

S-Adenosylmethionine Transport in *Rickettsia prowazekii*

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Rickettsia prowazekii, the causative agent of epidemic typhus, is an obligate, intracellular, parasitic bacterium that grows within the cytoplasm of eucaryotic host cells. Rickettsiae exploit this intracellular environment by using transport systems for the compounds available in the host cell's cytoplasm. Analysis of the *R. prowazekii* Madrid E genome sequence revealed the presence of a mutation in the rickettsial *metK* gene, the gene encoding the enzyme responsible for the synthesis of S-adenosylmethionine (AdoMet). Since AdoMet is required for rickettsial processes, the apparent inability of this strain to synthesize AdoMet suggested the presence of a rickettsial AdoMet transporter. We have confirmed the presence of an AdoMet transporter in the rickettsiae which, to our knowledge, is the first bacterial AdoMet transporter identified. The influx of AdoMet into rickettsiae was a saturable process with a K_T of 2.3 μ M. Transport was inhibited by S-adenosylethionine and S-adenosylhomocysteine but not by sinfungin or methionine. Transport was also inhibited by 2,4-dinitrophenol, suggesting an energy-linked transport mechanism, and by N-ethylmaleimide. AdoMet transporters with similar properties were also identified in the Breinl strain of *R. prowazekii* and in *Rickettsia typhi*. By screening *Escherichia coli* clone banks for AdoMet transport, the *R. prowazekii* gene coding for a transporter, RP076 (*sam*), was identified. AdoMet transport in *E. coli* containing the *R. prowazekii sam* gene exhibited kinetics similar to that seen in rickettsiae. The existence of a rickettsial transporter for AdoMet raises intriguing questions concerning the evolutionary relationship between the synthesis and transport of this essential metabolite.

Rickettsia prowazekii, the etiologic agent of epidemic typhus, is an obligate, intracellular, parasitic bacterium that grows within the cytoplasm of the eucaryotic host cell rather than within an intracytoplasmic vesicle. The rickettsiae exploit this environment by expressing distinctive transport systems for the high-energy intermediates available in the host cell cytoplasm. For example, *R. prowazekii* can transport such highly charged molecules as ATP/ADP, AMP, GMP, NAD, UMP, and UDPG (5, 6, 32–34). Due to the importance of transport systems in rickettsial intracellular parasitism, the identification and characterization of rickettsial transporters is critical to our understanding of how these unusual bacteria grow and cause disease.

The search for rickettsial transporters has benefited from the publication of the *R. prowazekii* Madrid E genome sequence (4). Many genes coding for putative membrane proteins can be identified and are now available for further study. In addition, the genome sequence identified mutations that point to the possible existence of specific transporters. For example, analysis of the *R. prowazekii* Madrid E genome sequence revealed the presence of numerous pseudogenes. These genes code for proteins with high homologies to characterized gene products of other organisms but contain mutations that would presumably preclude expression of functional products (4). One such gene is *metK*, coding for methionine adenosyltransferase (MAT). In the Madrid E strain of *R. prowazekii*, this gene contains a stop codon in the middle of the MAT coding sequence (1, 4). Interestingly, the *R. prowazekii* Breinl strain and a strain of *Rickettsia typhi* exhibit complete *metK* open reading frames, while the *metK* genes of the spotted

fever group rickettsiae possess numerous stop codons and frameshifts (2–4). MAT, the enzyme that catalyzes the synthesis of S-adenosylmethionine (AdoMet), has been identified in a wide variety of species of the bacterial, eucaryotic, and archaeal lineages (9, 27). AdoMet is an essential metabolite in both procaryotes and eucaryotes, where it serves as the primary methyl donor in a variety of methylation reactions (18, 27). In addition, the aminopropyl group of AdoMet serves as a substrate in the polyamine biosynthetic pathway (7, 18). Based on the facts that *R. prowazekii* has been shown to synthesize but not transport polyamines and that the *R. prowazekii* Madrid E strain contains a nonsense mutation within the coding sequence of the *metK* gene, a rickettsial transport system for AdoMet should be present (3, 26).

While AdoMet synthesis has been found in a wide variety of cells, AdoMet transport has been identified in only a few eucaryotes, including *Saccharomyces cerevisiae*, *Pneumocystis carinii*, *Trypanosoma brucei*, *Leishmania donovani*, and rat liver mitochondria (8, 11, 17, 19, 20, 22, 25). In this paper we identify and characterize a rickettsial AdoMet transporter, the first reported bacterial transporter for this essential metabolite.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The rickettsial strains used included the *R. prowazekii* Madrid E and Breinl strains and the *R. typhi* Wilmington strain. Rickettsiae were purified from the yolk sacs of embryonated hen eggs as described previously (32) and were suspended in a sucrose-phosphate-glutamate-magnesium solution (SPG-Mg; 0.218 M sucrose, 3.76 mM KH_2PO_4 , 7.1 mM K_2HPO_4 , 4.9 mM potassium glutamate, and 10 mM MgCl_2). For uptake assays, rickettsial suspensions were concentrated so that rickettsiae derived from 8 g of infected yolk sac were present in 1 ml. The concentrated suspensions ranged from 4 to 15 mg of protein per ml.

E. coli strain XL1-Blue (Stratagene, La Jolla, Calif.) was used as the standard recipient in these studies. An *R. prowazekii* cosmid clone bank constructed in *E. coli* DH1 was used in transport screening experiments (14, 15). *E. coli* strains

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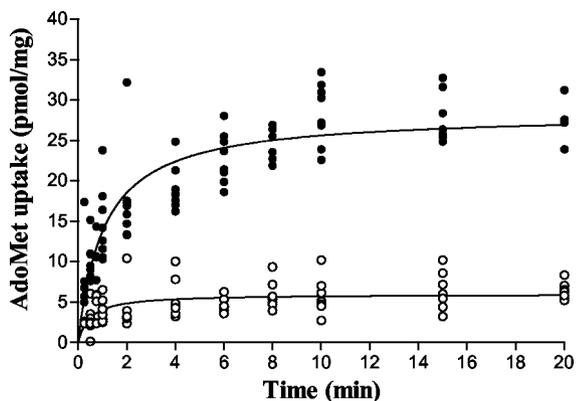


FIG. 1. Time course of AdoMet transport in *R. prowazekii* Madrid E. The uptake of AdoMet (10 μ M) was measured at 34°C (filled circles) or at 4°C (open circles).

were cultured in Luria-Bertani (LB) medium at 37°C. Where appropriate for selection of *E. coli* transformants, ampicillin was added to a final concentration of 50 μ g/ml.

Transport assays. Rickettsial AdoMet uptake assays were initiated by adding 1/10 the final volume of the concentrated rickettsial suspension to SPG-Mg containing *S*-adenosyl-L-[methyl- 14 C]methionine (Amersham Biosciences Corp., Piscataway, N.J.) at concentrations ranging from 0.5 to 20 μ M. For measuring the effect of substrate concentration on AdoMet uptake, solutions were incubated at 34°C for 15 s and 0.1 ml aliquots were placed on pretwetted membrane filters (25 mm; Durapore PVDF; Millipore Corp.) and were washed with 5 ml of 0.25 M sucrose. Filters were dried, and radioactivity was assayed by liquid scintillation. Intracellular accumulation of AdoMet was measured by using a microspace technique as previously described (31). To determine specificity, unlabeled, putative competitive inhibitors, at a final concentration of 25 μ M, were added to reaction mixtures containing 10 μ M labeled AdoMet. Sensitivity of transport to metabolic inhibitors was assayed similarly but at a final concentration of 1 mM, and they were preincubated with rickettsiae for 10 min. Assays were initiated by the addition of labeled AdoMet. All inhibitors were purchased from Sigma (St. Louis, Mo.). For *E. coli* assays, an overnight culture was used to inoculate fresh LB medium and the culture was grown to exponential phase. Bacteria were harvested by centrifugation, washed with 5 ml of 50 mM potassium phosphate buffer (pH 7.0), and finally suspended in SPG-Mg to a calculated optical density at 600 nm of 2.0 and assayed for AdoMet transport as described above for the rickettsiae. Kinetic parameters for all uptake experiments were calculated by nonlinear regression and were plotted by using GraphPad Prism software (GraphPad Software, Inc., San Diego, Calif.).

Clone bank screening and subcloning. A previously established cosmid clone bank was replicated to 96-well microtiter plates containing 100 μ l of LB medium plus ampicillin. After overnight incubation at 37°C, 100 μ l of LB medium plus ampicillin containing 20 μ M [14 C]AdoMet was added to each well. After a 4-h incubation the cells were placed in a 96-well vacuum manifold containing a nitrocellulose filter, the medium was removed, and the cells were washed with two 500- μ l aliquots of potassium phosphate buffer. The filters were dried, and the radioactivity was visualized by using a Cyclone Storage Phosphor System (PerkinElmer Life Sciences Inc., Boston, Mass.). Plasmid vectors used in the cloning of specific rickettsial fragments included pBluescript SKII(+) (Stratagene) and pSMART HCAmp (Lucigen Corp., Middleton, Wis.).

RESULTS

Transport of radiolabeled AdoMet in *R. prowazekii* Madrid E. Figure 1 shows the kinetics of radioactive AdoMet (10 μ M) uptake as a function of time. Uptake was linear for the first minute and reached a steady state within 8 min. A microspace assay was used to examine the accumulation of AdoMet by the rickettsiae at steady state (31). In two independent experiments, accumulation ratios (the ratio of the concentration inside to that outside) of 13 and 28 were obtained. Since uptake was linear for the first minute, subsequent uptake experiments

were sampled at periods of less than 1 min to minimize the possible effect of downstream processes on transport kinetics. The effect of substrate concentration on AdoMet uptake can be seen in Fig. 2. In contrast to nonspecific interactions that occur in the presence of 2,4-dinitrophenol (DNP) or when the rickettsiae are assayed at 4°C, accumulation of AdoMet by *R. prowazekii* at 34°C was a saturable process (Fig. 2A). A composite of *R. prowazekii* AdoMet uptake experiments that used four independent *R. prowazekii* preparations and that were normalized to a standard V_{max} revealed a K_T of AdoMet transport of 2.3 μ M (range of 0.9 to 4.2 μ M) (Fig. 2B). Due to the variability between rickettsial preparations it was impossible to determine the V_{max} for rickettsial AdoMet transport. In the series of experiments described above, the V_{max} spanned a range of 11 to 95 pmol $mg^{-1} min^{-1}$ after background subtraction.

The specificity of transport was examined by measuring rickettsial AdoMet transport in the presence of various AdoMet analogues (Table 1). The most effective inhibitor, comparable to that of unlabeled AdoMet, was *S*-adenosylethionine, which differs from AdoMet in the substitution of an ethyl group for the donor methyl group. *S*-adenosylhomocysteine, a byproduct of AdoMet methylation reactions, also inhibited uptake to a lesser extent, while sinfungin, an effective inhibitor of some eucaryotic AdoMet transporters (8, 11, 17), exhibited no significant inhibition. With inhibitor concentrations of 12.5, 25, 50, and 100 μ M, the calculated K_i values for *S*-adenosylethionine and *S*-adenosylhomocysteine were 6.4 ± 1.0 and 14.3 ± 6.9 μ M, respectively. Additional potential inhibitors (methionine, ethionine, adenosine, and methylthioadenosine) did not inhibit rickettsial AdoMet transport (data not shown).

The sensitivity of the *R. prowazekii* AdoMet transporter to metabolic inhibitors was also assessed (Table 1). DNP significantly inhibited accumulation of AdoMet, suggesting that AdoMet transport is an energy-dependent process. Rickettsial AdoMet transport was also sensitive to *N*-ethylmaleimide (NEM), a sulfhydryl group blocking agent.

AdoMet transport by other rickettsiae. The Breinl strain of *R. prowazekii* and the Wilmington strain of *R. typhi* do not have stop codons within their *metK* genes and thus may be able to synthesize AdoMet (2). In order to evaluate their transport capabilities, the kinetics of AdoMet transport were determined. The kinetics of uptake were found to be similar to those of the *R. prowazekii* Madrid E strain (Fig. 2C and D). The Breinl strain exhibited a K_T of 7.8 μ M while the *R. typhi* K_T was 5.2 μ M. Both substrate specificity and the sensitivity of these transporters to poisons were comparable to those of the *R. prowazekii* Madrid E strain (data not shown).

Identification of the AdoMet transporter gene. Since none of the genes contained in the *R. prowazekii* genome exhibited homology to any of the known eucaryotic AdoMet transporters, a genome screening method was used to identify the rickettsial transporter. A previously established cosmid clone bank (14, 15) was screened for clones that demonstrated uptake of radiolabeled AdoMet. One such clone that was identified contained a rickettsial *Sau3A* fragment encompassing the RP075-RP084 gene region (Fig. 3). A *Sna*BI-*Hpa*I fragment containing the complete gene sequence of the RP076 gene and partial sequences of the RP075 and *proP1* genes was subsequently

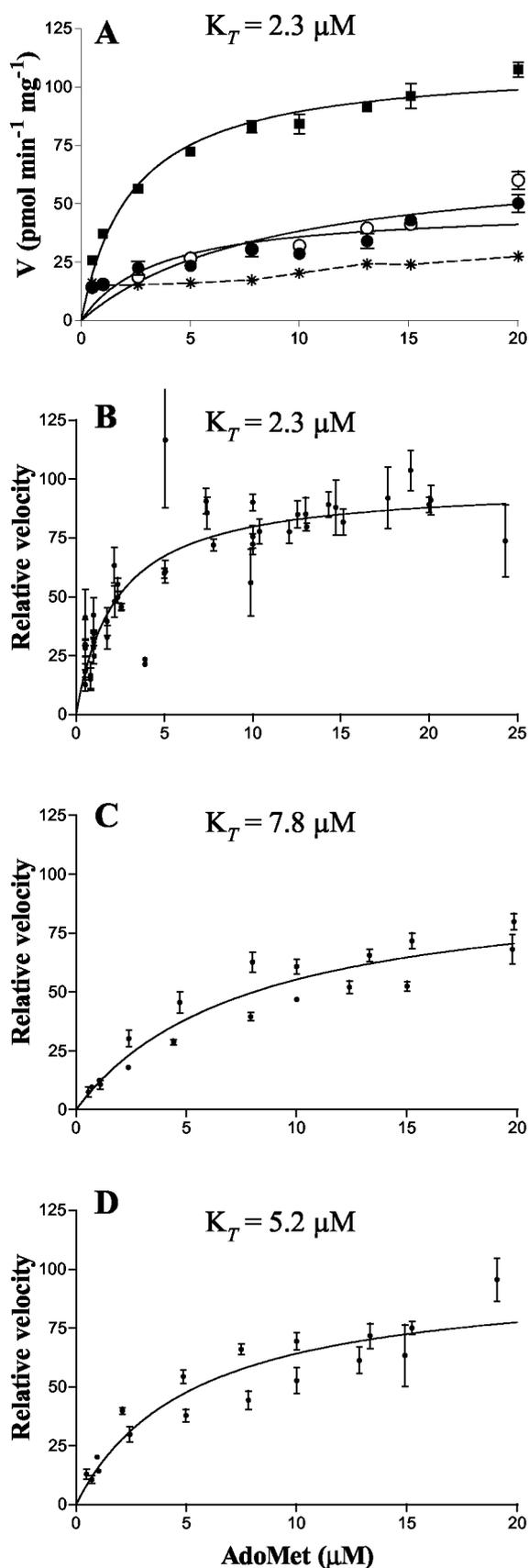


FIG. 2. Effect of substrate concentration on AdoMet transport in rickettsiae. K_T values were obtained by nonlinear regression analysis

TABLE 1. Effect of inhibitors on AdoMet uptake by *R. prowazekii* Madrid E and *E. coli* MOB 1402^a

AdoMet analogues	% Inhibition \pm SEM for	
	<i>R. prowazekii</i> Madrid E	<i>E. coli</i> MOB 1402
Unlabeled metabolites ^b		
AdoMet	62 \pm 2.8	36 \pm 1.8
SAE	50 \pm 3.2	36 \pm 2.2
SHC	34 \pm 4.0	31 \pm 4.2
Sinfungin	7 \pm 3.1	0
Methionine	6 \pm 5.6	25 \pm 3.8
No inhibitor at 4°C	64 \pm 2.8	66 \pm 3.7
Metabolic inhibitors ^c		
NEM	42 \pm 6.5	34 \pm 8.0
DNP	72 \pm 3.6	82 \pm 3.6

^a Two independent bacterial preparations were assayed three to five times for each inhibitor tested.

^b Assays contained 10 μ M labeled AdoMet and 25 μ M unlabeled inhibitor. SAE, S-adenosylethionine; SHC, S-adenosylhomocysteine.

^c Assays contained 10 μ M labeled AdoMet and inhibitors at a concentration of 1 mM added 10 min prior to assay.

identified as the region imparting AdoMet transport to *E. coli* clones (Fig. 3).

In order to conclusively prove that only the RP076 gene is necessary for AdoMet transport, the coding region of RP076, flanked by 56 bp upstream and 32 bp downstream, was PCR amplified and ligated into the blunt cloning vector pSMART, generating plasmid pMW1402. Originally, the pSMART vector was chosen to eliminate possible toxicity problems during the initial cloning of the RP076 PCR product. However, it was discovered that insertion of the RP076 coding region into pSMART in one orientation (pMW1402) resulted in constitutive AdoMet transport. Thus, this construction was used to investigate transport kinetics in *E. coli* (Fig. 4). The fragment inserted into the vector in the opposite orientation (pMW1410) served as a negative control. AdoMet uptake in *E. coli* cells containing pMW1402 remained linear over the 20-min assay, presumably due to the rapid metabolism of AdoMet within *E. coli*. To minimize the effect of downstream processes on the kinetics of transport, samples for determining the effect of substrate concentration on transport were taken at 30 s. A K_T of 4.7 μ M, similar to that found for rickettsial transport, was found under these conditions. In addition, the sensitivity of the cloned transporter to the inhibitors S-adenosylethionine and S-adenosylhomocysteine and to the metabolic inhibitors DNP and NEM was similar to that found for rickettsial transport (Table 1). Based on this data, we have assigned the gene designation *sam* to RP076.

with GraphPad Prism software. (A) Transport of AdoMet by *R. prowazekii* Madrid E at 34°C (■), at 4°C (○), and in the presence of DNP (●). Nonspecific background binding of labeled AdoMet to filters is also shown (*) and is subtracted from experimental values in panels B to D. (B) A composite of four independent *R. prowazekii* Madrid E AdoMet transport assays normalized to a V_{max} of 100 pmol min⁻¹ mg⁻¹. (C) A composite of two independent *R. prowazekii* Breinl AdoMet transport assays normalized to a V_{max} of 100 pmol min⁻¹ mg⁻¹. (D) A composite of two independent *R. typhi* Wilmington AdoMet transport assays normalized to a V_{max} of 100 pmol min⁻¹ mg⁻¹.

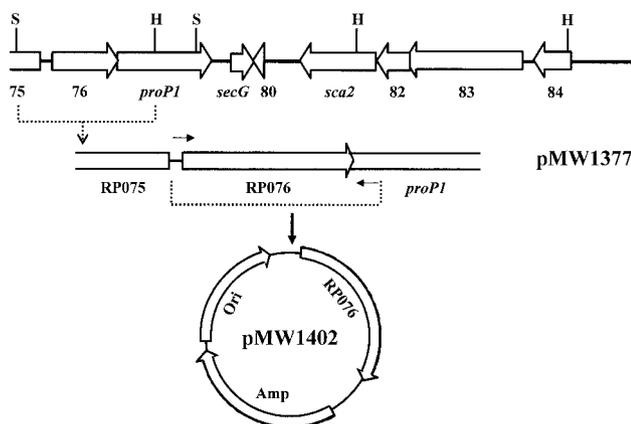


FIG. 3. Schematic maps of the cosmid insert containing the *R. prowazekii* RP075-RP084 gene region and subsequent recombinants. The orientation of the genes is indicated by open arrows. Dashed lines identify the regions cloned to generate specific recombinants. The indicated *SnaBI*-*HpaI* fragment was cloned into pBluescript SKII(+) to generate pMW1377. Small arrows represent primers used to amplify the RP076 gene. The amplified gene was cloned into pSMART to generate pMW1402. All the constructs shown transport AdoMet in *E. coli*. H, *HpaI*; S, *SnaBI*; Amp, ampicillin resistance gene; Ori, origin of replication.

The *R. prowazekii* *sam* gene exhibits a G+C content (32.4%) typical of *R. prowazekii* protein-coding genes (4). The transporter encoded by *sam* possesses characteristics of an integral membrane protein. The 294-amino-acid deduced protein has a calculated M_r of 33,092 and a pI value of 9.98. A Kyte-Doolittle hydrophobicity analysis (16) revealed a hydrophobic protein with 10 potential membrane-spanning regions (data not shown). Several topology models for transmembrane proteins also predicted 10 membrane-spanning regions (10, 13, 30). A homolog to *sam* (RC0106) with 90.5% identity at the amino acid sequence level can be identified in the *Rickettsia conorii* genome sequence (21). A BLAST search revealed that the rickettsial transporter exhibits a small but significant relationship (23 to 28% identity) to hypothetical proteins from a wide range of bacterial genera. The only annotated genes identified in the BLAST search, from *Brucella melitensis* and *Vibrio vulnificus*, are members of the DMT superfamily of drug/metabolite transporters. The *Brucella* transporter is also annotated as a member of the DME family, a subgroup of the DMT superfamily that contains integral membrane proteins with sizes ranging from 246 to 353 amino acids and having 10 membrane-spanning regions (12).

DISCUSSION

As obligate intracellular bacteria growing within the cytoplasm of the eucaryotic host cell, the rickettsiae are immersed in pools of metabolic intermediates. The rickettsiae have evolved to exploit this rich environment by expressing transport proteins specific for these metabolites. Consequently, the capability of rickettsiae to synthesize many of these compounds has been lost. Upon publication of the *R. prowazekii* genome sequence, an example of this reductive evolution process was encountered with the identification of a translational stop codon within the *metK* gene of *R. prowazekii* (1, 4). Since this gene codes for the enzyme responsible for synthesizing the

essential metabolite AdoMet, the identification of a translational stop within this gene led to the hypothesis that *R. prowazekii* Madrid E should transport AdoMet (3).

Our data demonstrate that rickettsiae transport AdoMet via a high-affinity system. This is the first bacterial AdoMet transporter identified, and the 2 to 8 μM K_T values for the rickettsial transporters are comparable to the values for the *S. cerevisiae* transporter (3.3 μM), the high-affinity transporter of *P. carinii* (4.5 μM), and the transporter found in rat liver mitochondria (8.9 μM) (11, 19, 20). In sensitivity to inhibitors, the rickettsial transporter is comparable to that of *S. cerevisiae*. However, there is no significant homology between any eucaryotic AdoMet transporter and the rickettsial transporter identified in this study. Thus, the rickettsial transporter offers a unique model for examining the transport of this essential metabolite.

Uptake of AdoMet by *E. coli* expressing the cloned transporter remained linear over 20 min, while rickettsial uptake reached a steady state within 8 min. This difference is likely due to a much lower metabolic demand for AdoMet in the

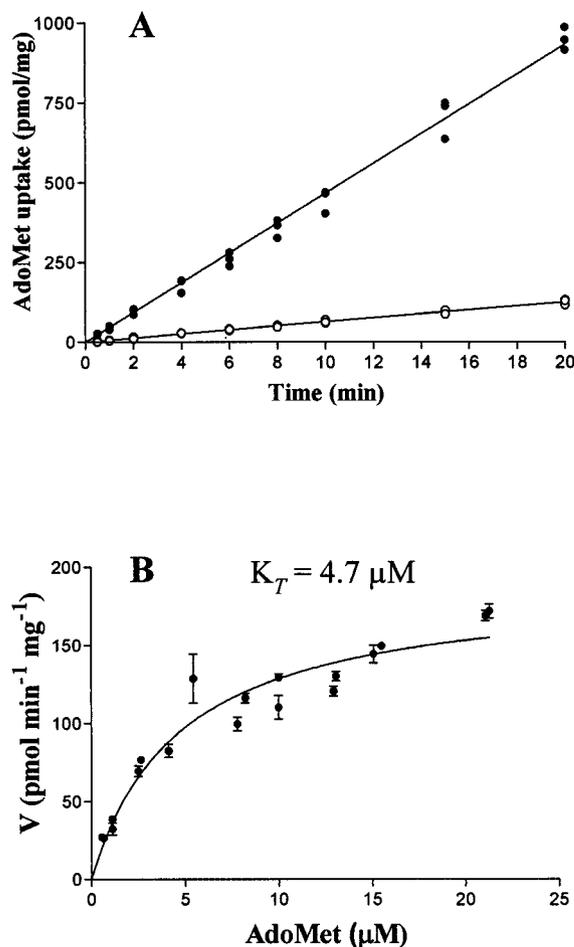


FIG. 4. Kinetics of AdoMet transport in MOB1402. (A) Time course of AdoMet uptake in *E. coli* MOB1402 and MOB1410. (B) Effect of substrate concentration on AdoMet transport in MOB1402. The data represent a composite of two independent bacterial preparations. The K_T value was obtained by nonlinear regression analysis with GraphPad Prism software.

rickettsiae. In rickettsiae, the only known use for AdoMet is in the synthesis of polyamines (26). AdoMet-dependent rickettsial methylation reactions have not been characterized. In addition, preliminary experiments examining the intracellular presence of AdoMet in rickettsiae by thin-layer chromatography identified AdoMet as the major labeled compound, suggesting a slower rate of AdoMet metabolism within the time frame of the experiments.

The existence of an AdoMet transporter in rickettsiae raises questions about the contribution of AdoMet synthesis in those rickettsiae containing an intact *metK* gene. Since AdoMet is an essential metabolite, it is obvious that a transporter must exist before the ability to synthesize this compound is lost completely. Thus, the hypothesis that the *R. prowazekii* Madrid E strain must have a transporter was logical when coupled to the fact that the Madrid E *metK* gene has a nonsense mutation within the coding sequence. However, the Breinl strain of *R. prowazekii* and the Wilmington strain of *R. typhi* possess complete open reading frames of the *metK* gene. While this does not preclude the existence of missense mutations within these *metK* genes, the presence of complete open reading frames raises the question of whether these strains would need to transport AdoMet in a comparable manner. Our data demonstrated that both strains transported AdoMet with kinetics similar to that seen for the Madrid E strain. While the K_T values of these strains were found to be slightly higher than that of Madrid E, this is insignificant considering the variability of rickettsial preparations. Obviously, the next step is to determine whether these rickettsial strains possess active MAT enzymes and how these rickettsial enzymes compare in activity with the well-studied MAT enzyme of *E. coli*. Preliminary data from whole-cell assays suggest that both the Breinl and Wilmington strains are expressing active enzyme, and analysis of the protein sequences reveals that both exhibit conservation of the active-site residues identified in *E. coli* (23, 24, 28, 29). Studies are under way to purify the MAT enzymes from the Breinl and Wilmington strains in order to directly compare and contrast the activities of these enzymes with those of the *E. coli* standard.

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