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# Spermidine Acetyltransferase Is Required To Prevent Spermidine Toxicity at Low Temperatures in *Escherichia coli*

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**Polyamines are required for optimal growth in most cells; however, polyamine accumulation leads to inhibition of cellular growth. To reduce intracellular polyamine levels, spermidine is monoacetylated in both prokaryotes and eukaryotes. In *Escherichia coli*, the *speG* gene encodes the spermidine acetyltransferase, which transfers the acetyl group to either the N-1 or N-8 position. In addition to polyamine accumulation, stress conditions, such as cold shock, cause an increase in the level of spermidine acetylation, suggesting an adaptive role for reduced polyamine levels under stressful growth conditions. The effect of spermidine accumulation on the growth of *E. coli* at low temperature was examined using a *speG* mutant. At 37°C, growth of the *speG* mutant was normal in the presence of 0.5 or 1 mM spermidine. However, following a shift to 7°C, the addition of 0.5 or 1 mM spermidine resulted in inhibition of cellular growth or cell lysis, respectively. Furthermore, at 7°C, spermidine accumulation resulted in a decrease in total protein synthesis accompanied by an increase in the synthesis of the major cold shock proteins CspA, CspB, and CspG. However, the addition of 50 mM $Mg^{2+}$ restored growth and protein synthesis in the presence of 0.5 mM spermidine. The results indicate that the level of spermidine acetylation increases at low temperature to prevent spermidine toxicity. The data suggest that the excess spermidine replaces the ribosome-bound $Mg^{2+}$, resulting in ribosome inactivation at low temperatures.**

Polyamines, such as spermidine and putrescine, are present in virtually all cells (25, 33). These polycations have pleiotropic properties, which can influence several cellular processes. They can bind to nucleic acids, stabilize membranes, and stimulate the activity of several enzymes, such as RNA polymerase (1, 31, 34). Found in the ribosomal fraction, polyamines enhance the synthesis of several proteins and stimulate the in vivo assembly of the *Escherichia coli* 30S ribosomal subunit (7, 13, 21, 35).

Although intracellular polyamine is required for optimal growth, polyamine accumulation can lead to inhibition of cellular growth (11, 25, 26, 33). The addition of spermidine to cell cultures of mouse FM3A cells results in a decrease in cell growth accompanied by inhibition of protein synthesis (11). To prevent polyamine toxicity in eukaryotes, polyamine catabolism is initiated by the monoacetylation of spermidine and spermine catalyzed by spermidine/spermine $N^1$-acetyltransferase (SSAT) (4). The acetylpolyamines are then either further oxidized by polyamine oxidase or excreted from the cell.

In *E. coli*, the *speG* gene encodes spermidine acetyltransferase (SAT), which transfers the acetyl group to either the N-1 or N-8 position of the polyamine (2, 8, 20). SAT is required to reduce the spermidine level, since the addition of exogenous spermidine to a *speG* mutant results in intracellular accumulation of spermidine (9). Furthermore, the excess spermidine causes decreased protein synthesis and cell viability during the stationary phase of growth (9). Acetylation serves to convert the polyamine to a physiologically inert form; acetylpolyamines cannot substitute for polyamines in RNA binding, in the enhancement of growth of an *E. coli* polyamine-deficient mutant, or in the stimulation of in vitro translation (18). Neither spermidine-deacetylating activity nor polyamine oxidase activity has been detected in *E. coli*, suggesting that the *N*-acetylspermidine is either excreted or kept in the inert acetylated form (14, 24). SAT is induced under nutrient-poor conditions, and its level is higher in cells growing in minimal medium than in those growing in rich medium (8). In addition to high polyamine levels, chemical and physical stresses result in an increased level of spermidine acetylation in *E. coli* and mammalian cells, suggesting a role for polyamine acetylation in adaptation to stress (2, 4, 10, 27, 32).

Shifting *E. coli* to low temperature results in inhibition of growth and protein synthesis accompanied by the onset of the cold shock response (16). The cold shock response consists of the induction of a set of proteins that has been proposed to aid in cellular adaptation to the low temperature. *E. coli* also responds to a downshift in temperature by increasing the level of spermidine acetylation (2, 32). However, the role of reduced spermidine levels in growth at low temperature is not known. In this study, we examined the effect of spermidine accumulation on adaptation to low temperatures. We found that the addition of spermidine to a *speG* mutant resulted in inhibition of growth and protein synthesis at low temperature. However, the inhibition of growth or protein synthesis caused by the addition of 0.5 mM spermidine could be suppressed by the presence of a $speG^+$ multicopy plasmid or the inclusion of magnesium in the growth medium. The data indicate that spermidine acetylation at low temperatures occurs to reduce the toxic effect of spermidine accumulation.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *E. coli* strains C600 (*supE44 hsdR thi thr leu lacY1 tonA21*) and CAG2242 (*speG supE44 hsdR thi thr leu lacY1 tonA21*) were obtained from E. W. Gerner (2). The $speG^+$ low-copy-number plasmid, pMWSAT (9), and the vector pMW119 (37) were provided by K. Igarashi. Cultures were grown in Luria-Bertani (LB) medium or in M9 medium (19) supplemented with 19 amino acids (no methionine) and 4 bases. For experiments with plasmids, ampicillin (25 μg/ml) was added.

**Two-dimensional electrophoresis of proteins.** Steady-state cultures of bacterial strains were grown at 37°C to an optical density at 420 nm of ca. 0.5. The cultures

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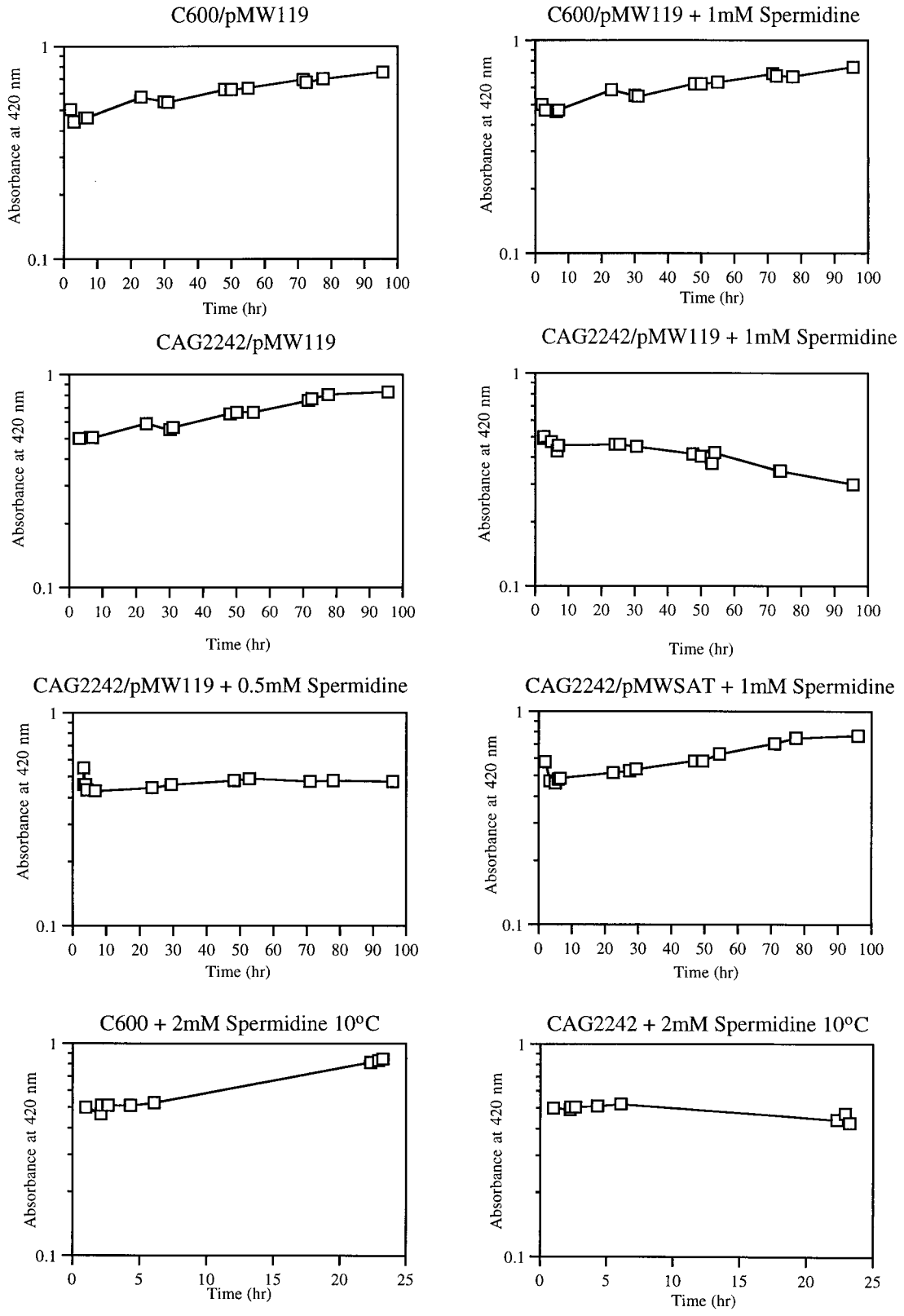


FIG. 1. Effect of spermidine on exponential growth following a shift from 37 to 7°C. The various cultures were grown in LB medium at 37°C to an optical density at 420 nm of ca. 0.5 followed by a shift to 7°C. Where indicated, various concentrations of spermidine were added at the time of the shift. Time zero represents the time of the shift to 7°C. Strains were transformed with pMWSAT (9), the *speG*$^+$ multicopy plasmid, or the vector pMW119 (36).

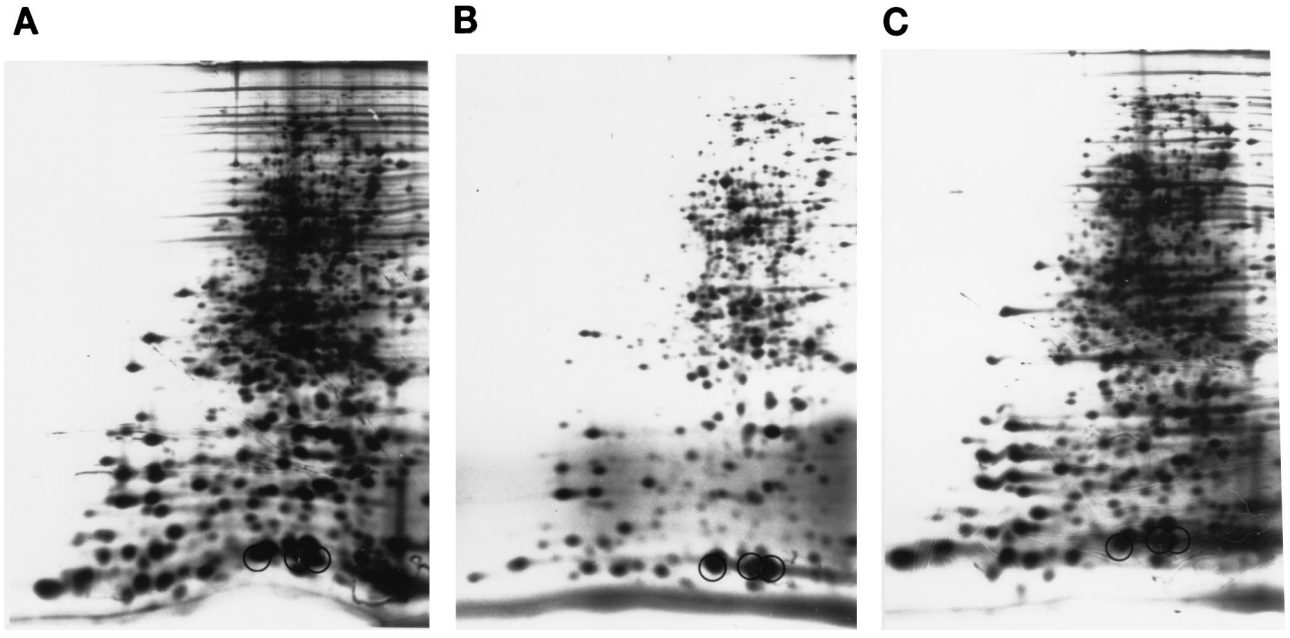


FIG. 2. Effect of spermidine on the synthesis of proteins following a shift from 37 to 7°C. Strains CAG2242/pMW119 (A), CAG2242/pMW119 in the presence of 1 mM spermidine (B), and CAG2242/pMWSAT in the presence of 1 mM spermidine (C) were labeled with [$^{35}$S]methionine at 68 h postshift, and extracts were processed by two-dimensional gel electrophoresis as described in Materials and Methods. Circles indicate cold shock proteins (from left to right) CspB, CspG, and CspA.

were shifted to 7°C, and 1 mM spermidine was added. At 68 h postshift, a 1-ml portion of the culture was labeled with [$^{35}$S]methionine (1,175 Ci/mmol, 100 μCi/ml; ICN Pharmaceuticals) for 2 h. Equal portions of the extracts were processed by two-dimensional gel electrophoresis (23).

**Preparation of the ribosome.** Steady-state cultures were grown at the indicated temperatures in LB medium. Ribosomal particles were isolated as described previously (6) from extracts prepared by the freeze-thaw method (29). The lysates corresponding to equal amounts of protein were layered on top of a 5 to 30% sucrose gradient in 10 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$–60 mM $NH_4Cl$–6 mM β-mercaptoethanol. The ribosomal particles were isolated by centrifugation at 151,000 × *g*.

**Measurement of protein synthesis.** At various times, 0.5-ml samples were pulse-labeled with [$^{35}$S]methionine (1,175 Ci/mmol, 150 μCi/ml). Following precipitation, trichloroacetic acid-insoluble radioactivity was counted using 0.1-ml aliquots.

## RESULTS

**Spermidine accumulation inhibits exponential growth at low temperatures.** The *speG* gene encodes SAT, which is required to prevent the intracellular accumulation of spermidine (8, 9). The addition of 0.5 mM spermidine to the *speG* mutant results in a threefold-increased level of intracellular spermidine without having any appreciable affect on exponential growth at 37°C (9). Introduction of the *speG*$^+$ plasmid, however, restores normal intracellular levels. Cold shock is a stress condition that increases the level of spermidine acetylation (2, 32). To determine the role of spermidine acetylation in adaptation of *E. coli* to low temperature, the effect of exogenous spermidine on the parental (C600) and *speG* mutant (CAG2242) strains was examined following a shift from 37 to 7°C. As shown in Fig. 1, the addition of 1 mM spermidine to the parental strain C600 did not have any noticeable effect on cellular growth at 7°C. In contrast, the addition of 1 mM spermidine to the *speG* mutant, strain CAG2242, resulted in inhibition of cellular growth accompanied by cell lysis. Furthermore, the addition of 0.5 mM spermidine to the *speG* mutant resulted in growth inhibition. However, as shown in Fig. 1, cellular growth in the presence of 1 mM spermidine was fully restored by the presence of the *speG*$^+$ multicopy plasmid pMWSAT. We also found that the growth inhibition caused by spermidine accumulation at 7°C could be reversed by shifting the mutant back to 37°C (data not shown). This is consistent with the previous observation that exponential growth of the *speG* mutant is not inhibited by spermidine accumulation at 37°C (9). Therefore, the data indicate that spermidine accumulation specifically inhibits exponential growth upon a shift to low temperatures. Although the addition of 1 mM spermidine inhibited the growth of the *speG* mutant at 7°C, we found that the addition of 1 mM spermidine did not inhibit the growth of the mutant following the shift from 37 to 10°C (data not shown). However, the addition of 2 mM spermidine to the mutant at 10°C resulted in inhibition of growth, in contrast to the effect on the parent (Fig. 1). The addition of 2 mM spermidine did not inhibit the exponential growth of the parent and *speG* mutant at 37°C or of the mutant transformed with pMWSAT at 10°C (data not shown). Therefore, at the lower temperatures, the toxic effect of spermidine accumulation increases as the temperature decreases. The data further indicate that SAT is required to prevent spermidine toxicity at low temperatures.

**Spermidine accumulation results in the preferential synthesis of cold shock proteins at low temperatures.** Spermidine accumulation in the *speG* mutant causes inhibition of protein synthesis at the stationary phase of growth (9). Furthermore, spermidine accumulation in mouse cells also results in a decrease in protein synthesis (11). Similarly, we found that the addition of 1 mM spermidine to the mutant at 7°C resulted in a dramatic decrease in the synthesis of several polypeptides, as shown in Fig. 2A and B. The presence of *speG*$^+$ plasmid pMWSAT restored normal protein synthesis (Fig. 2C). However, we found that spermidine addition resulted in the preferential synthesis of certain proteins (Fig. 2, circles). These proteins are the major cold shock proteins CspA, CspB, and CspG (17, 38). The data indicate that spermidine accumulation

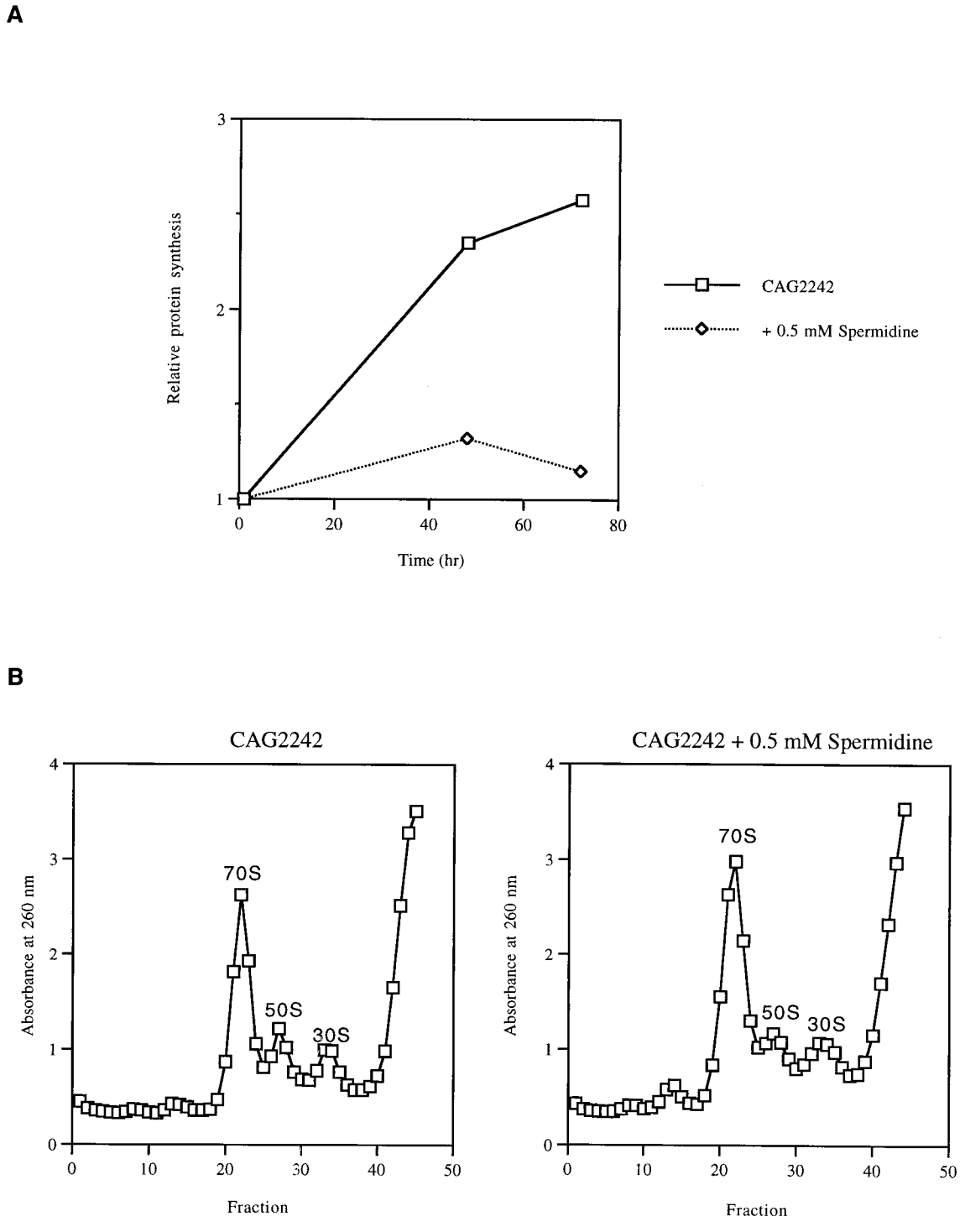


FIG. 3. Effect of 0.5 mM spermidine on protein synthesis (A) and on the levels of ribosomal particles (B) in the *speG* mutant. Strain CAG2242 was grown in LB medium to mid-log phase and shifted to 7°C. (A) Following the shift to 7°C, strain CAG2242 in the absence and presence of spermidine was pulse-labeled with [$^{35}$S]methionine (1, 48, and 72 h postshift) and radioactivity in the trichloroacetic acid-insoluble material was counted as described in Materials and Methods. The counts were normalized to the amount of protein present. The values were normalized to the value obtained at 1 h. Time zero represents the time of the shift to 7°C and/or the addition of 0.5 mM spermidine. (B) Sedimentation profiles of extracts of strain CAG2242 in the absence and presence of 0.5 mM spermidine were prepared as described in Materials and Methods. Extracts were prepared from cells growing for 72 h at 7°C.

in the *speG* mutant results in increased synthesis of the major cold shock proteins at low temperatures.

**Spermidine accumulation inhibits protein synthesis at low temperatures.** Our data suggest that spermidine accumulation inhibits protein synthesis at low temperatures. Following the shift to 7°C, total protein synthesis in the presence and absence of spermidine was monitored by the incorporation of [$^{35}$S]methionine into trichloroacetic acid-insoluble material. As shown

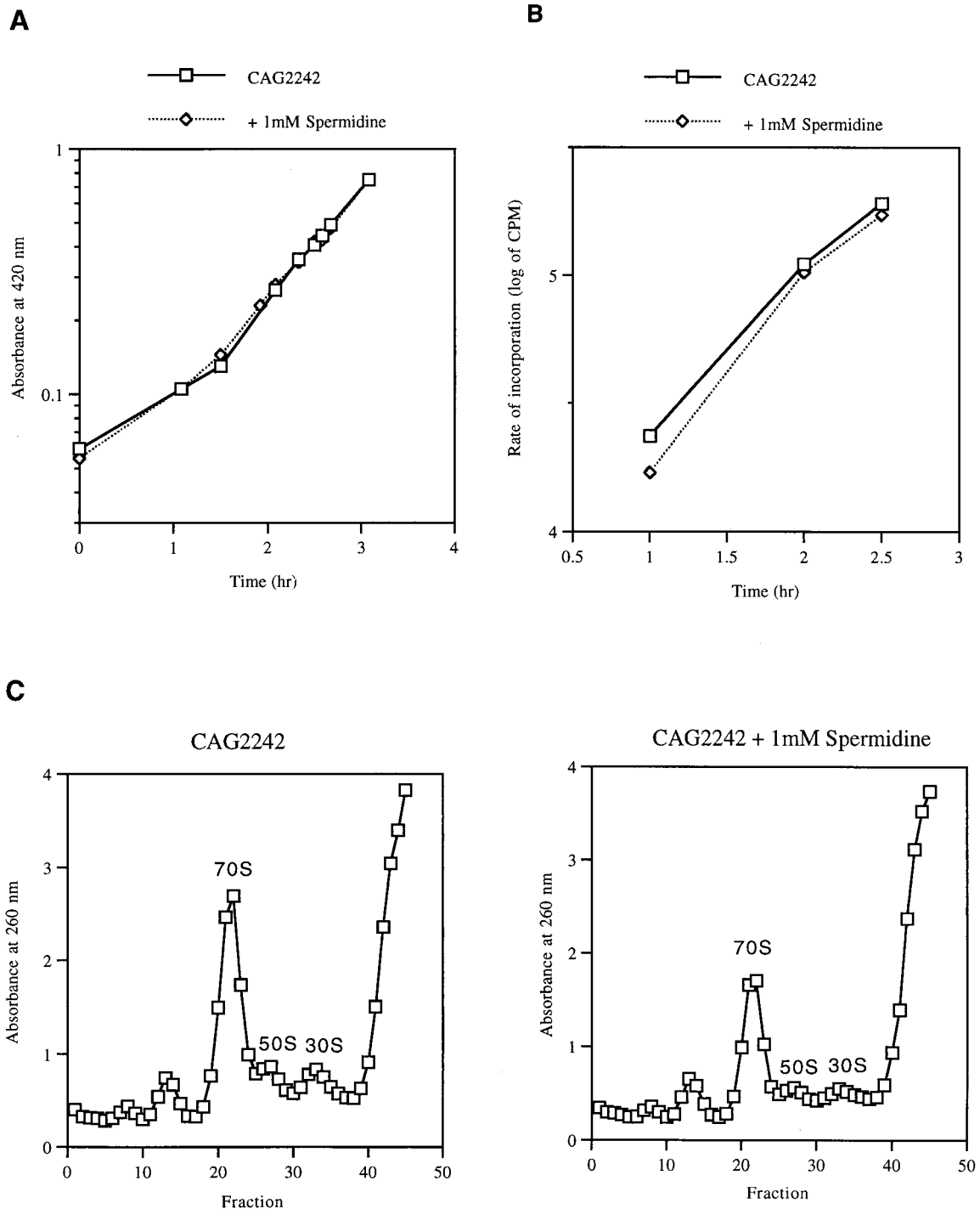


FIG. 4. Effect of spermidine on the growth (A), protein synthesis (B), and ribosomal levels (C) of strain CAG2242 at 37°C. (A) Strain CAG2242 was grown in LB medium in the absence and presence of 1 mM spermidine, which was added at time zero. (B) Samples were pulse-labeled to measure the rate of incorporation of [$^{35}$S]methionine into protein as described in Materials and Methods. (C) Sedimentation profiles of extracts of strain CAG2242 in the absence and presence of spermidine were prepared as described in Materials and Methods.

in Fig. 3A, spermidine accumulation resulted in inhibition of total protein synthesis at the low temperature.

Spermidine has been demonstrated in vitro to shift the equilibrium towards the formation of the 70S ribosome (30). Ribosome profiles of the *speG* mutant were analyzed to determine if excess spermidine results in an increase in the 70S ribosome level in vivo. Because spermidine accumulation inhibits growth at low temperatures, lysates corresponding to equal amounts of protein were loaded on the 5 to 30% sucrose gradient to compare the level of the 70S ribosome in the presence and absence of spermidine at 72 h postshift. As shown in Fig. 3B, the addition of 0.5 mM spermidine resulted

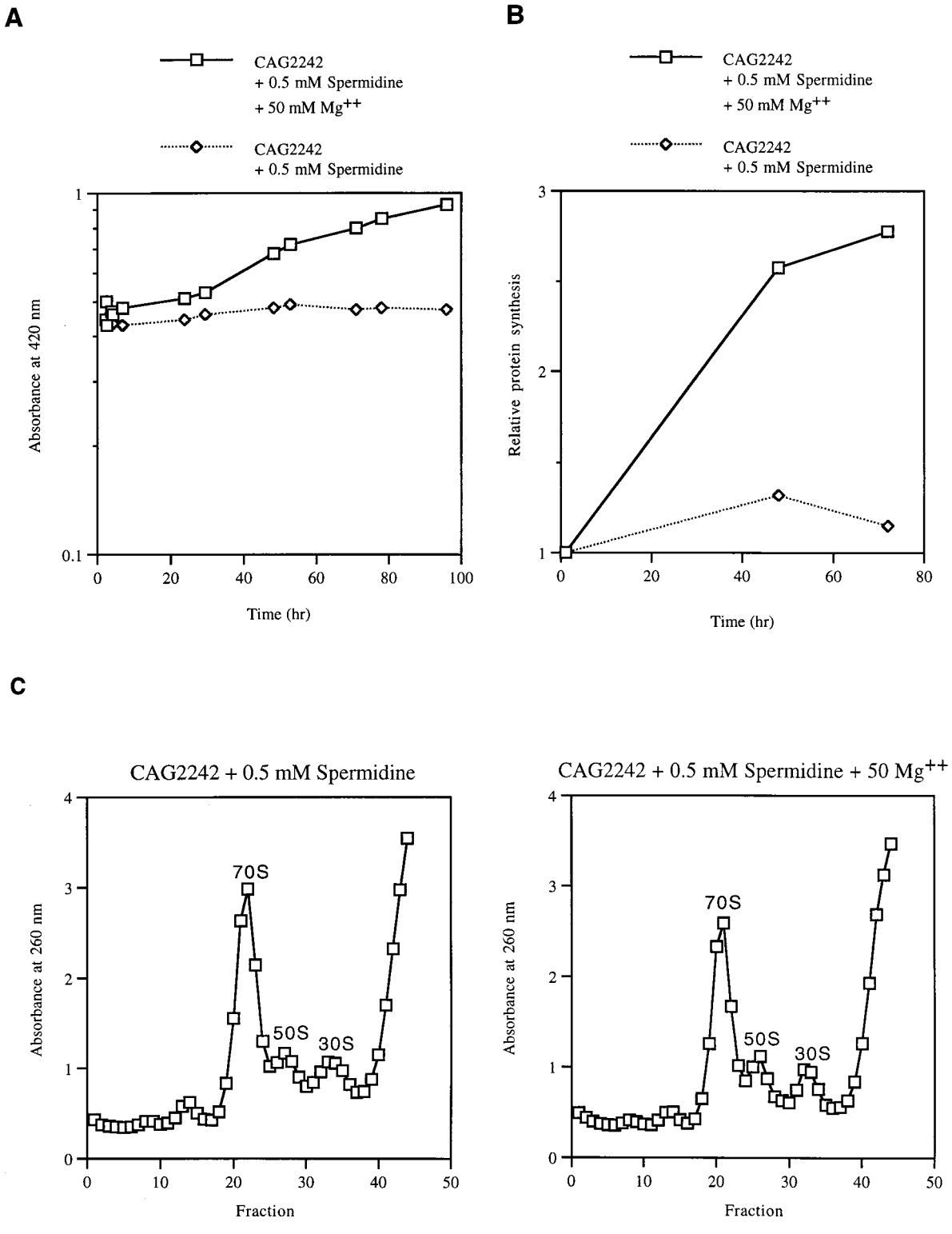


FIG. 5. Effect of $Mg^{2+}$ on the growth (A), protein synthesis (B), and ribosomal levels (C) of strain CAG2242. (A) Strain CAG2242 was grown in LB medium in the presence or absence of 50 mM $Mg^{2+}$ at 37°C to mid-log phase and then shifted to 7°C. Time zero represents the time of the shift to 7°C and the addition of 0.5 mM spermidine. (B) Following the shift to 7°C, strain CAG2242 was pulse-labeled with [$^{35}$S]methionine (1, 48, and 72 h postshift) and radioactivity in trichloroacetic acid-insoluble material was counted as described in Materials and Methods. The counts were normalized to the amount of protein present. The values were normalized to the value obtained at 1 h. (C) Sedimentation profiles of extracts of strain CAG2242 were prepared as described in Materials and Methods. Extracts were prepared from cells growing for 72 h at 7°C.

in a relatively higher 70S ribosome level, suggesting that excess spermidine stabilizes 70S ribosomes in vivo, as has been previously demonstrated in vitro (30).

It has been previously shown that spermidine accumulation in the *speG* mutant does not inhibit exponential growth at 37°C (9). The effects of spermidine accumulation on protein synthesis and the 70S ribosome level were also analyzed at 37°C. As shown in Fig. 4A and B, the exponential growth and protein synthesis of the *speG* mutant were not negatively affected by the addition of 1 mM spermidine. The doubling time of the *speG* mutant, regardless of the presence of spermidine, was 45 min. The addition of 1 mM spermidine to the mutant at 37°C also resulted in a decrease in the 70S ribosome level (Fig. 4C). The data indicate that spermidine accumulation inhibits exponential growth and protein synthesis only at low temperatures.

**Magnesium restores cellular growth in the presence of 0.5 mM spermidine.** An effect of polyamines in in vitro translation is the lowering of the $Mg^{2+}$ requirement (18). Since spermidine binds to sites on the ribosome similar to those bound by $Mg^{2+}$, it can replace ribosome-bound $Mg^{2+}$ (5, 28). However, the requirement for $Mg^{2+}$ binding to maintain ribosome function and stability cannot be abolished by spermidine binding (12, 36). To further determine if spermidine toxicity at low temperatures could be due to the replacement of $Mg^{2+}$ on the ribosome, experiments were done to ascertain if the inhibition of growth and protein synthesis could be suppressed by the inclusion of $Mg^{2+}$ in the growth medium. As shown in Fig. 5A and B, the addition of 50 mM $Mg^{2+}$ to the growth medium restored exponential growth and protein synthesis in the presence of 0.5 mM spermidine. As shown in Fig. 5C, the addition of 50 mM $Mg^{2+}$ also resulted in a relative decrease in the 70S ribosome level in the presence of 0.5 mM spermidine. However, in the presence of 1 mM spermidine, the addition of 50 mM $Mg^{2+}$ neither rescued the growth of the mutant nor restored protein synthesis (data not shown). The data suggest that spermidine accumulation at low temperatures replaces ribosome-bound $Mg^{2+}$, resulting in inhibition of growth and protein synthesis.

## DISCUSSION

Spermidine accumulation in the *speG* mutant does not inhibit exponential growth at 37°C (9). In this study, we have found that spermidine accumulation does inhibit exponential growth following a shift to a low temperature. However, growth can be restored by the presence of a plasmid carrying *speG*$^+$. Spermidine acetylation increases under cold shock conditions (2, 32). Therefore, our data indicate that an increase in the level of spermidine acetylation occurs to prevent polyamine toxicity at low temperatures.

Accompanying the inhibition of growth, spermidine accumulation in the mutant resulted in inhibition of protein synthesis at low temperatures. Spermidine accumulation has previously been shown to inhibit protein synthesis in mouse cells and during the stationary phase in *E. coli* (9, 11). Spermidine has also been demonstrated to cause an increase in the 70S ribosome level in vitro (30). We have found that spermidine accumulation at low temperatures results in a relatively higher 70S ribosome level, suggesting that excess spermidine stabilizes 70S ribosomes in vivo. Spermidine accumulation also resulted in the preferential synthesis of the major cold shock proteins CspA, CspB, and CspG. In addition to shifts to low temperatures, the synthesis of these cold shock proteins increases in response to other conditions where protein synthesis is inhibited (17). CspA, CspB, and CspG belong to a family of homologous proteins, which also include members that are not cold shock inducible (38). It has been proposed that these proteins, which contain RNA binding domains, function as RNA chaperones to increase the translational efficiency of the mRNA (15, 38). The data suggest that the spermidine-induced synthesis of the major cold shock proteins occurs to increase the level of protein synthesis.

A critical level of ribosome-bound $Mg^{2+}$ is required for ribosomal function and stability (12, 36). We found that the addition of $Mg^{2+}$ to the growth medium suppressed growth inhibition and restored protein synthesis in the presence of 0.5 mM spermidine. The data suggest that spermidine accumulation decreased the ribosomal binding of $Mg^{2+}$, resulting in a decrease in protein synthesis. This is consistent with previous reports indicating that one inhibitory effect of spermidine is the replacement of ribosome-bound $Mg^{2+}$ (11, 12, 22, 36). The replacement of more than 40 and 70% of the bound $Mg^{2+}$ by polyamines results in a decrease in protein-synthesizing activity in vitro using polysomes from rat liver and *E. coli*, respectively (12, 36). In addition, spermidine or spermine accumulation in the FM3A mouse cell line results in inhibition of growth and protein synthesis, which correlates with a reduction in the $Mg^{2+}$ content (11). However, cellular growth recovers on addition of $Mg^{2+}$ to the growth medium, suggesting that ribosome inactivation is due to the replacement of ribosome-bound $Mg^{2+}$ by the polyamines (11).

Our evidence suggests that acetylation occurs at low temperature to prevent excess spermidine from inhibiting the binding of $Mg^{2+}$ to the ribosome. Consistent with this is the finding that acetylpolyamines cannot substitute for polyamines in binding to RNA (18). Although the acetylpolyamine is still cationic, it has been suggested that the acetyl group may present a steric hindrance to the binding of nucleic acids. In *E. coli*, the level of spermidine acetylation increases in response to other stressful conditions, such as heat shock, high pH, and ethanol treatment (2). Furthermore, spermidine and spermine acetylation also increases in response to various chemical and physical stresses in mammalian cells (4, 10). However, the physiological role of polyamine acetylation in response to these stressful conditions has not yet been clearly defined. Because the induction of human SSAT by polyamine analogs is accompanied by growth inhibition and decreased cell viability of tumor cells, it has been suggested that stress-induced acetylation may occur to specifically inhibit cellular growth (3, 27). Furthermore, expression of human SSAT in *E. coli* results in the conversion of the spermidine to $N^1$-acetylspermidine, accompanied by inhibition of exponential growth at 37°C (14, 24). However, we have found that in response to spermidine accumulation at low temperature, acetylation occurs to promote exponential growth. Therefore, polyamine acetylation may be playing a similar physiological role in response to other stressful conditions.

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