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GENETICS AND MOLECULAR BIOLOGY

Methionine Availability in the Arthropod Intestine Is Elucidated through Identification of *Vibrio cholerae* Methionine Acquisition Systems

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ABSTRACT While only a subset of *Vibrio cholerae* strains are human diarrheal pathogens, all are aquatic organisms. In this environment, they often persist in close association with arthropods. In the intestinal lumen of the model arthropod *Drosophila melanogaster*, methionine and methionine sulfoxide decrease susceptibility to *V. cholerae* infection. In addition to its structural role in proteins, methionine participates in the methionine cycle, which carries out synthetic and regulatory methylation reactions. It is, therefore, essential for the growth of both animals and bacteria. Methionine is scarce in some environments, and the facile conversion of free methionine to methionine sulfoxide in oxidizing environments interferes with its utilization. To ensure an adequate supply of methionine, the genomes of most organisms encode multiple high-affinity uptake pathways for methionine as well as multiple methionine sulfoxide reductases, which reduce free and protein-associated methionine sulfoxide to methionine. To explore the role of methionine uptake and reduction in *V. cholerae* colonization of the arthropod intestine, we mutagenized the two high-affinity methionine transporters and five methionine sulfoxide reductases encoded in the *V. cholerae* genome. We show that MsrC is the sole methionine sulfoxide reductase active on free methionine sulfoxide. Furthermore, in the absence of methionine synthesis, high-affinity methionine uptake but not reduction is essential for *V. cholerae* colonization of the *Drosophila* intestine. These findings allow us to place a lower limit of 0.05 mM and an upper limit of 0.5 mM on the methionine concentration in the *Drosophila* intestine.

IMPORTANCE Methionine is an essential amino acid involved in both biosynthetic and regulatory processes in the bacterial cell. To ensure an adequate supply of methionine, bacteria have evolved multiple systems to synthesize, import, and recover this amino acid. To explore the importance of methionine synthesis, transport, and recovery in any environment, all of these systems must be identified and mutagenized. Here, we have mutagenized every high-affinity methionine uptake system and methionine sulfoxide reductase encoded in the genome of the diarrheal pathogen *V. cholerae*. We use this information to determine that high-affinity methionine uptake systems are sufficient to acquire methionine in the intestine of the model arthropod *Drosophila melanogaster* but are not involved in virulence and that the intestinal concentration of methionine must be between 0.05 mM and 0.5 mM.

KEYWORDS *Drosophila*, invertebrate host, microbiology, *Vibrio cholerae*, methionine, methionine sulfoxide

The Gram-negative, halophilic, diarrheagenic bacterium *Vibrio cholerae* is found in brackish and salt water environments where only a subset of strains carries the virulence determinants that are associated with the human diarrheal disease cholera

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(1). In these environments, *V. cholerae* is found in association with aquatic arthropods, such as zooplankton and other crustaceans. In the environment, *V. cholerae* colonizes the intestines of aquatic arthropods and, in the laboratory, the intestines of model arthropods (2–5). Studies in our laboratory demonstrated that the rate of stem cell division in the intestine of the model arthropod *Drosophila melanogaster* is linked to luminal methionine availability (6). In addition, these studies suggested that, in the intestinal environment, methionine sulfoxide (MetO), an oxidized form of methionine, plays a role in competitively inhibiting repair of damaged host proteins required for *V. cholerae* pathogenesis. We questioned whether *V. cholerae* uptake of methionine and MetO might promote virulence by decreasing the abundance of these amino acids in the arthropod intestine. Therefore, we set out to test the impact of intestinal methionine synthesis, methionine uptake, and MetO reduction on *V. cholerae* colonization and growth in the intestine of this host.

The sulfur-containing amino acid methionine participates in multiple essential synthetic and regulatory pathways central to bacterial physiology (7). Methionine exists in dextrorotatory (D) and levorotatory (L) forms due to its chiral α atom. These enantiomers have distinct functions in bacteria (8). L-Methionine is a building block of many proteins, and formyl-methionine is required to initiate protein translation. Furthermore, L-methionine and ATP are converted to *S*-adenosyl-L-methionine (SAM) through the action of SAM synthase. SAM is essential for synthetic methylation reactions that give rise to formylated Met-tRNA, pantothenate, thymidylate, thiamine, and purines and is responsible for glycine and serine interconversion. In addition, methylation of DNA, RNA, and proteins by SAM serves to regulate transcription, translation, and protein function. D-Methionine is also thought to play a role in bacterial physiology. It can be incorporated into peptidoglycan, and there is evidence that this inhibits peptidoglycan synthesis (9, 10).

Free methionine is easily oxidized by reactive oxygen species (ROS) to one of the diastereoisomers methionine-*S*-sulfoxide (Met-*S*-O) or methionine-*R*-sulfoxide (Met-*R*-O). Bacteria can use these compounds as a source of methionine after MetO reduction by methionine sulfoxide reductases (Msrs) (11–13). Protein function is also modulated by methionine oxidation, and a large body of evidence suggests that reversible oxidation of protein-associated methionine is a mechanism by which cells adjust their physiology in response to ROS (14). As one might predict, MetO reduction is essential for the virulence of many pathogens (15–20).

Because methionine participates in so many essential processes, bacteria have evolved methionine synthesis pathways, multiple uptake systems for this amino acid, and multiple Msrs (7, 21). Methionine synthesis from homocysteine is carried out by either MetE or MetH (22, 23). When environmental methionine is scarce, the LysR-type transcription factor MetR binds to the promoters of the *metE* and *metH* genes to activate their transcription and thus methionine synthesis (23, 24). Two high-affinity methionine uptake systems have been described in bacteria. The *metD* locus encodes an ATP-binding cassette (ABC) transporter composed of three proteins as follows: an ATPase (MetN), a permease (MetI), and a substrate-binding protein (MetQ) (25). This system transports both the L and D isomers of methionine as well as MetO (26, 27). The well-conserved MetT protein is a cation/methionine antiporter (28). MsrA, MsrB, and MsrC, three Msrs without sequence homology to each other, which are broadly conserved in bacteria, archaea, and eukaryotes, reduce MetO to methionine (29–31). MsrA, the first identified methionine sulfoxide reductase, is distinguished by a protein methionine sulfoxide reductase or peptide methionine sulfoxide reductase (PMSR) domain (32, 33). MsrA homologs are generally active on free and protein-associated Met-*S*-O (34). MsrB proteins possess SelR domains that coordinate zinc via a CXXC motif (35, 36). A subset of MsrBs utilize a rare catalytic selenocysteine amino acid for their function. MsrB homologs are generally active on protein-associated Met-*R*-O alone (37). Some organisms also encode fused forms of MsrA and B. MsrC proteins encode a GAF domain that is named for the proteins in which it is found, namely, cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA, the formate-hydrogen lyase transcrip-

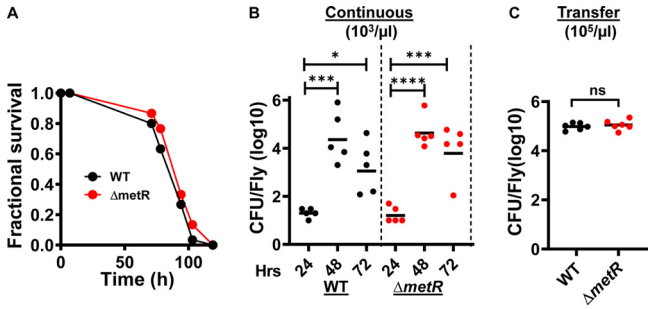

FIG 1 Methionine synthesis is not essential for *V. cholerae* growth in the fly gut. (A) Fractional survival of flies fed wild-type *V. cholerae* (WT) or a Δ*metR* mutant in LB broth. The difference in survival is not significant. (B) Colonization of the fly gut by wild-type *V. cholerae* (WT) and a Δ*metR* mutant during continuous feeding on PBS inoculated with *V. cholerae* at a density of 10^3 CFU/μl for 72 h. (C) Colonization of the fly gut by wild-type *V. cholerae* and a Δ*metR* mutant during continuous feeding on PBS inoculated with 10^5 CFU/μl of *V. cholerae* for 48 h followed by transfer to sterile PBS for 24 h. In panels B and C, each data point represents the *V. cholerae* burden of an individual fly. Data was log transformed. In panel B, the statistical significance was determined by applying a one-way ANOVA with Dunnett's multiple-comparison test to the log-transformed data. In panel C, a Student's *t* test was applied to the log-transformed data. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

tional activator protein (31, 38). In some of these proteins, the GAF domain has been demonstrated to bind cyclic nucleotides. MsrC homologs are active on free Met-*R*-O.

Very little is known about *V. cholerae* methionine synthesis and acquisition systems except that the *V. cholerae* genome encodes a MetR homolog that is essential for growth on defined medium lacking methionine (39). This transcription factor activates methionine synthesis by binding to promoters that direct methionine synthesis when environmental availability is inadequate. MetR was originally studied due to its role in colonization of the mammalian intestine (39). However, MetR activation of glycine catabolism rather than methionine synthesis was ultimately found to be critical in this environment.

To explore methionine acquisition by *V. cholerae* in the arthropod intestine, we first identified and deleted genes encoding two high-affinity methionine transporters and five Msrs. This allowed us to demonstrate that, similar to the mammalian intestine, methionine abundance in the arthropod gut is sufficient to support growth of *V. cholerae* in the absence of bacterial synthesis or dietary supplementation (39). Furthermore, we showed that, when synthesis is blocked by deletion of *metR*, high-affinity methionine transport but not MetO reduction is essential for colonization of the *Drosophila* intestine. Our findings suggest that the intestinal concentration of methionine is between 0.05 mM and 0.5 mM. Therefore, while methionine synthesis is required in the host environments of invasive pathogens (40, 41), this may not be the case for pathogens that cause disease from the intestinal lumen.

RESULTS

Methionine synthesis is not essential for virulence or growth in the *Drosophila* intestine. *V. cholerae* is a methionine auxotroph in the absence of the regulator MetR (39). To test the requirement for methionine synthesis in the arthropod gut, we constructed a strain carrying an in-frame deletion in *metR* and then fed wild-type *V. cholerae* as well as the Δ*metR* mutant resuspended in Luria-Bertani (LB) broth to the control *Drosophila* strain Oregon R (42). Flies infected with either wild-type *V. cholerae* or the Δ*metR* mutant died at the same rate (Fig. 1A). To determine whether MetR was essential for colonization of the fly intestine, we resuspended these strains at a density of 1,000 CFU/μl in phosphate-buffered saline (PBS), which is just above the infectious dose (5), and allowed the control *Drosophila* strain Oregon R to ingest this suspension. We quantified bacterial burden at 24, 48, and 72 h. As shown in Fig. 1B, at 24 h, the mean CFU/fly was only 20. Over the next 24 h, the intestinal load of wild-type *V. cholerae* increased to approximately 200,000 CFU/fly and then remained constant. The Δ*metR* mutant colonized the *Drosophila* intestine equally rapidly and abundantly. This

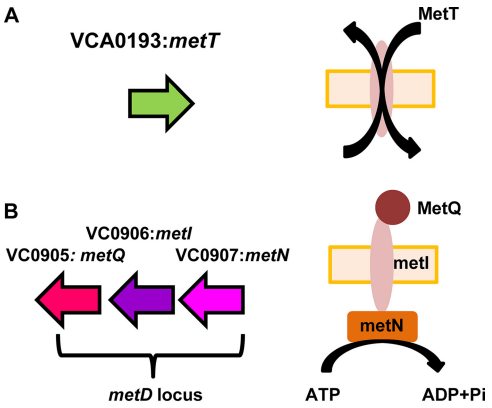

FIG 2 Two *V. cholerae* loci that encode homologs of bacterial methionine transport systems. (A) MetT is a cation/methionine antiporter. (B) The *metD* locus encodes an ABC transporter comprised of the periplasmic methionine-binding protein MetQ, the methionine inner membrane permease MetI, and the ATPase MetN. In this study, the *metD* locus is inactivated by an in-frame deletion in *metI*.

pattern of growth is similar to what has been observed previously (5). We hypothesize that minimal accumulation of *V. cholerae* in the fly gut on day 1 results from a bottle neck consisting of the innate immune response of the anterior midgut and the acidity of the middle midgut (43–45). *V. cholerae* bacteria that survive these compartments and reach the posterior midgut and rectum form biofilms and multiply on the surfaces of these compartments (5).

To ensure that bacteria that had accumulated as of day 2 were adherent to the intestine, we incorporated a 24-h washout period on sterile PBS at the end of our experiment. In this experiment, flies were fed *V. cholerae* at a density of 10^5 bacteria/μl, which is well above the minimum infectious dose, for 48 h prior to washout. We observed no difference in the ability of wild-type *V. cholerae* and the Δ*metR* mutant to adhere to the fly gut (Fig. 1C). Therefore, we conclude that methionine supplied by the host is adequate to support *V. cholerae* colonization and growth in the absence of methionine synthesis.

MetT and MetNIQ transport L-methionine. To identify high-affinity transporters of methionine that might be operative in the intestine, we set out to mutagenize all putative methionine transporters. The *V. cholerae* genome encodes two predicted methionine transport systems, MetNIQ and MetT (Fig. 2). Interestingly, the *V. cholerae* MetT homolog is more closely related to that of *Bacillus subtilis* (45% identity, 66% similarity) than to that of *Escherichia coli* (23% identity, 45% similarity).

To explore the role of MetNIQ and MetT in *V. cholerae* methionine uptake, we created in-frame deletions in *metI* and *metT* in a Δ*metR* mutant background. We then tested the growth of these mutants in a defined medium supplemented with various concentrations of L-methionine. As shown in Fig. 3A, the Δ*metR* mutant only grew in defined medium if supplemented with at least 0.05 mM L-methionine. The double mutants Δ*metR* Δ*metI* and Δ*metR* Δ*metT* demonstrated an L-methionine dependence very similar to that of the Δ*metR* mutant (Fig. 3B and C). However, the Δ*metR* Δ*metI* Δ*metT* triple mutant required 0.5 mM L-methionine for growth (Fig. 3D). Because the Δ*metR* Δ*metI* and Δ*metR* Δ*metT* mutants grow equally well with L-methionine supplementation, we conclude that they are both capable of transporting this substrate (Fig. 3E). Furthermore, because growth of the triple mutant is rescued by supplementation with high concentrations of L-methionine, we conclude that the observed growth defect results from the absence of high-affinity methionine transport and that one or more additional low-affinity transporters exists.

D-Methionine has opposing effects on *V. cholerae* growth. Periplasmic D-methionine has previously been reported to repress growth of *V. cholerae* as the result of incorporation into peptidoglycan (10). We hypothesized that D-methionine might also be transported into the cytoplasm by MetI and MetT and used to support growth. To

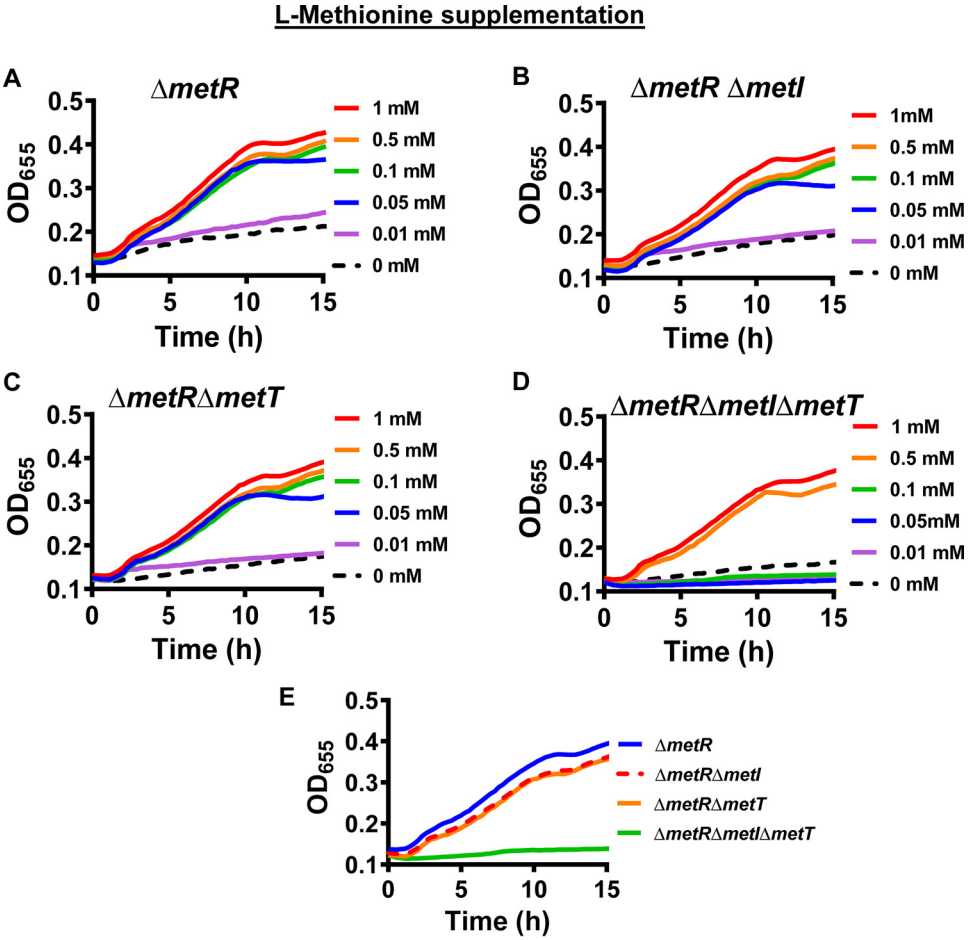

FIG 3 MetI or MetT is required for growth of a *V. cholerae* Δ*metR* mutant at concentrations of L-methionine less than or equal to 0.01 mM. (A to D) Growth of the indicated *V. cholerae* mutants in defined medium supplemented with L-methionine in the concentrations indicated at the right. (E) A comparison of the growth of indicated mutants in defined medium supplemented with 0.1 mM L-methionine. Growth curves represent the mean of technical duplicates and are representative of three biological experiments.

assess the periplasmic and cytoplasmic roles of D-methionine, we measured the growth of Δ*metR*, Δ*metR* Δ*metI*, Δ*metR* Δ*metT*, and Δ*metR* Δ*metI* Δ*metT* mutants over time in defined medium supplemented with this amino acid (Fig. 4). As shown in Fig. 4A, concentrations of 0.01 to 1 mM D-methionine decreased growth of the Δ*metR* mutant in defined medium. However, growth did not decrease steadily as the concentration of D-methionine was increased. Instead, the growth suppression was comparable for concentrations of 0.01 to 0.5 mM but less at the higher concentration of 1 mM D-methionine.

We then tested the impact on growth of D-methionine transport through MetI and MetT. In the Δ*metR* background, growth in unsupplemented defined medium was unchanged by deletion of *metI* (Fig. 4A and B) but decreased by deletion of *metT* (Fig. 4A and C), suggesting that MetT may play a minor role in transport of another amino acid. Furthermore, as compared with the parental Δ*metR* mutant, deletion of *metI* decreased growth in defined medium supplemented with 0.05 mM to 1 mM D-methionine (Fig. 4B to E). Growth inhibition by 0.01 mM D-methionine was observed both in the presence and absence of MetT and MetI (Fig. 4F). These observations are consistent with a model in which D-methionine has opposing effects on growth, inhibiting growth from the periplasm and increasing growth after MetI-mediated transport into the cytoplasm.

MetI and MetT transport methionine sulfoxide. In oxidizing environments, exogenous methionine may be present principally in the form of MetO. This oxidized form

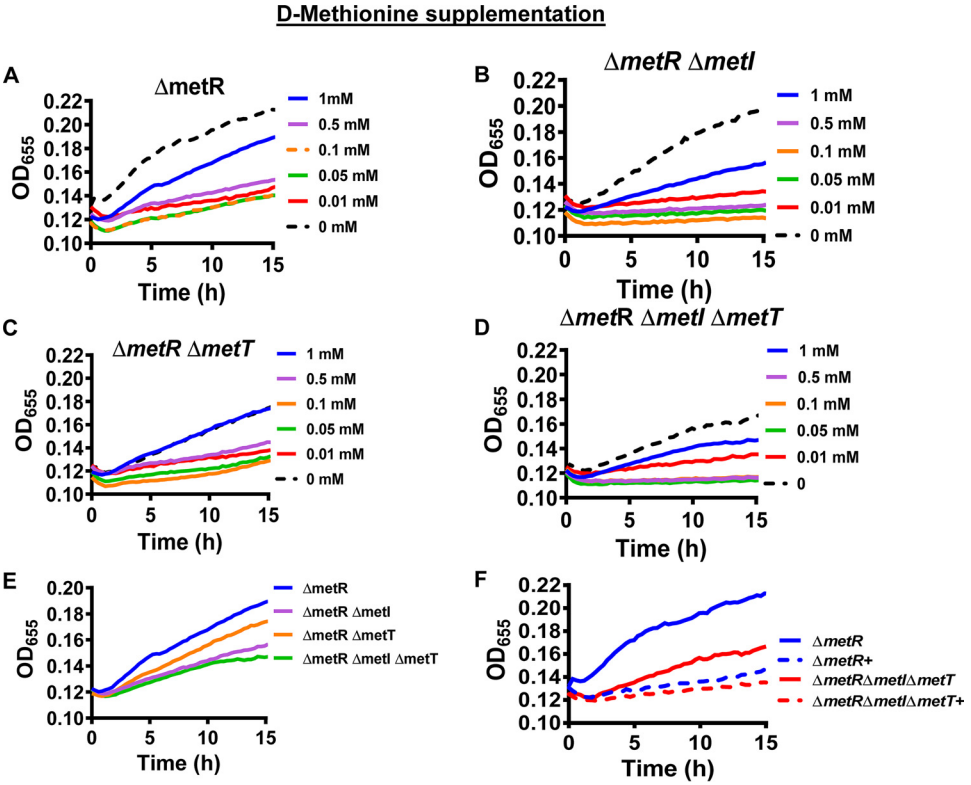

FIG 4 D-methionine decreases growth of a *V. cholerae* methionine auxotroph (Δ*metR*) in a transport-independent fashion. (A to D) Growth of the indicated mutants in defined medium supplemented with the indicated concentrations of D-methionine. (E) Growth of the indicated mutants in defined medium supplemented with 1 mM D-methionine demonstrating that transport by MetNIQ augments growth. (F) Growth of the indicated mutants in defined medium alone or supplemented with 0.01 mM D-methionine (+) illustrating transport-independent growth suppression by D-methionine. Growth curves represent the mean of technical duplicates and are representative of three biological experiments.

of methionine must be transported and reduced prior to use. *E. coli* MetNIQ has been shown to transport MetO as well as methionine (26). To determine whether *V. cholerae* MetI and MetT are involved in L-MetO transport, we measured growth of the Δ*metR* mutant as well as derivative transport mutants in the presence and absence of a variety of concentrations of L-MetO. As shown in Fig. 5A, the Δ*metR* mutant was able to grow on concentrations of L-MetO as low as 0.05 mM. The Δ*metR* Δ*metI* mutant could only grow on concentrations of 0.1 mM or higher, suggesting that, as previously observed for *E. coli*, *metT* transports L-MetO (Fig. 5B). Deletion of *metT* reduced *V. cholerae* growth only when MetI was absent (Fig. 5C and D). This suggests that, while MetI plays a more important role in transport of L-MetO at low substrate concentrations, MetT also transports L-MetO (Fig. 5E).

***V. cholerae* encodes five methionine sulfoxide reductases.** A previous publication identified the presence of one gene encoding a homolog of MsrA (VC2549), two genes encoding homologs of MsrB (MsrB1, VC1998; MsrB2, VC1236), and one gene encoding a fusion of MsrA and MsrB (MsrAB, VCA0615) (Fig. 6) (12). Upon further *in silico* research, we identified a homolog of MsrC (VC1498) in the *V. cholerae* proteome (Fig. 6). Only the MsrAB homolog included a signal sequence and transmembrane domain, suggesting that it is anchored in the inner membrane and functions in the periplasm. We discovered that in-frame deletions in the genes encoding the MsrAs and MsrBs either singly or in combination did not alter growth on L-Met-*R*,*S*-O (Fig. 7A to H). However, when *msrC* was deleted in any of these multiple deletion mutant backgrounds, *V. cholerae* was no longer able to grow on L-Met-*R*,*S*-O (Fig. 7I to K). Importantly, all mutants that were unable to grow on L-Met-*R*,*S*-O could be rescued by

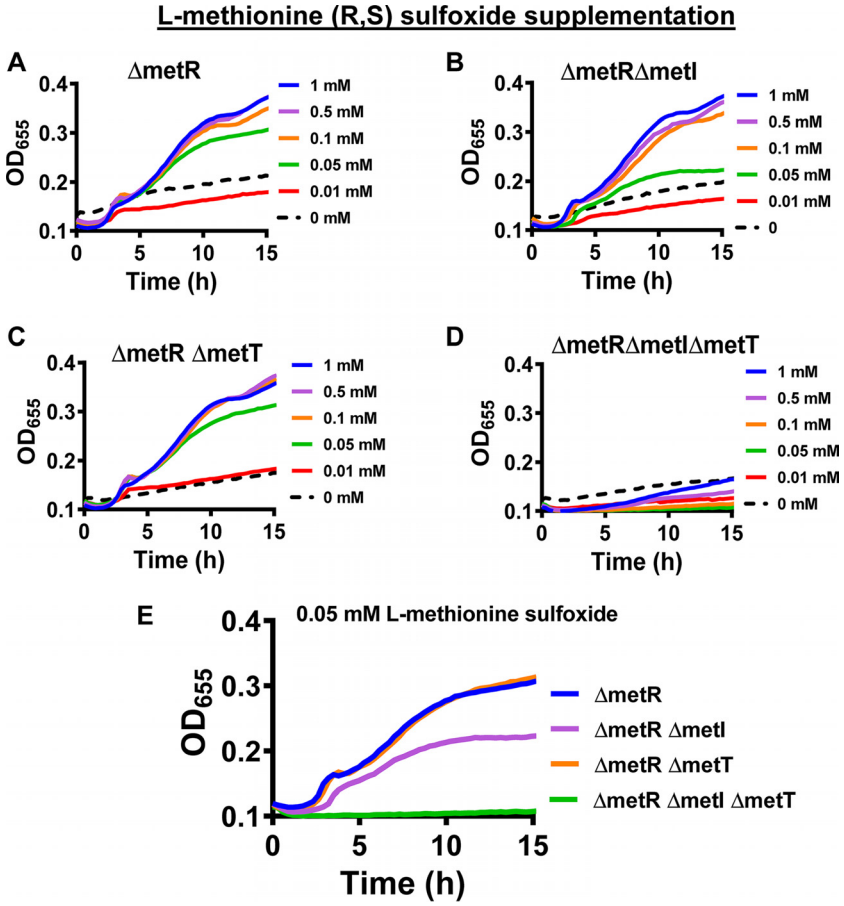

FIG 5 MetI and MetT are essential for growth of a *V. cholerae* methionine auxotroph on L-methionine (*R*,*S*) sulfoxide. (A to D) Growth of the indicated mutants in defined medium supplemented with the indicated concentrations of L-methionine sulfoxide. (E) Growth of the indicated mutants in defined medium supplemented with 0.05 mM L-methionine sulfoxide to illustrate the relative roles of MetI and MetT in transport. Growth curves represent the mean of technical duplicates and are representative of three biological experiments.

methionine supplementation (Fig. 7L), demonstrating that the growth defect is due to an inability to utilize L-Met-*R*,*S*-O. MsrA homologs are thought to be active on free and protein-associated Met-*S*-O, while MsrC homologs are active on free Met-*R*-O (34). Our results demonstrate that, of the five *V. cholerae* Msr homologs, only MsrC reduces

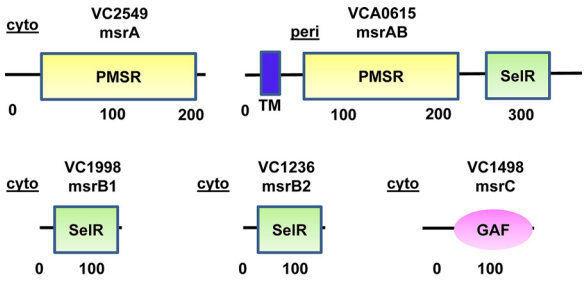

FIG 6 Five methionine sulfoxide reductases are encoded in the *V. cholerae* genome. *In silico* prediction of the domains and subcellular locations based on transmembrane domains and signal sequence predictions of the five methionine sulfoxide reductases of *V. cholerae*. MsrA, which has a peptide methionine sulfoxide reductase (PMSR) domain, is predicted to be active on free and protein-associated L-Met-*S*-O. MsrB has a SelR domain that coordinates Zn^{2+} via a CXXC motif and is predicted to be active on protein-associated L-Met-*R*-O. MsrC has a GAF domain that, in some proteins, binds cyclic nucleotides and is predicted to be active on free L-Met-*R*-O. Peri, periplasm; cyto, cytoplasm.

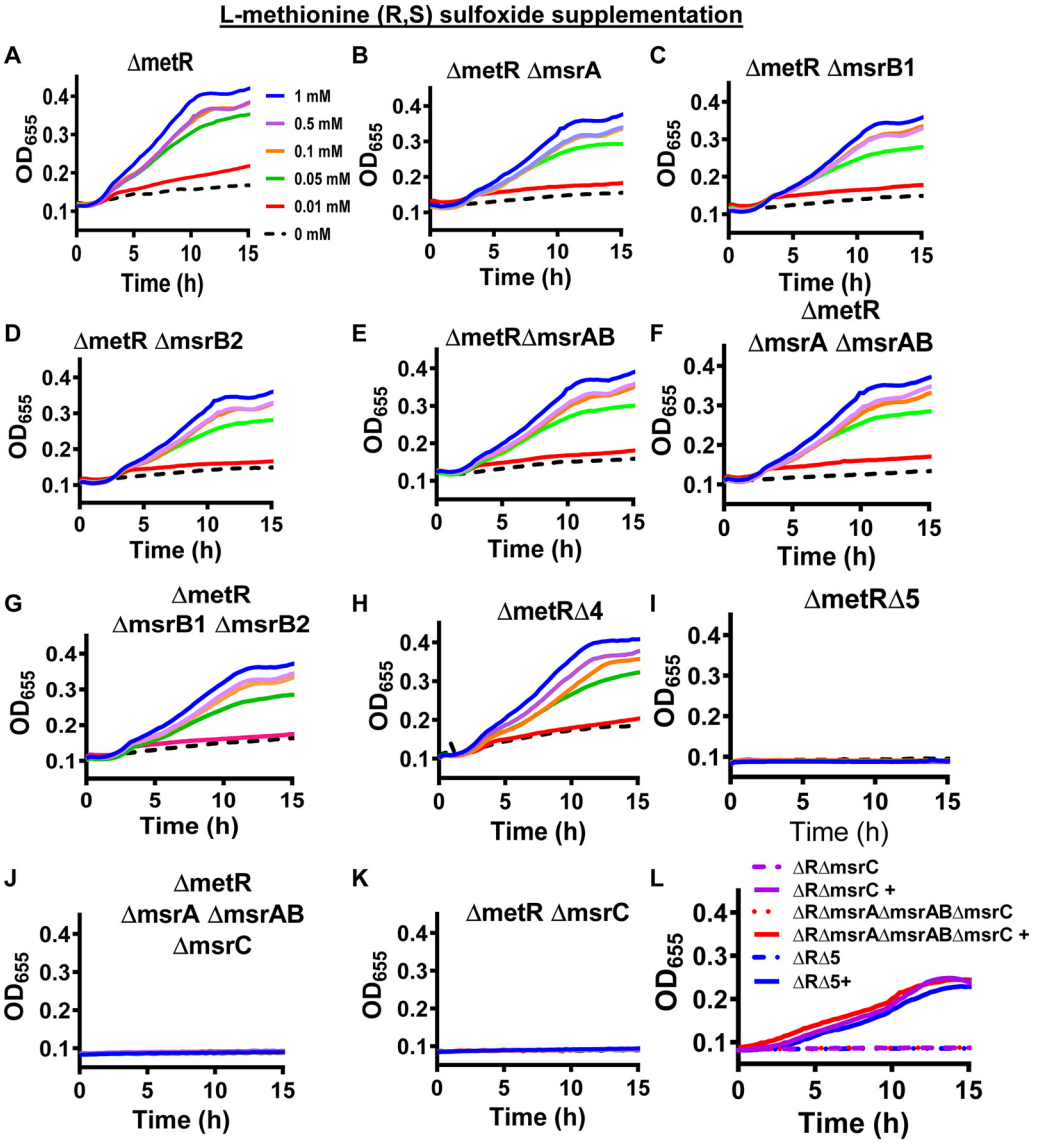

FIG 7 Methionine sulfoxide reductase C is essential for growth of a *V. cholerae* Δ*metR* mutant in defined medium supplemented with L-methionine-*R*,*S*-sulfoxide but not methionine. (A to K) Growth of the indicated *V. cholerae* mutants in defined medium supplemented with the indicated amount of L-methionine-*R*,*S*-sulfoxide. Δ*metR* Δ*msrA* Δ*msrB1* Δ*msrB2* Δ*msrAB*, Δ*metR* Δ4; Δ*metR* Δ*msrA* Δ*msrB1* Δ*msrB2* Δ*msrAB* Δ*msrC*, Δ*metR* Δ5. (L) Growth of the indicated mutants in the Δ*metR* background (ΔR) in defined medium alone or supplemented with 0.1 mM methionine (+). Growth curves represent the mean of technical duplicates and are representative of three biological experiments.

L-Met-O. Because we are supplementing our medium with the diastereomeric mixture L-Met-*R*,*S*-O, we cannot determine which diastereomer is reduced by MsrC.

High-affinity methionine transport but not methionine sulfoxide reduction is essential for *V. cholerae* replication in the *Drosophila* intestine. When *V. cholerae* is delivered in LB broth, its ingestion suppresses intestinal stem cell divisions and is lethal to the model arthropod host *Drosophila melanogaster* (42). Increased MetO concentrations in the intestinal lumen decrease host mortality, and methionine increases intestinal stem cell divisions, which has also been correlated with prolonged host survival (6, 46). We hypothesized that, if *V. cholerae* methionine uptake, MetO uptake, or MetO reduction significantly altered the concentrations of these amino acids in the *Drosophila* intestinal lumen, mutation of *V. cholerae* genes encoding transport systems and/or Msrs should decrease virulence. We, therefore, compared fly survival after infection with wild-type *V. cholerae*, a Δ*metR* mutant, a Δ*metR* Δ*metI* Δ*metT* mutant, a Δ*metR* Δ*msrC*

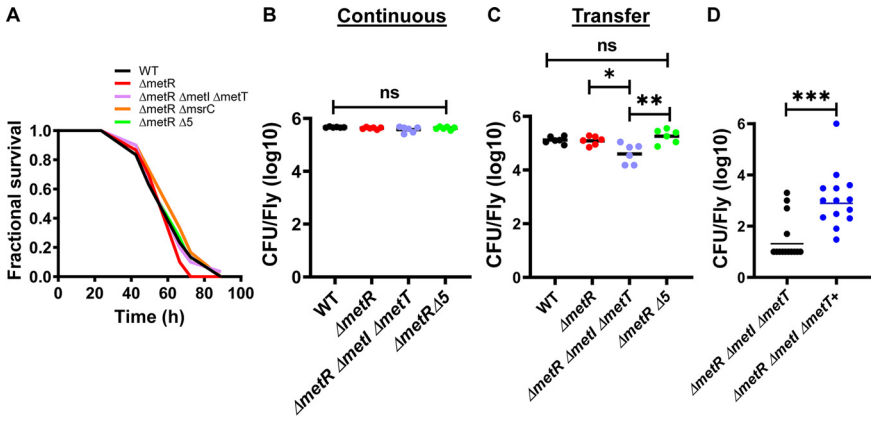

FIG 8 Methionine concentrations in the *Drosophila* intestine support colonization and growth of a *V. cholerae* methionine auxotroph capable of high affinity methionine transport. (A) Survival of *Drosophila* during ingestion of wild-type *V. cholerae* (WT), Δ*metR*, Δ*metR* Δ*metI* Δ*metT*, Δ*metR* Δ*msrC*, and Δ*metR* Δ*msrA* Δ*msrB1* Δ*msrB2* Δ*msrAB* Δ*msrC* (Δ*metR* Δ5) mutants administered in LB broth. Log-rank analysis was used to determine that these survival curves are not significantly different. (B) Colonization of the fly gut by wild-type *V. cholerae* and the indicated mutants during continuous feeding for 72 h on PBS inoculated with 10^5 CFU *V. cholerae*/μl. (C) Colonization of the fly gut by wild-type *V. cholerae* and the indicated mutants during continuous feeding for 48 h on PBS inoculated with 10^5 CFU *V. cholerae*/μl followed by transfer to sterile PBS for 24 h. (D) Colonization of the fly gut by the indicated *V. cholerae* mutants during continuous feeding at a concentration of 10^3 CFU/μl in PBS alone or supplemented with 1 mM methionine (+) for 72 h. A one-way ANOVA was used to calculate significance in panels B and C. A Mann-Whitney test was used in panel D. ***, $P < 0.001$; *, $P < 0.05$; ns, not significant. Each data point represents colonization of an individual fly.

mutant, and a *V. cholerae* Δ*metR* mutant also devoid of all Msrs (Δ5) in LB broth. As shown in Fig. 8A, none of these mutations resulted in a virulence defect. This suggests that *V. cholerae* methionine uptake, MetO uptake, and MetO reduction do not significantly impact the concentrations of these amino acids in the *Drosophila* intestinal lumen. We then questioned whether methionine and MetO uptake and MetO reduction are required for *V. cholerae* colonization in the *Drosophila* intestine. To test this, we compared colonization by the Δ*metR* Δ*metI* Δ*metT* and the Δ5 mutant with that by wild-type *V. cholerae* and the Δ*metR* mutant both during continuous feeding on 10^5 *V. cholerae*/μl in PBS and after a washout period. As shown in Fig. 8B, there was no difference in colonization between wild-type *V. cholerae* and these mutants after continuous feeding. In contrast, after a washout period, the Δ*metR* Δ*metI* Δ*metT* mutant but not the Δ5 mutant showed a colonization defect (Fig. 8C). To demonstrate that this colonization defect is the result of an inability to acquire methionine through the high-affinity methionine transporters MetI and MetT, we provided flies with 10^3 *V. cholerae*/μl in PBS alone or supplemented with 1 mM methionine and measured pathogen burden after 72 h. While few Δ*metR* Δ*metI* Δ*metT* mutants were detected in the *Drosophila* intestine under these conditions, provision of 1 mM methionine to this mutant significantly rescued intestinal numbers (Fig. 8D). We conclude that high-affinity methionine transport is essential for *V. cholerae* growth in the *Drosophila* intestine in the absence of synthesis, but reduction of MetO is not. Furthermore, because the high-affinity methionine transporters MetI and MetT allow for *V. cholerae* growth in the range of 0.05 to 0.5 mM methionine, we conclude that the concentration of methionine in the fly gut is between 0.05 and 0.5 mM.

DISCUSSION

The amino acid methionine is critical for many cellular functions including translation, transcription, and posttranscriptional methylation reactions. To understand how *V. cholerae* acquires methionine from its environment, we sought to identify methionine transporters and methionine sulfoxide reductases. In this work, we identify two transporters, MetI (VC0906) and MetT (VCA0193), which transport L-methionine, D-methionine, and MetO. Furthermore, we have identified five methio-

nine sulfoxide reductases (VC1236, VC1498, VC1998, VC2549, and VCA0615). Only one of these, MsrC, is essential for reduction of free MetO to methionine. The identification of these genes allows us to establish the importance of high-affinity methionine uptake but not methionine sulfoxide reduction for colonization and growth in the arthropod intestine.

Methionine is essential for *V. cholerae* viability regardless of whether the environment is an estuary or the intestinal lumen of a mammal or an arthropod. *V. cholerae* is capable of synthesizing methionine when necessary, but uptake is favored when methionine is available in the environment (39). Because synthesis is dispensable for survival in the infant mouse intestine, it is likely that this intestinal environment is not methionine limited (39). Similarly, we found that methionine synthesis was not essential for *V. cholerae* replication in the arthropod intestine, but that, in the absence of synthesis, high-affinity transporters were. This allowed us to place a lower limit of 0.05 mM and an upper limit of 0.5 mM on the concentration of L-methionine in the intestine. In both the infant mouse and arthropod infection models, *V. cholerae* is restricted to the intestinal lumen, where observations suggest that methionine is not limiting. However, methionine is limiting in some host environments. For instance, the concentration of methionine in human plasma is estimated to be 3 to 30 μM (41, 47, 48), and methionine biosynthesis is essential for intracellular replication of *Mycobacterium tuberculosis* in human macrophages and for pulmonary infection and dissemination in an immunocompromised mouse model (41). *Salmonella enterica* serovar Typhimurium infection of mice, which involves intracellular replication and dissemination from the intestine to the spleen and liver, is also dependent on methionine biosynthesis and high-affinity methionine acquisition (40, 49). For invasive pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, group B *Streptococcus*, and *E. coli*, proliferation in plasma is dependent on high-affinity methionine uptake systems (21, 47). For this reason, bacterial methionine synthesis and high-affinity uptake have been suggested as antibacterial targets.

Methionine is easily oxidized to MetO. Free MetO must be reduced to methionine prior to utilization, and reversible oxidation of methionine in proteins modulates their function. Msrs are, therefore, absolutely required for bacterial growth on MetO and for survival of oxidative stress, and the impact of Msr deletion on pathogen virulence reflects the level of oxidative stress in the host niche. Here, we show that *V. cholerae* acquires methionine and MetO through the same transporters. Furthermore, we show an unusual amount of redundancy in *V. cholerae* Msrs, suggesting that it is adapted to oxidizing environments. Msrs do not appear to be necessary for utilization of luminal methionine by a *V. cholerae* Δ*metR* mutant in the *Drosophila* intestine, suggesting that this is not a highly oxidizing environment. Furthermore, while we previously showed that luminal methionine and MetO attenuate *V. cholerae* virulence in this environment, deletion of *V. cholerae* MetI and MetT or all Msrs had no effect on virulence (6). This suggests that *V. cholerae* methionine uptake and MetO reduction do not significantly impact concentrations of these amino acids in the *Drosophila* gut under the conditions of our infection experiments. Survival of other pathogens in host gastrointestinal environment is dependent on Msrs. For instance, the genome of *Helicobacter pylori* encodes only one MsrAB protein found principally in the membrane. Upon deletion of MsrAB, *H. pylori* is no longer able to colonize the murine gastric mucosa (50). A *Lactobacillus reuteri* Δ*msrB* mutant displays a defect in competition with wild-type strains in the mouse intestine (51). Msrs may also be important in systemic bacterial infections. *S. aureus* produces three MsrAs and one MsrB (18, 52). However, only one of these, designated MsrA1, plays a role in survival in a mouse model of systemic infection. Defects in virulence upon deletion of Msrs have also been recorded for *Enterococcus faecalis* and *Francisella tularensis* (15, 53).

The amino acid methionine is an integral component of several essential bacterial synthetic, metabolic, and regulatory pathways. In this work, we have fully defined the pathways used by *V. cholerae* to acquire methionine. These pathways are characterized by remarkable redundancy. While methionine is a prerequisite for many essential

TABLE 1 Strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Genotype, description, or sequence	Reference or source
Bacterial strain		
PW724	*V. cholerae* MO10; Sm^r	60
PW1864	MO10 Δ*metI*; Sm^r	This study
PW1918	MO10 Δ*metT*; Sm^r	This study
PW1923	MO10 Δ*metI* Δ*metT*; Sm^r	This study
PW1865	MO10 Δ*msrAB*; Sm^r	This study
PW1866	MO10 Δ*msrA*; Sm^r	This study
PW1899	MO10 Δ*msrB1*; Sm^r	This study
PW1919	MO10 Δ*msrB2*; Sm^r	This study
PW1976	MO10 Δ*msrC*; Sm^r	This study
PW1874	MO10 Δ*msrAB* Δ*msrA*; Sm^r	This study
PW1924	MO10 Δ*msrB1* Δ*msrB2*; Sm^r	This study
PW1950	MO10 Δ*msrAB* Δ*msrA* Δ*msrB1* Δ*msrB2*; Sm^r	This study
PW1977	MO10 Δ*msrAB* Δ*msrA* Δ*msrB1* Δ*msrB2* Δ*msrC*; Sm^r	This study
PW1822	MO10 Δ*metR*; Sm^r	This study
PW1867	MO10 Δ*metR* Δ*metI*; Sm^r	This study
PW1944	MO10 Δ*metR* Δ*metT*; Sm^r	This study
PW1945	MO10 Δ*metR* Δ*metI* Δ*metT*; Sm^r	This study
PW1868	MO10 Δ*metR* Δ*msrAB*; Sm^r	This study
PW1986	MO10 Δ*metR* Δ*msrA*; Sm^r	This study
PW1952	MO10 Δ*metR* Δ*msrB1*; Sm^r	This study
PW2210	MO10 Δ*metR* Δ*msrC*; Smr	This study
PW1953	MO10 Δ*metR* Δ*msrB2*; Sm^r	This study
PW1895	MO10 Δ*metR* Δ*msrAB* Δ*msrA*; Sm^r	This study
PW2211	MO10 Δ*metR* Δ*msrAB* Δ*msrA* Δ*msrC*; Smr	This study
PW1946	MO10 Δ*metR* Δ*msrB1* Δ*msrB2*; Sm^r	This study
PW1955	MO10 Δ*metR* Δ*msrAB* Δ*msrA* Δ*msrB1* Δ*msrB2*; Sm^r	This study
PW1978	MO10 ΔmetR Δ*msrAB* Δ*msrA* Δ*msrB1* Δ*msrB2* Δ*msrC*; Sm^r	This study
SM10λpir	*E. coli thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (λpirR6K)	61
Plasmid		
pWM91	oriR6K mobRP4 *lacI* pTac *tnp* mini-Tn*10*; Km^r Ap^r	62
pWM91::Δ*metI*	pWM91 carrying an unmarked, in-frame deletion in VC0906	This study
pWM91::Δ*metT*	pWM91 carrying an unmarked, in-frame deletion in VCA0193	This study
pWM91::Δ*msrAB*	pWM91 carrying an unmarked, in-frame deletion in VCA0615	This study
pWM91::Δ*msrA*	pWM91 carrying an unmarked, in-frame deletion in VC2549	This study
pWM91::Δ*msrB1*	pWM91 carrying an unmarked, in-frame deletion in VC1998	This study
pWM91::Δ*msrB2*	pWM91 carrying an unmarked, in-frame deletion in VC1236	This study
pWM91::Δ*msrC*	pWM91 carrying an unmarked, in-frame deletion in VC1498	This study
pWM91::Δ*metR*	pWM91 carrying an unmarked, in-frame deletion in VC1706	This study
Oligonucleotide		
ΔVC0906		
metI#1	5′-GATGACGCAAATTGCTCGCA	This study
metI#2	5′-TAACGAGCGGCCGCACATAACCCAGCACCTCTACT	This study
metI#3	5′-TGCGGCCGCTCGTTATAAGCAAAACAAAAGCCTTT	This study
metI#4	5′-ATGCTGCGTTTGGTGTTACG	This study
ΔVCA0193		
metT#1	5′-CCTGCAGCCCGGGGGATCCACATCCCCACCGTATGTTCG	This study
metT#2	5′-CATGATTTGATATTTGCATGTGAATTAGGCGTTGCGATTG	This study
metT#3	5′-CAATCGCAACGCCTAATTCACATGCAAATATCAAATCATG	This study
metT#4	5′-ATTGACGGCTCTAGAACTAGGCTCTCTGCGGCGTTTGTA	This study
ΔVCA0615		
msrAB#1	5′-AGTGATAAAGCCGCACAGCA	This study
msrAB#2	5′-TAACGAGCGGCCGCACAAACCGGATCAGAAATTTC	This study
msrAB#3	5′-TGCGGCCGCTCGTTATAATTAGCTATACCAACCCT	This study
msrAB#4	5′-GTCGGATGATGTGGCAGTCA	This study
ΔVC2549		
msrA#1	5′-AATGCAACCCTGCGTCTGTA	This study
msrA#2	5′-TAACGAGCGGCCGCACATGATGTTTCCTTACTGAA	This study
msrA#3	5′-TGCGGCCGCTCGTTATAAAGACATCTCAATGAGGG	This study
msrA#4	5′-CCATCAGTGGCGCATTCATC	This study

(Continued on next page)

TABLE 1 (Continued)

Strain, plasmid, or oligonucleotide	Genotype, description, or sequence	Reference or source
ΔVC1998		
msrB1#1	5′-CCTGCAGCCCGGGGGATCCAAGCACGGAAAACGAAACGAC	This study
msrB1#2	5′-CAAGTTTTTCACAAAAAATGTGAATATTGCCAGACGACTT	This study
msrB1#3	5′-AAGTCGTCTGGCAATATTCACATTTTTTGTGAAAAACTTG	This study
msrB1#4	5′-ATTGACGGCTCTAGAACTAGAGCAATGGTCAACCCGTCAA	This study
ΔVC1236		
msrB2#1	5′-CCTGCAGCCCGGGGGATCCACCATTGCCGACGATAGCAAC	This study
msrB2#2	5′-CAACAAGAGGAGAGAGCAATGTAAATATACCCTTCCTACTTG	This study
msrB2#3	5′-CAAGTAGGAAGGGTATATTTACATTGCTCTCTCCTCTTGTTG	This study
msrB2#4	5′-ATTGACGGCTCTAGAACTAGGGCACAGTCTACTCGCTCTG	This study
ΔVC1498		
msrC#1	5′-CCTGCAGCCCGGGGGATCCACAATGGCGGTACCAATCACA	This study
msrC#2	5′-CACAGGCAAAGGGCAATTTACACTATTAGCCGCTCTTGGC	This study
msrC#3	5′-GCCAAGAGCGGCTAATAGTGTAAATTGCCCTTTGCCTGTG	This study
msrC#4	5′-ATTGACGGCTCTAGAACTAGTTCGCCTTTGGCTTGTTCG	This study
ΔVC1706		
metR#1	5′-CCTCAGCACATCGAAAGATG	This study
metR#2	5′-TAACGAGCGGCCGCACATGAACTCTCCTCACTTATC	This study
metR#3	5′-TGCGGCCGCTCGTTATAGGCATCACTACCGAGCA	This study
metR#4	5′-GGAGTGATGTGATGGATCTG	This study

bacterial processes, the redundant pathways for methionine synthesis and acquisition along with the availability of methionine or methionine sulfoxide in the mammalian intestine make this a poor target for the development of therapeutics against intestinal pathogens and an unlikely target for treatment or prevention of cholera.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in these experiments are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml) at 37°C. *Vibrio cholerae* strains were routinely cultured in LB broth (Difco) or on LB agar supplemented with streptomycin (100 μg/ml) at 27°C. Growth was assessed in defined medium. This previously described defined medium was prepared with the omission of methionine and supplementation with 0.5% glucose (54) (referred to as minimal medium in the previous publication). In addition, the indicated concentrations of L-methionine (M5308; Sigma), D-methionine (M9375; Sigma), or L-methionine-*R*,*S*-sulfoxide (M1126; Sigma) were added.

***V. cholerae* strain construction.** *V. cholerae* mutants used in this work were engineered by construction of a suicide plasmid carrying a mutant allele. This mutant allele was inserted into the chromosome by double homologous recombination as previously described (55). Briefly, approximately 500-bp fragments upstream and downstream of the target gene and including the start and stop codons, respectively, were amplified by the PCR using the primers listed in Table 1. For Δ*metR*, Δ*metI*, Δ*msrAB*, and Δ*msrA* mutant constructions, the overlapping fragments included 15-bp complementary sequences at their 3′ and 5′ ends. These were joined using the splicing by overlap extension (SOE) technique, resulting in the construction of a fragment with an in-frame deletion in the gene of interest (56, 57). The fragment containing the deletion was ligated into the suicide plasmid pWM91 to create the plasmids listed in Table 1. For Δ*metT*, Δ*msrB1*, Δ*msrB2*, and Δ*msrC*, inner primers included a complementary 20-bp sequence at their 3′ and 5′ ends to permit Gibson assembly. These two fragments were ligated with the pWM91 suicide vector by Gibson assembly as per the manufacturer's instructions (New England Biolabs) to create the plasmids listed in Table 1 (58). These plasmids were used to create gene deletions in the relevant strains by double homologous recombination and sucrose selection as previously described (59).

***Drosophila* virulence and colonization assays.** For virulence assays, ten 5- to 10-day-old Oregon R male flies were placed in each of three fly vials. These vials were prepared with a cellulose acetate plug infiltrated with 3 ml of LB broth inoculated with a 10-fold dilution of an overnight culture of the indicated *V. cholerae* strain. Mortality was assessed at least once each day.

For colonization assays, *V. cholerae* strains were prepared by culturing overnight in LB broth, washing in sterile PBS supplemented with 100 μg/ml streptomycin, and resuspending in an equal volume of PBS. The bacterial resuspension was diluted in a 1:10 ($10^5/\mu$l) or 1:1,000 ($10^3/\mu$l) ratio in PBS, and 3 ml of the diluted culture was added to a sterile cellulose acetate plug placed at the bottom of a standard fly vial. Ten male Oregon R flies aged between 5 and 10 days were placed in this vial inoculated with *V. cholerae* and either allowed to ingest this suspension for 72 h or transferred to sterile PBS supplemented with streptomycin after 48 h and maintained in these vials for 24 h. At this point, the flies in each vial were anesthetized with CO_2, and six live flies, chosen at random, were placed singly in Eppendorf tubes on ice. After addition of 200 μl of sterile PBS, flies were homogenized. The homogenate was allowed to settle briefly, and the supernatant was serially diluted in PBS. Dilutions were plated on LB agar plates supplemented with 100 μg/ml streptomycin, and CFU were counted after overnight incubation at 27°C.

The *V. cholerae* burden was measured for at least six infected flies for each test condition, and each experiment was repeated with similar results.

Growth curves. *V. cholerae* wild-type bacteria and mutants were grown overnight in LB at 27°C. One milliliter of culture was pelleted by centrifugation (2,300 × *g* for 6 min), washed 3 times in PBS, and then diluted to an optical density at 655 nm (OD_{655}) of 1 in PBS. The resulting suspension was diluted 1:100 in the indicated growth medium, and 100 μl was aliquoted into each of three wells of a 96-well plate to represent biological triplicates. The plates were incubated in a microplate reader (Infinite 200; Tecan), and the OD_{655} was measured every 15 min after agitation.

Statistical analyses. CFU were measured for at least 5 flies per condition. Each fly was considered to be an independent biological replicate. Experiments were repeated once. Horizontal lines shown represent the geometric mean, and log-transformed data were used in calculations of significance. Statistical significance was calculated using either a Student's *t* test or a one-way analysis of variance (ANOVA) followed by a Tukey's or Dunnett's multiple-comparison test as appropriate. Technical duplicates were performed for growth curves, and experiments were repeated three times. Graphs of growth curves represent the mean of technical duplicate measurements and are representative of the three experiments.

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