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Methylthioadenosine/S-adenosylhomocysteine nucleosidase, a critical enzyme for bacterial metabolism

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SUMMARY

The importance of Methylthioadenosine/S-adenosylhomocysteine (MTA/SAH) nucleosidase in bacteria has started to be appreciated only in the past decade. A comprehensive analysis of its various roles here demonstrates that it is an integral component of the activated methyl cycle, which recycles adenine and methionine through S-adenosylmethionine (SAM)-mediated methylation reactions, and also produces the universal quorum-sensing signal, autoinducer-2 (AI-2). SAM is also essential for synthesis of polyamines, N-acylhomoserine lactone (autoinducer-1), and production of vitamins and other biomolecules formed by SAM radical reactions. MTA, SAH and 5′-deoxyadenosine (5′dADO) are product inhibitors of these reactions, and are substrates of MTA/SAH nucleosidase, underscoring its importance in a wide array of metabolic reactions. Inhibition of this enzyme by certain substrate analogs also limits synthesis of autoinducers, and hence, causes reduction in biofilm formation and may attenuate virulence. Interestingly, the inhibitors of MTA/SAH nucleosidase are very effective against the Lyme disease causing spirochete, *Borrelia burgdorferi*, which uniquely expresses three homologous functional enzymes. These results indicate that inhibition of this enzyme can affect growth of different bacteria by affecting different mechanisms. Therefore, new inhibitors are currently being explored for development of potential novel broad-spectrum antimicrobials.

Keywords

Methylthioadenosine/S-adenosylhomocysteine nucleosidases; Activated methyl cycle; MTA; SAH (AdoHcy); S-adenosylmethionine; SAM (AdoMet); autoinducer; 5′ deoxyadenosine (5′dADO)

INTRODUCTION

S-adenosylmethionine (SAM) is an important nucleoside that serves as an activated group donor in a broad array of metabolic and biosynthetic reactions, including methylations, propylamine group transfer in polyamine synthesis, and SAM radical-mediated vitamin synthesis. The majority of cellular SAM is used in methylation of macromolecules of prokaryotes and eukaryotes, yielding S-adenosylhomocysteine (SAH) as a product. SAM involvement in synthesis of polyamine and acylhomoserine lactones results in the production of 5′-methylthioadenosine (MTA). I*n vitro* studies have shown that thionucleoside excess inhibits mammalian and bacterial methyltransferase and polyamine synthase activities. Therefore, inhibition of MTA/SAH nucleosidase activity is predicted to

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cause an accumulation of MTA and SAH within bacterial cells and lead to inhibition of methylase and polyamine synthase activities that are growth inhibitory (Beeston & Surette, 2002). In a recent study, the intracellular concentrations of SAM and SAH in the wild-type *E. coli* strain MG1655 (OD_{600}=1.62±0.16) were determined to be 0.4mM, and 1.3μM, respectively (Halliday *et al.*, 2010). A deletion of MTA/SAH nucleosidase in this strain caused an intracellular accumulation of SAH to levels that were approximately 50-fold greater than those found in the wild-type bacteria. These levels would be sufficient to inhibit a variety of bacterial methyltransferases *in vitro* (Reich & Mashhoon, 1991, Reich & Mashhoon, 1990, Simms & Subbaramaiah, 1991). In mammalian cells, buildup of MTA and SAH can affect cAMP metabolism, endothelial expression of adhesin molecules and cytokine secretion (Riscoe *et al.*, 1984, Cerri *et al.*, 1993). Therefore, breakdown of MTA and SAH is critical for the regulation of cellular processes in bacteria as well as in mammals, and these thionucleosides are quickly catabolized so that levels are typically maintained at submicromolar concentrations inside cells. In spite of this, the importance of MTA/SAH nucleosidase (EC 3.2.2.9) remains generally underappreciated. Only recently, it has been reported that the enzyme also catabolizes 5′ deoxyadenosine (5′dADO), the product of SAM radical reactions, suggesting that it is in fact a tri-substrate specific nucleosidase, broadly involved in bacterial metabolism (Choi-Rhee & Cronan, 2005, Challand *et al.*, 2009, Challand *et al.*, 2010). The crucial roles of this enzyme in bacterial metabolism are reviewed here.

Previous studies examined the presence of MTA/SAH nucleosidase and SAH hydrolase (EC 3.3.1.1) among bacterial species with completely sequenced genomes (Sun *et al.*, 2004, Winzer *et al.*, 2002a). Sun and coworkers showed that 51 of 138 bacterial species possess cytoplasmic MTA/SAH nucleosidase, 60 possess SAH hydrolase, while only a few possess both enzymes. Approximately 20% of the examined bacterial species lack both enzymes, but these are primarily symbionts and intracellular pathogens that probably rely on host enzymes for MTA, SAH and 5′dADO catabolism (Sun *et al.*, 2004, Winzer *et al.*, 2002a). *Borrelia burgdorferi* is the only known species of bacteria to possess three homologs of MTA/SAH nucleosidase, two of which are exported (Fraser *et al.*, 1997, Parveen & Leong, 2000, Parveen *et al.*, 2006). In fact, Bgp, a MTA/SAH nucleosidase that also displays affinity for heparin has been shown to be present on the surface of bacteria (Parveen & Leong, 2000, Parveen *et al.*, 2006). Furthermore, all three homologs are functional MTA/SAH nucleosidases [(Parveen *et al.*, 2006, Cornell *et al.*, 2009), and unpublished data], suggesting that these enzymes play a crucial role in the *B. burgdorferi* life cycle.

PRODUCTION OF MTA/SAH NUCLEOSIDASE SUBSTRATES

SAH, MTA and 5′dADO are the byproducts of four major pathways (Fig. 1) and they serve as substrates for MTA/SAH nucleosidase in bacteria. These reactions involve SAM, which is the primary methyl donor for methylation of macromolecules including nucleic acids, proteins, carbohydrates and lipids, and small molecules such as sterols and nucleosides. A comprehensive review of the SAM-dependent methyltransferases of both prokaryotes and eukaryotes has been previously published (Cheng & Blumenthal, 1999). A list of bacterial methyltransferases that play roles in the synthesis of secondary metabolites and other unique molecules is provided here (supplementary Table 1). SAM is also a critical activated group donor required for the synthesis of polyamines (predominantly spermidine in bacteria), N-acyl-homoserine lactone, and various vitamins (biotin, thiamine, etc) derived from SAM radical reaction mechanisms. Four pathways producing MTA/SAH nucleosidase substrates are as follows.

I. Methylation of Nucleic acids

SAM-dependent methyltransferases play critical roles in diverse biological reactions including the methylation of DNA. Methylation of cytosine (C-5 and N-4) and adenine (N-6) has regulatory effects on chromosomal structure, DNA mismatch repair and transcription. Methylation plays a key role in regulation of gene expression and the bacterial cell cycle (Cheng & Roberts, 2001, Jeltsch, 2002, Collier, 2009), and is also required for some nuclease recognition, such as the Type I restriction endonuclease EcoK1 (Loenen, 2006). Methylation of DNA at cytosine and adenine is also a common protective mechanism against damage by other restriction endonucleases (Scavetta *et al.*, 2000). In *Caulobacter crescentus*, cell cycle-regulated DNA methyltransferase (CcrM) is essential for bacterial viability, is transiently expressed at pre-division state, methylates adenine and releases SAH as a byproduct. The newly synthesized hemimethylated DNA is the preferred target of CcrM (Berdis *et al.*, 1998). Post-transcriptional modification of ribosomal RNA (rRNA), especially adenosine residue methylation facilitates bacterial resistance and survival in the presence of ribosome-targeting antibiotics (Vester & Long, 2009, Ero *et al.*, 2008, Yan *et al.*, 2010, Zelinskaya *et al.*, 2010). The X-ray crystal structure of the thiostrepton resistance RNA methyltransferase (Tsr) and *in vitro* analysis of the enzyme-target interactions show that two structural domains in the 23S rRNA contain methylation of specific nucleotides (Dunstan *et al.*, 2009). Methyltransferases are also involved in the transfer of methyl groups to the 16S rRNA (Gregory *et al.*, 2009) and methylation of tRNA of Gram-negative bacteria (Kealey *et al.*, 1994, Bujnicki *et al.*, 2004, Urbonavicius *et al.*, 2005, Zelinskaya *et al.*, 2010, Ta & Kim, 2010).

Methylation of other substrates

SAM is also the major methyl donor for methylation of various proteins, carbohydrates, lipids and other molecules and yields SAH as the byproduct (Fig. 1). For example, the methyltransferase CheR-mediated methylation of the cytoplasmic domain of the methyl-accepting chemotaxis protein (MCP) triggers an adaptive response in *E. coli* to external stimuli (Antommattei *et al.*, 2004). Recently, proteome analysis of the spirochete pathogen *Leptospira interrogans* demonstrated the presence of 155 proteins with arginine methylation (Cao *et al.*, 2010). Methylation of mycobacterial heparin-binding hemagglutinin and laminin-binding adhesin results in their resistance to proteolysis (Pethe *et al.*, 2002). The SAM-dependent methyltransferase Hma of *Mycobacterium tuberculosis* is essential for biosynthesis of both keto- and methoxymycolate components of the cell envelope (Boissier *et al.*, 2006). In *Geobacillus stearothermophilus*, SAM-dependent methylation of terminal L-rhamnose sugars is required for its incorporation into the S-layer glycan chain (Steiner *et al.*, 2008). Microbes under abiotic stress produce the methylation product betaine (N, N, N-trimethylglycine) as an osmoprotectant (Waditee *et al.*, 2003). The opportunistic pathogen *Pseudomonas aeruginosa* uses a SAM-dependent methyltransferase PhzM in pyocyanin pigment production (Parsons *et al.*, 2007). These reactions show SAH production by a variety of methylation reactions.

II. SAM-radical dependent enzymes

A superfamily of radical SAM dependent enzymes has been identified that uses SAM as an oxidizing agent to accomplish a diverse array of reactions including anaerobic oxidations, sulfur insertions, isomerizations, ring formation, and unusual methylations (Sofia *et al.*, 2001). The reactions proceed through a radical intermediate that yields methionine and 5′dADO, which is also a substrate for the nucleosidase (Fig. 1). These radical SAM enzymes are involved in biosynthesis of secondary metabolites, vitamins, antibiotics and DNA repair in bacteria (Wang & Frey, 2007). Biotin synthase (EC 2.8.1.6) is a radical SAM- dependent enzyme responsible for sulfur insertion into desthiobiotin to form biotin (Jarrett, 2005, Layer *et al.*, 2004, Marquet *et al.*, 2001). Lipoate synthase (EC 2.8.1.8) shows sequence homology

to biotin synthase suggesting that it could also be a radical enzyme. In several bacteria, the enzyme tyrosine lyase (EC 4.3.1.23) uses radical SAM to produce precursors of thiamine (Challand *et al.*, 2010). In addition, the *Klebsiella pneumoniae* enzyme PqqE is a SAM radical enzyme involved in the initial step in the biosynthesis of Pyrroquinoline quinone (PQQ) (Wecksler *et al.*, 2009). Accumulation of 5′dADO inhibits activities of these radical SAM enzymes (Choi-Rhee & Cronan, 2005), further emphasizing the importance of MTA/SAH nucleosidase in its metabolism (Fig. 2).

III. Polyamine synthesis

In prokaryotic polyamine synthesis, SAM is first decarboxylated and the resulting 5′ propylamine group is then added to putrescine by spermidine synthases (EC 2.5.1.16) to produce spermidine and the corresponding MTA nucleoside (Fig. 1). MTA is recycled to methionine through different paths depending on the species of the organism (Fig. 2). The levels of spermidine and putrescine are tightly controlled in bacteria, and their concentrations within *E. coli* were reported to be 4.2mM and 28.4mM, respectively (Davis *et al.*, 1992). Polyamines were suggested to exist bound to nucleic acids in the intact cell with negligible amount existing as the free form in the cytoplasm (Kashiwagi *et al.*, 1986). Therefore, the effect of intracellular MTA accumulation on polyamine production in bacteria is difficult to assess. However, mutation in the *pfs* gene in *E. coli* resulted in a significant growth defect (Cadieux *et al.*, 2002). Furthermore, MTA accumulation was proposed to inhibit polyamine synthases in bacteria, but the experimental evidence was not provided (Beeston & Surette, 2002).

IV. Production of N-acylhomoserine lactone

MTA is also a byproduct of N-acylhomoserine lactone synthases (EC 2.3.1.184) in Gram-negative bacteria, as previously described in two comprehensive reviews (Winans & Bassler, 2002, Fuqua *et al.*, 2001). N-acylhomoserine lactone (autoinducer 1, AI-1) was identified as the first cell-cell communication or quorum-sensing (QS) signal in bacteria, where it functions in a largely species-specific manner (Nealson & Hastings, 1979).

EFFECT OF ACCUMULATED MTA, SAH, and 5′dADO

Accumulation of MTA, SAH and 5′dADO can have wide-ranging physiological consequences. SAH is a potent feedback inhibitor of SAM-dependent methylation reactions, both in bacteria and mammals (Borchardt, 1986). While data on the *in vivo* effect of SAH inhibition on methyltransferase activity in bacteria is sparse, the assumption has been made that the bacterial methyltransferases will be affected in a similar fashion to the more extensively studied eukaryotic methyltransferases. This is supported by data from *in vitro* enzymology demonstrating that a number of bacterial methyltransferases are susceptible to SAH inhibition at low micromolar concentrations (Reich & Mashhoon, 1991, Simms & Subbaramaiah, 1991, Reich & Mashhoon, 1990). This assumption is further validated by several reports of the development of colorimetric assays that use MTA/SAH nucleosidase to alleviate product inhibition of methylation reactions *in vitro* (Dorgan *et al.*, 2006, Hendricks *et al.*, 2004). More recently, an *E. coli* MTA/SAH nucleosidase knockout strain has been shown to accumulate SAH to more than 50μM, a concentration that is approximately 50-fold greater than that of the isogenic wild-type. Conversely, SAM, SRH, and methionine levels were decreased in the knockout strain, probably due to the loss of the salvage pathway (Halliday *et al.*, 2010).

MTA is also a potent product inhibitor of polyamine synthase reactions at low micromolar concentrations (Pajula & Raina, 1979, Raina *et al.*, 1982). The subsequent limitation of polyamine availability influences DNA replication, leading to growth arrest. In addition,

MTA is a potent feedback inhibitor of AI-1 synthase, inhibiting 50% of the enzyme activity at concentrations as low as 5μM (Hanzelka *et al.*, 1999). Although this latter effect probably has little influence on growth rates in Gram negative bacteria, MTA inhibition of AI-1 synthase could significantly alter quorum sensing-dependent phenotypes such as biofilm formation and virulence.

The buildup of 5′dADO resulting from radical SAM reactions reduces vitamin availability required for numerous metabolic reactions and probably alters flux rates through central glycolytic pathways, resulting in reduced cellular growth (Choi-Rhee & Cronan, 2005). To prevent these growth inhibitory effects, all organisms that produce SAH, MTA, and 5′dADO also possess or exploit mechanisms for removal of these compounds.

CATABOLISM OF THIONUCLEOSIDES BY MTA/SAH NUCLEOSIDASE

After examination of 138 bacterial species for which the genomes have been completely sequenced and annotated, the majority of bacterial species (80%) were predicted to use either Pfs (MTA/SAH nucleosidase) or SAH hydrolase to breakdown SAH (Sun *et al.*, 2004, Winzer *et al.*, 2002a) emphasizing the importance of SAH hydrolysis in cellular metabolism. Sun et al (2004) suggested that the remaining 20% of bacterial species, which mainly represent symbionts and intracellular parasites, depend on the host to recycle these toxic intermediates. In 51 bacterial species, including the majority of the pathogens with reductive genome evolution, a MTA/SAH nucleosidase enzyme is responsible for the metabolism of both MTA and SAH (Cornell *et al.*, 1996a, Cornell *et al.*, 1996b, Ferro *et al.*, 1976, Della Ragione *et al.*, 1985, Sun *et al.*, 2004). Bacterial MTA/SAH nucleosidases show comparable efficiency in hydrolyzing the glycosidic bond in these substrates to yield adenine and the corresponding sugar (Lee *et al.*, 2005a, Cornell & Riscoe, 1998, Cornell *et al.*, 1996a, Parveen *et al.*, 2006). In addition, the MTA/SAH nucleosidase substrate 5′dADO is produced as a result of SAM radical enzyme activities (Figs. 1 and 2). In *P. aeruginosa, L. interrogans,* several Archaea, and a number of other bacterial species that possess relatively large genomes (>6Mb) and exhibit complex metabolism, MTA phosphorylase (EC 2.4.2.28) converts MTA to 5-methylthioribose-1-phosphate (MTRP) in a reversible phosphate dependent reaction (Sekowska *et al.*, 2004, Cacciapuoti *et al.*, 2003) with adenine as a byproduct. In these organisms, SAH is broken down by SAH hydrolase and results in the production of homocysteine and adenosine. Similarly, in various eukaryotes, including mammals, MTA phosphorylase and SAH hydrolase catabolize MTA and SAH, respectively (Sufrin *et al.*, 1995, Riscoe, 1989, Stepkowski *et al.*, 2005). These separate enzymes have narrow substrate specificities in both environmental bacteria and eukaryotic counterparts, but are more efficient in metabolizing MTA and SAH. Efficient recycling of MTA and SAH probably facilitates the survival of these versatile bacteria under varying environmental conditions (Stepkowski *et al.*, 2005).

In a variety of organisms, quick removal of MTA and salvage of the methionyl moiety is facilitated by hydrolysis of MTA into 5-methylthioribose (MTR) and subsequent phosphorylation of MTR to MTRP by MTR kinase (EC 2.7.1.100), as shown in the Fig. 2. Four to five enzymes then convert MTRP into methionine in a sequential manner that has been best elucidated in *Klebsiella* and *Bacillus*, but appears to be common to all organisms with a complete salvage pathway (Albers, 2009,Cornell *et al.*, 1996b,Gianotti *et al.*, 1990,Sekowska *et al.*, 2004,Furfine & Abeles, 1988). Other organisms that exist in sulfur rich environments, such as *E. coli*, often do not possess MTR kinase, and thus do not salvage methionine from MTA, but rather secrete MTR (Hughes, 2006).

SAH CATABOLISM THROUGH ACTIVATED METHYL CYCLE

The activated methyl cycle (AMC) is responsible for producing SAM required for methylations, and the recycling of the product SAH back to methionine (Fig. 3). Methionine is converted by SAM synthetase (MetK, EC 2.5.1.6) to SAM using ATP as a substrate and energy from the hydrolysis of high-energy phosphate bonds to drive the reaction. In several environmental bacteria, and some plant and animal pathogens/symbionts, such as *Caulobacter*, *Pseudomonas*, *Xanthomonas*, *Rhizobium* and *Brucella* spp., SAH hydrolase is used to directly convert SAH to homocysteine (Sun *et al.*, 2004). However, in a majority of eubacteria, detoxification of SAH is carried out by MTA/SAH nucleosidase to produce S-ribosylhomocysteine (SRH) (Sun *et al.*, 2004,Winzer *et al.*, 2002a,Markham & Pajares, 2009). The enzyme LuxS (EC 4.4.1.21) further cleaves SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2 (Fig. 3). Homocysteine is then recycled back to methionine using cobalamine-dependent MetH (EC 2.1.1.13) or cobalamine-independent MetE (EC 2.1.1.14) methionine synthases (Kamarthapu *et al.*, 2008). Only strains of *H. pylori*, *Streptococcus pyogenes* and *Enterococcus faecalis* lack MetE and MetH enzymes (Winzer *et al.*, 2002a,Sun *et al.*, 2004). In several bacterial species, AMC is also the sole sulfur source, such that either methionine or homocysteine is converted to cysteine through a series of enzymatic reactions (Hullo *et al.*, 2007,Doherty *et al.*, 2010,Sewald *et al.*, 2007,Markham & Pajares, 2009).

MTA/SAH NUCLEOSIDASE IN QUORUM-SENSING

Quorum-sensing (QS) is a cell density-dependent communication system of bacteria. Some Gram-negative bacteria are known to secrete as many as three signaling molecules or autoinducers (AI): AI-1, AI-2, and AI-3. The accumulation of these autoinducers as a function of culture cell density results in signaling cascades that ultimately change cellular gene expression profiles and lead to population wide adaptive responses (Winzer *et al.*, 2002a, Chen *et al.*, 2002, Sun *et al.*, 2004, Miller *et al.*, 2002, Zhu *et al.*, 2002, Waters & Bassler, 2005, Ng & Bassler, 2009). QS systems regulate pathogen-host cell interactions, bacterial virulence, and the formation of bacterial biofilms. N-Acylhomoserine lactone or AI-1 was first discovered in the marine bioluminescent bacterium *Vibrio fischeri* (Nealson & Hastings, 1979). AI-1 is produced by acylhomoserine lactone synthase (LuxI) in *V. fischeri*, or by equivalent enzymes in other bacteria. SAM is a substrate and hexanoyl Acyl carrier protein (ACP) acts as the acyl donor in a reaction that generates AI-1 and MTA as the byproduct (Fig. 1). After reaching a critical threshold, AI-1 interacts with the receptor LuxR and this complex then induces expression of the *lux* operon encoding luciferase (Fuqua & Greenberg, 2002).

Both Gram-positive and Gram-negative bacteria possess AI-2-mediated QS systems derived from SAH catabolism. Therefore, AI-2 is often known as the “universal” QS-signaling molecule (Winzer *et al.*, 2002a, Sun *et al.*, 2004). In addition to methyltransferases, three enzymes, SAM synthase (MetK), MTA/SAH nucleosidase (Pfs) and LuxS in AMC are involved in the synthesis of 4,5-dihdroxy-2,3-pentanedione from methionine (Fig. 3). SAH hydrolysis by Pfs (MTA/SAH nucleosidase) yields S-ribosylhomocysteine (SRH), a precursor of autoinducer-2 synthesis, a quorum-sensing signal that governs a variety of bacterial phenotypes such as virulence and biofilm formation as reviewed previously (Gospodarek *et al.*, 2009, Federle, 2009, Federle & Bassler, 2003). AI-2 is produced as a result of spontaneous cyclization of 4,5-dihdroxy-2,3-pentanedione to methyltetrahydroxy furan, and in boron rich environments on to the corresponding borate diester (Fig. 3). In the Lyme disease causing spirochete, *B. burgdorferi*, the three genes of the AI-2 pathway (*metK, pfs, luxS*) are present in one operon producing all three as cytoplasmic enzymes, while the other two Pfs homologs, Bgp and MtnN, in *B. burgdorferi* are exported proteins. Genes

encoding *bgp* and *mtnN* are located elsewhere in the spirochete genome and are not part of any operon (Riley *et al.*, 2007, Fraser *et al.*, 1997, Winzer *et al.*, 2002a). In *Porphyromonas gingivalis*, the operon contains only *pfs* and *luxS* genes, indicating simultaneous production of the encoded enzymes (Riley *et al.*, 2007, Fraser *et al.*, 1997, Winzer *et al.*, 2002a). Indeed, transformation of *P. aeruginosa* with the *pfs-lux* operon of *P. gingivalis*, reconstitution of *luxS* mutant *E. coli* strains with *luxS* gene from other genera, and magnetic nanofactories cell capture modules assembled with Pfs-LuxS fusion chimeras, all showed that these two proteins are sufficient to produce AI-2 from SAH (Winzer *et al.*, 2002a, Fernandes & Bentley, 2009).

Pfs is an integral component of the AI-2 synthesis pathway. Indeed, AI-2 production shows a tighter correlation with Pfs transcription than *luxS* gene expression in several bacteria including *Salmonella enterica serovar typhimurium* and *Streptococcus suis* (Beeston & Surette, 2002, Han & Lu, 2009, Kim *et al.*, 2006). In *B. burgdorferi*, *luxS* mutations affect transcription of several genes but this change in the transcriptome did not seem to affect transmission of bacteria through ticks or pathogenesis of the spirochete in the mouse model of infection (Babb *et al.*, 2005, Stevenson & Babb, 2002, Hubner *et al.*, 2003). The *luxS* mutant of *H. pylori* also did not show any detectable change in its gene expression pattern relative to the wild-type strain (Joyce *et al.*, 2000, Forsyth & Cover, 2000). However, knock-out mutants of *luxS* genes in V. cholerae, S. pyogenes, S. pneumoniae, N. meningitidis, and *C. perfringens* exhibited severe defects in the expression of genes encoding virulence factors (Miller *et al.*, 2002, Zhu *et al.*, 2002, Lyon *et al.*, 2001, Marouni & Sela, 2003, Stroeher *et al.*, 2003, Winzer *et al.*, 2002c, Ohtani *et al.*, 2002). In the meningitis and septicemia causing bacterium *Neisseria meningitidis*, AI-2 release was completely eliminated in the *pfs* mutant (Heurlier *et al.*, 2009). These results are further supported by the report that cultures of *V. cholerae* and enterohemorrhegic *E. coli* (EHEC) strain O157:H7 treated with MTA/SAH nucleosidase inhibitors did not synthesize AI-2 and showed reduced biofilm formation (Gutierrez *et al.*, 2009). However, the growth defects observed in the *N. meningitidis pfs* mutant, and probably also of the *luxS* mutant, were not attributable to loss of AI-2 synthesis but rather due either to the accumulation of toxic SAH and MTA or to metabolic imbalances within the bacteria (Heurlier *et al.*, 2009, Winzer *et al.*, 2002a, Winzer *et al.*, 2002b, Dove *et al.*, 2003, Li *et al.*, 2008).

The majority of bacteria possess either the single step SAH hydrolase that yields adenosine and homocysteine, or the two-step enzymatic system mediated by Pfs and LuxS to recycle the toxic SAH metabolite produced by the AMC. However, a few bacteria appear to possess both systems (Sun *et al.*, 2004). Winzer and coworkers (2002) suggested that the physiological and metabolic state of the organism determines the uptake of different products using ABC transporters or equivalent import systems, after breakdown of SRH by LuxS. In addition, both uptake and degradation of these molecules occurs in a controlled manner (Surette & Bassler, 1999, Surette *et al.*, 1999, Surette & Bassler, 1998, Lyon *et al.*, 2001, Winzer *et al.*, 2002a, Winzer *et al.*, 2002b, Wang *et al.*, 2005, Xavier & Bassler, 2005, Taga *et al.*, 2003, Taga *et al.*, 2001). Therefore, the primary role of LuxS was predicted to be in metabolic pathways, especially in the AMC to produce homocysteine for conversion back to methionine. Several reports have now provided supporting evidence for the metabolic function of Pfs and LuxS in recycling the sulfur group. Although AI-2 production has been evaluated in a wide variety of bacteria by using *V. harveyi* reporter strains, the proof of a functionally active set of proteins from sensor/receptor to response regulators required for AI-2-signalling cascade has not yet been reported in organisms other than *Vibrio* spp. (Sun *et al.*, 2004). Therefore, there is no direct evidence that the majority of bacteria actually respond to AI-2 as a QS signal. However, Pfs expression shows a strong correlation with AI-2 synthesis, purine and nutrient availability, and a *pfs*-mutant of *E. coli* failed to grow in the absence of exogenous methionine (Cadieux *et al.*, 2002, Kim *et al.*,

2006, Han & Lu, 2009). Furthermore, exogenous supply of AI-2 to cultures failed to rescue the growth defect and competitiveness of *N. meningitidis pfs* and *luxS* mutants. These results indicate that intracellular metabolite imbalance rather than the lack of AI-2-mediated QS signaling was responsible for the growth defect of the *N. meningitidis* mutants (Heurlier *et al.*, 2009). Since SAH was not detectable in the wild-type strain, accumulation of this metabolite, or potentially MTA, was suggested to be responsible for the growth defect of the *pfs* mutant of *N. meningitidis*. Furthermore, the *luxS* mutant of *Campylobacter jejuni* exhibited metabolic effects and inhibition of methionine cycle gene transcription was reported (Holmes *et al.*, 2009).

MTA/SAH NUCLEOSIDASE AS TARGET OF NOVEL ANTIMICROBIALS

Soon after the first purification of MTA nucleosidase from *E. coli*, even before SAH was identified as its second substrate, MTA analogs were recognized as effective competitive inhibitors of this enzyme (Ferro *et al.*, 1976). A further analysis of 25 analogs of naturally occurring thioesters showed that several were potent inhibitors of the nucleosidase activity (Della Ragione *et al.*, 1985). MTA/SAH nucleosidase and MTA phosphorylase have been purified and characterized from a variety of bacteria (Appleby *et al.*, 2001, Cacciapuoti *et al.*, 2007, Cacciapuoti *et al.*, 2003, Cacciapuoti *et al.*, 1999, Lee *et al.*, 2001, Lee *et al.*, 2005c). Extensive x-ray crystallographic analysis (Lee *et al.*, 2003, Lee *et al.*, 2001, Lee *et al.*, 2005c, Lee *et al.*, 2005b, Singh *et al.*, 2007, Singh & Schramm, 2007, Singh *et al.*, 2005) unequivocally showed that the MTA/SAH nucleosidase enzyme exists as a dimer. Importantly, the active site of each subunit contains a hydrophobic pocket that appears to play a role in the discrimination of different adenosyl nucleoside substrates and is partially composed of residues from the second subunit. The acidic residues involved in catalysis are highly conserved across species, and the substrate hydrolysis appears to be essentially irreversible (Cornell *et al.*, 1996a, Lee *et al.*, 2005c, Singh *et al.*, 2005). In contrast, MTA phosphorylases appear to be trimeric and display reversible reactions (Cacciapuoti *et al.*, 2003, Cacciapuoti *et al.*, 1999, Cacciapuoti *et al.*, 2007). MTA analog(s) interaction with the purified *E. coli* enzyme indicated the transition state of the enzyme-substrate complex (Allart *et al.*, 1998). Lee and coworkers solved the crystal structure of the enzyme complexed with adenine and with the substrate analog 5′-methylthiotubercidin (MTT) and transition state analog, formycin A (FMA) (Lee *et al.*, 2003, Lee *et al.*, 2001) to determine the molecular interactions involved in substrate recognition. Additional analysis using a series of early and late transition state analogs and kinetic isotope effects, allowed differentiation of the transition states of *N. meningitides* and *H. pylori* nucleosidases that showed an early dissociative transition state and was more similar to bovine purine nucleoside phosphorylases (PNP, EC 2.4.2.1). In contrast, the nucleosidases from *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* demonstrated a fully dissociative transition state that was shared with human PNP and MTA phosphorylase (Singh *et al.*, 2007, Singh & Schramm, 2007, Singh *et al.*, 2005, Gutierrez *et al.*, 2009, Luo & Schramm, 2008).

Despite similarities in the enzyme transition states, crystallographic evidence points to distinct structural differences in the enzyme active sites between the bacterial nucleosidase and mammalian phosphorylase. A study by Lee *et al.* (2004) compared the crystallographic structures of the *E. coli* MTA/SAH nucleosidase to the mammalian MTA phosphorylase (Lee *et al.*, 2004). The active site in the nucleosidase was shown to have a larger 5′ alkylthio binding pocket than MTA phosphorylase, supporting the observation that the bacterial enzyme functions on thionucleosides such as SAH that have extended 5′ structures, whereas the mammalian enzyme has a much narrower substrate specificity. In addition, electrostatic maps showed a negatively charged region in MTA/SAH nucleosidase around the substrate 2′ hydroxyl recognition site, which was positively charged in MTA phosphorylase. These combined differences could be exploited for drug design. More recent work has reported

potent late stage transition state analogs with bulky 5′ substitutions (DADMe-immucillin 54; DADMe-immucillin 57) that can indeed discriminate between the *E. coli* and human enzymes (Longshaw *et al.*, 2010). These analogs show either no activity against MTA phosphorylase and picomolar dissociation constants for MTA/SAH nucleosidase (DADMe-immucillin 54), or nanomolar dissociation constants for the human enzyme that are greater than a thousand-fold higher than for the bacterial enzyme (DADMe-immucillin 57).

Structure-based design was also employed to identify highly potent indazole, purine, and deazapurine-based inhibitors for MTA/SAH nucleosidase by screening virtual compound libraries (Li *et al.*, 2003, Tedder *et al.*, 2004). The most potent of these inhibitors displayed low nanomolar concentration dissociation constants for the enzyme. However, the antimicrobial activity of the inhibitors was only modest. Reported MIC values against *N. meningitides*, *S. pneumoniae*, and *S. pyogenes* were at best 1-2 micromolar, and more frequently were greater than 20-30 micromolar.

Antimicrobial activity reported for immucillin-based transition state analogs also appears modest, with little effect on bacterial planktonic cell growth, possibly due to poor drug transport (Gutierrez *et al.*, 2009). Addition of a million fold concentrations of picomolar transition state inhibitors did not affect the growth of *V. cholerae.* However, these inhibitors bound tightly to MTA/SAH nucleosidase in *V. cholerae* and EHEC *E. coli* O157:H7, and significantly disrupted AI-2 production and biofilm formation at picomolar concentrations (Gutierrez *et al.*, 2009, Schramm *et al.*, 2008). These results demonstrate that sufficient drug influx is occurring to effect metabolic changes. These drugs could be developed into novel antimicrobials based upon their mechanism of action in disrupting AI-2 dependent pathogenicity, rather than direct bactericidal activity. Furthermore, the inhibitors may exert their effects by restricting degradation of 5′ dADO, leading to altered rates of vitamin synthesis that ultimately affect central carbon metabolism. This may explain some of the observations on changes to bacterial growth in response to pharmacologic or genetic blockade of MTA/SAH nucleosidases made by Cadieux *et al*. (2002), Gutierrez *et al.* (2009) and Heurlier *et al.* (2009). Evaluation of the substrate analogs and transition state analogs effective against MTA/SAH nucleosidases of *B. burgdorferi* led to identification of compounds that either inhibited growth of these spirochetes or showed bactericidal activities (Cornell *et al.*, 2009). Some of these inhibitors showed more potent activities than those exhibited on *E. coli*, perhaps due to the underlying purine auxotrophy in *B. burgdorferi*, which makes it more critical for these spirochetes to salvage methionine and adenine through the nucleosidase. In addition, there are three homologous MTA/SAH nucleosidases in *B. burgdorferi*, two of which are exported and at least one, Bgp, which is present on the surface of the spirochete, presumably to help scavenge purines from the host or the vector (Parveen & Leong, 2000, Parveen *et al.*, 2006). Therefore, in *B. burgdorferi* drug permeability may not be an issue.

Overall, these reports suggest that inhibitors of MTA/SAH nucleosidase could possibly become new anti-infective drugs against various bacterial pathogens. Interestingly, Rrp1, which produces the secondary messenger cyclic diguanylate (c-di-GMP) and the hybrid histidine kinase-response regulator (Hpk1) are involved in the upregulation of expression of Bgp at the cusp of infection, i.e., immediately after tick takes bloodmeal from the mammals (Rogers *et al.*, 2009). Thus, these novel drugs could inhibit infection in the early state in some organisms and restrict QS systems and biofilm formation later in infection in others.

CONCLUDING REMARKS

SAM is an essential nucleoside required for growth of all living organisms due to its involvement in critical biological methylations, polyamine synthesis, and a broad array of

other reactions. MTA, SAH, and 5′dADO are toxic byproducts of these reactions that can even be lethal. Most prokaryotes possess either MTA/SAH nucleosidase and/or a combination of SAH hydrolase and MTA phosphorylase to remove these inhibitory nucleosides. In addition, MTA/SAH nucleosidase is an integral component of the AMC responsible for recycling methionine from homocysteine, and is involved in AI-2 synthesis. Indeed, transcription of the MTA/SAH nucleosidase gene, *pfs*, and not *luxS*, is tightly correlated with AI-2 production in different bacteria. Therefore, MTA/SAH nucleosidase is being explored as a target for potential novel drugs that could show anti-infective activity due to accumulation of toxic byproducts within the cells or by prevention of AI-2-mediated processes, such as virulence factor synthesis and biofilm formation by the pathogen later in infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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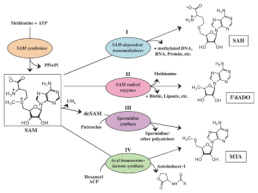

Fig. 1. Four metabolic pathways produce substrates of MTA/SAH nucleosidase

Four pathways involving SAM: (I) methylation of macromolecules, (II) reactions producing different metabolites by SAM radical enzymes, (III) polyamine (including spermidine) synthesis, and (IV) autoinducer-1 (N-acylhomoserine lactone) synthesis. All result in production of nucleosidase substrates: SAH, MTA or 5′dADO (boxed). Methylation of various macromolecules using specific transmethylases produces SAH as a byproduct. SAM radical enzymes produce vitamins, metabolites and antibiotics with methionine and 5′dADO as byproducts. Polyamine synthesis is a two-step process, (i) decarboxylation of SAM followed by (ii) conversion of putrescine and decarboxylated SAM to spermidine and MTA. Hexanoyl Acyl carrier protein (ACP) donates theAcyl group to SAM to form N-acylhomoserine lactone.

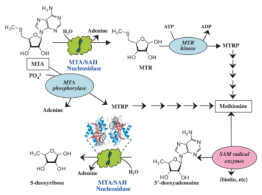

Fig. 2. Recycling of MTA and 5′dADO by MTA/SAH nucleosidase

In the majority of bacteria, MTA is first converted by MTA/SAH nucleosidase into methylthioribose (MTR). In species with a complete methionine salvage cycle, MTR is subsequently phosphorylated by MTR kinase to 5-methylthioribose-1-phosphate (MTRP). The thiomethyl group from MTRP is recycled in a multistep pathway back to methionine. This critical amino acid is also produced as a byproduct by SAM radical enzyme reactions during synthesis of vitamins and other metabolites. The byproduct, 5′dADO, is also a substrate of MTA/SAH nucleosidase and this enzymatic reaction produces 5′deoxyribose and adenine. In eukaryotes and some environmental bacteria, MTA is catabolized by MTA phosphorylase to MTRP in a single step, which is then converted through the same enzymatic steps to methionine. A ribbon diagram of the MTA/SAH nucleosidase with bound substrate, MTA, (marked by oval) is shown.

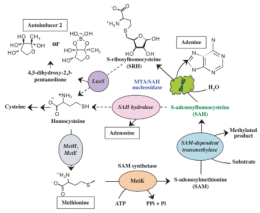

Fig. 3. MTA/SAH nucleosidase is involved in Activated Methyl Cycle

Methionine is converted into SAM by SAM synthetase (MetK). Donation of the methyl group of SAM to a variety of methyl acceptors results in SAH, which is recycled back to homocysteine in either a one-step or two-step process. In several bacterial species SAH hydrolase directly converts SAH into homocysteine. However, in the majority of eubacteria, MTA/SAH nucleosidase first converts SAH into S-ribosylhomocysteine (SRH), which is then recycled back to homocysteine by LuxS. As a byproduct of this reaction, 4,5 dihydroxy 2,3-pentanedione is produced which spontaneously forms autoinducer-2 (AI-2). In some bacteria, cysteine is produced from homocycteine by multi-step enzymatic reactions since this cycle is the only source of sulfur-containing compounds. Homocycteine is recycled back to methionine in different organisms by the Met E or MetH methionine synthases.