factor-mediated conjugation with a set of Hfr strains (KL series) (17). Then, a fine mapping was done by means of P1 transduction with a large set of Tn10 insertions in the chromosome (xx series::Tn10) (3). The mutation was mapped to a position between 67 and 82 min on the E. coli chromosome and then to a position between 76.9 and 77.8 min.
This result indicated that the phenotypic alteration observed for the putative mutant resulted from a single mutational event (the mutant allele was tentatively designated sirA1, for σS regulation). As a result of these experiments, we have established the following two strains: GY47, which carries only the sirA1 allele, and GY49, which carries the sirA1 allele as well as the Δhns allele (note that without these modifications, these strains have isogenic backgrounds derived from MC4100, which carries the katE-lacZ fusion gene).

The sirA1 mutation affects the stability of σS. σS production was examined in GY47 (sirA1) and GY49 (sirA1 Δhns). It is known that the regulation of σS production in E. coli is modulated during transcription and translation and that it affects stability (11). For both GY47 and GY49, the possible transcriptional control of rpoS was examined by employing an rpoS-lacZ transcriptional fusion gene whereas the possible translational control of rpoS mRNA was examined by means of pulse-labeling with [35S]methionine for a short period (2 min), as described previously (18). The results showed that neither the transcriptional nor the translational efficiency of rpoS mRNA was affected by the sirA1 mutation in both GY47 and GY49 (data not shown). However, it was found that the sirA1 mutation affects significantly the stability of σS, particularly during the logarithmic growth phase (Fig. 2A). As demonstrated previously (2, 18), σS becomes more stable in the Δhns background than in the wild-type background. The sirA1 mutation caused a destabilization of σS in both the Δhns and wild-type backgrounds. Based on this finding, the phenotypes shown in Fig. 1 can be reasonably explained. However, it should be noted that this effect of the sirA1 lesion was not observed for the cells grown to the stationary phase (Fig. 2B). In any case, from these results, we concluded that the sirA1 mutation affects, albeit moderately, σS stability at the logarithmic growth phase.

Cloning of the sirA gene. We then wanted to clarify the nature of the sirA gene. To this end, we attempted to clone this putative gene. Based on the finding that the sirA gene is lo-
formed with the plasmid mixture, and then SirA
mid vector. GY49, which carries the
sequence. Therefore, we conclude that the
yhhP
gene corresponds to the
region. The DNA inserts from these λ phages were digested partially with Sau3A1 and then cloned randomly onto a plasmid vector. GY49, which carries the sirA1 allele, was transformed with the plasmid mixture, and then SirA+ transformants were screened by spreading them on M9 glucose agar plates containing X-Gal, based on the rationale described above. One of the positive clones showing dark-blue color was found to harbor a recombinant plasmid (named pSIRA9), shown in Fig. 3A. Based on the latest version of the E. coli genome structure, a number of open reading frames (ORFs) were predicted within this region. Most of them, except for that of the fisY gene, are hypothetical. We further constructed several subclones from the original plasmid and found that pSIRA9-5 carrying the short BsmI-BclI fragment is able to complement the sirA1 mutation (Fig. 3A). According to sequences in the E. coli databases, this segment should encompass only a single complete ORF (or gene), named yhhP. We determined the entire nucleotide sequence for the cloned BsmI-BclI fragment from pSIRA9-5, and the sequence was confirmed to be identical to those in the databases. The yhhP gene is predicted to encode a protein of only 81 amino acids. To determine whether the yhhP gene corresponds to the sirA gene, we sequenced the yhhP gene from the chromosome of the sirA1 mutant with appropriate oligonucleotide PCR primers. The sirA1 mutant allele was found to have a single base substitution (G to A), which results in an amino acid substitution (Glu to Lys) at amino acid position 18 in the yhhP coding sequence. Therefore, we conclude that the sirA gene corresponds to the yhhP gene (hereafter, sirA will be referred to as yhhP).

Characterization of a yhhP deletion mutant. To gain further insight into the function of the yhhP gene product, the HpaI-HpaI chromosomal region of E. coli MC4100 was replaced by the chloramphenicol cassette (Cm') to yield GY51 (o732-yhhP::Cm') (Fig. 3A). This substitution was carried out by the established method of a linear DNA transformation in a recD mutant background (16). This construct should lack both the functional yhhP and o732 genes on the chromosome. Keeping this fact in mind, we characterized this strain in terms of yhhP's function. We first examined GY51 with special reference to σ5 stability. Essentially the same experiment as that conducted for the sirA1 mutant shown in Fig. 2A was done for GY51, and essentially the same result as that observed for GY49 (sirA1) was obtained (data not shown). It should be emphasized that this altered property of GY51 was completely complemented by the introduction of pSIRA9-5 carrying only the yhhP gene. This result fully supported our previous notion that this gene is implicated in σ5 stability.

The yhhP gene product is crucial for cell viability. Surprisingly, we were unable to isolate the o732-yhhP::Cm' construct on Luria agar rich medium (i.e., we used M9 glucose minimal medium). In addition, although we wanted to construct the o732-yhhP::Cm' deletion in the Δhns background, such a stable construct was never generated. We interpreted these results by assuming that either the o732 or the yhhP gene plays a crucial physiological role under standard conditions for growth. Typical growth curves for MC4100 (wild type) and GY51 (o732-yhhP::Cm') in M9 glucose minimal medium and in Luria broth rich medium at 37°C are shown in Fig. 4A and B, respectively. It was found that when GY51 cells grown in M9 glucose medium were inoculated into the rich medium, cell growth completely ceased almost immediately. Essentially the same result was obtained with agar plates containing rich medium. This phenomenon was seen even when the cells were grown at various temperatures. This typical phenotype is the so-called rich medium sensitivity for growth. We then examined cell morphology for GY51, at the growth points indicated in Fig. 4A. When the GY51 cells were incubated for several hours in the rich medium, they started to show an extremely elongated cell morphology, probably due to a defect in cell division (Fig. 4F) (4, 6-diamidino-2-phenylindole [DAPI]-stained cells). Note that chromosome segregation appeared to occur normally and that each filamentous cell contained multiple nuclei positioned regularly in the compartment. In any case, the rich medium's sensitivity for growth and filamentous-
It was thus revealed that the properties, though to a slightly more moderate extent (i.e., very slow growth on the rich medium), it also exhibited the same characteristic phenotypes. GY51 mutant cells, along with wild-type MC4100 cells, were grown either in M9 glucose medium (A) or Luria broth (B) at 37°C. The growth curves were monitored by measuring turbidity. At the times indicated, the cells were stained with DAPI and then examined by fluorescence microscopy, (C) MC4100 grown in M9 glucose medium; (D) MC4100 grown in Luria-broth; (E) GY51 grown in M9 glucose medium; (F) GY51 grown in Luria broth. Note that scale bars are not indicated because the wild-type cells (C and D) were found to be of average sizes (around 2 μm in length), and all micrographs were prepared with the same magnification as that used for the wild type.

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sequence of *E. coli* revealed that, of 4,288 protein-coding genes annotated, 38% (1,632) have no attributed function (4). Furthermore, of the 4,288 genes, 9% (381) have protein products smaller than 100 amino acids (14). Blattner and his colleagues (4) mentioned that it was difficult in general to assign functions to small ORFs, unless they had been characterized genetically or biochemically. In this sense, our results proved that the small ORF is indeed functional. As recently emphasized by Moxon and Higgins (15), we may not know as much about the biology of *E. coli* as we believe.

We thank K. Ito (Institute for Virus Research, Kyoto University) for kind gifts of *E. coli* strains, (KL series of Hfr strains and those carrying zxx series: Tn7).

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan.

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