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Allosteric Inhibitors of Cell-Cycle-Regulated Methyltransferase for Novel Antibiotic Development

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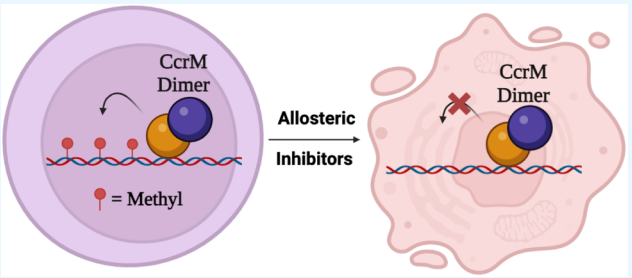
ABSTRACT: Cell-cycle-regulated methyltransferase (CcrM) plays a crucial role in regulating important cellular processes that are essential for proper cell division and growth; disruptions of these processes can attenuate the bacteria's viability. Notably, CcrM homologs are present across a set of diverse human pathogens, suggesting that selective inhibition of CcrM over human DNA methyltransferases (DNMT's) could offer a new strategy for combating human bacterial pathogens, leading to the development of novel antibiotics. Herein, we report the screening of two open-access chemical libraries—the National Cancer Institute Developmental Therapeutic Program Diversity Set VII and Medicines for Malaria Venture Global Health Priority Box—and identified four structurally diverse inhibitors of CcrM. Among these, two inhibitors displayed both micromolar affinity and high selectivity for CcrM over human DNA methyltransferase 3A, highlighting their potential as leads for a new class of antibiotics.



INTRODUCTION

The rise of bacterial resistance to traditional antibiotics has created an urgent need for new treatments with novel modes of action. Factors such as overuse of current medications, lack of new bacterial drug targets, and reduced drug development efforts by the pharmaceutical industry have all contributed to the ongoing antibiotic resistance crisis.[1] Novel antibiotic criteria should consist of selective inhibition of bacterial processes and new treatments should not be affected by existing resistance mechanisms.[2,3] Accordingly, bacterial DNA methyltransferases are a novel bacterial drug target that could lead to the development of a new class of antibiotics with alternative modes of action than those from traditional antibiotics.

Bacterial and mammalian DNA methyltransferases are responsible for regulating cellular processes including development, host-protection, DNA repair, differentiation, and cell-cycle regulation.[4−6] Among the human DNMTs, DNA methyltransferase, DNMT1, is essential for maintenance methylation patterns, and like all DNMTs, uses the cofactor S-Adenosyl methionine (AdoMet).[4] In contrast, DNMT3A and DNMT3B are de novo enzymes that determine the patterns of cell and tissue specific patterns of methylation.[4] Inhibition of these human DNMTs can lead to disastrous consequences on human health.

The bacterial N6-adenine Cell-cycle regulated DNA methyltransferase, CcrM, from *Caulobacter crescentus* (*C. crescentus*) plays a vital role in regulating the expression of genes that are essential for bacterial cell division.[7] Normal CcrM-dependent methylation patterns are essential for proper cell division and growth; disruption or aberration in these methylation patterns can attenuate bacterial viability.[8] Therefore, DNA methyltransferases that are important for bacterial cell viability are validated targets for novel antibiotic development.[9,10]

Recent efforts in combatting the antibiotic resistance crisis have focused on the development of AdoMet analogs which have displayed novel antibiotic mechanism inhibition of another bacterial N6-adenine methyltransferase, CamA.[11] CamA has been shown to be essential in pathogenesis for the human bacterial pathogen *Clostridioides difficile* (*C. difficile*)[11] which is deemed as a hospital-acquired "superbug" linked to severe and potentially fatal pseudomembranous colitis, largely due to its resistance to existing treatments.[12] The CDC lists it as an urgent threat,[13] prompting the exploration of nontraditional antibiotics like those targeting bacterial DNMTs.

CcrM orthologs are found across a wide range of bacterial species, including several human pathogens. These orthologs

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share high sequence similarity and may also have structural and functional similarities to CcrM from *C.crescentus*. (Figures 1 and 2). Notable CcrM-containing pathogens include *Brucella abortus*, *Brucella melitensis*, and *Haemophilus influenzae*, which have developed increased resistance to current antibiotics.[14,15] Therefore, inhibitors targeting CcrM in *C. crescentus* should have conserved effects across other CcrM orthologs found in human pathogens and could potentially lead to the development of a new class of antibiotics.

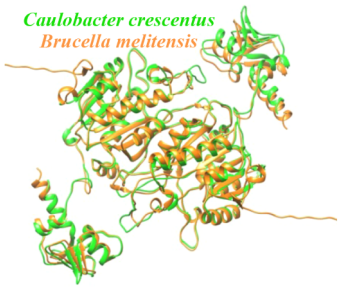

Figure 1. Superimposition of CcrM from *Caulobacter crescentus* and *Brucella melitensis* reveals structural homology. Alphafold was used to predict the Apo dimers of CcrM from *Caulobacter crescentus* (green) and *Brucella melitensis* (orange). The superimposition and structural image were made in UCSF Chimera.

Herein we report the screening of two chemical libraries, the National Cancer Institute (NCI) Developmental Therapeutic Program (DTP) Diversity Set VII and the Methods for Malaria (MMV) Venture Global Health Priority Box (GHP) comprising of 1581 and 240 chemical compounds, respectively. The NCI Diversity Set VII is a collection of structurally diverse compounds derived of nearly 140,000 compounds available, selected based on unique pharmacophores (e.g., structures containing five or more pharmacophores).[17] The MMV GHP set consists of compounds in various stages of drug discovery and development, tested against various vector species and screened against neglected and zoonotic diseases.[18] All 1,821 compounds from both libraries were screened at a

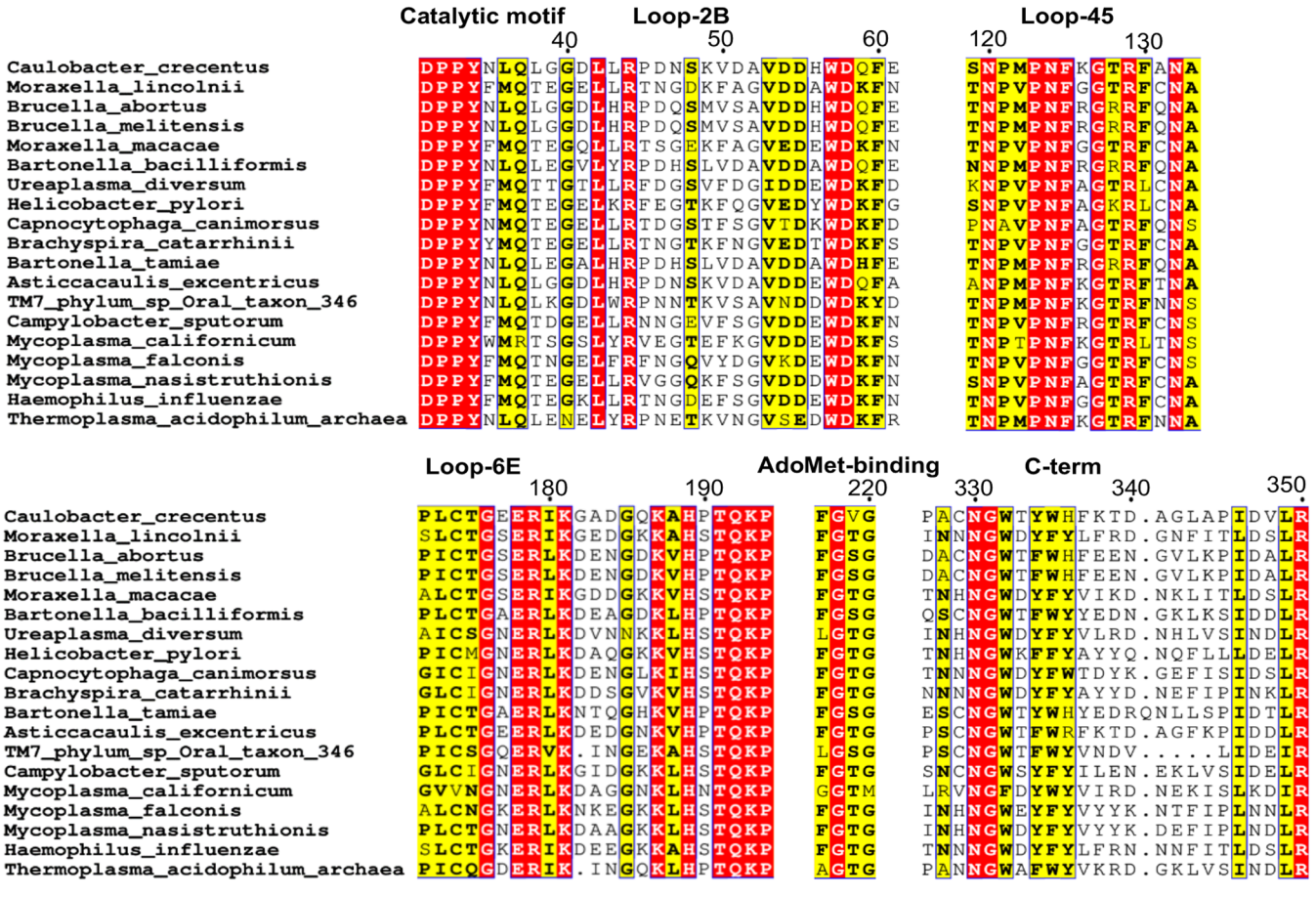

Figure 2. CcrM from *Caulobacter crescentus* (top) is highly conserved across pathogenic orthologs.[16] CcrM in all organisms are β-class DNA methyltransferases based on the sequential ordering of a conserved catalytic motif (D31-Y34) and a conserved AdoMet binding motif (F216-G220).[16] Uniquely, the catalytic motif is located at the N-terminus of catalytic Loop-2B (D31-E61).[16] Loop-2B, Loop-45 (S119-A133), and Loop-6E (P172−P194) participate in base-specific recognition of DNA.[16] CcrM's unique C-terminal domain (P271−N358) is essential for DNA binding and strand-separation.[16] The 88aa C-terminal sequence was used as the BLAST search seed. The MSA was performed with Clustal Omega and visualized using ESPript 3.0 with a similarity color scheme global score of 0.7. Numbering is based on the CcrM sequence from *Caulobacter crescentus*. Red residues: 100% conserved, yellow residues: 70−100% conserved, white residues: not conserved. Figure adapted with permission from Konttinen O, Carmody J, Pathuri S, Anderson K, Zhou X, Reich N. Cell cycle regulated DNA methyltransferase: fluorescent tracking of a DNA strand-separation mechanism and identification of the responsible protein motif. Nucleic Acids Res. **2020**, 48 (20), 11589−11601. DOI: 10.1093/nar/gkaa844. Copyright 2020 Oxford University Press.

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concentration of 100 μM using a modified version of our radiochemical assay deploying triturated AdoMet to measure DNA methylation (**Biochemical Methods, SI**).[10]

RESULTS AND DISCUSSION

Four compounds were identified (Figure 3) from screening of the combined libraries, which, were then subjected to a second

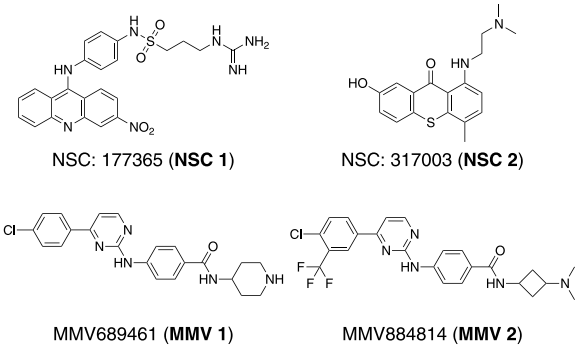

Figure 3. Inhibitors identified from initial screening from the NCI Diversity Set VII and MMV Global Health Priority Box that showed greater than 50% inhibition of CcrM at 100 μM.

screening to verify their inhibitory activity (Figure S1). Among these, the two originating from the NCI library manifested different scaffolds, while the remaining two from the MMV library shared several structure similarities.

NSC177365 (**NSC 1**) contains an acridine motif found among therapeutics with various modes of action, ranging from DNA intercalation, endonuclease mimicry, and enzymatic inhibition.[19] It has also been shown to reverse several neurodegenerative disorders and serve as an anticancer agent.[19] NSC317003 (**NSC 2**), also known lucanthone, contains a thioxanthone scaffold. This is a known pharmaceutical used for treatment of parasitic diseases such as bilharziasis.[20] On the other hand, the inhibitors originating from the MMV GHP library, **MMV 1** and **2**, contain no scaffold of reported bioactivity.

After completing the initial CcrM screening, we then counter screened the four compounds (Figure 3) for any inhibitory activity against CcrM from *Moraxella lincolnii* (*M. lincolnii*) at 100 μM, using the identical radiochemical assay deployed for the identification of CcrM inhibitors. *M. lincolnii* is an aerobic human bacterial pathogen that has been isolated mainly from the respiratory tract of humans[21] and contains a CcrM homolog displaying highly conserved protein motifs to that of CcrM from *C. crescentus* (Figure 2).[16] The criteria of counter-screening these inhibitors against CcrM *M. lincolnii*was 2-fold: 1) to support our claim based on our bioinformatic study (Figure 2) that CcrM orthologs share high sequence similarity and may have structural and functional similarities and 2) inhibition of CcrM from *C. crescentus* could have conserved effects across other CcrM-containing bacterial pathogens. Much to our satisfaction, all four compounds displayed inhibitory activity against *M. lincolnii* at 100 μM.

NSC 1 and **NSC 2** contain different scaffolds and displayed single digit micromolar affinity against CcrM *C. crescentus* while both exhibited a 6-fold loss in potency against CcrM *M. lincolnii* (Table 1, entries 1−2). **MMV 1** and **MMV 2** are structurally related compounds that demonstrated markedly different inhibition toward CcrM from both *C. crescentus* and

Table 1. IC_{50} Values of Compounds for CcrM from C. Crescentus and M. Lincolnii[a]

Entry	Compound	Library ID	*C. crescentus* IC_{50} (μM)	*M. lincolnii* IC_{50} (μM)
1	**NSC 1**	NSC177365	2.3 ± 0.4[b]	14.6 ± 4.4[b]
2	**NSC 2**	NSC317003	7.3 ± 1.3[b]	45.8 ± 3.2[b]
3	**MMV 1**	MMV689461	21.6 ± 3.6[b]	122 ± 6[b]
4	**MMV 2**	MMV884814	65.5 ± 14.3[b]	>200[b]

[a]All Assays Performed in Either Triplicate or Duplicate, with Additional Repetitions Added as Necessary [b]Mean ± standard deviation, n = 3.

M. lincolnii (Table 1, entries 3−4). We speculate the increase in hydrophobicity from the additional trifluoromethyl group *ortho* to the chlorine, or the loss of a hydrogen acceptor from the replacement of the piperidine group with a cyclobutyl dimethyl amine (Figure 2, MMV 2), may have contributed to the loss in potency toward CcrM. Further structural activity relationship (SAR) studies can substantiate these claims.

When comparing the IC_{50} values of all four compounds against CcrM from both *C. crescentus* and *M. lincolnii*, these inhibitory values against CcrM *M. lincolnii* were five to 6-fold weaker (Table 1, entries 1−4). Nonetheless, given that these inhibitors display inhibitory activity against two CcrM orthologs (*C. crescentus* and *M. lincolnii*) supports our prediction of the structural similarity between the two enzymes (Figure 2). Furthermore, these findings substantiate our claim that inhibitors of CcrM from *C. crescentus* could also inhibit antibiotic resistant CcrM-containing bacterial pathogens such as *Brucella abortus*,[14] *Brucella melitensis*,[14] and *Hemophilis influenzae*.[15]

The inhibitory mechanisms of these compounds were next investigated by varying both DNA and AdoMet concentrations, and the data analyzed using *Prism 10* software to identify the best-fitting inhibition models. Nonlinear Michaelis−Menten curves and corresponding double-reciprocal plots were generated (Figure 4) and subsequently fit to classical inhibition models. Remarkably, all four inhibitors exhibited allosteric inhibition with respect to AdoMet, indicating that they all bind away from the active site, and do not compete with the cofactor that is conserved across both human and bacterial DNA methyltransferases. Double-reciprocal plots showed that **NSC 1** and **NSC 2** best fit a noncompetitive inhibition model against AdoMet, suggesting that these compounds can bind equally well to the free enzyme or the enzyme-AdoMet complex. Additionally, **NSC 1** fit a competitive inhibition model against DNA, implying that it disrupts DNA binding. If **NSC 1** binds to the DNA directly, this could lead to competitive inhibition, which is supported by findings showing that acridines, such as **NSC 1**, intercalate DNA.[19] In contrast, **NSC 2** fits an uncompetitive inhibition model suggesting that it does not interfere with DNA binding and prefers to bind the enzyme-DNA complex (Figure 4).

The studies of both **MMV 1** and **MMV 2** best fit a noncompetitive inhibition model toward AdoMet, which indicates these inhibitors also bind outside of the active site. Interestingly, **MMV 1** also fits an uncompetitive inhibition model toward DNA, signifying a preference for its binding to the enzyme-DNA complex. This preference could be advantageous, as CcrM bound to DNA is likely to be the dominant species in cellular conditions. In contrast, **MMV 2** fits a noncompetitive inhibition model against DNA. Nevertheless,

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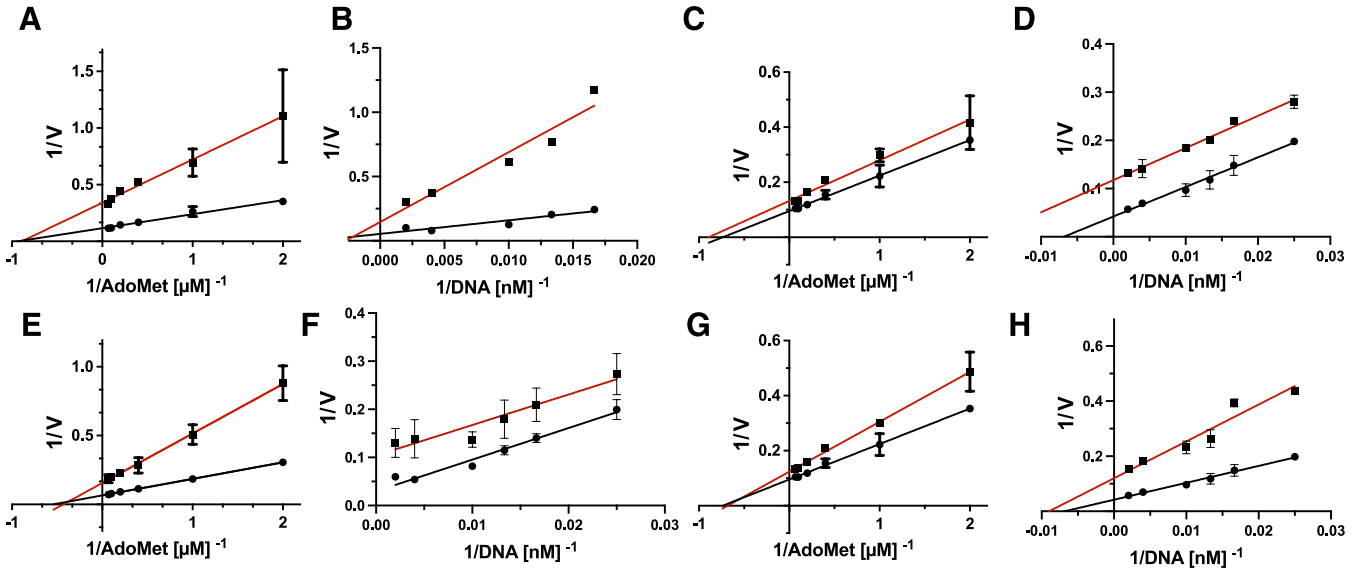

Figure 4. Double reciprocal plots for NSC 1 (A, B), NSC 2 (C, D), MMV 1 (E, F), and MMV 2 (G, H) with DNA and AdoMet. For A-D, the data points represent the mean and standard deviation of three independent reactions with added inhibitor (red line: A, C = 40 μM; B, D = 10 μM) and without inhibitor (black line). For E-H, the data points represent the mean and standard deviation of two independent reactions with added inhibitor (red line: E, F = 50 μM; G, H = 75 μM) and without inhibitor (black line). Reactions were done at initial velocity conditions using 50 nM CcrM, and varied concentrations of one substrate while the other was kept at saturating conditions.

both **MMV** compounds **1** and **2** exhibited allosteric inhibition for both AdoMet and DNA. Given the structural similarities between these two pyrimidines, it would seem that both share allosteric mechanisms. It is worth noting that other bacterial DNA methyltransferase inhibitors have displayed mechanistically divergent behaviors, for example by interfering directly with DNA or AdoMet binding.[10]

Our additional inhibition studies were focused on determining whether these compounds were selective for CcrM over human DNA methyltransferase 3A (DNMT3A). We used the catalytic domain (C-terminal) of DNMT3A (DNMT3A_CD), which is functional and highly conserved across all DNMTs,[22] using 100 μM concentration of the inhibitors. Based on our prior mechanistic inhibition studies, we were not surprised to find that two of the four compounds showed great selectivity for CcrM over DNMT3A (Figure 5).

Curiously, both **MMV 1** and **2** exhibited no inhibition against DNMT3A_CD at concentrations up to 150 μM. Their allosteric mode of inhibition, as explained by Figure 3, and selectivity for CcrM over human DNMT3A makes these compounds promising for continued development. Despite these promising findings, their potency for CcrM requires further optimization. When comparing **NSC 1** and **2** to **MMV 1** and **2**, the MMV compounds display structures that are more easily modified, offering opportunities for synthetic alterations leading to improve their potencies while retaining their selectivity (Figure 6). The drawbacks due to **NSC 1**'s history as a DNA intercalator, as well as **NSC 2**'s limited capacity for structural alteration are compounded by the lack of selectivity observed by both compounds, limiting their potential for further development.

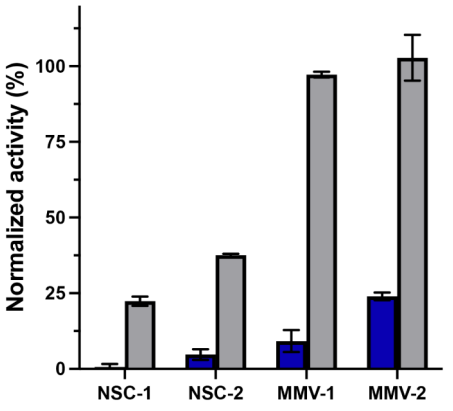

Figure 5. Selectivity screening of inhibitors against human DNMT3A_CD. All inhibitors were screened against both DNMT3A_CD (gray) and CcrM (blue) at 100 μM and the normalized activity of each enzyme was measured. For all compounds, the data represents the mean and standard deviation of three independent reactions.

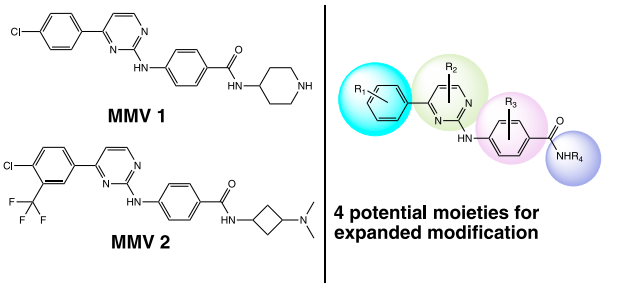

Figure 6. Potential structural zones of the MMV compounds for synthetic derivatization and improvement of potency while retaining selectivity.

CONCLUSION

In summation, the screening of two chemical libraries (National Cancer Institute Developmental Therapeutic Program Diversity Set VII and Medicines for Malaria Venture Global Health Box) identified four CcrM inhibitors. Three of the four compounds exhibited binding outside of the active site with only **NSC 1** displaying a competitive mode of inhibition

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against DNA. **NSC 1** and **2** displayed low selectivity between CcrM and DNMT3A compared to **MMV 1** and **2**. The modular scaffold of our MMV compounds offers various moieties for further optimization and could provide a basis for a new group of bacterial epigenetic inhibitors. Thus, our findings provide a new strategy for combatting various CcrM-containing bacterial pathogens and could lead to the development of novel antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.5c01540.

Full details of all materials and methods used in this work including protein production and assay methods (PDF)

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Author Contributions

The manuscript was written through contributions of all authors./All authors have given approval to the final version of the manuscript.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS

CcrM	Cell-cycle regulated methyltransferase
DNMT3A,	DNA methyltransferase 3A; DNMT's, DNA methyltransferases
National Service Center	NSC
National Cancer Institute	NCI
Developmental Therapeutic Program	DTP
MMV	Medicines for Malaria Venture
AdoMet	S-adenosyl methionine
GHP	Global Health Priority Box
M. lincolnii	*Moraxella lincolnii*
C. crescentus	*Caulobacter crescentus. C. difficile, Clostridioides difficile*

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