Identification and Characterization of the *Chlamydia trachomatis* L2 *S*-Adenosylmethionine Transporter

Rachel Binet, Reinaldo E. Fernandez, Derek J. Fisher, and Anthony T. Maurelli

Department of Microbiology and Immunology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA

ABSTRACT Methylation is essential to the physiology of all cells, including the obligate intracellular bacterium *Chlamydia*. Nevertheless, the methylation cycle is under strong reductive evolutionary pressure in *Chlamydia*. Only *Parachlamydia acanthamoebae* and *Waddlia chondrophila* genome sequences harbor homologs to *metK*, encoding the *S*-adenosylmethionine (SAM) synthetase required for synthesis of SAM, and to *sahH*, which encodes the *S*-adenosylhomocysteine (SAH) hydrolase required for detoxification of SAH formed after the transfer of the methyl group from SAM to the methylation substrate. Transformation of a conditional-lethal Δ*metK* mutant of *Escherichia coli* with a genomic library of *Chlamydia trachomatis* L2 identified CTL843 as a putative SAM transporter based on its ability to allow the mutant to survive *metK* deficiency only in the presence of extracellular SAM. CTL843 belongs to the drug/metabolite superfamily of transporters and allowed *E. coli* to transport *S*-adenosyl-L-[methyl-^{14}C]methionine with an apparent K_m of 5.9 μM and a V_{max} of 32 pmol min^{-1} mg^{-1}. Moreover, CTL843 conferred a growth advantage to a Δ*pfs E. coli* mutant that lost the ability to detoxify SAH, while competition and back-transport experiments further implied that SAH was an additional substrate for CTL843. We propose that CTL843 acts as a SAM/SAH transporter (SAMHT) serving a dual function by allowing *Chlamydia* to acquire SAM from the host cell and excrete the toxic by-product SAH. The demonstration of a functional SAMHT provides further insight into the reductive evolution associated with the obligate intracellular lifestyle of *Chlamydia* and identifies an excellent chemotherapeutic target.

IMPORTANCE Obligate intracellular parasites like *Chlamydia* have followed a reductive evolutionary path that has made them almost totally dependent on their host cell for nutrients. In this work, we identify a unique transporter of a metabolite essential for all methylation reactions that potentially bypasses the need for two enzymatic reactions in *Chlamydia*. The transporter, CTL843, allows *Chlamydia trachomatis* L2 to steal *S*-adenosylmethionine (SAM) from the eukaryotic host cytosol and to likely remove the toxic *S*-adenosylhomocysteine (SAH) formed when SAM loses its methyl group, acting as a SAM/SAH transporter (SAMHT). In addition to reflecting the adaptation of *Chlamydia* to an obligate intracellular lifestyle, the specific and central roles of SAMHT in *Chlamydia* metabolism provide a target for the development of therapeutic agents for the treatment of chlamydial infections.

Chlamydiae live as obligate intracellular parasites in specialized vacuoles within eukaryotic cells. The vast majority of species within this phylum of Gram-negative bacteria cause diseases in animals and/or humans and belong to the genus *Chlamydia*, although the list of *Chlamydia*-like organisms capable of causing disease continues to expand (1). *Chlamydia trachomatis* causes severe ocular and urogenital infections in humans and was the first *Chlamydia* species to be fully sequenced (2). Since that time, the sequences of 26 additional chlamydial genomes have been released in public databases (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). All chlamydiae show signs of incomplete biosynthetic pathways, similar to other organisms that have adapted to parasitic/symbiotic lifestyles, such as mycoplasmas, phytoplasmas, and alpha- and gammaproteobacteria, including rickettsiae. Although novel enzymes have evolved to bypass some of these metabolic "holes" (3), the reduced

genome size of obligate intracellular organisms is possible mostly because many metabolites do not need to be synthesized by the complex pathways characteristic of free-living bacteria but instead are transported from the substrate-rich host cell cytoplasm by novel transport systems absent in free-living bacteria (4). For example, a specific nucleotide transport protein allows *Protochlamydia amoebophila* to acquire the universal electron carrier NAD (NAD$^+$) from the host in exchange for bacterial ADP, rather than synthesizing this compound (5).

S-Adenosyl-L-methionine (also known as AdoMet or SAM) is an essential intermediate in the physiology of all cells (6). The majority of SAM is used for methyltransferase reactions in which the S-methyl group of SAM is transferred to acceptor substrates, including nucleic acids, proteins, phospholipids, biological amines, and a long list of small molecules. During the transmethylation reaction, SAM is converted to *S*-adenosylhomocysteine

(also known as AdoHcy or SAH) by the removal of the S-methyl group. SAH is a potent inhibitor of SAM-dependent methyltransferases and is therefore rapidly detoxified either by a SAH hydrolase or by a methylthioadenosine (MTA)/SAH nucleosidase (7, 8). Although SAM is not likely to be used as a methyl group donor for DNA in *Chlamydia* due to the absence of DNA methyltransferases (9), we recently showed that methylation of *C. trachomatis* 16S rRNA by the bacterial KsgA methylase is critical for bacterial fitness (10), confirming an earlier report that SAM-dependent methylation reactions take place in chlamydiae (11).

In this study, we examined the methylation cycle in *Chlamydia*. Comparative genome analysis showed that the methylation cycle is under reductive evolutionary pressure in *Chlamydia*. Only two *Chlamydia*-like organisms, *Parachlamydia acanthamoebae* and *Waddlia chondrophila*, appear to possess both an *S*-adenosylmethionine synthetase, the conserved but energetically demanding enzyme that catalyzes the only known route of SAM synthesis (12), and a recognized SAH catabolizing enzyme. Due to the lack of genetic tools for manipulating chlamydiae, we used *Escherichia coli* as a surrogate host to identify the strategy employed by the majority of chlamydiae to acquire SAM and eliminate SAH. By use of *C. trachomatis* serovar L2 strain 434/Bu as a model, CTL843 was identified through a genetic screen in a SAM synthetase-deficient *E. coli* strain and shown to mediate transport of SAM and likely SAH, acting as a SAMHT (SAM/SAH transporter). While serving as another example of the specialization of *Chlamydia* within its eukaryotic cell niche, the discovery of a novel means of *Chlamydia* host-cell interaction also presents an excellent chemotherapeutic target for the treatment of chlamydial infections.

RESULTS

The methylation cycle is under reductive evolutionary pressure in *Chlamydia*. We recently showed that the conserved dimethyltransferase KsgA plays an important role in *Chlamydia* biology (10). KsgA transfers two methyl groups from the activated methyl donor SAM to the 16S rRNA molecules in ribosomes, generating the toxic by-product SAH. *S*-Adenosylmethionine synthetase (MAT or AdoMet synthetase [EC 2.5.1.6]) is the enzyme that synthesizes SAM from methionine and ATP. MAT is a highly conserved protein that is well studied at the primary, secondary, and tertiary structural levels and is an essential enzyme in prokaryotes and eukaryotes (12, 13). Comparative analysis of *Chlamydia* genome sequences identified open reading frames (ORFs) sharing 60% identity with *E. coli* MAT in *P. acanthamoebae* and *W. chondrophila* (i.e., pah_c014o152 and wcw_0127, respectively) and harboring all the conserved amino acid residues required for SAM synthetase activity (see Fig. S1 in the supplemental material). On the other hand, no MAT homolog was found in the other chlamydial genome sequences available (as of March 2011), with the exception of *P. amoebophila*, where a gene encoding a truncated and likely nonfunctional version of MAT (i.e., Pc1819) was identified.

Because SAH formed from SAM during methylation reactions needs to be eliminated due to its inhibitory effect on methyltransferases, we also looked for the presence of genes encoding putative SAH-detoxifying enzymes in *Chlamydia* genome sequences. Two different classes of enzymes degrade SAH in living cells. Most microbes, including *E. coli*, harbor an MTA/SAH nucleosidase (MTAN; EC 3.2.2.9) to metabolize SAH to adenine and

S-ribosylhomocysteine, whereas mammals and some microbes employ a specific SAH hydrolase (SahH) (EC 3.3.1.1) to metabolize SAH into homocysteine and adenosine. Although putative MTAN ORFs are identified in the genome annotation of *Chlamydia felis* (i.e., CF0410), *Chlamydia caviae* (i.e., CCA0593), and *Chlamydia pneumoniae* (i.e., Cpn0232 for *C. pneumoniae* TW-183), sequence alignments revealed that most of the conserved residues required for MTAN activity in *Arabidopsis thaliana*, *E. coli*, and *Staphylococcus aureus* were missing in these chlamydial homologs (Fig. S2). Further examination revealed that the products of these three chlamydial ORFs belong to the PNP_UDP_1 superfamily (PF01048), which includes purine nucleoside phosphorylase (PNP), uridine phosphorylase (UdRPase), and 5′-methylthioadenosine phosphorylase (MTA phosphorylase). Thus, they are unlikely to be involved in SAH hydrolysis. Consistent with the presence of MAT in *P. acanthamoebae* and *W. chondrophila*, SahH homologs possessing the conserved motifs representative of active SahH enzymes were identified in these organisms, i.e., pah_c022o240 and wcw_1005, respectively (Fig. S3). Since out of 26 chlamydial genomes analyzed, only two, *P. acanthamoebae* and *W. chondrophila*, appear to have the genes for a complete methylation cycle (SAM synthesis, methylation activity, and SAH detoxification), the question is raised as to how the majority of *Chlamydia* species (which all possess at least one SAM-utilizing enzyme), including *C. trachomatis*, synthesize SAM and detoxify SAH.

Positive selection of *C. trachomatis* L2 *metK*-complementing ORF in *E. coli*. *metK* is essential in *E. coli*, and *E. coli* cells are impermeable to extracellular SAM (14–16). To construct a conditional-lethal Δ*metK* mutant of *E. coli*, the rescuing copy of *metK* needs to be tightly controlled. Although *metK* deletion was first obtained in ATM770 (Table 1), this strain was still able to grow in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside), indicating that the lactose promoter controlling *metK* in pREF71 was still leaky in the absence of inducer (data not shown), as previously encountered with the *ara* promoter (16). On the other hand, by placement of a copy of the *metK* gene containing an alternate GUG start codon (to reduce translational efficiency) under the control of the arabinose-inducible, glucose-repressible *ara* promoter, the growth and/or survival of the Δ*metK* ATM778 mutant was made dependent on the presence of arabinose (Fig. 1A). We screened a *C. trachomatis* serovar L2 genomic DNA library in ATM778 in the presence of glucose and identified several colonies on medium supplemented with 1 mM SAM, while no colonies were detected under the same conditions in the absence of extracellular SAM. Two of 12 independent colonies characterized displayed consistent SAM-dependent growth in the presence of glucose (i.e., repression of *E. coli metK* expression) (Fig. 1B). Rescuing plasmids were isolated and analyzed by restriction mapping and sequence analyses. All library clones revealed the same 2,528-bp insert harboring *C. trachomatis* L2 CTL843 and 123 bp of upstream sequence in the same orientation as the lactose promoter of pUC, followed by CTL842 with 7 bp of upstream sequence, in reverse orientation. Subcloning of this ORF into pBluescript (i.e., pRAK368, Table 1) confirmed that CTL843 conferred a SAM-dependent growth phenotype to ATM778 in the presence of glucose and IPTG (Fig. 1B), suggesting that CTL843 functions as a transporter for SAM.

Kinetic analysis of SAM uptake in whole cells expressing recombinant CTL843. To investigate CTL843 function, uptake of

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype/description	Reference or source
E. coli strains		
DH5α	F⁻ φ80 Δ(*lacZY-argF*)U169 *deoR recA1 endA1 phoA hsdR17 supE44* λ⁻ *thi-1 gyrA96 relA1* Δ(*lacZ*)M15	54
MG1655	F⁻ λ⁻ *ilvG rfb-50 rph-1*	*E. coli* Genetic Stock Collection
MC4100	F⁻ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 deoC1 rbsR fthD5301 fruA25* λ⁻	55
BW25113	Δ(*araD-araB*)567 Δ*lacZ4787* (::*rrnB-3*) λ⁻ *rph-1* Δ(*rhaD-haB*)568 *hsdR514*	56
ATM609	BW25113 transformed with pKD46	56
ATM770	Δ*metK*::*kan*/pREF71	Allelic exchange mutant of ATM609/pREF71
ATM777	MC4100/pREF73	Cmʳ transformant of MC4100 with pREF73
ATM778	Δ*metK*::*kan*/pREF73	Kmʳ transductant of ATM777 with P1 grown on ATM770
NC13	MC4100 Δ*pfs*(8-226)::*kan*	17
ATM1113	Δ*metK*::*kan*/pREF73/pREF77	Apʳ transformant of ATM778 with pREF77
ATM1114	Δ*metK*::*kan*/pREF73/pRAK368	Apʳ transformant of ATM778 with pRAK368
ATM1115	MG1655 Δ*pfs*::*kan*	Kmʳ transductant of MG1655 with P1 grown on NC13
ATM915	MG1655/pRAK368	Apʳ transformant of MG1655 with pRAK368
ATM1116	Δ*pfs*::*kan*/pRAK368	Kmʳ transductant of ATM915 with P1 grown on NC13
ATM1117	Δ*metK*::*kan*/pREF73/pUC18	Apʳ transformant of ATM778 with pUC
Plasmids		
pKD46	P_{ara} *gam bet exo oriR101 repA101*(Ts); Apʳ	56
pAM238	IPTG-inducible expression vector; Spcʳ; pSC101 derivative; low copy number	57
pREF71	pAM238::AUG-*metK*$_{Ec}$	This work
pBAD33	Arabinose-inducible expression vector; Cmʳ; p15A derivative; low copy number	58
pREF73	pBAD33::GUG-*metK*$_{Ec}$	This work
pCtL2	10-fold coverage of *C. trachomatis* serovar L2 genome, 2.2-kb average insert size; Apʳ; pUC19 derivative, 500–700 copies per cell	3
pREF77	pUC19::*ctl843*$_{Ct}$; isolate 9	This work
pGEMT	PCR cloning vector; Apʳ; high copy number	Promega
pRAK367	pGEMT::*lacI*^{q}	This work
pBluescript II SK(+)	IPTG-inducible expression vector; Apʳ; pUC derivative; 300–500 copies per cell	Stratagene
pRAK368	pBluescript II SK(+)::*samht*$_{Ct}$ *lacI*^{q}	This work

[¹⁴C]SAM into intact cells of *E. coli* was analyzed by a rapid filtration assay. Control cells showed a slow, low linear uptake of SAM (Fig. 2A). In contrast, expression of CTL843 in ATM915 showed a marked increase in the amount of intracellular label over time in a process that was linear during the first minute and then reached saturation indicative of carrier-mediated transport (Fig. 2A). Cell suspensions were also incubated with 5 to 100 μM [¹⁴C]SAM, and uptake was measured after 30 s to estimate the kinetic constants of recombinant CTL843 (Fig. 2B). From these data, the calculated apparent K_m values (reflecting SAM affinity for CTL843) and V_{max} values (reflecting CTL843 activity) were 5.88 ± 0.11 μM and 31.57 ± 0.09 pmol min⁻¹ mg⁻¹, respectively. To assess the role of the proton gradient in CTL843 function, ATM915 was pretreated for 5 minutes with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which acts as a channel through the inner membrane to dissipate the H⁺ gradient. CCCP treatment reduced [¹⁴C]SAM intracellular uptake by 50%.

SAH is a possible substrate for recombinant CTL843. The specificity of CTL843 for SAM was determined by measuring the effect of structurally related derivatives on SAM transport. Competition studies were performed in the presence of a 10-fold excess of unlabeled putative competitive inhibitors (Table 2). As expected, addition of excess cold SAM competed for binding and uptake of [¹⁴C]SAM in induced ATM915 cells. While we observed

a slight increase in SAM uptake in the presence of methionine or homocysteine, only SAH, the molecule formed during the course of SAM-dependent methylation reactions, strongly inhibited SAM uptake. Other structural analogs (adenosine, MTA [formed from SAM during spermidine synthesis], and sinefungin [a synthetic analog of SAH]) had negligible effects on the ability of CTL843 to transport SAM. The uptake of [¹⁴C]SAM was measured in the presence of various concentrations of SAH to estimate the apparent K_i value of SAH for CTL843, which was determined to be 4.12 μM.

In the absence of a commercially available source of radioactive SAH to follow the direct transport of SAH by CTL843 (see the supplemental material), we utilized a genetic assay instead. Elevated levels of SAH are toxic for cells, and loss of *pfs* (which encodes the MTA/SAH nucleosidase responsible for detoxification of both MTA and SAH in *E. coli*) leads to diminished bacterial growth (7, 17). Although this Δ*pfs* phenotype was relatively unstable, based on the high-frequency (i.e., ~10⁵), spontaneous appearance of mutants with fitness comparable to that of the wild-type strain, first-passage colonies formed when the Δ*pfs*::*kan* mutation was transduced into MG1655 (see Text S1 in the supplemental material) were always about 60% larger in diameter in the strain expressing CTL843 (ATM988, Table 1), indicating that the chlamydial transporter offered an immediate fitness advan-
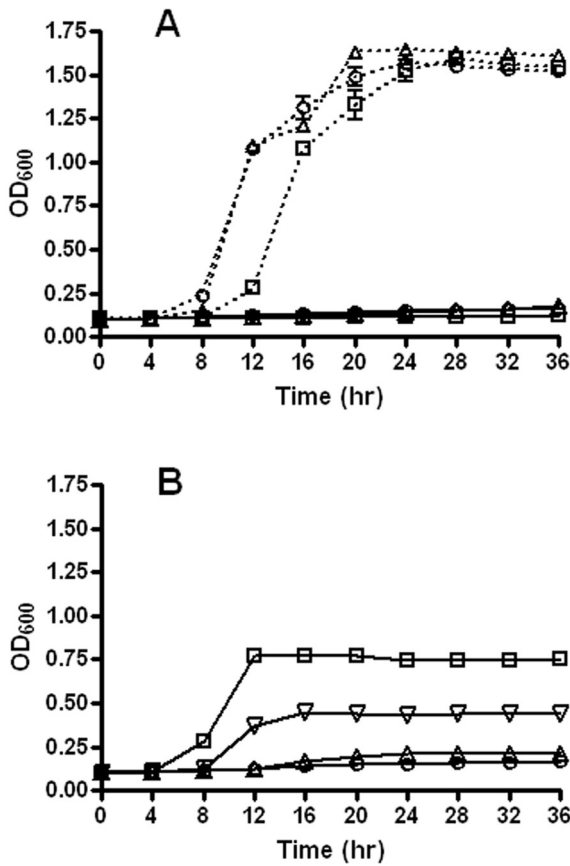
FIG 1 Growth of *E. coli* ΔmetK mutant in the presence or absence of extracellular SAM. ATM778 (ΔmetK) transformed with the indicated plasmids was grown at 37°C in LB supplemented with Ap, Cm, 0.2% glucose (solid lines), or 0.2% arabinose (dotted lines), in the absence (A) or presence (B) of 1 mM SAM. Absorbance (OD$_{600}$) was measured in a Bioscreen growth curve analyzer and plotted against time (hours). Error bars represent the standard deviations from four replicates. Symbols: ○, pUC-empty vector; □, pREF77-ctl843; △, pRAK368-ctl843; ▽, pRAK368-ctl843 with IPTG.

FIG 2 Kinetics of SAM transport in *E. coli*. (A) Time course of [^{14}C]SAM uptake in *E. coli* at 37°C as a function of time. The time course assay was carried out in transport buffer supplemented with 10 μM [^{14}C]SAM. Time point assays were done in triplicate. (B) Effect of substrate concentration on [^{14}C]SAM uptake at 37°C. The data represent six independent experiments done in triplicate. The background transport seen with MG1655 (○) was subtracted from the transport seen with ATM915 (MG1655 expressing *ctl843* [△]) with GraphPad Prism software, and then nonlinear regression analysis was performed to obtain the K_m and V_{max} values.

tage to the *pfs* mutant (Fig. S4). The partial complementation of the growth phenotype in ATM988 likely reflects the ability of CTL843 to recognize and export SAH out of the cells and the inability of CTL843 to recognize MTA (Table 2) and is consistent with the data indicating that SAH is a competitive inhibitor of SAM transport.

Recombinant CTL843 mediates specific counterexchange of SAM with SAM and SAH. In order to further characterize CTL843 as an SAM/SAH antiporter, back-exchange studies were performed. Cultures of ATM915 expressing CTL843 were preloaded with labeled SAM, washed to remove external radioactivity, and resuspended in M9 minimal salts supplemented with putative counterexchange substrates at a 10-fold excess over labeled SAM and incubated at 37°C. After 10 min, the cells were centrifuged and radioactivity was counted in the cells and in the supernatant. Quantification of exported radioactivity allows differentiation between counterexchange and unidirectional transport. *E. coli* expressing CTL843 and preloaded with [^{14}C]SAM released significant amounts of internal label (~80% of the initial amount) after resuspension in buffer medium supplemented with unlabeled SAM (thus, against the SAM concentration gradient) or SAH.

CCCP treatment did not affect the counterexchange of labeled SAM with SAM or SAH (Fig. 3), indicating that exchange of internal SAM with external SAM or SAH in excess was energy independent. On the other hand, maintenance of the cellular SAM gradient observed in the absence of "competitors" required an intact proton motive force, as SAM concentration reached equilibrium across the membranes in the presence of CCCP (Fig. 3). Thus, CTL843 is active in both directions and can function as an active symporter or energy-independent antiporter (SAM uptake coupled to labeled SAM exit). The similitude between SAM and SAH in the ability to promote the efflux of labeled SAM from the cells strongly suggests that SAH is also being transported into the cells in exchange for SAM. Consequently, we propose that CTL843 be renamed *C. trachomatis* L2 SAMHT for SAM/SAH transporter.

Comparative analysis of SAMHT. An ORF similar to CTL843 was found in all genome sequences available for the order *Chlamydiales*, with amino acid identity of >90% for *Chlamydia* homologs, 60 to 67% for *Chlamydophila* homologs, and 34 to 38%

TABLE 2 Effect of putative inhibitors on SAM uptake by *E. coli* ATM915 expressing CTL843

Unlabeled SAM analogues[a]	Molecular structure	Change (%)[b]
S-Adenosyl-L-methionine (SAM)		−74 ± 8
S-Adenosyl-L-homocysteine (SAH)		−84 ± 10
5′-Deoxy-5′-(methylthio)adenosine (MTA)		15 ± 5
Adenosylornithine (sinefungin)		−10 ± 13
L-Adenosine		6 ± 8
L-Homocysteine		23 ± 5
L-Methionine		32 ± 9

[a] One hundred micromolar of the unlabeled compound.

[b] Change in radioactivity observed in presence of unlabeled compound compared to control with only 10 μM [^{14}C] SAM.

for the *Chlamydia*-like homologs. All CTL843 homologs contain a duplication of the evolutionarily conserved domain EamA, also known as DUF6 (PF00892; http://pfam.sanger.ac.uk/family /PF00892). While EamA is a signature for transporters belonging to the drug and metabolite transporter (DMT) superfamily, the presence of two EamA domains further subclassifies these chlamydial ORFs in the drug/metabolite exporter (DME) family exhibiting 10 alpha-helical transmembrane spanners (TMSs) (http: //www.tcdb.org). Interestingly, this family of transporters also contains the SAM transporters belonging to *Rickettsieae*, another group of obligate intracellular bacteria. However, *Rickettsia prowazekii* RP076 (15) shares only about 20% identity with *C. trachomatis* L2 CTL843 (Fig. 4). Additional phylogenetic anal-

ysis failed to support any evolutionary relationship between the rickettsial and the chlamydial SAM transporters (Fig. S5).

DISCUSSION

Bacterial evolution toward obligate intracellular parasitism in a eukaryotic host is thought to be associated with loss of genetic information, especially for genes that become redundant within the host niche (18). These losses are not deleterious for the organism provided that the missing genes, such as those encoding biosynthetic functions, can be compensated for by an increased repertoire of transport functions that allow the organism access to essential nutrients in the intracellular environment. In this work,

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FIG 3 Efflux of [^{14}C]SAM from *E. coli* cells expressing CTL843. Preloading of labeled SAM (10 μM) in ATM915 was performed initially for 1 min at 37°C and then stopped by dilution and washes at 4°C. Samples were resuspended in duplicate in M9 minimal salts with or without 100 μM cold SAM or cold SAH and incubated at 37°C in the presence or absence of 20 μM CCCP. After 10 min, the partition of labeled SAM was determined by counting radioactivity in the supernatants and in the pellets after centrifugation at 13,000 rpm for 90 s.

we used genetic and biochemical strategies to identify a *C. trachomatis* transporter, CTL843, for the metabolites SAM and likely SAH. We propose that CTL843 and its respective *Chlamydia* homologs be named SAMHT to clarify their role as a SAM and possible SAH transporter.

Our attempts to genetically complement an *E. coli* Δ*metK* mutant, ATM778, failed to identify a gene in *C. trachomatis* L2 that encodes SAM synthetase activity. Complementation of ATM778 with *C. trachomatis* L2 CTL843 ORF was dependent on the presence of extracellular SAM and was related to the expected level of expression in the cells (compared to pRAK368, pREF77 is expected to yield 200 extra copies per cell during replication). CTL843 allows SAM to enter cells, therefore bypassing the *E. coli* membrane permeability barrier for SAM. A similar effect has been reported previously for the *Rickettsia prowazekii* SAM transporter (14, 15). However, this transporter shares only ~20% amino acid

homology with CTL843. In addition, we showed that *C. trachomatis* L2 CTL843 expression partially complemented the growth defect of an *E. coli* Δ*pfs* mutant lacking MTA/SAH nucleosidase activity, suggesting that recombinant CTL843 was able to reduce the internal buildup of toxic SAH in the mutant cells. Whole-cell transport assays further supported CTL843 as a transporter for SAM and SAH: (i) uptake of [^{14}C]SAM into recombinant *E. coli* cells was saturable, exhibiting kinetic constants indicative of a high-affinity carrier with a K_m value below 6 μM; (ii) uptake of [^{14}C]SAM was specifically inhibited by SAH at an equivalently high affinity; and (iii) internal [^{14}C]SAM was expelled out of recombinant cells in the presence of extracellular SAM or SAH following a countertransport mechanism. Although we were unable to monitor direct transport of SAH in *E. coli* cells expressing CTL843 due to the lack of commercially available labeled SAH and to the limit of detection of SAH by high-performance liquid chromatography (HPLC) (see Text S1 in the supplemental material), we believe that our genetic and biochemical data strongly support the idea that both SAH and SAM are substrates for *C. trachomatis* L2 CTL843.

Unlike rickettsiae, chlamydiae multiply in the cytoplasm of the host cell inside a specialized compartment termed an inclusion. Because the inclusion membrane is permeable to molecules smaller than 520 Da (19), SAH (384.4 Da) and SAM (399.4 Da) are expected to diffuse freely between the host cytoplasm and the bacteria expressing SAMHT. The pI value of SAM is 7.24, and SAH is neutral between pI values of 3.5 and 9.2 (20). Consequently, both molecules are neutral in the mammalian cell cytoplasm and in the chlamydial inclusion, where the pH values are calculated to be 7.29 ± 0.07 and 7.25 ± 0.19, respectively (21). Although the glucose present in the transport assay was probably partially compensating for the deleterious effect of CCCP on membrane potential (22), we still observed inhibition of SAM import in *E. coli* expressing SAMHT in the presence of CCCP. This shows that *C. trachomatis* L2 SAMHT acts as a secondary active symporter for SAM, similarly to the rickettsial SAM transporter (15). Conversely, exchange of internal SAM with external SAM or SAH is not affected by CCCP and is therefore energy independent (Fig. 3). This mechanism of antiport transport driven by the inward substrate (SAM) gradient and the outward product (SAH) gradient is most favorable for the chlamydiae, which are energy parasites (23). A similar transport mechanism has been described for the *E. coli* putrescine-

FIG 4 Sequence alignments of *C. trachomatis* L2 CTL843 and *R. prowazekii* RP076 SAM transporter. Amino acid sequences were first aligned on a web interface of MAFFT (53) and were subsequently aligned via Clustal W2 to illustrate the similarity. Identical residues are denoted with an asterisk and are highlighted in black boxes. Strongly conserved residues and weakly conserved residues are marked with a colon and a period, respectively, and are displayed in gray boxes.

ornithine antiporter PotE, which can excrete putrescine as the result of the antiport activity between putrescine and ornithine, in an energy-independent manner, and can also catalyze putrescine uptake in a process that is energy dependent without excretion of ornithine (24).

Although SAM transporters have been found so far in four evolutionarily diverse transporter families, identification of SAM carriers using sequence similarity is of limited value because their degree of homology inside a family is generally not much higher than that between the different members of the same family. In *Saccharomyces cerevisiae* (25), humans (26), and plants (27, 28), members of the well-characterized mitochondrial carrier protein (MCP) family (29) transport external SAM in counterexchange with SAM or SAH with less affinity. In *Saccharomyces cerevisiae* (30) and *Leishmania* (31), transporters belonging to the amino acid permease superfamily or to the folate biopterin transporter family, respectively, seem more specific to SAM, with affinities in the nanomolar ranges. Like the rickettsial SAM transporter (15), the chlamydial SAMHT homologs to CTL843 belong to the drug/metabolite exporter (DME) family (32) and appear to transport both SAM and SAH with affinities in the micromolar range. Note that we hypothesize that SAH is also a substrate for the rickettsial SAM transporters, based on the inhibition of SAM uptake observed in the presence of SAH (15) and the apparent lack of SAH hydrolase homologs in their genomes (data not shown). Although the DME family was recognized 10 years ago and now has >500 sequenced members in bacteria and archaea (33), only three additional transporters from *E. coli* have been functionally characterized to date (34–36), and the molecular mechanisms driving the activity of DME transporters are not known. The growth dependence of the *E. coli* Δ*metK* mutant on the activity of SAMHT in the presence of SAM might be the ideal platform to screen for antimicrobial compounds targeting this transport system. In addition to improving our knowledge on the mode of action of DME transporters, SAMHT inhibitors may well lead to the development of a new antichlamydial specific therapy.

The 40 chlamydial species, identified mostly by 16S rRNA sequencing, are classified into seven families (37, 38), among which the *Chlamydiaceae* (including *C. trachomatis*) are the most studied due to their importance in human and veterinary medicine. Nevertheless, "*Chlamydia*-like" organisms have been receiving more attention lately, in particular those residing in free-living amoebae, since an intraprotozoal lifestyle has likely contributed significantly to the adaptation of intracellular bacterial pathogens to higher eukaryotes (39–42). Escobar-Páramo et al. (43) suggested that "ancient" genes will not persist if they do not carry a "lasting adaptive value" to populations. The presence of a CTL843 SAMHT homolog in the nine *Chlamydiaceae* representatives and the three "*Chlamydia*-like" organisms that have been sequenced suggests that SAMHT confers adaptive functions that allow the exploration of new niches such as mammalian cells. Since an obligate intracellular lifestyle limits the chance of gene acquisition by horizontal gene transfer, SAMHT may have initially been acquired by an ancestral, facultative intracellular form of *Chlamydia*. Subsequently, the constant supply of metabolites (i.e., SAM) from the host relaxed the selective pressure to maintain the metabolic pathways involved in SAM synthesis (MAT) and SAH degradation (MTAN or SahH) and resulted in the complete loss or pseudogenization of these genes in all *Chlamydiaceae* and in *P. amoebophila* UWE25, respectively.

The isolated asexual reproductive cycle of *Chlamydia* and the evolutionary bottleneck observed at each transmission or passage to a new host are expected to favor the process of genome degradation (44, 45). Moreover, considering that MAT requires ATP and that chlamydiae parasitize their host for ATP (46), loss of MAT is expected to make the microbe more fit in its interaction with the host and MAT could therefore be under strong pathoadaptive pressure (47). Consequently, it is somewhat surprising that the two emerging pathogens (18, 48) *W. chondrophila* and *P. acanthamoebae* have maintained apparently functional MAT and SahH enzymes. In the absence of a genetic system to test the contribution of the three genes to bacterial fitness, we do not know if this reflects a greater need for metabolic versatility, a more effective selection for the maintenance of weakly beneficial genes, or simply a less advanced stage in the process of the reductive evolution of their genomes (49). The answers to these questions will add to a better understanding of the adaptive evolution of *Chlamydia* towards pathogenicity.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* strains and plasmids used in this study are listed in Table 1, and their construction is described in the supplemental material. *E. coli* strain DH5α was used for cloning. Strains were grown in Luria-Bertani (LB) broth with aeration or on LB agar, unless indicated otherwise. Medium was supplemented with ampicillin (Ap; 50 μg/ml), kanamycin (Km; 50 μg/ml), chloramphenicol (Cm; 10 μg/ml), spectinomycin (Spc; 100 μg/ml), arabinose (0.2%), glucose (0.5%), isopropyl-β-D-1-thiogalactopyranoside (IPTG; 1 mM), and mannitol (0.5%) as needed.

Whole-cell transport assays. (i) SAM import. Whole-cell transport assays were performed as described in references 14 and 50 with slight modifications. Overnight LB cultures of MG1655 or ATM915 (Table 1) were subcultured 1:100 into 20 ml LB supplemented with IPTG to induce expression of CTL843 (plus appropriate selective agents as needed) and grown at 37°C with shaking to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.7). Cultures were standardized to an OD_{600} of 0.5 and concentrated to 2×10^9 bacteria/ml. Cells were centrifuged and washed with M9 minimal salts (51). Washed cells were suspended in cold M9 minimal salts plus 0.5% glucose (i.e., transport buffer), yielding approximately 0.6 to 0.8 mg/ml total protein and about 1×10^8 bacteria per 50-μl reaction mixture. A 10 μM concentration of *S*-adenosyl-L-[*methyl*-^{14}C]methionine ([^{14}C]SAM) (GE Healthcare UK Limited) was added to the bacterial suspension for a final volume of 60 μl per reaction mixture, and uptake was initiated at 37°C and followed up to 5 min. Samples were periodically removed, transferred to 20 ml ice-cold M9 minimal salts to stop transport, filtered through a 0.45-μm Durapore membrane filter (Millipore Corp.), and washed once with the same buffer. Filters were placed in scintillation vials, and radioactivity was measured using 5 ml of ReadySafe liquid scintillation cocktail (Beckman Coulter). Disintegration-per-minute values determined by sample counting were normalized to the activity of [^{14}C]SAM (55 mCi/mmol) and to the protein concentration of the sample to express counts as pmol SAM · minute^{-1} · mg total protein^{-1}.

Kinetic analysis was accomplished by incubating cell suspensions with increasing concentrations of [^{14}C]SAM for 30 s. Data were plotted in Prism 4 (GraphPad Software), and apparent K_m and V_{max} values were determined using the included Michaelis-Menten linear regression template. To examine the dependence of SAM transport on membrane potential, samples were pretreated with 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 5 minutes prior to the start of the transport assay. All uptake data are the results of experiments performed in triplicate unless otherwise indicated.

(ii) SAMHT$_{Ct}$ specificity. The substrate specificity of recombinant CTL843 was assessed by measuring the capacity of nonradioactive effec-

tors structurally related to SAM to inhibit the uptake of 10 μM [^{14}C]SAM by *E. coli* strain ATM915. Values obtained from 5 min of preincubation with 100 μM effectors were expressed as percent inhibition relative to the absence of inhibitor (15, 50). Additionally, the dissociation constant for the binding of SAH to CTL843 (i.e., K_i value) was determined by two methods using Prism 4 software. First, the kinetics of SAM uptake were determined in the presence of 5 and 20 μM cold SAH. Second, the K_i value of SAH was calculated from the half-maximal inhibitory concentration (IC$_{50}$) estimated by iterative curve fitting for sigmoidal equations describing SAM uptake velocity in the presence of 0 to 100 μM SAH, using the equation of Cheng and Prusoff (52).

(iii) Reversibility of SAM transport. To determine the capacity of recombinant CTL843 to transport SAM in two directions, induced cells were incubated with 10 μM [^{14}C]SAM for 1 min at 37°C to allow for uptake of labeled SAM and then washed twice in 25 ml cold M9 minimal salts and resuspended in transport buffer to a density of 1×10^9 bacteria/ 100 μl of bacterial suspension. Nonradioactive SAM or SAH was added to 100 μl of bacteria to a final concentration of 100 μM and incubated at 37°C. Reactions were stopped after 10 min by centrifugation at 13,000 rpm for 90 s, and radioactivity (disintegration-per-minute values) was measured in the supernatants and the pellets. To examine the dependence of SAM exchange on membrane potential, samples were pretreated with 20 μM CCCP for 5 minutes prior to addition of the cold competitive inhibitors. The two independent experiments performed showed the same trend.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00051-11/-/DCSupplemental.

Text S1, DOC file, 0.043 MB.
Figure S1, PPT file, 0.105 MB.
Figure S2, DOC file, 0.083 MB.
Figure S3, PPT file, 0.125 MB.
Figure S4, PPT file, 0.310 MB.
Figure S5, PPT file, 0.056 MB.

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