

# A Dominant Negative Effect of *eth-1'*, a Mutant Allele of the *Neurospora crassa* S-Adenosylmethionine Synthetase-Encoding Gene Conferring Resistance to the Methionine Toxic Analogue Ethionine

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## ABSTRACT

*eth-1'*, a thermosensitive allele of the *Neurospora crassa* S-adenosylmethionine (AdoMet) synthetase gene that confers ethionine resistance, has been cloned and sequenced. Replacement of an aspartic amino acid residue (D<sup>48</sup> → N<sup>48</sup>), perfectly conserved in prokaryotic, fungal and higher eukaryotic AdoMet synthetases, was found responsible for both thermosensitivity and ethionine resistance conferred by *eth-1'*. Gene fusion constructs, designed to overexpress *eth-1'* *in vivo*, render transformant cells resistant to ethionine. Dominance of ethionine resistance was further demonstrated in *eth-1<sup>+</sup>/eth-1'* partial diploids carrying identical gene doses of both alleles. Heterozygous *eth-1<sup>+</sup>/eth-1'* cells have, at the same time, both the thermotolerance conferred by *eth-1<sup>+</sup>* and the ethionine-resistant phenotype conferred by *eth-1'*. AdoMet levels and AdoMet synthetase activities were dramatically decreased in heterozygous *eth-1<sup>+</sup>/eth-1'* cells. We propose that this negative effect exerted by *eth-1'* results from the *in vivo* formation of heteromeric *eth-1<sup>+</sup>/eth-1'* AdoMet synthetase molecules.

**E**THIONINE is an analogue of methionine that is toxic to cells (ALIX 1982). Mechanisms underlying ethionine toxicity have been mainly explored in simple cellular systems by the isolation of mutant organisms resistant to this drug. Particularly informative have been the studies made on Eth<sup>r</sup> (ethionine-resistant) mutants of the enteric bacteria *Escherichia coli* and *Salmonella thyphimurium* (LAWRENCE *et al.* 1968; GREENE *et al.* 1970; HOLLOWAY *et al.* 1970; HOBSON and SMITH 1973; HAFNER *et al.* 1977; SAINT-GIRONS *et al.* 1988), the yeast *Saccharomyces cerevisiae* (CHEREST *et al.* 1969, 1973; THOMAS and SURDINKERJAN 1991), the filamentous fungus *Neurospora crassa* (METZENBERG *et al.* 1964; KERR and FLAVIN 1970; BURTON and METZENBERG 1975) and the plant *Arabidopsis thaliana* (INABA *et al.* 1994). Mutations to ethionine resistance occur mainly in the gene encoding S-adenosyl-L-methionine synthetase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6; CANTONI 1953). This enzyme catalyzes the conversion of L-methionine and ATP to S-adenosylmethionine (AdoMet), which is the major cellular donor of methyl groups in a variety of biochemical pathways (TABOR and TABOR 1984).

The *eth-1'* mutant of *N. crassa* has been exhaustively analyzed at the genetic and biochemical levels (MET-

ZENBERG *et al.* 1964; KAPPY and METZENBERG 1965; METZENBERG and PARSON 1966; BURTON and METZENBERG 1975; JACOBSON *et al.* 1977). It was recently proved (MAUTINO *et al.* 1996a) that this locus encodes AdoMet synthetase, as first suggested by KERR and FLAVIN (1970). A phenotypic feature associated with this mutation, which appears to be crucial for the mechanism underlying ethionine resistance, is the overproduction of methionine (KAPPY and METZENBERG 1965). It has been proposed that AdoMet synthetase mutants overproduce methionine by the additive effect of both a lower rate of conversion of methionine to AdoMet and a derepression of some enzymes involved in the methionine biosynthetic pathway normally repressed by AdoMet (KAPPY and METZENBERG 1965; GREENE *et al.* 1970; KERR and FLAVIN 1970; BÖERJAN *et al.* 1994). There is strong evidence coming from Eth<sup>r</sup> mutants of the yeast *S. cerevisiae* that a variety of additional reasons may be involved in conferring resistance to ethionine (ALIX 1982; SHIOMI *et al.* 1991; THOMAS and SURDINKERJAN 1991; HEILAND and HILL 1993).

In this work we report the molecular cloning and sequence of the *N. crassa eth-1'* allele. In addition, we show that ethionine resistance conferred by *eth-1'* is dominant. Analyses of heterozygous strains suggest that dominant ethionine resistance could be mediated by the *in vivo* formation of heteromeric (*eth-1<sup>+</sup>/eth-1'*) AdoMet synthetase molecules.

## MATERIALS AND METHODS

**Strains, growth conditions and crosses:** *N. crassa* strains *eth-1' A* and *a* (FGSC #1212 and #1220), wild type 74-OR23-1A

**This work is dedicated to the memory of Dr. Federico A. Cumar.**

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(FGSC #987) and 74-OR8-1a (FGSC #988), *T(I→II)39311 tol trp-4*, A (FGSC #2985) were obtained from the Fungal Genetics Stock Center (FGSC). Standard *N. crassa* methodologies were used according to DAVIS and DE SERRES (1970). Growth rates were determined in race tubes (RYAN *et al.* 1943). Construction of partially duplicated strains, *Dp39311(eth-1<sup>+</sup>/eth-1<sup>-</sup>) tol trp-4 A/a*, was done essentially as described (PERKINS 1972). The *tol* mutation (PERKINS *et al.* 1982) was included to suppress the vegetative incompatibility associated with the mating type alleles A and a. DL-ethionine (Sigma) was added to solid and liquid media at 50–250 µg/ml, when indicated. L-tryptophan was added at 250 µg/ml.

**DNA manipulations and protoplast transformation:** Total *N. crassa* DNA was purified and subjected to Southern blot analysis as described (HAEDO *et al.* 1992). Plasmid DNA was purified and analyzed using standard procedures (SAMBROOK *et al.* 1989). Protoplasts were prepared and transformed following the protocol of VOLLMER and YANOFSKY (1986). Co-transformations experiments were carried out using 1 µg of pBT6 DNA (ORBACH *et al.* 1986; MAUTINO *et al.* 1993). Benomyl at 0.75 µg/ml was included in the bottom agar to select for transformant cells. Probes were oligolabelled according to the method of FEINBERG and VOGELSTEIN (1983). [ $\alpha$ -<sup>32</sup>P]-dATP (3000 Ci/mmol) was from NEN-DuPont.

**PCR amplification, molecular cloning and DNA sequencing of the *eth-1<sup>-</sup>* allele:** Degenerate oligonucleotides *d1/d3* and PCR were used to amplify a *N. crassa* genomic fragment corresponding to almost all of the open reading frame of the AdoMet synthetase encoded by the *eth-1<sup>-</sup>* allele. The sequence of these oligonucleotides, which were designed for the cloning of the AdoMet synthetase gene of *A. immersus* (MAUTINO *et al.* 1996b), are 5'CGGGATCCGTIGGWAIGGTCAYCCI3' and 5'CCCTCGAGAAGCTTSAIAGYCTTIGGCTT3'. PCR reactions were performed with *Taq* polymerase (Perkin Elmer) using 100 ng of genomic DNA from the mutant *eth-1<sup>-</sup>*. Reactions were carried out for 35 cycles at 94° for 45 sec, 60° for 30 sec, and 72° for 1 min 20 sec, in a 50-µl reaction volume. An amplified fragment of 1150 bp, identical in size to that obtained when using wild-type DNA as the template, was gel purified (Prep-A-Gene; BioRad), digested with *Bam*HI and cloned into the *Bam*HI-*Eco*RV sites of pBKS(+) (Stratagene). Unidirectional deletions of several independent clones were made with Exonuclease III (HENIKOFF 1987), after digestion with the enzymes *Hind*III and *Apa*I, which do not cut into the insert fragment. Deletion clones were sequenced by the chain termination method (SANGER *et al.* 1977) using the reverse sequencing primer and Sequenase (USB). Multiple sequence alignment was performed with the program PILEUP and PRETTYBOX of the GCG sequence analysis package (DEVEREUX *et al.* 1984). The nucleotide sequence reported in this work has been submitted to GenBank with accession number U36761.

**Construction of the pETH expression plasmids carrying the mutant AdoMet synthetase (*eth-1<sup>-</sup>*) gene fused to heterologous promoters:** Two alternative constructs, pETH-N and pETH-A, carrying *eth-1<sup>-</sup>* coding sequences under the control of the gene promoters from either the *N. crassa cfp* (pyruvate decarboxylase) (ALVAREZ *et al.* 1993; E. TEMPORINI and A. ROSA, unpublished results) or the *A. nidulans trpC* (STABEN *et al.* 1989) genes, respectively, were made from plasmids pSGT-N and pSGT-A that were previously designed to overexpress the wild-type allele of the AdoMet synthetase gene (MAUTINO *et al.* 1996a). To construct pETH-N and pETH-A, a 315-bp *Bst*EII restriction fragment from the wild-type AdoMet synthetase gene was replaced by the corresponding fragment of the *eth-1<sup>-</sup>* allele that includes a G → A transition in position +142.

**Measurements of AdoMet synthetase activity and cellular AdoMet contents:** AdoMet synthetase was partially purified

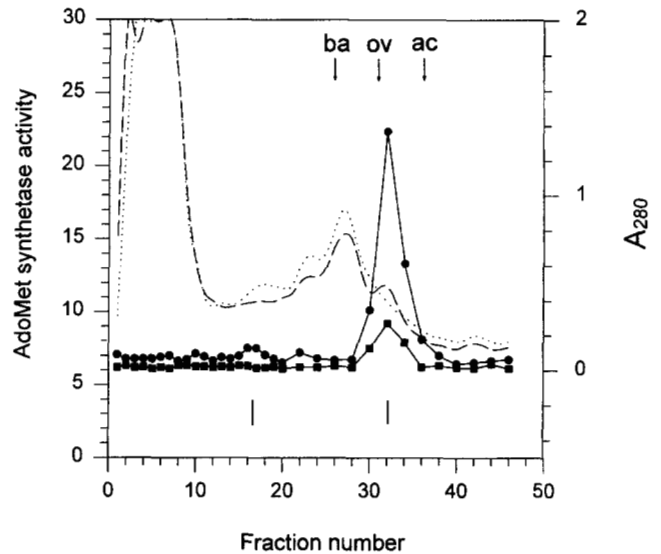


FIGURE 1.—FPLC chromatography of AdoMet synthetases from *eth-1<sup>+</sup>* and *eth-1<sup>-</sup>* strains. Elution profile of enzymatic activities (arbitrary units) of wild type (●—●) and *eth-1<sup>-</sup>* (■—■) strains are shown.  $A_{280}$  values corresponding to the wild-type (— — —) and *eth-1<sup>-</sup>* (· · · · ·) chromatographic fractions are also indicated. Vertical bars, at fractions ~17 and ~32, indicate the position of AdoMet synthetase activities of  $M_r$  150,000 and  $M_r$  41,000, respectively. Molecular weight standards were anhydrase carbonic (*ac*), ovalbumin (*ov*) and bovine albumin (*ba*).

by FPLC (Pharmacia; Sweden) as follows: *N. crassa* mycelium (1.5 g) was harvested, frozen, powdered in a mortar under liquid nitrogen, and extracted with 2 vol of buffer (10 mM KCl, 30 mM MgCl<sub>2</sub>, 100 mM Tris-HCl pH 7.5, 5 mM β-mercaptoethanol). The homogenate was clarified by microcentrifugation (13,000 × g, 20 min at 4°) and the protein extract (4 mg of protein) was chromatographed in a Superdex-200 column. Aliquots (250 µl) were taken and used to determine AdoMet synthetase activity as described (MAUTINO *et al.* 1996a). Cellular AdoMet levels were determined according to MAUTINO *et al.* (1996a).

## RESULTS

**Characterization of AdoMet synthetase from *N. crassa* wild-type and *eth-1<sup>-</sup>* strains:** The primary amino acid sequence of AdoMet synthetase is highly conserved throughout evolution (KOTB and GELLER 1993). The deduced sequence of the *N. crassa* enzyme (MAUTINO *et al.* 1996a) predicts a molecular size of ~43 kDa, which is similar to that of all other AdoMet synthetases characterized so far (KOTB and GELLER 1993). Identical FPLC elution profiles, corresponding to an experimental  $M_r$  41,000, were observed for the wild-type and *eth-1<sup>-</sup>* enzymes (Figure 1). Even when chromatography was performed in the presence of AdoMet, a condition that appears to stabilize the oligomeric state of AdoMet synthetases (CHEREST and SURDIN-KERJAN 1981), only a minor fraction of the *N. crassa* enzyme was recovered in a putative tetrameric form, which is the main aggregation state of the yeast enzyme (THOMAS and SURDIN-KERJAN 1987). As previously reported (JACOBSON *et al.* 1977),

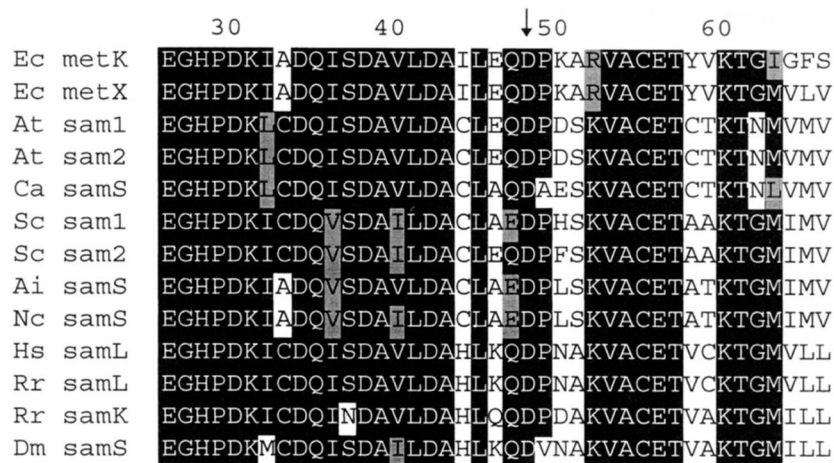


FIGURE 2.—Multiple alignment of AdoMet synthetase sequences surrounding the D<sup>48</sup> amino acid residue. AdoMet synthetase sequences from bacteria, plants, fungi, mammalian and insect are compared. Amino acid residues that are identical in at least eight of the 13 analyzed sequences are indicated with black boxes. Conservative amino acid changes are shown in shaded boxes. The arrow indicates the conserved aspartic residue. GenBank accession numbers are given below in parentheses: *E. coli* (*Ec*) *metK* (K02129) and *metX* (M98266), *A. thaliana* (*At*) *sam1* (M55077) and *sam2* (M33217), *D. caryophyllus* (*Ca*) (M61882), *S. cerevisiae* (*Sc*) *sam1* (J03477) and *sam2* (M23368), *A. immersus* (*Ai*) (U21548), *N. crassa* (*Nc*) (U21547), human liver (*Hs*) (D11332), rat liver (*Rr*) *samL* (X15734), rat kidney (*Rr*) *samK* (J05571) and *D. melanogaster* (*Dm*) (X77392).

the *eth-1<sup>r</sup>* mutant has reduced AdoMet synthetase activity (Figure 1). Analyses of substrate (*e.g.*, methionine and ethionine) affinities confirmed previous evidence (JACOBSON *et al.* 1977), indicating that the *eth-1<sup>+</sup>* and *eth-1<sup>r</sup>* enzymes have similar kinetic properties *in vitro* (not shown).

**Molecular cloning and sequence of the *eth-1<sup>r</sup>* allele:** PCR was used to amplify the genomic region corresponding to the *eth-1<sup>r</sup>* allele. DNA products from independent amplification reactions were isolated, cloned and sequenced. Two nucleotide substitutions, G → A and C → T, in positions +142 and +945, respectively, were consistently found in several sequenced clones. Only the G → A substitution results in an amino acid change (D<sup>48</sup> → N<sup>48</sup>). Multiple amino acid alignment of AdoMet synthetases (Figure 2) shows that D<sup>48</sup> is completely conserved in all the available prokaryotic, fungal and higher eukaryotic enzyme sequences. Interestingly, D<sup>48</sup> follows a perfectly conserved periodic arrangement of acidic amino acids (EX<sub>3</sub>DX<sub>3</sub>DX<sub>3</sub>DX<sub>3</sub>DX<sub>3</sub>D<sup>48</sup>) (Figure 2). This sequence corresponds to an  $\alpha$ -helix domain located close to the active site of the enzyme (TAKUSAGAWA *et al.* 1996). Searching in the SWISSPROT database (Rel. 31.0) with alternative peptide sequences obtained from the AdoMet consensus sequence A-V-L-D-A-C/H-L-X-Q/E-D-P-X-X-K/R-V-A-C-E-T (Figure 2) did not detect homologous sequences, which could suggest a conserved functional role for the region including D<sup>48</sup>.

**Analysis of *eth-1<sup>+</sup>/eth-1<sup>r</sup>* heterozygous strains:** Strains carrying the wild-type *eth-1<sup>+</sup>* allele, plus ectopically integrated copies of *eth-1<sup>r</sup>* (plasmids pETH-N and pETH-A; see MATERIALS AND METHODS) were constructed by protoplast transformation. Presence of plasmid sequences, in several *eth-1<sup>+</sup>/eth-1<sup>r</sup>*<sub>n</sub> transformant cell lines (where *n* means different copy numbers of the transgene), was determined by Southern blot (not shown). *In vivo* functionality of similar fusion constructs carrying the wild-type AdoMet synthetase gene has been demonstrated by transformation of *eth-1<sup>r</sup>* protoplasts

and complementation of the thermosensitive phenotype associated with *eth-1<sup>r</sup>* (MAUTINO *et al.* 1996a).

Previous results obtained with either forced primary heterocaryons (*eth-1<sup>+</sup> + eth-1<sup>r</sup>*), or *eth-1<sup>r</sup>* strains transformed with the wild-type AdoMet synthetase gene indicated that thermosensitivity associated with *eth-1<sup>r</sup>* is overcome in the presence of the wild-type allele (MAUTINO *et al.* 1996a). All of the selected *eth-1<sup>+</sup>/eth-1<sup>r</sup>*<sub>n</sub> strains were able to grow at the temperature restrictive for the allele *eth-1<sup>r</sup>* (39°) (see T1 and T2 in Figure 3, A and C). However, it was not known if the transgene was being expressed, as the recipient cells (*eth-1<sup>+</sup>*) were thermoresistant before transformation. In an attempt to determine the functionality of the *eth-1<sup>r</sup>* transgenes, we tested if *eth-1<sup>+</sup>/eth-1<sup>r</sup>*<sub>n</sub> strains were able to grow at the permissive temperature for the *eth-1<sup>r</sup>* allele in media containing DL-ethionine. All of the selected *eth-1<sup>+</sup>/eth-1<sup>r</sup>*<sub>n</sub> strains were able to grow in a medium containing high levels of DL-ethionine, at 30° (Figure 3B). Interestingly, these strains were also able to grow in this medium at 39° (Figure 3D), a nonpermissive temperature for the mutant allele. Thus, even when it was expected that the *eth-1<sup>r</sup>* molecules should be inactivated at 39°, heterozygous strains display at the same time the combined phenotypes of thermoresistance and ethionine resistance associated with *eth-1<sup>+</sup>* and *eth-1<sup>r</sup>*, respectively. Simultaneous and functional expression of the *eth-1<sup>+</sup>* and *eth-1<sup>r</sup>* alleles should be required to confer this "thermotolerant-ethionine resistance" in heterozygous strains. Loss or counterselection of either the wild-type or the mutant copies of the gene, which could change the proportion of the respective alleles in the transformants, should result in strains that are not thermotolerant and ethionine resistant at the same time. As discussed below, these results can be explained if an interaction of *eth-1<sup>+</sup>* and *eth-1<sup>r</sup>* AdoMet synthetase molecules take place in heterozygous strains.

**Analysis of partial diploid strains:** We have confirmed and extended the results obtained with *eth-1<sup>+</sup>/*

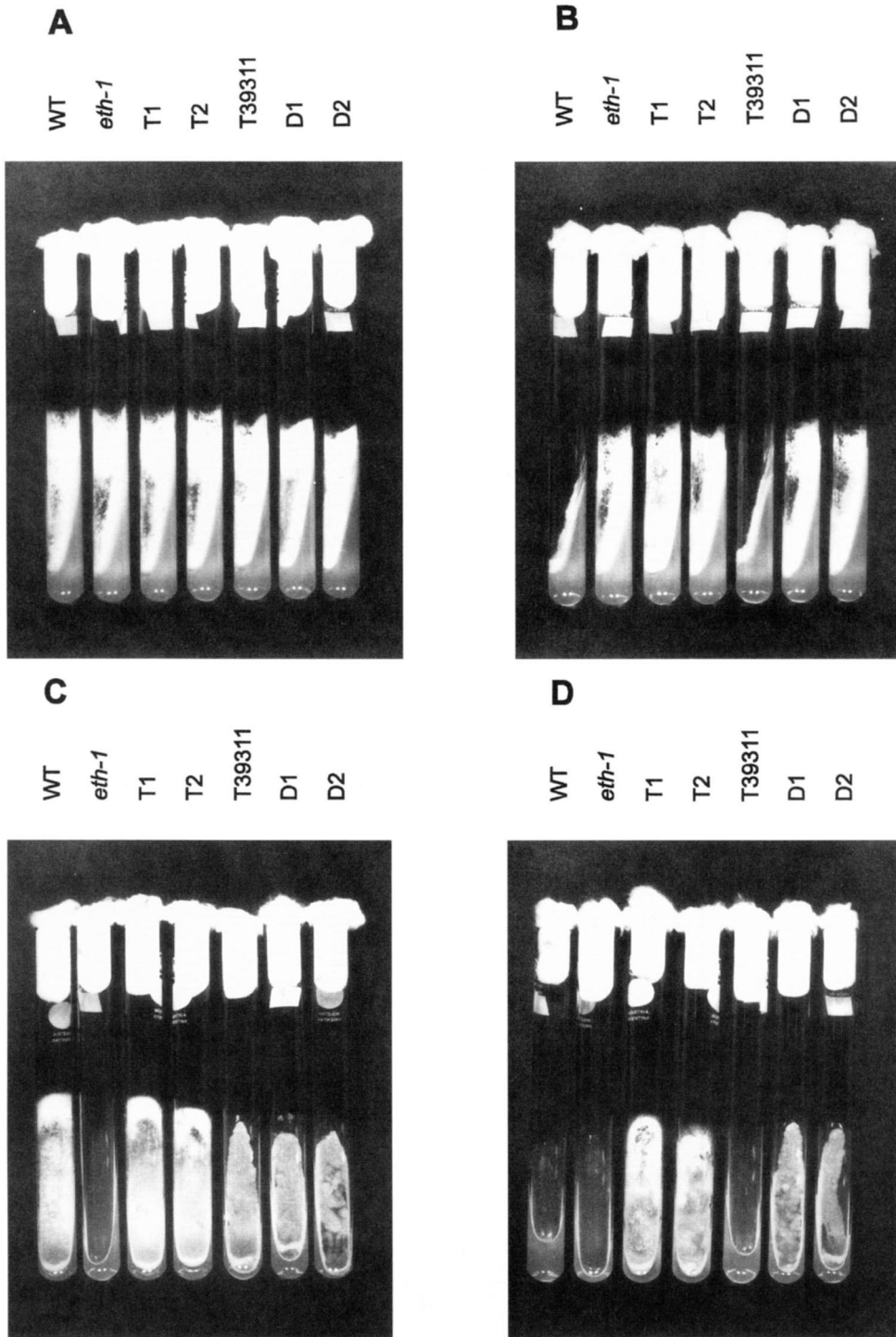


FIGURE 3.—Thermosensitivity and ethionine-resistance analyses of heterozygous *eth-1*<sup>+</sup>/*eth-1'* strains. Wild type (WT), *eth-1'* (*eth-1*), pETH-N and pETH-A transformants (*T1* and *T2*, respectively), *T(I → II) 39311 tol trp-4* (*T39311*) and partial diploids (*D1* and *D2*) strains were grown for 3 days in test tube slants at 30° (A and B) or 39° (C and D) in media supplemented (B and D) or not (A and C) with 100 µg/ml of DL-ethionine. For details see text.

(*eth-1'*)<sub>n</sub> heterozygous strains by studying chromosome-duplicated strains having identical gene doses of the *eth-1*<sup>+</sup> and *eth-1'* alleles in every nucleus. Although the partial diploids have an unwanted morphological temperature-sensitive phenotype, which is similar to the *T(I → II) 39311* translocated parent itself (J. L. BARRA and A. ROSA, unpublished observations), they were highly advantageous for these studies. The growth of *eth-1*<sup>+</sup>/*eth-1'* partial diploids resemble that of their *eth-1*<sup>+</sup> (*T(I → II) 39311*) parent at 39° (strains *D1* and *D2*

in Figure 3C), confirming the recessiveness of the *eth-1'* thermosensitive phenotype. Partial diploids were further analyzed for their ethionine resistance properties. As was observed for *eth-1*<sup>+</sup>/*(eth-1')*<sub>n</sub> strains, *eth-1*<sup>+</sup>/*eth-1'* partial diploids are resistant to ethionine at both permissive and nonpermissive temperature conditions for the *eth-1'* allele (Figure 3, B and D), thus confirming the dominant nature of this phenotype. The ability of the mutant *eth-1'* allele in partial diploids to confer ethionine resistance at 39° indicates that thermosensitivity

TABLE 1

Ethionine resistance in *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> partial diploids

DL-ethionine ( $\mu\text{g/ml}$ )	Strain		
	<i>eth-1</i> <sup>+</sup>	<i>eth-1</i> <sup>+</sup> / <i>eth-1</i> <sup>-</sup> (D1)	<i>eth-1</i> <sup>+</sup> / <i>eth-1</i> <sup>-</sup> (D2)
None	4.02	3.82	3.45
50	2.82	3.77	3.34
250	0	2.95	2.85

Growth rates (mm/hr) of the mentioned strains were determined at 30° in race tubes containing minimal medium supplemented with 250  $\mu\text{g/ml}$  of L-tryptophane and the indicated concentrations of DL-ethionine.

associated with *eth-1*<sup>-</sup> is being overcome in the presence of *eth-1*<sup>+</sup>. Thus, partial diploids show simultaneously the combined phenotypes of thermoresistance and ethionine resistance associated with *eth-1*<sup>+</sup> and *eth-1*<sup>-</sup>, respectively. These observations support the above proposed interaction between *eth-1*<sup>+</sup> and *eth-1*<sup>-</sup> molecules (see DISCUSSION).

**Quantitative analysis of ethionine resistance in *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains:** A more accurate measurement of the ethionine-resistance properties of *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> partial diploids was obtained by scoring growth rates in race tubes (RYAN *et al.* 1943). Table 1 shows that *eth-1*<sup>+</sup> and *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains have similar growth rates when growing in a medium without DL-ethionine. As little as 50  $\mu\text{g/ml}$  of DL-ethionine (0.3 mM) produces a partial inhibition of the growth rate of the wild-type strain without affecting that of the *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains. At 250  $\mu\text{g/ml}$  DL-ethionine, growth of the *eth-1*<sup>+</sup> strain was totally inhibited while growth rates of the *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains were only slightly affected (Table 1), being similar to that of strains carrying a single *eth-1*<sup>-</sup> allele (not shown). The morphological temperature-sensitive phenotype of partial diploids (Figure 3, C and D) did not allow measurement of growth rates at 39°.

**AdoMet synthetase activities in *eth-1*<sup>+</sup>, *eth-1*<sup>-</sup>, *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> (*eth-1*<sup>-</sup>)<sub>n</sub> and *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains:** It has been proposed that ethionine resistance, conferred by the *eth-1*<sup>-</sup> allele, results from the low cellular levels of AdoMet in this mutant (KAPPY and METZENBERG 1965; KERR and FLAVIN 1970; JACOBSON *et al.* 1977). To test if there is a correlation between ethionine resistance and cellular AdoMet levels, we determined AdoMet synthetase activities and cellular levels of AdoMet, in *eth-1*<sup>+</sup>, *eth-1*<sup>-</sup>, *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> (*eth-1*<sup>-</sup>)<sub>n</sub> and *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> strains. Figure 4A shows that, in agreement with previous reports (JACOBSON *et al.* 1977; MAUTINO *et al.* 1996a), the *eth-1*<sup>-</sup> mutant strain has 20–30% of AdoMet synthetase activity when compared with the wild type (see also Figure 1). Interestingly, *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> (*eth-1*<sup>-</sup>)<sub>n</sub> (T1 and T2 in Figure 4A) and *eth-1*<sup>+</sup>/*eth-1*<sup>-</sup> (D1 and D2 in Figure 4A) strains have reduced AdoMet synthetase activities, comparable to those present in the *eth-1*<sup>-</sup> mutant. The cellular content of AdoMet was deter-

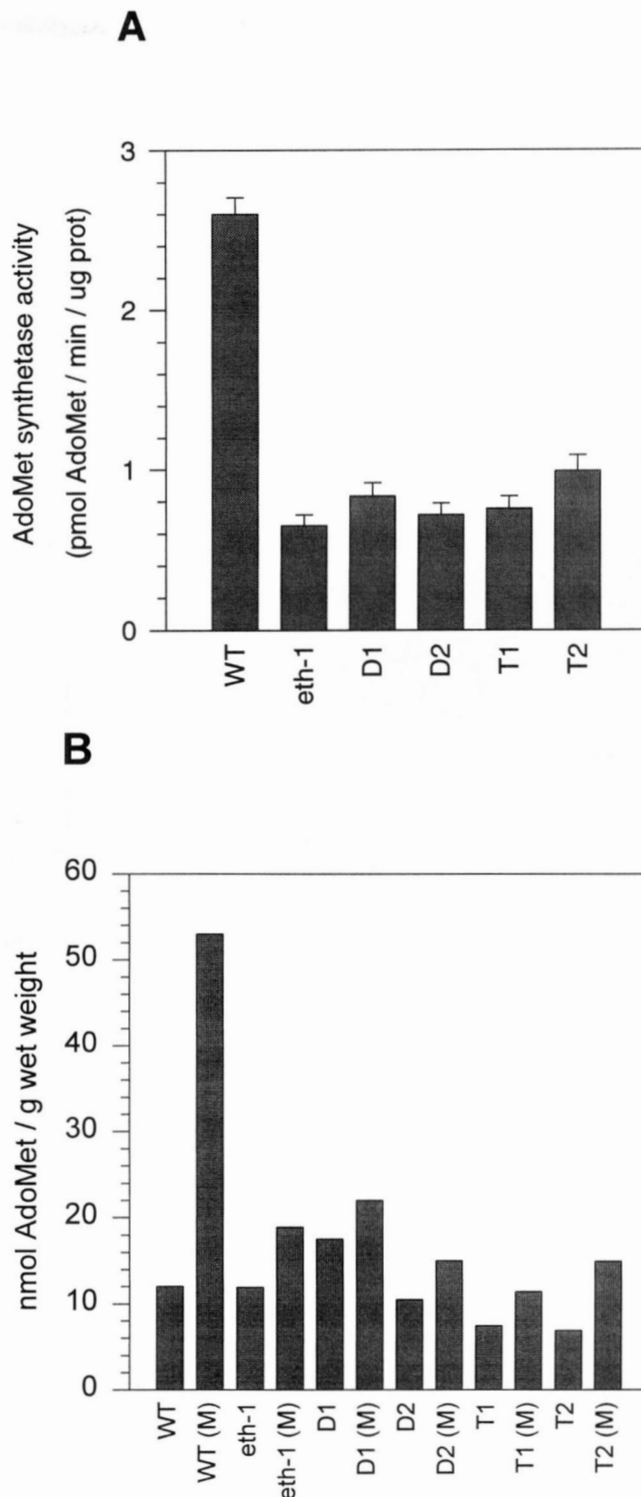


FIGURE 4.—Dominant effect of the *eth-1*<sup>-</sup> mutation. AdoMet synthetase activity (A) and cellular AdoMet levels (B) were determined for the wild type (WT), *eth-1*<sup>-</sup> (*eth-1*), partial diploids (D1 and D2), and pETH-N and pETH-A transformants (T1 and T2, respectively) strains. Enzymatic activities were determined on protein extracts obtained from mycelia grown at 25° in minimal medium as described in MATERIALS AND METHODS. Error bars represent  $\pm$ SD of two independent experiments. AdoMet levels were determined in mycelia grown at 25° in media supplemented, indicated as (M), or not with 2.5 mM L-methionine.

mined in these strains (Figure 4B). Heterozygous *eth-1<sup>+</sup>/(eth-1<sup>-</sup>)<sub>n</sub>* strains, as well as *eth-1<sup>+</sup>/eth-1<sup>-</sup>* partial diploids, have levels of AdoMet similar to those of the *eth-1<sup>-</sup>* mutant, whether or not media are supplemented with 2.5 mM L-methionine (Figure 4B). Thus, in contrast with the wild-type strain, strains carrying the *eth-1<sup>-</sup>* allele fail to increase their endogenous levels of AdoMet when growing in a medium supplemented with L-methionine. Additional evidence was obtained in experiments performed with *eth-1<sup>-</sup>* strains carrying gene fusion constructs designed to overexpress the wild-type AdoMet synthetase gene (MAUTINO *et al.* 1996). These transformants, which have the thermotolerant character conferred by the wild-type transgene, were unable to overproduce cellular AdoMet though maintaining the ethionine-resistance character conferred by the endogenous *eth-1<sup>-</sup>* allele (not shown).

#### DISCUSSION

Ethionine toxicity has been largely explored in simple cellular systems by the isolation of mutant organisms resistant to the drug (ALIX 1982). An emerging picture from these studies is that mutations in AdoMet synthetase genes, which result in cells with low levels of AdoMet and high levels of endogenous methionine, are the main cause of resistance to ethionine. Certainly, cellular "dilution" of toxic analogues has been the cause of resistance in well documented examples (UMBARGER 1971; RICCARDI *et al.* 1981). Studies performed several years ago with the *N. crassa* mutant *eth-1<sup>-</sup>* established that it has diminished AdoMet synthetase activity (KERR and FLAVIN 1970; JACOBSON *et al.* 1977) and accumulates methionine (KAPPY and METZENBERG 1965; BURTON and METZENBERG 1975). A plausible mechanism was proposed to explain ethionine resistance in this mutant based on methionine accumulation, and dilution of ethionine in methionine-requiring reactions, due to a defect in the feedback inhibition of cystathionine  $\gamma$ -synthase by AdoMet (JACOBSON *et al.* 1977). In addition to feedback regulation by AdoMet, transcriptional regulation of the genes governing the pathway of methionine biosynthesis could take place (see THOMAS *et al.* 1992). Recent evidence, obtained from transgenic plants, carrying "silenced" AdoMet synthetase genes (BÖERJAN *et al.* 1994), indicates that higher eukaryotic cells also accumulate methionine when AdoMet synthetase activity and/or cellular levels of AdoMet are severely diminished. Nevertheless, results from the analysis of the *N. crassa* double mutant *me-4* (now *cys-10*) *eth-1<sup>-</sup>* (KAPPY and METZENBERG 1965) show that methionine overproduction is not the only requisite to confer ethionine resistance. Thus, cellular levels of L-methionine would not necessarily correlate with the phenotype of ethionine resistance. JACOBSON *et al.* (1977) showed that diminished activity of AdoMet synthetase in the *eth-1<sup>-</sup>* mutant avoids accumulation of S-

adenosylethionine, which is a toxic compound derivative of ethionine (ALIX 1982). Thus, ethionine dilution due to methionine overproduction, as well as diminished cellular production of the toxic S-adenosylethionine, should result in a phenotype of ethionine resistance.

Although the present work does not deal directly with the underlying mechanism of ethionine resistance, our finding that *eth-1<sup>-</sup>* confers this phenotype in a dominant fashion was unexpected when considering the above proposed model of ethionine resistance. This model predicts that the presence of wild-type AdoMet synthetase activity should increase the cellular levels of AdoMet that, in turn, should restore the normal flux of the methionine biosynthetic pathway, rendering an ethionine-sensitive phenotype. Accordingly, one would expect the ethionine-resistance phenotype conferred by *eth-1<sup>-</sup>* to be recessive. The apparent contradiction between this model of ethionine resistance and the dominant ethionine resistance found for *eth-1<sup>-</sup>* could be explained if *eth-1<sup>-</sup>* molecules exert a dominant negative effect. An interesting observation in our experiments was that the dominance of the ethionine-resistance phenotype associated with *eth-1<sup>-</sup>* was also present in heterozygous strains at 39°, a nonpermissive temperature for the *eth-1<sup>-</sup>* mutant (METZENBERG 1964), which has a heat-labile AdoMet synthetase activity measurable *in vitro* (JACOBSON *et al.* 1977). The finding of ethionine resistance in heterozygous strains at 39° strongly supports the contention that this allele is being expressed. This result was not only observed when analyzing *eth-1<sup>+</sup>/(eth-1<sup>-</sup>)<sub>n</sub>* strains, where gene doses may be partially altered during vegetative growth, but also in the partial diploids where an identical gene ratio of both alleles is maintained. In addition, this effect could not be explained as a trivial codominant phenotype because in this case the coexpressed phenotypes should be mutually exclusive. That is, in heterozygous strains the putative homomeric *eth-1<sup>-</sup>* molecules should be inactivated at 39° (JACOBSON *et al.* 1977), thus losing their associated ethionine-resistance character. Moreover, in the case of a codominant phenotype, AdoMet synthetase activity levels should be intermediate between those present in the *eth-1<sup>-</sup>* mutant and the wild-type strain at the permissive temperature. Based on these observations, and the current model of ethionine resistance, we propose that *eth-1<sup>-</sup>* confers dominant ethionine resistance by a negative effect (HERSKOWITZ 1987). A tentative explanation for this negative effect is an *in vivo* interaction of the *eth-1<sup>+</sup>* and *eth-1<sup>-</sup>* gene products [e.g., the *in vivo* formation of heteromeric (*eth-1<sup>+</sup>/eth-1<sup>-</sup>*) AdoMet synthetase molecules with diminished enzymatic activity]. Measurements of AdoMet synthetase activity levels in *eth-1<sup>+</sup>/eth-1<sup>-</sup>* heterozygous strains additionally support the existence of this dominant negative effect. The occurrence of a similar phenomenon could also explain why some ethionine-resistant putative point mutant alleles

in the *S. cerevisiae* SAM2 (*eth-2*) are dominant (CHEREST *et al.* 1978), while gene-disrupted (null) alleles of this locus are recessive (THOMAS *et al.* 1988). Partial dominance has also been documented for a particular mutant allele of AdoMet synthetase in *S. cerevisiae* (MERTZ and SPENCE 1972). However, in this case it was concluded that partial dominance was the consequence of a gene dosage effect.

There are not differences in the cellular AdoMet levels between wild-type and *eth-1'* strains growing in minimal medium (see Figure 4; ROBERTS and SELKER 1995; MAUTINO *et al.* 1996a). A marked increase in the AdoMet levels was observed when the wild-type strain was challenged by exogenously added L-methionine. However, neither the *eth-1'* mutant nor the *eth-1'+/(eth-1')*<sub>n</sub> or partial diploid strains were able to increase their cellular AdoMet levels when media were supplemented with L-methionine. This effect agrees with previous observations (JACOBSON *et al.* 1977) indicating that the wild-type strain produces 20-fold higher amounts of the toxic compound S-adenosylethionine (ALIX 1982) than the *eth-1'* mutant. Thus, although the kinetic properties of the wild type and mutant enzymes are similar (JACOBSON *et al.* 1977; J. L. BARRA and A. L. ROSA, unpublished results), there is a marked deficient response *in vivo* of strains carrying the *eth-1'* allele to overproduce either AdoMet or S-adenosylethionine when the medium is supplemented with L-methionine or L-ethionine, respectively. According to these observations, we favor the model in which reduced production of the toxic S-adenosylethionine, due to reduced AdoMet synthetase activity, as well as dilution of ethionine due to accumulation of methionine, are the combined basis of ethionine resistance in strains carrying *eth-1'*.

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