A surprising range of modified-methionyl S-adenosylmethionine analogues support bacterial growth

Mojun Zhao,1 Yasanandana S. Wijayasinghe,1† Pravin Bhansali,1 Ronald E. Viola1 and Robert M. Blumenthal2

1Department of Chemistry and Biochemistry, University of Toledo, Toledo, OH 43606, USA
2Department of Medical Microbiology and Immunology, and Program in Bioinformatics, University of Toledo Health Sciences Campus, Toledo, OH 43614, USA

S-Adenosyl-L-methionine (AdoMet) is an essential metabolite, serving in a very wide variety of metabolic reactions. The enzyme that produces AdoMet from L-methionine and ATP (methionine adenosyltransferase, MAT) is thus an attractive target for antimicrobial agents. We previously showed that a variety of methionine analogues are MAT substrates, yielding AdoMet analogues that function in specific methyltransfer reactions. However, this left open the question of whether the modified AdoMet molecules could support bacterial growth, meaning that they functioned in the full range of essential AdoMet-dependent reactions. The answer matters both for insight into the functional flexibility of key metabolic enzymes, and for drug design strategies for both MAT inhibitors and selectively toxic MAT substrates. In this study, methionine analogues were converted in vitro into AdoMet analogues, and tested with an Escherichia coli strain lacking MAT (AmetK) but that produces a heterologous AdoMet transporter. Growth that yields viable, morphologically normal cells provides exceptionally robust evidence that the analogue functions in every essential reaction in which AdoMet participates. Overall, the S-adenosylated derivatives of all tested L-methionine analogues modified at the carboxyl moiety, and some others as well, showed in vivo functionality sufficient to allow good growth in both rich and minimal media, with high viability and morphological normality. As the analogues were chosen based on incompatibility with the reactions via which AdoMet is used to produce acylhomoserine lactones (AHLs) for quorum sensing, these results support the possibility of using this route to selectively interfere with AHL biosynthesis without inhibiting bacterial growth.

INTRODUCTION

S-Adenosyl-L-methionine (AdoMet) is an essential metabolite in all cellular organisms, and is required by a wide variety of enzymes, including everything from macromolecule methyltransferases to ribonucleotide reductase (Cheng & Blumenthal, 1999; Fontecave et al., 2004). AdoMet is produced from L-methionine (L-Met) and ATP by a methionine adenosyltransferase (MAT; Pajares & Markham, 2011). MAT is thus an attractive potential target for both anti-cancer and antimicrobial agents (Taylor et al., 2009). Our original interest in the range of AdoMet derivatives that can support growth derived from the fact that AdoMet is also required for production of bacterial quorum signalling molecules, such as acylhomoserine lactones (Churchill & Chen, 2011) and furanosyl borate diesters (Galloway et al., 2011), that control virulence in many Gram-negative pathogens. There is particular interest in identifying agents that would reduce bacterial pathogenicity [possibly without strong selection for resistance (Gerdt & Blackwell, 2014)] by interfering with quorum sensing but not with growth or viability (Defoirdt et al., 2010; Hodgkinson et al., 2012; Kalia, 2013; Rutherford & Bassler, 2012). Our approach has been to identify methionine analogues that are substrates for the bacterial MAT orthologues (called MetK in many bacteria), and that yield AdoMet analogues which are selectively non-functional in acylhomoserine lactone (AHL) biosynthesis. In this regard, we have structurally and functionally characterized a

1Present address: Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, MO 63110, USA.

Abbreviations: AdoXXX, S-adenosyl derivative of XXX (e.g. AdoNAM is S-adenosyl-N-acetyl-L-methionine); D-Met, d-methionine; D-MEE, d-methionine ethyl ester; D-MME, d-methionine methyl ester; L-Met, L-methionine; MAT, methionine adenosyltransferase; MEE, L-methionine ethyl ester; MetK, bacterial methionine adenosyltransferase; MME, L-methionine methyl ester; MPE, L-methionine phenyl ester; NAM, N-acetyl-L-methionine; NNDM, N,N-dimethyl-L-methionine.

Four supplementary figures are available with the online Supplementary Material.
number of MetK orthologues from bacterial pathogens as different as Pseudomonas and Campylobacter, and tested their respective abilities to form methylation-competent AdoMet analogues from analogues of L-Met (Wijayasinghe et al., 2014; Zano et al., 2013). However, those analyses have assessed only individual functions and not the wide range of biochemical reactions, aside from methyl transfers, in which AdoMet participates.

In the current study, we made use of a ΔmetK strain of Escherichia coli that carries an AdoMet transporter from Rickettsia prowazekii, such that this strain is completely dependent upon exogenous AdoMet for growth (Driskell et al., 2005). We report here that a surprising range of AdoMet variants, produced in vitro from methionine analogues (Fig. 1), are able to support growth of this E. coli strain, yielding viable and (in several cases) morphologically normal cells. Such growth support means that the full complement of essential AdoMet-dependent enzymes are quite tolerant of some AdoMet structural modifications. These results validate the strategy of identifying methionine analogues that yield AdoMet forms competent for all of its essential roles, but not capable of serving as substrates for AHL biosynthesis. Such an approach complements (and may be synergistic with) attempts by others to directly inhibit AHL synthases.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** E. coli K-12 strain MOB1490, a derivative of BW25113 (lacfl rnap716 lacZ+β16 hsdR514 araBAD+ rhaBAD-1278), was kindly provided by Dr D. O. Wood (Driskell et al., 2005). MOB1490 carries ΔmetK (metK::kan), plasmid pMW1402 specifying an AdoMet transporter (and Ampβ), and pMW1484, a pBAD-derived plasmid carrying rpoB (Rifβ).

![Fig. 1. Summary of AdoMet analogues used. The positions, substitutions and abbreviations are provided for the AdoMet analogues containing N-acetyl methionine (AdoNAM), N’N’-dimethyl methionine (AdoNNDM), the methyl, ethyl and phenyl esters of methionine (AdoMME, AdoMEE and AdoMPE, respectively), and the D-methionine methyl and ethyl ester analogues (AdoDMME and AdoDME, respectively).](http://mic.sgmjournals.org)
ruling (Hauser Scientific), with unstained cells. Photographs of stained cells were analysed using ImageJ (W. S. Rasband, NIH; http://imagej.nih.gov/ij/index.html). Images were first converted to binary form and the ‘analyse particle’ module was used in outline mode. This allowed calculation of cell areas in the plane of the micrograph. As E. coli cell widths are essentially constant at a given growth rate (Pierucci, 1978; Robert et al., 2014), area is proportional to length. For E. coli strains B/r and MG1655 (K-12) growing in rich media, those studies reported widths of 0.87 and up to 0.95 μm, respectively. In comparison to the haemocytometer results, our strain in LB + 64 μM AdoMet gave uniform widths of ~1.2 μm. From this, and length to width ratios, we estimated cell lengths. Direct measurement of cell length was also determined for strain MOB1490 and its parent (PS2209) by spotting a dilution of stationary-phase cultures onto the haemocytometer. Objects with the largest areas were tangles or clusters of cells (not shown), for which the length extrapolation is meaningless. For this reason, both area and extrapolated length are indicated. To create the relative cell length plots, the first 500 cells from each image were ranked. This is a random sampling, as the software does not analyse and number the imaged cells in order of size. In some cases, where only low culture densities were achieved, the population was scaled up to 500 so the distributions could be compared.

Cell viability analysis. Bacterial cultures from the microtitre plates at the end of the growth were serially diluted 1:10 (eight times), and 10 μl of each dilution was spotted onto an LB plate containing 32 μM AdoMet (New England Biolabs) to rescue the viable bacteria cell from the AdoMet analogue-supported growth over time.

RESULTS

Assessment of AdoMet utilization

An important goal of our research into quorum sensing inhibitors is to identify methionine analogues that act as substrates for bacterial AdoMet synthetase (MetK), resulting in AdoMet analogues that function in all essential biochemical reactions despite being incompetent for AHL biosynthesis. To test this functionality, we used a strain of E. coli, MOB1490 (Driskell et al., 2005), from which the endogenous metK gene has been deleted. In every tested case, metK deletion is lethal; however, some intracellular parasites produce transport proteins that allow them to use AdoMet produced by their hosts (Perez-Leal et al., 2011; Tucker et al., 2003). Strain MOB1490 carries the gene for a rickettsial AdoMet transporter, and can grow essentially normally in the presence of sufficient exogenous AdoMet. For our studies of AdoMet analogue utilization, it is essential that there are no confounding effects due to competition from endogenous AdoMet. This discrimination has been achieved because there is no cellular MAT activity in this strain.

The control studies, with unmodified AdoMet, are shown in the top row of Fig. 2. For the purposes of the later AdoMet analogue studies, we assessed growth in both rich medium (left column of Fig. 2), which places a higher demand on the rate of AdoMet utilization, and in minimal medium (right column of Fig. 2), which requires that the full complement of AdoMet-dependent biosynthetic enzymes be able to use the AdoMet variant. The leftmost panel in each column shows the OD₆₀₀ versus time growth curve. In the context of strain MOB1490, the rickettsial AdoMet transporter had previously been shown to have a Kₗ for AdoMet uptake of 4.7 μM (Tucker et al., 2003). Our results with unmodified AdoMet are consistent with those findings, and with earlier studies on the effects of AdoMet levels on E. coli growth (El-Hajj et al., 2013; Posnick & Samson, 1999). In the rich medium the maximal culture density was reached at the maximal rate at AdoMet concentrations ≥ 4 μM. Under our conditions (microtitre wells at 37 °C without shaking), this doubling time was ~60 min. Cultures grown in the presence of 1 μM AdoMet reached lower maximal density following slower growth than they did at higher AdoMet concentrations, although even 0.5 μM exogenous AdoMet supported very slow growth (data not shown).

In glucose minimal medium, there was no improvement in growth at ≥2 μM AdoMet (Fig. 2, top right). This is also shown in Fig. S1(a, b) (available in the online Supplementary Material), a parallel experiment with direct comparison of growth in rich and minimal media at the same AdoMet concentrations. In subsequent experiments, AdoMet (or analogue) levels were chosen based on growth requirements in the respective media. In minimal medium there was a clear toxic effect of higher concentrations of AdoMet, reflected in both lower growth rates and reduced maximum culture densities as [AdoMet] increased. This was not due to starvation for methionine; in minimal media, methionine as well as AdoMet must be provided for MOB1490 growth, to overcome AdoMet co-repression of methionine biosynthesis (El-Hajj et al., 2013), and our minimal medium was supplemented with 0.2 mM L-Met. The apparent toxicity was also not due to components from the MetK reactions used to make AdoMet from L-Met and ATP, as we saw the same phenomenon (but to a lesser extent) with purified commercial AdoMet (Fig. S2). A possible explanation for this phenomenon is that, at higher concentrations, AdoMet either co-represses the gene or allosterically inhibits a biosynthetic enzyme that is required only in minimal medium to make something other than methionine. For example, E. coli cannot make cysteine from L-Met (Seiflein & Lawrence, 2001), and relies on reduction of sulphate in the medium, although we are not aware of AdoMet regulatory effects on sulphate reduction. It is also possible that some of this toxicity is due to non-enzymic alkylation by AdoMet. Direct methyl transfer from AdoMet to DNA and protein has been reported (Barrows & Magee, 1982; Truscott et al., 2012), although some specific DNA modifications were not observed (Posnick & Samson, 1999). Irrespective of the apparent AdoMet toxicity in minimal medium, our results provide a baseline against which the AdoMet analogues can be compared.

Ability of AdoMet analogues to support growth of E. coli

Strain MOB1490 was next examined in the presence of different AdoMet analogues, each prepared in vitro by
using the purified MetK orthologue with the highest efficiency for adenosylation of each methionine analogue (Zano et al., 2013). We tested eight AdoMet analogues (Fig. 1) over a nearly two-log concentration range, continuously monitoring growth in a temperature-controlled multiwell plate reader. The AdoMet analogues tested included adenosylated derivatives of L-Met modified at the carboxyl (MME, MEE, MPE) or amino moieties (NNDM, NAM), as well as D-Met or its carboxyl derivatives (DMEE, DMME).

To assess the possible effects of methionine modifications on AdoMet stability, we examined the three analogues that had been produced in greatest abundance due to the high catalytic rates for MetK orthologues with these methionine analogues (Zano et al., 2013). These AdoMet analogues were quite stable to degradation, with 60 % or higher levels still present after storage at 4 °C for 5 days (data not shown). The corresponding demethylated adenosylhomocysteine analogues were the major breakdown products identified. These AdoMet analogues are therefore likely to be stable over the 1–2 day period of the cell growth studies. The results of these growth studies are shown in Figs 2 and 3.

The carboxyl ester derivatives AdoMME and AdoMPE (Fig. 2), and AdoMEE (Fig. S1c) gave similar results to those obtained with AdoMet. In rich medium, the lowest

Fig. 2. Ability of AdoMet and its carboxyl-ester analogues to support growth of AdoMet-dependent E. coli. For each AdoMet analogue, growth of strain MOB1490 is indicated in the rows; the left columns indicate growth in rich medium (LB), while the right columns represent growth in MOPS-glucose minimal medium (MG). MG cultures were supplemented with 0.2 mM L-Met (see text). The leftmost panel in each row shows culture density versus time in the presence of varying concentrations (1–64 μM for LB, 2–128 μM for MG) of authentic or modified AdoMet. Darker shades correspond to higher concentrations, as indicated. The same time scale was used in both rich and minimal media to facilitate comparison of growth kinetics. The middle panels show culture viability. For this, 10 μl of tenfold serial dilutions of culture samples, taken at the end of the growth experiment, were spotted onto LB agar plates containing 32 μM authentic AdoMet. Dilutions shown ranged from 10^-3 (top) to 10^-8 (bottom). The rightmost panels show crystal violet-stained photomicrographs of the undiluted samples, taken with a Zeiss Axiovert inverted microscope using the 20× objective. Images were uniformly contrast enhanced; representative fields are shown.
tested concentration (1 μM) gave slightly poorer growth with AdoMME and AdoMEE than was seen with 1 μM AdoMet, but otherwise the results with these analogues were nearly identical to those with AdoMet. However, surprisingly, AdoMPE appeared to support somewhat better growth than AdoMet, in both media (in rich medium at all tested concentrations; in minimal medium at 128 μM). In minimal medium the toxicity previously observed with AdoMet was again apparent with increasing analogue concentrations. Spotting culture dilutions onto LB agar containing 32 μM AdoMet (just to the right of the growth plots) revealed no obvious differences in culture viability between AdoMet and the analogues. In summary, the carboxyl ester AdoMet analogues support cell growth and viability comparable in extent to AdoMet.

Results with amino-modified AdoMet analogues are shown in the top two rows of Fig. 3. In rich medium, 32 μM AdoNAM supported substantial growth, but with a longer doubling time and lower saturation density than with AdoMet. However, the lower concentrations of this analogue supported only slow cell growth. AdoNNDM supported some growth, but very poorly. In minimal
medium, these two analogues showed little if any growth, and the resulting cells were morphologically abnormal (see below). The poor utilization of the amino-modified derivatives of L-Met could be a consequence of a defect in polyamine biosynthesis. AdoMet decarboxylase uses a Schiff base between a pyruvoyl co-factor and the z-amino group of AdoMet to catalyse the first step in the synthesis of polyamines (Bale & Ealick, 2010). Methionine analogues with a derivatized z-amino group would be impaired for participation in this reaction. To test this hypothesis we grew rich medium cultures of the AdoMet-dependent E. coli strain in the presence of amino-derivatized AdoMet analogues, with or without supplementation using the polyamine spermidine (Fig. S3). The spermidine did have a small but significant effect on growth in AdoNAM, but did not improve growth in the presence of AdoNNDM. These results suggest that impaired polyamine synthesis may be one of the contributing factors in the relatively poor growth by this strain in the presence of amino-modified AdoMet analogues.

Results with D-Met derivatives were similar to those with L-Met amino derivatives (bottom two rows of Fig. 3). In rich medium, AdoDMet supported growth about as well as AdoNAM, although all tested concentrations of AdoDMet gave the same growth kinetics. AdoDME (Fig. 3) and AdoDMME (Fig. S1d) are each carboxyl esters and both supported growth, although poorly, with the 32 μM cultures showing many abnormally long cells. In minimal medium none of the D-Met derivatives yielded significant growth.

Effects of AdoMet analogues on cell morphology

The morphology of cells grown in these cultures also provides useful information, as E. coli cells restricted for AdoMet exhibit a division defect that leads to abnormally long cells (El-Hajj et al., 2013; Wang et al., 2005). Culture samples from the end points of each growth curve experiment were spotted onto slides, stained with crystal violet, and examined under a microscope. Representative image sections are shown in the right panels in each column in Figs 2 and 3, and the cell size distributions from the full images are presented in Fig. 4. In each case, to facilitate comparison, 500 cells are plotted (see Methods for details), and their size is plotted versus their rank from smallest to largest. Variation in size is expected, with cells immediately pre-division having twice the length (but the same width) as cells immediately post-division (Robert et al., 2014).

The left panels in Fig. 4 show the results for unmodified AdoMet, with representative images from each size class. The right panels indicate the distribution of cells among the three size classes, following growth in the highest tested analogue concentration. Particularly in the minimal medium (lower panels), the distribution with AdoMet shows very few small or large cells. A similar result was obtained in rich medium (upper panels), although the distribution was shifted by an increased number of very small cells. Using this as the baseline for comparison yields conclusions similar to those described from the growth rate measurements: the carboxyl ester derivatives of AdoMet (AdoMME, AdoMEE and AdoMPE) give the most similar results to those of AdoMet. As with cell growth, AdoNAM came next in similarity to the control results with AdoMet, but only in rich medium. The D-Met analogues gave distributions that were heavily dominated by small cells. This may reflect the known role of d-amino acids in modulating peptidoglycan structure in some bacteria (Lam et al., 2009), and the possible association of altered peptidoglycan with formation of micellins (Obermann & Höltje, 1994). In addition, a number of cell stresses and environments can affect E. coli morphology (Darmon et al., 2014; Hill et al., 2013). In this respect, while not affecting comparison of MOB1490 cells in the presence of different AdoMet analogues, it is noteworthy that the MOB1490 cells tended to be longer than those of the parental strain PS2209 even in the presence of 32 μM AdoMet (Fig. S4).

DISCUSSION

AdoMet plays many central roles in cell physiology, and among metabolites is second only to ATP for the number of reactions in which it participates (Cantoni, 1975; Fontecave et al., 2004). For this reason, MAT (or MetK in many bacteria) has attracted substantial interest as a target for inhibition by anti-cancer or antibiotic agents (e.g. Taylor et al., 2009). However, our long-term goal is somewhat more challenging. We are seeking methionine analogues that are good substrates for MetK and will be converted efficiently into AdoMet analogues, and that these AdoMet analogues will support all essential aspects of bacterial metabolism but not production of the AHLs required for quorum sensing. Our strategy relies on the fact that very few AdoMet-dependent reactions appear to involve the participation of both the z-amino and z-carboxyl moieties in catalysis. However, these moieties are indeed involved in some reactions (Breen et al., 2003; Pegg, 2009), and are likely to be required for AdoMet binding by some enzymes. If any of these z-amino- or z-carboxyl-dependent reactions are essential under the conditions used, then the analogues that we have examined would not support cell growth. In addition, even if these analogues can function in critical AdoMet-dependent reactions, the resulting products may accumulate if they are not substrates for downstream processing enzymes such as methylthioadenosine/S-adenosylhomocysteine nucleosidase (Parveen & Cornell, 2011), and this might have toxic effects.

In this study, we have demonstrated that several of the tested methionine analogues yielded AdoMet analogues that supported E. coli growth, using a strain that is completely dependent upon exogenous AdoMet. Particularly when conducted in minimal medium, this is an exceptionally robust test of AdoMet functionality. Lack of growth is not fully informative, as it may result from either poor uptake by the cloned rickettsial AdoMet transporter or limited
functionality inside the cell. But AdoMet analogues that do support growth of this E. coli strain must be functioning in every essential AdoMet-dependent reaction. For the carboxyl esters, there is some possibility that endogenous E. coli carboxylesterases (e.g. Lescat et al., 2009) generate a certain amount of unmodified AdoMet. However, this is unlikely to explain the growth results for several reasons. First, these analogues function in vitro with two different methyltransferases under conditions where no AdoMet is available (see Wijayasinghe et al., 2014). Second, even the lowest tested concentrations (1–2 μM) supported growth comparable to that of unmodified AdoMet (Figs 2 and 3), an unlikely result if hydrolysis is required before they become viable for cell growth. Also, the phenyl ester of AdoMet is somewhat better at supporting cell growth than AdoMet.

The relatively poor utilization of D-Met derivatives compared to that of carboxyl esters of AdoMet may reflect the fact that some classes of methyltransferases bind AdoMet in bent rather than extended conformations (Schubert et al., 2003), and at least one of these enzymes is required for essential processing of tRNAs (Masuda et al., 2013). The poor use of D-Met derivatives is also consistent with mechanistic studies of a family of AdoMet-dependent radical enzymes. AdoMet-mediated radical chemistry is involved in a wide range of essential processes, including protein and nucleic acid modification reactions, lipid metabolism and co-factor synthesis. This enzyme family uses a bidentate interaction between the α-carboxyl and α-amino groups of AdoMet and a bound iron–sulphur cluster to generate an AdoMet-derived 5′-deoxyadenosyl radical intermediate (Walsby et al., 2002). Changing the stereochemistry around the α-carbon of L-Met would significantly alter the orientation of the AdoMet substrate in this binding site.

Where an AdoMet analogue does support growth, the analogue must not only be transported into the cells, but must also be functioning in every AdoMet-dependent reaction that is essential under the growth conditions used.
The results shown here indicate that, particularly during growth in rich medium, many tested l-isomers (AdoMEE, AdoMME, AdoMPE and AdoNAM) can substitute for AdoMet and support E. coli growth quite well. Our results thus allow us to progress with tests of interference with AHL-dependent quorum sensing, and to identify additional candidate selective inhibitors. This strategy is distinct from, and possibly synergistic with, directly inhibiting AHL synthases. More broadly, it is quite striking that such a variety of modified AdoMet molecules can support the full range of essential AdoMet-dependent reactions in E. coli.

ACKNOWLEDGEMENTS

We thank Dr David Wood (University of Southern Alabama) for providing E. coli strain MOB1490. We also thank Dr Randall Worth (University of Toledo) for training on and use of the Zeiss microscope, and Drs Akira Takashima and Jyl Matson (University of Toledo) for use of the FLUOstar and CLARIOstar plate readers. This work was supported by an NIH grant (AI098702) to R.E.V. and R. M. B.

REFERENCES


Edited by: G. Unden