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Vibrio cholerae Colonization of Soft-Shelled Turtles

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ABSTRACT *Vibrio cholerae* is an important human pathogen and environmental microflora species that can both propagate in the human intestine and proliferate in zooplankton and aquatic organisms. Cholera is transmitted through food and water. In recent years, outbreaks caused by *V. cholerae*-contaminated soft-shelled turtles, contaminated mainly with toxigenic serogroup O139, have been frequently reported, posing a new foodborne disease public health problem. In this study, the colonization by toxigenic *V. cholerae* on the body surfaces and intestines of soft-shelled turtles was explored. Preferred colonization sites on the turtle body surfaces, mainly the carapace and calipash of the dorsal side, were observed for the O139 and O1 strains. Intestinal colonization was also found. The colonization factors of *V. cholerae* played different roles in the colonization of the soft-shelled turtle's body surface and intestine. Mannose-sensitive hemagglutinin (MSHA) of *V. cholerae* was necessary for body surface colonization, but no roles were found for toxin-coregulated pili (TCP) or *N*-acetylglucosamine-binding protein A (GBPA). Both TCP and GBPA play important roles for colonization in the intestine, whereas the deletion of MSHA revealed only a minor colonization-promoting role for this factor. Our study demonstrated that *V. cholerae* can colonize the surfaces and the intestines of soft-shelled turtles and indicated that the soft-shelled turtles played a role in the transmission of cholera. In addition, this study showed that the soft-shelled turtle has potential value as an animal model in studies of the colonization and environmental adaption mechanisms of *V. cholerae* in aquatic organisms.

IMPORTANCE Cholera is transmitted through water and food. Soft-shelled turtles contaminated with *Vibrio cholerae* (commonly the serogroup O139 strains) have caused many foodborne infections and outbreaks in recent years, and they have become a foodborne disease problem. Except for epidemiological investigations, no experimental studies have demonstrated the colonization by *V. cholerae* on soft-shelled turtles. The present studies will benefit our understanding of the interaction between *V. cholerae* and the soft-shelled turtle. We demonstrated the colonization by *V. cholerae* on the soft-shelled turtle's body surface and in the intestine and revealed the different roles of major *V. cholerae* factors for colonization on the body surface and in the intestine. Our work provides experimental evidence for the role of soft-shelled turtles in cholera transmission. In addition, this study also shows the possibility for the soft-shelled turtle to serve as a new animal model for studying the interaction between *V. cholerae* and aquatic hosts.

KEYWORDS *Vibrio cholerae*, soft-shelled turtle, colonization, mannose-sensitive hemagglutinin, toxin-coregulated pili, *N*-acetylglucosamine-binding protein A

Cholera is a life-threatening infectious disease that affects millions of people and kills thousands annually (1). Over 200 serogroups have been identified in *Vibrio cholerae*, but only the toxigenic O1 and O139 serogroups have been responsible for cholera

epidemics to date. Seven cholera pandemics have been recorded historically. According to etiological evidence, the sixth pandemic was caused by the classical biotype of serogroup O1, and the seventh pandemic, which began in 1961 and is ongoing, was caused by the O1 El Tor biotype of serogroup O1 (2). In 1992, cholera caused by serogroup O139 emerged in India and spread to many countries in Asia (3, 4). In 1993, cholera from serogroup O139 was found in China, and since then, sporadic O139 cholera cases and local outbreaks have appeared in some provinces.

In addition to human hosts, *V. cholerae* naturally inhabits aquatic environments, particularly estuarine water, by colonizing zooplankton (5, 6). It can use chitin as a carbon and nitrogen source, which may also benefit the carbon and nitrogen cycles in aquatic environments (7). Water and seafood contaminated with *V. cholerae* are common sources of infections and outbreaks. In addition, foodborne infections and outbreaks of cholera, mainly of serogroup O139, mediated by soft-shell turtles have been frequently reported in China. Some investigations have reported that the rates of detection of *V. cholerae* on soft-shelled turtles were higher than for other aquatic products (8, 9). In some areas, the detection rate has reached 8.9% (9). Epidemiological and laboratory investigations of some cholera outbreaks showed that *V. cholerae* strains isolated from turtle samples had molecular subtyping patterns indistinguishable from those of patient strains, which suggested that the turtles served as the vehicle (10–13). *V. cholerae* was also found in soft-shelled turtles imported from Malaysia to China and from Bangladesh to Japan (14, 15). *V. cholerae* strains have been isolated from turtles' surfaces, cloacae, intestines, and eggs (9, 10, 13, 16–18). Taken together, these results provide strong evidence that *V. cholerae* can adhere to and colonize soft-shelled turtles. Thus, since no appearance or weight changes have been observed in turtles carrying *V. cholerae*, this colonization may result in the wide spread of the strains through markets.

Soft-shelled turtles are reptiles that inhabit standing or slow-flowing bodies of water, such as reservoirs, ponds, and rivers, and they are known as a nutrient-rich food in Asian countries, particularly, China (19). The large-scale breeding of soft-shelled turtles has been rapidly expanding, particularly, in eastern and southeastern China. Soft-shelled turtle consumption has reached approximately 100 to 150 million kilograms annually (20). *Pelodiscus sinensis* (commonly known as Chinese soft-shelled turtles), *Palea steindachneri*, and *Pelochelys bibroni* are three species of soft-shelled turtles in China. Among them, *P. steindachneri* and *P. bibroni* are rare animals protected by Chinese law, and only Chinese soft-shelled turtles can be sold in markets for consumption. The relationship of cholera cases or outbreaks to soft-shelled turtles has mainly been identified through epidemiological investigations and the isolation of strains. Direct observation of *V. cholerae* colonizing soft-shelled turtles is rare, except for an experiment that showed that *V. cholerae* could adhere to soft-shelled turtle eggshells and invade eggs (21). Such studies will provide new insight into the colonization of soft-shelled turtles by *V. cholerae* and will have profound significance for human health and foodborne infectious disease control and prevention.

Mannose-sensitive hemagglutinin (MSHA), toxin-coregulated pili (TCP), and *N*-acetylglucosamine-binding protein A (GBP) are three important factors of *V. cholerae* colonization that have been identified in animal models. MSHA, which is a member of the family of type IV pili and is associated with hemagglutinating activity, plays a significant role in biofilm formation on abiotic or biotic surfaces (22) and promotes adherence to zooplankton (23). No apparent role for MSHA in *V. cholerae* colonization of human or mouse intestines has been reported (24, 25). TCP is another type IV pilus that is necessary for colonization of the mammalian intestine and for pathogenesis (24, 26, 27). GBP is a chitin-binding protein that is involved in the attachment to chitin and mammalian intestine cells (28, 29). Although these three colonization factors have been widely studied, most studies are based on the mammalian intestine, zooplankton, and chitin surfaces. Their roles on the surfaces and in the intestines of soft-shelled turtles have not been investigated.

In this study, we estimated the colonization of the soft-shelled turtle by toxigenic *V. cholerae*. The colonization ability was certified on the body surface and in the intestine,

and the roles of various colonization factors were identified. This study suggests that, aside from its epidemiological significance in the spread of cholera, the soft-shelled turtle can also be developed as a model for *V. cholerae* colonization.

RESULTS

Colonization of *V. cholerae* strains on the surface of the soft-shelled turtle. To observe *V. cholerae* colonization on the soft-shelled turtle's surface and identify colonization sites, we constructed a bioluminescent strain, VC4251, of the *V. cholerae* serogroup O139 using the *lux* reporter plasmid pXEN-pmdh-luxCDABE. Soft-shelled turtles were infected via immersion in phosphate-buffered saline (PBS) containing approximately 10^7 CFU/ml bioluminescent cells. Because *V. cholerae* strains grow slowly in aquatic environments and too many rounds of anesthesia will lead to turtle deaths, bioluminescence was measured 24 h postinfection and observed every 24 h. At 96 h, the entire dorsal sides of the turtles emitted photons. Hence, the soft-shelled turtles were not analyzed for bacterial colonization after 96 h postinfection. After the bioluminescence imaging analysis, the surfaces of the soft-shelled turtles were sampled with sterile cotton swabs, and the experimental strains were obtained.

The surface bioluminescence imaging results showed that strong bioluminescent signals could be detected at 24 h and that the bioluminescence increased gradually with time (Fig. 1). Compared with that on the ventral sides, the dorsal sides emitted more photons. Photon emission was strongest and most stable on the calipashes during the experiments, showing that *V. cholerae* had the strongest ability to colonize the calipash. In addition, relatively strong bioluminescent signals could also be monitored on the dorsal sides of the carapaces, and these signals increased gradually with time (Fig. 1). The entire dorsal sides of the soft-shelled turtles' carapaces emitted photons at 96 h. The bioluminescent signals could also be detected on the dorsal sides of the limbs and necks. Poor photon emission was detected on the ventral sides, and the main bioluminescent sites on this side were on the limbs. Lower photon emission was detected on the plastrons, calipashes, and necks on the ventral sides (Fig. 1). A bioluminescent strain of *V. cholerae* serogroup O1 (VC1898) was also constructed based on the bioluminescent plasmid pXEN-pmdh-luxCDABE to estimate the colonization by *V. cholerae* O1 on soft-shelled turtles. Using the same infection and bioluminescence imaging procedures, the colonization by this serogroup O1 strain on the soft-shelled turtle surface was observed. The colonization sites and the expanded bioluminescent areas were similar to those of O139 strain VC4251.

To determine the possible outer membrane factors of *V. cholerae* involved in its colonization on the body surfaces of soft-shelled turtles, mutations were made in the genes encoding TCP, MSHA, and GBP. The mutant strains were then tested for their ability to colonize and were compared with their corresponding isogenic wild-type strains. We first determined the colonization functions of these gene mutants with competition tests in the infant mouse intestine, the commonly used colonization model for evaluation of these factors in *V. cholerae*, and confirmed their effects for strain VC4251 (Fig. 2). In addition, no obvious growth defects were found between the wild-type strain and mutants carrying the pXEN-pmdh-luxCDABE plasmid, and all strains produced similar levels of bioluminescent light at the same time points (see Fig. S1C and D in the supplemental material). Using reverse transcription-PCR (RT-PCR) assays, the transcription of these three genes at 28°C was also confirmed, whereas the knockout mutants did not express mRNAs for the deleted genes (data not shown). These strains were used in the following experiments. Bioluminescence imaging of the turtles' body surfaces showed that in comparison to the wild-type strain, VC4251, the *gbpA* and *tcpA* mutant strains had similar photon emission trends, i.e., expanded bioluminescent areas on the dorsal sides and stable areas on the ventral sides of the turtles (Fig. 1). However, the *mshA* mutant strain had poor photon emission after infection, and after 48 h, photon emission from the strain could barely be detected on the soft-shelled turtles' surfaces (Fig. 1). These data suggested that compared with the wild-type strain, VC4251, the *gbpA* and *tcpA* mutant strains had similar colonization
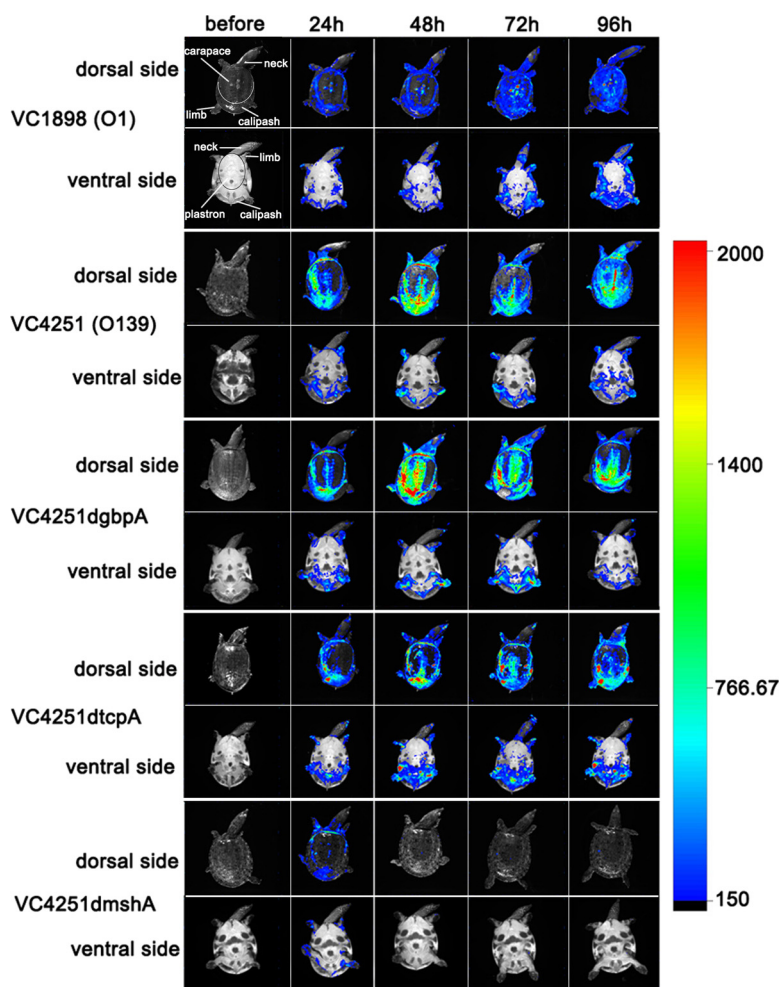
FIG 1 Body surface bioluminescence imaging of soft-shelled turtles infected with *V. cholerae* strains. The soft-shelled turtles were separately immersed in bacterial suspensions of bioluminescence-labeled O1 strain VC1898, O139 strain VC4251, and three VC4251-derived gene mutant bioluminescent strains. All the dorsal and ventral body surfaces were scanned before infection (as negative controls) and at 24, 48, 72, and 96 h postinfection. The figure shows one representative turtle for each tested strain. The color bar on the right shows the intensity of bioluminescence, representing increasing densities of bacteria. The anatomical structures on the dorsal side are labeled in the upper left corner image.

abilities on the body surfaces of the soft-shelled turtles, whereas the deletion of *mshA* led to a colonization defect.

Colonization competition assays of *V. cholerae* on the turtle surface. To accurately evaluate the colonization abilities of VC4251dlacZ and *mshA*, *gbpA*, and *tcpA* mutant strains, colonization competition assays were performed. According to the surface bioluminescence imaging results, the main colonization sites for *V. cholerae* on soft-shelled turtles' surfaces (the dorsal side carapace, calipash, and limbs) were sampled in the colonization competition assays. We measured the optical density at 600 nm (OD_{600}) of these strains in LB medium, and no obvious growth difference was found between the VC4251 wild-type strain and any mutant (Fig. S1C). According to surface bioluminescence imaging, the dorsal side carapace, calipash, and limbs were colonized; *t* tests were performed to compare the differences between the output ratios and input ratios for each competition strain pair. The results showed that for *gbpA* and *tcpA* mutants, from day 0 to day 10 on the carapace, calipash, or limbs, no significant differences between the input and output ratios of the competition pairs ($P > 0.05$ by *t* test) were observed. The competitive indexes of the *gbpA* and *tcpA* mutants were always close to 1 (Fig. 3, shown with the $\log_{10}$ values). However, for the *mshA* mutant
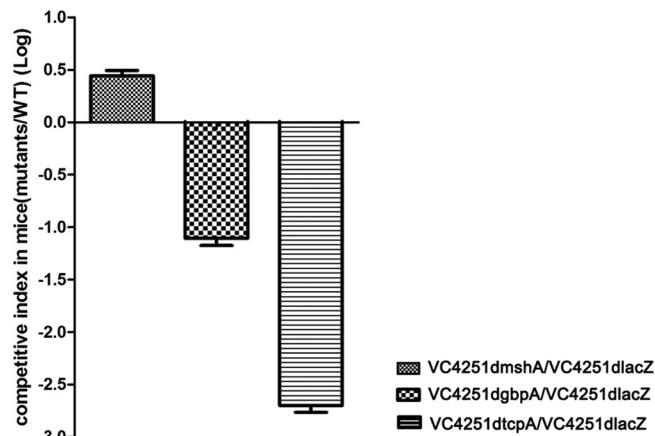
FIG 2 Small intestine colonization competition assays in infant mice with O139 strain VC4251dlacZ and *mshA*, *gbpA*, and *tcpA* deletion mutants from strain VC4251. The competition indexes were measured in the small intestines of infant mice; the *y* axis represents the log values of the competition index (mutant/VC4251dlacZ). The bars represent competition pairs of strain VC4251dlacZ and the mutants.

strain, significant colonization differences were observed on the carapaces, calipashes, and limbs ($P < 0.05$ by *t* test). From day 2, the competitive indexes were relatively stable and showed that loss of *mshA* decreased colonization by 10- to 81-fold compared with that of the wild type (Fig. 3).

We also tested the colonization abilities of the three colonization factor gene mutants which were restored by introducing the plasmids pBADmshA, pBADgbpA, and pBADtcpA to express *mshA*, *gbpA*, and *tcpA*, respectively, on the carapaces, calipashes, or limbs, from day 0 to day 10. The colonization ability for the *mshA*-complemented strain was restored and even 2.5- to 11.4-fold higher than for VC4251dlacZ(pBAD), the wild-type strain characterized by the lack of *lacZ* and carrying the control plasmid pBAD24, on the carapace, calipash, and limbs (Fig. 3). For *gbpA* and *tcpA* complementary strains, the colonization competitive indexes were always close to 1 and showed no colonization differences from strain VC4251dlacZ(pBAD) ($P > 0.05$ by *t* test).

Colonization of *V. cholerae* in turtle intestine. To detect whether *V. cholerae* can colonize the intestines of soft-shelled turtles, the animals were intragastrically inoculated with the bioluminescent O139 strain, VC4251. It was previously found that the food digestion time of soft-shelled turtles weighing approximately 150 g was between 34.03 and 56.56 h at 28°C (30). Thus, we chose 72 h as the colonization time. Because soft-shelled turtles have thick carapaces, bioluminescent signals could not be directly detected by our *in vivo* imaging system. Thus, at 72 h postinfection, the soft-shelled turtles were euthanized, and the internal organs, including the hearts, livers, lungs, kidneys, spleens, and digestive tracts, were separated and placed on the observation platform of the imaging system. *In vivo* bioluminescence imaging assays showed that bioluminescent signals could only be detected in the intestines (Fig. 4), and the *V. cholerae* strain was isolated only from these segments. No bioluminescent photons were emitted from other organs and no *V. cholerae* strains were isolated in other organs.

To determine the colonization by the O1 serogroup *V. cholerae* strain and the possible outer membrane factors involved in the *in vivo* colonization of soft-shelled turtles, the turtles were also intragastrically inoculated with the bioluminescent-labeled serogroup O1 strain, VC1898, and all VC4251-derived gene mutant bioluminescent strains. For the bioluminescence-labeled serogroup O1 strain, VC1898, and VC4251dmshA gene mutant bioluminescent strains, the same colonization sites (i.e., only in the intestinal tracts) were suggested by bioluminescence imaging and bacterial isolation. However, for the VC4251dgbpA and VC4251dtcpA gene mutant bioluminescent strains, no bioluminescent photons were emitted from the intestines,
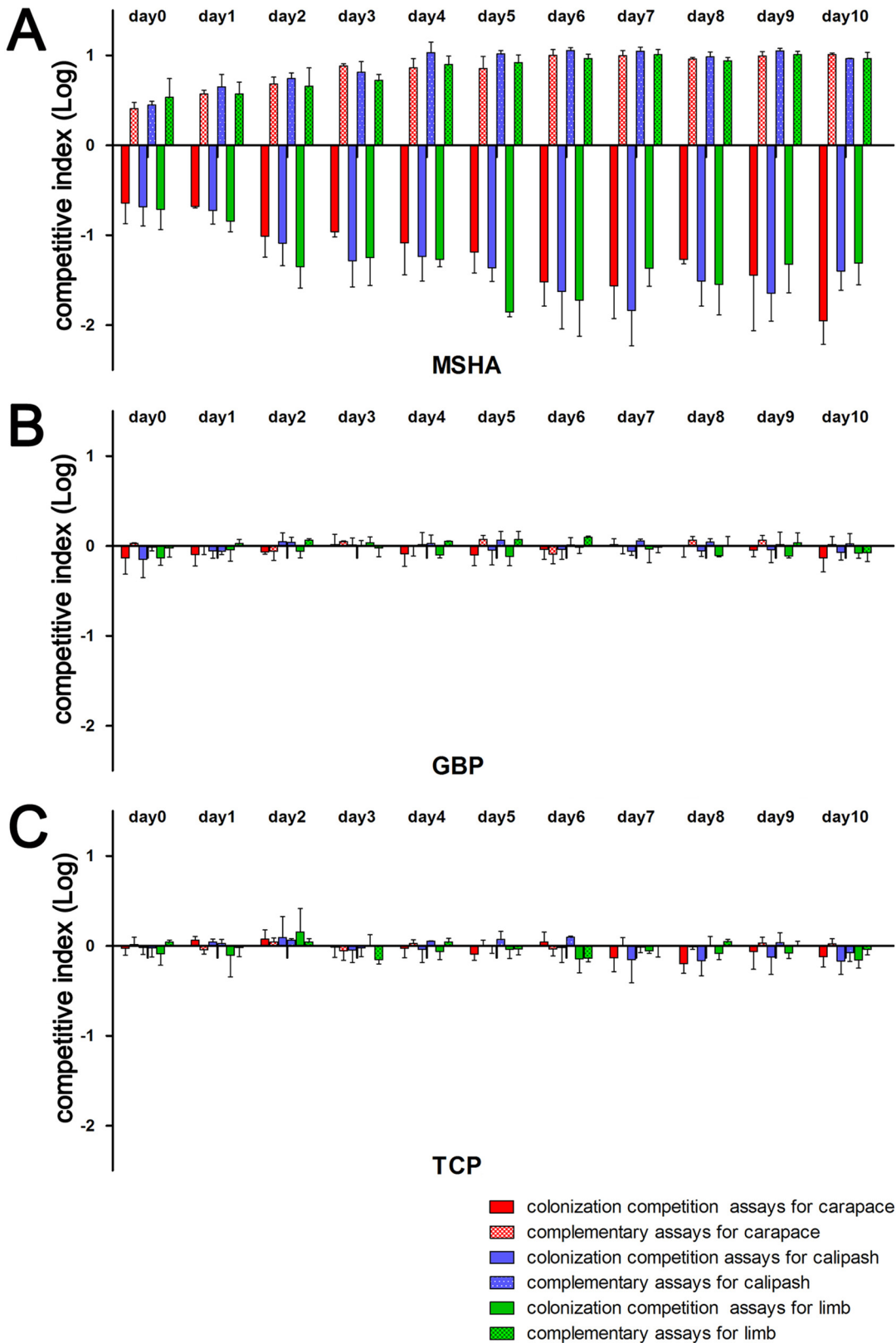
FIG 3 Colonization competition and complementary assays of *V. cholerae* strains on turtle body surfaces. Competition indexes were measured at different sites on the soft-shelled turtles' body surfaces. Colonization competition and complementary assays for *mshA* (A), *gbpA* (B), and *tcpA* (C) strains. The *y* axis represents log values of the competitive index [mutants/VC4251dlacZ or complementation strains/VC4251dlacZ(pBAD)]. Bars with different colors indicate competitive indexes for different competition pairs. For the *mshA* gene competition and complementary assays, the results always showed statistically significant differences (day 0 to day 10, regardless of carapace, calipash, or limbs; all $P < 0.05$ by *t* test). For the *gbpA* and *tcpA* genes, no competition pairs showed significance (all $P > 0.05$).
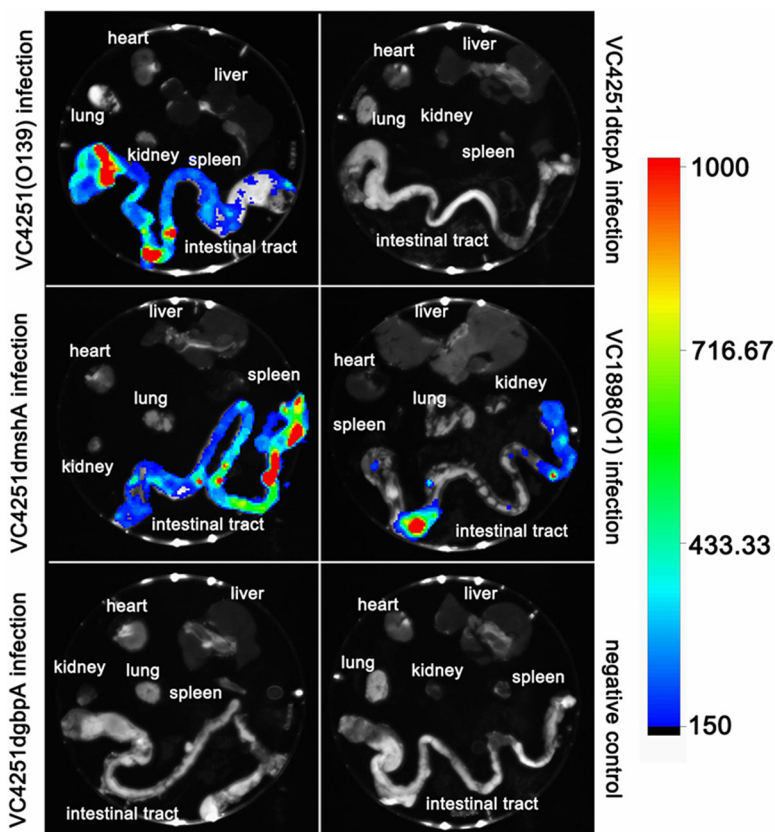
FIG 4 Bioluminescence imaging of organs of soft-shelled turtles intravenously infected with bioluminescence-labeled *V. cholerae* strains. Six groups of soft-shelled turtles (three turtles per group) were intravenously infected with bioluminescence-labeled serogroup O1 strain VC1898, serogroup O139 strain VC4251, and VC4251-derived gene mutant bioluminescent strains. The negative control was injected with sterile PBS. All soft-shelled turtles were euthanized at 72 h, and internal organs, including the hearts, livers, lungs, spleens, kidneys, and digestive tracts, were separated and scanned. The experiments were repeated three times, and this figure shows one representative test. The color bar on the right shows bioluminescence intensity, representing increasing densities of bacteria. Anatomical structures are labeled in each picture.

and relatively few VC4251dgbpA or VC4251dtcpA strains were isolated. In other organs, both the bioluminescence imaging detection results and bacterial isolation results were negative.

Colonization competition assays of *V. cholerae* in the turtle intestine. To accurately evaluate the roles of MSHA, TCP, and GBP in the intestinal colonization by *V. cholerae*, colonization competition assays were performed by coinfection of the strain VC4251dlacZ and *tcpA*, *mshA*, and *gbpA* deletion mutants. After 72 h, the entire intestines were isolated for plate counting of the competition strain pairs. Similar competition effects were observed with the *gbpA* and *tcpA* groups, and the comparisons between the ratios of the inputs and outputs were all statistically significant ($P < 0.05$ by t tests). Competition index calculations showed that mutations of *gbpA* and *tcpA* decreased the colonization abilities of the mutants by 44- and 225-fold, respectively (Fig. 5, shown with the $\log_{10}$ values). The colonization abilities of the *gbpA* and *tcpA* mutants were restored by the complemented plasmids pBADgbpA and pBADtcpA, respectively, and were 2.69- and 3.57-fold higher than that for VC4251dlacZ(pBAD), respectively, as deduced by the overexpression of these two complementary genes resulting from the strong promoter induced by arabinose. For *mshA*, comparisons of the ratios between the inputs and outputs of the VC4251dmshA-VC4251dlacZ pairs were statistically significant ($P < 0.05$ by t test). The competition index analysis showed that colonization by the *mshA* mutant was 3.71-fold higher than that by the wild-type strain and 1.96-fold lower when *mshA* was complemented (Fig. 5).
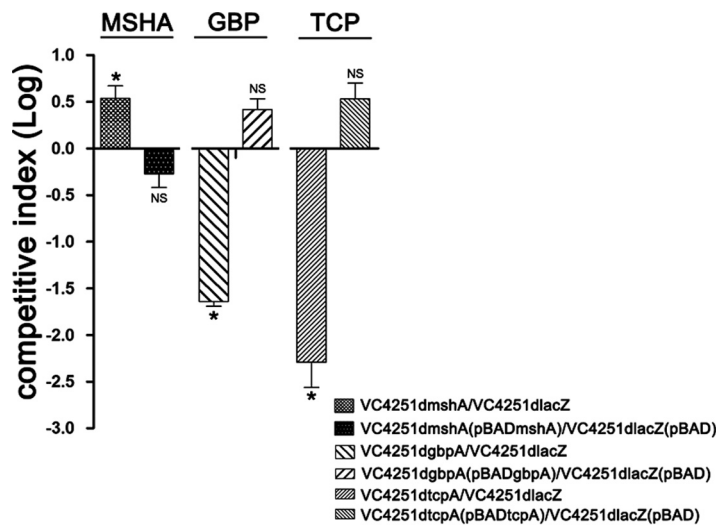
FIG 5 Colonization competition assays and complementary assays of *V. cholerae* strains in turtle intestines. Competition indexes were calculated for isolated intestines of soft-shelled turtles. The *y* axis represents the log values of the competitive index [mutants/VC4251dlacZ or complementation strains/VC4251dlacZ(pBAD)]. The columns indicate competitive indexes for different competition pairs. *, $P <$ 0.05 by *t* test). NS, not significant.

DISCUSSION

V. cholerae has two typical living environments, namely, an aquatic habitat and the human intestine (31, 32). The organism possesses different colonization and proliferation mechanisms in each environment (33). Soft-shelled turtles have already caused several foodborne cholera outbreaks (10–13) and have become a notable spreading factor for cholera. In this study, we demonstrated that the soft-shelled turtle could be colonized by *V. cholerae* on the surface and in the intestine, therefore playing a role in *V. cholerae* transmission as a medium.

Using bioluminescence-labeled *V. cholerae* strains and bioluminescence imaging techniques, the colonization sites on the soft-shelled turtle surface and the proliferation of *V. cholerae* were observed directly and successively. Combined with competition assays, these data were used to estimate the roles of the colonization factors of *V. cholerae* on the soft-shelled turtle surface and in the digestive tract. Both methods showed good repeatability. In *V. cholerae*, the animal models commonly used for colonization and pathogenesis studies are infant mice and rabbits, as well as the ileal loop of the adult rabbit (34–37). Compared with those using mammalian animal models, studies with soft-shelled turtles provide a new method for evaluating *V. cholerae* colonization on aquatic animal surfaces; animals can be continuously observed without euthanization. In addition, zooplankton, fish, shellfish, chironomid egg masses, waterfowl, and crustaceans are environmental hosts of *V. cholerae* (6, 38–43), and fish and shellfish are proven vectors of *V. cholerae* (44). In studies of colonization in aquatic organisms, soft-shelled turtles can be anesthetized and sampled out of water for relatively long periods without the risk of hydropenia and death, due to their amphibious nature, making them a better model than zebrafish and other fish or shrimp models. Therefore, it should be possible to develop specific-pathogen-free soft-shelled turtles as a model for studying *V. cholerae* survival mechanisms in aquatic environments.

From bioluminescence imaging assays, we found the colonizations by *V. cholerae* on soft-shelled turtles surfaces were uneven and that the main colonization sites were the carapace, calipash, limbs, and the dorsal side of the neck. Soft-shelled turtle muscle and skin (45), particularly the calipash (46), are highly abundant in collagen. *V. cholerae* produces collagenase (47), and collagen may be a nutrient source for the growth of *V. cholerae*. The carapace and plastron have different anatomical and physiological struc-

tures, which possibly lead to the different colonization abilities of *V. cholerae* at these two sites.

MSHA, TCP, and GBP are the major colonization factors of *V. cholerae* and are recognized as such in mouse models (24, 26–29) and in zooplankton (23). In the soft-shelled turtle's intestine and on the body surface, we found different effects of these factors. In the soft-shelled turtle's intestine, effects similar to those seen in the mouse intestine were observed for these colonization factors. TCP was important in the colonization by *V. cholerae* in the turtle intestine, similar to that in the mouse model (24–26). MSHA pili showed an extremely important role in colonization on the turtle body surface. In the bioluminescence imaging assays, the weak bioluminescence of the *mshA* mutant strain was found in the first 24 h but hardly observed later, showing the colonization loss caused by MSHA. We suspected it may represent nonspecific binding in the first 24 h because of the higher bacterial concentration, and the mutant with the defective colonization was washed away, since every 24 h the turtles were washed and the sterile PBS was replaced during the experiments. The complement of intact *mshA* to the *mshA* mutant restored the colonization ability and produced a slightly increased colonization. MshA is the major pilin subunit of the MSHA pili, and we suspect that the overexpressed MshA from the complementary plasmid could promote the assembly of MSHA pili and result in the slightly increased colonization compared with that of the wild-type strain. However, in the intestine, this appeared to depress colonization. A similar effect was also found in the mouse model (25). In the suckling mouse intestine, MSHA pili bound secretory immunoglobulin A (sIgA) and reduced the colonization ability of *V. cholerae* by preventing the bacteria from penetrating the mucus barrier and adhering to epithelial cells (48). As reptiles, soft-shelled turtles can also produce sIgA (49), and reptilian sIgA is evolutionarily related to mammalian sIgA (50). *V. cholerae* has a low level of MSHA gene expression in the intestine (48). Therefore, we hypothesize that the slightly lower colonization of the wild type compared with that of the *mshA* deletion mutant might result from the interaction between sIgA and weakly expressed MSHA in the wild-type strain; cells could bind to sIgA to some degree, whereas the *mshA* mutant should lack MSHA pili.

Compared with that of the wild-type strain, the colonization ability of the *gbpA* deletion mutant obviously decreased in the soft-shelled turtle intestine, similar to that in the mouse intestine colonization model (51). However, on the body surfaces of soft-shelled turtles, inactivation of *gbpA* has no significant effect on colonization. Previous results have shown that deletion of the *gbpA* gene of *V. cholerae* led to a significant decrease in colonization on the shells of crustaceans, such as crab, shrimp, and shellfish (28, 29). Chitin is the main element of crustacean shells (52, 53), and its β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues are receptors for GBP-mediated binding (54). Thus, GBP interacts with the chitinous exoskeleton and promotes the colonization ability of *V. cholerae*. Soft-shelled turtles are reptiles with endoskeletons and are distinct from crustaceans with exoskeletons (55). Epidermis, dermis, and subcutaneous tissues cover the outside of the carapace skeleton, and these tissues are rich in collagen (56). GBP had no effect on the *V. cholerae* colonization of soft-shelled turtle shells, possibly because of this difference in structure and material composition.

In summary, we studied the colonization ability, colonization sites, and roles of major colonization-related factors for *V. cholerae* on soft-shelled turtles. Soft-shelled turtles' surfaces and intestines carried *V. cholerae* and allowed it to proliferate. These outer membrane accessory structures play different but important and even aquatic animal-specific roles in the colonization by *V. cholerae* on the body surfaces and in the intestines of soft-shelled turtles. The results from this study strongly implicate soft-shelled turtles in cholera transmission in areas with high soft-shelled turtle consumption. These soft-shelled turtles are thus an important risk factor for foodborne cholera outbreaks. In addition, soft-shelled turtles could be developed as a valuable animal model to study the interaction between *V. cholerae* and aquatic hosts.

TABLE 1 Bacterial strains used in this work

Strain	Relevant properties[a]	Source
VC1898	O1 serogroup, Sm^r, PB^r	Lab strain
VC4251	O139 serogroup, Sm^r, PB^r	Lab strain
VC4251dmshA	Strain VC4251 with *mshA* deleted	This work
VC4251dgbpA	Strain VC4251 with *gbpA* deleted	This work
VC4251dtcpA	Strain VC4251 with *tcpA* deleted	This work
VC4251dlacZ	Strain VC4251 with *lacZ* deleted	This work
VC4251dlacZ(pBAD)	VC4251dlacZ containing pBAD24	This work
VC4251dmshA(pBADmshA)	VC4251dmshA containing pBADmshA	This work
VC4251dgbpA(pBADgbpA)	VC4251dgbpA containing pBADgbpA	This work
VC4251dtcpA(pBADtcpA)	VC4251dtcpA containing pBADtcpA	This work
VC4251(pXEN-pmdh-luxCDABE)	VC4251 containing pXEN-pmdh-luxCDABE	This work
VC4251dmshA(pXEN-pmdh-luxCDABE)	VC4251dmshA containing pXEN-pmdh-luxCDABE	This work
VC4251dgbpA(pXEN-pmdh-luxCDABE)	VC4251dgbpA containing pXEN-pmdh-luxCDABE	This work
VC4251dtcpA(pXEN-pmdh-luxCDABE)	VC4251dtcpA containing pXEN-pmdh-luxCDABE	This work
SM10 *λpir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *λpir*	Lab strain

[a]Sm, streptomycin; PB, polymyxin B.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* serogroup O139 strain VC4251 and serogroup O1 strain VC1898 were isolated from soft-shelled turtles in markets in Jiangxi and Guizhou provinces in China, respectively. Both strains have natural resistances to streptomycin (100 μg/ml) and polymyxin B (5 μg/ml). The *mshA*, *gbpA*, *tcpA*, and *lacZ* mutants were constructed from strain VC4251. A list of all strains used in this study is presented in Table 1.

These strains were obtained from swab samples of the turtles and identified according to the Manual for Prevention and Control of Cholera, 6th edition (57). The serogroups were determined with diagnosis typing sera (Wantai Biotech, Zhengzhou, China). The *Escherichia coli* strain SM10 *λpir* was constructed from SM10 (58) by lysogenizing with *λpir* to allow the replication of an R6K suicide plasmid (http://www.openwetware.org/wiki/E._coli_genotypes) used to construct a mutant allele in allelic exchange to create a gene mutation, which was obtained from the strain collection in our lab.

Soft-shelled turtles. Soft-shelled turtles were obtained from an aquaculture company in Shanghai. Turtles weighing 150 g and 9 cm in length (diameter of calipash) were selected for use in these studies. To ensure that the turtles used in this study did not carry *V. cholerae* before the experiments, we screened the turtles with PCR and with isolation for *V. cholerae*. Surface and anus swabs were collected from the turtles. PCR for *V. cholerae* was performed as previously described (*ompW* and *ctxA* were detected as the targets of *V. cholerae* [59]). The surface and anus swabs were also placed in alkaline peptone water for *V. cholerae* enrichment, and *V. cholerae* was isolated and identified according to the Manual for Prevention and Control of Cholera, 6th edition (57). Turtles positive for any gene in the PCR and/or isolation were excluded, and turtles negative for both detections were used in subsequent experiments.

Each selected soft-shelled turtle was carefully washed with sterile distilled water and kept in sterile distilled water for 48 h to remove mud from the surface and intestine. The soft-shelled turtles were kept in sterile PBS for another 48 h to maintain osmotic pressure for the subsequent experiments. For anesthesia, the soft-shelled turtles were placed in a sealed beaker with five ether cotton balls (containing 10 ml of ether) for 10 min. For euthanasia, the dose of ether was doubled, and the soft-shelled turtles were kept in the sealed beaker for 60 min.

This study was performed in strict accordance with animal protocols approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, China CDC, in accordance with the medical research regulations of the Ministry of Health, China.

Construction of bioluminescent *V. cholerae* strains. To enhance the expression of *luxCDABE* and bioluminescence intensity, the promoter of the housekeeping gene *mdh* (malate dehydrogenase gene), a highly transcribed gene (by transcriptome analysis, data not shown), from strain VC4251 was amplified (primers are listed in Table 2). The amplicons were digested with KpnI and BamHI and cloned into pXEN-luxCDABE (PerkinElmer) through the multiple cloning site, to generate the recombinant plasmid pXEN-pmdh-luxCDABE. O1 strain VC1898, O139 strain VC4251, and all mutants generated from strain VC4251 were transformed with the plasmid pXEN-pmdh-luxCDABE. These strains were plated on LB (Luria-Bertani) agar plates containing ampicillin (100 μg/ml), and lux-expressing clones were selected with the In-vivo FX Pro imaging system (Bruker) and were verified by sequencing.

Construction of mutants. The *mshA* mutant strain was constructed from strain VC4251 using a homologous recombination method. We designed primers for a region upstream of the *mshA* gene (*mshA*-1 and *mshA*-2) and introduced a XhoI endonuclease recognition site into the primer *mshA*-1. Downstream primers (*mshA*-3 and *mshA*-4) were also designed, and a NotI endonuclease recognition site was introduced into the primer *mshA*-4. The upstream and downstream DNA fragments flanking the target sequences within *mshA* were amplified from VC4251 genomic DNA using the primer pairs *mshA*-1/*mshA*-2 and *mshA*-3/*mshA*-4, respectively. Because portions of the primers *mshA*-2 and *mshA*-3 were complementary to one another, the upstream and downstream amplicons were mixed at equimolar concentrations and used as the template to amplify the chromosomal fragment, excluding the target-

TABLE 2 Primers for bioluminescent plasmid and mutant strain construction

Primer	Sequence[a]	Restriction enzyme
gbpA-1	5'-CCGCTCGAGAGCGCATGGTGCTCCTTA-3'	XhoI
gbpA-2	5'-AGGATAACTTCACAGACTCTTCTTTGTTAGCTG-3'	
gbpA-3	5'-AGAGTCTGTGAAGTTATCCTCCCTCTTACA-3'	
gbpA-4	5'-ATTTGCGGCCGCGCCTTGGGATGTTCTACG-3'	NotI
mshA-1	5'-CCGCTCGAGGGTGGAACTGGTCATCGT-3'	XhoI
mshA-2	5'-AGCCTATGTCCTCTCTTTCATGTGAATACGCA-3'	
mshA-3	5'-TGAAAGAGAGGACATAGGCTTCAATGGTTA-3'	
mshA-4	5'-ATTTGCGGCCGCGCCTAAACTATCGAAATCAA-3'	NotI
tcpA-1	5'-CCCTCGAGTCCACAGTCAAAGTGAC-3'	XhoI
tcpA-2	5'-GTGATATTAGATTTATATAACTCCACCATTTGT-3'	
tcpA-3	5'-TTATATAAATCTAATATCACGCATGTTGAG-3'	
tcpA-4	5'-ATTTGCGGCCGCTAATCAGCATCACAAGCA-3'	NotI
lacZ-1	5'-CCGCTCGAGCCACCACGATGATAACCAAT-3'	XhoI
lacZ-2	5'-GAGTGAGCAACCCTCAAGCCGAGGAGTAAA-3'	
lacZ-3	5'-GGCTTGAGGGTTGCTCACTCAGCCGCACTA-3'	
lacZ-4	5'-ATTTGCGGCCGCGCGAACAGGCGATGACTAAC-3'	NotI
prom-mdh-F	5'-CGGGGTACCAGCCAAAGCGTTCTTCTTTTAGTAA-3'	KpnI
prom-mdh-R	5'-CGCGGATCCCGTAAATCTCCTTGAGAGTAATCTC-3'	BamHI
gbpC-F	5'-CCGGAATTCATGAAAAAACAACCTAAAATGACCG-3'	EcoRI
gbpC-R	5'-CTAGTCTAGATTAACGTTTATCCCACGCCATTTCC-3'	XbaI
mshC-F	5'-CCGGAATTCATGGTAATAATGAAAAGACAAGGTG-3'	EcoRI
mshC-R	5'-CTAGTCTAGATTATTGCGCTGGTTTACCACAAGCA-3'	XbaI
tcpC-F	5'-CCGGAATTCATGCAATTATTAAAAACAGCTTTTTA-3'	EcoRI
tcpC-R	5'-CTAGTCTAGATTAACTGTTACCAAAAGCTACTGTG-3'	XbaI

[a]Restriction endonuclease sites are underlined.

deleted fragment, using the primer pair *mshA*-1/*mshA*-4. After digestion with XhoI and NotI, the resulting fragment was cloned into the suicide plasmid, pWM91, containing a *sacB* counterselectable marker (60) to generate the plasmid pWM91-Δ*mshA*. The resulting suicide plasmid was maintained in *E. coli* SM10 λ*pir* and transformed to the strain VC4251 by conjugation. The transconjugants were selected on LB agar plates containing streptomycin (100 μg/ml) and ampicillin (100 μg/ml), and the clones were streaked on LB agar plates with 8% sucrose and without NaCl at 22°C. After 48 h, double-crossover recombination mutants were selected and verified by sequencing to generate the mutant strain, VC4251dmshA. The *gbpA*, *tcpA*, and *lacZ* mutants were constructed using the same method described above. The primers used in the mutant construction of these genes are listed in Table 2.

Construction of complementary strains. The complementation plasmids carrying *mshA*, *gbpA*, and *tcpA* were constructed with plasmid pBAD24 (61). The open reading frames (ORFs) of *mshA*, *gbpA*, and *tcpA* were amplified from VC4251 chromosomal DNA using the primer pairs mshc-F/mshC-R, gbpC-F/ gbpC-R, and tcpC-F/tcpC-R, respectively. After digestion with EcoRI and XbaI, the resulting fragments were cloned downstream of the pBAD promoter in the plasmid pBAD24 (as a result, all transcriptions of these genes were driven by the pBAD promoter), to generate the complementation plasmids pBADmshA, pBADgbpA, and pBADtcpA, respectively, which were verified further by sequencing. Then, the mutant strains VC4251dmshA, VC4251dgbpA, and VC4251dtcpA were transformed with the complementation plasmids pBADmshA, pBADgbpA, and pBADtcpA, respectively, to generate the complementation strains VC4251dmshA(pBADmshA), VC4251dgbpA(pBADgbpA), and VC4251dtcpA(VC4251dtcpA). Strain VC4251dlacZ was transformed with the control plasmid pBAD24 to generate strain VC4251dlacZ (pBAD).

Small intestine colonization competition assays in infant mice. VC4251dlacZ and *mshA*, *gbpA*, and *tcpA* mutant strains were cultured overnight on LB agar plates at 37°C. The colonies were scraped and resuspended in 3 ml of PBS to an OD_{600} of 1.0 (the amount of each strain was measured by plate counting), which was approximately equal to 10^9 CFU/ml. Approximately 10^5 cells of *mshA*, *gbpA*, and *tcpA* mutants were mixed with VC4251dlacZ at a 1:1 ratio and were inoculated intragastrically into 5-day-old CD-1 infant mice. The mixtures were diluted for enumeration to calculate the input ratios (mutant to wild type). After 24 h, the mice were euthanized and the small intestines were removed and homogenized. Serial dilutions were made and plated on LB agar plates supplemented with polymyxin B (5 μg/ml), streptomycin (100 μg/ml), and X-Gal ([5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside] 50 μg/ml) for enumeration and calculation of the output ratios (mutant to wild type). Referring to the colony counting method from the previous study (62), the *lacZ* mutant strain was also constructed from strain VC4251. Because *lacZ*-positive *V. cholerae* strains (all colonization gene mutants, including VC4251dmshA, VC4251dgbpA, and VC4251dtcpA) show blue colonies on LB agar plates in the presence of X-Gal and *lacZ*-negative *V. cholerae* strain colonies are white, the colony numbers of different strains for colonization competition assays can be calculated directly. The competitive index was calculated from the output ratio and normalized against the input ratio. Three mice were used for each group of competition assays.

Turtle body surface bioluminescence imaging assays. The bioluminescence-labeled serogroup O1 strain VC1898, serogroup O139 strain VC4251, and VC4251-derived *mshA*, *gbpA*, and *tcpA* mutants were

cultured overnight on LB agar plates containing 100 μg/ml ampicillin at 37°C. The colonies were scraped and resuspended in 30 ml of PBS containing 100 μg/ml ampicillin to obtain an OD_{600} value of 1.0 (the amount of each strain was measured by plate counting), which was approximately 10^9 CFU/ml. The suspensions were diluted in 3 liters of PBS containing 100 μg/ml ampicillin. Soft-shelled turtles were immersed in the bacterial suspension for 2 h for surface colonization. Each soft-shelled turtle was washed twice with 2 liters of sterile PBS to remove cells that were not attached. The soft-shelled turtles were kept in 3 liters of sterile PBS containing 100 μg/ml ampicillin at room temperature (28°C). At 24, 48, 72, and 96 h postinfection, the soft-shelled turtles were washed and placed in fresh sterile PBS containing 100 μg/ml ampicillin. After administration of anesthesia, the soft-shelled turtles were analyzed for bioluminescent bacteria with an In-vivo FX Pro imaging system. Bioluminescent signals were also detected before infection as negative controls. After bioluminescence imaging, the surfaces of the soft-shelled turtles were sampled with sterile cotton swabs for *V. cholerae* isolation. In these assays, five turtles were immersed in the bacterial suspensions of the above five strains, and the tests were repeated three times (15 turtles in total were used).

Body surface colonization competition assays. The *mshA*, *gbpA*, and *tcpA* mutants and VC4251dlacZ were cultured overnight on LB agar plates at 37°C, and the colonies were scraped and resuspended in PBS to an OD_{600} of 1.0. VC4251dlacZ was mixed with the *mshA*, *gbpA*, and *tcpA* mutants at a 1:1 ratio. The mixtures were diluted for enumeration to calculate the input ratios. Then, 30 ml of the mixtures was diluted with 3 liters of PBS. The soft-shelled turtles were immersed in the bacterial suspension for 2 h for surface infection; the turtles were separated into three groups to detect the three gene mutants. Each soft-shelled turtle was washed twice with 2 liters of sterile PBS to remove cells that were not attached. The dorsal carapace, calipash, and limbs of each turtle were sampled with sterile cotton swabs. The swabs were washed with 300 μl of sterile PBS. Serial dilutions were made and plated on LB agar plates supplemented with polymyxin B (5 μg/ml), streptomycin (100 μg/ml), and X-Gal (100 μg/ml) for enumeration and calculation of the output ratios (this detection time point was assigned as day-0 time point). The soft-shelled turtles were then kept in 3 liters of sterile PBS at 28°C. Every 24 h postinfection, the soft-shelled turtles were washed, sampled, and placed in fresh sterile PBS. Bacterial enumerations were performed as described above to calculate the output ratios. The detections were continuously performed for 10 days (day-1 time point to day-10 time point). The competitive index was calculated from the output ratio and normalized against the input ratio. Three soft-shelled turtles were used for each group in the competition assays, and the assays were repeated three times.

In the competition assays using strain VC4251dlacZ(pBAD) and the complementation strains VC4251dmshA(pBADmshA), VC4251dgbpA(pBADgbpA), and VC4251dtcpA(pBADtcpA), the LB agar plates and PBS were supplemented with ampicillin and arabinose to final concentrations of 100 μg/ml and 0.5%, respectively, to culture these strains. LB agar plates for the selective isolation of these strains were supplemented with the following components: ampicillin (final concentration of 100 μg/ml), polymyxin B (5 μg/ml), streptomycin (100 μg/ml), and X-Gal (50 μg/ml). Other procedures of the competition assay with these strains were performed as described above.

Bioluminescence imaging assays of the internal organs. The bioluminescence-labeled serogroup O1 strain VC1898, serogroup O139 strain VC4251, and VC4251-derived *mshA*, *gbpA*, and *tcpA* mutant bioluminescent strains were cultured overnight on LB agar plates containing 100 μg/ml ampicillin at 37°C. The next day, the strains were scraped and resuspended in 3 ml of PBS to obtain an OD_{600} of 1.0. Soft-shelled turtles were anesthetized as described above. Next, 200-μl bacterial suspensions were inoculated intragastrically into the soft-shelled turtles' stomachs. To ensure the entry of all bacterial suspensions into the stomachs, polyethylene tubes that were at least 15 cm long were inserted into the turtles' mouths. In the assays, six turtles were inoculated intragastrically with the above six strains, and the assays were repeated three times (18 turtles in total were used).

The turtles were cultured in 3 liters of sterile PBS containing 100 μg/ml ampicillin at 28°C. Every 24 h postinfection, the soft-shelled turtles were placed in new sterile PBS containing 100 μg/ml ampicillin. At 72 h, the soft-shelled turtles were euthanized, and the internal organs (including the hearts, livers, lungs, spleens, kidneys, and digestive tracts) were analyzed for bioluminescent bacteria. Bioluminescent signals were also detected for the soft-shelled turtles inoculated with sterile PBS as negative controls. The internal organs were homogenized with 5 ml of sterile PBS. Serial dilutions were made and plated on selective LB agar plates supplemented with polymyxin B (5 μg/ml), streptomycin (100 μg/ml), and ampicillin (100 μg/ml) for *V. cholerae* isolation.

Intestinal colonization competition assays. In the competition assays with the gene mutant strains/VC4251dlacZ and complementary strains/VC4251dlacZ(pBAD), the strains were cultured overnight on LB agar plates (100 μg/ml ampicillin was used when strains carrying plasmids were cultured) at 37°C. The next day, the colonies were scraped and resuspended in 3 ml of PBS to obtain an OD_{600} of 1.0. The paired strains used for the competition assays were mixed at approximately a 1:1 ratio. The mixtures were diluted for enumeration to calculate the input ratios. Soft-shelled turtles were inoculated with 200 μl of the bacterial suspensions and kept as described above. At 72 h, the soft-shelled turtles were euthanized, the intestines were aseptically removed, and the intestinal tubes were washed with sterile PBS three times to remove cells that were not attached and homogenized with 5 ml of sterile PBS. Serial dilutions were made and plated on LB agar plates supplemented with the corresponding antibiotics as above and X-Gal for enumeration of the corresponding strains and the calculation of output ratios. The competitive index was calculated from the output ratio and normalized against the input ratio. Three soft-shelled turtles were used for each competition strain pair in these colonization competition assays, and the assays were repeated three times.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .00713-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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