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A Novel Methyltransferase Catalyzes the Methyl Esterification of *trans*-Aconitate in *Escherichia coli**

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We have identified a new type of *S*-adenosyl-L-methionine-dependent methyltransferase in the cytosol of *Escherichia coli* that is expressed in early stationary phase under the control of the RpoS σ factor. This enzyme catalyzes the monomethyl esterification of *trans*-aconitate at high affinity (K_m = 0.32 mM) and *cis*-aconitate, isocitrate, and citrate at lower velocities and affinities. We have purified the enzyme to homogeneity by gel-filtration, anion-exchange, and hydrophobic chromatography. The N-terminal amino acid sequence was found to match that expected for the *o252* open reading frame at 34.57 min on the *E. coli* genomic sequence whose deduced amino acid sequence contains the signature sequence motifs of the major class of *S*-adenosyl-L-methionine-dependent methyltransferases. Overexpression of the *o252* gene resulted in an overexpression of the methyltransferase activity, and we have now designated it *tam* for *trans*-aconitate methyltransferase. We have generated a knock-out strain of *E. coli* lacking this activity, and we find that its growth and stationary phase survival are similar to that of the parent strain. We demonstrate the endogenous formation of *trans*-aconitate methyl ester in extracts of wild type but not *tam*$^-$ mutant cells indicating that *trans*-aconitate is present in *E. coli.* Since *trans*-aconitate does not appear to be a metabolic intermediate in these cells but forms spontaneously from the key citric acid cycle intermediate *cis*-aconitate, we suggest that its methylation may limit its potential interference in normal metabolic pathways. We have detected *trans*-aconitate methyltransferase activity in extracts of the yeast *Saccharomyces cerevisiae*, whereas no activity has been found in extracts of *Caenorhabditis elegans* or mouse brain.

Our laboratory has been interested in the protein L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77), an enzyme that catalyzes the methyl esterification of spontaneously altered residues in a pathway that can lead to the conversion of isomerized aspartyl residues to normal aspartyl residues in a net repair reaction (1). We have postulated that the physiological role of this methyltransferase is to preserve the integrity of the polypeptide chain in the face of age-dependent non-enzymatic reactions that lead to alterations in its configuration (2, 3). In the bacterium *Escherichia coli*, this enzyme is required for optimal survival of stationary phase cells against environmental stresses (4, 5), presumably functioning to maintain proteins in active configurations under conditions where protein synthesis to replace damaged proteins is limited.

In the course of our studies of the protein L-isoaspartate methyltransferase encoded by the *pcm* gene in *E. coli*, we found an activity in cytosolic extracts that appeared to catalyze methyl ester formation but was not dependent upon the *pcm* gene product. We have now traced this activity to that of a previously undescribed small molecule methyltransferase that is active on *trans*-aconitate, an apparently non-enzymatically formed derivative of the citric acid cycle intermediate *cis*-aconitate. Since both types of methyltransferase activities are directed to substrates that can be formed by spontaneous age-related processes, we were interested in characterizing the *trans*-aconitate methyltransferase. We found that the expression of this activity is dependent on the stationary phase specific σ factor RpoS. We have purified and characterized this enzyme, identified its gene, and characterized a knock-out strain lacking this activity. We were able to show that *trans*-aconitate is an endogenous substrate for the enzyme and that the product is its monomethyl ester.

EXPERIMENTAL PROCEDURES

Preparation of E. coli Cytosol for Methyltransferase Assay

E. coli strains and plasmids used in this study are described in Table I. For analytical studies, *E. coli* cells were grown to stationary phase (20 h) in 5 ml of Luria-Bertani (LB) broth or M9 medium containing D-glucose supplemented with thiamine (18 μg/ml) and leucine (40 μg/ml) (Ref. 10, section A.3). When appropriate, 100 μg/ml ampicillin, 50 μg/ml kanamycin, 20 μg/ml chloramphenicol, or 20 μg/ml tetracycline were added. Cells were collected by centrifugation at 5,000 × *g* at 4 °C for 10 min. The cell pellet was resuspended in 0.5 ml of buffer containing 5 mM disodium EDTA, 10% glycerol, 25 μM phenylmethylsulfonyl fluoride in 5 mM potassium phosphate buffer at a final pH of 7.0. Cells were lysed by sonication in an ice bath using the microtip of a Branson model W350 instrument at an output control setting of 4 for three sets of 5 pulses separated by 30-s cooling pauses. The extract was centrifuged at 12,000 × *g* at 4 °C for 10 min, and the supernatant was used as a cytosolic fraction. The protein concentration was determined by the method of Lowry *et al.* (11) after 10% trichloroacetic acid precipitation of the samples.

trans-Aconitate Methyltransferase Assay

Enzyme activity was measured using a modification of the protein carboxyl methyltransferase assay (7). Unless otherwise stated, the assay mixture consisted of 2 μl of 20 mM *trans*-aconitic acid (Sigma) in 0.4 M sodium HEPES, pH 7.5, 5–15 μl of the enzyme preparation, 5 μl of 80 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]AdoMet[1]; specific radioactivity about 110 cpm/pmol, 53 mCi/mmol, Amersham Pharmacia Biotech), 10 μl 0.4 M sodium HEPES, pH 7.5, and water to a total volume of 40 μl. Samples were incubated at 37 °C for 5–30 min, and the

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[1] The abbreviations used are: [^{14}C]AdoMet, *S*-adenosyl-L-[*methyl*-^{14}C]methionine; [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; AdoMet, *S*-adenosyl-L-methionine; PCR, polymerase chain reaction; kb, kilobase pair; MES, 2-(*N*-morpholino)ethanesulfonate; HPLC, high pressure liquid chromatography.

TABLE I
Strains and plasmids used

Strain/plasmid	Genotype/description	Ref.
Strains		
MC1000	λ⁻ e14⁻ *araD*139 Δ(*araA-leu*)7697 *galE*15 *galK*16 Δ(*codB-lac*)3 *rpsL150 mcrB1 relA spoT1*	6
CL1010	MC1000 Δ*pcm*(Δ*Mlu*I-*Cla*I)::Km^r *rpoS396*	7
JV1012	MC1000 *rpoS13*::Tn*10*	8
HC1011	MC1000 Δ*pcm*(Δ*Mlu*I-*Cla*I)::Km^r *rpoS396 attB*::*rpoS*$^+$	4
HC1014	MC1000 *tam* (*o252*)::*cat*	This study
JC7623	AB1157 *recC22 recB21 sbcB15 sbcC201*	9
Plasmids		
pHC107	Overexpression vector for *tam* (*o252*), containing *tam* (*o252*) between the *Nde*I and *BamH*I site in the multicloning site of pT7–7; chloramphenicol-resistant	This study
pHC108	pUC19 containing *tam* (*o252*) and its flanking region (3.1 kb) in the *BamH*I site; ampicillin-resistant	This study
pHC109	pHC108 containing a chloramphenicol resistance gene at the *Age*I site within *tam* (*o252*); ampicillin-resistant, chloramphenicol-resistant	This study

reactions were quenched by adding 40 μl of freshly prepared 2 M NaOH. Sixty μl of this mixture was immediately spotted onto an accordion-pleated 1.5 × 8-cm piece of thick filter paper (Bio-Rad 165-0962), and the paper was placed in the neck of a 20-ml vial containing 5 ml of Safety-Solve scintillation fluid (Research Products International Corp.), capped, and incubated for 2 h at room temperature. Radioactivity was determined by liquid scintillation counting in a Beckman LS6500 counter after removal of the filter paper.

In initial experiments, citrate was used as a methyl-accepting substrate. Here, the reaction contained 20 μl of 0.2 M sodium citrate, pH 6.0 (Fisher, ACS-certified), 15 μl of enzyme preparation, and 5 μl of 80 μM [^{14}C]AdoMet as described above. Samples were incubated at 37 °C for 20 min and analyzed as described above. Other substrates tested included *cis*-aconitic acid, DL-isocitrate (*threo*-D_sL_s-isocitrate; trisodium salt, 93–98%), (2*R*,3*S*)-isocitrate (threo-D_s(+)isocitrate; monopotassium salt, approximately 99%), fumaric acid, tricarballylic acid, and malic acid, all obtained from Sigma, and succinic acid and oxalacetic acid obtained from Fisher.

Purification of the E. coli trans-Aconitate Methyltransferase

Preparation of E. coli Cytosol—Four flasks each containing 2 liters of LB media were each inoculated with 2 ml of an overnight culture of *E. coli* strain MC1000 and were grown to stationary phase at 37 °C for 20 h with shaking. Cells were collected by centrifugation at 5,000 × *g* at 23 °C for 15 min. The cell pellet (29.1 g) was washed three times with 400 ml of buffer A (50 mM Tris-HCl, 5 mM disodium EDTA, 300 mM NaCl, pH 8.0) and then resuspended in 50 ml of buffer B (50 mM Tris-HCl, 5 mM disodium EDTA, 25 μM phenylmethylsulfonyl fluoride, pH 8.0) at 4 °C. Cells were disrupted by passing them twice through a French press cell (SLM Aminco) at 20,000 pounds/square inch. The cytosolic fraction was obtained by centrifugation at 23,000 × *g* for 30 min at 4 °C. This supernatant was then further centrifuged at 100,000 × *g* for 60 min at 4 °C to remove any residual membrane material.

Ammonium Sulfate Precipitation—An equal volume of 90% saturated ammonium sulfate (4 °C) was gradually added to the cytosol with stirring, followed by additional stirring at 4 °C for 30 min. The mixture was then centrifuged at 23,000 × *g* at 4 °C for 30 min. The protein pellet was redissolved in 20 ml of buffer B to a protein concentration of approximately 28 mg/ml.

Superdex S-200 Chromatography—An aliquot (5 ml) of the redissolved ammonium sulfate pellet was loaded onto a Superdex-200 (Amersham Pharmacia Biotech) gel filtration column (1.5 cm in diameter × 58 cm in height, 102-ml bed volume), pre-equilibrated at 4 °C with buffer C (50 mM Tris-HCl, 5 mM disodium EDTA, pH 8.0). The column was eluted at 18 ml/h, and fractions of 1.2-ml were collected. The activity was eluted at fractions 49–58, and these fractions were pooled and stored at 4 °C. The material from 4 column runs was combined to use in the following step.

DEAE-cellulose Anion-exchange Chromatography—The active pool from the Superdex-200 column (total volume of 48 ml) was loaded onto a DE52 anion-exchange column (Whatman; 2 cm in diameter × 12.7 cm in height, 40-ml bed volume) pre-equilibrated at 4 °C with buffer C. After sample loading, the column was washed with 3 column volumes of the equilibration buffer. The enzyme was then eluted with a linear sodium chloride gradient (0–0.8 M in the equilibration buffer, total of 250 ml) followed by a 5 column volumes of a high salt wash (1.0 M sodium chloride in the equilibration buffer) at 4 °C. The flow rate was 19.5 ml/h, and 2.8-ml fractions were collected. Activity was found to elute between sodium chloride concentrations of 300 and 400 mM between fractions 102 and 112. These active fractions were pooled and stored at 4 °C.

Hydrophobic Interaction Chromatography—-Potassium monobasic phosphate was dissolved in concentrated buffer C, the pH was adjusted to 8.1 with KOH, and the solution was diluted to give a final concentration of 1 M phosphate in buffer C. This solution was added to the active pool from the DE52 anion-exchange column to bring the potassium phosphate concentration to 0.6 M. This material was then applied to a phenyl-Sepharose column (Amersham Pharmacia Biotech; 1 cm diameter × 10 cm height, 7.8-ml bed volume) pre-equilibrated with 0.6 M potassium phosphate in buffer C. The flow rate was 18 ml/h, and 2.5-ml fractions were collected. After loading the sample, the column was washed with 5 column volumes of the equilibration buffer and then eluted with a linear gradient of potassium phosphate (0.6–0 M in the equilibration buffer). Enzyme activity was found to elute between 0 and 0.1 M potassium phosphate concentrations.

Amino Acid Sequencing

N-terminal amino acid sequence analysis was performed by Dr. Audree Fowler at the UCLA Protein Microsequencing Facility with a Porton 2090E gas-phase sequencer with on-line HPLC detection. The active pool from the DE52 column (fractions 103–110, total volume of 19.6 ml) was added to an equal volume of 25% (w/w) trichloroacetic acid, mixed well by vortexing, and incubated at 4 °C while rotating slowly overnight. The mixture was centrifuged at 15,000 × *g* for 30 min at 4 °C, and the protein pellet was then dissolved in 100 μl of 1× sample buffer for SDS gel electrophoresis (Ref. 10, section 18.47–18.55). After polyacrylamide gel electrophoresis, the separated polypeptides were electroblotted onto a polyvinylidene difluoride membrane in 25 mM Tris base, 10 mM glycine, 0.5 mM dithiothreitol, in 10% methanol, 90% water (v/v) at pH 9. This membrane was then stained with Coomassie Brilliant Blue R250, and the polypeptide band at 29 kDa corresponding to the *trans*-aconitate methyltransferase was excised and subjected to automated Edman sequencing.

Cloning and Disruption of the trans-Aconitate Methyltransferase Gene in E. coli

A 3.1-kb DNA fragment containing the entire *trans*-aconitate methyltransferase gene (*o252*) and flanking regions was amplified by polymerase chain reaction (PCR) from template DNA in MC1000 cells (12) using *Taq* polymerase (Promega) at 2.5 mM magnesium chloride and an annealing temperature of 60 °C. The primers were KO-5 (5′-TATGACTACGAAGCGGATCCTAATGGCA, corresponding to bases −1362 to −1335 from the translation start site of *o252* with the underlined nucleotides changed to create a *Bam*HI site) and KO-3 (5′-GCGTATTGAGAATGGGATCCTAATCACG corresponding to the reverse complement of bases 1714–1741 with the underlined nucleotides changed to prevent hairpin formation and to create another *Bam*HI site). This fragment was purified by gel electrophoresis using Geneclean II (Bio 101), cut with *Bam*HI, and then ligated into the *Bam*HI site within the multicloning site of the pUC19 vector to generate the plasmid pHC108 (Table I). This plasmid, pHC108, was then used to construct a null mutation in the *o252* gene by blunt-end ligation of a 1.5-kb chloramphenicol resistance (*Cm*^{r}) cassette (13) at the unique *Age*I site within

the gene to create pHC109. The chromosomal *o252* gene was then replaced with the Cm^r disrupted gene in pHC109 by homologous recombination in strain JC7623, which does not support plasmid replication (14). pHC109 was transformed into $CaCl_2$-competent JC7623 cells, and Cm^r colonies were selected on a plate containing 20 μg/ml chloramphenicol. The loss of the vector in the recipient strain was confirmed by screening for ampicillin sensitivity. This disrupted *o252* gene was subsequently transduced into the MC1000 background by P1 transduction (15). The disruption of the *o252* gene was confirmed by the PCR amplification of a 3.96-kb product using a primer (5′-GATTCAGTACGCCAAATGTG) corresponding to genomic sequence upstream of the KO-5 primer described above and a primer OE-3 (described below) corresponding to a sequence downstream of the stop codon for *o252* gene. We also confirmed the disruption by the detection of the expected 3.44-kb *Eco*RV, 6.35-kb *Hin*dIII, and 0.60- and 1.35-kb *Eco*RI fragments using a random-primed probe corresponding to the Cm^r gene in Southern blot hybridization (data not shown).

Overexpression of trans-Aconitate Methyltransferase

The *trans*-aconitate methyltransferase gene was PCR-amplified from colonies of *E. coli* strain MC1000 as described above using the primers OE-5 (5′- CGGGAGTAAACATATGTCTGACTGG; corresponding to bases −13 to +12 from the translation start site with the underlined bases changed to create a 5′ *Nde*I site) and OE-3 (5′-ACCACTGGATCCCATATGCAACGC; corresponding to the reverse complement of bases +848 to +871 with the underlined based changed to create a *Bam*HI site and to prevent hairpin formation; the stop codon is located at bases +757 to +759). The 884-base pair PCR fragment was cleaved with *Nde*I and *Bam*HI and the large fragment purified as described above. This fragment was then cloned into the corresponding sites in the multicloning site of the pT7-7 vector (16) to generate pHC107. DNA sequence analysis using both oligonucleotides described above as primers showed that no mutations were introduced during the cloning procedure. The plasmid was then transferred into BL21(DE3) cells (Invitrogen) for expression. An aliquot of an overnight culture of the transformed cells (20 μl) was diluted into 20 ml of fresh LB medium, incubated with shaking at 37 °C, and cultured to an $A_{600\ nm}$ of 1.0. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to the culture, and the cells were incubated with shaking for another 2 h. Cytosolic fractions were then prepared and assayed for *trans*-aconitate methyltransferase activity as described above. The enzymatic activity of this preparation was 100–150 nmol/min/ml (specific activity 12–22 nmol/min/mg protein), and it was used as a concentrated source of enzyme in the enzyme kinetic assays and in the experiments to characterize the methyl acceptors. A control extract was prepared where the same strain was grown in the absence of plasmid. The specific activity of this extract was 0.020 nmol/min/mg protein, indicating that the degree of overexpression was about 630-fold.

High Performance Liquid Chromatography

Analysis of the substrates and products was carried out with a Waters HPLC system. An Alltech Partisil SAX anion-exchange column (250 mm length × 4.6 mm inner diameter; 10-μm resin bead diameter) was used for initial separations. The column was equilibrated with 50 mM potassium phosphate, pH 4.5, and eluted at 1 ml/min at room temperature. After each run, the column was regenerated with 500 mM potassium phosphate, pH 4.5, prior to re-equilibration with the starting buffer. For reverse-phase separations, an Alltech Econosphere C18 column (250 mm length × 4.6 mm inner diameter; 5-μm spherical resin bead diameter) was used in a two-solvent system. Solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid, 99.5% acetonitrile, and 0.4% water. The column was eluted at room temperature at a flow rate of 1 ml/min for 20 min in solvent A, followed by a linear gradient over 20 min from 100% solvent A to 100% solvent B, followed by 10 min of 100% solvent B. The column was re-equilibrated with 100% buffer A. In each case, 1-min fractions were collected.

The UV profile was monitored by Waters model 441 absorbance detector at 214 nm, and chromatographs were recorded with the PowerChrom system from ADInstruments. Radiolabeled products were detected by counting 100 μl of each in 5 ml of scintillation fluid.

Thin Layer Chromatography

Thin layer chromatography was also used to analyze the products of the methylation reaction after the method of Otten and Mehltretter (17). Polyester-based 60-Å silica gel-coated plates were used (Whatman PE SIL G, 250 μm layer). The solvent is benzene/methanol/acetic acid (45:16:4, v/v/v) and is prepared fresh daily and used to pre-equilibrate the chamber for 2 h before each run at room temperature. Samples (1–5 μl) were spotted in each lane in 1-μl aliquots, and each spot was allowed to air-dry before further application of sample or chromatography. The solvent front was allowed to migrate approximately 18 cm on the plate, then its position was marked and the plate was air-dried in a hood, followed by baking at 105 °C in a vacuum oven for 1 h. Carboxylic acid-containing compounds were detected as yellow spots on a blue background after spraying the plate with 0.04% bromcresol green dissolved in 95% ethanol, pH 8.0.

Synthesis and Characterization of trans-Aconitate Methyl Esters

Chemical Methylation—trans-Aconitic acid (4.8 mg) was incubated with 60 μl of methanol and 1 μl of concentrated (12 M) HCl for 16 h at room temperature. The sample was vacuum-dried in Speedvac apparatus and dissolved in 138 μl of H_2O to give a final concentration of *trans*-aconitate derivatives of 0.2 M. An aliquot of the sample (10 μl) was chromatographed on the SAX HPLC anion-exchange column as described above. The peaks were collected, and an aliquot of each peak was re-chromatographed on the C18 reverse-phase column as described.

Enzymatic Methylation—The reaction mixture contains 5 μl of 0.02 M *trans*-aconitate in 0.4 M sodium HEPES, pH 7.5, 10 μl of 0.4 M sodium HEPES, pH 7.5, 1 μl of a preparation of cytosol from BL21 cells overexpressing the *trans*-aconitate methyltransferase (7.72 μg protein, 12.6 pmol/min/μg protein), 17.5 μl of 8.16 mM *S*-adenosyl-L-methionine (AdoMet) in water, and 2.5 μl [^{14}C]AdoMet to a total volume of 42.5 μl made up by H_2O. The reaction was carried out at 37 °C for 24 h. Forty μl of the sample was purified as described above. The methylated *trans*-aconitate was followed by radioactivity.

Mass Spectroscopy—Mass spectroscopy was performed by Dr. Kym Faull at the UCLA Mass Spectrometry Facility. HPLC fractions from the C18 reverse-phase column were collected and dried in a SpeedVac. The dried HPLC samples were redissolved in 20 μl of water/acetonitrile/triethylamine (50:50:0.1, v/v/v), and aliquots were injected into an electrospray ionization source attached to a quadrupole mass spectrometer (Perkin-Elmer, Thornhill, Canada, API III; −3.5 Kv ion spray voltage, spray nebulization with hydrocarbon-depleted air ("zero" grade air, 40 pounds/square inch, 0.6 liters/min; Zero Air Generator, Peak Scientific, Chicago, IL), curtain gas (0.6 liters/min) from the vapors of liquid nitrogen; mass resolution set so the isotopes of the polypropylene glycol/NH_4^+ singly charged ion at m/z 906 were resolved with 40% valley) scanning from m/z 120–250 in the negative ion mode. Spectra were collected (step size 0.3 Da, dwell time 20 ms/step, 6.7 s/scan, orifice at −60 V), and the resulting spectra were summed then background subtracted with software supplied with the instrument.

RESULTS

Identification of a Novel Methyltransferase Activity in E. coli—In the course of studies quantitating protein L-isoaspartate *O*-methyltransferase activity in various strains, we measured methyl esterification in cytosolic extracts in the presence and absence of added L-isoaspartyl-containing methyl-accepting peptide KASA(isoD)LAKY. [^{14}C]Methyl esters formed when extracts are incubated with [^{14}C]AdoMet in a sodium citrate buffer were hydrolyzed in base to generate volatile [^{14}C]methanol that can be separated from unreacted [^{14}C]AdoMet and other non-volatile species and quantitated. Although we found that the L-isoaspartyl peptide-dependent activity correlated well with the presence of the *pcm* gene for the isoaspartyl methyltransferase, we were surprised to find that the "endogenous" activity in the absence of added peptide was much higher in extracts from strains with an intact *rpoS* gene than in extracts from strains mutated in this gene (Table II). The *rpoS* gene is located about a kilobase downstream from the *pcm* gene and codes for a specific σ factor that is required for the expression of a number of genes in stationary phase cells (for a review, see Ref. 18). This result suggested that either *rpoS* or one of the genes it regulates might also have a methyl esterification activity.

We then began to investigate the nature of the RpoS-dependent endogenous methyl esterification activity using extracts of the strain HC1011 that lacks the *pcm* gene so there would be no contribution of the L-isoaspartyl methyltransferase to the

TABLE II
Endogenous and isoaspartyl peptide-dependent methyl esterification activity

E. coli cytosolic fractions were made as described under "Experimental Procedures." Endogenous activity was measured in the citrate buffer as described. For the endogenous + peptide-dependent activity, 100 μM KASA-(isoD)-LAKY was included in the reaction mixture. Peptide-dependent activity was calculated as the difference between the endogenous and peptide-containing activities. Results are expressed with the standard deviations obtained from three parallel experiments.

Cytosols	Relevant genotype	Endogenous methyltransferase activity	Endogenous + peptide-dependent methyltransferase activity	Peptide-dependent methyltransferase activity
		pmol/min/mg[a]	*pmol/min/mg*	*pmol/min/mg*[b]
MC1000	pcm^+ $rpoS^+$	4.1 ± 1.0	7.0 ± 2.0	3.3 ± 0.2
JV1012	pcm^+ $rpoS^-$	0.2 ± 0.2	4.4 ± 0.3	3.6 ± 0.5
CL1010	pcm^- $rpoS^-$	0.5 ± 0.3	0.8 ± 0.1	0.1 ± 0.1
HC1011	pcm^- $rpoS^+$	3.2 ± 0.4	3.6 ± 0.8	0.05 ± 0.04

[a] Activity was calculated as picomoles of methyl groups transferred per min per mg of cytosolic protein.
[b] Peptide-dependent activities were calculated for each pair of samples and were then averaged.

methylation activity. We found that about 78% of the total activity was localized in the cytosolic fraction, and 22% of the activity was found in the membrane pellet fraction. The combination of membrane and cytosolic fractions did not increase the activity over that of cytosolic fractions alone (data not shown). The production of the endogenous methylated product from cytosolic extracts was found to be linear with time and with the amount of cytosolic extract used (data not shown). The activity appeared to be a protein because treatment of the extract with proteinase K resulted in a complete loss of activity and because heating the extract greatly reduced the activity. Finally, we showed that the activity could be inhibited by *S*-adenosyl-L-homocysteine, a product of the reaction and an effective inhibitor of most AdoMet-dependent methyltransferases (19). We found 72% inhibition with 0.38 mM *S*-adenosyl-L-homocysteine and essentially complete inhibition at 0.94 mM (data not shown).

Initial Characterization of Substrates and Products—The formation of volatile radioactivity by the RpoS-dependent methyltransferase in the assay described above was dependent upon hydrolysis of the reaction products, suggesting that a methyl ester linkage was formed. No radioactivity was detected when water replaced the 2 M NaOH quenching solution. There was no increase in activity when 7 M NaOH was used as a quench; 74% of the activity was detected with 1 M NaOH and only 49% activity found with 0.2 M NaOH. Only 2, 3, and 8% of the maximum activity was found when 1, 2, and 7 M HCl was used as a quench (data not shown).

No loss of activity was seen after dialysis of the extract, initially suggesting that a macromolecule may be the methyl acceptor. However, we found that the endogenous activity was dependent upon the presence of the citrate buffer in the assay mixture. When the citrate was replaced by Tris, phosphate, acetate, or MES buffers of similar pH, no activity was seen (data not shown). The role of citrate in the reaction did not appear to be that of a metal chelator because there was no activity in a buffer containing 5 mM sodium EDTA. These results suggested that citrate could itself be the substrate for the reaction where one or more of its three carboxyl groups could be methyl-esterified. To ask whether compounds structurally related to citrate would be better or worse substrates, we assayed extracts with a variety of tricarboxylates and dicarboxylates. While we found that succinate, fumarate, malate, oxalacetate, and tricarballylate gave no activity when substituted for citrate, even better activity was found with *cis*-aconitate, DL-isocitrate, (2*R*,3*S*)-isocitrate, and *trans*-aconitate. From the concentration dependence of the activity, we estimated an apparent K_m value for *trans*-aconitate of about 0.3 mM, a value at least 16-fold lower than that measured for citrate (7.1 mM), *cis*-aconitate (33 mM), (2*R*,3*S*)-isocitrate (9.1 mM), or DL-isocitrate (5 mM). We found that the reaction with citrate had a maximal velocity of only 7.5% that of *trans*-aconitate, whereas the reaction with *cis*-aconitate, (2*R*,3*S*)-isocitrate, DL-isocitrate had comparable maximal velocities (108, 49, and 78%) (data not shown). By using *trans*-aconitate as a substrate, we found that the apparent K_m for [^{14}C]AdoMet is about 5 μM, whereas the pH dependence of the enzyme activity demonstrated a broad maximum from pH 6 to 8 decreasing at higher or lower pH values with half-maximal activities at about pH 5.5 and pH 8.5 (data not shown).

trans-Aconitate-dependent Methyltransferase Activity Rises as Cells Enter Stationary Phase and Then Decreases—Because the methyltransferase activity required the presence of the RpoS stationary phase σ factor (Table II) whose concentration increases near the end of exponential growth phase (20), we assayed this enzyme during various stages of cell growth using *trans*-aconitate as a substrate. We found that the specific activity of the methyltransferase increases dramatically in late exponential phase and peaks at the transition into stationary phase (Fig. 1*A*), paralleling the known accumulation of RpoS (21). The specific activity of the methyltransferase then decreases dramatically after 24 h in stationary phase (Fig. 1*B*), which also correlates with the decrease in the concentration of RpoS (21). There is little or no detectable activity after 72 h in stationary phase. We also observed that the specific activity of *trans*-aconitate-dependent methyltransferase is 3–4-fold higher in the cytosolic fractions obtained from cells cultured in rich LB media than from cell cultured in minimal (M9) media with glucose (data not shown). This difference may also reflect the higher level of RpoS in rich media compared with minimal media (20, 21).

Purification of trans-Aconitate Methyltransferase—From the results presented above, it is possible that either RpoS has a methyltransferase activity or that it is essential for the transcription of the methyltransferase gene. To clarify this issue, the methyltransferase was purified by ammonium sulfate precipitation, gel filtration chromatography on Superdex 200, anion-exchange chromatography on DE52 resin, and hydrophobic chromatography on phenyl-Sepharose resin as described under "Experimental Procedures." A single peak of activity was found in each chromatographic step suggesting that isozymes are not present (Fig. 2). Characterization of the polypeptide composition of each fraction by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) revealed a polypeptide of about 29 kDa that is progressively enriched during the purification (Fig. 3). In the final step of hydrophobic chromatography, only a single band at 29 kDa was observed whose concentration corresponded directly to the methyltransferase activity (data not shown). The overall purification of 594-fold (Table III) suggested that the enzyme made up approximately 0.2% of the total cytosolic protein in early stationary phase. Additional native gel filtration experiments showed that the methyltrans-

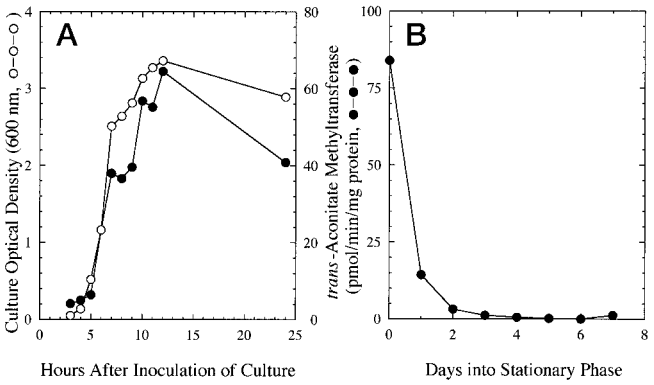

FIG. 1. **Expression of *trans*-aconitate methyltransferase during growth of *E. coli*.** An overnight culture of strain MC1000 was diluted 1:100 into 50 ml of fresh LB media and incubated with shaking at 37 °C. At various time points, the optical density of the culture was measured at 600 nm (*A*, *open circles*), and 1-ml aliquots of the culture were centrifuged at 12,000 × *g* for 1 min. The cytosolic fraction prepared from these cells was assayed for *trans*-aconitate methyltransferase activity as described under "Experimental Procedures" (*A and B*, *closed circles*).

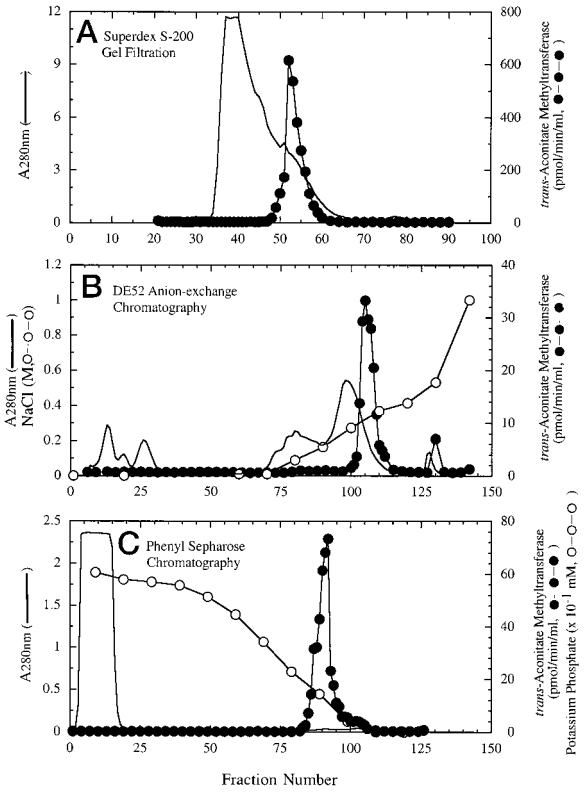

FIG. 2. **Chromatographic purification of the *E. coli trans*-aconitate methyltransferase from strain MC1000.** An ammonium sulfate-precipitated fraction of *E. coli* MC1000 cytosol was prepared as described under "Experimental Procedures." Column effluents from the three chromatographic procedures were analyzed for protein concentration by absorbance at 280 nm (*straight lines*) and *trans*-aconitate methyltransferase activity (*closed circles*). *A,* Superdex S-200 gel filtration chromatography. *B,* DEAE-cellulose chromatography. A DE52 anion-exchange column was eluted with a linear sodium chloride gradient (0–0.4 M) (*open circles*). *C*, phenyl-Sepharose hydrophobic chromatography. The column was eluted with a reverse linear potassium phosphate gradient (0.6–0 M) (*open circles*).

ferase eluted between the molecular weight markers ovalbumin (43 kDa) and the human L-isoaspartyl methyltransferase (25 kDa) and thus appears to be composed of a single polypeptide chain.

Identification of the E. coli Gene Encoding the trans-Aconitate Methyltransferase—Microsequencing of the purified 29-kDa polypeptide demonstrated an N-terminal amino acid sequence of SD*X*NKQLYLQFMAEMS, where the assignment of residues 1, 2, and 4 was more certain than residues 5–16, and no assignment could be made for residue 3. This sequence, including an assumed N-terminal methionine residue, was used to search against the GenBank™ protein data base using ungapped BLAST (22). The best match corresponded to the N terminus of the deduced product of the *o252* open reading frame in the newly sequenced *E. coli* genome (23) where identities were found at 11 of the 17 positions (Fig. 4). This previously uncharacterized gene, at 34.57 min on the chromosome,[2] potentially encodes a polypeptide of 28,876.4 Da (lacking the initiator methionine which would be expected to be removed (25)) that corresponds to the 29-kDa polypeptide purified. The deduced sequence also contains the four well conserved motifs (I, post-I, II, and III) in a variety of methyltransferases (Ref. 26; Fig. 4).

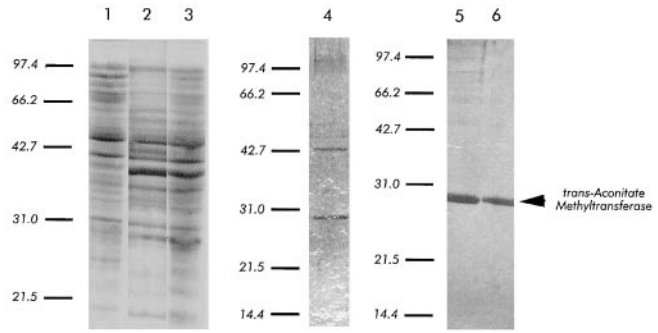

FIG. 3. **Analysis of the polypeptide composition of fractions from the purification of the *trans*-aconitate methyltransferase by SDS-gel electrophoresis.** *Lane 1*, 0.5 μl of French press supernatant; *lane 2*, 0.5 μl of the resuspended pellet from ammonium sulfate precipitation; *lane 3*, 5 μl of Superdex S-200-combined fractions; *lane 4,* 25 μl of DE52-combined fractions; and *lanes 5* and *6*, 500 μl of phenyl-Sepharose fractions 91 and 92. The proteins in *lane 5* and *6* were precipitated with 12.5% trichloroacetic acid prior to loading as described under "Experimental Procedures." *Lanes 1–3* were Coomassie-stained, and *lanes 4–6* were silver-stained. Bio-Rad low range molecular mass standards included phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa), and their migration positions are indicated by the *lines* at the *side* of the lane. The *arrow* on the *right* marks the position of *trans*-aconitate methyltransferase. A 12% acrylamide gel was used (Ref. 10, section 18.47–18.55).

Overexpression of the trans-Aconitate Methyltransferase and Methyl-accepting Substrate Characterization—The *o252* gene was PCR-amplified and cloned into a pT7-7 expression vector as described under "Experimental Procedures." We found that the specific activity of the *trans*-aconitate methyltransferase was increased 630-fold in extracts of BL21(DE3) cells compared with extracts of this strain lacking the plasmid. This result suggests that the *o252* gene does indeed encode the methyltransferase activity, and we have now named it *tam* for *trans*-aconitate methyltransferase.

The availability of the overexpressed enzyme allowed us to characterize its substrate specificity. Initial velocity measurements at a variety of substrate concentrations confirmed that *trans*-aconitate was the best substrate with a K_m value of 0.32 mM (Table IV). The catalytic efficiencies (V_{max}/K_m) of the enzyme for *cis*-aconitate, (2*R*,3*S*)-isocitrate, and DL-isocitrate were less than 3% that of *trans*-aconitate, whereas the catalytic efficiency for citrate was only about 0.4% that of *trans*-aconi-

[2] *E. coli* Genetic Stock Center, Yale University, New Haven, CT. The Web site address is as follows: http://www.cgsc.biology.yale.edu.

TABLE III

Purification of trans-aconitate methyltransferase from E. coli strain MC1000

For each purification step, an aliquot of the active pool was removed and analyzed in duplicate for protein content and enzymatic activity with *trans*-aconitate, as described under "Experimental Procedures."

Fractionation step	Total volume	Protein concentration	Total protein	Specific activity	Total activity	Yield	Purification
	ml	*mg/ml*	*mg*	*pmol/min/mg*	*pmol/min*	*%*	*-fold*
French press supernatant	58	32	1867	38.1	71,200	100	1.0
45% ammonium sulfate precipitation	20	28	558	104	58,000	81.6	2.8
Superdex S-200	48	2.2	106	140	14,800	20.8	3.7
DE52	22	0.2	5.1	2280	11,600	16.3	60
Phenyl-Sepharose	3	0.0087	0.026	22,600	588	0.8	594

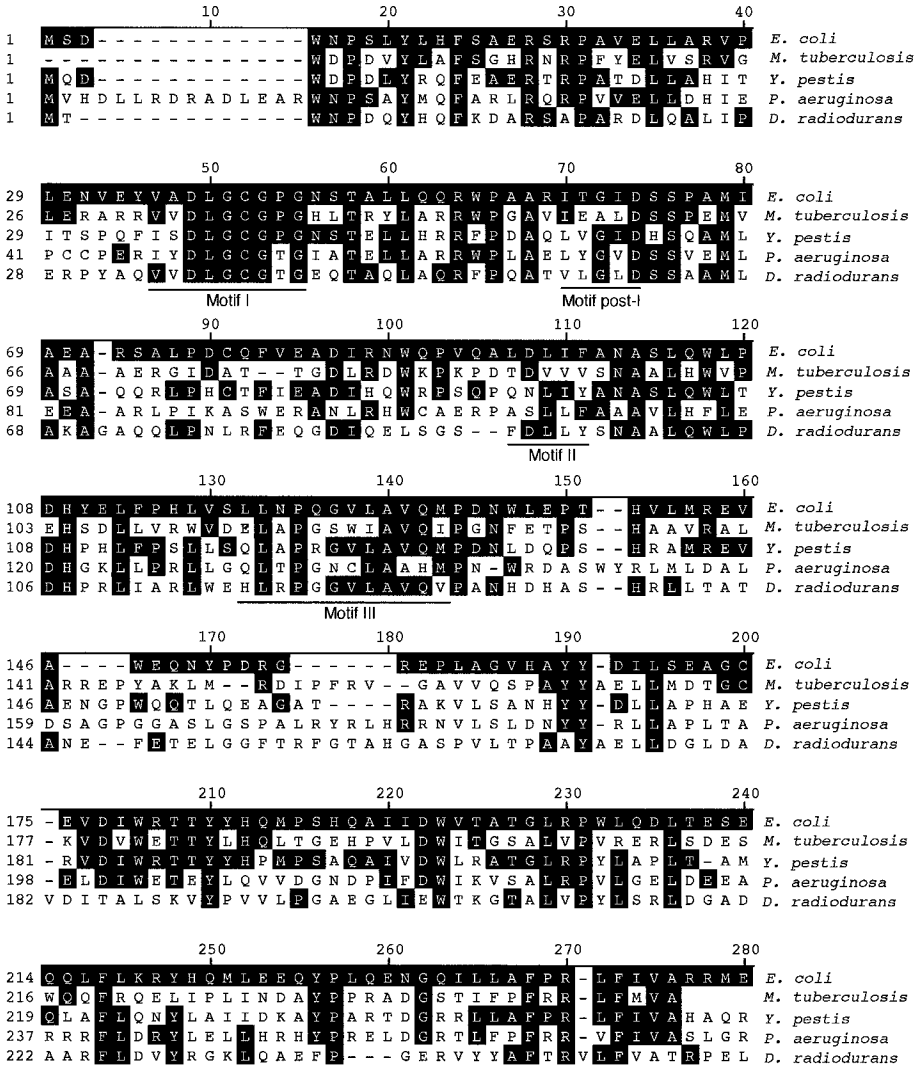

FIG. 4. **Protein sequence identity between the products of the *trans*-aconitate methyltransferase gene of *E. coli*, gene *Rv0294* of *M. tuberculosis*, a gene in contig899 of *Y. pestis*, a gene in contig226 of *P. aeruginosa*, and a gene in gdr_28 of *D. radiodurans*.** The *E. coli* sequence and the four open reading frames were aligned using the Cluster method in the MegAlign program (DNAStar). Residues identical with the *E. coli* sequence are *shaded*. Overall, the *M. tuberculosis* sequence was 35% identical with the *E. coli* sequence, and the comparable figures for the *Y. pestis*, *P. aeruginosa*, and *D. radiodurans* sequences were 51, 34, and 34%, respectively.

tate (Table IV). The values in Table IV obtained for the overexpressed enzyme are generally similar to those found when the cytosol of wild type cells was used as a source of enzyme (see above). Finally, we found that the K_m for AdoMet with *trans*-aconitate was 4.8 μM, a value also similar to that obtained with the non-overexpressed cytosol.

To exclude the possibility that minor contaminants in the commercial preparations of *trans*-aconitate, *cis*-aconitate, (2*R*,3*S*)-isocitrate, or citrate could represent all or part of the methyl-accepting activity observed in the experiments described above, we fractionated each these compounds by HPLC high resolution anion-exchange chromatography (Fig. 5). Individual fractions were then assayed as potential methyl-accepting substrates for the preparation of overexpressed *trans*-aconitate methyltransferase. When *trans*-aconitate was fractionated, we found that the methyl-accepting activity exactly paralleled the elution of *trans*-aconitate (monitored by its UV absorbance), suggesting that this was in fact the methyl acceptor (Fig. 5*A*). On the other hand, when the commercial preparation of *cis*-aconitate was fractionated, a more complex pattern was seen. Here, UV analysis indicated that a small contaminant (approximately 5%) of *trans*-aconitate was pres-

TABLE IV

Substrate specificity of the E. coli trans-aconitate methyltransferase

Methyltransferase activity was measured in 0.1 M HEPES buffer at pH 7.5 as described under "Experimental Procedures," except that 5 μl of 0.36 mM [^{14}C]AdoMet was used. Substrates were dissolved in 0.1 M HEPES at pH 7.5, and the pH was readjusted to 7.5. The enzyme source was a cytosolic extract of overexpressed enzyme. Substrates were used over a concentration range of 1–64 mM, except for *trans*-aconitate where a range of 20 μM to 2 mM was used. Assays were done in triplicate at both 5- and 10-min incubation times. K_m and V_{max} values were calculated by fitting the data to the Michaelis-Menten equation using the DeltaGraph program. The standard deviation of each measurement is indicated.

Substrate	K_m	V_{max}	V_{max}/K_m
	mM	*nmol/min/mg protein*	
trans-Aconitate	0.32 ± 0.06	21.3 ± 2.1	66.6
cis-Aconitate	4.6 ± 1.1[a]	7.1 ± 0.8	1.5
(2*R*,3*S*)-Isocitrate	6.0 ± 1.2	10.6 ± 1.0	1.8
DL-Isocitrate	8.1 ± 3.4	10.3 ± 1.0	1.3
Citrate	6.9 ± 1.1	1.7 ± 0.1	0.2

[a] Since *cis*-aconitate is contaminated with a small amount of *trans*-aconitate (Fig. 5), this K_m value represents a minimal estimate.

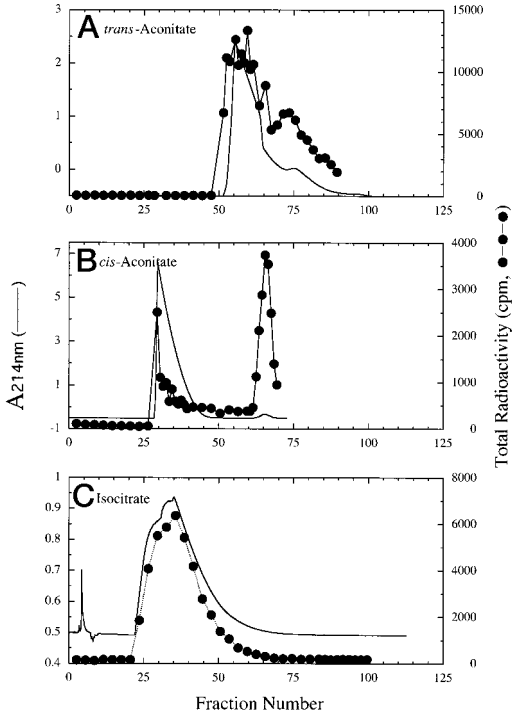

FIG. 5. **Methyl acceptor activity with fractionated *trans*-aconitate, *cis*-aconitate, and (2*R*,3*S*)-isocitrate.** Commercial preparations of *trans*-aconitate, *cis*-aconitate, and (2*R*,3*S*)-isocitrate (10 μl of 0.2 M) were each HPLC-purified on an anion-exchange SAX column as described under "Experimental Procedures," and 1-ml fractions were collected. An aliquot of each fraction (0.1 ml) was assayed with 15 μl of an *E. coli* cell cytosol containing recombinant *trans*-aconitate methyltransferase (36,000 pmol/min/ml; 11,300 pmol/min/mg protein) and 1 μl of 365 μM [^{14}C]AdoMet (53 mCi/mmol). The reaction mixture was incubated at 37 °C for 1 h, followed by addition of 116 μl of 2 N NaOH. An aliquot (100 μl) of the quenched mixture was spotted on filter paper for the vapor phase determination of [^{14}C]methanol as described under "Experimental Procedures." Assays on each fraction were done in duplicate, and the average is shown in *closed circles*. The absorbance at 214 nm is given as the *continuous line*.

ent. This material was a major methyl acceptor, however, as would be expected from the results described above (Fig. 5*B*). Significantly, however, there was still a methyl-accepting peak corresponding to the *cis*-aconitate peak. Thus, *cis*-aconitate appears to also be recognized by the methyltransferase but at even lower affinity than suggested by the data in Table IV.

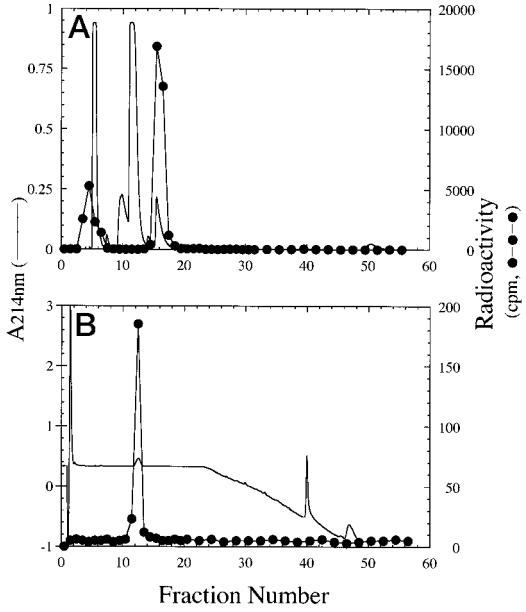

FIG. 6. **A monomethyl ester of *trans*-aconitate is the product of the enzymatic reaction.** *A,* a mixture of the methyl esters of *trans*-aconitate was prepared chemically and fractionated by anion-exchange chromatography on a SAX column as described under "Experimental Procedures." The elution position of the products are indicated by the absorbance at 214 nm (*solid line*). In a parallel experiment, the elution position of the radiolabeled products of the *trans*-aconitate methyltransferase was determined on the same chromatography system. *E. coli* HC1011 cytosol (125 μg protein) was incubated with 3 μl of 0.2 M *trans*-aconitate and 5 μl of [^{14}C]AdoMet in 0.1 M sodium HEPES, pH 7.5, at 37 °C for 2 h. The sample was then chromatographed under the same conditions as for the chemically methylated species. *B,* the major ^{14}C-methylated product from the column shown in *A* (150 μl of fraction 16) was mixed with the corresponding peak from the fractionation of the chemically synthesized methyl esters and rechromatographed on a C18 reverse-phase column as described under "Experimental Procedures."

When we fractionated (2*R*,3*S*)-isocitrate we found that all of the methyl-accepting activity co-eluted with the major peak of isocitrate (Fig. 5*C*), suggesting that isocitrate is also a methyl-accepting substrate of this enzyme. Finally, fractionation of citrate showed that the methyl-accepting activity followed the peak of citrate (data not shown).

Analysis of the Methyltransferase Reaction Products in Vitro—trans-Aconitate can be methyl-esterified to produce one trimethyl-, three structurally distinct dimethyl-, and three structurally distinct monomethyl esters of *trans*-aconitate. We first analyzed the products of the chemical methyl esterification of *trans*-aconitate by anion-exchange HPLC at pH 4.5. We found UV-absorbing products eluting at 5.5, 9.9, 11.5, and 15.6 min and a very small peak of residual *trans*-aconitate was found at 50.4 min (Fig. 6*A*). Mass spectral analyses demonstrated that the 5.5-min peak contained both mono- and dimethyl derivatives of *trans*-aconitate and that the 11.5- and 16-min peaks contained monomethyl *trans*-aconitate derivatives. No *trans*-aconitate derivatives were detected in the 9.9-min peak. In a parallel experiment, we then compared the elution position of the enzymatically generated [^{14}C]methyl esters formed by the *E. coli* enzyme and *trans*-aconitate with [^{14}C]AdoMet (Fig. 6*A*). About 27% of the radioactivity was found in the flow-through fractions at 4–8 min in the position expected for residual [^{14}C]AdoMet. The remaining 73% of the radioactivity was found to elute in a distinct peak at 16 min in an amount expected for the methyl ester product of the reaction. When the material eluting at 16 min was treated with 2 N NaOH, 95% of the total radioactivity was base-labile, consistent with a methyl ester product (data not shown). This radio-

labeled peak eluted in the same position as one of the peaks of the chemically synthesized methyl esters corresponding to a monomethyl ester (Fig. 6*A*). To determine the nature of this material, the peaks of material eluting at 16 min from the chromatographic separations of the enzymatically and chemically synthesized material were then mixed and further fractionated on a C18 reverse-phase column (Fig. 6*B*). Here, the radioactivity was found to elute in a single peak at about 11 min that also corresponded to a peak of absorbance of 214 nm. Analysis of this material by mass spectroscopy indicated that it has an m/e of 187 consistent with one or more of the monomethyl esters of *trans*-aconitate. Since the analysis of the other UV-absorbing peaks in the experiment shown in Fig. 6*A* showed that monomethyl esters of *trans*-aconitate are also present in the 5.5- and 11.5-min peaks, these results suggest that enzymatic reaction may be specific for a single carboxylic acid group of *trans*-aconitate. In additional experiments, we prepared the enzymatic product on a larger scale with the overexpressed enzyme as described under "Experimental Procedures" and confirmed these results with mass spectral analysis of the product separated by anion-exchange and reverse-phase chromatography.

Characterization of E. coli Cells Lacking the trans-Aconitate Methyltransferase—To confirm further our identification and to study the physiological function of the methyltransferase, we constructed an *E. coli* strain where a chloramphenicol resistance element was inserted into the middle of the *tam* gene as described under "Experimental Procedures." Extracts of this strain, designated HC1014, demonstrated no methyltransferase activity using *trans*-aconitate, *cis*-aconitate, citrate, or DL-isocitrate as substrates (data not shown). Because this gene is expressed under the control of the RpoS σ factor in stationary phase (Table II and Fig. 1), we measured the survival rates of the wild type and mutant strains in stationary phase and under environmental stresses of heat shock, osmotic stress, ethanol treatment, and oxidative stress in experiments similar to those described in Visick *et al.* (4). We found no detectable differences between the wild type and mutant strains under any of the conditions tested. We also showed that the growth rates were similar in LB media and in minimal media containing either D-glucose or acetate as a carbon source (data not shown). Finally, neither strain was able to grow in a solid or liquid media containing *trans*-aconitate as the sole carbon source (27), and we could detect no aconitate isomerase activity in wild type or mutant cells using the method of Watanabe *et al.* (27).

Endogenous Substrates of the trans-Aconitate Methyltransferase in E. coli—To characterize the endogenous substrates of this enzyme, we incubated cytosolic extracts of the parent MC1000 strain (tam^+) and the mutant HC1014 strain (tam^-) with *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) in the absence of any exogenous methyl acceptors. We analyzed the extracts for radioactivity in compounds present in the parent but not in the mutant strain lacking the *trans*-aconitate methyltransferase. We first ether-extracted the acidified reaction mixtures and chromatographed the ether-soluble phase on anion-exchange HPLC (Fig. 7*A*). We found that a peak of radioactivity was present in the position of the *in vitro* enzymatically formed monomethyl ester of *trans*-aconitate in the parent strain but no radioactivity at this position in the mutant strain. To confirm that the endogenous material from the parent strain was the methyl ester of *trans*-aconitate, we pooled the radioactive peak and subjected the material to reverse-phase HPLC. Here we found that the material again chromatographed as the methyl ester of *trans*-aconitate (Fig. 7*B*). Finally, we pooled the reverse-phase radioactive peak and sub-

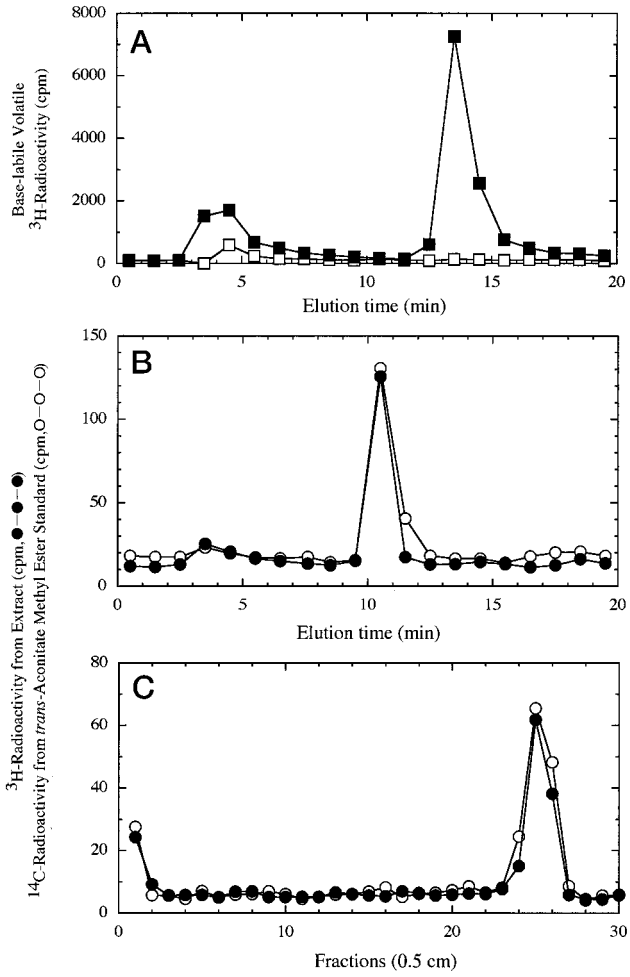

FIG. 7. **Detection of *trans*-aconitate as a substrate in MC1000 cytosol.** Cytosols from the wild type strain (MC1000) and the knockout strain (HC1014) of the *trans*-aconitate methyltransferase gene (75 μg of protein) were used for *in vitro* labeling. Twenty five μl of [^{3}H]AdoMet (7.06 μM, 77.9 Ci/mmol, in 10 mM sulfuric acid solution/ethanol (9:1), DuPont) and 20 μl of, 0.4 M sodium HEPES, pH 7.5, were added to the cytosols, and the reactions were incubated at 37 °C for 2 h. The reaction mixtures were then acidified with 20 μl 10 N sulfuric acid, and 200 μl ether was used to extract the products three times. The ether phase from the three-time extraction was combined and back-extracted with 100 μl of water. The resulting ether phase was air-dried and dissolved into 100 μl of water. *A*, 20 μl of the ether phase was applied to a SAX anion-exchange column eluted with 50 mM potassium phosphate at pH 4.5. Fractions of 1 ml were collected; 100-μl aliquots were mixed with 100 μl of 2 N NaOH and base-labile volatile radioactivity determined as described. Radioactivity from the wild-type extract is shown with *closed squares* and that from the mutant extract with *open squares*. The enzymatic methylated product of a standard of *trans*-aconitate was found to elute at 13.5 min in a parallel experiment. *B*, fraction 14 (100 μl) containing ^{3}H radioactivity from the wild-type incubation in *A* above was mixed with a standard of *trans*-aconitate enzymatically methylated with [^{14}C]AdoMet and the mixture rechromatographed on a C18 reverse-phase column as described under "Experimental Procedures." Fractions of 1 ml were collected, and 0.1 ml was directly added into 5 ml of scintillation fluid and counted. The ^{3}H radioactivity derived from fraction 14 is shown in *closed circles*, and the ^{14}C radioactivity derived from the standard is shown in *open circles*. *C*, 0.5 ml of fraction 11 from the reverse-phase column was dried down in SpeedVac and dissolved in 5 μl of H_2O. Thin layer chromatography was carried out as described under "Experimental Procedures." After the run, without staining, the lane was cut into 0.5-cm pieces, and the silica from each piece was scrapped into a microcentrifuge tube. 100 μl of H_2O was added to each tube, and the tube was vortexed vigorously for 30 s. The tube was then centrifuged in a microcentrifuge, and the supernatant was counted for ^{3}H radioactivity (*closed circles*) and ^{14}C radioactivity (*open circles*).

TABLE V

trans-Aconitate methyltransferase activities in several organisms

Cytosolic extracts from various organisms were assayed for citrate, DL-isocitrate, and *trans*-aconitate-dependent activities as described under "Experimental Procedures." For the endogenous activities, no tricarboxylic acid was added. For substrate-dependent activity, final concentrations of 50 mM citrate, 20 mM DL-isocitrate, or 1 mM *trans*-aconitate were added, and the endogenous activities were subtracted. Yeast cytosol (23 mg of protein/ml) from strain CH9100–2 was prepared by Dr. Jonathan Gary, nematode cytosol (10 mg of protein/ml) from strain CB1489 by Dr. Agnieszka Niewmierzycka, and mouse brain cytosol (8 mg of protein/ml) by Dr. Jonathan Lowenson. The values are averages of two experiments. ND, not determined.

Cytosols	Endogenous activity	Citrate-dependent activity	DL-Isocitrate-dependent activity	*trans*-Aconitate-dependent activity
		pmol/min/mg		
E. coli	0.09	3.92	31.9	41.0
Yeast (*S. cerevisiae*)	0.06	0.25	14.5	20.3
Nematodes (*C. elegans*)	0.08	ND	−0.04	−0.05
Mice (brain)	0.86	ND	−0.02	−0.08

jected it to thin layer chromatography (Fig. 7*C*). Once again the radioactivity co-migrated with the methyl ester of *trans*-aconitate. These experiments demonstrate that *trans*-aconitate is present in *E. coli* extracts and is an endogenous substrate of the methyltransferase.

Presence of the Activity and Gene in Other Organisms—We assayed the citrate-, DL-isocitrate-, and *trans*-aconitate-specific activity in cell extracts of *Saccharomyces cerevisiae, Caenorhabditis elegans,* and mouse brain. The methyltransferase activity is present in yeast but absent in nematodes and mouse brain (Table V). The specific activity of the enzyme in the yeast extract used was about half that seen in extracts of *E. coli* in stationary phase. Analysis of the GenBank™ data base revealed a homolog of the *E. coli trans*-aconitate methyltransferase gene in *Mycobacterium tuberculosis* but, surprisingly, not one in the complete genome of *S. cerevisiae*. However, examination of several unfinished microbial genomes available through the National Center for Biotechnology Information indicated that there are apparent homologs of *trans*-aconitate methyltransferase in *Deinococcus radiodurans, Pseudomonas aeruginosa,* and *Yersinia pestis* (Fig. 4).

DISCUSSION

We have identified a novel *O*-methyltransferase in *E. coli*. It methylates one of the three carboxyl groups of *trans*-aconitic acid to form a monomethyl ester. The enzyme also recognizes *cis*-aconitate, (2*R*,3*S*)-isocitrate, and citrate but with much higher K_m values and/or much lower V_{max} values. No reaction is seen with other related metabolites such as succinate, fumarate, malate, or oxalacetate. The fact that it is not active on tricarballylate (the saturated form of aconitate) suggests the importance of an olefinic or hydroxyl function in the recognition of the substrate for the methyl transfer reaction. Further work will be required to identify which carboxyl group is modified in *trans*-aconitate.

trans-Aconitate appears to be present in *E. coli* because we can isolate the radiolabeled methyl ester after incubation of cell extracts with [^{3}H]AdoMet. Its origin, however, is unclear. Wild type *E. coli* cells are reported to be unable to use *trans*-aconitate as a carbon source (28), and we are unaware of any pathways where it is a product or substrate for an enzymatic reaction in this organism. We have confirmed that *E. coli* cells cannot grow on *trans*-aconitate nor do they contain an aconitate isomerase activity that can convert the citric acid cycle intermediate *cis*-aconitate to *trans*-aconitate. Nevertheless, *trans*-aconitate can be formed spontaneously from *cis*-aconitate (29–32).

What advantage might the ability to methylate *trans*-aconitate give *E. coli* cells? At least in mammalian systems, *trans*-aconitate is an inhibitor of two central enzymes of the citric acid cycle, aconitase (33, 34) and fumarase (35, 36). The possibility thus exists that the enzymatic methylation of *trans*-aconitate can attenuate its inhibition of these crucial enzymatic reactions in energy metabolism. This may occur either because the methyl ester of *trans*-aconitate is inherently less inhibitory to central metabolic reactions or by a novel type of pathway that might convert *trans*-aconitate methyl ester to a less toxic species. It is even possible that the methylation of *trans*-aconitate might initiate a pathway that could result in its net conversion to *cis*-aconitate and its return to the citric acid cycle. These alternatives are presently under investigation in our laboratory. Significantly, the methyltransferase is expressed in early stationary phase when the cessation of rapid cell division may allow altered metabolites to accumulate (see below). Nevertheless, we have been unable to demonstrate a growth phenotype in the methyltransferase-deficient strain we have constructed in this work.

An interesting aspect of *trans*-aconitate methyltransferase is that its expression appears to be regulated by the stationary phase specific σ factor RpoS (20). Upon starvation, *E. coli* cells embark upon a developmental program resulting in metabolically less active and more resistant cells (37, 38). The starvation-induced expression of many genes is controlled by RpoS, and an intact *rpoS* allele is crucial for maintaining cell shape, resistance to multiple stresses, synthesis of glycogen, and long term survival in stationary phase cells. The fact that the expression of the *trans*-aconitate methyltransferase is dependent on the presence of an intact *rpoS* gene and the activity of the enzyme in different growth phases correlates with that of RpoS suggests that the ability to metabolize *trans*-aconitate is most important when cell division is limited and potential inhibitors might be expected to accumulate. The loss of enzyme activity after extended stationary phase is likely to reflect both the loss of RpoS protein and the instability of the enzyme under these conditions, but it is not clear what the physiological significance of this decrease is.

Although we have detected an active *trans*-aconitate methyltransferase activity in the yeast *S. cerevisiae*, no activity has been found in extracts from nematodes or mouse brain. Interestingly, although potential homologous open reading frames have been found in the genomes of a number of procaryotes, there is no clear homolog in the complete genome sequence of yeast. This latter result indicates that this activity may have arisen independently in yeast and bacteria or that the sequence may have diverged rapidly.

In certain plants, *trans*-aconitate is made in relatively large amounts and can represent up to 12% of tissue dry weight (39, 40). The enzyme responsible for this conversion is aconitate isomerase that catalyzes the formation of *trans*-aconitate from *cis*-aconitate (27, 41). The function of the accumulation of *trans*-aconitate in plants is not clear. It does present a problem for animals that consume it such as ruminants where it is associated with "grass tetany," a calcium-magnesium defi-

ciency linked to the chelation properties of a ruminal bacterial metabolite of *trans*-aconitate, tricarballylate (24, 42).

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