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# Polyamines Are Not Required for Aerobic Growth of *Escherichia coli*: Preparation of a Strain with Deletions in All of the Genes for Polyamine Biosynthesis▽

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**A strain of *Escherichia coli* was constructed in which all of the genes involved in polyamine biosynthesis—*speA* (arginine decarboxylase), *speB* (agmatine ureohydrolase), *speC* (ornithine decarboxylase), *spe D* (adenosylmethionine decarboxylase), *speE* (spermidine synthase), *speF* (inducible ornithine decarboxylase), *cadA* (lysine decarboxylase), and *ldcC* (lysine decarboxylase)—had been deleted. Despite the complete absence of all of the polyamines, the strain grew indefinitely in air in amine-free medium, albeit at a slightly (ca. 40 to 50%) reduced growth rate. Even though this strain grew well in the absence of the amines in air, it was still sensitive to oxygen stress in the absence of added spermidine. In contrast to the ability to grow in air in the absence of polyamines, this strain, surprisingly, showed a requirement for polyamines for growth under strictly anaerobic conditions.**

Polyamines are highly abundant in essentially all organisms, ranging from bacteria to humans, and there have been a large number of studies from this and many other laboratories reporting a variety of phenotypic effects resulting from changes in the concentration of polyamines in both in vitro and in vivo experiments. In particular, polyamines have been associated with such biological processes as nucleic acid and protein biosynthesis and structure, cell growth, and differentiation (reviewed in references 5, 22, and 23). Therefore, it was surprising that, in our earlier studies (24), we found that a mutant of *Escherichia coli* that had mutations in the genes for the biosynthesis of the polyamines (Δ*speA*, Δ*speB*, Δ*speC*, Δ*speD*, Δ*speE*, and *cadA*) still grew indefinitely in a polyamine-free medium, albeit at a decreased growth rate (ca. 30% of the normal growth rate).

The strain used in our previous studies still had trace amounts of putrescine and significant amounts of cadaverine. To study whether these small amounts of amines could account for the slow growth of these strains, we have now constructed a new strain that is completely deficient in these amines by including deletions of *cadA* (inducible lysine decarboxylase), *ldcC* (constitutive lysine decarboxylase), and *speF* (inducible ornithine decarboxylase) to the strain described above. We found that this strain which is completely deficient in all of the amines still grows well (40 to 50% of normal growth rate) in purified medium in air. This indicates that, at least for this organism, the various physiological functions attributed to polyamines are not required for growth in air. In contrast, we have found that polyamines are required for growth of this strain in 95% oxygen and under anaerobic conditions.

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## MATERIALS AND METHODS

**Strain construction.** P1 transductions were carried out essentially as described by Miller (17). The strains used for the P1 transduction were obtained from the Keio collection (1) from the Yale *E. coli* Genetics Center. The deleted genes in this collection contain a kanamycin insert, which was used for the selection process. For use in subsequent transduction experiments, the kanamycin insert was excised from the transduced strain by the FLP recombinase, as described by Baba et al. (1) and by Datsenko and Wanner (6).

**Culture conditions.** Strains were maintained on LB-agar plates. Prior to use, the strains were grown in amine-free medium for 24 to 48 h to deplete the cells of carryover polyamines. The amine-free medium was either VBC (25) or M9 (17) containing proline and threonine. All incubations were performed at 37°C with shaking.

**Oxygen incubation.** For the experiments on the effect of incubation in oxygen, the amine-deficient cells were diluted to an optical density at 600 nm ($OD_{600}$) of 0.01 in 30 ml of VBC in each of two 250-ml flasks; to one flask spermidine was added to a final concentration of $10^{-4}$ M. The flasks were shaken at 37°C with continual flow of 95% oxygen and 5% $CO_2$ passing over each flask as described earlier (4). Periodically, aliquots were drawn for making cell counts.

**Anaerobic incubation.** The amine-depleted cells were grown in either a polyamine-free medium (M9) or in the same medium containing $10^{-5}$ M spermidine for about 6 h. Each culture was then diluted to a calculated $OD_{600}$ of 0.0001 to 0.0005. Then, as previously described (2), 5-ml aliquots of each culture were placed into multiple 18-by-150-mm acid-washed glass tubes that were stoppered with nonabsorbent cotton plus circles of filter paper, to which 0.4 ml of 40% pyrogallol was added. These tubes were placed in a vacuum desiccator that was then subjected to several cycles of evacuation and exposure to 95% $N_2$ and 5% $CO_2$. Then, 0.4 ml of 40% sodium carbonate was added to the filter papers in each tube. The tubes were quickly stoppered with turnover rubber stoppers (Fischer). Each tube also contained, just under the cotton plug, a strip of anaerobic indicator strips (Becton Dickinson). The tubes were then shaken at 37°C. Periodically, individual tubes were removed from the incubator for determination of the cell counts.

**Amine analyses.** After preliminary incubation in amine-free medium (see above), the culture was diluted into 100 ml of M9 or VBC medium to give a calculated $OD_{600}$ of 0.001. The cultures were then incubated with shaking in air until the $OD_{600}$ was 0.4 to 0.5. The cells were collected by centrifugation. The cell pellet was weighed and extracted with 5 volumes of 10% perchloric acid. Then, 50 μl of this extract, representing 10 μg of cells (wet weight) were then analyzed by ion-exchange chromatography essentially as described by Murakami et al. (18) by using a Shim-pack column (Shimadzu, catalogue no. ISC-05/S0504), except that the eluting buffer was 1 M NaCl–0.2 M sodium citrate. Postcolumn fluorometric determination of polyamines was performed by reaction with *o*-phthalal-

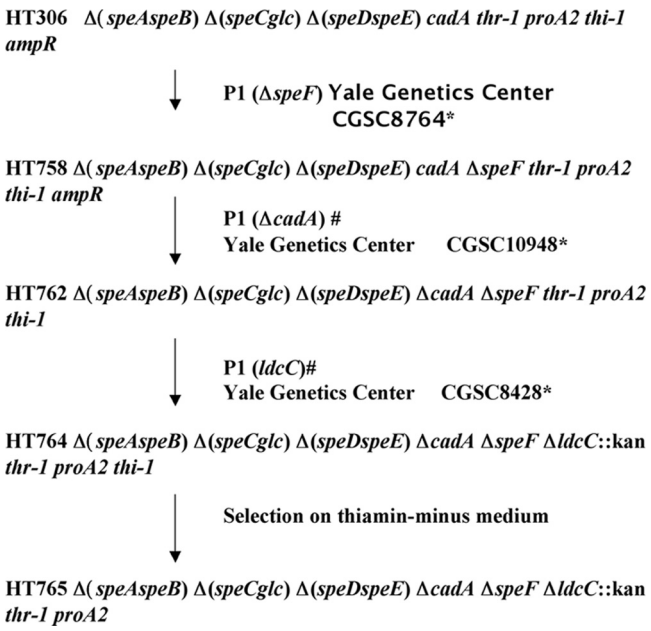


FIG. 1. Steps involved in the construction of strain HT765, completely deficient in all polyamines. *, *speF* inducible ornithine decarboxylase (9, 13); *, *cadA* inducible lysine decarboxylase (16, 23, 24); *, *ldcC* constitutive lysine decarboxylase (7, 14, 27); #, prior to this P1 transduction the *kanR* insertion was removed from the recipient strain by the use of FLP recombinase (1, 6).

dehyde, and data were collected by using Powerchrome hardware and software (AD Instruments). Under these conditions, we could detect 0.25 nmol of putrescine in a 50-μl volume.

## RESULTS

**Construction of a polyamine-free strain.** Strain HT306, which was used in our earlier experiments (4, 24) still grew at ca. 30% of the normal growth rate in purified medium even though it contained mutations in the major genes required for polyamine biosynthesis (Δ*speA*, Δ*speB*, Δ*speC*, Δ*speD*, Δ*speE*, and *cadA*). We now find that this strain still had trace amounts of putrescine and substantial amounts of cadaverine. Since strain HT306 only had a point mutation in *cadA* and was not mutated in gene *speF* (inducible ornithine decarboxylase) or in gene *ldcC* (constitutive lysine decarboxylase), we have now constructed a strain (HT764, Fig. 1) with these additional deletions. We then converted HT764 into HT765 by incubation in a thiamine-free medium and selecting a strain that no longer required thiamine. We have also converted the thiamine-requiring HT764 into a thiamine prototroph more directly by transduction of the wild-type $thi^+$ genes using a P1 preparation prepared from a strain (CGSC 10846) carrying the Δ*yjaZ*::*kan* marker which maps close to the *thi*+ genes at 90.26 min. We found no difference in the growth curves in air between these two thiamine prototrophs.

**Amine levels.** In Table 1 we summarize the amine analyses for strain HT306 and strain HT765 grown in M9 medium to an OD of 0.4. HT765 contained no putrescine, cadaverine, or spermidine, whereas strain HT306 contained 20 μmol of cadaverine per g of wet weight. In this table we have also presented the results of the amine analysis for strain JIL539, which was used by Jung et al. (10) and contained the same mutations in the polyamine pathway as HT306 but in a different background.

Strain HT765 has the wild-type *adiA* gene that codes for an inducible arginine decarboxylase; no agmatine was present in the cultures of this strain when collected in log phase, but a small amount of agmatine was detected in the cultures harvested after long incubation in stationary phase. We also constructed a derivative of strain HT765 that also contains a deletion of the adi*A* gene; i.e. the gene coding for the inducible arginine decarboxylase (19, 21). This deletion was constructed by a P1 transduction of *adiA*Δ::*kan* (from strain CGSC 11452) into HT765 (after the kanamycin marker had been removed from the latter strain). This strain did not contain any agmatine (or other amines) even after long incubation in the stationary phase. There was no difference in the growth rate of this strain in amine-free medium compared to the growth rate of HT765 in the same medium.

**Growth in air.** Even though strain HT765 contains no amines, it still grew in purified medium at ca. 40% of the growth rate found in cultures supplemented with spermidine (Fig. 2A). Upon repeated subculture of the deficient cells into purified medium, the cells continued to grow at the same growth rate for at least 10 days (Fig. 2B).

**Growth in oxygen.** In contrast to the above findings of good growth in air, we found that the polyamine-deficient HT765 cultures were sensitive to incubation in 95% oxygen–5% $CO_2$ (Fig. 3). A large decrease in cell counts was observed in a culture grown without any amines, whereas the culture grown in the presence of spermidine was protected from oxygen stress. These results are similar to those we previously reported for strain HT306 (4).

**Anaerobic growth.** When polyamine-deficient HT765 cells were grown anaerobically in 95% nitrogen–5% $CO_2$, we found that this polyamine-deficient strain had a marked requirement for spermidine for growth (Fig. 4). There was little or no growth within 48 h in the absence of added spermidine. In contrast in the presence of $10^{-5}$ M spermidine good growth was observed with a doubling time of 2 to 3 h. However, after

TABLE 1. Amine content of mutant strains

| Strain | Mutated genes (polyamine related) | Amine content (μmol/g [wet wt])[a] | | |
|---|---|---|---|---|
| | | Putrescine | Cadaverine | Spermidine |
| HT306 | Δ(*speA speB*) Δ(*speC glc*) Δ(*speD speE*) *cadA* | ~2* | 20 | 0 |
| JIL539 | Δ(*speA speB*) Δ(*speC glc*) | ~1* | 124 | 0 |
| HT765 | Δ(*speA speB*) Δ(*speC glc*) Δ(*speD speE*) Δ*cadA* Δ*speF* Δ*ldcC*::*kan* | 0 | 0 | 0 |

[a] No spermine was found in any of the strains. *, Limit of detection = 1 to 2 μmol per g (wet weight).

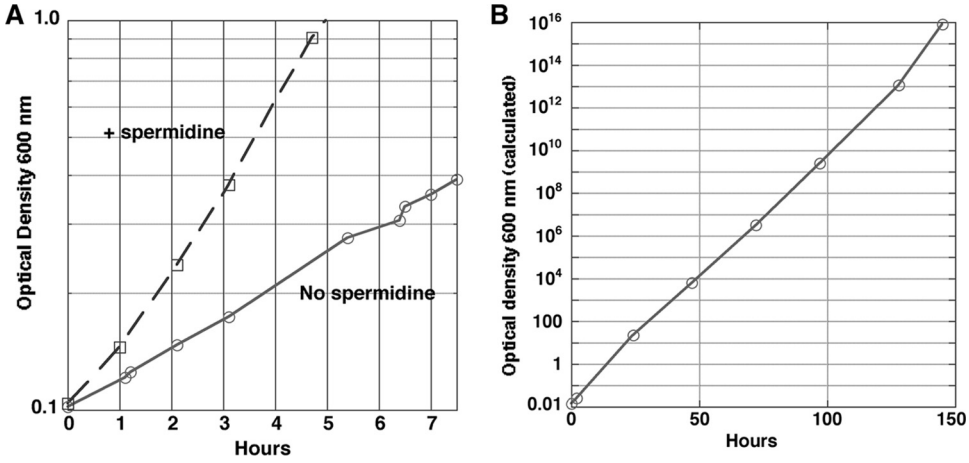


FIG. 2. Growth rate ($OD_{600}$) of amine-deficient mutant (HT765) in air in the presence or absence of spermidine. (A) Short-term growth with or without spermidine. (B) Demonstration that the amine-deficient cultures are able to grow indefinitely in an amine-free medium. Each day the cultures were diluted 1:100 into fresh medium; incubation of the diluted cultures was continued, and the $OD_{600}$ values have been corrected for the dilutions.

incubation for 3 to 4 days full growth was observed in the amine-deficient cultures even though the indicator paper showed that the atmosphere was still anaerobic; when multiple tubes were incubated in parallel, the different tubes showed growth at different times (all >3 days), indicating the possibility of a genetic bypass.

## DISCUSSION

The most important finding of the experiments described here is that strain HT765, which does not have any putrescine, cadaverine, or spermidine, can grow indefinitely at 40% of the normal growth rate (Fig. 2). These results are particularly significant in view of the many reports in the literature on the effect of polyamines in various systems (see reviews by Cohen [5] and Igarashi and Kashiwagi [8]). Thus, it is clear that the roles for polyamines postulated by many authors are not essential for the growth of *E. coli*. Of course, the growth experiments reported in the present study do not exclude other important physiological functions for polyamines that are not reflected in growth of *E. coli* or the physiological functions of polyamines in other organisms. In yeast and other eukaryotes, for example, spermidine is clearly required for growth because of its conversion to hypusine in the eukaryote protein synthesis factor eIF-5A (3, 26).

Even though strain HT765 grew indefinitely in air, we found that the strain was sensitive to incubation in 95% $O_2$ and 5% $CO_2$ (Fig. 3). Thus, these results are consistent with our previous findings on the toxicity of oxygen in the polyamine-deficient cells with strain HT306 (4, 24) and with the reports of Jung and Kim (11) with strain JIL539.

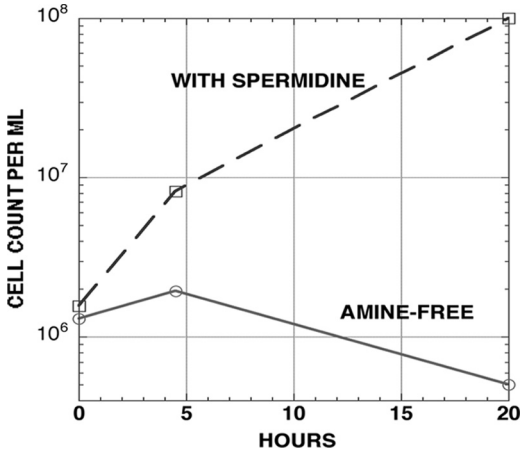


FIG. 3. Growth rate (viable cell counts) of amine-deficient mutant (HT765) in the presence of 95% oxygen and 5% $CO_2$ with or without spermidine. Cells were depleted of amines by growing in amine free medium as mentioned in Materials and Methods. Cultures were diluted and incubated in the presence or absence of spermidine. Periodically, cultures were assayed for viable cell counts per milliliter.

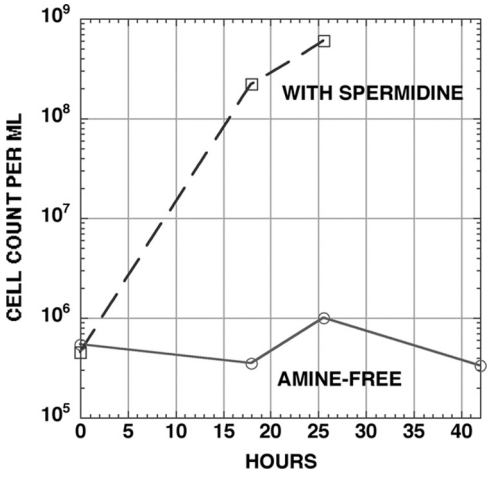


FIG. 4. Growth rate (viable cell counts) of amine-deficient mutant HT765 under anaerobic conditions (95% nitrogen and 5% $CO_2$) with or without spermidine. Cultures were incubated under strictly anaerobic conditions in multiple tubes with or without spermidine. Periodically, individual tubes were removed from the incubator for determination of cell counts.

Even though both HT765 (the present study) and JIL539 (obtained from I. G. Kim) were sensitive to 95% oxygen, the two strains differed in their sensitivities to oxidative stress in air when grown in the absence of polyamines. In the experiments of Jung et al. (10) strain JIL539 showed inhibition of growth when the cultures were shaken rapidly in air, but almost no inhibition if the cultures were shaken slowly or were grown without shaking. Further evidence for oxidative stress in their cultures was indicated by decreased inhibition if thiamine or other antioxidants was present in the medium or if the medium was supplemented with certain amino acids or sucrose or sorbitol. We have confirmed most of these effects with strain JIL535 but did not find any evidence for oxidative stress in cultures of strain HT765 grown in air. Presumably, these differences are related to different genetic backgrounds. Perhaps strain HT765 has an increased background level of one or more of the enzymes involved in protection against oxidative stress. Indeed, Jung and Kim (12) have recently reported the selection of polyamine mutants that no longer show oxygen stress in air. We want to emphasize that our results and those of Jung et al. do not negate the importance of polyamines in protecting cells from oxygen stress.

In view of the observations that polyamine-deficient cultures of both HT765 and JIL539 are sensitive to incubation in oxygen, and the findings of Jung et al. (10) that JIL539 had evidence of oxygen stress even when grown in air, we expected that polyamine-free cultures of HT765 would grow well anaerobically. Therefore, we were surprised to find that there was little growth for several days when the cultures of HT765 were incubated under strictly anaerobic conditions. Since there have been many studies showing that shifting a culture from air to nitrogen triggers many adaptive changes in metabolism (15, 20), further work is needed to clarify the key role played by polyamines during the shift from aerobic to anaerobic growth and whether the growth that is observed after several days of anaerobiosis represents adaptation or a suppressor mutation.

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