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RESEARCH ARTICLE

Methylation-Independent Chemotaxis Systems Are the Norm for Gastric-Colonizing *Helicobacter* Species

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ABSTRACT Many bacteria and archaea rely on chemotaxis signal transduction systems for optimal fitness. These complex, multiprotein signaling systems have core components found in all chemotactic microbes, as well as variable proteins found in only some species. We do not yet understand why these variations exist or whether there are specific niches that favor particular chemotaxis signaling organization. One variation is in the presence/absence of the chemotaxis methylation adaptation enzymes CheB and CheR. Genes for CheB and CheR are missing in the gastric pathogen *Helicobacter pylori* but present in related *Helicobacter* that colonize the liver or intestine. In this work, we asked whether there was a general pattern of CheB/CheR across multiple *Helicobacter* species. *Helicobacter* spp. all possess chemotactic behavior, based on the presence of genes for core signaling proteins CheA, CheW, and chemoreceptors. Genes for the CheB and CheR proteins, in contrast, were variably present. Niche mapping supported the idea that these genes were present in enterohepatic *Helicobacter* species and absent in gastric ones. We then analyzed whether there were differences between gastric and enterohepatic species in the CheB/CheR chemoreceptor target methylation sites. Indeed, these sites were less conserved in gastric species that lack CheB/CheR. Lastly, we determined that *cheB* and *cheR* could serve as markers to indicate whether an unknown *Helicobacter* species was of enterohepatic or gastric origin. Overall, these findings suggest the interesting idea that methylation-based adaptation is not required in specific environments, particularly the stomach.

IMPORTANCE Chemotaxis signal transduction systems are common in the archaeal and bacterial world, but not all systems contain the same components. The rationale for this system variation remains unknown. In this report, comparative genomics analysis showed that the presence/absence of CheR and CheB is one main variation within the *Helicobacter* genus, and it is strongly associated with the niche of *Helicobacter* species: gastric *Helicobacter* species, which infect animal stomachs, have lost their CheB and CheR, while enterohepatic *Helicobacter* species, which infect the liver and intestine, retain them. This study not only provides an example that a chemotaxis system variant is associated with particular niches but also proposes that CheB and CheR are new markers distinguishing gastric from enterohepatic *Helicobacter* species.

KEYWORDS chemotaxis, CheB, CheR, *Helicobacter*, gastric, enterohepatic, adaptation, methylation

Chemotaxis signal transduction systems are critical for the survival of bacteria and archaea in their complex and various environments. Indeed, 50% of species in the Bacteria and Archaea domains contain these multiprotein systems, which are the most complex prokaryotic signaling systems (1). Work on *Escherichia coli* characterized the basic molecular players, but our understanding changed when pioneering studies in *Rhodobacter sphaeroides* and *Bacillus subtilis* suggested that chemotaxis systems have various components (2, 3). Subsequent groundbreaking genomic studies identified

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17 types of chemotaxis systems, each defined by protein conservation and often a distinct combination of signaling proteins (1). While we know that chemotaxis systems can vary, we do not yet know why or whether there are specific niches that favor particular chemotaxis signaling organizations.

Chemotaxis signal transduction systems comprise both common and variable proteins (1). The common core proteins generally control the signaling phosphotransfer reactions, while the variable ones are more often involved in signal adaptation. The core phosphotransfer components are the chemoreceptors, the CheA histidine kinase, the CheW scaffold/coupling protein, and the CheY response regulator. Chemoreceptors are at the front of the pathway, detecting attractant and repellent ligands and forming a ternary complex with CheW and CheA that controls CheA activity. This ternary complex can be in a conformation that either activates or inactivates the CheA kinase, depending on whether the chemoreceptors detect changes in attractants or repellents. For example, in most bacteria, including *E. coli* and *Helicobacter pylori*, an increase of repellents or a decrease of attractants promotes the ternary complex to adopt the conformation that activates CheA. In these cases, CheA phosphorylates itself and then passes phosphate to the response regulator CheY, resulting in phosphoryl CheY (CheY-P). CheY-P binds to the flagellar motor and switches the flagellar rotational direction from counterclockwise to clockwise. This outcome in turn creates bacteria that change direction, sampling new courses to move further from the harmful environment. The opposite happens with increased attractants: the complex adopts the CheA inactive form, resulting in loss of CheY-P and a flagellar motor that rotates counterclockwise, the straight-swim direction (4).

The variable chemotaxis proteins are found in a subset of chemotaxis systems (1). Two of the most widespread are the CheR methyltransferase and the CheB methylesterase, which are missing from only 10% of chemotactic microbes. These proteins modify the chemoreceptors and allow the ternary complex to adapt to the current conditions. CheB and CheR work on the same region of chemoreceptors, with CheR adding methyl groups to chemoreceptors to create the CheA-active form and CheB removing them (4). For example, as described above, when a chemoreceptor binds attractant ligands, the CheA kinase is initially inactive. Over time, CheR adds methyl groups to the chemoreceptor, and it switches the ternary complex to the CheA-active form (4). CheB can be activated through phosphorylation via CheA. As time continues, CheB removes the methyl groups, allowing the system to reset (5, 6). The action of CheR and CheB are required for bacteria to be able to migrate over large attractant concentration gradients. Without CheR/CheB, as soon as all the receptors are either ligand bound or not bound, it is hard to reset the system (7). Thus, numerous studies have shown that CheR and CheB are critical for a bacterium's ability to respond to attractants and repellents over multiple orders of magnitude of concentration (8). In addition to CheB/CheR, *in silico* analysis showed that adaptation to stimuli could be provided by CheV, a coupling protein with an additional receiver domain (9).

We were intrigued about the bacteria that lack CheR and CheB, as they are relatively rare (1). In particular, we and others have noted that one group of bacteria that lack CheR and CheB are in the *Helicobacter* genus (10, 11). Most *Helicobacter* species are pathogens, infecting the gastrointestinal tracts of different animals. The best known is *H. pylori*, which infects the human stomach. This microbe infects more than half of the human population worldwide and causes various diseases, including gastritis, peptic ulcer, and cancer (12–14). *H. pylori* has a complete chemotaxis system but lacks CheR and CheB (10, 15). In addition to *H. pylori*, there are other kinds of *Helicobacter* species infecting different animals and different parts of the body. For example, *Helicobacter hepaticus* infects the intestine and liver of mice and causes colitis (16). In contrast, *Helicobacter enhydrae* infects the stomachs of sea otters and is correlated with gastric ulcers (17, 18). Indeed, *Helicobacter* species can be divided into gastric or enterohepatic species (19). Gastric *Helicobacter* species colonize the stomachs of mammals, including humans, dogs, cats and other felines, pigs, dolphins, sea otters, and whales, while enterohepatic *Helicobacter* species colonize parts of the gastrointestinal tract outside the stomach, including the liver and intestine of mammals, birds, and reptiles (20).

The specificity of *Helicobacter* to colonize different habitats likely results from long-term adaptation and evolution (21). Many environmental factors vary between the different regions of the gastrointestinal tract, including oxygen, mucus thickness, nutrient availability, and immune factors (22). In this study, we queried whether there was any pattern to the presence/absence of CheB/CheR in *Helicobacter* spp. We analyzed the predicted niches and variations within chemotaxis systems. We identified that all known gastric *Helicobacter* species lack genes for CheB/CheR and correspondingly have poorly conserved chemoreceptor methylation sites. In contrast, enterohepatic *Helicobacter* spp. retain *cheB/cheR*, and have highly conserved methylation sites. This finding provides the first evidence that occupation of a particular niche correlates with a lack of specific chemotaxis signaling proteins. Furthermore, we identified that *cheB* and *cheR* have additional utility as markers for distinguishing gastric versus nongastric *Helicobacter* species.

RESULTS

CheB and CheR are the two major variations in *Helicobacteraceae* chemotaxis machinery. Our first goal was to evaluate the chemotaxis proteins in *Helicobacter* spp. We took a broad view of the *Helicobacter* relatives and compared *Helicobacteraceae* with other families within the entire order *Campylobacterales*, using Aquerium, a program developed by Adebali and Zhulin for tracing the evolutionary history of proteins by providing their taxonomic distribution (23). We evaluated the presence/absence of CheA, CheW, the alternate scaffold protein CheV, CheB, and CheR in this order. Most bacteria belonging to the *Campylobacter* or *Helicobacter* genera have CheA, suggesting most of them have chemotaxis ability (Fig. 1). When we counted the number of each kind of chemotaxis protein in Campylobacterales, all except *Wolinella succinogenes* had only one CheA and one CheW, suggesting that most have only one chemotaxis system (Table S1). There were variations, however, in three attributes: the number of chemoreceptor genes, the number of *cheV* genes, and the presence/absence of *cheR/cheB*. Chemoreceptor numbers varied from 0 to 31 and also varied in the types found (Table S1). Chemoreceptors are typed by signaling domain length, according to the number of helical heptads (H), so called 24H, 28H, 36H, 40H, 44H, and 52H (24); all of these types are found in the *Helicobacteraceae*, in various numbers between species (Table S1). The number of *cheV* genes in *Helicobacteraceae* family members also varied, ranging from 1 to 4 (Table S1). In contrast, *Campylobacteraceae* family members have only one *cheV* gene (data not shown). Lastly, a significant fraction of *Helicobacteraceae* family members lacked genes for *cheB* and *cheR*, while other members of the Campylobacterota phylum retained them, including *Campylobacteraceae* species (Fig. 1) (15). Interestingly, some non-*Helicobacter* species, e.g., *Sulfurimonas denitrificans*, *Campylobacter mucosalis*, and *Campylobacter hominis*, have CheR but not CheB (Fig. 1). Overall, this analysis showed there were three major variations in the *Helicobacteraceae* chemotaxis machinery: the type and number of chemoreceptors, the number of CheVs, and the presence/absence of CheR and CheB. For the remainder of this study, we focused on the CheB and CheR variation to understand it in more depth.

Chemotaxis systems can be divided into 17 classes based on the phylogenetic tree of CheA-CheB-CheR (1). Usually, a microbe's chemotaxis system has only one class of proteins, but this is not always the case, so we analyzed whether the classes of chemotaxis systems match the classes of CheB and CheR in *Helicobacter* species. This analysis was accomplished by searching each microbe in the MiST database (25) and noting the CheA, CheB, and CheR designation. Unexpectedly, there was a mismatch between the classification of the chemotaxis system and the CheB and CheR in numerous members of this phylum (Table 1). For example, *Helicobacter bilis* has an F3 system but F7 CheB and CheR, while *Desulfurella* spp. have an F1 system but an F9 CheR. We additionally analyzed the location of *cheB* and *cheR* on the chromosome, using TREND (26) and the "Gene" database of NCBI (27). *cheB* and *cheR* were always located in one polycistronic operon, but this operon was distant from other chemotaxis core genes and did not conserve the neighboring genes (Fig. S1). This analysis raised the possibility that *cheB* and *cheR* in some *Helicobacter* species could have resulted from horizontal

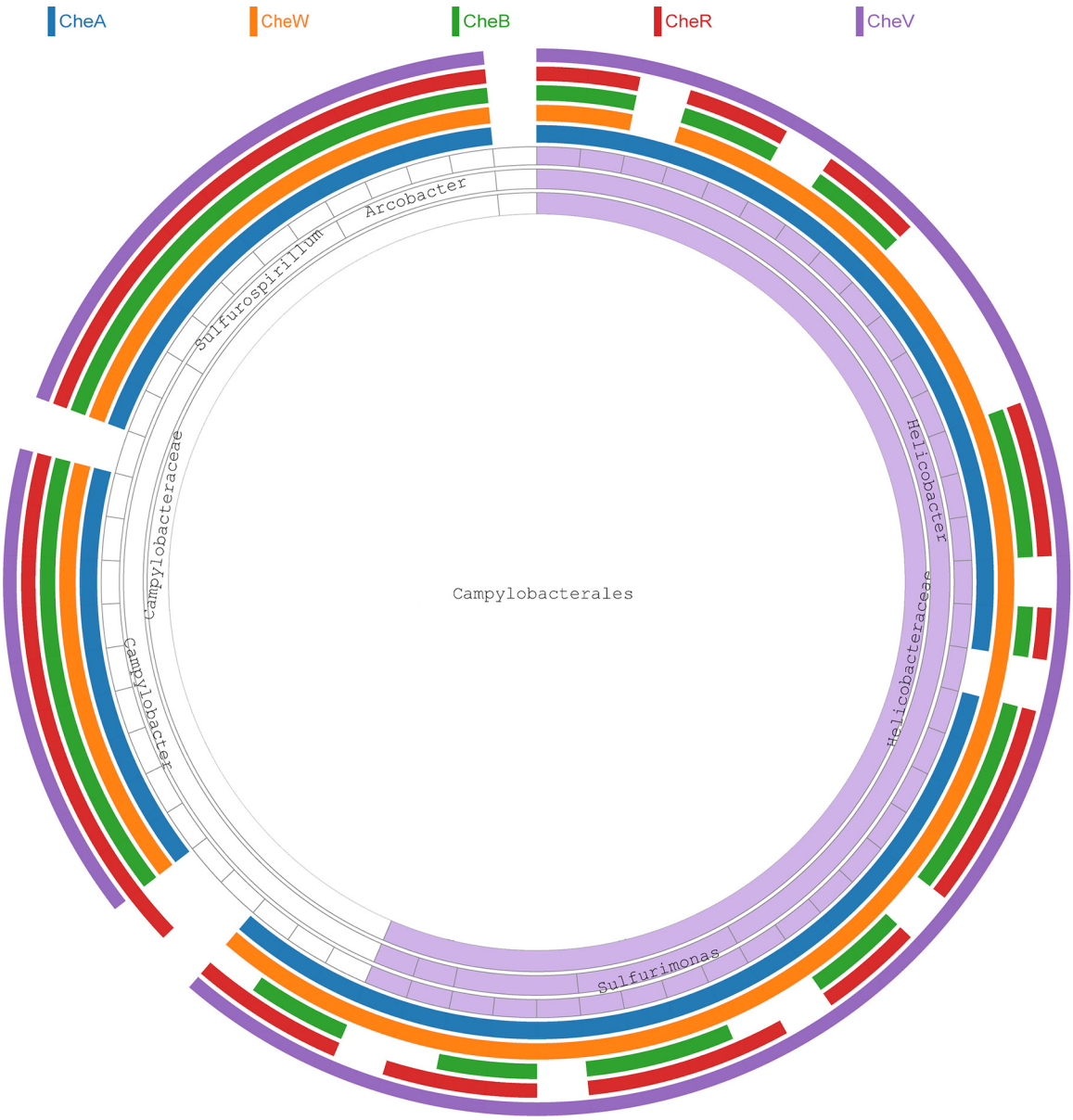

FIG 1 Phyletic distribution of five chemotaxis proteins in the *Campylobacterales* order: CheA (blue), CheW (orange), CheB (green), CheR (red), and CheV (purple). *Helicobacteraceae* are indicated by light purple shading. In the ring just inside the CheA ring, individual species are denoted by separating lines, with 35 species in the *Helicobacteraceae* family (purple area) and 26 in the *Campylobacteraceae* one. The figure was created using Aquerium.

gene transfer. To test this idea, we built and compared phylogenetic trees based on the protein sequences of either CheA, CheB, or CheR (Fig. S2). For the microbes with all three proteins, the trees had similar topologies, with the same microbes grouping together. This analysis indicated the ancestor had all three genes, consistent with an analysis of the Campylobacterota phylum (15). This analysis suggests that *cheB/cheR* evolved vertically but developed enough variation to be classified into different chemotaxis families. Analysis of the Campylobacterota phylum has suggested that it is monophyletic with a common ancestor that had *cheB* and *cheR* (15). Modern host-associated members underwent gene loss, resulting in species that do not have *cheB*/*cheR* now, possibly as the result of evolution or environmental adaptation.

CheB and CheR correlate with *Helicobacter* ecological niche. We next analyzed whether particular *Helicobacteraceae* branches retained or lost CheB/CheR by building a phylogenetic tree and mapping *cheB/cheR* status on it. The phylogenetic tree was built

TABLE 1 Chemotaxis systems of the Campylobacterota phyla[a]

GTDB representative species	Taxon	Chemotaxis system	CheR	CheB
GCF_001010585.1	*Arcobacter butzleri*	F7 system	1(F7)	1(F7)
GCF_000092245.1	*Arcobacter nitrofigilis*	F7 system	1(F7)	1(F7)
GCF_000935065.1	*Arcobacter anaerophilus*	F7 system	1(F7)	1(F7)
GCF_002723485.1	*Arcobacter canalis*	F7 system	1(F7)	1(F7)
		F9 system	1(F9)	1(F9)
GCF_001878855.1	*Arcobacter lekithochrous*	F7 system	1(F7)	2(F7)
		F8 system	1(F8)	
GCF_900475935.1	*Campylobacter fetus*	F3 system	1(F3)	1(F3)
GCF_001298465.1	*Campylobacter concisus*	F3 system	1(F3)	1(F3)
GCF_002220755.1	*Campylobacter sputorum* bv. *paraureolyticus*	F3 system	2(F3)	2(F3)
GCF_001457695.1	*Campylobacter jejuni*	F3 system	1(F3)	1(F3)
GCF_900478295.1	*Helicobacter pylori*	F3 system		
GCF_000686565.1	*Helicobacter bilis*	F3 system	1(F7)	1(F7)
GCF_000507865.1	*Helicobacter canis*	F3 system	1(F7)	1(F8)
GCF_000007905.1	*Helicobacter hepaticus*	F3 system	1(F7)	1(F7)
GCF_000687535.1	*Helicobacter rodentium*	F3 system	1(F7)	1(F7)
GCF_000200595.1	*Helicobacter felis*	F3 system		
GCF_001602095.1	*Helicobacter himalayensis*	F3 system		1(F8)
GCF_900198475.1	*Helicobacter cholecystus*	F3 system		
GCF_900199585.1	*Helicobacter* sp. "house sparrow 1"	F3 system		
GCF_002272925.1	*Helicobacter* sp. 13S00401-1	F3 system	1(F7)	1(F7)
GCF_002272795.1	*Helicobacter* sp. 13S00482-2	F3 system	1(F3)	1(F3)
GCF_000196135.1	*Wolinella succinogenes*	F3 system	1(F7)	1(F7)
GCF_900115615.1	*Hydrogenimonas thermophila*	F3 system	1(F3)	1(F3)
		F7 system		
		F8 system	1(F8)	1(F8)
GCF_900176045.1	*Nitratiruptor tergarcus*	F14 system	1(F3)	1(F3)
			1(F9)	1(F9)
GCF_000183725.1	*Sulfuricurvum kujiense*	F3 system	1(F3)	1(F3)
		F7 system	1(F7)	2(F7)
		F8 system	1(F8)	
GCF_000147355.1	*Sulfurimonas autotrophica*	F3 system		1(F7)
		F8 system	1(F8)	
GCF_000024885.1	*Sulfurospirillum deleyianum*	F3 system	1(F3)	1(F3)
		F7 system	1(F7)	1(F8)
GCF_000597725.1	*Sulfurospirillum arcachonense*	F3 system	1(F3)	1(F3)
		F7 system	1(F7)	1(F7)
GCF_000186245.1	*Nitratifractor salsuginis*	No chemotaxis		
GCF_000170735.1	*Caminibacter mediatlanticus*	F3 system		
GCF_002335445.1	*Lebetimonas natsushimae*	F3 system		
GCF_000021725.1	*Nautilia profundicola*	F3 system		
GCF_000517565.1	*Desulfurella acetivorans*	F1 system	1(F9)	1(F1)
GCF_002119425.1	*Desulfurella amilsii*	F1 system	1(F9)	1(F1)
GCF_000194135.1	*Hippea maritima*	F3 system	1(F9)	1(F5)
GCF_000744435.1	*Hippea jasoniae*	F3 system	1(F9)	1(F5)

[a]Chemotaxis systems in members of the Campylobacterota phyla have class mismatches between CheA, CheB, and CheR. The mismatches are indicated with underlined type. GTDB, Genome Taxonomy Database.

using the Genome Taxonomy Database (GTDB) (28), an initiative to establish a standardized microbial taxonomy based on genome phylogeny via 120 single-copy marker proteins. This approach has been suggested to be more accurate than 16S/23S RNA alone (28). Lineages that lacked *cheB/cheR* were found in clusters that were not monophyletic (Fig. 2). We therefore examined whether the presence or absence of CheB and CheR correlated with the niche, specifically stomach/gastric or non-stomach/enterohepatic. Not all the *Helicobacter* species have known niches, particularly if they were collected from fecal, rectal, or cloacal swabs. We therefore selected well studied representative *Helicobacter* species with clear reports of isolation directly from gastric or enterohepatic samples (Table 2). *Helicobacter mustelae* and *Helicobacter pametensis* are controversial. For example, *H. mustelae* has been characterized as a gastric (29), enterohepatic (30), and gastrointestinal (31).

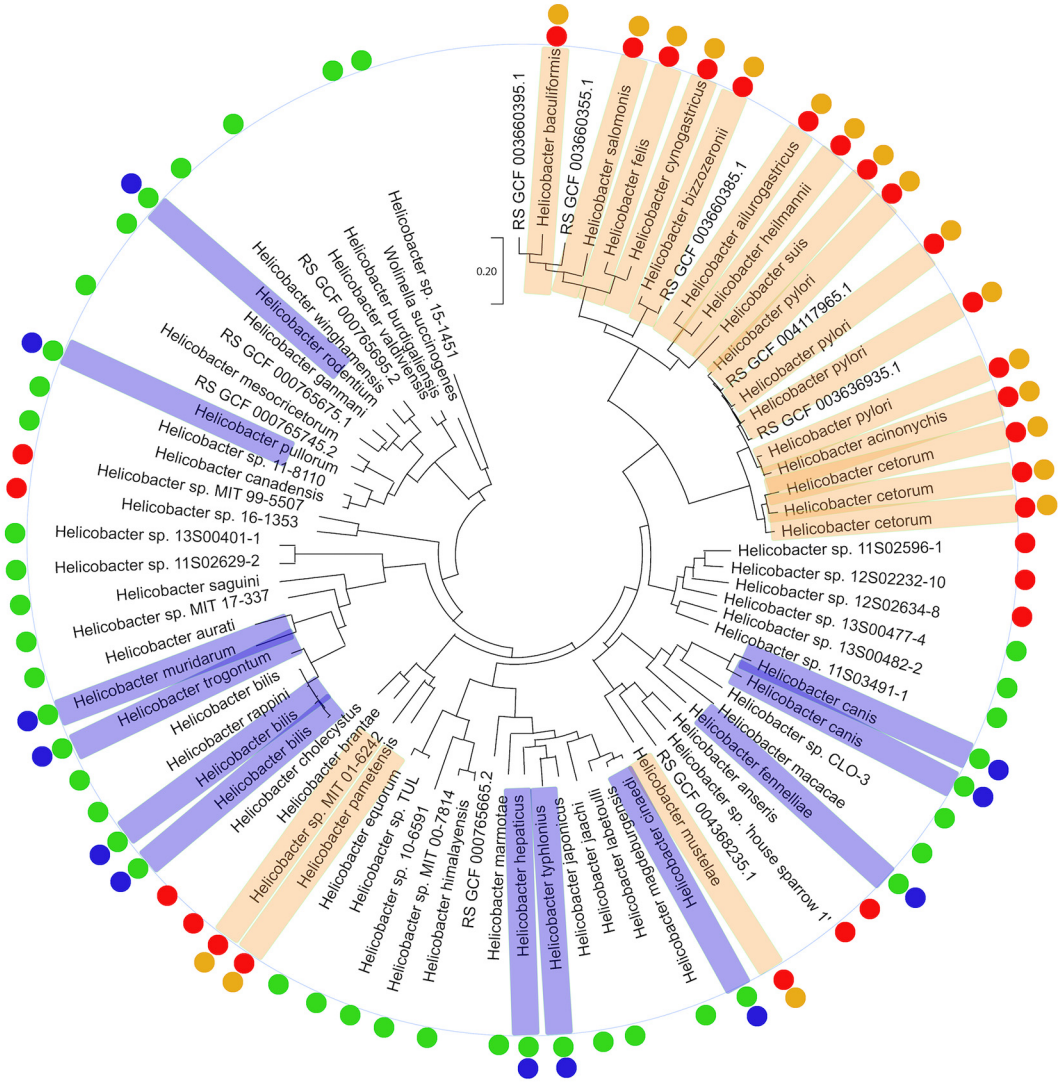

FIG 2 Phylogenetic tree of *Helicobacteraceae* species combined with niche mapping and *cheB*/*cheR* presence/absence. The outer-most layer shows the *cheB*/*cheR* presence. Red dots indicate genomes without *cheB*/*cheR*, and green dots indicate genomes with *cheB*/*cheR*. The second layer indicates *Helicobacter* species with known gastric (orange) or enterohepatic (blue) niches, indicated by both dots and shading. The species name with RS GCF indicates the representative species (RS) number in Genome Taxonomy Database (GTDB), when it does not have a NCBI organism name. The tree was built using 120 single-copy marker proteins and was collected from the GTDB (28) and Annotree (81).

However, because *H. mustelae* and *H. pametensis* have been isolated from actual stomach tissue (Table 2), we categorized them as gastric *Helicobacter*. This analysis resulted in a set of 24 *Helicobacter* species with robust niche characterization.

We then analyzed the evolutionary relationship between the 24 species on the phylogenetic tree of *Helicobacteraceae*. This analysis identified that some gastric *Helicobacter* species or enterohepatic *Helicobacter* species clustered together, but many species are distributed interlocally (Fig. 2). This result is consistent with the observation that some species are difficult to classify, given gastric and enterohepatic *Helicobacter* species do not cluster separately.

Finally, we mapped the presence or absence of *cheB* and *cheR* onto the niche and phylogeny information (Fig. 2). Surprisingly, all known enterohepatic *Helicobacter* species have *cheB* and *cheR*, while confirmed gastric *Helicobacter* species lack *cheB* and *cheR* (Fig. 2). These results indicate, on the one side, that the *cheB* and *cheR* genes could be used as markers to

TABLE 2 Gastric and enterohepatic *Helicobacter* species[a]

GTDB Rs	Taxon	Niche	Isolated source	References
GCF_900197685.1	*Helicobacter baculiformis*	**Gastric**	Feline stomach	86
GCF_900197775.1	*Helicobacter salomonis*	**Gastric**	Dog stomach	87, 88
GCF_000200595.1	*Helicobacter felis*	**Gastric**	Cat stomach	89
GCF_900197855.1	*Helicobacter cynogastricus*	**Gastric**	Dog stomach	90
GCF_000237285.1	*Helicobacter bizzozeronii*	**Gastric**	Dog stomach	91
GCF_001282945.1	*Helicobacter ailurogastricus*	**Gastric**	Cat stomach	92
GCF_001283065.1	*Helicobacter heilmannii*	**Gastric**	Cat stomach	93
GCF_000187625.1	*Helicobacter suis*	**Gastric**	Pig stomach	94
GCF_000277405.1	*Helicobacter pylori*	**Gastric**	*Homo sapiens* stomach	95
GCF_900461455.1	*Helicobacter acinonychis*	**Gastric**	Cheetah stomach	96
GCF_000259255.1	*Helicobacter cetorum*	**Gastric**	Cetaceans stomach	97
GCF_000507865.1	*Helicobacter canis*	Enterohepatic	Dogs/fecal samples	98
GCF_000509365.1	*Helicobacter fennelliae*	Enterohepatic	*Homo sapiens* rectal swab	99
GCF_900476215.1	*Helicobacter mustelae*	**Gastric**	Mammal (ferret) stomach	100
GCF_000155475.1	*Helicobacter cinaedi*	Enterohepatic	*Homo sapiens* blood, rectal swab	99
GCF_001460635.1	*Helicobacter typhlonius*	Enterohepatic	Mouse cecum	101
GCF_000007905.1	*Helicobacter hepaticus*	Enterohepatic	Rat fecal/colons	102
GCF_000518225.1	*Helicobacter pametensis*	**Gastric**	Feces of wild bird, domestic swine	103
GCF_001693335.1	*Helicobacter* sp. MIT 01-6242	**Gastric**	*Enhydra lutris* sp. *nereis* stomach	17
GCF_000686565.1	*Helicobacter bilis*	Enterohepatic	Mouse colon, cecum	104
GCF_000765905.1	*Helicobacter trogontum*	Enterohepatic	Rat colon	105
GCF_000765805.2	*Helicobacter muridarum*	Enterohepatic	*Rattus* intestines	106
GCF_000155495.1	*Helicobacter pullorum*	Enterohepatic	Human gastrointestinal tract, chicken	107
GCF_000687535.1	*Helicobacter rodentium*	Enterohepatic	Mouse feces, colon, cecum	108

[a]The table shows the known gastric and enterohepatic *Helicobacter* species and their isolation sources. The information of known gastric and enterohepatic *Helicobacter* species was collected from the NCBI-BioSample database and cited articles. The niches of gastric and enterohepatic *Helicobacter* species are indicated with bold type and underlined type, respectively. Rs, representative species.

distinguish gastric and enterohepatic *Helicobacter* species. On the other side, they suggest that CheB and CheR might be important for *Helicobacter* species to adapt to different niches.

Predicted *Helicobacter* CheB and CheR retain their key catalytic sites. Given that many *Helicobacter* spp. have lost the *cheR* and *cheB* genes, we next explored whether the retained CheB and CheR in enterohepatic *Helicobacteraceae* species are likely to keep their methyltransferase and methylesterase activities, by analyzing the key sites involved in enzyme activity. CheB contains a methylesterase domain, and in some bacteria, such as *E. coli* and *S. enterica* Typhimurium, CheB also has an N-terminal response regulator domain (Fig. S3). CheB in *Helicobacter* species has only a methylesterase domain, as do those in *Campylobacter* species, including *Campylobacter jejuni* and *Campylobacter coli* (Fig. S3). The lack of a response regulator domain results in CheB proteins that are not regulated by phosphorylation but that do affect chemotaxis (32), suggesting that the presence of the response regulator domain is not essential for CheB's function in chemotaxis. We aligned the *Helicobacter* CheB amino acid sequences, built a WebLogo, and located the active site amino acids involved in methylesterase activity (Ser, His, and Asp) (33). These sites were conserved in CheB of *Helicobacter* species (Fig. 3A). For CheR, we also found that the key sites (such as Asp, Ile, and Val) involved in catalysis and *S*-adenosylmethionine (AdoMet) binding (34) were conserved in *Helicobacter* species (Fig. 3B). These results show that CheB and CheR in *Helicobacteraceae* have key sites involved in catalysis and likely retain their enzymatic activities.

Methylation sites of chemoreceptors in gastric *Helicobacter* species are degraded. Given that some *Helicobacter* species lack genes for CheB and CheR, we wondered whether the CheB and CheR chemoreceptor interaction sites would show similar patterns of loss and retention. Both CheB and CheR interact with two chemoreceptor sites: a C-terminal NWETF chemoreceptor pentapeptide (35–38) and the methylation sites themselves. The C-terminal pentapeptide acts to increase the local concentration of CheR and CheB to improve their activity (39–41), and is conserved on approximately 10% of bacterial chemoreceptors, particularly in host-infecting bacteria (42). The pentapeptide sequence, however, was not conserved in chemoreceptors from either gastric

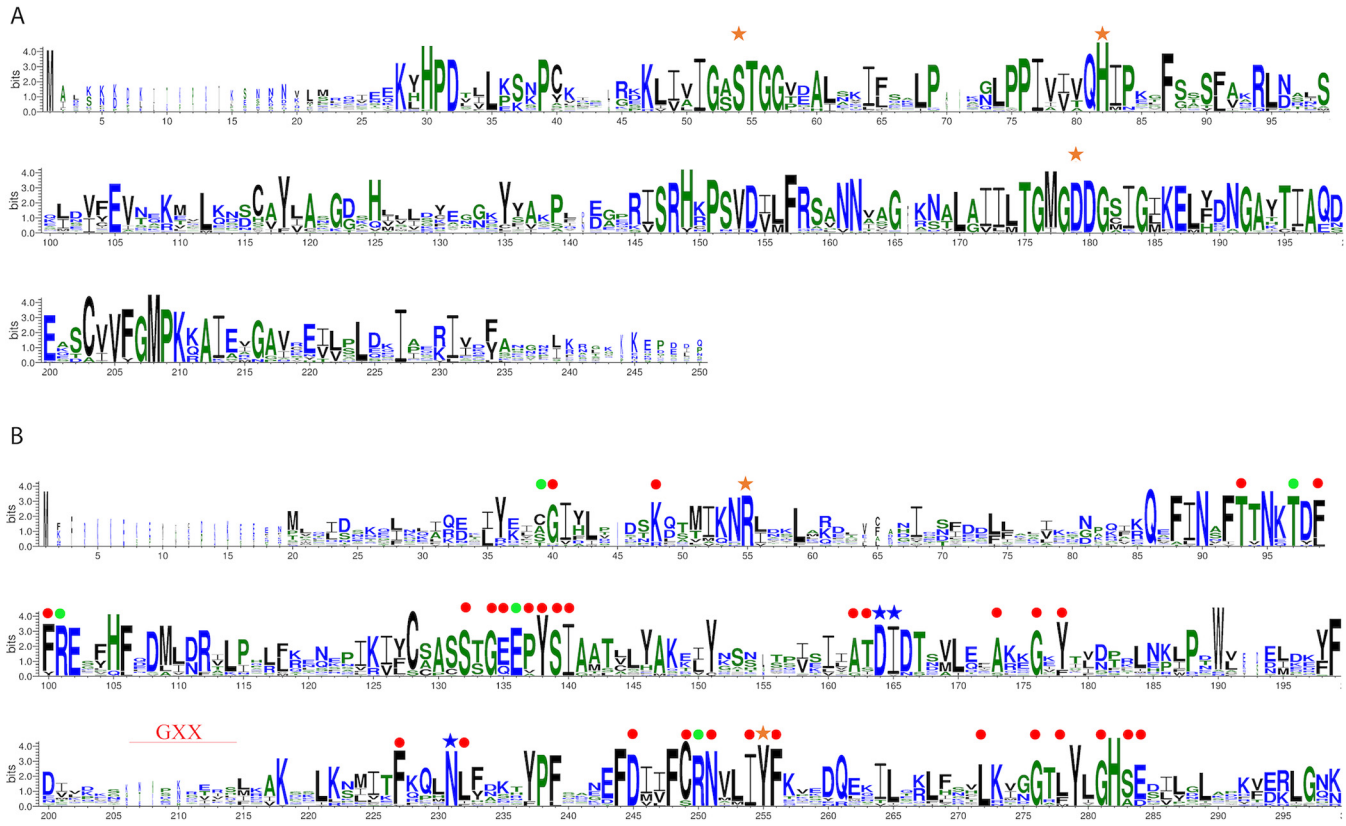

FIG 3 Protein sequence alignments of predicted CheB (A) and CheR (B) protein sequences from enterohepatic *Helicobacter* species. In both panels, predicted active sites residues are indicated with orange stars. In panel B, red dots indicate sites conserved in CheR from other non-*Helicobacter* bacteria; blue stars indicate interaction sites with AdoMet; and green dots indicate residues that form hydrogen bonds with AdoMet. GXX indicates the motif at the β-subdomain, which present only in CheR that interacts with pentapeptides. Multiple sequence alignments were performed by EBI-MAFFT (77), analyzed with Jalview (78), and visualized using WebLogo3 (80).

or enterohepatic *Helicobacter* species, suggesting that these chemoreceptors do not bind CheB/CheR via this region (data not shown). We then analyzed whether the predicted CheR and CheB proteins retained their pentapeptide-binding motifs. CheR binds the pentapeptide via a GXX motif at the β-subdomain (43). This GXX motif was absent in CheR from *Helicobacter* species (Fig. 3B), suggesting that they are unable to bind the pentapeptide. The pentapeptide-binding region of CheB spans the N-terminal end of the receiver domain and the C-terminal part of CheB domain (44). *Helicobacter* species CheB proteins, however, lack the receiver domain (Fig. S3), suggesting that they would not be able to bind the pentapeptide either. These results suggest that *Helicobacter* CheR and CheB do not have the ability to bind the pentapeptide and are consistent with the observation that *Helicobacter* chemoreceptors do not have this sequence.

We next analyzed the methylation site conservation patterns in the *Helicobacter* chemoreceptors. Chemoreceptors typically have two to five methylation sites spaced throughout their coiled-coil cytoplasmic signaling domain. The first three sites are adjacent, while the others can be spread throughout the protein. Within these sites, methylation occurs either on glutamine (Q) or on a deamidated glutamate (E). Thus, these residues tend to be most conserved. When mapped onto a wrap of a seven-amino acid coiled coil with positions a to e, the Q or E at position c or d tends to be highly conserved, along with several other amino acids that create the coiled-coil register. The methylation sites can be recognized by the conserved motif shown in Fig. 4, based on the three adjacent methylation sites (1–3) found in chemoreceptors from *E. coli*, *S. enterica*, and *Thermotoga maritima* (45–47). We then compared these putative methylation sites between gastric and enterohepatic chemoreceptors. Those found in enterohepatic species had clear conservation signals, conserving the Q or E at positions

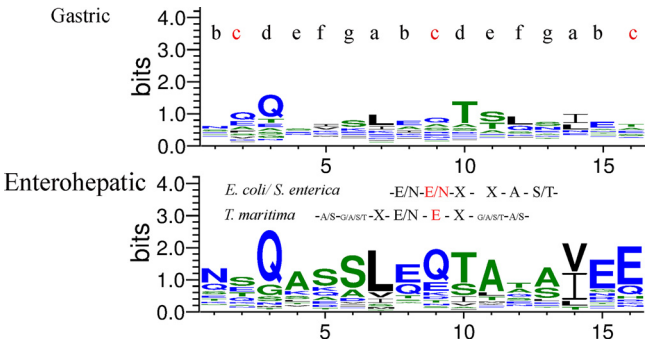

FIG 4 Analysis of conservation patterns in methylation sites 1, 2, and 3, comparing chemoreceptors from gastric (top) or enterohepatic (bottom) *Helicobacter* species. The seven-residue coiled coil repeat positions (a to g) are indicated at the top. The methylation sites are located at position c or d and indicated with red color. The consensus sequence for methylation sites from *E. coli*, *S. enterica*, and *T. maritima* are indicated as insets in the bottom panel. Multiple sequence alignments were performed by EBI-MAFFT (77) and visualized using WebLogo3 (80). Methylation sites were identified by aligning of *Helicobacter* species chemoreceptors with *E. coli* and *C. jejuni* chemoreceptors with known methylation sites.

c/d in sites 1, 2, and 3 (Fig. 4). Gastric *Helicobacter* species, in contrast, showed less conservation of the methylation sites (Fig. 4). The only hint of conservation was in the retention of a Q at the first methylation site (Fig. 4). In *E. coli*, the second and third methylation sites in the Tar chemoreceptor have more important effects on methylation and demethylation kinetics than other sites (48, 49). These results suggest that there is a higher level of methylation site conservation in enterohepatic species chemoreceptors compared to gastric ones. These results are consistent with the idea that gastric species chemoreceptors do not use methylation.

28H chemoreceptors often retain methylation sites. We next analyzed the enterohepatic *Helicobacter* spp. chemoreceptors in more detail, to ascertain whether particular subsets retained or lacked methylation sites. As mentioned above, chemoreceptors are typed according to the number of heptads (H) in the cytoplasmic domain (24) or the sequence of the ligand-binding domain (50). Most enterohepatic *Helicobacter* species' chemoreceptors are 24H, 28H, 40H, or 44H (Fig. 5; Table S1). We then evaluated which of these classes have methylation sites, by aligning the enterohepatic *Helicobacter* chemoreceptor signaling domain to *E. coli* chemoreceptors (Tar, Tsr, Tap, and Trg) with experimentally determined sites and *C. jejuni* chemoreceptors (Tlp1, Tlp2, Tlp3, and Tlp4) identified by *in*

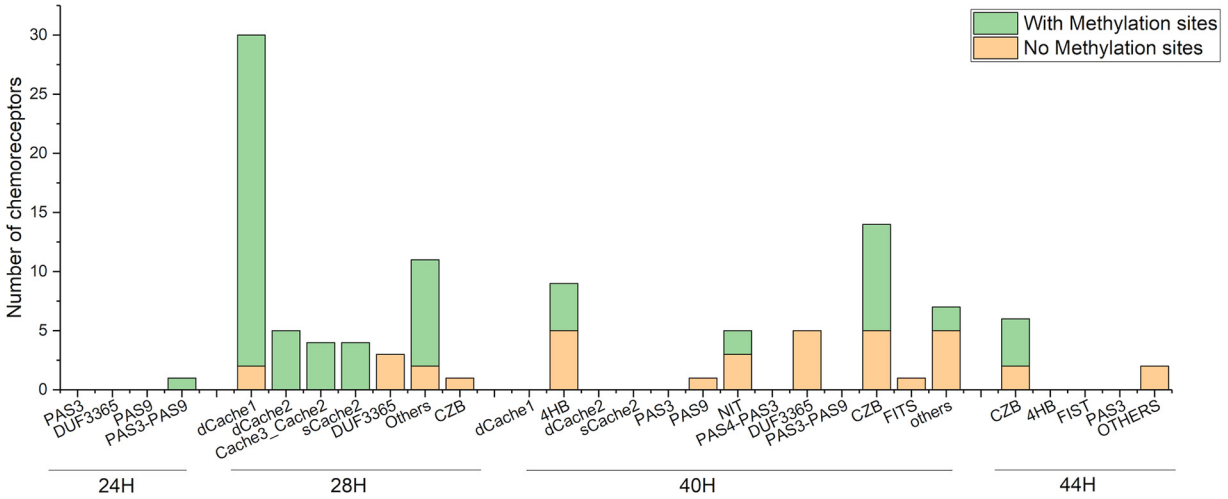

FIG 5 Analysis of prevalence of different types of enterohepatic *Helicobacter* chemoreceptors with or without methylation sites. Chemoreceptor types were collected from the MiST database and named 24H through 44H according to the number of heptads (H). PAS, Cache, 4HB, CZB, NIT, etc., indicate the ligand-binding domain of cognate chemoreceptors and were also collected from MiST.

silico analysis (45, 51–53), followed by methylation site confirmation according to the consensus (Fig. 4). Methylation sites were found often in the 28H class (Fig. 5). We then evaluated whether particular types of ligand-binding domains were correlated with retained methylation sites. Within the 28H class, methylation sites were retained with most ligand-binding types without an obvious pattern. These results hint that there may be subsets of chemoreceptors, the 28H type, that are more often targeted by CheB/CheR in *Helicobacter* species.

***cheB* and *cheR* presence/absence and genome size may have utility to differentiate gastric from enterohepatic *Helicobacter* species.** One ongoing challenge is to predict whether a newly isolated *Helicobacter* sp. is native to the gastric or enterohepatic niche, e.g., when one obtains the sample from feces, rectum, or cloaca. Various approaches have historically failed to distinguish gastric from enterohepatic *Helicobacter* species, including analysis of the 16S rRNA sequences, analysis of other single genes, omics analyses, or urease activity (30, 31, 54). The phylogeny also is not conclusive: although most gastric or enterohepatic *Helicobacter* species cluster into distinct clades, there are some exceptions (Fig. 2). Thus, there is a need for better markers to separate the two groups. We wondered whether *cheB*/*cheR* could be used in a predictive way. We first asked how many genes were unique to gastric or enterohepatic species, using the BioCyc comparative analysis. Two genes were present in 100% of gastric *Helicobacter* species and absent from all enterohepatic ones: the gene encoding the NCS2 family permease and the gene encoding WaaL, an O antigen ligase. Thirty genes were unique to enterohepatic species, including *cheB* and *cheR* (Table S2). These results indicate that *cheB* and *cheR* could be good markers to distinguish enterohepatic and gastric *Helicobacter* species. We thus asked whether *cheB*/*cheR* could predict the origin of several *Helicobacter* spp. whose origin is unknown. As a first test, we challenged our hypothesis using several newly isolated gastric *Helicobacter* species (*Helicobacter labacensis*, *Helicobacter mehlei*, and *Helicobacter vulpis* [55]) and enterohepatic *Helicobacter* species (*Helicobacter didelphidarum* [56]), which were not used in the analysis presented in Fig. 2. We found that all three of the test gastric *Helicobacter* species did not have *cheB/cheR*, while the test enterohepatic *H. didelphidarum* did have them (data not shown). This analysis provided further support that *cheB/cheR* could be a good predictive tool. We then analyzed several *Helicobacter* spp. with unknown origins. Based on the absence of *cheB* and *cheR*, we predict that *Helicobacter brantae*, *Helicobacter anseris*, and *Helicobacter* sp. "house sparrow 1" might be gastric *Helicobacter* species (Fig. 2). Interestingly, these species are all embedded with enterohepatic *Helicobacter* species on phylogenetic tree and so have been difficult to predict (Fig. 2).

Compared to the intestine, the stomach is a harsh environment for bacteria. Other microbes that live in harsh environments have been reported to have smaller genome sizes than those who live in less challenging niches, as shown with *Methylophilaceae* species (57). We hypothesized that the genome size of gastric *Helicobacter* species would be streamlined compared to enterohepatic ones. We therefore analyzed *Helicobacteraceae* genome size using information from GTDB and NCBI genomes. On average, the genome size of gastric *Helicobacter* species was around 1.65 Mbp, while the genome size of enterohepatic *Helicobacter* species was around 2.05 Mbp (Fig. 6, inset). The significant differences in genome size between gastric and enterohepatic *Helicobacter* are consistent with our hypothesis that the gastric *Helicobacter* genomes are streamlined. When we mapped the genome size of each species to the phylogenetic tree of *Helicobacteraceae*, the genome size of gastric *Helicobacter* species is much smaller than that of their nearby enterohepatic *Helicobacter* species (Fig. 6). Indeed, genome streamlining in gastric *Helicobacter* species might underlie the loss of *cheB* and *cheR*. Finally, we used the *Helicobacter* species genome size as an additional tool to distinguish gastric and enterohepatic *Helicobacter* species. *H. brantae* (1.731 Mbp), *H. anseris* (1.624 Mbp), and *Helicobacter* sp. "house sparrow 1" (1.429 Mbp) all had smaller genomes than their nearby enterohepatic species *H. bilis* (2.62 Mbp) or *Helicobacter fennelliae* (2.159 Mbp). This approach further supports they are gastric *Helicobacter* species.

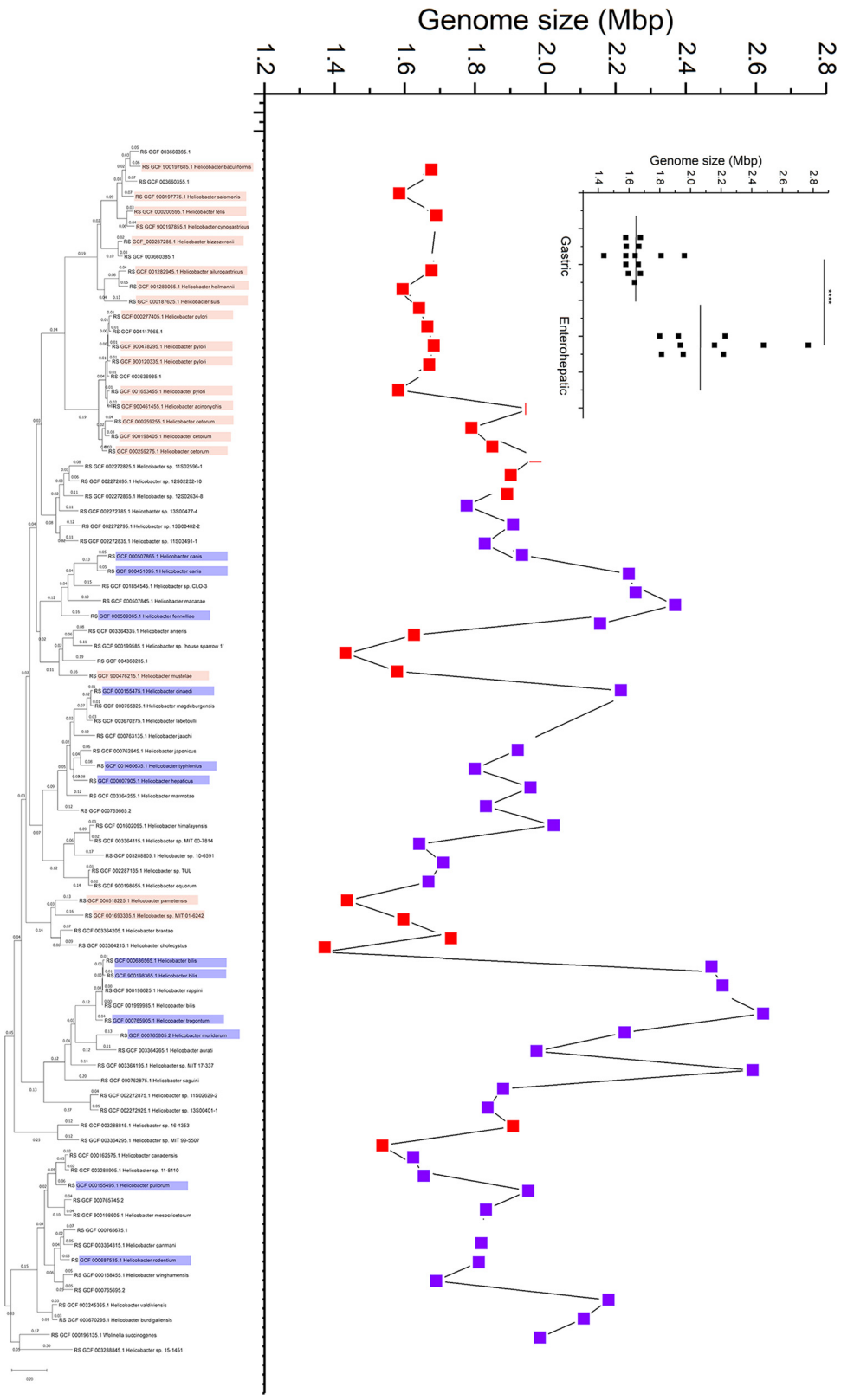

FIG 6 Mapping the genome size of *Helicobacter* species with or without CheB/CheR to the phylogenetic tree of Helicobacteraceae. Red squares and red highlighting indicate genomes without CheB/CheR, while the blue squares and blue highlighting indicate genomes with CheB/CheR. The inset shows the genome size in gastric or enterohepatic *Helicobacter* species, with each dot representing a species and the solid lines indicating the average. Comparison between these two groups was done using a Student's *t* test, yielding a *P* value of <0.0001, indicated by ****.

DISCUSSION

We report here the surprising finding that bacterial species in a specific niche, the stomach, have chemotaxis systems that lack methylation-based adaptation. This observation was made by determining both that gastric *Helicobacter* species lack the *cheB* and *cheR* genes and that their chemoreceptors have poorly conserved methylation consensus sequences.

Helicobacter species are animal-associated microbes. However, different *Helicobacter* species colonize different positions in gastrointestinal tract, creating gastric and enterohepatic *Helicobacter* species. There are likely substantial differences between the stomach and intestine that necessitate different colonization mechanisms. Chemotaxis is one critical colonization factor, important for many bacteria to infect both animals and plants (58). Indeed, studies in *H. pylori*, *C. jejuni*, and *C. coli* have established that *Campylobacteraceae* and *Helicobacteraceae* require chemotaxis for normal colonization. In *H. pylori*, loss of chemotaxis results in poor initial colonization and less occupation of a known *H. pylori* niche, the gastric glands (59–62). Here, we identified that the chemotaxis systems of gastric and enterohepatic *Helicobacter* species have a fundamental variation in that systems in gastric *Helicobacter* species lack CheB and CheR, the methylation-adaptation system. Our results suggest that methylation-based chemotaxis adaptation may not be needed for chemotaxis in the stomach niche.

The vast majority of chemotaxis systems possess CheB and CheR. The chemotaxis system of *E. coli* works optimally with CheB and CheR, but studies with mutants generated to lack *cheB* and *cheR* have highlighted that chemotaxis can occur without them. Specifically, loss of *cheB* and *cheR* significantly impairs the chemotaxis response regulated by the Tar, Tap, Trg, and Tsr chemoreceptors (63–65), but not Aer chemoreceptor-regulated aerotactic behavior (66). Indeed, aerotaxis via Aer is independent of methylation adaptation (66). The benefit of CheB and CheR methylation adaptation is that the cell's swimming behavior can be finely adjusted. Without CheB and CheR, the activity of CheA is retained at a high or low level, causing extreme clockwise-biased or counterclockwise-biased flagellar rotation and resulting in a delayed chemotaxis response. Although aerotaxis is methylation-independent in *E. coli*, aerotaxis of *Azospirillum brasilense* is dependent on the presence of CheB and CheR (8). These reports suggest that there is no strict relationship between methylation-dependent or independent adaptation and aerotaxis, and instead the need for methylation might be unique for particular chemoreceptors.

The lack of CheB and CheR in gastric *Helicobacter* species may be due to distinct aspects of the stomach niche. Two major differences between the stomach and intestine are the thickness and pH of mucus, with the stomach mucus layer being thicker and more acidic (22). Loss of CheB and CheR is predicted to allow cells to sustain a response, e.g., extend long-term straight runs with attractants. Longer runs have been shown to benefit *Vibrio cholerae* intestinal colonization (67). This finding suggests that run bias via counterclockwise flagellar rotation is beneficial in some settings, perhaps for gastric *Helicobacter* species to cross the thick mucus. Acid acts as chemorepellent for *H. pylori* and causes higher reversal frequency (68, 69). Loss of CheB and CheR might act to blunt the signal response to low pH and let bacterial cells move forward for a relatively long time. Gastric *Helicobacter* species must cross the mucus to colonize near the epithelial cells and within glands. The loss of CheB and CheR may decrease the frequency of flagellar directional changes, promoting long straight runs and possibly conferring more efficient colonization.

Chemoreceptors have multiple methylation sites, but the exact number varies between chemoreceptors. Transmembrane chemoreceptors in *T. maritima* have two, three, or four methylation sites (46). In *E. coli*, Trg has five methylation sites (45), while Tar has four (70). We did not analyze the exact number of methylation sites in this work, instead focusing on the sites that are adjacent (1–3) because they are more readily identified bioinformatically. Previous work has shown that these sites are critical but not of equal importance. A mathematical model of methylation site sequential

methylation and experimental substitution of methylation sites in Tar both suggest that the second and third methylation sites are more important than the first one (48, 49, 71). The degradation of these key methylation sites in gastric *Helicobacter* species chemoreceptors is consistent with the idea that methylation does not occur in this chemotaxis system. Our data show that only some enterohepatic species chemoreceptors had methylation sites to begin with. Thus, loss of CheB and CheR may indicate the essential roles of their cognate chemoreceptors on stomach colonization. It is worth noting, however, that there may be rare conserved methylation sites that are hidden by building a consensus with all receptors.

In addition to variation in genes for CheB and CheR, we also found that gastric *Helicobacter* species have streamlined genomes. The reason that gastric *Helicobacter* species have a smaller genome size remains unknown. One possible reason is faster evolution rates under harsh environments, like low pH in the stomach. There is a correlation between faster evolution rates and smaller genomes (72), and previous studies indicate that the gastric *Helicobacter* species *H. pylori* has a high rate of gene recombination and unusual genetic flexibility (73–75), and those traits were considered to be helpful for the adaption to the dynamic environment. Low pH tolerance might not be the direct reason underlying *cheB/R* loss, as some free-living acidophilic bacteria lack *cheB/cheR*, e.g., *Acidithiobacillus ferrivorans*, *Acidithiobacillus caldus*, and *Acidithiobacillus sulfuriphilus*, while others do have them, e.g., *Acidithiobacillus albertensis* (data not shown).

Chemotaxis is well known to contribute to bacterial fitness, including colonizing hosts or special niches found in soil (76). Although deletion of *cheB*, *cheR*, or both could reduce the chemotaxis ability of bacteria significantly, they are not essential for bacterial chemotaxis (8, 32). Within the stomach, the acidic niche prevents most bacteria from competing with gastric *Helicobacter* species. In other words, maintaining competitive chemotaxis ability for gastric *Helicobacter* species may not be as important as soil or enterohepatic bacteria. Thus, it may be possible that gastric *Helicobacter* species do not need efficient or rapid chemotaxis. In this scenario, *cheB* and *cheR* could be lost during the gastric *Helicobacter* genome streamlining with minimal negative consequences.

In summary, we report here that gastric *Helicobacter* species lack genes for CheB and CheR. Our data suggest that *cheB* and *cheR* are new markers for distinguishing gastric and enterohepatic *Helicobacter* species. The reason behind the loss of CheB and CheR remains to be determined. Our results do suggest the idea that there are specific environments where efficient methylation-based adaptation is not needed. These same gastric *Helicobacter* species also have streamlined genomes, suggesting that loss of *cheB* and *cheR* happened during the trimming of genes that were nonessential for gastric colonization.

MATERIALS AND METHODS

Selection of *Helicobacter* species and their niches. The representative *Helicobacter* species with strain ID were collected from the Genome Taxonomy Database (GTDB) (28). The genome size of selected *Helicobacter* species was collected from NCBI Genome and GTDB. The isolation source of each representative *Helicobacter* species was collected from NCBI-BioSample and double checked with published articles.

Collection of chemotaxis protein data and cooccurrence analysis. The amino acid sequences of chemotaxis proteins and their classifications were collected from MiST3 database (25). The GTDB strain ID was first used to search each strain in the MiST3 database, and then the sequences of CheA, CheV, and chemoreceptors were collected from the table showing chemosensory systems proteins. The classification of CheA was used to identify the class of chemotaxis system. The cooccurrence analysis of chemotaxis proteins, CheA, CheW, CheV, CheB, and CheR was analyzed with the webserver Aquerium, focusing in Campylobacterales (23). The query statements below were used to explore the coocurrence of chemotaxis proteins CheA, CheW, and CheV: CheA, "CheW' in p and 'HATPase_c' in p'; CheW, "CheW' in p and 'HATPase_c' not in p and 'Response_reg' not in p'; CheV, "CheW' in p and 'HATPase_c' not p and 'Response_reg' in p'. CheB and CheR were identified with CheB and CheR domains, respectively.

Sequence alignment and phylogenetic analysis. The EBI-MAFFT program (77) was employed to perform multiple sequence alignment of chemotaxis proteins. The results of multiple sequence alignment were then analyzed with Jalview (78) and used to build a phylogenetic tree by MEGA with the maximum likelihood method (79). The frequency of conserved residues was visualized using WebLogo3 (80). The phylogenetic tree of *Helicobacteraceae* built by 120 single-copy marker proteins was collected from the GTDB (28) and Annotree (81).

Analysis of gene neighborhoods and protein domains. The gene neighborhoods were analyzed by the MiST3, TREND, and MicrobesOnline databases (82). The domains including the ligand-binding domains of chemoreceptors CheB, and CheR were analyzed by TREND (26) and CD-VIST (83).

Analysis of pentapeptide and methylation site of chemoreceptors. The pentapeptide of chemoreceptors at C-terminal ends was screened as reported (84). The chemoreceptors in *Helicobacter* species were aligned with known chemoreceptors with or without pentapeptides in *E. coli*. The chemoreceptors with a C-terminal tail 10 amino acid residues longer than chemoreceptors lacking pentapeptides were selected first. If the selected chemoreceptors have the XZXXZ motif (where Z can be F, W, or Y at positions 2 and 5 of the pentapeptide), then they were seen as chemoreceptors with pentapeptides.

For identifying the methylation sites, *Helicobacter* species chemoreceptors were aligned with *E. coli* and *C. jejuni* chemoreceptors with known methylation sites to confirm the possible region with methylation sites. Then, the presence of methylation sites was checked with the methylation site motifs shown in *E. coli*, *S. enterica*, and *T. maritima*: -E/N-E/N-X-X-A-S/T- or -A/S-G/A/S/T-X-E/N-E-X-G/A/S/T-A/S- (45–47). The presence of the conserved E/N sites in the motif indicates that there are methylation sites in cognate chemoreceptors.

Comparative genome analysis. The comparative genome analysis was analyzed by BioCyc comparative analysis tools (85). Six genomes of enterohepatic *Helicobacter* species (*H. bilis*, *H. canis*, *H. cinaedi*, *H. hepaticus*, *H. pullorum*, and *H. typhlonius*) and 10 genomes of gastric ones (*H. ailurogastricus*, *H. bizzozeronii*, *H. acinonychis*, *H. cetorum*, *H. felis*, *H. heilmannii*, *H. mustelae*, *H. pylori*, *H.* sp. MIT 01-6242, and *H. suis*) were selected to analyze the orthologs shared among them or unique to an organism. The protein orthologs unique to each group were then identified by hand.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 6.6 MB.

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We declare no conflict of interest.

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