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Enhancement of the Synthesis of RpoN, Cra, and H-NS by Polyamines at the Level of Translation in *Escherichia coli* Cultured with Glucose and Glutamate▽†

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Proteins whose synthesis is enhanced by polyamines at the level of translation were identified in a polyamine-requiring mutant cultured in the presence of 0.1% glucose and 0.02% glutamate instead of 0.4% glucose as an energy source. Under these conditions, enhancement of cell growth by polyamines was almost the same as that in the presence of 0.4% glucose. It was found that synthesis of RpoN, Cra, and H-NS was enhanced by polyamines at the level of translation at the early logarithmic phase of growth (A_{540} of 0.15). The effects of polyamines on synthesis of RpoN, H-NS, and Cra were due to the existence of unusual Shine-Dalgarno sequences (RpoN and H-NS) and an inefficient GUG initiation codon (Cra) in their mRNAs. Thus, *rpoN*, *cra*, and *hns* genes were identified as new members of the polyamine modulon. Because most of the polyamine modulon genes thus far identified encode transcription factors (RpoS [σ^{38}], Cya, FecI [σ^{18}], Fis, RpoN [σ^{54}], Cra, and H-NS), DNA microarray analysis of mRNA expressed in cells was performed. At the early logarithmic phase of growth, a total of 97 species of mRNAs that were up-regulated by polyamines more than twofold were under the control of seven polyamine modulon genes mentioned above.

Polyamines (putrescine, spermidine, and spermine) are present at millimolar concentrations in both prokaryotic and eukaryotic cells and play regulatory roles in cell growth (5, 14, 15). Because polyamines interact with nucleic acids and exist mostly as polyamine-RNA complexes in cells (23, 33), their proliferative effects are presumed to be caused by stimulation of nucleic acid and protein synthesis. We have previously studied whether the translation of a defined set of proteins is enhanced by polyamines by using *Escherichia coli* MA261, a polyamine-requiring mutant. The MA261 strain is unable to synthesize putrescine, and cell growth slows down in the absence of exogenous putrescine (6). When putrescine is added to the culture medium, it is taken up into cells (13) and spermidine is synthesized from putrescine, leading to the recovery of cell growth (6, 13). Thus, these polyamine effects in a polyamine-requiring mutant mimic the polyamine effects in normal cells which can synthesize putrescine. By comparing the protein expression patterns in MA261 cultured with and without putrescine, we found that polyamines increase the synthesis of several proteins at the level of translation (8, 12, 36–38). These include OppA, a periplasmic substrate-binding protein of the oligopeptide uptake system (12); adenylate cyclase (Cya) (36); RpoS (σ^{38}) sigma factor, for transcription of stationary-phase genes (37); FecI sigma factor (σ^{18}), for transcription of the iron transport operon (38); Fis, a global regulator of transcription of some growth-related genes including those for rRNA and some tRNAs (38); and RF2, polypeptide releasing factor 2 (8). Taking all these observations together, we proposed that the genes whose expression is enhanced by polyamines at the level of translation can be classified as the "polyamine modulon" (38).

There appear to be several mechanisms underlying polyamine stimulation of the synthesis of various members of the polyamine modulon. First, polyamine stimulation of protein synthesis can occur when a Shine-Dalgarno (SD) sequence in the mRNA is obscure or is distant from the initiation codon AUG. Polyamines cause structural changes in a region of the SD sequence and the initiation codon AUG of the mRNA, facilitating formation of the initiation complex. This is the case for OppA, FecI, and Fis (38, 39). Second, polyamines enhance the inefficient initiation codon UUG-dependent fMet-tRNA binding to *cya* mRNA ribosomes (36). Third, polyamines stimulate readthrough of the amber codon UAG-dependent Gln-tRNA^{supE} on ribosome-associated *rpoS* mRNA (37) or stimulate a +1 frameshift at the 26th UGA codon of *prfB* mRNA encoding RF2 (8). On the basis of these observations, we can predict that translation of some mRNAs with these characteristics can be enhanced by polyamines.

The polyamine modulon members have thus far been identified in *E. coli* grown in minimal 0.4% glucose medium. Under these conditions, polyamines did not influence strongly the synthesis of RpoN (σ^{54}), whose mRNA has an unusual SD sequence. RpoN (σ^{54}) is involved in the transcription of genes for nitrogen metabolism (18, 22). We expected that the pattern of genome expression might change depending on the culture conditions. In this study, we searched for new members of the polyamine modu-

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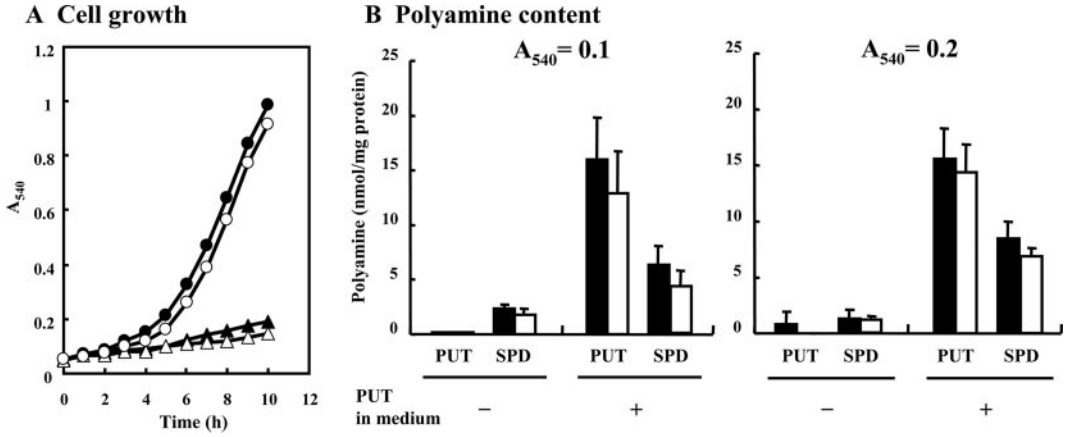

FIG. 1. Growth and polyamine content of *E. coli* MA261. (A) Culture of *E. coli* MA261 cells. ●, 0.1% glucose and 0.02% glutamate with 100 μg/ml putrescine; ▲, 0.1% glucose and 0.02% glutamate without putrescine; ○, 0.4% glucose with 100 μg/ml putrescine; △, 0.4% glucose without putrescine. (B) Polyamine content was measured as described in Materials and Methods. ■, polyamine content of cells cultured in the presence of 0.1% glucose and 0.02% glutamate; □, polyamine content of cells cultured in the presence of 0.4% glucose. Data are shown as means ± SEs of triplicate determinations. PUT, putrescine; SPD, spermidine.

lon by changing nutritional conditions from 0.4% glucose to 0.1% glucose and 0.02% glutamate and found that the *rpoN*, *cra*, and *hns* genes are members of the polyamine modulon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A polyamine-requiring mutant of *Escherichia coli* (6), MA261 (*speB speC gly leu thr thi*), which was kindly provided by W. K. Maas, New York University School of Medicine, and *E. coli* MA261 *lacZ*::Em (20) were cultured in medium A [0.4% glucose (22.2 mM), 40.2 mM K_2HPO_4, 22.1 mM KH_2PO_4, 1.7 mM sodium citrate, 7.6 mM $(NH_4)_2SO_4$, 6 μM thiamine, 40 μM biotin, 0.8 mM leucine, 0.8 mM threonine, 0.7 mM methionine, 1 mM serine, 1 mM glycine, 0.6 mM ornithine, pH 6.8] in the presence and absence of 100 μg/ml (0.6 mM) putrescine dihydrochloride. Where indicated, 0.1% glucose (5.6 mM) and 0.02% glutamic acid (1.4 mM) were added instead of 0.4% glucose per liter of water. Cell growth was monitored by measuring the absorbance at 540 nm.

Plasmids. Total chromosomal DNA from *E. coli* W3110 was prepared according to the method of Wilson et al. (34). To make the *rpoN-lacZ* fusion gene, PCR was performed using total chromosomal DNA as template and 5′-TGACCCG ACCCGGGTTATCGACATTAAACG-3′ (P1) and 5′-GATTTGCTCAACCC GGGGATTACTCTCCAG-3′ (P2) as primers. The amplified *rpoN* gene (a 259-nucleotide 5′ upstream region and a 135-nucleotide open reading frame) was digested with XmaI and inserted into the same restriction site of the medium-copy-number vector pMC1871 (29) to make the pMC*rpoN-lacZ* fusion plasmid. For construction of pMW*rpoN-lacZ*, the BamHI fragment containing the *rpoN-lacZ* gene was obtained from pMC*rpoN-lacZ* and then inserted into the same restriction site of the low-copy-number vector pMW119 (Nippon Gene). Site-directed mutagenesis for the construction of a mutated fusion gene with modified SD sequences was performed by overlap extension using PCR (9). To make the *rpoN*(SD)-*lacZ* fusion gene, the initial PCR was performed using total chromosomal DNA from *E. coli* W3110 as template and P1, 5′-GTTCAGCTCCGTAC TGACGTGCTAAAACGT-3′ (P3), 5′-TTTAGCACGTCAGTACGGAGCTG AACATGA-3′ (P4), and P2 as primers. Then the second PCR was performed using the initial PCR products as template and P1 and P2 as primers. Plasmid pMW*rpoN*(SD)-*lacZ* was prepared using the same method used for the construction of pMW*rpoN-lacZ*.

To make the *cra-lacZ* fusion gene, PCR was performed using total chromosomal DNA as template and 5′-AATACGGAAATATCCCGGGGGCAAATT ATC-3′ (P5) and 5′-GCACCACAGCCCCGGGTTTTTCAACGGTTT-3′ (P6) as primers. The amplified *cra* gene (a 346-nucleotide 5′ upstream region and a 114-nucleotide open reading frame) was digested with XmaI and inserted into the same restriction site of pMC1871 to make the pMC*cra*(GTG)-*lacZ* fusion plasmid. For construction of pMW*cra*(GTG)-*lacZ*, the SalI fragment containing the *cra*(GTG)-*lacZ* gene was obtained from pMC*cra*(GTG)-*lacZ* fusion plasmid and then inserted into the same restriction site of pMW119. Plasmid pMW*cra*(ATG)-*lacZ* was prepared as described above with site-directed mutagenesis by overlap extension using PCR (9). The initial PCR was performed using P5, P5(ATG) (5′-AGCGATTTCATCCAGTTTCATAATTGCCCC-3′), P6(ATG) (5′-ACGCAAGGGGCAATTATGAAACTGGATGAA-3′), and P6 as primers.

To make the *hns-lacZ* fusion gene, PCR was performed using total chromosomal DNA as template and 5′-ATAGGGAATTCCCGGGAACACAACTAA TAC-3′ (P7) and 5′-GCCGCGCTTTCTTCCCCGGGACGTTCGTTA-3′ (P8) as primers. The amplified *hns* gene (a 287-nucleotide 5′ upstream region and a 123-nucleotide open reading frame) was digested with XmaI and inserted into the same restriction site of pMC1871 to make the pMC*hns-lacZ* fusion plasmid. For construction of pMW*hns-lacZ*, the SalI fragment containing the *hns-lacZ* gene was obtained from pMC*hns-lacZ* fusion plasmid and then inserted into the same restriction site of pMW119. Plasmid pMW*hns*(SD)-*lacZ* was prepared as described above with site-directed mutagenesis by overlap extension using PCR (9). The initial PCR was performed using P7, P7(SD) (5′-TGTAGTAACCTCAA ACTTATATTGGGGTGG-3′), P8(SD) (5′-TAAGTTTGAGGTTACTACAAT GAGCGAAGC-3′), and P8 as primers.

The nucleotide sequence of the plasmids was confirmed by the CEQ8000 DNA genetic analysis system (Beckman Coulter).

Dot blot and DNA microarray analysis. *E. coli* MA261 cells were cultured at an A_{540} of 0.03 in the presence and absence of putrescine and harvested at an A_{540} of 0.15. Total RNA was prepared from these cells by the method of Emory and Belasco (7). Dot blot analysis was performed according to the method of Sambrook et al. (27). PCR products of the *rpoN* gene (primers 5′-TTTAGCAC GTCAGTAGGAGGCTGAACATGA-3′ and 5′-CGCATTAAAGTGCGGAA GTC-3′), the *cra* gene (primers 5′-GTGAAACTGGATGAAATCGC-3′ and 5′-ATCATCCTGATCGGCACCAA-3′), and the *hns* gene (primers 5′-TAAGT TTGAGGTTACTACAATGAGCGAAGC-3′ and 5′-TGCTTGATCAGGAAA TCGTC-3′) were labeled with [α-^{32}P]dCTP by using the BcaBEST labeling kit (Takara Shuzo Co.) and used as probe. The probe used for the *cya* gene was synthesized as described previously (36). The sizes of the probes used for *rpoN*, *cra*, *hns*, and *cya* genes were 736, 498, 429, and 2,547 nucleotides, respectively. The radioactivity on the blot was quantified with a BAS2000II imaging analyzer (Fuji Film). DNA microarray experiments were carried out according to the method of Oshima et al. (25) with total RNA isolated from *E. coli* MA261 harvested at an A_{540} of 0.15 by using TaKaRa IntelliGene *E. coli* CHIP, version 2.0 (Takara Shuzo Co.). Data were analyzed with ImageQuant (Molecular Dynamics) (35). Data in Table S1 in the supplemental material are shown as means of triplicate determinations. The standard error (SE) of all data was less than 10%.

Western blot analysis. Western blot analysis was performed by the method of Nielsen et al. (24) using ECL Western blotting reagents (GE Healthcare Bio-Sciences). Antibodies to OppA, Cya, RpoS (σ^{38}), FecI, Fis, RpoN (σ^{54}), cyclic AMP (cAMP) receptor protein (CRP), and RpoF (σ^{28}) were prepared as described previously (1, 12, 18, 19, 36). Antibody to Cra was prepared using purified

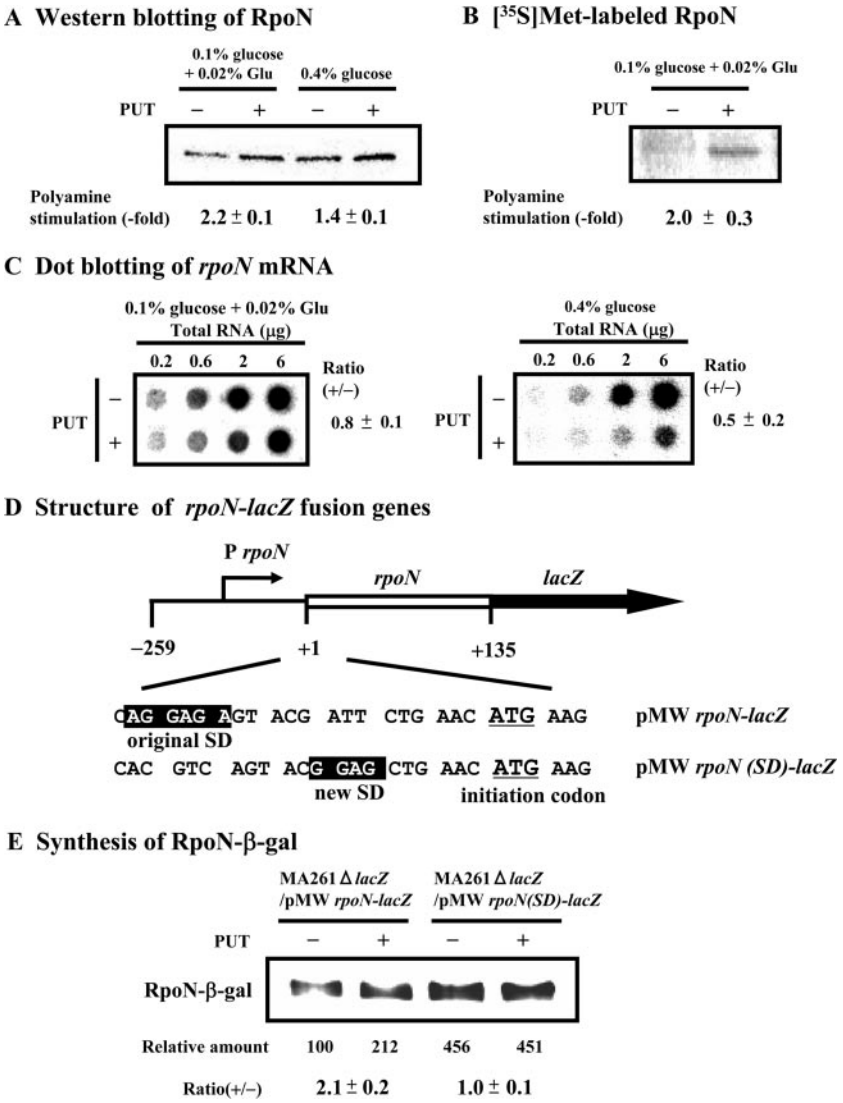

FIG. 2. Effect of polyamines on synthesis of RpoN in *E. coli* MA261. (A) Cells were cultured in the presence and absence of 100 μg/ml putrescine and harvested at an A_{540} of 0.15. Western blotting of RpoN was performed using 10 μg of protein of cell lysate. (B) Measurement of [^{35}S]methionine-labeled RpoN was performed using 1,000,000 cpm of [^{35}S]methionine-labeled protein and antibody to RpoN. (C) Dot blot analysis of *rpoN* mRNA was performed as described in Materials and Methods. (D) Schematic of the *rpoN-lacZ* fusion genes. The *rpoN* gene containing a 259-nucleotide 5′ upstream region with an unmodified or modified SD sequence and a 135-nucleotide open reading frame was fused to the *lacZ* gene. (E) Western blotting of RpoN–β-Gal fusion protein was performed using 20 μg of protein of cell lysate. The levels of *rpoN-lacZ* mRNA in cells cultured with and in those cultured without 100 μg/ml putrescine were nearly equal judging from the dot blot analysis. Values are means ± SEs of triplicate determinations. PUT, putrescine.

Cra (30). Antibodies to RF2 (17) and H-NS (32) were kindly supplied by Y. Nakamura, University of Tokyo, and T. Mizuno, Nagoya University, respectively. Antibody to β-galactosidase was obtained from Sigma. The level of protein was quantified with a LAS-1000 Plus luminescent image analyzer (Fuji Film).

Measurement of [^{35}S]methionine-labeled RpoN. *E. coli* MA261 cells were cultured in medium A containing 0.03 mM methionine in the absence of putrescine. At the cell density of 0.15 A_{540}, the culture was divided into 5-ml aliquots and continued to grow in the presence (100 μg/ml) and absence of putrescine. After 10 min, [^{35}S]methionine (1 MBq) was added to each 5-ml aliquot, and the cells were allowed to grow for an additional 20 min. After the addition of unlabeled methionine at a final concentration of 20 mM, the cells were harvested, resuspended in 1 ml of buffer A (10 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1% Triton X-100, and 0.1% sodium dodecyl sulfate), and disrupted with a French pressure cell at 20,000 lb/in^2. The amount of radioactive RpoN was determined using whole-cell lysate containing 1,000,000 cpm of [^{35}S]methionine-labeled proteins and antiserum to RpoN as described previously (36). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the radioactivity associated with RpoN was quantified using a BAS2000II imaging analyzer (Fuji Film).

Measurement of polyamine and cAMP content in whole cells. Polyamines and cAMP in whole cells harvested at an A_{540} of 0.1, 0.15, or 0.2 were extracted by treatment of the cells with 10% trichloroacetic acid at 70°C for 15 min with occasional shaking. Polyamine content was determined by high-pressure liquid chromatography as described previously (11). The cAMP content in cells was measured using a cAMP enzyme immunoassay system (GE Healthcare Bio-Sciences) after trichloroacetic acid was removed with ether. Protein content was determined by the method of Bradford (4).

RESULTS

Stimulation of cell growth by polyamines during the culture of *E. coli* in the presence of 0.1% glucose and 0.02% glutamate. Previously we analyzed the influence of polyamines on cell growth and the gene expression pattern for *E. coli* grown in the presence of 0.4% glucose as a sole carbon source (12, 38). In

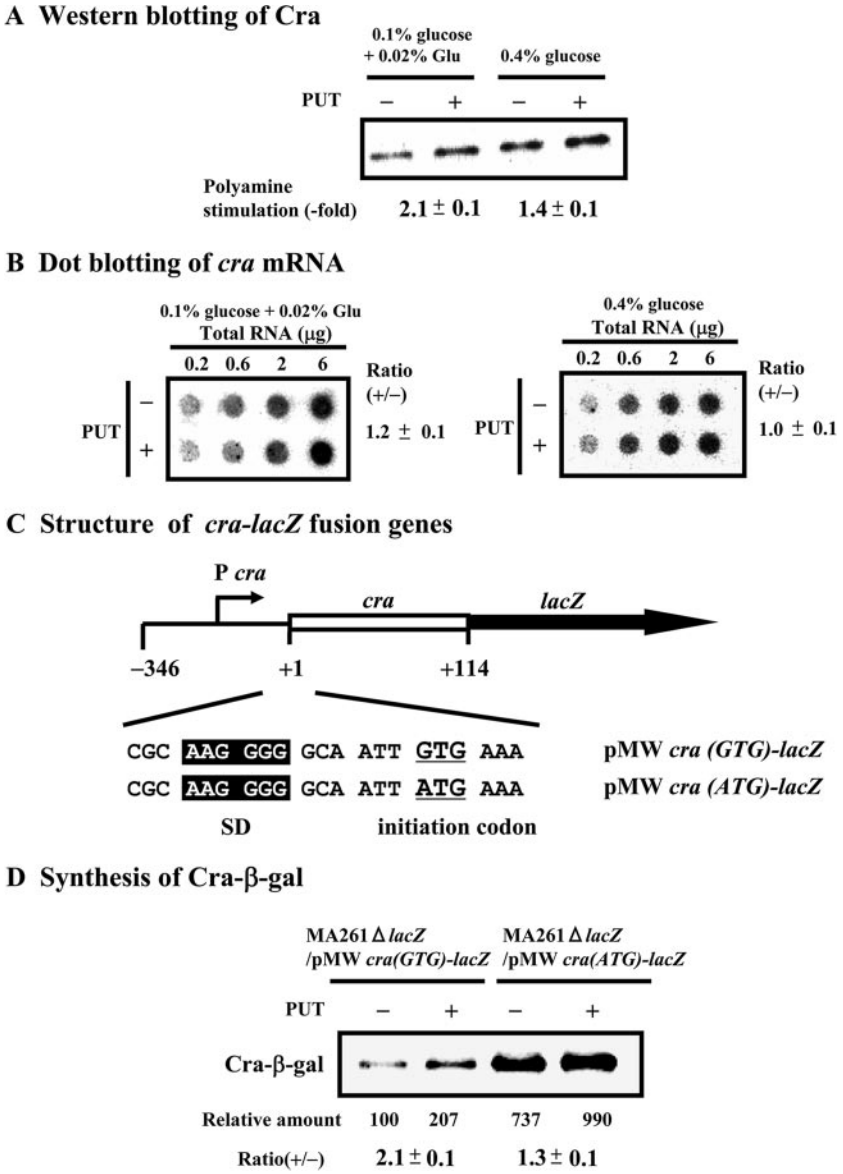

FIG. 3. Effect of polyamines on synthesis of Cra in *E. coli* MA261. (A) Western blotting of Cra was performed using 10 μg of protein of cell lysate. (B) Dot blot analysis of *cra* mRNA was performed as described in Materials and Methods. (C) Schematic of *cra-lacZ* fusion genes. The *cra* gene containing a 346-nucleotide 5′ upstream region and a 114-nucleotide open reading frame with a GUG or AUG initiation codon was fused to the *lacZ* gene. (D) Western blotting of Cra–β-Gal fusion protein was performed using 20 μg of protein of cell lysate. The levels of *cra-lacZ* mRNA in cells cultured with and in those cultured without 100 μg/ml putrescine were nearly equal judging from the dot blot analysis. Values are means ± SEs of triplicate determinations. PUT, putrescine.

nature, amino acids are also used as an energy source together with glucose. In this study, we analyzed the effect of external addition of putrescine on cells of a polyamine-requiring mutant, *E. coli* MA261, grown in the presence of 0.1% glucose and 0.02% glutamate. As shown in Fig. 1A and 1B, stimulation of cell growth by polyamines and polyamine contents in cells cultured with putrescine and those in cells cultured without putrescine were nearly equal regardless of the energy source.

Stimulation of synthesis of RpoN and Cra by polyamines at the level of translation. To identify the polyamine modulon members, we focused on mRNAs with the sequence characteristics of the polyamine modulon as noted above. Glutamate is a key molecule for nitrogen metabolism supplied by the external environment. RpoN (σ^{54}) is one of the key regulatory factors involved in transcription of a wide variety of stress response genes including those involved in nitrogen metabolism (18, 22). On the other hand, Cra (or FruR) is a global transcription factor for a large number of genes involved in glycolysis and glyconeogenesis (26, 30). Furthermore, the SD sequence of the *rpoN* mRNA is distant from the initiation codon AUG, and the initiation codon of *cra* mRNA is GUG instead of AUG. Thus, we first examined the possible influence of polyamines on the synthesis of these two global regulators, RpoN and Cra, in the presence of both glucose and glutamate.

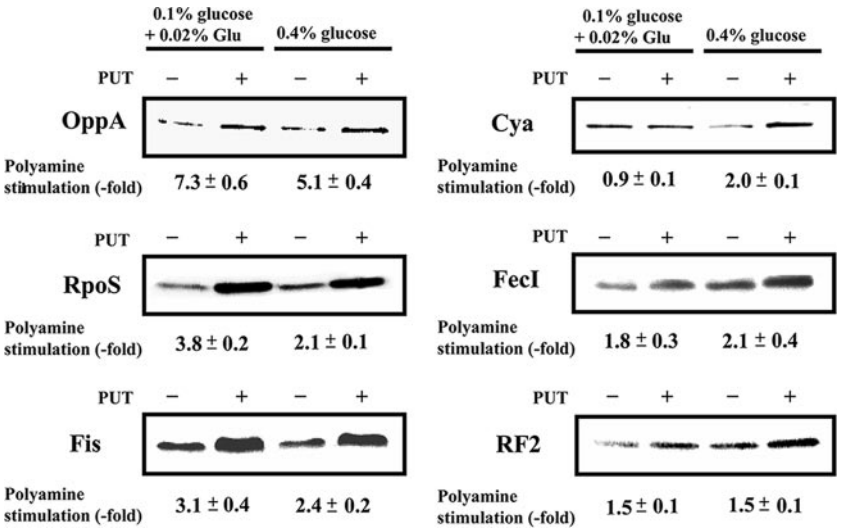

FIG. 4. Levels of OppA, Cya, RpoS, FecI, Fis, and RF2 in *E. coli* MA261 cultured with and without putrescine. Western blotting of OppA, Cya, RpoS, FecI, Fis, and RF2 was performed using 1, 10, 10, 50, 10, and 10 μg of protein of cell lysate, respectively. Values are means ± SEs of triplicate determinations. PUT, putrescine.

When *E. coli* MA261 cells were cultured in the presence of 0.4% glucose and harvested at an A_{540} of 0.2, polyamines did not stimulate the synthesis of RpoN significantly (36). However, when these cells were harvested at an earlier logarithmic phase of cell growth (A_{540} of 0.15), polyamines slightly enhanced the synthesis of RpoN (1.4-fold) (Fig. 2A). When MA261 cells were cultured in the presence of both 0.1% glucose and 0.02% glutamate and harvested at an A_{540} of 0.15, polyamines significantly enhanced synthesis of RpoN (2.2-fold) as determined by Western blotting (Fig. 2A). This was confirmed by pulse-labeling of proteins with [^{35}S]methionine and immunoprecipitation with antibody to RpoN (Fig. 2B). The level of *rpoN* mRNA in cells cultured in the presence of putrescine was 50 to 80% of the level in the absence of putrescine (Fig. 2C). The results suggest that the efficiency of *rpoN* mRNA translation increases in the presence of polyamines. One characteristic of the *rpoN* mRNA is that an SD sequence (AGGAGA) is present 15 nucleotides upstream of the initiation codon AUG of *rpoN* mRNA. This is similar to the sequence found in *oppA* mRNA, the translation of which is enhanced by polyamines (39). To determine whether the abnormal distance of the SD sequence from the initiation codon AUG is involved in polyamine stimulation of RpoN synthesis, it was replaced by a typical SD sequence 7 nucleotides upstream from the initiation codon AUG (Fig. 2D), and the level of RpoN–β-galactosidase (RpoN–β-Gal) fusion protein was measured by Western blotting. Synthesis of an RpoN–β-Gal fusion protein from the original SD sequence-containing mRNA was stimulated 2.1-fold by polyamines, whereas the polyamine stimulation was not observed after replacement with the consensus SD sequence even though the basal level of protein synthesis in the absence of polyamines was enhanced by 4.6-fold (Fig. 2E). The results indicate that the synthesis of RpoN was enhanced by polyamines at the level of translation by modulating the conformation of *rpoN* mRNA in the region containing the unusual SD sequence.

We next examined the effect of polyamines on Cra synthesis. When *E. coli* MA261 cells were cultured in the presence of 0.4% glucose and harvested at an A_{540} of 0.15, polyamines slightly enhanced the synthesis of Cra (1.4-fold) (Fig. 3A). When the cells were cultured in the presence of 0.1% glucose and 0.02% glutamate and harvested at an A_{540} of 0.15, polyamines stimulated the synthesis of Cra by 2.1-fold (Fig. 3A). The levels of *cra* mRNA in cells cultured with and in those cultured without 100 μg/ml putrescine were nearly equal (Fig. 3B), suggesting that polyamines stimulate the synthesis of Cra at the level of translation. The *cra* mRNA has a GUG initiation codon instead of AUG. This is similar to *cya* mRNA, the translation of which is enhanced by polyamines (36). To determine whether the inefficient GUG codon is related to polyamine stimulation of Cra synthesis, the initiation codon GUG was replaced by AUG (Fig. 3C), and the translation efficiency of *cra-lacZ* fusion mRNA was measured. Synthesis of Cra–β-Gal fusion protein from the mRNA containing the GUG initiation codon was stimulated by polyamines 2.1-fold, whereas the polyamine stimulation was reduced to 1.3-fold after replacement with an AUG initiation codon even though the basal level of protein synthesis in the absence of polyamines was strongly enhanced (7.4-fold) (Fig. 3D). The results indicate that the synthesis of Cra was also stimulated by polyamines at the translational level due to the existence of an inefficient initiation codon GUG in *cra* mRNA.

Synthesis of other proteins encoded by polyamine modulon genes in the presence of 0.1% glucose and 0.02% glutamate. We then determined whether synthesis of other proteins encoded by the hitherto-identified polyamine modulon genes is stimulated by polyamines in *E. coli* MA261 cultured in the presence of both 0.1% glucose and 0.02% glutamate instead of 0.4% glucose. As shown in Fig. 4, synthesis of OppA, RpoS (σ^{38}), Fis, FecI (σ^{18}), and RF2 was enhanced by polyamines. However, polyamine stimulation of Cya synthesis was lost when the cells were cultured in the presence of 0.1% glucose and 0.02% glutamate. As shown in Fig. 5A, the level of *cya* mRNA decreased greatly in the presence of polyamines, when

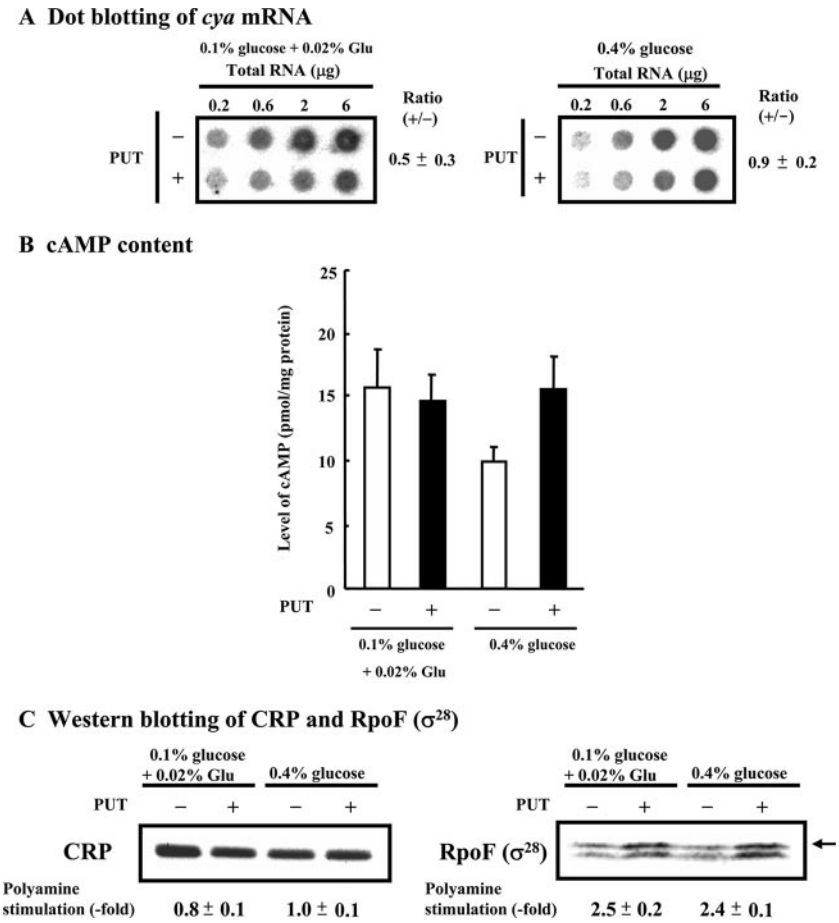

FIG. 5. Measurement of *cya* mRNA (A), cAMP (B), and CRP and RpoF (C) levels in *E. coli* MA261 cultured with or without putrescine. These were measured as described in Materials and Methods. Western blotting of CRP and RpoF (σ^{28}) was performed using 10 and 20 μg of protein of cell lysate, respectively. The arrow indicates the position of intact RpoF, and the second band is a degradation product of RpoF. Values are means ± SEs of triplicate determinations. PUT, putrescine.

cells were cultured in the presence of 0.1% glucose and 0.02% glutamate. When cells were cultured in the presence of 0.4% glucose, the levels of *cya* mRNA were nearly equal in cells cultured with and in those cultured without putrescine. This suggests that polyamine stimulation of *cya* mRNA translation operates even in the presence of glutamate, but the *cya* mRNA level significantly decreased when cells were cultured in the presence of 0.1% glucose and 0.02% glutamate.

CRP is a global regulator that controls transcription of hundreds of *E. coli* genes, in particular those involved in carbon metabolism. The levels of both CRP and its effector cAMP, however, stay nearly constant in cells cultured with and in those cultured without putrescine (Fig. 5B and 5C). However, the level of RpoF (σ^{28}) factor, whose synthesis is controlled by cAMP/CRP or H-NS (see Fig. 6),was higher when cells were cultured in the presence of putrescine (Fig. 5C). Thus, the effect of polyamines on synthesis of H-NS was examined.

Stimulation of H-NS synthesis by polyamines in the presence of 0.1% glucose and 0.02% glutamate. Synthesis of RpoF (σ^{28}) factor is positively regulated by the flagellar master operon (*flhDC*) (2), and the expression of *flhDC* is regulated by both cAMP/CRP and H-NS (Fig. 6A) (21, 31). Because the levels of cAMP and CRP did not change in the presence and absence of putrescine, the effect of polyamines on synthesis of H-NS was examined. The level of H-NS was significantly enhanced by polyamines in cells cultured with 0.1% glucose and 0.02% glutamate at the early logarithmic phase (A_{540} of 0.15) (Fig. 6B). However, the *hns* mRNA levels in cells cultured with and in those cultured without 100 μg/ml putrescine were nearly equal (Fig. 6C), suggesting that polyamines stimulate the synthesis of H-NS by enhancing the efficiency of its translation. Synthesis of H-NS was also slightly enhanced by polyamines at the level of translation when cells were cultured with 0.4% glucose. The *hns* mRNA has a weak SD sequence 10 nucleotides upstream of the initiation codon AUG, as in the case of *rpoN* mRNA.

To determine whether the weak and distant SD sequence is involved in polyamine stimulation of *hns* mRNA translation, it was replaced by a typical SD sequence 9 nucleotides upstream of the initiation codon AUG (Fig. 6D), and the translation efficiency of *hns-lacZ* fusion mRNA was measured. Synthesis of H-NS–β-Gal fusion protein from the original SD sequence-containing mRNA was stimulated 2.0-fold by polyamines, whereas polyamine stimulation was reduced to 0.9-fold after replacement with the consensus SD sequence even though the basal level of protein synthesis in the absence of polyamines

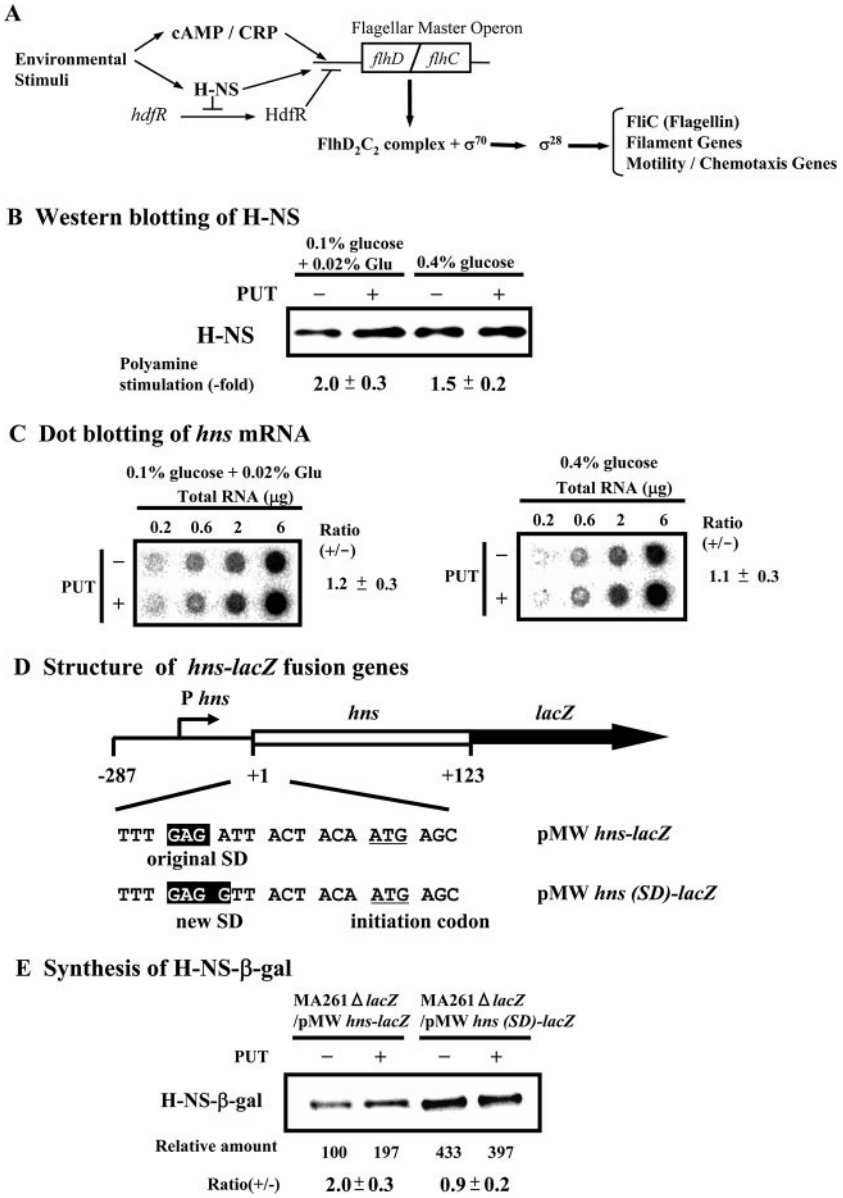

FIG. 6. Effect of polyamines on synthesis of H-NS in *E. coli* MA261. (A) Genes that regulate σ^{28} synthesis. (B) Western blotting of H-NS was performed using 10 μg of protein of cell lysate. (C) Dot blot analysis of *hns* mRNA was performed as described in Materials and Methods. (D) Schematic of *hns-lacZ* fusion genes. The *hns* gene containing a 287-nucleotide 5′ upstream region with an unmodified or a modified SD sequence and a 123-nucleotide open reading frame was fused to the *lacZ* gene. (E) Western blotting of H-NS–β-Gal fusion protein was performed using 20 μg of protein of cell lysate. The levels of *hns-lacZ* mRNA in cells cultured with and in those cultured without 100 μg/ml putrescine were nearly equal judging from the dot blot analysis. Values are means ± SEs of triplicate determinations. PUT, putrescine.

was enhanced by 4.3-fold (Fig. 6E). The results indicate that the synthesis of H-NS was enhanced by polyamines at the level of translation due to the existence of a unique SD sequence in the *hns* mRNA.

Our results indicate that *rpoN*, *cra*, and *hns* genes are new members of the polyamine modulon. Including these three genes, a total of nine genes (*oppA*, *rpoS*, *cya*, *fecI*, *fis*, *rpoN*, *cra*, *hns*, and *prfB*) have now been identified as members of the polyamine modulon.

Identification of mRNAs whose synthesis is regulated by polyamines at the early logarithmic phase. To identify the whole set of mRNAs whose synthesis is regulated by polyamines at the early logarithmic phase, we next performed microarray analysis (28). When *E. coli* MA261 cells were cultured in the presence of 0.4% glucose, 347 out of 2,714 genes were up-regulated (>2-fold increase) and 285 genes were down-regulated (>2-fold decrease) by polyamines (8). When cells were cultured in the presence of 0.1% glucose and 0.02% glutamate, 313 among 2,879 genes were up-regulated (>2-fold increase) and 259 genes were down-regulated (>2-fold decrease) by polyamines. The number of total genes expressed was slightly different in the two culture conditions. This may be

TABLE 1. Typical genes up- and down-regulated by RpoS, Cya, FecI, Fis, RpoN, Cra, and H-NS in the presence of putrescine[a]

Regulator	Gene(s)
σ^{38} (σ^{S})	Up: *acnA*,[b] *appC*, *cbpA*, *cfa*, ***dps***, *elaB*, *entD*, *fic*, *ftsZ*, *gabD*, *gabP*, *gadA*, ***gadB***, *glcG*, *glgA*, *hdeA*, *hdeB*, *hyaC*, *hyaF*, *katG*, ***narY***, *osmE*, *otsB*, *phnP*, ***poxB***, *slp*, *sufS*, *talA*, *treA*, *wrbA*, ***xasA***, ***yahO***, *ybaT*, ***ybgA***, *ybgS*, *ybhP*, *ycaC*, *ycfH*, *ycgB*, *ydeI*, *yeaG*, *yebF*, *ygiS*, *yhcO*, *yhiE*, ***yhiU***, *yiaG*, *yjbJ*, *yidI*, *yjdJ*, ***yjgR***, *ytfQ* Down: ***appY***,[c] *argH*, *artI*, *artP*, *glgA*, *uspA*, *ybiO*, *yggE*
Cya	Up: *aer*, *flgM*, ***flhC***,[d] ***flhD***,[d] ***fliA***, ***fliD***, *fliI*, ***fliN***, ***manX***, ***manZ***, *ptsG*,[e] *ptsI*, ***sdhA***, ***sdhD*** Down: ***argG***, *ompR*
FecI (σ^{18})	Up: ***fecA***
Fis	Up: *adhE*, *nuoA*, *nuoB*, *nuoD*, ***nuoG***, ***nuoI***, ***nuoK***, ***nuoL***, *ptsG*[e]
σ^{54} (σ^{N})	Up: *astB*, *atoD*, *atoE*, *fhlA*, *glnK*, *hypC*, *hypE*, *pspA* Down: ***hisJ***, *hisQ*, ***glnH***, ***glnP***, *glnQ*, *hycF*
Cra	Up: *acnA*[b] Down: *fruB*, *yahA*
H-NS	Up: *flgI*, *flhA*, ***flhC***,[d] ***flhD***,[d] ***rplA***, ***rplB***, *rplC*, *rplF*, *rplJ*, *rplK*, *rplL*, *rplN*, ***rplQ***, *rpmG*, ***rpsN***, ***rpsS*** Down: ***appY***,[c] *aslB*, *csgA*, *feoB*, *gnd*, *spy*, *wcaI*, *ydbD*, *ygaP*

[a] The 97 kinds of genes up-regulated more than twofold and the 26 kinds of genes down-regulated more than twofold by polyamines in the presence of 0.1% glucose and 0.02% glutamate are shown. Boldface indicates genes regulated more than twofold by polyamines in both culture conditions (0.1% glucose and 0.02% glutamate, and 0.4% glucose). Underlining indicates genes regulated more than 1.5-fold by polyamines in the presence of 0.4% glucose.
[b] Regulated by both RpoS (σ^{38}) and Cra.
[c] Regulated by both RpoS (σ^{38}) and H-NS.
[d] Regulated by both cAMP-CRP and H-NS.
[e] Regulated by both cAMP-CRP and Fis.

due to the difference of the nutritional conditions or the batch of chips used. The details on up- and down-regulated genes in the presence of 0.1% glucose and 0.02% glutamate can be seen in the supplemental material. Among nine proteins encoded by the polyamine modulon, seven (RpoS [σ^{38}], Cya, FecI [σ^{18}], Fis, RpoN [σ^{54}], Cra, and H-NS) were involved in transcription. It was found that 97 kinds of mRNAs up-regulated by polyamines and 26 kinds of mRNAs down-regulated by polyamines more than twofold were under the control of the seven polyamine modulons at the early logarithmic phase of cells cultured in the presence of 0.1% glucose and 0.02% glutamate (Table 1). There were 28 kinds of common mRNAs up-regulated more than twofold by the polyamine modulon in the presence of either 0.1% glucose plus 0.02% glutamate or 0.4% glucose (boldface in Table 1), but the number of commonly up-regulated mRNAs increased to 47 (boldface and underlining) when the level of up-regulation was set at more than 1.5-fold in cells cultured in the presence of 0.4% glucose. Among 26 kinds of mRNAs down-regulated by polyamines, many mRNAs were related to the amino acid metabolism. These results support our ideas concerning polyamine stimulation of cell growth (15): Polyamines stimulate some kinds of protein synthesis at the level of translation through polyamine interaction with specific regions of mRNA. These proteins then enhance the expression of approximately 300 kinds of mRNAs, rRNA, and some kinds of tRNAs which are important for cell growth.

DISCUSSION

In this study, we tried to find new members of the polyamine modulon by using *E. coli* cells cultured in the presence of both glucose and glutamate instead of glucose alone. The addition of glutamate influences the expression pattern of a number of the genes involved in carbon and nitrogen metabolism. Transcription of the genes for these metabolic enzymes is under the control of many transcription factors. We thus looked for new members of the polyamine modulon from a set of the genes encoding a total of about 150 species of the transcription factor (16).

It has been reported elsewhere that the distribution of start codons is as follows: ATG, 3,542; GTG, 612; and TTG, 130 (3). However, there is no information about unusual SD sequences. When we searched the nucleotide sequence of the mRNAs encoding 162 kinds of transcription factors, 25 kinds of mRNAs have a nonconsensus SD sequence and 12 kinds of mRNA have either GUG or UUG initiation codons. Among the genes encoding these transcription factors, it was found that *rpoN* and *cra* were new members of the polyamine modulon. *rpoN* mRNA has a nonconsensus SD sequence while *cra* mRNA has an inefficient initiation codon. Synthesis of RpoN and Cra was slightly enhanced by polyamines (1.4-fold) in the presence of 0.4% glucose as a sole carbon source. It is reasonable that the polyamine effect became greater in the presence of both 0.1% glucose and 0.02% glutamate, because RpoN (σ^{54}) controls the expression of a wide variety of stress response genes involved in nitrogen metabolism (18, 22). On the other hand, Cra (or FruR) controls a large number of the genes for glycolysis and glyconeogenesis (26, 30). Since the expression level of Cra was influenced by the presence of glutamate, Cra may be one key regulator of the balance between carbon and nitrogen metabolism. As for RpoN synthesis, *rpoN* mRNA transcription was slightly inhibited by polyamines in the presence of 0.4% glucose. Thus, the polyamine effects at the level of translation of RpoN synthesis may be nearly equal in the two culture conditions.

Both Cya and H-NS enhance the synthesis of RpoF (σ^{28}), which controls the genes involved in the downstream cas-

cades (2). Synthesis of H-NS was stimulated by polyamines in both culture conditions, while Cya synthesis was enhanced by polyamines only in the presence of glucose. Thus, H-NS may be more important than Cya for the synthesis of RpoF. H-NS functions as a positive regulator of expression of genes involved in flagellin synthesis (20, 31) and also ribosomal protein synthesis (10). In fact, we found that the expression of mRNAs encoding flagellin and ribosomal proteins was enhanced by polyamines (Table 1). Since polyamines function at the level of translation (15), it is noted that polyamines stimulate the synthesis of Fis, which enhances rRNA synthesis (38), and also H-NS, which enhances the synthesis of ribosomal protein mRNA in addition to the synthesis of flagellin mRNAs.

Among the hitherto-identified nine members of the polyamine modulon, seven proteins are involved in the regulation of transcription. Microarray analysis indicated that polyamines stimulated the expression of approximately 300 kinds of mRNAs more than twofold. Among these mRNAs, 97 mRNAs were under the control of these seven transcription factors (RpoS, Cya, FecI, Fis, RpoN, Cra, and H-NS) encoded by the polyamine modulon. Through further identification of the polyamine modulon members, we would like to clarify the physiological functions of polyamines at the molecular level.

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