Published in final edited form as:

Biochem Biophys Res Commun. 2015 May 22; 461(1): 65–69. doi:10.1016/j.bbrc.2015.03.170.

Role of methylthioadenosine/S-adenosylhomocysteine nucleosidase in *Vibrio cholerae* cellular communication and biofilm development

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Abstract

In *Vibrio cholerae*, the genes required for biofilm development are repressed by quorum sensing at high cell density due to the accumulation in the medium of two signaling molecules, cholera autoinducer 1 (CAI-1) and autoinducer 2 (AI-2). A significant fraction of toxigenic *V. cholerae* isolates, however, exhibit dysfunctional quorum sensing pathways. It was reported that transition state analogs of the enzyme methylthioadenosine/S-adenosylhomocysteine nucleosidase (MtnN) required to make AI-2 inhibited biofilm formation in the prototype quorum sensing-deficient strain N16961. This finding prompted us to examine the role of both autoinducers and MtnN in biofilm development and virulence gene expression in a quorum sensing-deficient genetic background. Here we show that deletion of *mtnN* encoding methylthioadenosine/S-adenosylhomocysteine nucleosidase, *cqsA* (CAI-1), and/or *luxS* (AI-2) do not prevent biofilm development. However, two independent *mtnN* mutants exhibited diminished growth rate and motility in swarm agar plates suggesting that, under certain conditions, MtnN could influence biofilm formation indirectly. Nevertheless, MtnN is not required for the development of a mature biofilm.

1. Introduction

The formation of biofilm communities pose a challenge to the prevention and treatment of infectious diseases. It is well established that bacterial biofilms are recalcitrant to elimination with biocides, classical antibiotics and disinfectants [1-3]. The enhanced resistance of bacterial biofilms to innate host defenses and antibiotic treatment encourages chronic and/or relapsing infections [4-5] and calls for long-term antibiotic therapies that

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favor the acquisition of antibacterial drug resistance. Thus, novel compounds capable of inhibiting biofilm formation are commonly received with enthusiasm.

In numerous organisms, biofilm development is regulated by quorum sensing. Quorum sensing is a process by which bacteria communicate with one another by secreting and responding to extracellular signaling molecules termed autoinducers [6]. In *V. cholerae*, two autoinducer systems have been identified. System 1 consists of the metabolically-related molecules 3-aminotridec-2-en-4-one (Ea-CAI-1), tridecane-3,4-dione (DK-CAI-1) and (S)-3-hydroxytridecan-4-one (CAI-1) sensed by the receptor CqsS [7-9] . Ea-CAI-1 is synthesized by the activity of CqsA that catalyzes the reaction between S-adenosylmethionine (SAM) and decanoyl-coenzyme A to yield Ea-CAI-1 and methylthioadenosine [9]. Ea-CAI-1 is spontaneously converted to DK-CAI-1, which can be enzymatically reduced to CAI-1 [9]. System 2 consists of autoinducer 2 (AI-2), a set of interconverting molecules derived from the shared precursor (4S)-4,5-dihydroxypentane-2,3-dione (DPD). DPD is synthesized from *S*-ribosylhomocysteine (SRH) by the activity of LuxS [10]. CqsS and the AI-2 receptor LuxPQ feed cell density sensory information through a phosphorelay system to the regulator LuxO [7]. At low cell density, the autokinase domains of CqsS and LuxPQ become phosphorylated and phosphorus is transferred to LuxO [7]. Phospho-LuxO acts to destabilize the *hapR* mRNA encoding the master quorum sensing regulator HapR [11-12]. When the concentration of autoinducers produced by growing bacteria reaches a threshold, CqsS and LuxPQ switch from kinase to phosphatase and the flow of phosphorus is reversed. Inactivation of LuxO allows the expression of the HapR which functions to represses biofilm formation [13-17]. In *V. cholerae* C7258, a strain that expresses a functional HapR-dependent quorum sensing pathway, deletion of *luxS* enhanced biofilm formation while exogenous DPD diminished biofilm formation in a dose dependent manner [18]. Not all choleragenic *Vibrios*, however, express HapR. A study involving 16 geographically diverse O1, O139, nonO1/nonO139 serogroup *V. cholerae* strains revealed a high rate (62 %) of isolates with dysfunctional quorum sensing systems [19]. One example is the prototype strain N16961 that contains a natural frame shift mutation in *hapR* [20]. It has been shown that *V. cholerae* strains containing a natural frame shift mutation in *hapR* respond to autoinducers through a separate pathway involving the diguanylate cyclase VCA0939 [21].

The *V. cholerae mtnN* gene (VC2379) encodes an N-glycosidase with dual substrate specificity for methylthioadenosine and S-adenosylhomocysteine. Recently, transition state analogs of methylthioadenosine/S-adenosylhomocysteine nucleosidase (MtnN) were suggested to diminish biofilm formation in *V. cholerae* strain N16961 [22-23]. The *luxS* and *mtnN* gene products belong to a pathway that functions to recycle S-adenosylhomocysteine (SAH) [24]. MtnN converts SAH to adenine and SRH. Then, the product of *luxS* (S-ribosylhomocysteine lyase) converts SRH into L-homocysteine and DPD. Consequently, inhibition of MtnN activity blocks the production of the AI-2 quorum sensing signal. Since accumulation of autoinducer molecules at high cell density represses biofilm formation, the claim that blocking the biosynthesis of AI-2 inhibits biofilm formation [22-23] challenges this regulatory model.

Little knowledge exists on the role of quorum sensing and AI-2 in biofilm development in strain N16961. Thus, we undertook a genetic approach to determine the role of each autoinducer and MtnN in biofilm development in this genetic background. Our data shows that lack of both autoinducers or MtnN do not prevent nor enhance biofilm formation in this prototype cholera strain.

2. Materials and methods

2.1. Strains and media

The strains used in study were derived from *V. cholerae* N16961 (O1, El Tor biotype). *Escherichia coli* strains TOP10 (Life technologies) and S17-1λpir [25] were used for plasmid propagation and mutant construction. Bacterial strains were grown in LB medium at 37°C with agitation (250 rpm). *V. cholerae* MM920 [7] and *V. harveyi* BB170 [26] were used to measure CAI-1 and AI-2 activities, respectively. For the AI-2 assay, strain BB170 was grown in AB medium [26]. To detect the expression of the toxin co-regulated pilus (TCP), *V. cholerae* was grown under ToxR-permissive condition in AKI medium [27]. Ampicillin (Amp, 100 μ g/mL), polymyxin B (PolB, 100 units/mL) or kanamycin (Km, 50 μ g/mL) were added to the medium as required. The MtnN transition state analog MT-DADMe-Immucillin-A was kindly provided by Verrn Schramm (Albert Einstein College of Medicine) and added to the culture medium at a final concentration of 10 μ g/mL.

2.2. Construction of mutants

All mutants were constructed in strain N16961 by allelic exchange using derivatives of the suicide vector pCVD442 [28] as described previously [29]. The suicide vectors pCVDΔCqsA [29], pCVDluxS-Km [29], and pCVDΔluxO [30] harbor chromosome fragments containing deletions of *cqsA*, *luxS* and *luxO*, respectively. To construct *cqsA*, *luxS* and *luxO* mutants the corresponding suicide vector was transferred by conjugation from *E. coli* S17-1λpir to N16961. Exconjugants were selected in LB medium containing Amp and PolB. Finally, allelic exchange products containing the corresponding deletion in the N16961 background were obtained by sucrose selection as previously described [29]. To construct N16961*luxO*^{D47E}, the wild type *luxO* gene was amplified by PCR from strain N16961 genomic DNA using the Advantage 2 PCR kit (Biosciences Clontech) and the primer combination 5'-ATGGTAGAAGACACGGC/5'-TTACCGTTCCTTCTCTT. The PCR product was cloned in pUC19 and the D47E amino acid substitution generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's instructions. The mutant allele was transferred to pCVD442 and N16961*luxO*^{D47E} isolated by allelic exchange as described above. To construct N16961 derivatives lacking MtnN activity, chromosome fragments flanking the *mtnN* open reading frame were amplified by PCR using the primer combinations 5'-GCCGAGCTCCAGTCAGGGAGTCGATTC/5'-AAAGGATCCGATGCCGATTTTCATAGGG and 5'-GAAGGATCCGCTCTTCCGCTATGGTGCTG/5'-TAAGCATGCAGGTTTCCACTCCCGCCTTG and sequentially cloned in pUC19. A Km resistance cassette from plasmid pUC4K (GenBank accession number X06404) was inserted in place of the *mtnN* gene. Finally, the *mtnN* flanking DNA fragments and Km cassette was

transferred to pCVD442 to yield pCVDΔmtnN. The above suicide vector harboring the *mtnN* deletion and Km insertion was used to construct the N16961ΔmtnN strains by conjugation and sucrose selection as described above. All mutations and deletions were confirmed by PCR and DNA sequencing.

2.3. Autoinducer bioassays

To measure the production of CAI-1 activity, *V. cholerae* strains were grown in LB medium to late log phase and the cultures were centrifuged at 12,000 rpm for 10 min. The supernatants were filtered through a 0.22-μm syringe filter and tested for the presence of CAI-1 activity by inducing light production in the *V. cholerae* reporter strain MM920 [7]. The reporter strain was grown overnight with shaking at 30°C, diluted 1:10 in fresh medium and 70 μ L aliquots transferred to an opaque-wall 96-well microtiter plate. Cell-free culture fluids were added to a final concentration of 30% (v/v). The plates were incubated at 30°C with agitation and light production was measured at 30 min intervals in a Synergy 2 BioTek plate reader. Results are expressed as light fold induction relative to a sterile medium control. For AI-2 activity, cell-free culture supernatants were tested for light production in the AI-2 reporter strain *V. harveyi* BB170 as described in [26].

2.4. Measurement of MtnN activity

To determine MtnN activity cell pellets were resuspended in 10 mM HEPES (pH 7.4) - 1 mM dithiothreitol (DTT) and disrupted by sonication. The cleared cell extracts were dialyzed against buffer containing 100 mM HEPES, 1 mM DTT, 20% glycerol. Reactions mixtures (1 mL) consisted of 100 mM HEPES, 50 mM potassium phosphate, 100 μM methylthioadenosine and crude extract so that linear increase in product formation could be followed over time. Reactions were incubated for 0, 0.25, 0.5, 1, and 2 hours at 37 °. At each time point 150 μl of reaction mixture was removed, mixed with 150 μl of water, and the reaction was stopped by boiling. The cell debris was removed by filtration and the sample was injected onto a 5 μm BDS Hypersil C-18 column (150 × 4.6 mm) (Keystone Scientific Inc., State College, PA) equilibrated with 50 mM ammonium dihydrogen phosphate buffer (pH 4.5) containing 2.5% acetonitrile (flow rate of 1 ml/minute). Adenine and methylthioadenosine were eluted from the column by isocratic elution for 10 min with 50 mM ammonium dihydrogen phosphate buffer (pH 4.5) containing 2.5% acetonitrile followed by 20 min linear gradient to 50 mM ammonium dihydrogen phosphate buffer (pH 4.5) containing 50% acetonitrile. Adenine and methylthioadenosine were detected as they eluted from the column by their absorbance at 254 nm.

2.5. Biofilm and motility assays

Biofilm formation was measured by the crystal violet staining method and results were normalized for growth and expressed as the OD_{570} nm/OD_{600} nm ratio [13]. For motility detection, swarm agar plates consisting of LB medium containing 0.3% agar were inoculated by stabbing with overnight cultures grown from single colonies in LB medium. The plates were incubated for 24 h at 30°C, imaged and the swarm halo (mm) was measured.

2.6. Western blot detection of the toxin co-regulated pilus production

TcpA, the major TCP subunit, was detected in Western blots using a rabbit anti-TcpA serum kindly provided by Biao Kan (CDC Beijing). Cell pellets corresponding to the same number of cells based on OD_{600} readings were boiled in 100 μ L of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and proteins were separated in a 12% polyacrylamide gel. Gels were transferred to polyvinylidene difluoride (PVDF) membranes, and TCP protein was detected with the BM chemiluminescence kit (Roche Applied Sciences, Indianapolis, IN) following the manufacturer's recommended procedure.

3. Results and discussion

In this study we first examined if quorum sensing could activate rather than repress biofilm formation in *V. cholerae* strains exhibiting a dysfunctional quorum sensing system. To this end, we constructed a set of isogenic quorum sensing deletion mutants starting from strain N16961 (**Fig. 1**). Since SAM serves as a precursor for the biosynthesis of both autoinducers, we first examined the possibility that a blockage in the biosynthesis of one autoinducer could indirectly affect the production of the other. As expected, Δ*cqsA* and Δ*luxS* mutants did not produce detectable CAI-1 and AI-2 activity, respectively (**Fig. 1**). Further, the Δ*cqsA* deletion mutant produced wild type levels of AI-2 while the Δ*luxS* mutant produced wild type levels of CAI-1 indicating that blocking the biosynthesis of one autoinducer does not impact the production of the second (**Fig. 1**). We also constructed a Δ*luxO* mutant in which quorum sensing is permanently active and a *luxO*^{D47E} mutant. The D47E mutation in LuxO renders this protein active irrespective of its phosphorylation stage to permanently turn off quorum sensing [7]. We next examined the effect of these mutations on biofilm formation using the crystal violet staining assay. As shown in **Fig. 2**, none of these mutations prevented biofilm formation and the modest differences observed between strains did not reach statistical significance. In *V. cholerae*, biofilm development and the expression of virulence factors are both negatively regulated by quorum sensing [13]. Thus, we asked if the N16961 quorum sensing mutants expressed elevated virulence factors. To this end, we chose to measure the expression of TCP. Expression of TCP is co-regulated to that of cholera toxin and the pilus is required for surface adherence to the intestinal mucosa, microcolony formation and attachment to chitinous surfaces [31-32]. As shown in **Fig. 3**, alteration of quorum sensing by mutation did not significantly impact TCP expression. From the above results, we conclude that quorum sensing does not regulate biofilm development and virulence gene expression in strain N16961 that lacks the HapR regulator.

Based on the above findings, our data is not consistent with inhibition of MtnN diminishing biofilm formation by disrupting quorum sensing. The fact that *mtnN* is required for the biosynthesis of AI-2 and at the same time is part of a metabolic pathway that recycles SAH can complicate the interpretation of *mtnN* phenotypes. Thus, we examined if MtnN could affect biofilm formation by a mechanism independent of quorum sensing. To this end, we constructed two independent *mtnN* deletion mutants. The MtnN activity of wild type strain N16961 was 2717 ± 535 nmoles/mg/h. In contrast, both *mtnN* mutants exhibited activities < 10 nmoles/mg/h under identical conditions. This result indicates that *mtnN* is the only gene

encoding this enzyme activity in the *V. cholerae* genome. As expected, we could not detect AI-2 activity in the supernatants of Δ*mtnN* mutants (data not shown). As shown in **Fig. 4A**, deletion of *mtnN* did not prevent the formation of wild type biofilms. Loss of *mtnN*, however, resulted in diminished growth rate, which could be due to the accumulation of potentially toxic SAH (**Fig. 4B**). Further, the *mtnN* deletion mutants exhibited reduced motility in swarm agar plates (**Fig. 4C**). We suggest that the reduced swarm halo could be due to SAH inhibition of SAM-dependent methylation of methyl-accepting chemotaxis proteins required to transduce chemotactic signals to the flagellar motor. Consistent with the above genetic data, strain N16961 was capable of developing a wild type biofilm in the presence of the transition state analog MT-DADMe-Immucillin-A. Addition of the transition state analog to the medium, however, resulted in 80 % inhibition of MtnN activity. This level of inhibition suggests that a significant fraction of MT-DADMe-Immucillin-A remained bound to MtnN after cell disruption and dialysis. This result is consistent with MT-DADMe-Immucillin-A binding to MtnN with a dissociation constant in the picomolar range [23].

Our results don't rule out the possibility that deletion/inhibition of MtnN could affect biofilm development under certain experimental conditions. If any, the effect of MtnN on biofilm formation could result from reduced motility/chemotaxis and/or growth rate rather than disruption of quorum sensing. Indirect effects of this kind could be subject to significant variability in the crystal violet microtiter plate assay. For instance, it has been shown that flagellum and pili accelerate surface attachment and facilitates bacterial spreading along abiotic surfaces [33-34]. Nevertheless, non-motile mutants can still develop normal biofilms provided sufficient time. Another source of variability is the strength with which biofilm cells are anchored to the surface. This could vary with strain and growth medium due to the expression of matrix proteins such as Bap1 [35]. An additional complicating factor is the non homogenous distribution of adherent cells in the crystal violet microtiter plate assay. In addition to adhering to the bottom and walls of a well, many *V. cholerae* strains form a thick biofilm at the liquid-air interface that could be difficult to quantitate or could detach with the washings. Our study, nevertheless, shows that *V. cholerae* mutants lacking MtnN activity, can form a biofilm similar to wild type.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This study was supported by National Institutes of Health Research Grants AI081039 to AJS, AI090397 to JAB and by a Southern Research Institute endowment fund to JAB.

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Highlights

- Quorum sensing and autoinducer 2 modulate biofilm formation in *Vibrio cholerae*.
- S-adenosylhomocysteine nucleosidase activity is required to produce autoinducer 2.
- S-adenosylhomocysteine nucleosidase was not required for biofilm development.
- Lack of this enzyme resulted in reduced growth rate and flagellar motility.

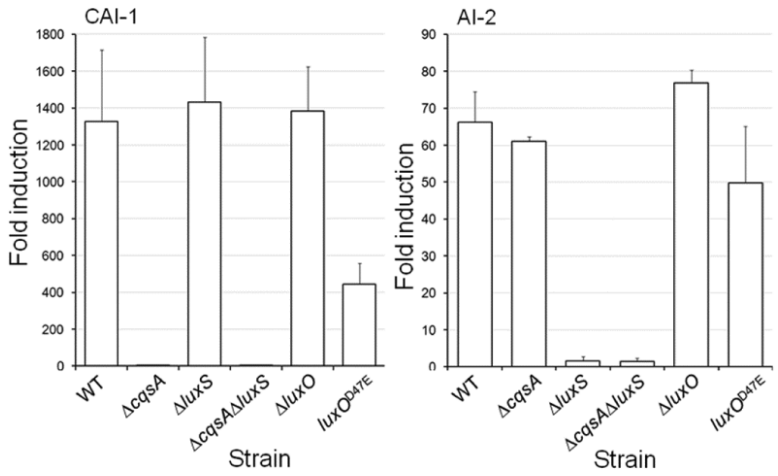

Fig.1. Production of CAI-1 and AI-2 in quorum sensing mutants

Strain N16961 (WT) and its isogenic mutants were grown in LB medium at 37°C to late log phase and the corresponding autoinducer activity measured as described in materials and methods. Each value represents the mean of three independent experiments. Error bars denote the standard deviation (STDEV).

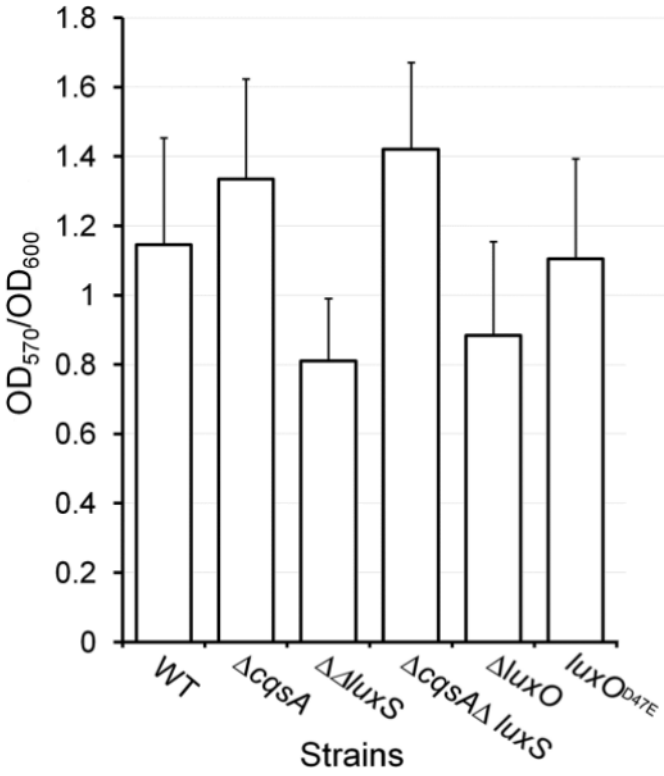

Fig.2. Biofilm formation in quorum sensing mutants

Strain N16961 (WT) and its isogenic mutants were grown in LB medium at 37°C to stationary phase. The cultures were diluted 100-fold in fresh medium and biofilms allowed to develop under static conditions for 24 h in 96-well microtiter plates. Biofilm formation was measured using the crystal violet straining method (OD_{570}) and the data normalized for growth (OD_{600}). Each value represents the mean of three independent experiments. Error bars denote the standard deviation (STDEV).

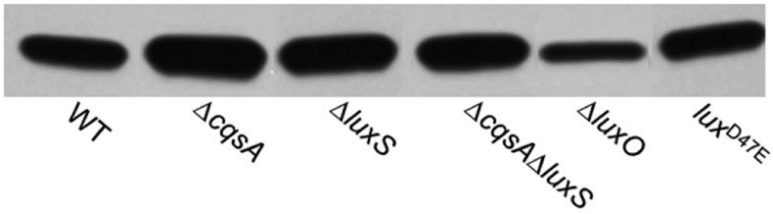

Fig.3. Expression of the toxin co-regulated pilus in quorum sensing mutants

Strain N16961 (WT) and its isogenic mutants were grown in AKI cultures and production of TcpA, the TCP major subunit, was determined by Western blot as described in materials and methods.

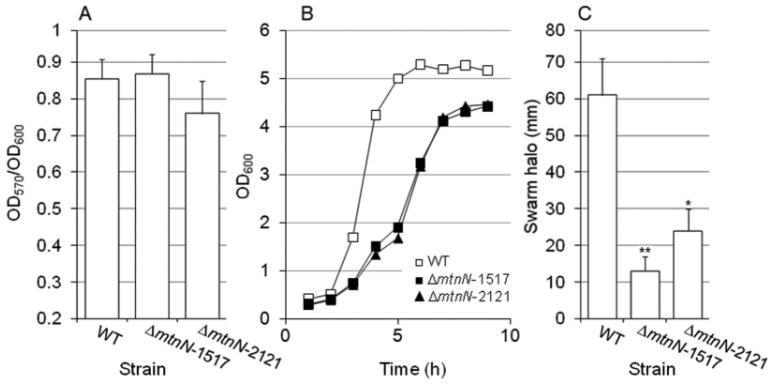

Fig.4. Phenotypic analysis of *mtnN* mutants

Strain N16961 (WT) and two independent isogenic Δ*mtnN* mutants were grown in LB medium at 37°C. **A. Biofilm formation**. The cultures were diluted 100-fold in fresh medium and biofilm formation measured as described for the quorum sensing mutants in 96-well microtiter plates. Each value represents the mean of three independent experiments. Error bars denote the standard deviation (STDEV). **B. Growth curve**. Overnight cultures were diluted 1000-fold in fresh LB medium and incubated with agitation (250 rpm) at 37°C. At different time points, growth was estimated by measuring the OD_{600}. **C. Motility**. Wild type and two independent isogenic Δ*mtnN* mutants were grown in LB medium to stationary phase at 37°C. Swarm (soft) agar plates were stabbed with cultures of each mutant and the plates were incubated 24 h. Motility was estimated by measuring the swarm halo (mm). Each value represents the mean of three independent experiments. Error bars denote the standard deviation (STDEV). *Swarm significantly different than wild type ($p < 0.05$); ** swarm significantly different than wild type ($p< 0.01$).