Expression of the human spermidine/spermine N^1-acetyltransferase in spermidine acetylation-deficient *Escherichia coli*

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A cDNA encoding the human spermidine/spermine N^1-acetyltransferase (N^1SSAT) was conditionally expressed in a strain of *Escherichia coli* deficient in spermidine-acetylating activity. Conditional expression of this cDNA was performed under the control of the *lac* promoter, by addition of the non-hydrolysable lactose analogue isopropyl β-D-thiogalactoside. Expression of the N^1SSAT cDNA oriented in the sense direction resulted in the acetylation of spermidine at the N^1 but not the N^8 position and a decrease in endogenous spermidine contents and growth rates in these bacteria. When this cDNA was expressed in the antisense orientation, spermidine acetylation was not detected and endogenous spermidine contents and growth rates were unaffected. Increasing the endogenous N^1-acetylspermidine concentration by addition of this amine to the culture medium did not suppress growth, and increasing endogenous spermidine pools by exogenous addition was not sufficient to restore optimal growth in cells expressing the human N^1SSAT. Exogenous spermidine, but neither N^1- nor N^8-acetylspermidine, stimulated cell growth in strains unable to synthesize spermidine. These results suggest that one physiological consequence of spermidine acetylation in *E. coli* is growth inhibition. The mechanism of this inhibition seems to involve the formation of acetylspermidine, and is not simply due to a decrease in the intracellular concentration of non-acetylated spermidine.

INTRODUCTION

Polyamines are essential for optimal proliferation of both prokaryotic and eukaryotic cells [1]. Mutant strains of *Escherichia coli* and *Saccharomyces cerevisiae* that are incapable of synthesizing the diamine putrescine, the first amine in the polyamine pathway, do not grow [1,2]. Null mutants of *S. cerevisiae*, which make putrescine but not the triamine spermidine owing to the deletion of the gene encoding *S*-adenosylmethionine decarboxylase, also do not grow [3]. *E. coli* apparently lacks this spermidine requirement for growth [4]. Spermidine is acetylated in *E. coli* and animal cells [5], but the physiological consequence of this modification is not clear. In *E. coli*, spermidine acetylation is not required for growth, as mutants lacking spermidine acetyltransferase activity grow at rates similar to wild-type cells [6]. In *E. coli* the spermidine acetyltransferase is encoded by a single gene and acetylates spermidine at both the N^1 and N^8 positions [7]. Animal cells express two different spermidine acetyltransferases. One of these enzymes is nuclear in origin and acetylates spermidine at the N^8 position [8,9], whereas the other enzyme, the spermidine/spermine N^1-acetyltransferase (N^1SSAT), is predominantly cytoplasmic and acetylates spermidine or the tetra-amine spermine at the N^1 position [10]. In animal cells, N^1-acetylspermidine and N^1-acetylspermine are substrates for the FAD-dependent polyamine oxidase (PAO), which catabolizes these amines to the shorter-chain amines putrescine and spermidine respectively [11].

In human and rodent cells, a variety of growth-inhibiting stresses induce N^1SSAT activity [12–14]. These stresses also induce spermidine acetylation in bacteria [6]. The ability of polyamine analogues to induce N^1SSAT activity has been correlated with ability to inhibit growth [15] and viability [16] of human tumour-derived cells. Thus spermidine acetylation might be a strategy used by both bacteria and animal cells for inhibiting growth in response to environmental stresses. To test this hypothesis a human N^1SSAT cDNA [17] was conditionally expressed in an *E. coli* strain previously shown to lack endogenous spermidine acetyltransferase activity [6]. The human enzyme was used in the studies reported here because the bacterial acetyltransferase had not yet been cloned when this work was initiated.

EXPERIMENTAL

Bacterial strains and growth

Three bacterial strains were used in this study. The C600 strain, an *E. coli* K-12 derivative, is capable of acetylating spermidine at both the N^1 and N^8 positions in response to a variety of stresses [6]. CAG2242, which was constructed and kindly provided by Carol Gross, was found to be deficient in acetylating spermidine [6]. The HT653 strain is unable to synthesize putrescine (it is Δ[speAspeB]ΔspeC) and was graciously provided by Herbert and Celia Tabor. Bacteria were grown in Luria–Bertani (LB) medium or Mops medium supplemented with potassium phosphate (1.32 mM), glucose (4%, w/v), thiamine and riboflavin (each 1 mg/l), all 20 α-amino acids (4 mg/l) and tetracycline (20 μg/ml) [6]. Ampicillin (50 μg/ml) was added to the cultures of *E. coli* containing plasmid pTrc99A. Growth was monitored by measuring the attenuation at 540 nm (D_{540}) of cultures.

N^1SSAT cDNA and expression vectors

A cDNA encoding the human N^1SSAT, kindly provided by Bob Casero [17], was removed from pSATH and inserted into the bacterial conditional expression vector pTrc99A (Pharmacia) in the sense and anti-sense orientations. This vector contains the *lac* promoter element upstream of the multiple cloning site, so that inserted genes can be conditionally expressed in response to the

Abbreviations used: IPTG, isopropyl β-D-thiogalactoside; N^1SSAT, spermidine/spermine N^1-acetyltransferase; PAO, flavine adenine dinucleotide-dependent polyamine oxidase; D_{540}, attenuation at 540 nm.

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non-hydrolysable lactose analogue isopropyl β-D-thiogalactoside (IPTG) (5 mM). CAG2242 (spermidine acetylation-deficient strain) were then transformed with plasmid pTrc99A without insert and with N^1SSAT inserted in the sense and anti-sense orientations. Positive colonies were isolated and inserts and orientations were confirmed by restriction enzyme digests.

N^1SSAT enzyme activity and polyamine analysis

N^1SSAT activity was measured as previously described [6], with [^{14}C]acetyl-CoA. Polyamine contents were measured by the HPLC method of Seiler and Knodgen [18].

RESULTS

Expression of the human N^1SSAT in spermidine acetylation-deficient bacteria

Multiple clones transformed with either the human N^1SSAT cDNA in the sense and anti-sense orientations, or pTrc without the N^1SSAT insert, were isolated and characterized for N^1SSAT enzyme activity when grown in the presence or absence of IPTG. Figure 1 shows N^1SSAT enzyme activity as a function of time after culture dilution in one CAG2242 clone transformed with the human N^1SSAT cDNA in the sense orientation and another transformed with that cDNA in the anti-sense orientation. Enzyme activity in the parental C600 cells is shown for comparison. pTrc contains promoter elements capable of expressing inserted genes in the absence of IPTG, as advertised by the vendor. CAG2242 (results not shown) or CAG2242 cells transformed with N^1SSAT in the anti-sense orientation do not express detectable N^1SSAT activity. This same strain, transformed with the cDNA in the sense orientation, expresses N^1SSAT activities ranging from 100 to 600 pmol/min per mg of protein in the absence of IPTG, and enzyme activity increases 2–6-fold when IPTG is present in the culture medium. N^1SSAT activity increases within the first 1 h after dilution of the transformed cells, and then decreases. Endogenous bacterial spermidine acetyltransferase activity in the parental C600 cells (25–100 pmol/min per mg of protein) is less than that expressed by CAG2242 cells transformed with human N^1SSAT in the sense orientation. However, C600 cells display a similar pattern of increase and decrease in enzyme activity over this time interval, as do the CAG2242 (pSATH/sense) cells.

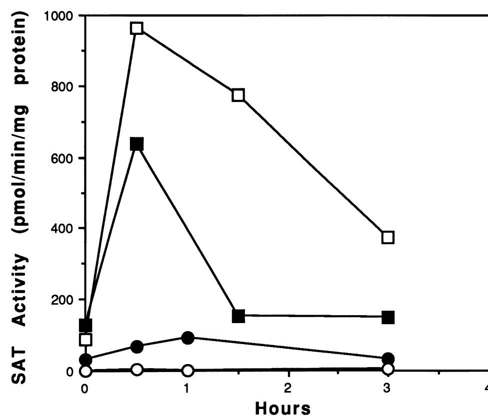

Figure 1 N^1SSAT activity in bacterial strains C600 (●), or CAG2242 cells transformed with cDNAs encoding the human N^1SSAT in the sense (■, □) or anti-sense (○) orientations

Cultures were grown in the absence (■) or presence □) of IPTG in the culture medium. Enzyme activity was determined in triplicate from a single cell lysate in an individual experiment, which was replicated three times. Mean values from a representative experiment are shown here. Standard errors of the mean are smaller than the symbols.

To confirm that the human N^1SSAT was actually acetylating spermidine in these bacteria, CAG2242 (pSATH/sense) and CAG2242 (pSATH/anti-sense) were grown for 3 h, with and without IPTG, in medium containing [*methylene*-^{3}H]spermidine to radiolabel the endogenous spermidine pool. As seen in Figure 2, N^1-, but not N^8-, acetylspermidine was found in acid extracts of CAG2242 (pSATH/sense) cells. IPTG stimulates the amount of labelled N^1-acetylspermidine formed relative to labelled spermidine. No acetylspermidines are found in CAG2242 (pSATH/anti-sense) strains, and IPTG does not change this result.

Expression in CAG2242 cells of the human N^1SSAT cDNA in the sense orientation suppresses endogenous spermidine pools and increases endogenous N^1-acetylspermidine contents (Table 1). Expression of N^1SSAT also increases putrescine contents, relative to those of spermidine, in cells expressing the sense compared with the anti-sense N^1SSAT cDNA.

Effect of human N^1SSAT expression on bacterial cell growth

CAG2242, CAG2242 (pSATH/sense), CAG2242 (pSATH/anti-sense), CAG2242 (pTrc) and C600 cultures were diluted into fresh Luria–Bertani medium, with and without IPTG, at D_{540} of 0.1; growth was assessed by measuring D_{540} (Figure 3). The parental, spermidine acetylation-proficient C600 cells grew at a slightly higher rate than any of the CAG224 strains over the first 3 h, but reached similar saturation densities. CAG224 strains not expressing spermidine-acetylating activity [CAG2242, CAG2242 (pSATH/anti-sense) and CAG2242 (pTrc)] all displayed similar growth rates and saturation densities. CAG2242 (pSATH/sense) strains, cultured in medium containing IPTG and expressing high levels of human N^1SSAT activity, grew at the lowest rate. These cultures also consistently reached saturation densities that were less than those reached by CAG224 strains not expressing N^1SSAT, although the magnitude of this effect was small (approx. 25% decrease compared with controls). CAG2242 (pSATH/sense) strains growing in the absence of IPTG, but expressing lower levels of N^1SSAT, displayed a lesser but consistent decrease in growth compared with these same cells growing in medium containing IPTG.

It was possible that the growth inhibition of cultures, caused as a result of N^1SSAT expression, was due to the depletion of endogenous spermidine pools. To test this hypothesis, CAG2242 (pSATH/sense) cells were titrated with exogenous spermidine to increase endogenous spermidine contents in cells expressing N^1SSAT. As seen in Figure 4, enhanced expression of N^1SSAT (resulting from treatment with IPTG) caused decreased growth of these cultures. Addition of spermidine (50–200 μM) to the culture medium did not restore optimal growth to these cultures, even though endogenous spermidine pools were increased to over three times untreated control values [e.g. 24.06 ± 2.62 (S.D.) nmol/mg of protein when the medium is adjusted to 200 μM spermidine, compared with 7.18 ± 1.17 nmol/mg of protein without the addition of exogenous spermidine to culture medium] (Table 2). Exogenous spermidine also caused a 2–3-fold increase in intracellular pools of N^1-acetylspermidine in CAG2242 (pSATH/sense) cultures growing in IPTG, although the increase in the N^1-acetylspermidine pool was quantitatively larger than the increase in the non-acetylated spermidine pool. The N^1-acetylspermidine pool increased from 116 nmol/mg of protein in cultures growing in medium without additional spermidine to 230–325 nmol/mg of protein in cultures growing

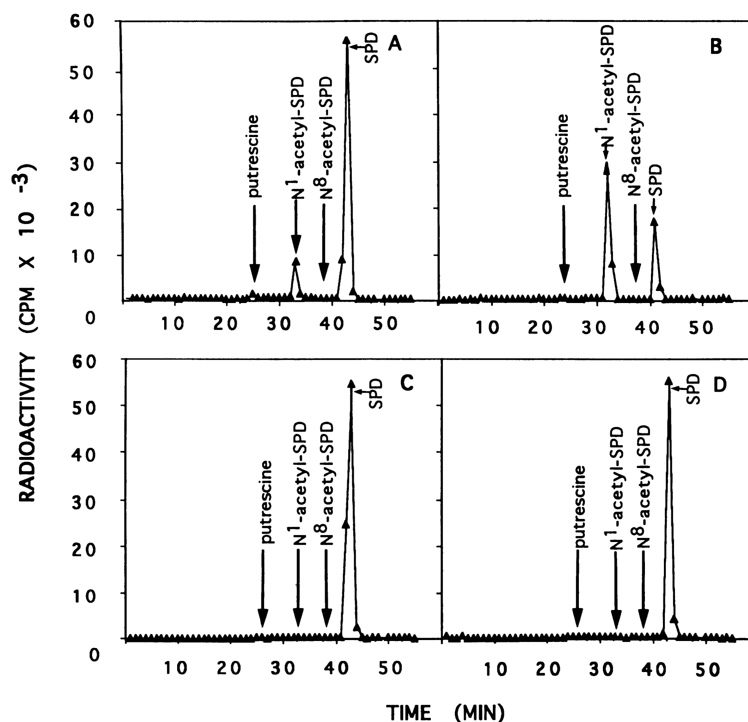

Figure 2 Identification of radiolabelled spermidine and spermidine derivatives in transformed cells by HPLC

CAG2242 was transformed with cDNAs encoding the human N^1SSAT in the sense (**A**, **B**) or anti-sense (**C**, **D**) orientations. Transformed cultures were then grown in medium lacking (**A**, **C**) or containing (**B**, **D**) IPTG to induce expression of N^1SSAT. Radiolabelled spermidine was added to the cultures along with IPTG. After 3 h of incubation, cultures were harvested, cell lysates were extracted with perchloric acid and acid extracts were analysed by HPLC. Equivalent counts were analysed from parallel cultures. Standard preparations of authentic putrescine and spermidine and acetylspermidine derivatives were used to identify peaks of radioactivity. Positions of the standard amines are shown.

Table 1 Polyamine contents in acetylation-deficient bacteria transformed with cDNAs encoding the human N^1SSAT in the sense and anti-sense orientations

Cultures were incubated in the presence and absense of IPTG for 3 h. Values shown are means ± S.D. for triplicate determinations from three representative experiments. Abbreviation: n.d., not detectable; limit of detection was 0.05 nmol/mg of protein.

		Polyamine contents (nmol/mg of protein)		
N^1SSAT orientation	IPTG	Putrescine	Spermidine	N^1-Acetylspermidine
Sense	No	203.4 ± 5.8	6.2 ± 0.5	39.2 ± 1.1
Sense	Yes	207.9 ± 3.1	n.d.*	80.5 ± 3.0
Anti-sense	No	42.8 ± 1.1	75.5 ± 1.1	n.d.
Anti-sense	Yes	36.0 ± 0.9	68.4 ± 3.4	n.d.

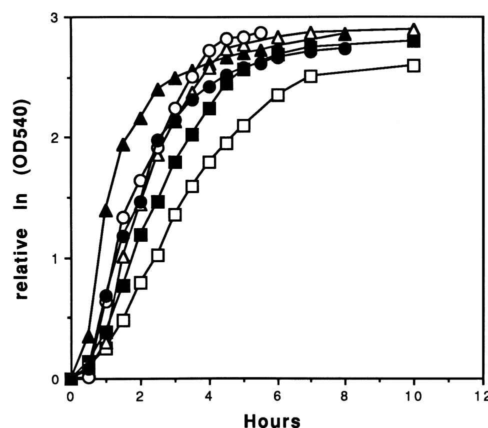

Figure 3 Effect of N^1SSAT expression on growth of transformed cells

CAG2242 was transformed with cDNAs encoding the human N^1SSAT in the sense (■, □) or anti-sense (△) orientations, and grown in the presence (□) or absence (■) of IPTG. Controls included untransformed CAG2242 (●) and this same strain transformed with the vector minus the N^1SSAT insert (○). C600 growth is shown (▲) for reference. Relative $\ln D_{540}$ [ln (OD540)] was determined by normalizing culture D_{540} measurements at various times to the D_{540} value at zero time and then taking the natural logarithm of this normalized value. Values shown derive from a single representative experiment, which was replicated three times.

in medium with spermidine, whereas the spermidine pool only increased from 7 to 24 nmol/mg of protein in the same cultures. In this series of experiments, expression of the N^1SSAT, by addition of IPTG to culture medium without other amines, decreased endogenous spermidine levels, but not as dramatically as seen in experiments described in Table 1.

Because the growth inhibition of CAG2242 (pSATH/sense) cells caused by N^1SSAT expression in the presence of IPTG was not simply due to the depletion of intracellular spermidine pools, we examined whether N^1-acetylspermidine was inhibitory to growth of these bacteria. Addition of 200 μM N^1-acetylspermidine to medium lacking IPTG did not suppress the growth of cultures (Figure 4), even though it increased endogenous N^1-acetylspermidine pools to 97.73 ± 4.85 nmol/mg of protein (Table 2). This elevation was similar to the 116.11 ± 11.87 nmol/

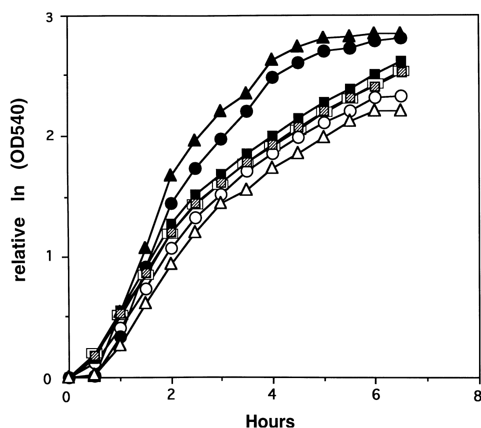

Figure 4 Inability of exogenous spermidine to restore optimal growth in cultures expressing N^1SSAT

CAG2242 (pSATH/sense) cultures were grown in the absence (●,▲) or presence (all other symbols) of IPTG. N^1-acetylspermidine (200 μM) (▲,△) or spermidine [50 μM (■), 100 μM (open rectangles) or 200 μM (hatched squares)] was added to the medium and D_{540} (OD540) was determined for up to $6\frac{1}{2}$ h after amine addition. ○, IPTG alone with no other additions.

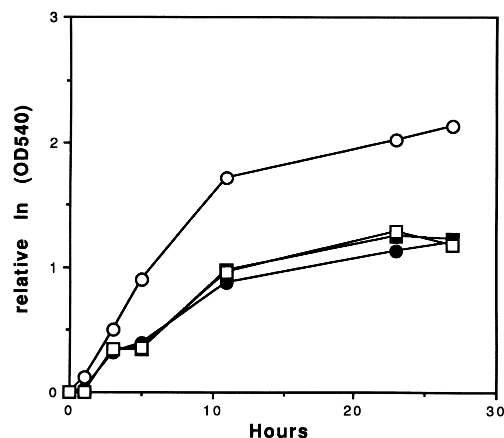

Figure 5 Effect of spermidine and spermidine derivatives on growth of polyamine-deficient HT653 cells

Spermidine (○), N^1-acetylspermidine (■) or N^8-acetylspermidine (□) was added at culture initiation and growth was monitored for up to 27 h thereafter. ●, Growth of control cultures to which no amines were added. Relative lnD_{540} [ln (OD_{540})] was determined as described in Figure 3. Values shown derive from a single representative experiment, which was replicated three times.

Table 2 Polyamine contents in acetylation-deficient bacteria transformed with a cDNA encoding the human N^1SSAT in the sense orientation and titrated with spermidine or N^1-acetylspermidine

Cultures were incubated with the amines, with and without IPTG, for 3 h. Values shown are means ± S.D. for triplicate determinations from three representative experiments.

		Polyamine content (nmol/mg of protein)		
Addition	IPTG	Putrescine	Spermidine	N^1-Acetylspermidine
None	No	107.53 ± 10.30	7.18 ± 1.17	49.60 ± 9.65
None	Yes	164.94 ± 14.42	5.44 ± 1.67	116.11 ± 11.87
Spermidine				
50 μM	Yes	110.78 ± 7.51	18.88 ± 3.54	325.90 ± 15.33
100 μM	Yes	98.83 ± 9.61	24.42 ± 7.78	312.62 ± 8.99
200 μM	Yes	108.37 ± 14.64	24.06 ± 2.62	231.42 ± 15.22
N^1-Acetylspermidine				
200 μM	No	76.33 ± 8.30	8.78 ± 1.35	97.73 ± 4.85
200 μM	Yes	124.67 ± 8.88	7.60 ± 1.13	220.43 ± 13.70

mg of protein of this amine caused by enhanced expression of the human N^1SSAT by addition of IPTG to culture medium lacking N^1-acetylspermidine, which was sufficient to suppress growth in these cultures. Addition of both the acetylated spermidine and IPTG caused a minor additional decrease in cell growth compared with IPTG alone. The elevation in N^1-acetylspermidine was similar to that observed in cells titrated with non-acetylated spermidine and growing in medium with IPTG, although the levels of spermidine were lower in these latter cultures.

Effects of exogenously supplied acetylspermidine derivatives on bacterial cell growth

The decrease in growth rate associated with N^1SSAT expression in CAG2242 cells could be due to one of several mechanisms. Spermidine acetylation could inhibit growth if (1) acetyl derivatives were ineffective, compared with spermidine, in stimulating cell proliferation or (2) they exhibited growth inhibitory activity. The results presented in the previous section show that elevation of N^1-acetylspermidine pools by addition of this amine to the culture medium did not suppress growth of CAG2242 cells, and that restoration of endogenous spermidine pools by exogenous spermidine was not sufficient to restore optimal growth of these cultures. To determine whether acetylspermidine derivatives could stimulate cell proliferation, bacteria deficient in polyamine synthesis were grown in media containing spermidine or acetylspermidines. Exogenous spermidine (10 μM) stimulated the growth of polyamine-deficient HT653 cells (Figure 5). Equivalent concentrations of N^1- or N^8-acetylspermidine had no measurable effect, either stimulatory or inhibitory, on the growth of this strain.

DISCUSSION

The results presented here demonstrate that expression of a cDNA encoding the human N^1SSAT in a bacterial strain deficient in spermidine acetylation causes a decrease in cell growth. Acetylated spermidine derivatives lack the growth-stimulatory activity of non-acetylated spermidine, as they are unable to stimulate growth in polyamine-deficient bacteria. Spermidine acetylation decreases the endogenous pool size of spermidine, and spermidine stimulates cell growth. However, N^1-acetylspermidine might possess growth inhibitory activity, as restoration of endogenous spermidine pools with exogenous spermidine is unable to return optimal growth to cultures expressing N^1SSAT activity. Together these results suggest that growth suppression caused by N^1SSAT expression is not simply due to a decrease in the size of the spermidine pool but is rather a consequence of spermidine acetylation. Spermidine acetylation could affect intracellular spermidine location or further metabolism of this amine. Alternatively the ratio of acetylated to non-acetylated spermidine could affect cell growth. The results provided here are not able to distinguish between these possible mechanisms.

Our results on the effects of spermidine acetylation on growth corroborate the findings of previous studies. Kakegawa et al. [19] reported that the N^1-acetylated derivative of spermine, a longer-

chain amine not normally found in bacteria, does not stimulate the growth of another polyamine-deficient strain of bacteria. Parry et al. [20] overexpressed a human N^1SSAT cDNA in a bacterial strain that also expressed an endogenous spermidine acetyltransferase, and observed growth inhibition. Our experiments with bacterial mutants that lack the ability to synthesize putrescine and spermidine extend these studies and demonstrate for the first time that the physiologically significant amine N^1-acetylspermidine does not replace spermidine as a growth stimulator.

Expression of a human cDNA encoding N^1SSAT might have relevance for the physiological action of the bacterial spermidine acetyltransferase. The polyamines are highly conserved polycations [1]. Expression of a mammalian ornithine decarboxylase cDNA in bacteria incapable of synthesizing putrescine relieved their dependence on exogenous polyamines for growth [21]. Because the human N^1SSAT acetylates bacterial spermidine, as shown here, it is likely that the suppression of growth rates that occurs as a consequence of this acetylation is the same consequence that occurs as a result of expression of the bacterial enzyme. After the completion of the work reported here, Fukuchi et al. [22] reported that expression of the *E. coli* spermidine acetyltransferase protected CAG2242 cells from toxicity due to high endogenous spermidine contents.

Null mutants of *S. cerevisiae*, lacking the gene encoding *S*-adenosylmethionine decarboxylase and therefore unable to synthesize spermidine, do not grow in the absence of exogenous spermidine [3]. However, null mutants of *E. coli* lacking the *S*-adenosylmethionine decarboxylase gene grow normally, indicating that spermidine is not essential for *E. coli* growth [4]. Our findings, considered along with those of Xie et al. [4], could indicate that acute changes in spermidine pools, such as those caused by acetylation, do affect growth, but that bacteria can adapt over time to the lack of spermidine. Presumably, bacteria, but not eukaryotes, are able to substitute the shorter-chain diamine putrescine for the triamine spermidine.

One mechanism that could explain the effects of spermidine acetylation on growth could relate to the role of polyamines on translation. Differential expression of ornithine decarboxylase, and subsequent putrescine production, in *S*-adenosylmethionine decarboxylase deletion mutants results in significant changes in the ratio of putrescine to spermidine and ribosomal frameshifting in yeast [23]. Acetylated spermidine affects protein translation to a different extent than spermidine, at least in some cell-free systems [24]. Thus acute changes in spermidine pools, caused by acetylation, could have effects on translation that could disappear as a consequence of changes in putrescine pools in bacteria. This adaptation would not occur in eukaryotes, which have evolved the requirement for the hypusine modification in the putative eukaryotic translation initiation factor 5A [25,26].

Animal cells have evolved two mechanisms for reutilizing acetylated spermidine. Spermidine acetylated at the N^8 position by the nuclear spermidine acetyltransferase can be deacetylated to yield spermidine [11]. Inhibitor studies suggest that this acetylation/deacetylation reaction occurs early in growth [8]. Spermidine acetylated at the N^1 position by the cytosolic spermidine/spermine acetyltransferase is not deacetylated, but is a substrate for PAO. The peroxisomal PAO oxidizes N^1-acetylspermidine and N^1-acetylspermine to form putrescine and spermidine respectively [11]. Amine oxidases capable of utilizing acetylputrescine as a substrate have been identified in micro-organisms, including bacteria [27]. We have been unable to detect spermidine deacetylating activity in our *E. coli* strains. We have also been unable to detect a polyamine oxidase activity in these bacteria capable of forming putrescine from N^1-acetylspermidine. As seen in Figure 2, radiolabel from spermidine appears in N^1-acetylspermidine, but not putrescine, in these bacteria. We have performed similar labelling protocols in the C600 strain, which is capable of acetylating spermidine. As in the CAG2242 cells, we find no evidence of polyamine-oxidizing activity in the C600 strain. Thus it appears that acetylation of spermidine in *E. coli* does not lead to further catabolism to putrescine or other polyamines, and is a mechanism for depleting the functional pool of spermidine. The mechanism by which spermidine acetylation causes growth inhibition, however, remains undefined, as repletion of endogenous spermidine pools is not sufficient to restore optimal growth in cells actively acetylating spermidine.

The endogenous spermidine acetyltransferase of *E. coli* acetylates spermidine at both the N^1 and N^8 positions and is constitutively expressed [6]. Pools of acetylated spermidine are only detectable, however, after cells are subjected to stress. It remains a possibility that the accumulation of acetylspermidine derivatives is due to an inhibition of subsequent metabolism, even though we have been unable to identify two routes of subsequent change (deacetylation and oxidation) known to occur in eukaryotes. In spite of this uncertainty these results show that spermidine acetylation can be sufficient to suppress growth in bacteria. Inhibiting reutilization of acetylspermidine in the maintenance of spermidine pools might have the same effect in animals. Brasitus and co-workers [28] have reported that inhibitors of PAO cause a decrease in tumour formation in one animal model of colon carcinogenesis. Future studies variably expressing N^1SSAT in yeast and animal cells will be needed to define the role of this gene in eukaryotes.

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