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TlpD mediates chemotactic repellent responses to reactive oxygen species that are relevant to Helicobacter pylori gastric gland colonization

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TlpD mediates chemotactic repellent responses to reactive oxygen species that are relevant to *Helicobacter pylori* gastric gland colonization

A dissertation submitted in partial satisfaction of the requirements for the degree of

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in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

Kieran D. Collins

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Abstract

TlpD mediates chemotactic repellent responses to reactive oxygen species that are relevant to *Helicobacter pylori* gastric gland colonization

Kieran Collins

The human gastric pathogen *Helicobacter pylori* relies upon chemotaxis during early infection, although the system appears dispensable during later stages of infection. This body of work describes efforts to characterize the signals interpreted by the cytoplasmic chemoreceptor TlpD and gain insight into the role the chemoreceptor plays during infection. Results of this work suggest that TlpD mediates chemotactic repellent responses to stimuli that evoke reactive oxygen species (ROS) formation in the cytoplasm and that these responses are involved in *H. pylori* colonization of gastric glands.

The first chapter serves as a review on chemotactic responses, and focuses on cytoplasmic chemoreceptors. This group of chemoreceptors are understudied relative to their transmembrane counterparts and are prevalent in both bacteria and archaea. An emphasis is placed on describing cytoplasmic chemoreceptors as a group, including likely ligand binding domains and their localization.
The ensuing chapters describes basic aspects of TlpD chemotactic responses and their relevance to colonization of the gastric epithelium. TlpD was found to sense and respond to stimuli that provoke cytoplasmic ROS production independently of transmembrane chemoreceptors, which include extracellular iron, hydrogen peroxide and superoxide generators. These findings suggest that the chemoreceptor senses and responds to a yet unknown signal generated by oxidative stress imparted by ROS, which likely involve labile cytoplasmic iron pools. We go on to describe colonization defects of a tlpD mutant, which appears to be connected to gastric gland colonization. The gastric gland colonization defects noted in wild type hosts for a tlpD mutant were rescued in hosts that were deficient in ROS production at the gastric epithelium. These results suggested that TlpD-mediated chemotactic responses to ROS were involved in *H. pylori* gastric gland colonization, and could be involved in the spread of the bacteria between glands in response to ROS production. Finally we discuss mechanistic issues concerning TlpD recognition of ROS.

Taken together these results have bolstered our basic understanding of TlpD signal transduction and the role of the chemoreceptor in host colonization.
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Chapters one and two are multi-author publications. I am responsible for all figures except 1, 3 and 4 of chapter two.
Internal sense of direction: Sensing and signaling from cytoplasmic chemoreceptors

Kieran D. Collins, Jesus Lacal, and Karen M. Ottemann

Summary

Chemoreceptors sense signals and drive the chemotactic response in Bacteria and Archaea. There are two main classes of chemoreceptors: integral inner membrane and soluble cytoplasmic proteins. The latter were identified more recently than integral membrane chemoreceptors, and have been studied much less thoroughly. These cytoplasmic chemoreceptors are the subject of this review. Our analysis determined that 14% of Bacterial and 43% of Archaeal chemoreceptors are cytoplasmic, based on currently sequenced genomes. There appear to be three Archaeal species that have solely cytoplasmic chemoreceptors. Cytoplasmic chemoreceptors appear to share the same key structural features as integral membrane chemoreceptors, including formation of homodimers, trimer-of-dimers, and 12 nm hexagonal arrays within the cell. Cytoplasmic chemoreceptors exhibit varied subcellular locations, with some localizing to the poles, like integral membrane chemoreceptors do, and others appearing both cytoplasmic and polar. Some cytoplasmic chemoreceptors adopt more
exotic locations, including formation of exclusively internal clusters, or moving
dynamic clusters that coalesce at points of contact with other cells. Cytoplasmic
chemoreceptors presumably sense signals within the cytoplasm, and bear diverse
signal input domains that are mostly N-terminal to the domain that defines
chemoreceptors, the so-called MA domain. Similar to transmembrane receptors, our
analysis suggests that the most common signal input domain is PAS (Per-Arnt-Sim),
but a variety of other N-terminal domains exist. It is also common, however, for
cytoplasmic receptors to have C-terminal domains that may function for signal input.
The most common of these is the recently identified Chemoreceptor Zinc Binding
(CZB) domain, found in 8% of all cytoplasmic chemoreceptors. The widespread
nature and diverse signal input domains suggests that these chemoreceptors can
monitor a variety of cytoplasmically-based signals, most of which remain to be
determined.

**Introduction**

Chemotaxis is a motility-based response that biases cell movement toward beneficial
molecules, called attractants, and away from harmful molecules, also known as
repellents. Chemotaxis is initiated through the recognition of attractants and
repellents by chemoreceptors, which are the signal-sensing proteins of the bacterial
chemotaxis system. The chemoreceptors transduce this information to the central
regulator of bacterial chemotaxis, the CheA kinase (Fig. 1) (Reviewed in (1, 2)),
which in turn leads to the regulation of flagellar rotation. This paradigm has been well
studied in integral membrane chemoreceptors of *Escherichia coli*, and has lead to many key insights into signal transduction. There are, however, entire classes of bacterial chemoreceptors that are fundamentally different from those of *E. coli* (3, 4). Studying these chemoreceptors likely will generate even more insights into the fundamental properties of signal recognition and transduction. We focus here on one type of distinct bacterial chemoreceptor: those that lack transmembrane domains and operate strictly cytoplasmically.

Chemoreceptors can be classed as either integral inner membrane proteins or soluble cytoplasmic ones (Fig. 1). All chemoreceptors are identified by the presence of a highly conserved cytoplasmic signaling domain called “MA” or “MCP signal”, which interacts with the CheW coupling protein and the CheA histidine kinase. Often chemoreceptors are called methyl accepting chemotaxis proteins (MCPs) because of their ability to be methylated, but we will use the term chemoreceptor because most so-called MCPs have not been experimentally tested for methylation. A substantial fraction of chemoreceptors are cytoplasmic. Specifically, our analysis of 8384 chemoreceptor proteins in the SMART protein database (5) found that 14.5% lack transmembrane regions and are predicted to reside in the cytoplasm. These cytoplasmic chemoreceptors presumably function similarly to transmembrane chemoreceptors, but detect intracellular ligands, although there is little known regarding how they actually sense and transmit signals (6, 7). Furthermore, chemoreceptors can function in processes other than motility, e.g. gene regulation.
Cytoplasmic chemoreceptors however, are known to play important roles in many microbial processes including pathogenesis (8, 9), fruiting body formation (10), as well as mediating taxis in response to cellular energy stores (11, 12), redox (13) (14), and metabolites (15). In this review, we start first with a discussion of basic chemoreceptor attributes, and then summarize the current state of understanding about signal recognition, signal transduction, and subcellular localization of cytoplasmic chemoreceptors.

**Chemoreceptors signal via interactions with the key signaling proteins**

**CheW and CheA**

Bacterial chemoreceptors contain signal output regions as well as signal input regions that are, in some cases, clearly defined (6, 16). In all types of chemoreceptors, the signal output region is cytoplasmic and highly conserved because it interacts with the downstream signal transduction proteins, CheW and CheA (Fig. 2). Chemoreceptors mediate chemotaxis by controlling CheA’s kinase activity (2). CheA phosphorylates CheY, which in turn interacts with the flagellar motor and affects the frequency at which the motor changes direction of rotation.

Chemoreceptors interact with both CheW and CheA. CheW is a coupling or scaffold protein that is essential to form connections between chemoreceptors and CheA (Fig 2). Interactions between chemoreceptors and CheW/CheA occur in a subregion of the MA domain called the protein interaction region (PIR) (2, 17). In transmembrane chemoreceptors, the PIR is the most distal portion of the
chemoreceptor from the membrane (Fig. 1). The PIR is a four-stranded coiled-coil, with two strands originating from each chemoreceptor monomer. Evidence suggests the PIR adopts the same structure in all receptors, cytoplasmic or transmembrane (18). The interaction of CheW with the PIR has been known for a long time and verified by numerous methods including NMR chemical shifts (19), targeted disulfide cross-linking (20) and genetic suppressor mutations (21, 22). The interaction of CheA and the PIR, in contrast, has only been appreciated recently but has been documented by several methods, including targeted disulfide cross-linking and mutagenesis (23-25). Interactions between chemoreceptors and CheW, as well as those between chemoreceptors and CheA, are both important for controlling CheA kinase activity (24, 26). Cytoplasmic chemoreceptors appear to participate in similar interactions based on observations between CheW, CheA and the PIR within the Tm14 cytoplasmic chemoreceptor from *Thermotoga maritime* (24). Based on these similar interactions, the output of ligand binding in all receptors is likely to trigger conformational changes at the PIR, and in turn affect CheA activation.

All chemoreceptors contain the MA domain, which acts to transduce signal input information to CheA. Only one copy of the MA domain is present in transmembrane chemoreceptors, while more than one copy of the MA domain can be found in cytoplasmic chemoreceptors. For instance, we identified 20 cytoplasmic chemoreceptors with two MA domains. All 20 chemoreceptors consist of an N-terminal region followed by two consecutive MA domains. Eighteen of these chemoreceptors have only small N-terminal regions (Class IVb), described in more
detail below, while two have longer N-terminal regions (Class IVa). None of these chemoreceptors has an annotated sensing domain and their functions are not yet known. Interestingly, while the MA domains are similar for all these receptors, their N-terminal regions are not suggesting they are not strict orthologs. These receptors were found only in Bacteria, within the Actinobacteria, Clostridia, Alpha-, Beta- and Gamma-Proteobacteria. The genome of *Caldicellulosiruptor bescii* DSM 6725 is the only one containing more than one (two) of these receptors. On the other hand, we found that the four *Vibrio cholerae* species have the same cytosolic chemoreceptor containing two MA domains (Supplementary Table 1). The relevance of a chemoreceptor having more than one MA domain is not yet known.

**Signal transduction in chemoreceptors**

The signal input region of chemoreceptors is extremely variable, in contrast to the highly conserved signal output region. Transmembrane chemoreceptors typically present an easily-identifiable region for signal input outside the cytoplasmic membrane. In contrast, it is often difficult to identify the signal input domain of cytoplasmic chemoreceptors, and it is not yet clear if it always lies N-terminal to the MA domain. Lacal and colleagues reported that some cytoplasmic chemoreceptors have a large N-terminal region, with a portion containing known or predicted sensing domains, while other cytosolic chemoreceptors have only a very short N-terminal domain that has no identifiable sensing domains (7).
In transmembrane chemoreceptors, several different organizations have been
described (6, 7). The most common organization is typified by the E. coli receptors
Tar and Tsr, and consists of: (1) a short N-terminal sequence that acts as a
transmembrane region; (2) a poorly-conserved periplasmic region; (3) a second
transmembrane region; and (4) a cytoplasmic region containing at least the highly-
conserved MA domain (Fig. 1). The periplasmic region is therefore flanked by two
transmembrane regions, is very diverse in length and sequence, and is responsible for
sensing ligands directly or through the interaction with ligand-binding proteins (1).
Ligand binding is thought to trigger a conformational change in the chemoreceptor
that consists of an ~1.4 Å downward slide of the long transmembrane helix that
connects the periplasmic and cytoplasmic regions (17, 27, 28). There have been no
studies of ligand-driven conformational changes in cytoplasmic chemoreceptors.
Numerous cytoplasmic chemoreceptors contain putative N-terminal ligand binding
domains, but C-terminal ones also exist as we describe in this review. It is unknown
whether cytoplasmic receptors detect ligand by direct binding, operate through
protein partners, or both.

A frequently found signal transduction domain in E. coli chemoreceptors is
the HAMP domain, which lies between the signal input and output domains (Fig. 1)
(29). HAMP domains are thought to function to transmit transmembrane ligand-
binding signal to the MA domain, although the exact mechanism is not yet clear.
HAMP domains are small 50 amino acid homodimeric folds, that adopt a four-helix
bundle structure of two amphipathic helices joined by a linker segment (29). HAMP
domains have been observed in crystal structures to adopt multiple conformations, varying by helix rotation, helix translation, and helix–helix crossing angles (30, 31). These different conformations are proposed to underlie signal transduction. About 80% of transmembrane chemoreceptors contain a membrane-proximal HAMP domain, based on our analysis of MA containing proteins in the SMART database (5). Sometimes more than one copy of the HAMP domain is found in chemoreceptors. However, the HAMP domain may not be essential since we found that almost 20% of the transmembrane chemoreceptors do not have an annotated HAMP domain. Given that HAMP domains are canonically thought to function in transmembrane signaling, it was not clear or known whether cytoplasmic chemoreceptors would possess them. To fill this gap, we determined the abundance of the HAMP domain in cytoplasmic chemoreceptors using proteins obtained from the SMART database that lack transmembrane domains (5). 5% of the 1217 cytoplasmic chemoreceptors contained a HAMP domain. In these chemoreceptors, the HAMP domain was always N-terminal to the MA domain and was frequently accompanied with the PAS domain (~ 30% of cytoplasmic chemoreceptors), and more rarely with the CHASE, Hemerythrin, NIT, and Cache_1 domains (these domains are discussed below). Generally, HAMP domains were prevalent in cytoplasmic chemoreceptors with long N-terminal domains, so called class IVa (7). One possibility is that cytoplasmic chemoreceptors with long N-terminal regions are more likely to contain a signal sensing domains in this region, and thus utilize a HAMP domain similar to transmembrane chemoreceptors in coupling conformational changes from a ligand
binding domain in the receptor to altering CheA kinase activity (29). This possibility, however, remains to be confirmed.

**Adaptation in chemoreceptors**

Chemoreceptors are capable of responding to a broad range of chemoeffector concentrations through a process known as adaptation (1, 17). Adaptation is a system of post-translational modifications affecting a chemoreceptor’s ability to activate CheA in conditions of prolonged stimulation. In the case of prolonged attractant stimulation, a bacterium’s swimming behavior will return to the prestimulation direction change frequency after a period of smooth swimming triggered by attractant addition. The best understood mechanism of adaptation occurs via the reversible methylation of glutamate residues located in the signal output region of the chemoreceptors. Methyl groups are added by the methyltransferase CheR, and removed by the methylesterase CheB (Fig. 2). Methylation of the chemoreceptors increases their ability to activate CheA. There is complex feedback between the CheA kinase and the CheB portion of methylation system. Specifically, CheA phosphorylates CheB but on a slower time scale than it does CheY. Phosphorylation of CheB activates its esterase activity and leads to demethylation of chemoreceptors, diminishing their capacity to activate CheA (32) (17, 33). A similar system appears to operate in at least some cytoplasmic chemoreceptors, based on the observation that these cytoplasmic chemoreceptors possess a methylation consensus in the adaptation
region (15, 34-36). In most cases, however, methylation has not been confirmed nor is the specific role of methylation known.

Although methylation is the best understood adaptation method, there are at least two additional adaptation systems utilized by chemoreceptors. These systems have only been well characterized in *B. subtilis*, so these proteins may function differently outside of that system (37). Nonetheless, it’s useful to keep in mind that there are additional non-methylation adaptation systems that may be used by cytoplasmic chemoreceptors. The first system utilizes the CheV proteins, which are chimeras of CheW plus a phosphorylatable receiver (REC) domain (38). CheV proteins allow additional chemoreceptor-kinase control, dictated by the variable phosphorylation state of the CheV REC domain. The second alternative system relies on the CheD and CheC proteins. Both of these proteins have enzymatic activity, but additionally perform adaptation via protein-protein interactions (39). CheD interacts directly with chemoreceptors at the methylation region, and can deamidate them, thereby increasing their ability to activate CheA (39). CheD availability is modulated by CheC (40). A model has been proposed whereby CheY-P causes CheC to sequester CheD from the chemoreceptors, inducing adaptation of the chemotaxis system (41). Many species that possess CheD lack CheC, which supports the possibility of variation and diversity in these adaptation systems. To date, no studies about the role(s) of CheV or CheD/C have been done with cytoplasmic chemoreceptors, so it is not yet known whether these types of receptors utilize these diverse adaptation systems.
Higher order structures of cytoplasmic chemoreceptors

Chemoreceptors exist as homodimers, clustered into trimers-of-dimers. Homodimers form extensive anti-parallel four-helix bundle coiled-coil interactions that have been seen in structures of cytoplasmic chemoreceptors (42) as well as in soluble cytoplasmic fragments of the Tsr and Tm1143c chemoreceptors (22, 43). These features are shared between different chemoreceptors, supporting that all chemoreceptors are likely to form homodimers (3).

Trimers-of-dimers are a higher order form of chemoreceptors that seem to be a universal state of chemoreceptors. Briegel et al. reported that Tm14, a cytoplasmic chemoreceptor of *T. maritima*, forms trimers-of-dimers in crystals (18). Recently, this work was extended to show that cytoplasmic chemoreceptors from two different species formed trimer-of-dimer structures in cells (44). Similar to transmembrane chemoreceptor arrays, cytoplasmic clusters contain trimers-of-receptor-dimers organized in 12-nm hexagonal arrays. In contrast to transmembrane arrays, however, cytoplasmic clusters comprise two CheA/CheW baseplates sandwiching two opposed receptor arrays. They further show that cytoplasmic fragments of normally transmembrane *E. coli* chemoreceptors form similar sandwiched structures in the presence of molecular crowding agents. Together these results suggest that the 12-nm hexagonal architecture is fundamentally conserved, and that sandwiching and crowding can replace the stabilizing effect of the membrane.
Chemoreceptors furthermore form extensive multi-chemoreceptor arrays, composed of many trimers-of-dimers connected by CheW and CheA at the chemoreceptor PIR (18, 45, 46). In accordance with these observations, CheW and CheA are essential for extensive multi-chemoreceptor array formation. To gain higher resolution information of the relative positions of each protein, a crystal structure of a cytoplasmic chemoreceptor— *Thermotoga* Tm14— was employed (18, 24). The high resolution structure of Tm14 in complex with CheA and CheW shows an unusual unzipped conformation of the chemoreceptors, into trimers of tetramers, with CheW and CheA engaged in a ring around the PIRs. This unusual unzipped conformation has not been observed in native situations and is believed to be a non-native feature. However, evolutionary analysis of sequence conservation and mutation patterns suggests that the contacts among the receptor, CheA and CheW displayed by the structure are relevant to the native chemosensory system (24). This structural model bears strong similarity to interactions predicted and observed for intact transmembrane chemoreceptors using cryotomography, supporting the accuracy of this idea (18, 47, 48).

**Cytoplasmic chemoreceptors are prevalent in Bacteria and Archaea**

Although there are several examples of cytoplasmic chemoreceptors, their prevalence was unknown. We therefore conducted an analysis of 8384 chemoreceptors in complete genomes of the SMART database (5). These chemoreceptors were defined by the presence of the MA domain, and included 207
from Archaea and 8177 from Bacteria (Fig. 3). Of the bacterial chemoreceptors, 14% (1129) were cytoplasmic, based on lack of transmembrane domains. Cytoplasmic chemoreceptors were more abundant in Archaea, with nearly 43% of all MCPs identified being cytoplasmic (Fig. 3). We found three Archaeal species with exclusively cytoplasmic receptors including *Thermofilum pendens Hrk 5* (one chemoreceptor), *Archaeoglobus fulgidus DSM 4304* (two chemoreceptors) and *Candidatus Methanoregula boonei 6A8* (four chemoreceptors). Interestingly, in the Archaea *Methanosphaerula palustris E1-9C*, 11 out 12 of its chemoreceptors are cytoplasmic. Most genomes, however, have transmembrane chemoreceptors plus some cytoplasmic ones.

Cytoplasmic chemoreceptors had previously been called Class IV chemoreceptors (49) and subsequently had been further divided into two different classes according to the length of the polypeptide N-terminal to the MA domain (7). Specifically, Class IVa chemoreceptors contain an N-terminal domain of at least 108 amino acids, whereas Class IVb contains a N-terminal region smaller than 108 amino acids. Many of the Class IVa receptors have predicted ligand binding motifs as described in the next section, but for the most part, we do not yet know the functional significance of long and short N-terminal domains. However, Class IVa are about 2-3-fold more abundant than Class IVb chemoreceptors (Fig. 3).

**Ligand-binding domains found in cytoplasmic chemoreceptors**
How cytoplasmic chemoreceptors sense their ligands and respond to them it is a major remaining question in bacterial chemotaxis. To help fill this gap, we used both SMART and PFAM to analyze the domain content of our set of 1129 cytoplasmic chemoreceptors obtained from SMART database. We determined the prevalence of individual domains in cytoplasmic chemoreceptors, the frequency of co-occurrences of domains, and the position of these domains relative to the MA domain. Roughly a quarter of cytoplasmic chemoreceptors had no identifiable domain other than the MA domain (Fig. 4). Several types of domains were found fairly commonly in cytoplasmic chemoreceptors, including domains that sense small molecules, or bind redox-active cofactors and thus are predicted to drive a tactic response to redox or cellular energy levels. Each of these domains is discussed below. Interestingly, some domains were found N-terminal to the MA, as is typical in transmembrane chemoreceptors, while others were found C-terminal to the MA domain. The significance of this positioning, however, is not yet known. Additionally, we do not yet know whether ligand sensing at these domains controls the conformation or availability of the PIR as has been suggested for transmembrane chemoreceptors (17, 24).

PAS domains are by far the most abundant sensing domains annotated in cytoplasmic chemoreceptors, occurring in 47% of analyzed proteins, and in most cases several PAS domains were found per protein (Fig. 4). PAS domains are found in many signaling proteins, from human to bacteria, where they function to bind small molecules (50). Many PAS-domain proteins bind a small molecule that in turn detects
the signal. For example, heme binding confers oxygen detection, and FAD binding allows redox detection. In all cytoplasmic chemoreceptors, the PAS domain was located N-terminal to the MA domain (Table 1). A handful of PAS-containing cytoplasmic chemoreceptors have been implicated in energy taxis, a motility-based response for positioning cells in an optimum microenvironment for energy generation (12, 15) (Table 1).

The second most common domain is the CZB domain, found in about 8% of all cytoplasmic chemoreceptors (Fig. 4). In all cases, the CZB domain was C-terminal to the MA domain (Table 1). CZB was first identified in the \textit{H. pylori} TlpD chemoreceptor (51). It consists of a set of conserved histidines and one cysteine, and binds zinc (51). The CZB domain is also found in non-chemoreceptor proteins including the diguanylate cyclase DgcZ/YdeH. In that protein, it responds to zinc and exhibits zinc-dependent allostERIC control on the associated domain (52). The CZB domain was typically the sole identifiable domain in the chemoreceptors probed, although in a few cases a protoglobin domain was also found.

The third most common sensing domain found in cytoplasmic chemoreceptors is the protoglobin domain, which occurred in 7% of proteins analyzed (Fig. 4). The protoglobin domain is a member of the hemoglobin superfamily, and binds heme. The \textit{B. subtilis} cytoplasmic chemoreceptor HemAT has a heme-bound protoglobin domain that drives aerotaxis (14). The protoglobin domain was found exclusively N-terminal to the MA domain (Table 1), and was typically the sole identifiable domain in
chemoreceptors with rare exceptions containing it in addition to the C-terminal CZB or PilZ domains.

Several other domains were found in about 1% of cytoplasmic chemoreceptors. Several were N-terminal to the MA domain, including the FIST, GAF, Diacid_REC, and PocR domain. Two were C-terminal to the MA domain, the SBP_bac_5, and PilZ domains. One was found either N- or C-terminally placed, the Cache_1 domain (Fig. 4, Table 1). The first N-terminal domain, FIST, has been postulated to bind small ligands, such as amino acids, based on the chromosomal proximity of FIST-encoding genes to those coding for proteins involved in amino acid metabolism and transport (53). Interestingly when this domain is present the HAMP domain is not, and this domain was only detected in proteobacteria. Another N-terminal domain is GAF. The GAF domain is named based on the proteins it was originally found in: cGMP-specific phosphodiesterases, adenylyl cyclases and FhIA (54, 55). The GAF domains were often found in multiple copies per chemoreceptor (Table 1). The Diacid_rec domain is found in several proteins characterized as carbohydrate diacid regulators (56). It is always located N-terminal to the MA domain (Table 1). Lastly, the PocR domain is a variant of the PAS domain, and predicted to bind hydrocarbons (57). When present, it was the only annotated domain outside of the MA domain, and always N-terminal to it (Table 1).

The Cache_1 domain was the only domain found located either N- or C-terminal to the MA domain (Table 1). Cache_1 is a typically extracellular domain that is predicted to have a role in small-molecule recognition in a wide range of proteins,
including the animal dihydropyridine-sensitive voltage-gated Ca\(^{2+}\) channel alpha-2δ subunit, and various bacterial chemotaxis receptors (58). The name Cache comes from CAlcium channels and CHEmotaxis receptors.

Lastly, the SBP_bac_5 and PilZ domains were found exclusively C-terminal to the MA domain (Table 1). The SBP_bac_5 domain was originally characterized as a extracellular domain that binds small molecules and participates in high affinity transport (59). Members include proteins such as the periplasmic oligopeptide-binding proteins (OppA), the periplasmic murein peptide-binding protein of *E. coli* (MppA), the periplasmic nickel-binding protein (NikA) of *E. coli*, and the heme-binding lipoprotein (HbpA or DppA) from *H. influenza*. In cytoplasmic chemoreceptors, SBP-bac_5 was found only in Clostridial spp. The PilZ domain, in comparison, binds the bacterial second messenger cyclic-di-GMP (60). C-di-GMP is associated with the regulation of biofilm formation, the control of exopolysaccharide synthesis, flagellar- and pili-based motility, gene expression, and other aspects of bacterial physiology in diverse bacteria (60). The PilZ domain was found in soluble chemoreceptors combined with a variety of other domains, e.g. PAS and protoglobin N-terminal to the MA domain, and CZB C-terminal to the MA domain. PilZ domains were only found in cytoplasmic chemoreceptors from alpha-proteobacteria. Recently, the Alexandre group showed that the PilZ domain at the C-terminal end of a transmembrane chemoreceptor bound c-di-GMP and modulated chemotactic signaling, supporting that ligand binding at C-terminal domains can influence chemoreceptor function (61).
The diversity of domains in cytoplasmic chemoreceptors suggests that these proteins sense and integrate a wide group of intracellular signals. Furthermore, these domains may be divided presumptively into those that sense redox, such as the PAS domain, those that sense oxygen such as the protoglobin domain, and those that respond to small molecules such as PAS and PilZ. It remains unknown how cytoplasmic chemoreceptors convert signals into a tactic response.

**Subcellular localization of cytoplasmic chemoreceptors**

Transmembrane chemoreceptors localize predominantly at or around the vicinity of the cell pole, but also can be found in the lateral membrane (62). In both locations, they form extensive multi-chemoreceptor arrays, although the polar ones tend to be larger (46). Cytoplasmic chemoreceptors, in contrast, appear to have a wider subcellular distribution, ranging from colocalization with transmembrane chemosensory clusters, diffuse cytoplasmic distribution, and cytoplasmic cluster localization, as described in more detail below. One point to consider in analyzing chemoreceptor localization is whether a particular microbe has only one, or more than one set of chemotaxis signaling proteins. Many bacteria have multiple sets of chemotaxis signaling proteins that can act during distinct conditions or even function in processes such as twitching motility or transcription (32) (Table 2). Presumably if there is only one set of signaling proteins, all chemoreceptors must share these and therefore would be more likely to be colocalized. Localization of cytoplasmic
chemoreceptors is by far the best-studied attribute of these proteins, so below we summarize a significant body of work in this area.

**Cytoplasmic chemoreceptors with nearly exclusive localization at the pole with the transmembrane chemoreceptor arrays**

Some cytoplasmic chemoreceptors are found predominantly at the pole of the cells. One such protein is *Pseudomonas aeruginosa* McpS (we use the genus/species abbreviation as a subscript to help differentiate the many similarly named chemoreceptors, e.g. McpS\textsubscript{PA}) (Table 2, Fig. 5). The *P. aeruginosa* PAO1 genome encodes four chemotaxis pathways with twenty six chemoreceptors, and three of which lack transmembrane regions and are therefore predicted to be soluble (34). McpS\textsubscript{PA} contains an N-terminal PAS domain and predicted methylation sites, but it is not yet known what it responds to or whether it is methylated (34). McpS\textsubscript{PA} localizes to the pole along with several other chemoreceptors, based on immunofluorescence (34). Increasing McpS\textsubscript{PA} levels disrupted the polar localization of transmembrane chemoreceptors but McpS\textsubscript{PA} was still observed at the pole. Increased levels of McpS\textsubscript{PA} also caused decreased motility in the soft agar chemotaxis assay. There have been no studies on the proteins required for McpS\textsubscript{PA} polar localization. Taken together, this cytoplasmic chemoreceptor appears to reside in a polar complex with transmembrane chemoreceptors and this macromolecular structure is sensitive to the concentration of McpS\textsubscript{PA}, perhaps through displacing interactions between receptors or signaling proteins (34).
IcpA and McpY of *Sinorhizobium meliloti* (IcpASM and McpYSM) also are found nearly exclusively at the pole (Table 2, Fig. 5). IcpASM and McpYSM are the two cytoplasmic chemoreceptors in the nine-chemoreceptor/two pathway system of *S. meliloti*. IcpASM contains a predicted protoglobin domain at its N-terminus, and McpYSM contains two PAS domains in tandem at its N-terminus; the input signals of these two, however, are not known (63). IcpASM and McpYSM are both located at the pole, based on analysis of GFP fusion proteins (63). They furthermore colocalized with the transmembrane chemoreceptor McpX and fluorescently tagged CheA, suggesting they are part of a large chemoreceptor signaling complex. Interestingly, McpYSM and IcpASM required different sets of proteins to localize to the pole. Specifically, IcpASM was not affected by loss of the other chemoreceptors, CheA or both CheW1 and CheW2, while McpYSM polar localization decreased without these proteins (63). Fractionation of *S. meliloti* cells followed by western blot revealed that McpYSM was found in the cytosolic fraction, while IcpASM was present exclusively in the membrane fraction. This membrane association was probed by mild treatment of membrane fractions with detergent, after which IcpASM still showed association with the membrane fraction (63). The localization of IcpASM is intriguing, as it suggests that cytoplasmic chemoreceptors may interact at the pole with partners apart from chemoreceptor arrays and chemotaxis signaling proteins.

*B. subtilis* HemAT is yet another example of a polarly-localized cytoplasmic chemoreceptor (Table 2, Fig. 5). *B. subtilis* encodes one chemotaxis system with ten chemoreceptors, two of which are soluble: HemAT and YfmS (64). HemAT mediates
aerotaxis via oxygen binding through a coordinated heme group at its N-terminus within a protoglobin domain (65). The ligand of YfmS is unknown; the sole annotated domain in this protein is the MA domain. The localization of YFP-tagged HemAT and YfmS was studied by fluorescent microscopy, and both localize primarily to the pole, similar to the transmembrane receptor McpB (66). HemAT is also found in *H. salinarum*, but its localization in this microbe is not known. It has been suggested that these receptors may be part of the large membrane chemoreceptor array and undergo conformational changes upon ligand binding that propagates to neighboring receptors. This hypothesis could explain the fact that aerotaxis is more efficient when HemAT is not the sole receptor present (64).

**Cytoplasmic chemoreceptors with both polar colocalization with transmembrane receptor clusters and diffuse cytoplasmic localization**

The localization of some cytoplasmic chemoreceptors seems to be multifaceted, present in both soluble and membrane fractions. This is the case of *Azospirillum brasilense* AerC (AerC\textsubscript{AB}) and *Helicobacter pylori* TlpD (TlpD\textsubscript{HP}) (Table 2, Fig. 5). *A. brasilense* codes for four chemotaxis pathways, with 51 chemoreceptors. AerC\textsubscript{AB} is a cytoplasmic chemoreceptor that possesses two N-terminal PAS domains and a C-terminal MA domain (12). AerC\textsubscript{AB} binds FAD at each of two N-terminal PAS domains and is postulated to sense intracellular redox—via the bound FAD— and direct an oxygen repellent response to support nitrogen fixation (12). AerC\textsubscript{AB}-YFP localization was determined by fluorescence microscopy,
and found to be both diffuse in the cytoplasm and localized in foci at the pole, depending on growth conditions (Fig. 5) (12). In conditions promoting nitrogen fixation, as opposed to growth in the presence of ammonia, AerC_{AB} was predominantly at the pole (12). In contrast, growth in the presence of ammonia created dimmer polar foci. Polar localization required proteins of the Che1 operon (CheA1, CheW1, CheB1, CheY1) (12, 67) as well as specific nitrogen-fixing growth conditions. FAD-binding was not required for AerC_{AB} polar localization, but did alter migration in a soft agar assay and abolish aerotaxis in oxygen gradients (12).

Nitrogen fixation is an energetically intensive process, and the enzyme responsible for splitting N\textsubscript{2}, nitrogenase, is inhibited by oxygen. In this manner AerC_{AB} would help \textit{A. brasilense} restrict nitrogen fixation to low oxygen environments.

TlpD\textsubscript{HP} of \textit{H. pylori} is found both at the pole with the other chemoreceptors, and throughout the cytoplasm (11). \textit{H. pylori} possesses three transmembrane chemoreceptors in addition to TlpD\textsubscript{HP}, all part of one chemotaxis pathway. TlpD\textsubscript{HP} binds zinc at its conserved C-terminal CZB zinc binding domain (51). The direct signal of TlpD\textsubscript{HP} is unknown, but the receptor has been reported to mediate a repellent response to inhibitors of the electron transport chain (11). TlpD\textsubscript{HP} has been detected in cytoplasmic and membrane portions of fractionated cells in approximately equivalent amounts (11). Membrane localization of TlpD\textsubscript{HP} depends on the transmembrane chemoreceptors, CheA, CheW, and CheV1 when assayed by subcellular fractionation and western blotting (K.D. and K.M.O., unpublished). This finding suggests that
TlpD may exist in a complex with the other chemotaxis proteins, but also is able to reside in the cytoplasm.

**Cytoplasmic chemoreceptors that form clusters distinct from the transmembrane polar receptors**

The study of *Rhodobacter sphaeroides* chemotaxis has provided an intriguing case of cytoplasmic chemoreceptors that localize exclusively internal to the cytoplasm, away from the polar transmembrane chemoreceptors (Table 2, Fig. 5). *R. sphaeroides* possesses three chemotaxis pathways with 13 chemoreceptors, including four that lack predicted transmembrane domains. Two of these, TlpC<sub>Rs</sub> and TlpT<sub>Rs</sub>, have been studied. TlpC<sub>Rs</sub> and TlpT<sub>Rs</sub> localize to a cytoplasmic cluster (36) that contains a repertoire of chemotaxis signaling proteins (68). Localization of the cytoplasmic cluster is cell-cycle dependent (36) and duplication of the cluster occurs prior to cell division, allowing segregation of each duplicated cluster to a daughter cells. Mechanisms controlling the duplication of the cluster remain unknown. Clusters contain TlpC<sub>Rs</sub>, TlpT<sub>Rs</sub>, CheA<sub>3</sub>, CheA<sub>4</sub>, CheW<sub>4</sub>, CheR<sub>3</sub>, and a ParA homolog called PpfA (all of which are encoded in the same *cheOp3* operon) (68). Cluster formation relies on the presence of TlpT<sub>Rs</sub> and CheW<sub>4</sub> (69), while the positioning and segregation of the cluster relies on the presence of the N-terminus of TlpT<sub>Rs</sub> and the activity of PpfA (70, 71). Cells lacking *ppfA* do not form a second cytoplasmic cluster during cell division and only one daughter inherits a cluster as a result. Daughter cells lacking a cluster are nonchemotactic until they synthesize new cluster components.
PpfA interacts with TlpT$_{RS}$ via its N-terminus, which is thought to stimulate PpfA ATPase activity. Additional PpfA interactions with chromosomal DNA may fix the cluster localization and allow the cluster to be segregated during cell division with chromosomal DNA (72). This localization mechanism may be widespread, given that many cytoplasmic chemoreceptors are encoded in the same operon as a protein with PpfA homology (46) and positioning of chemosensory arrays in Vibrio also seems to depend on the presence and activity of a ParA-like protein, ParC (73, 74).

### Cytoplasmic chemoreceptors with dynamic cytoplasmic localization

The bacterium *Myxococcus xanthus* presents an interesting case for soluble chemoreceptors in social behaviors and developmentally-regulated processes. This gram-negative soil bacterium demonstrates social behavior in coordinating movement during predatory hunting and also undergoes fruiting body formation during nutrient limitation, both of which are controlled in part by chemosensory systems (10, 75). *M. xanthus* contains eight chemotaxis pathways that perform a variety of functions. It possesses two soluble chemoreceptors, FrzCD and Mcp7 (Table 2). FrzCD controls vegetative swarming and starvation-induced aggregation prior to fruiting body formation (10), while Mcp7 regulates coupling between aggregation and sporulation (76). FrzCD shows a dynamic localization that appears to be sensitive to cell-cell contacts and is organized in helical filaments spanning the length of the cell (Table 2, Fig. 5). *M. xanthus* employs two forms of gliding motility that both rely on cellular reversals but are not flagellar-based (10). The Frz pathway, including FrzCD, along
with chemotaxis signaling proteins including the FrzE histidine kinase controls the frequency of reversals. Mutations in the Frz pathway alter the frequency of reversals. Fluorescent microscopy revealed that FrzCD localizes in helical filaments that cover the cell length, and that FrzCD is dynamic within these structures, continuously changing its position, number, and intensity (77, 78). FrzCD in one cell transiently aligns with that in another, as cells make side-to-side contacts. An intact Frz signaling pathway is required for FrzCD localization, as a \textit{frzE} mutant showed more diffuse FrzCD clusters that were also less abundant than in wild type. Clusters within the \textit{frzE} mutant were still dynamic though, constantly changing their number, position, and intensity. FrzCD is proposed to operate as a regulator of reversal frequency through its tracking along a cytoskeletal filament from one pole of the cell to the other, and cell-cell contacts increase the frequency at which FrzCD stimulates reversals (77).

FrzCD appears to respond to the presence of attractants and repellents through levels of methylation, and the modification appears to have consequences on fruiting body formation (10). The second \textit{M. xanthus} cytoplasmic chemoreceptor, Mcp7, displays subpolar localization at either one or both poles, and was the most mobile of the \textit{M. xanthus} chemoreceptors (78). This chemoreceptor is encoded in the \textit{che7} locus with cognate copies of CheA, CheW, CheY, CheB, CheR and the accessory protein Cpc7. Mcp7 mutants prematurely sporulate before cell populations aggregate. Mcp7 activity, specifically the phosphorylation of the CheY encoded in the \textit{che7} locus (CheY7), negatively regulates sporulation. Phosphorylated CheY7 physically interacts with Cpc7 and this complex negatively regulates sporulation until cell
aggregation is complete (76). These two soluble chemoreceptors provide an important reminder that not all chemosensory modules are strictly involved with motility, and may provide a connections between sensation of a cell's environment and developmentally controlled behaviors or processes.

**Cytoplasmic chemoreceptors with unknown cytoplasmic localization**

Several cytoplasmic receptors have been studied only by subcellular fractionation, and clearly localize to the cytoplasmic fraction of cells. It is not known, however, where within the cell (e.g. polar, clustered) these chemoreceptors reside. *Caulobacter crescentus* McpB (McpB<sub>CC</sub>) and *Halobacterium salinarum* Car are examples of this class of cytoplasmic chemoreceptors (Table 2). The *C. crescentus* chemotaxis system consists of two chemosensory pathways of nineteen predicted chemoreceptors, with McpB<sub>CC</sub> being one of five cytoplasmic chemoreceptors. McpB<sub>CC</sub> contains a predicted N-terminal protoglobin domain, and appears to be methylated based on the migration of the protein in strains lacking either CheB or CheR (35). The localization of McpB<sub>CC</sub> was determined by cellular fractionation to be exclusively in the cytoplasmic fraction (35). The presence of an N-terminal protoglobin domain, similar to HemAT, suggests that McpB<sub>CC</sub> may play a role in aerotaxis, although this role has not been validated (35).

*H. salinarum* is a halophilic archaea encoding one chemosensory system with eighteen chemoreceptors, six of which are predicted to be cytoplasmic. Car is one of these cytoplasmic chemoreceptors and possesses a PAS domain at its N-terminus.
Strains lacking Car lose the ability to respond to arginine in a soft-agar chemotaxis assay, but retain the ability to respond to other amino acids, suggesting that this cytoplasmic chemoreceptor mediates a tactic response to arginine (15). Car localizes to the cytoplasmic fraction, based on subcellular fractionation (15). Car is believed to monitor the concentrations of arginine in the cytoplasm, possibly as part of a metabolism-dependent motility response (15).

**Conclusions**

Cytoplasmic chemoreceptors are common amongst microbes of the Bacterial and Archaeal domains, comprising 14% and 43% of chemoreceptors, respectively. Existing studies, although limited, suggest that cytoplasmic chemoreceptors share the same key structural features as transmembrane chemoreceptors: homodimeric trimer-of-dimer configuration, organized into 12 nm hexagonal arrays. However, the locations of the cytosolic chemoreceptor arrays vary. Certain cytoplasmic chemoreceptors have been observed to localize to the pole with transmembrane chemoreceptors, and signaling could occur through interactions with other receptors in that array. Other chemoreceptors show diffuse cytoplasmic and polar localization, suggesting trafficking between these two locations. Localization of *R. sphaeroides* cytoplasmic chemoreceptors in internal clusters offers the most well studied example of solely cytoplasmic chemoreceptors, and these receptors seem to be engaged in a complex that is capable of signaling independently of transmembrane signaling centers. The case of FrzCD offers an interesting alternative to other cytoplasmic chemoreceptor
signaling as part of a dynamic structure that operates along the length of *Myxococcus*. This paradigm of signaling may not be unique to this bacterium and instead may offer insight into cytoplasmic chemoreceptors in contexts outside of flagellar-driven chemotaxis. The driving force for distinct cellular locations is not yet known. It may be the location of the signal, some aspect of the signal transduction, another factor, or some combination of these that determines whether a receptor is cytoplasmic or polar, static or dynamic. Cytoplasmic chemoreceptors bear diverse signal input domains, which are mostly N-terminal to the MA domain. Similar to transmembrane receptors, the most common signal input domain is PAS, and a variety of other N-terminal domains exist. It is also common, however, for cytoplasmic receptors to have C-terminal domains. The most common of these is the recently identified CZB domain, found in 8% of all cytoplasmic chemoreceptors. The reason that some domains are N-terminal, while others are C-terminal to the MA domain, is not yet known. Some cytosolic chemoreceptors do not seem to contain an input signal region in their sequence, suggesting that these chemoreceptors might play some sort of structural role instead of receiving signals, or that there as-yet unidentified input domains. Many open questions about this interesting group of chemoreceptor proteins remain. Future studies of these chemoreceptors will undoubtedly generate significant insights into the fundamental properties of signal recognition and transduction.

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Figure 1. Domain structure of transmembrane and soluble chemoreceptors.

Domain structure of transmembrane (left) and soluble chemoreceptors (right). Only one type of transmembrane receptor is shown, the so-called Class I with two transmembrane regions and a periplasmic ligand-binding region as these are the best studied type of transmembrane receptor. Different transmembrane receptor topologies have been described (6, 7). * indicates domain not always present.
Chemoreceptors exist in a ternary complex with the CheW coupling protein and the CheA kinase. Chemoreceptor in the absence of ligand activates CheA, which in turn phosphorylates the response regulator CheY. Phosphorylated CheY interacts with the flagellar motor and affects the direction of motor rotation.

CheR is a methyltransferase that acts upon conserved glutamates in the adaptation region of the receptors; methylation at these sites enhances CheA activation by chemoreceptors. CheB is a methylesterase that catalyzes the
removal of methyl groups. Its activity is enhanced by phosphorylation via CheA. Additional adaptations proteins, CheV, CheC and CheD are described in the text.
Figure 3. Abundance of cytoplasmic chemoreceptors

Abundance of cytoplasmic chemoreceptors in Archaea and Bacteria are shown in the left-side pie charts. Chemoreceptors were identified from complete genomes in the SMART database (5) as proteins with annotated MA domains, and further narrowed by identifying those that had no transmembrane domains, to create a set of 1217 cytoplasmic chemoreceptors. The right side charts show results from analyzing only the cytoplasmic chemoreceptors, manually, to determine which
are Class IVa, those with N-terminal domains 108 amino acids or longer, or Class IVb, those with N-terminal domains shorter than 108 amino acids.
Figure 4. Most common domains found in soluble chemoreceptors.

The set of cytoplasmic chemoreceptors (1217 proteins) were identified as described in the Fig. 3 legend, and used as in input for both the SMART (5) and PFAM databases (79) to identify all additional annotated domains. Domains with several subtypes were combined as follows: GAF contains GAF and GAF_2; FIST contains FIST and FIST_C; PAS contains PAS_3, PAS_4, PAS_8 and PAS_9; CACHE contains CACHE_1 and CACHE_2. Annotated domains with 3 or less hits are not shown here, but are included in Table 1. Brief descriptions of domain functions are described in Table 1.
Figure 5. Examples of the diverse subcellular locations of cytoplasmic chemoreceptors.

The top panel shows chemoreceptors that are primarily polar, as distinct bright polar spots. (A) B. subtilis HemAT visualized as HemAT-YFP. Image courtesy of George Ordal, reproduced with permission. Similar images are published in (64). (B) B. subtilis YfmS visualized as YfmS-YFP. Image courtesy of George Ordal, reproduced with permission. Similar images are published in (64). (C) S. meliloti IcpA-EGFP. Reprinted from (63), with permission. (D) P. aeruginosa McpS, visualized using immunofluorescence with anti-His antibody to recognize His-McpS. Reprinted from (34), with permission. The bottom panels show chemoreceptors that are (i) diffuse or polar under different environmental conditions; (ii) that reside in an internal cytoplasmic cluster that is distinct from...
the polar transmembrane chemoreceptors; or (iii) dynamic. (E) A. brasilense
AerC, visualized as AerC-YFP, under different growth conditions. Image courtesy
of Gladys Alexandre, reproduced with permission. Similar images are published
in (12). (F) E) R. sphaeroides TlpC visualized as TlpC-GFP Image courtesy of
Judith Armitage, reproduced with permission. Similar images are published in
(68). (G). M. xanthus FrzCD, visualized with anti-FrzCD, and bacterial cells
stained with FM4-64 Reprinted from reference (77) with permission.
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Table 1. Domains identified in cytoplasmic chemoreceptors.

NH3 indicates the percent found on the amino terminal side of the MA domain, with the total number in parenthesis. COOH indicates the percent found on the carboxy terminal side of the MA domain, with the total number in parenthesis. Class IVa are cytoplasmic receptors with N-terminal domains 108 amino acids or larger, while Class IVb have N-terminal regions smaller than 108 amino acids (7). Database indicates whether the domains were identified by SMART or PFAM.

The following domains were included in this table: PAS-domain proteins interact and respond to small molecules through bound cofactors including FAD and heme groups (50). CZB domains bind zinc through conserved histidines and cysteines and in some cases are known to sense zinc, although this is not confirmed for other CZB proteins (51, 52). PilZ domains bind and mediate a response to the secondary messenger c-di-GMP (80). Protoglobin domains coordinate a heme group and can respond to oxygen (81). FIST domains are proposed to bind small ligands including amino acids (53). GAF domains interact and respond to 3’, 5’ cyclic guanosine monophosphate (cGMP) (55, 82). The Diacid_rec domain is proposed to bind and respond to carbohydrates (56). The PocR domain is a variant of the PAS domain, and is predicted to bind hydrocarbons (57). Cache_1 and SBP_bac_5 domains are predicted to have a role
in small-molecule recognition (59). PBPb domains are high affinity small molecule binding domains characterized in ABC transporters (59). Hemerythrin bind and respond to oxygen through coordinated iron atoms (83, 84), and have also been reported to mediate responses to nitric oxide (85). NMT1 domains have been characterized in their role in the synthesis of the pyrimidine moiety of thiamine and are regulated by thiamine (86, 87). The HNOB domain coordinates heme and is predicted to interact with and respond to gaseous ligands including nitric oxide (88). CBS domains bind and respond to molecules with adenosyl groups such as AMP and ATP, or s-adenosylmethionine (89). The NIT domain binds and responds to nitrate and nitrite (“The NIT domain: a predicted nitrate-responsive module in bacterial sensory receptors,” 2003). The CHASE3 domain has been characterized as an extracellular sensory domain, although the perceived ligand is unknown (90). Bac_globin coordinates heme as a prosthetic group and binds oxygen reversibly (14, 91). DUF3365 domains are present in bacteria but are functionally uncharacterized.
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<td>PAS2 (2)</td>
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<td>ND</td>
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<td>Protoglobin</td>
<td>P</td>
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<td>Protoglobin</td>
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<td>CC</td>
<td>No</td>
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<td>D, F</td>
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Table 2. Characteristics of well-studied cytoplasmic chemoreceptors.

Domains are given as either amino-terminal (NH3) or carboxy-terminal (COOH) relative to the MA domain. Localization is given as: P, polar; P/C, polar and cytoplasmic; CC, cytoplasmic clusters; D, cytoplasmic and dynamic; F, filaments; C, cytoplasmic but exact location unknown; ND, not determined. Methylation consensus indicates whether methylation consensus sequences have been identified in the protein, while verified indicates whether these were experimentally confirmed (yes) or not yet determined (ND). Number of chemotaxis operons and encoded CheA proteins was obtained from (1) Porter et al. (32), (2) Russell et al. (61), the MIST Database (96) or MicrobesOnline (97) as indicated. This number is usually estimated based on the number of encoded CheA proteins.
The *Helicobacter pylori* CZB cytoplasmic chemoreceptor TlpD forms an autonomous polar chemotaxis signaling complex that mediates a tactic response to oxidative stress

Kieran D. Collins, Tessa M. Andermann, Jenny Draper, Lisa Sanders, Susan M. Williams, Cameron Araghi, and Karen M. Ottemann
Abstract

Cytoplasmic chemoreceptors are widespread among prokaryotes but are far less understood than transmembrane chemoreceptors, despite being implicated in many processes. One such cytoplasmic chemoreceptor is Helicobacter pylori TlpD, which is required for stomach colonization and drives a chemotaxis response to cellular energy levels. Neither the signals sensed by TlpD nor its molecular mechanisms of action are known. We report that TlpD functions independently of the other chemoreceptors. When TlpD is the sole chemoreceptor, it is able to localize to the pole, and recruits CheW, CheA, and at least two CheV proteins to this location. It loses normal membrane association that appears to be driven by interactions with other chemoreceptors as well as CheW, CheV1, and CheA. These results suggest that TlpD can form an autonomous signaling unit. We further determined that TlpD mediates a repellent chemotaxis response to conditions that promote oxidative stress including iron, hydrogen peroxide, paraquat, and metronidazole. Lastly, we find that all tested H. pylori strains express TlpD, whereas other chemoreceptors are variably present. Our data suggest a model in which TlpD coordinates a signaling complex that responds to oxidative stress and may allow H. pylori to avoid stomach areas with high concentrations of reactive oxygen species.
Introduction

Chemoreceptors operate at the front line of the bacterial chemotaxis response, sensing signals and initiating attractant or repellent responses. Chemoreceptors can be divided into two classes based on whether they reside in the inner membrane or are soluble cytoplasmic proteins. The cytoplasmic class presumably senses signals that occur intracellularly. Cytoplasmic chemoreceptors are relatively understudied, but represent 14% of Bacterial and 43% of Archaeal chemoreceptors (98). As a group, cytoplasmic chemoreceptors are reported to mediate tactic responses to diverse conditions, including to cellular energy stores (11, 12), redox conditions (13, 14), and metabolites (15), although significant gaps remain in our understanding of what they sense and how they function (98). One such cytoplasmic chemoreceptor is Helicobacter pylori’s TlpD. TlpD plays a critical role during H. pylori’s infection of the mammalian stomach (8, 9). In wild-type mice or gerbils, mutant strains of H. pylori that lack TlpD display colonization defects during early stages of infection that are more severe than those of mutant strains that lack any other individual chemoreceptor (8, 9).

TlpD, which was previously referred to as HP0599 or HylB, does not have transmembrane domains and resides in both soluble and membrane-associated subcellular fractions (11). TlpD possesses the canonical chemoreceptor domain, called MA or MCP-signal, which typically interacts with the signal transduction proteins CheW and CheA. TlpD additionally has a C-terminal zinc-binding
domain called CZB (11, 12, 51, 52), which is the second most prevalent domain found in cytoplasmic chemoreceptors (13, 14, 98). Numerous cytoplasmic chemoreceptors contain a CZB domain and thus share a domain structure with TlpD (51, 98). Chemoreceptors like TlpD are widespread, found in many bacterial genera, including gastric and non-gastric Helicobacters. CZB domains have been shown to function as zinc-responsive allosteric regulators in diguanylate cyclases (15, 52), but their function in TlpD and other chemoreceptors is not known. Information relating to the regulation of transcription or translation of tlpD is sparse. There is some evidence that tlpD, as well as tlpB, are upregulated in gerbil infections. The regulatory mechanisms, however, are not yet known (99).

The H. pylori chemotaxis signal transduction system is reasonably well understood. It contains the typical core signaling proteins: the CheA kinase, the CheW coupling protein, and the CheY response regulator, all of which are required for chemotaxis (6, 98, 100). H. pylori encodes three transmembrane chemoreceptors called TlpA (HP0099), TlpB (HP0103), and TlpC (HP0082) (8, 9, 100, 101). H. pylori also possesses a handful of accessory chemotaxis signaling proteins that supplement the core ones. In addition to CheW, H. pylori expresses three coupling proteins called CheV1, CheV2, and CheV3. CheV proteins combine the CheW coupling domain with an additional phosphorylatable response regulator domain (REC) (8, 9, 38). H. pylori CheV proteins all function in chemotaxis, but their exact roles are unclear (102-105). In B. subtilis, CheV
modulates CheA activity depending on the covalent modification state of the chemoreceptors and the phosphorylation state of CheV (11, 38, 103, 105, 106). *H. pylori* also encodes two more proteins that are critical for chemotaxis. The first is a CheZ phosphatase, called CheZ$_{HP}$, capable of dephosphorylating CheY, CheA, and CheV2 (107). The second is a poly-glutamate-rich protein called ChePep. ChePep contains a putative response regulator domain and is conserved in Epsilon proteobacteria (108).

Chemotaxis proteins in *H. pylori* are organized into two distinct polar complexes. One complex contains the chemoreceptors, CheA, CheV1 (109) while the other contains CheZ$_{HP}$ and ChePep (109). CheZ$_{HP}$ and ChePep physically interact with each other, and form a distinct complex at the cell pole that is independent of the one formed by the chemoreceptors and other signaling proteins (109). It is not known why *H. pylori* organizes its chemotaxis proteins into two distinct complexes. Chemotaxis appears to have the greatest impact on colonization within the first three weeks of infection, consistent with the idea that this signal transduction system plays a particularly important role in early *H. pylori* colonization (8, 104, 110-114). *tlpD* mutants are substantially affected in their ability to colonize the stomach at early time points (8, 9), presumably because *tlpD*-deficient strains are unable to sense signals that are generated by exposure to the host environment and are therefore unable to locate a preferred niche in the stomach. At later time points, colonization defects due to chemotaxis and TlpD are less
apparent (102, 104). TlpD is reported to mediate a tactic response to electron transport chain inhibitors in vitro, a behavior ascribed to energy taxis (11), but it is not yet clear what signal TlpD responds to during such treatment. Studies have shown that H. pylori does not display aerotaxis, so a direct oxygen response appears unlikely (102). TlpD has no identifiable domains that would bind FADH or FMN at either its N- or C- terminus, but it has been reported to interact with iron to a small degree (51). Given the important role TlpD plays during host colonization, we sought to understand the function of this chemoreceptor in greater detail. Our results suggest that TlpD is able to operate independently of transmembrane chemoreceptors and coordinates a chemotaxis complex at the pole that contains at least CheA, CheW, CheV1, and CheV3, confirming it is a bona fide chemoreceptor. Furthermore we report that TlpD mediates a repellent response to conditions that promote oxidative stress, including iron, hydrogen peroxide, paraquat, and metronidazole. We propose a model in which TlpD-mediated repellent responses to oxidative stress are vital to H. pylori avoiding mammalian niches with elevated oxygen, such as near to the epithelial surface (115, 116), or reactive oxygen species (ROS) that arise from sites of inflammation (117, 118).
Methods

Bacterial strains and culture conditions.

_H. pylori_ strains mG27, G27, or SS1 were used for strain construction and experimental studies (Table 1). Other _H. pylori_ strains were used to analyze chemoreceptor diversity as noted in Table 1. _H. pylori_ was cultured on either Columbia horse blood agar (CHBA), Brucella broth with 10% Fetal Bovine Serum (FBS, Life Technologies) (BB10), or 0.8X HAMS-F12 with 10% FBS (HAMS10). CHBA consisted of Columbia agar (BD) with 5% defibrinated horse blood (Hemostat Labs, Davis, CA), 50 µg/ml cycloheximide, 10 µg/ml vancomycin, 5 µg/ml cefsulodin, 2.5 Units/ml polymyxin B and 0.2% (w/v) β-cyclodextrin. Cultures were incubated at 37°C, under 5-7% O₂, 10% CO₂ and balance N₂. For selection of _H. pylori_ mutants, sucrose was used at 10% (w/v), chloramphenicol was used at 5-10 µg/ml, and kanamycin was used at 15 µg/ml. For selection of plasmid-bearing _E. coli_, chloramphenicol was used at 20 µg/ml, kanamycin at 30 µg/ml, and ampicillin at 100 µg/ml. All media, chemicals and antibiotics were from BD, Fisher, Sigma, Gold Biosciences, or ISC Bioexpress.

Creation of mG27 mutants

Construction of the ∆cheA, ∆tlpA, ∆tlpB, ∆tlpC and ∆tlpD mutants has been described (119) (Table 1). Construction of the multiple receptor mutants has been described recently (109). In brief, mG27 ∆tlpA or ∆tlpB were used as starting strains, and then _tlpC_ was eliminated by transforming with plasmid pK0150, or _tlpD_ was eliminated by transforming with ∆_tlpD::cat_ chromosome.
from strain KO1006. In all cases, the resulting mutations were verified using PCR.

**Motility and Chemotaxis Analysis**

Soft agar assays were performed using Brucella broth, 2.5% vol/vol FBS, and 0.35% Bacto agar, as previously described (112). Soft agar plates were incubated under microaerobic conditions, at 37ºC, for five to seven days. To obtain films of swimming *H. pylori*, bacteria were inoculated from plates into 5 mL of HAMS-10 in a 50 mL flask and then incubated with shaking for approximately 15-17 hours prior to filming. To inspect for tactic responses to extracellular iron or hydrogen peroxide liquid cultures were checked for motility and then diluted to an OD$_{600}$ of 0.12 into prewarmed HAMS-10 containing Fe$_2$(SO$_4$)$_3$, FeCl$_3$, or H$_2$O$_2$ that had been prepared immediately prior to use, at concentrations indicated in the text, and filmed immediately for a maximum duration of five minutes. To inspect for tactic responses to iron chelation or superoxide production cultures were similarly diluted into 50 μM dipyridyl, 10 μg/ml metronidazole or 10.5 μM paraquat followed by incubation for fifteen minutes in microaerobic conditions. After this period, the cultures were either filmed immediately or treated with Fe$_2$(SO$_4$)$_3$, FeCl$_3$, or H$_2$O$_2$ and subsequently filmed immediately for a maximum duration of five minutes. Treatments with dipyridyl were 15 minutes to allow adequate time for the chelator to act, using previously determined time points. Treatments with metronidazole or paraquat were extended compared to that of iron or hydrogen peroxide treatment based on observations, and with the
understanding that the effects of either treatment would not be diminished, and
instead enhanced by an extended treatment. EC$_{50}$ values were determined by
nonlinear regression of direction change frequencies obtained by tracking WT
mG27 exposed to a range of FeCl$_3$ concentrations using GraphPad Prism 6
(GraphPad Software Inc., San Diego, CA). After mixing, cultures were filmed
immediately for a maximum duration of five minutes using phase contrast
microscopy with Simple PCI software (version 5.2.1.1609 (Compix 2003) using a
Hamamatsu Digital Camera C4742-95 mounted on Nikon Eclipse E600 at 400x
magnification. Videos were recorded at maximum acquisition speed at
(approximately 16 frames/second) and exported into .AVI format and then
relabeled to blind the analyzer to the condition.

For reversal frequency analysis, individual bacterial swimming paths
were analyzed by hand tracing the path of a randomly selected cell that was
visible for at least three seconds, with each clear direction change counted, as
described previously (120).

**Plug-bridge spatial chemotaxis assay**

For the spatial agarose-in-plug bridge chemotaxis assay, *H. pylori* SS1 was grown
to an O.D.$_{600\text{nm}}$ of 0.2-0.8 in BB10 with shaking, and then checked for motility.
Cells were collected by centrifugation at 2,000 rpm for 5 minutes at room
temperature, washed in phosphate buffered saline (PBS), and resuspended in
PBS plus 10% fetal bovine serum (FBS) which had been treated by boiling for 30
minutes in the presence of 50mM EDTA, followed by freezing overnight, filtering the liquid portion through a 0.2µm filter and dialyzing 2 times against a 3500 kDa dialysis membrane in PBS, as described previously (120). Motility was assessed by microscopy as above. The agarose-in-plug bridge assay was carried out as described by (121), except that the inherent precipitation of the FeCl₃ was used in place of agarose immobilization. Briefly, two plastic coverslips were placed about 16mm apart on a glass slide and covered by a glass coverslip. 90-100µL of washed and prepared motile cells were pipetted under the top glass coverslip. Immediately following, 2µL of 100mM FeCl₃ was pipetted at the edge of the top glass coverslip. The slides were incubated for 15 minutes under microaerobic conditions, as this duration was empirically determined to lead to a robust chemotactic response. The area immediately surrounding the crystallized iron was observed for the formation of chemotactic bands at 25X magnification using a Zeiss Axiovert 200 inverted microscope and images were recorded with under bright field illumination using a Zeiss AxioCam digital camera.

**Protein Preparation from Whole Cell Lysates and Subcellular Fractionation**

For whole cell lysates, total cell proteins were prepared from *H. pylori* cultured on CHBA plates for two days. Cells were resuspended in ice cold PBS. The optical density at 600 nm was determined. The suspension was then mixed with 2X Laemmli sample buffer to give a final OD₆₀₀ of 1.0, and then boiled for 10
minutes. 5-10 µl of each sample was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

For subcellular fractionation, *H. pylori* strains were grown in 100 ml BB10 for 24-48 hours, generating OD$_{600}$s, ranging from 0.24-0.55. All cultures were verified for purity and motility by light microscopy, and diluted in BB10 to matching OD$_{600}$s. *H. pylori* cells were collected by centrifugation at 8500 g for 10 minutes at 4ºC, and resuspended in 1-2 ml lysis buffer (50mM Tris-Cl pH 7.0, 10% glycerol, 1mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10 mM dithiothreitol (DTT)). Samples were kept at 4ºC/ice for all future steps. Resuspended cells were lysed in bursts by sonication until lysate appeared clear (3-5 minutes). The lysate was then spun at 349,000g for 15 minutes at 4ºC in a Beckman TL-100 ultracentrifuge to separate the cytoplasm & membrane fractions. The supernatant (cytoplasm) was removed, and the pellet (membrane fraction) was rinsed three times with 2 ml “high-salt buffer” (lysis buffer + 2M KCl). The membrane pellet was then completely resuspended in 2 ml high-salt buffer using an 18 gauge needle and syringe, and then collected by centrifugation at 217,000g for 15 minutes at 4ºC. This step was repeated once more, and then repeated using lysis buffer instead of “high-salt” buffer. The final membrane pellet was resuspended in an equal volume of lysis buffer as the original sample and stored at -20ºC. Protein concentration was measured using the Bio-Rad Protein Assay with bovine serum albumin (BSA) as a standard.
Samples were boiled in Laemmli sample buffer for 5 minutes and loaded onto 
10% SDS-PAGE gels, with equal total protein concentration in each lane.

**Western Blotting**

Samples run on SDS-PAGE gels were transferred to immunoblot polyvinylidene 
difluoride (PVDF) membranes (Bio-Rad). Successful transfer and relative protein 
loading was visualized by staining the membrane with DB71 dye prior to 
antibody binding. Membranes were incubated with a 1:2000-1:5000 dilution of 
anti-glutathione transferase (GST)-TlpA22 (104) or a 1:60 dilution of anti-CheV1 
(109). For visualization, the blots were incubated with the horseradish 
peroxidase conjugated secondary antibodies ‘chicken anti-rabbit’ or ‘goat anti-
rabbit’ (Santa Cruz Biotech) at a dilution of 1:2000, followed by incubation with 
luminol, p-coumaric acid, and hydrogen peroxide. Luminescent blots were then 
exposed to Biomax Light film (Kodak) or a Biorad Chemidoc MP.

**Immunofluorescence**

Liquid cultures of *H. pylori* wild type mG27 and *tlpABC* (TlpD-only) grown in 
BB10 to a final OD$_{600}$ of 0.6 – 1.0 were inspected for motility and purity by 
microscopy prior to fixation on poly-L-lysine coated slides. Bacteria were fixed 
and permeabilized as described previously (122) (109). Antibodies were pre-
absorbed as described previously (109). Pre-adsorbed anti His-CheAY (1:200) 
(109), anti-GST-TlpA22 (1:200) (104), anti-CheV1 (1:100) (109), anti-CheZ
(1:200) (109), anti-CheV3 (1:100) (Castellon, Lertsethtakarn, and Ottemann, in preparation), anti-CheW (1:100) (this work, see below), or chicken anti H. pylori (AgriSera AB) (1:500) were added and incubated at room temperature for 30 minutes, and washed with blocking buffer (3% BSA, 0.1% TritonX-100 in 1x PBS) three times. Goat anti-guinea pig Alexa Fluor 594 or goat anti-chicken Alexa fluor 488 (Abcam) were added at 1:300 or 1:500 respectively, and incubated in the dark at room temperature for 30 minutes. The samples were washed with blocking buffer as described above and a drop of Vectashield (Vector Laboratories) was added to the samples prior to samples being sealed with coverslips. Imaging was performed on Zeiss LSM 5 PASCAL Confocal microscope. Images were taken separately in each channel and merged in Adobe Photoshop CS2.

**Protein purification and antibody production**

*cheW* was amplified from SS1 genomic DNA with the primers cheW623bam (5’-CCCCGGATCCGTGAGCAACCAATTA-3’) and cheW624ecoR1 (5’-CACAGAATTTCTAGAAGTCTTTTTTGAATTTT-3’), digested with BamH1 and EcoR1, and ligated into pGEX6P2 (GE Healthcare) to create pGEX6P2-CheW. The resulting plasmid was sequenced to confirm its identity. pGEX6P2-CheW was transformed into Arctic Express *E. coli* (Agilent), grown to OD\textsubscript{600} 0.6, induced with 1 mM IPTG, and expressed at 10ºC for 24 hours. After over-expression, GST-CheW was purified essentially as described for other GST chemotaxis
proteins (107). Protein concentrations were determined by the Bradford assay (Bio Rad) using BSA as a standard. Anti-CheW antibodies were generated in guinea pigs with purified CheW (Cocolico).

Results

Although certain cytoplasmic chemoreceptors have been reported to respond to specific ligands or conditions, it has remained unclear whether those chemoreceptors can function independently of transmembrane chemoreceptors. To begin our studies into TlpD, we first constructed an H. pylori strain that expresses TlpD as its sole chemoreceptor, and for comparison, strains with all other combinations of the chemoreceptors. We confirmed that each chemoreceptor is expressed in the absence of the others by western blotting using an antibody raised against the conserved MA domain of TlpA (104) (Fig. 1A). As reported before for G27-derived strains (119), mG27 expresses only three of the four chemoreceptors: TlpA, TlpB, and TlpD, and these were each expressed at similar levels regardless of which other chemoreceptors were present, although there were some degradation products detected in TlpB-only strains (Fig. 1A). Strains lacking all chemoreceptor genes displayed no antibody cross-reactivity, consistent with the notion that the known chemoreceptors are the only MA domain-containing proteins in mG27 (Fig. 1A). With these strains in hand, we then analyzed whether the sole chemoreceptors were able to confer chemotactic function, using the common Brucella broth (BB) plus fetal bovine
serum (FBS) soft agar chemotaxis assay, which has been a valuable method to characterize the chemotaxis system in *H. pylori* (102, 110, 123). Mutant strains that retained only TlpD, referred to as TlpD-only, retained near wild-type chemotaxis, similar to mutants that retained TlpB-only (Fig. 1B). These findings are consistent with previous work reporting that TlpD-only mutants retain chemotaxis function in a different assay consisting of conditions that alter cellular energy (11). Strains expressing TlpA only, in contrast, had poor chemotaxis ability (Fig. 1B). Mutants lacking all of the chemoreceptors did not form expanded colonies in this assay, similar to a mutant lacking the key chemotaxis kinase CheA (Fig. 1B), suggesting that there are no other chemotaxis receptors in *H. pylori* that function in this medium. These results suggest that TlpD is able to function on its own to confer chemotaxis.

**TlpD has all the information necessary to localize to the pole and assemble a multiprotein chemotaxis signaling complex**

The finding that TlpD is able to confer soft agar migration suggests that it is sufficient to assemble the required chemotaxis signaling proteins. To determine what proteins TlpD assembles, we utilized immunofluorescence of whole cells to visualize the subcellular localization and TlpD dependence of the CheA kinase, the CheW and CheV coupling proteins, and the CheZHP phosphatase. Previous published work showed that chemoreceptors and chemotaxis signaling proteins are all polar in wild-type *H. pylori* (109). In TlpD-only strains, we observed that
TlpD localized to the pole, suggesting it contains the information needed for this subcellular localization (Fig. 2). CheA, CheW, CheV1, and CheV3 likewise localized to the cell pole in a TlpD-dependent manner (Fig. 2). In contrast, localization of CheZH
 did not require chemoreceptors, consistent with its localization to a independent complex as shown previously (109). These results suggest that TlpD has the information necessary to establish and maintain polar localization of itself and to form a chemotaxis signaling complex containing at least CheW, CheA, CheV1, and CheV3.

**TlpD membrane association depends on transmembrane chemoreceptors, CheA, CheW, and CheV1**

TlpD has been reported previously to reside in both the membrane and soluble subcellular fractions (11). It has remained unclear, however, how TlpD associates with the membrane, as it does not have transmembrane domains. Given that TlpD as well as chemotaxis proteins in general exist in multiprotein complexes (Fig. 2) (18), we examined whether any known chemotaxis proteins were required for TlpD to localize with the membrane fraction. *H. pylori* whole-cell lysate was separated into cytoplasmic and washed membrane fractions, and probed using an anti-chemoreceptor antibody (104). TlpD was found in both soluble and membrane-associated fractions in wild-type cells (Fig. 3A), consistent with previous reports using a distinct *H. pylori* strain (11). Both types of subcellular fractions appeared substantially pure based on the lack of
transmembrane chemoreceptor proteins in the cytoplasmic fraction, and the absence of anti-CheV1 cross-reacting cytoplasmic proteins in the membrane fraction (Fig. 3A). In mutants that lack all transmembrane chemoreceptors, the vast majority of TlpD is cytoplasmic (Fig. 3A), suggesting membrane chemoreceptors promote TlpD membrane association. Consistent with this possibility, TlpD’s membrane association was substantially reduced in strains lacking the core chemotaxis signaling proteins CheA or CheW (Fig. 3B), as well as the CheV1 coupling protein (Fig. 3C). Taken together, these data suggest that TlpD membrane association is indirect, e.g. that TlpD itself does not interact with the membrane, and instead relies on the chemotaxis signaling and chemoreceptor proteins.

**TlpD drives a chemotaxis response to conditions generated by iron**

The specific parameters sensed by TlpD remain unclear. Prior work had shown that this chemoreceptor responds to conditions generated by blockage of the electron transport chain, but the exact TlpD signal is unknown (11). We thus sought additional conditions sensed by TlpD. For this approach, we employed the agarose-in-plug bridge spatial gradient-based chemotaxis assay described by Yu and Alam (121) to test many compounds, including metals, amino acids, and organic acids. We detected a positive response with FeCl₃. Specifically, we observed that wild-type *H. pylori* formed a sharp band near the edge of a FeCl₃ precipitate allowing for the bacteria to respond to gradients emerging from the
source (Fig. 4). In this assay there were two features. The first was the sharp bacterial band (indicated by arrows in Fig. 4), which was dependent on chemotactic motility, while the other was a clearing zone, which was independent of chemotactic motility (Fig. 4). We therefore focused on the sharp band as an indicator of chemotaxis. Mutants lacking tlpD did not form the sharp bacterial band, whereas other single receptor mutants lacking tlpA, tlpB, or tlpC formed sharp bands similar to the one seen with wild-type cells. No band formed in response to MnCl$_2$, suggesting that the cells respond to the iron and not the chloride portion of FeCl$_3$. We considered the possibility that tlpD mutants are generally defective for chemotaxis, but concluded this is not the case because they retain nearly normal soft agar migration (Fig. 1B) and retain the ability to respond chemotactically to acid (120). We determined that iron is released from the immobilized FeCl$_3$ in this assay, using inductively coupled plasma mass spectrometry analysis of the media around the FeCl$_3$. We found levels that were 2.5-fold higher than background, equivalent to 92 nM. We do not know the form of this iron, as the media contains FBS proteins so the iron may be chelated by some of these. Additionally, the media surrounding the iron has a pH of ~5.5 (data not shown), because iron interacts readily with water to form iron hydroxide to liberate protons. The TlpB pH chemoreceptor, however, does not appear to play a role in these conditions. Taken together, these data show that iron is released from FeCl$_3$ in our chemotaxis assay, causes _H. pylori_ to form a chemotaxis-dependent band near but not directly adjacent to the immobilized
iron, and that TlpD is the only receptor required for this response. These data however do not indicate whether the TlpD-dependent chemotactic response to FeCl₃ is an attractant or repellent response. The position of the bacterial band a bit away from the iron source, however, suggests that iron may have some repellent properties, as observed previously for other bacteria in their spatial response to oxygen (124).

**Iron acts as a TlpD-detected repellent**

To determine whether iron was acting as a chemotaxis attractant or repellent, we employed a well-established microscopic assay for *H. pylori* in which the number of bacterial reversals is monitored in response to specific conditions (11, 119, 120). Repellents trigger high numbers of bacterial reversals, while attractants suppress reversals; changes in flagellar rotation have been used in many instances as an accurate indicator of a chemotaxis response (108, 119, 125, 126). This assay has been used to monitor an *H. pylori* repellent response to acid and AI-2; these two conditions cause an 1.5-2-fold increase in bacterial reversals (11, 119, 120). Wild-type *H. pylori* showed a statistically significant increase in the frequency of direction changes upon FeCl₃ exposure, with an apparent half maximal effective concentration (EC₅₀) for this response of 100 µM (Fig. 5a). The magnitude of this response—a 1.5-fold increase—is similar to those reported for other *H. pylori* repellents including acid and AI-2 (119). 100 µM Fe(II) iron sulfate evoked a similar response as 100 µM Fe(III)Cl₃ (data not
shown). TlpD and chemotaxis signaling were required for this iron-triggered increase in reversals (Fig. 5b). TlpD-only *H. pylori* retained the ability to respond to 100 μM FeCl₃ as a repellent, although the change effected by iron was muted compared to wild type and complicated by the fact that this strain already displays a high frequency of reversals (Fig. 5b). Taken together, these results suggest that TlpD mediates a repellent response to iron exposure.

**TlpD mediates a repellent response to conditions that promote oxidative stress**

While iron is an essential nutrient, it is also toxic because it can react with cellular hydrogen peroxide via Fenton-mediated chemistry to generate reactive oxygen species (ROS) including hydroxyl radical and hydroxide ion (127). Given that iron acted as a repellent, we examined whether ROS might also be a chemotaxis signal for *H. pylori*. We employed the same microscopic swimming assay, and assessed the response of *H. pylori* to chemicals shown to generate ROS in *H. pylori*: hydrogen peroxide (128), paraquat (129), and metronidazole (130).

Hydrogen peroxide exposure at 1mM evoked a repellent response that was TlpD-dependent and was retained in a TlpD-only strain (Fig. 5B). A similar response was noted for lower concentrations of hydrogen peroxide at 50 μM, 100 μM and 500 μM (data not shown). To assess a contribution of Fenton-mediated chemistry in generating conditions sensed by TlpD, *H. pylori* was incubated with the membrane-permeable iron chelator dipyridyl prior to
exposure to hydrogen peroxide, using an exposure shown previously to result in intracellular affects within this time frame (131). Dipyridyl pretreatment abolished the TlpD-mediated response to hydrogen peroxide (Fig. 5B). This data suggests that TlpD mediates a chemotaxis response to ROS, and that cytoplasmic iron pools contribute to this response.

To further confirm whether TlpD mediates a response to ROS, we next tested the ability of two superoxide generators to generate TlpD-dependent repellent responses, metronidazole and paraquat. *H. pylori* strains were incubated in the presence of either metronidazole or paraquat and swimming behavior was recorded and tracked. Both metronidazole and paraquat exposure induced a TlpD-dependent increase in direction change frequency (Fig. 5C) which was insensitive to the addition or chelation of iron (data not shown). The diluted metronidazole and paraquat did not affect the pH of the media which *H. pylori* was tracked, removing the possibility that repellent responses induced involved pH sensing. Taken together, this data suggests that TlpD responds ROS generated by Fenton chemistry, as well as superoxide.

**TlpD is expressed in all *H. pylori* strains**

TlpD is critical for animal infection (8, 9), but previous reports had suggested that expression of TlpD—as well as TlpA and TlpC—varied between *H. pylori* isolates. Specifically, G27 strains express only three chemoreceptors (Fig. 1), and previous studies had suggested that *H. pylori* strains can lack the genes for either
tlpC or tlpD (132), or have an interrupted tlpA gene (133). It thus was not clear whether TlpD was present, and therefore potentially utilized, by all H. pylori strains. We therefore examined the expression of TlpD across different H. pylori strains, using western blotting. Of 14 strains examined, all strains expressed TlpD (Fig. 6). All strains also expressed TlpB, but the amount varied substantially (Fig. 6), consistent with a report showing TlpB expression varies due to a G repeat in the 5’ untranslated region (134). Several strains lacked either TlpC (28%) or TlpA (14%), but no strains lacked both (Fig. 6). The ubiquity of TlpD suggests it plays critical roles in human infection, in line with its importance in animal models.

Discussion

We report here that the cytoplasmic chemoreceptor TlpD assembles a chemotaxis signaling complex at the pole independently of the transmembrane chemoreceptors, suggesting that TlpD has all the information required for polar localization and signaling complex formation. We furthermore determined that TlpD mediates a repellent response to compounds that generate ROS. Oxygen and ROS are ubiquitous in the stomach where H. pylori resides, arising from both epithelial and immune cells (117, 135). Our findings thus suggest a model in which TlpD is used to help H. pylori avoid regions with high ROS, as discussed below.
Previous studies had not addressed whether cytoplasmic chemoreceptors are able to function on their own (98). Here we show clearly that TlpD is capable of independent function. Specifically, we report that TlpD-only strains can mediate chemotaxis in the soft-agar assay in the absence of transmembrane chemoreceptors, and TlpD-only strains furthermore retain TlpD-mediated chemotaxis responses to ROS. Previous work had shown that TlpD-only strains in a different *H. pylori* background retained ability to chemotactically respond to electron transport chain inhibition (11), in line with what we report here. We also report that TlpD is sufficient for the formation of polar complexes that contain multiple chemotaxis signaling proteins. Taken together, this work provides strong evidence that TlpD is able to carry out all the necessary functions of a chemoreceptor.

Recent work has visualized cytoplasmic chemoreceptors inside intact cells. Briegel and colleagues identified clusters of cytoplasmic chemoreceptors in *Rhodobacter sphaeroides* and *Vibrio cholerae* that were positioned adjacent to and separate from the transmembrane receptors (136). These structures showed high similarity in many respects to those produced by transmembrane chemoreceptors: the clusters contained trimers of chemoreceptor dimers organized in 12 nanometer hexagonal arrays (136). These studies, however, were done in wild-type cells that possess multiple chemoreceptors. Our work shows that a soluble chemoreceptor can be sufficient to organize a signaling complex, consistent with the observation that some prokaryotes have only
soluble chemoreceptors (98). Polar localization of chemoreceptors is achieved using a variety of mechanisms, including stochastic self assembly, the use of polar landmark proteins, or ParA orthologues (137). Presumably TlpD can utilize one or more of these mechanisms, although it is not known which operates in *H. pylori*.

TlpD exists in both soluble and membrane-associated forms, as reported previously (11) and similar to other cytoplasmic chemoreceptors (98). We found that association of TlpD with the membrane depends on the transmembrane chemoreceptors, CheA, CheW, and CheV1. This finding suggests that TlpD is normally part of the *H. pylori* chemoreceptor signaling complex, interacting with the chemoreceptors via the signaling proteins. Studies in other microbes have also found a role for CheA and CheW in chemoreceptor organization, but the role of the CheV proteins was not well understood. CheV of *B. subtilis* promotes inter-chemoreceptor clustering of the ten *B. subtilis* chemoreceptors (66), suggesting it acts similarly to *H. pylori* CheV1. Of the cytoplasmic chemoreceptors reported to localize to the cell pole, some remain primarily co-localized with transmembrane chemoreceptors (34, 63, 64) and others cycle between a diffuse cytoplasmic distribution and a defined polar location (12). It appears that a large fraction of TlpD is soluble in wild-type *H. pylori* strains. It is not clear whether TlpD exists in equilibrium between soluble and membrane-associated states or if membrane association is affected by any signals.
We report here that TlpD mediates a repellent response to conditions that promote oxidative stress including iron, hydrogen peroxide, paraquat, and metronidazole. Iron was the initial TlpD chemotaxis signal we identified. Chemotactic responses to iron, mediated by TlpD in the agarose-in-bridge assay, were confounded by the fact that these tactic responses could be interpreted as a repellent, attractant, or mixed attractant and repellent. To clarify the nature of the response, we studied the swimming behavior of *H. pylori* in the presence of iron and noted that behaviors elicited were consistent with a repellent response. Correlating swimming behavior and flagellar motor direction changes to chemotactic responses has been used in numerous studies of bacterial chemotaxis, across many species, (108, 119, 125, 126) and leant confidence to our conclusion that *H. pylori* was responding to extracellular iron as a repellent. It is possible that iron is a repellent at high concentration and an attractant at lower concentrations, but we could not detect any such an attractant response even at low concentrations of iron (Fig. 5A).

Because iron acted as a repellent, we focused our attention on its toxic effects, namely generating ROS. Extracellular iron has been documented to rapidly enter the *H. pylori* cytoplasm (138, 139). Specifically, *H. pylori* had significantly higher levels of intracellular iron within five minutes of treatment with either iron(III) or iron(II) (138, 139), suggesting that our exposure here would result in intracellular iron increases. Excess iron has been shown carry out Fenton chemistry with endogenous hydrogen peroxide to generate hydroxyl
radical and hydroxide anion. Thus one hypothesis is that iron exposure causes cytoplasmic ROS that is in turn the signal sensed by TlpD. Consistent with the idea that elevated cytoplasmic iron propagates ROS, others have found that iron causes a transcriptional response in *H. pylori* genes for ROS detoxifying enzymes (140). In line with this hypothesis, we found that hydrogen peroxide similarly triggered a repellent response, and this response was dependent on free iron (Fig. 5). Iron-ROS connections have been noted additionally in that oxidative stress increases the free cytoplasmic iron pool in *H. pylori* (141). Further support for the idea that TlpD senses ROS came from analysis of other compounds that generate ROS, paraquat and metronidazole, which similarly resulted in a TlpD-mediated repellent response. This latter data suggests that TlpD can respond to superoxide as well as the hydroxyl radical generated by Fenton chemistry. Although these stresses may be linked (142), TlpD appears to respond to both oxidative stresses independently in our analyses because iron chelation did not affect the response to superoxide. Therefore our results support a model in which TlpD detects several forms of cytoplasmic oxidative stress and leads to a repellent chemotaxis response.

Previous work had shown that TlpD additionally mediates a repellent response to electron transport chain inhibitors (11). Interestingly, electron transport chain inhibition is known to generate both hydrogen peroxide and superoxide (142, 143), so it is possible that electron transport inhibitors and ROS affect chemotaxis via the same mechanism. Furthermore, Behrens and
colleagues report that tlpD mutants are more sensitive to oxidative damage (9), providing an additional connection between TlpD and oxidative stress.

While we know that TlpD is necessary for the ROS response, we do not yet know whether it’s sufficient. Chemoreceptors are known to sense signals either directly or via interacting proteins. TlpD has a reported protein interaction partner, HP0697, a predicted subunit of acetone carboxylase (144). This or other unidentified proteins may be required for TlpD’s ROS response. Alternatively, TlpD may sense ROS directly, or possibly respond to a small molecule whose concentration changes during the oxidative stress. Other proteins use redox active cofactors such as FADH, FMN, or iron to sense alterations in redox state. These cofactors are typically bound by domains such as the PAS domain, that are located N-terminal to the MA domain. (98). TlpD has no identifiable domains that would bind FADH or FMN at either its N- or C-terminus, but it has been reported to interact with iron (51). There are no identifiable domains in the TlpD N-terminal region at all (51), but it does possess a CZB domain at its C-terminus, which binds zinc via histidine and cysteine residues (51). As some of us have noted previously (51), other ROS sensing proteins use cysteine as a way to sense redox status, so this mechanism may operate in TlpD (51).

TlpD is critical during colonization in animal models (8, 9), particularly in the first weeks of infection. H. pylori is exposed to ROS produced in the stomach by both epithelial and phagocytic cells (117, 135, 145). The ability to survive
ROS appears critical during *H. pylori* colonization, as antioxidant enzymes are highly expressed by this microbe, and mutants lacking them are attenuated in colonizing animal hosts (128, 146). Others have documented that epithelial cells of the stomach release hydrogen peroxide that exposes the related *Helicobacter felis* to ROS, consistent with the idea that gastric *Helicobacters* are regularly exposed to ROS (135). Additionally, there is a recently appreciated radial oxygen gradient emanating from the tissue to the lumen of the gastrointestinal tract. While not yet measured in the stomach, the intestinal oxygen gradient is extremely steep, ranging from a tissue level of 160mmHg (5.25%) to <1 mmHg (116). These and other findings suggest that near-tissue bacteria are exposed to higher oxygen than those in the lumen (115, 147). Indeed, our swimming analysis suggests that room oxygen (21%) acts as a modest *H. pylori* repellent, because dipyridyl decreased the basal bacterial reversal frequency (Fig. 5B), which could be explained by prior reports suggesting that *H. pylori* experiences oxidative stress in atmospheric oxygen (141). ROS affect *H. pylori* cytoplasmic iron concentrations (148), suggesting that the oxidative stress that *H. pylori* experiences is sensitive to oxygen levels in which the bacterium resides. *H. pylori* has been measured to localize within 15 µm of the epithelial surface (149), and it may maintain this distance to limit the oxidative stress it experiences from oxygen and/or reactive oxygen species emanating from the epithelial surface. In summary, we report that the cytoplasmic chemoreceptor TlpD possesses the key features of a bona fide chemoreceptor: ability to drive a chemotaxis
response and to organize a chemotaxis signaling complex at the pole. TlpD mediates a repellent response to cytosolic ROS. Our work thus adds substantially to our understanding of cytoplasmic chemoreceptors, by clearly showing that they retain properties such as polar localization and signaling complex organization independently, and can promote responses to specific signals.
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Fig. 1. TlpD is stably produced when the sole chemoreceptor and confers chemotactic function in a soft agar assay.

A. Whole cell lysates were analyzed by western blotting with the anti-TlpA22 antibody that recognizes all four *H. pylori* chemoreceptors (104). Lanes are as follows; 1, wild type mG27 (WT); 2, mG27 ΔtlpA; 3, mG27 ΔtlpB; 4, mG27 ΔtlpC; 5, mG27 ΔtlpD; 6, mG27 ΔtlpA ΔtlpB (ΔtlpAB); 7, mG27 ΔtlpA ΔtlpC (ΔtlpAC); 8, mG27 ΔtlpA ΔtlpD (ΔtlpAD); 9, mG27 WT; 10, mG27 ΔtlpB ΔtlpC (ΔtlpBC); 11,
mG27 ΔtlpB ΔtlpD (ΔtlpBD); 12, mG27 ΔtlpC ΔtlpD (ΔtlpCD); 13, mG27 ΔtlpA ΔtlpB ΔtlpC (ΔtlpABC); 14, mG27 ΔtlpA ΔtlpC ΔtlpD (ΔtlpACD); 15, mG27 ΔtlpA ΔtlpB ΔtlpD (ΔtlpABD); 16, mG27 ΔtlpB ΔtlpC ΔtlpD (ΔtlpBCD). Not shown is mG27 ΔtlpA ΔtlpB ΔtlpC ΔtlpD, which looks identical to lane 15, as both lack tlpC. Marker sizes are given in kilodaltons on the left side. The migration position of TlpA, TlpB and TlpD are indicated at the right with arrows. Western blots are representative of 2-5 replicates for each strain.

B. Brucella broth-FBS soft agar chemotaxis assays with the colonial diameter of the indicated *H. pylori* strains was measured after five days. Error bars represent standard error of the mean. Each strain was analyzed in two to five biological replicates, with ≥ 3 technical replicates each time. * indicates p<0.05, ** indicates p<0.01 as compared to wild type using Student’s T test.
Fig. 2. TlpD localizes to the pole and coordinates a chemotaxis complex there

Immunofluorescence images of wild-type (WT), TlpD-only (D only), and a strain lacking all chemoreceptors (receptorless, ΔtlpABCD) mG27 *H. pylori* strains. *H. pylori* whole cells were detected with chicken anti-*H. pylori* antibodies, followed by staining with goat-anti chicken IgG conjugated to AlexaFluor 588 (red). Specific chemotaxis proteins were detected as indicated on the left side of the figure, with antibodies as described in Materials and Methods, and subsequently stained with secondary antibodies conjugated to AlexaFluor 494 (green). The TlpA antibody recognizes all *H. pylori* chemoreceptors. Scale bar indicates 4 µm in all panels.
Fig. 3. TlpD associates with the membrane fractions via interactions with chemoreceptors and chemotaxis signaling proteins.

A. TlpD subcellular localization in wild-type strains and strains lacking *H. pylori* chemoreceptors, top panel, assessed by immunoblot with anti-TlpA22. W, whole cells; S, soluble; and M, membrane in each panel. The position of the *H. pylori* chemoreceptors TlpA, TlpB, TlpC, and TlpD are shown at the right. Bottom panel, control blot, stripped and reprobed with an anti-CheV1 antibody that
additionally recognizes several cytoplasmic proteins (arrowheads). B. TlpD subcellular localization in strains lacking chemotaxis signaling proteins CheA and CheW, top panel, assessed as described in panel A. Bottom panel shows the control blot as described in panel A. C. TlpD subcellular localization in strains lacking chemotaxis signaling proteins CheV1, CheV2, and CheV3, assessed as described in panel A. In all panels, the amount of protein loaded was normalized based on Bradford assay and confirmed by staining the blots with DB71 (not shown).
Fig. 4. TlpD mediates tactic response to iron in the agarose-in-plug bridge assay.

Modified agarose-in-plug-bridge assays were carried out using *H. pylori* strain SS1 and its isogenic mutants. Arrows mark chemotactic bacterial rings, and asterisks mark crystallized FeCl₃ or MnCl₂ in an agarose matrix. Clearing around the plugs is non-specific, as it appears independently of chemotaxis or motility. Images are representative of the following number of replicates: wild-type n=17; cheW, n=1; cheA, n=4; cheY, n=4; tlpA, n=2; tlpB, n=4; tlpC, n=2; tlpD, n=14; motB, n=1.
A) 

![Graph showing direction changes/three seconds vs. log [FeCl₃] M](image)

B) 

![Bar graph showing direction changes/three seconds for WT, ΔtlpD, TlpD-only, and ΔcheA](image)

C) 

![Bar graph showing direction changes/three seconds for WT, ΔtlpD, TlpD-only with different additions](image)
Fig. 5. *H. pylori* displays a repellent chemotaxis response to oxidative stress

A) WT mG27 grown overnight in HAMS-FBS were exposed to various concentrations of FeCl₃ immediately before being filmed and tracked. Direction changes were tracked over a three second window. Error bars represent standard error of the mean. * indicates p<0.05, ** indicates p<0.01 as compared to wild type using Student’s T test.

B) mG27 strains grown overnight in HAMS-FBS were exposed to 100 mM FeCl₃, 50 μM dipyridyl, or 1 mM hydrogen peroxide prior to being filmed and tracked. For dipyridyl exposure, cultures were incubated with 50 mM dipyridyl for 15 minutes prior to addition of FeCl₃ or H₂O₂. Direction changes were tracked over a three second window. Error bars represent standard error of the mean. * indicates p<0.05, ** indicates p<0.01 as compared to wild type using Student’s T test.

C) mG27 strains were incubated for fifteen minutes in the presence of either 10 μg/ml metronidazole or 10.5 μM paraquat and swimming behavior was recorded and direction changes were tracked over a three second window. Error bars represent standard error of the mean. * indicates p<0.05, ** indicates p<0.01 as compared to wild type using Student’s T test.
Fig 6. TlpD is expressed by all examined *H. pylori* strains

Chemoreceptor content was analyzed from total cell lysate of the indicated strains using western blotting with the TlpA22 antibody that recognizes the conserved domain present in all *H. pylori* chemoreceptors (104). Marker size is given in kilodaltons on left side. The migration positions of TlpA, TlpB, TlpC and TlpD are indicated at the right with arrows. Western blots are representative of > three replicates for each sample.
**Table 1. Bacterial strains and plasmids used in this study**

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Chemotaxis allows bacteria to overcome host-generated reactive oxygen species that constrain gland colonization

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Abstract:
The epithelial layer of the gastrointestinal tract contains invaginations, called glands or crypts, which are colonized by symbiotic and pathogenic microorganisms and may function as a designated niche for certain species. Factors that control gland colonization are poorly understood, but bacterial chemotaxis aids occupation of these sites. We report here that a Helicobacter pylori cytoplasmic chemoreceptor, TlpD, is required for gland colonization in the stomach. tlpD mutants demonstrate gland colonization defects characterized by a reduction in the percent of glands colonized, but not in number of bacteria per gland. tlpD mutants showed hallmarks of exposure to large amounts of reactive oxygen species (ROS), consistent with TlpD's reported role in ROS avoidance. To assess the role of host-generated ROS in TlpD-dependent gland colonization, we utilized mice that lack either the ability to generate epithelial hydrogen peroxide or immune cell superoxide. tlpD gland colonization defects were rescued to wild-type H. pylori levels in both of these mutants. These results suggest that multiple types of innate immune generated ROS production limit gland colonization and
that bacteria have evolved specific mechanisms to migrate through this gauntlet to establish in the glands.

**Introduction:**

The epithelium of the gastrointestinal (GI) tract contains invaginations, called glands in the stomach and crypts in the intestine, which are thought to serve as a niche for particular microbes and in turn, promote chronic colonization. Our knowledge of the factors that control the colonization of these structures is incomplete. Host factors that have been implicated in controlling gland colonization include the production of mucus (160), the production of antimicrobial peptides (161), and the presence of resident immune cells in the lamina propria (162). Gland colonization, therefore, requires microbes to bypass these defensive strategies.

Bacteria too appear to have special adaptations to the gland niche. These include the ability to use certain carbohydrates (163) and perform chemotaxis (108, 164, 165). The chronically-colonizing gastric pathogen *Helicobacter pylori* is one such microbe that requires chemotaxis for gland colonization (108, 164, 165). Chemotaxis permits bacteria to sample their environment via chemoreceptors that use ligand-binding signals to alter the autophosphorylation of a complexed histidine kinase CheA. Ultimately, this pathway alters flagellar motility to allow bacteria to follow or repel themselves from gradients of specific
signals (100). *H. pylori* expresses four chemoreceptors, three of which (TlpA, TlpB, and TlpC) are embedded the inner membrane, and one that is fully cytoplasmic (TlpD). The relevance of individual chemoreceptors on overall gastric colonization has been gauged previously by the level of colonization defect that a particular mutant displays. Among individual *H. pylori* chemoreceptor mutants, *tlpD* mutants display the most severe colonization attenuation in two animal models of infection (8, 9). The exact nature of the *tlpD* mutant colonization deficit, however, has remained unclear, as has the role of specific signals and chemoreceptors in gland colonization.

TlpD has been linked to a chemotactic response to multiple stress-related signals including electron transport chain inhibitors (11), acid (165), and reactive oxygen species (ROS) including hydrogen peroxide (H\(_2\)O\(_2\)) or superoxide generators (metronidazole and paraquat) (166). One hypothesis is that these signals are connected because they all affect oxidative stress experienced in the cytoplasm (166). Gastric Helicobacter are known to encounter host-generated ROS derived from both epithelial and immune cells during infection and must cope with the stress to successfully colonize the gastric epithelia (117, 135).

ROS are produced by both gastric epithelial cells and innate immune cells, and include hydrogen peroxide (H\(_2\)O\(_2\)), superoxide (O\(_2^{-}\)) and hypochlorous acid (HOCl) (117). To counter these stresses, *H. pylori* possesses a suite of ROS detoxification systems including catalase, superoxide dismutase, and
peroxiredoxins (167). *H. pylori* mutants lacking these systems are sensitive to ROS and are also attenuated in the host (167). ROS production limits colonization at epithelial surfaces in the stomach and intestine (135, 168); in agreement with this idea, mouse mutants that lack the epithelial DUOX enzyme produce less H$_2$O$_2$ and allow elevated colonization by a relative of *H. pylori*, *Helicobacter felis* (135). ROS production may serve to drive microbes away from the epithelial surface, as microbial adherence to intestinal epithelial cells promotes H$_2$O$_2$ production and hosts respond to *H. pylori* infection with elevated ROS (167, 168). However, it is not clear how ROS affects colonization within the glands.

To define the contribution of TlpD in gastric colonization, we first determined its effect on distribution in the stomach. We found that *tlpD* mutants showed specific deficits in colonizing a broad swathe of gastric glands, and displayed hallmarks of exposure to elevated ROS. This result raised the possibility that gland colonization defects could be due to an inability of *tlpD* mutants to successfully migrate in response to ROS. To assess whether host-generated ROS impacted *H. pylori* colonization, we compared the colonization and distribution of wild type (WT), *tlpD* and nonchemotactic cheY mutants in mice deficient in either epithelial dual oxidases (Duoxa-/-) or phagocytic NOX2 NADPH oxidase (Cybb-/-). Infection of either Duoxa-/- or Cybb-/- mutant mice rescued the gland colonization defects of *tlpD* mutants noted in WT hosts. Interestingly, we found that when a *tlpD* mutant was able to access a gland, it
multiplied to levels that were at or above those of wild type. Our results suggest that ROS production impacts \textit{H. pylori} gland transit, and that TlpD-mediated chemotactic responses are needed to thread this restricted gland entrance.

\textbf{Results}

\textit{tlpD \textit{H. pylori} show antral colonization defects in WT hosts}

To begin our analysis of TlpD's role in colonization, we orally infected WT C57BL6 mice with WT, \textit{tlpD}, or \textit{cheY} mutant variants of \textit{H. pylori} that all expressed GFP. \textit{cheY} encodes the central chemotaxis signaling proteins, so mutants that lack it are fully non-chemotactic, while mutants that lack \textit{tlpD} lose only responses sensed by that receptor and thus are partially chemotactic. After two weeks of infection, the total colonization levels in tissue of the stomach corpus and antrum were determined. \textit{tlpD} mutants showed a significant colonization defect of the antrum of WT mice (Fig. 1A), which was previously reported (8). Colonization of the corpus by \textit{tlpD} mutants was reduced relative to WT \textit{H. pylori}, although the reductions were not statistically significant. These results suggested that \textit{tlpD} GFP+ \textit{H. pylori} behaved similarly to \textit{tlpD} infections lacking GFP described previously (8), and encouraged the analysis of gland colonization by the mutant.

\textbf{The loss of \textit{tlpD} increases corpus gland load, while the loss of chemotaxis signaling increases gland loads throughout the stomach.}
We next sought to examine TlpD’s role in gland colonization. To monitor gland colonization, we employed the bacterial localization in isolated glands (BLIG) approach in which gastric glands are isolated from the infected corpus or antrum tissue, epithelial cells labeled with Hoechst DNA stain, and glands examined for the presence of GFP+ *H. pylori* by fluorescent microscopy (164). Bacteria within glands were counted manually, and two parameters of gland colonization were compared between *H. pylori* strains. The first parameter was gland bacterial load, the number of bacteria per infected gland. Our calculation of gland bacterial load excludes non-infected glands. The second parameter was gland occupancy, the percent of glands infected.

In WT mice, WT *H. pylori*-colonized the glands of both the corpus and the antrum to similar levels, averaging 10 bacteria/infected gland as reported previously (Fig. 1B) (164). Loss of TlpD did not affect gland load in the antrum but caused a ~1.8-fold increase in gland load in the corpus compared with WT (Fig. 1B). Full loss of chemotaxis (*cheY* mutants) also resulted in elevated gland loads of 2- to 3-fold in both the corpus and the antrum relative to WT *H. pylori* (Fig. 1B). These results suggest that chemotactic defects did not impair growth within glands. Instead, it appears that if an *H. pylori* gains access to a gland, it multiplies to high levels and may not be able to leave the gland.

**The loss of *tlpD* or chemotaxis results in a reduction in gland occupancy throughout the stomach in WT hosts**
Because \( tlpD \) and \( cheY \) mutants appeared to have altered gland phenotypes, we next analyzed gland occupancy to thus determine the percentage of glands infected by \( H. pylori \). This frequency likely reflects both the initial population of glands infected by \( H. pylori \) as well as the ability to spread and colonize new glands. In WT mice, WT \( H. pylori \) had colonized 40-50% of corpus and antral glands by two weeks of infection, and was found in similar proportions in both regions (Fig. 1C). \( tlpD \) mutants showed an \(~3\)-fold reduced occupancy in both the corpus and antrum relative to WT \( H. pylori \) (Fig. 1C). \( cheY \) mutant gland occupancy was also decreased relative to WT \( H. pylori \), with significant reductions in both the corpus and the antrum (Fig. 1C). These results suggest that chemotaxis generally and TlpD specifically is required for \( H. pylori \) to occupy new glands.

**\( tlpD \) mutants show evidence of increased ROS exposure relative to WT \( H. pylori \)**

We reported recently that TlpD mediates chemotactic repellent responses to multiple ROS (166). Combining this information with our data above suggested that \( tlpD \) mutant gland colonization defects could be due to an inability of these mutants to sense and repel themselves away appropriately from ROS. We therefore asked whether \( tlpD \) mutants experienced differential oxidative stress \textit{in vivo}. For this approach, we used quantitative real-time PCR of mRNA isolated from infected mouse tissue. We examined the expression of the
catalase gene (\textit{katA}) mRNA by \textit{H. pylori} strains, whose expression has been shown to be sensitive to several oxidative stresses \cite{140, 169}. We determined that this gene was modestly upregulated \textit{in vitro} in our strains following exposure to 1 mM H$_2$O$_2$ for twenty minutes (Fig. 2A). This result suggested that \textit{katA} mRNA could serve as a reasonable proxy for H$_2$O$_2$ exposure \textit{in vivo}. We next assessed whether the expression of \textit{katA} mRNA differed between WT, \textit{tlpD}, or \textit{cheY} \textit{H. pylori} during infection of WT mice. \textit{tlpD} mutants expressed significantly more \textit{katA} mRNA than WT \textit{H. pylori} in the antrum, and modestly more in the corpus (Fig. 2B). These results suggest that \textit{tlpD} mutants experience elevated oxidative stress during infection. Conversely, \textit{cheY} mutants did not express elevated catalase mRNA (Fig 2B). This outcome suggests that the loss of TlpD specifically leads \textit{H. pylori} to be exposed to conditions that are different than those encountered by WT, consistent with high exposure to oxidative stress.

**Gland colonization defects of \textit{tlpD} are rescued in hosts deficient in H$_2$O$_2$ production by gastric epithelial cells.**

The results presented above suggest that TlpD helps to mitigate exposure of \textit{H. pylori} to oxidative stress in the mouse. In order to follow up on oxidative stress and its role in TlpD-mediated colonization, we next infected two mutant mouse hosts that were deficient in the production of H$_2$O$_2$ and O$_2^-$ production. The first of these lacks the dual oxidase (Duox) heterodimeric enzyme complex by virtue of loss of the \textit{Duoxa}-encoded subunit \cite{135}. Duox is expressed by
gastric epithelial cells and generates extracellular H$_2$O$_2$ that may serve to limit physical interactions between microbes and the epithelial surface (168). Duox has been implicated in limiting the colonization of a related Helicobacter species in the stomachs of mice (135).

To examine whether Duox impacted H. pylori colonization, Duoxa$^{-/-}$ mice were infected similarly to WT mice for two weeks, at which point the mice were sacrificed and colonization of WT, cheY, and tlpD GFP$^+$ H. pylori was compared. All H. pylori strains colonized the Duoxa$^{-/-}$ mutants to levels that were a bit elevated but not significantly different from those in WT mouse hosts (Fig. 3A). Gland loads were also generally similar between WT and Duoxa$^{-/-}$ glands, across WT and tlpD mutant H. pylori in both locations, and cheY mutants in the corpus (Fig. 3B). There was a modest increase in gland load in the antrum of the tlpD mutant and a very large decrease in loads of the cheY mutant, suggesting the effect of Duox was greatest in the antrum.

We next assessed how the loss of Duoxa$^{-/-}$ would alter gland occupancy. WT H. pylori gland occupancy was seemingly unaffected by the loss of Duoxa$^{-/-}$, as ~50% of glands were infected in this background as well as in WT mice (Fig. 3C). Interestingly, the tlpD mutant showed an increase in gland occupancy compared to its levels in a WT mouse, moving from <15% occupied to over 40% (Fig. 3C). Indeed, the tlpD mutant achieved gland occupancy levels in the corpus and antrum that were not different from WT H. pylori (Fig. 3C). In contrast, the cheY mutant was not rescued, suggesting the loss of Duoxa$^{-/-}$ rescue is specific to
signals sensed by TlpD and requires chemotaxis. This apparent rescue in tlpD gland occupancy suggests that the loss of H$_2$O$_2$ production by gastric epithelial cells allows for tlpD mutants to move more readily into new gastric glands in both the corpus and the antrum.

**Gland colonization defects of tlpD are rescued in hosts deficient in O$_2^-$ production by phagocytes.**

We next assessed the contribution of phagocyte ROS production to *H. pylori* gland colonization. Phagocyte ROS production was assessed in *Cybb*/- mice that lack the catalytic subunit of phagocyte oxidase (Phox). *Cybb*/- mice were infected similarly to WT and *Duoxa*/- mice, and the same colonization parameters were compared between WT, *cheY*, and *tlpD* GFP+ *H. pylori*.

The overall colonization of the corpus and antrum was seemingly unaffected by loss of *Cybb* for all three *H. pylori* strains, showing no significant differences from WT mouse infections (Fig. 4A). Gland loads, on the other hand, were affected in *Cybb*/- hosts. Both WT and *tlpD* *H. pylori* showed elevated gland loads in the corpus and the antrum relative to WT BL6 infections, achieving 20-30 bacteria/gland in both regions. *cheY* mutants did not follow this trend in *Cybb*/- hosts and instead showed reduced gland loads in the corpus and the antrum relative to WT BL6 infections (Fig 4B). This outcome suggests that superoxide may limit *H. pylori* numbers in a chemotaxis-dependent way. Lastly, we compared gland occupancy in *Cybb*/- hosts. Strikingly, the *tlpD* mutant gland
occupancy in both the corpus and antrum climbed to levels that were not different from WT *H. pylori*. This finding suggests that, similarly to *Duoxa*<sup>-/-</sup> infections, gland occupancy defects of *tlpD* were rescued by loss of host ROS. As seen with *Duoxa*<sup>-/-</sup> infections, *cheY* gland occupancy did not appear to benefit from the loss of *Cybb*<sup>-/-</sup> (Fig 4C). These results suggest that the loss of superoxide production by phagocytes rescues gland colonization defects of *tlpD* *H. pylori*, as was observed in *Duoxa*<sup>-/-</sup> hosts. Chemotaxis appears necessary for this rescue, as *cheY* mutants showed similar gland colonization values observed in WT mice. Taken together these results suggest that host-generated ROS serves as a barrier for gland colonization by *H. pylori* that the bacteria overcome via TlpD-mediated chemotactic responses. Furthermore, *tlpD* colonization defects can be attributed to low gland occupancy in the corpus and the antrum, which can be rescued to WT levels by disrupting ROS production by the host.

**Discussion**

We report here the *H. pylori* requires repellent ROS chemotaxis to be able to successfully colonize glands. Factors that control gland colonization throughout the GI tract are poorly understood, although it appears that an interplay exists between host and microbe to regulate gland access. Host factors known to limit gland colonization include mucus production (160), oxygen gradients emanating from the epithelial surface (170), and antimicrobial peptide production (161). Microbial adaptations that have been reported to aid gland
colonization include chemotaxis (108, 164, 165), sugar transport systems (171), and the ability to dampen host immune responses (172). Therefore it seems reasonable to posit that glands represent a desired but protected niche for some microbes in the GI tract. Our work adds that ROS limits gland access, and chemotaxis helps overcome this barrier.

Host ROS generation has been implicated in limiting microbial adhesion in the intestine, and Duoxa/- mice showed elevated mucosal penetrance by a subset of the microbiota (168). Our data suggest that host ROS plays an important role in restricting gland access in the stomach, and that bacteria can use chemotaxis to overcome this barrier. Gland colonization defects observed for tlpD mutants in WT hosts were effectively rescued in hosts with ROS production defects. Low gland occupancy could be due to very low initial colonization, or to low gland-gland spread, as both of these processes require chemotaxis (164). Our results lend support to a prior report suggesting that TlpD mediates chemotactic repellent responses to ROS treatments in vitro (166), and defines the nature of colonization defects of tlpD mutants which have been described in the past (8, 9).

Our work additionally suggests that chemotaxis is not required for growth once bacteria are in glands, because we observed here that non-chemotactic and tlpD mutants obtained high numbers/gland in WT mice. These results are not in conflict with a prior report which presented the average number of bacteria/gland, and included uninfected glands in that calculation.
Excluding uninfected glands from data reported in Keilberg et al. concerning gland loads for WT and cheY H. pylori would produce similar values as those described in this report. It is not yet known what sets gland load, but it has been observed that this number varies over the course of a mouse infection, climbing to an average of ~15-25 bacteria/gland within the first month, and then dropping to less than 5 by six months of infection (164). Our results show that chemotaxis can affect these within-gland levels, somewhat surprisingly playing a role to limit bacterial numbers. Our data suggest the possibility that chemotaxis plays a critical role in gland exit, such that without chemotaxis or TlpD specifically, bacterial numbers rise in the glands but bacteria cannot effectively leave. This in turn creates poor gland occupancy. The exact signals being sensed are not yet known, but appear to affected by ROS as the high gland numbers are abrogated by loss of the ability to produce H$_2$O$_2$ or superoxide.

Previous work showed that TlpD drives chemotactic repellent responses in vitro (11, 165, 166), and our data is consistent with the idea that it also mediates chemotactic repellent responses in the host. Specifically, we found that tlpD mutants display hallmarks of high ROS exposure, in agreement with the idea that these mutants cannot avoid ROS. One role for this response in vivo comes from the observation that hosts upregulate defensive ROS production upon H. pylori infection (167). Thus, H. pylori may experience a delay from initially colonizing glands to experiencing stress imparted by the host. Therefore a
repellent response mediated by TlpD could limit the detrimental effect of these stresses.

In conclusion, we have described host ROS generation as an additional host limitation on gland colonization in the stomach that is overcome by *H. pylori* chemotaxis. We implicate the cytoplasmic chemoreceptor TlpD in ROS-dependent gland colonization effects in the host and show that colonization defects noted for a *tlpD* mutant in WT hosts is relieved in ROS-production deficient hosts. TlpD appears to be involved in the dispersal of *H. pylori* between glands in a ROS-dependent fashion.

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Methods:

Bacterial strains and culture conditions. WT and cheY SS1 GFP+ *H. pylori* described previously were employed for mouse infections (164). *H. pylori* was cultured on either Columbia horse blood agar (CHBA), or brucella broth with 10% fetal bovine serum (FBS; Life Technologies) (BB10). CHBA consisted of Columbia agar (BD) with 5% defibrinated horse blood (Hemostat Labs, Davis, CA), 50 µg/ml cycloheximide, 10 µg/ml vancomycin, 5 µg/ml cefsulodin, 2.5 U/ml polymyxin B, and 0.2% (wt/vol) α-cyclodextrin. All chemicals were from Thermo Fisher or Gold Biotech. Cultures were incubated at 37°C under 5 to 7% O2, 10% CO2, and balance N2.

Creation of *tlpD* GFP+ *H. pylori* mutants

*tlpD* GFP+ *H. pylori* strain SS1 (KO1614) was constructed by transformation of Δ*tlpD::cat* SS1 (KO914) (104, 166) with the plasmid pTM115 (164, 173) isolated from *H. pylori* strain SS1, and selected on CHBA plates containing 15 µg/ml kanamycin (8, 164).

Animal infections and *H. pylori* colonization calculations.

The University of California, Santa Cruz Institutional Animal Care and Use Committee approved all animal protocols and experiments. Cybb-/- targeted homozygous null mice in a B6.129S background were obtained from Jackson Laboratory (JAX stock #002365, Bar Harbor, ME)(174); Duoxa-/- mice lacking
functional dual oxidase enzymes by virtue of loss of the duoxa1-duoxa2 maturation subunits (175) were obtained as heterozygotes on the B6 background from the University of Michigan. All mice were obtained as breeding pairs, and bred at UC Santa Cruz. Duoxa-/- mice were generated, screened, and maintained as previously described (135). In brief, Duoxa genotyping was performed by isolating genomic DNA from tail tissue with the Qiagen DNeasy Blood & Tissue Kit, followed by PCR with a common primer (DA-WT/KO), a WT allele-specific primer (DA-WT-R) and a knockout allele-specific primer (DA-KO-R) (135). Genotypes were judged by the presence of the WT allele as a 381-basepair fragment, and the knockout allele as a size of 568 basepair fragment (175).

Six to eight-week-old mice (male and female) were infected intraorally by allowing the animals to drink a 50 microliter suspension from a pipette tip containing *H. pylori* grown to mid-exponential phase and concentrated to an optical density at 600 nm of 3.0 (~ 5x10⁷/50 μl) in BB10 medium, as done previously (108). At the end of an infection period, mice were sacrificed by CO2 narcosis. The stomach was removed, opened along the lesser curvature and washed in phosphate-buffered saline (PBS) to remove food. The corpus and antrum were divided based on tissue coloration, cut into pieces that were then processed to analyze total bacterial colonization, gland isolation, or for RNA extraction. For total bacterial colonization, corpus and antral tissue was weighed, homogenized with the Bullet Blender (Next Advance) with 1.0-mm zirconium
silicate beads, and then plated to determine the number of colony forming units (CFU) per gram of stomach tissue on CHBA with the addition of 20 µg/ml bacitracin, 10 µg/ml nalidixic acid, and 15 µg/ml kanamycin.

Gland isolation and microscopy

Glands were isolated by incubating dissected gastric tissue in Dulbecco’s phosphate-buffered saline (DPBS) (Millipore) plus 5 mM EDTA at 4°C for 2 hours with agitation, as described previously (164, 176). The tissue was subsequently transferred to DPBS containing 1% sucrose and 1.5% sorbitol and shaken for thirty seconds. Glands were labeled with 10 ng/ml Hoechst DNA stain (Life Technologies). Glands were kept on ice and examined as soon as possible. Ten microliters of shaken tissue were placed on glass slides and visualized with a Nikon Eclipse E600 microscope with fluorescence filters for 4’,6’-diamidino-2-phenylindole (DAPI), GFP, and RFP. For each time point and infection, 100 glands each were imaged for the corpus and antrum, and the number of *H. pylori* cells inside the gland was counted manually for each gland. Gland load levels were calculated by averaging the number of bacteria observed in colonized glands per mouse and *H. pylori* strain. Gland occupancy was calculated as the frequency of glands occupied per mouse host and averaged over at least three mice. Gland colonization comparisons were made for at least three mice per genotype and *H. pylori* strain.
RNA isolation and qPCR:

Gastric tissue was flash frozen in liquid nitrogen, homogenized in TRIzol (Invitrogen) and RNA was isolated following the TRIzol RNA isolation protocol (GIBCO). DNA was removed by following the TURBO DNA-free kit protocol (Life technologies). cDNA was produced with the High-Capacity cDNA Reverse Transcription Kit (Life technologies) using random primers. qPCR was performed using the SensiFAST SYBR No-ROX kit (Bioline) using the primers listed below. Primer efficiency was calculated by amplifying serial dilutions of WT *H. pylori* genomic DNA, plotting the Ct values obtained per dilution and calculating the slope. Efficiencies were derived from the slope with the equation Efficiency = -1 + 10(-1/slope) (177). Relative fold changes were calculated using the ΔΔCt method with Pfaffl correction for PCR amplification efficiency, using 16S and gapB as reference genes with primers listed 5’-3’ below (177). 16S forward: GGAGGATGAAGGTTTTAGGATTG; 16S reverse: TCGTTTAGGGCGTGGACT; katA forward: AGAGGTTTTGCGATGAAGT; katA reverse: CGTTTTTGAGTGTGGATGAA; gapB forward: GCCTCTTGCACGACTAACGC; gapB reverse: CTTTGCTCACGCCGGTGCTT.

*In vitro* treatment of *H. pylori* with H2O2:

Overnight cultures of *H. pylori* strains were adjusted to OD600 = 0.2, split into two cultures with one receiving treatment with 1 mM H2O2 for twenty minutes. RNA isolation and qPCR protocols were identical to that described above.
Figure 1. *tlpD* mutants have deficits in gland occupancy in WT mice but not colonization of total tissue or individual glands

Comparison of colonization of WT mice by *H. pylori* GFP+ SS1 WT (*n* = 4), *tlpD* (*n* = 4) and *cheY* (*n* = 3). Mice were orally infected, and stomachs were collected and analyzed for tissue and gland colonization after 2 weeks of infection. (A)
CFU/gram for corpus or antrum regions. (B) Gland loads in the isolated corpus and antral glands. These numbers are the average number of bacteria counted per gland, excluding uninfected glands. Infected gland numbers are: WT corpus (436 glands from 6 mice), WT antrum (508 from 6 mice), tlpD corpus (67 glands from five mice), tlpD antrum (48 glands from five mice), cheY corpus (58 glands from four mice), cheY antrum (24 glands from four mice). (C) Gland occupancy in the isolated corpus and antral glands, representing the percentage of glands infected with the indicated *H. pylori* strain. Error bars represent standard error of the mean (SEM) for all panels. Numbers of mice infected are the same as described for gland loads. Statistical differences are indicated by * (*P* < 0.05) and ** (*P* < 0.01) as analyzed by Student T-test.
A

![Graph A](image)

B

![Graph B](image)
Figure 2. *tlpD* mutants show evidence of ROS exposure in vivo

Comparison of catalase mRNA expression *in vitro* and *in vivo* between *H. pylori* strains. (A) Mean +/- SEM of fold change increases in *katA* mRNA of *H. pylori* strains exposed to 1 mM H$_2$O$_2$ for twenty minutes, normalized to *gapB*. (B) Comparison of mean +/- SEM of *katA* expression by *H. pylori* strains in three WT mice, normalized to *gapB*. Statistical differences are indicated by * (P < 0.05) and ** (P < 0.01) as analyzed by Student T-test, with actual p values indicated above the bar. *gapB* expression was insensitive to H$_2$O$_2$ exposure based on comparison to 16S rRNA.
Figure 3. Loss of epithelial H₂O₂ rescues tlpD mutant gland defects

Colonization of Duoxa⁻/⁻ mice by WT (n = 4), tlpD (n = 5) and cheY (n = 5) GFP+ H. pylori SS1 strains at two weeks post-infection. Mice were orally infected, and stomachs were collected and analyzed for tissue and gland colonization. (A) CFU/gram at two weeks post-infection for corpus or antrum regions. Data for WT mice are the same as in Fig. 1, and are reshown here for comparison. (B)
Gland loads in the isolated corpus and antral glands, representing the average number of bacteria counted per gland, excluding uninfected glands. Infected gland numbers are: WT corpus (313 glands from six mice), WT antrum (472 glands from 6 mice), tlpD corpus (132 from six mice), tlpD antrum (149 glands from three mice), cheY corpus (24 glands from three mice). (C) Gland occupancy in the isolated corpus and antral glands, representing the percentage of glands infected with the indicated *H. pylori* strain. Error bars represent SEM for all panels. Numbers of mice infected are the same as described for gland loads. Statistical differences are indicated by * (\(P < 0.05\)) and ** (\(P < 0.01\)) as analyzed by Student T-test.
A

![Graph A](image)

- WT corpus
- WT antrum
- tlpD corpus
- tlpD antrum
- cheY corpus
- cheY antrum

B

![Graph B](image)

- Corpus
- Antrum

C

![Graph C](image)

- Corpus
- Antrum
Figure 4. Loss of immune superoxide rescues tlpD mutant gland

Colonization of Cybb−/− mice by WT (n = 6), tlpD (n = 14) and cheY (n = 6) GFP+ H. pylori SS1 strains at two weeks post-infection. Mice were orally infected, and stomachs were collected and analyzed for tissue and gland colonization. (A) CFU/gram at two weeks post-infection for corpus or antrum regions. Data for WT mice are the same as in Fig. 1, and are reshown here for comparison. (B) Gland loads in the isolated corpus and antral glands, representing the average number of bacteria counted per gland, excluding uninfected glands. Infected gland numbers are: WT corpus (69 glands from three mice), WT antrum (89 glands from 3 mice), tlpD corpus (107 glands from three mice), tlpD antrum (60 glands from three mice), cheY corpus (31 glands from three mice), cheY antrum (37 glands from three mice). (C) Gland occupancy in the isolated corpus and antral glands, representing the percentage of glands infected with the indicated H. pylori strain. Error bars represent SEM for all panels. Numbers of mice infected are the same as described for gland loads. Statistical differences are indicated by * (P < 0.05) and ** (P < 0.01) as analyzed by Student T-test.
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Exploring possible mechanisms of TlpD ROS sensing

Introduction:

TlpD responds to a set of stimuli that likely involve reactive oxygen species (ROS) formation in either the cytoplasm or the extracellular environment. These conditions include extracellular iron, hydrogen peroxide (H$_2$O$_2$), superoxide generators (166) and electron transport chain inhibitors (11). Although these stimuli may not represent the entirety of the signals sensed by TlpD, they do suggest at a minimum that the chemoreceptor is capable of sensing and responding either directly or indirectly to a consequence of oxidative stress imparted by cytoplasmic ROS. Despite the recognition that the chemoreceptor likely responds to conditions that involve ROS, little is known about how TlpD senses and responds to these stimuli. To analyze how TlpD could sense and respond to ROS, we have explored mechanisms employed by other ROS sensors and how these responses could be tied to chemotaxis receptors.

The most clearly understood ROS sensors in bacteria are transcription factors. Transcription factors have been described in bacteria that respond to H$_2$O$_2$, superoxide and reactive electrophiles (178). Although these proteins sense and respond to ROS differently, some commonalities exist between their responses. The first common theme among these sensors is that ROS is sensed
by the protein by direct oxidation at sensitive amino acids including cysteines (179), and in rare cases histidines (180). Alternatively oxidation can occur at metal centers including mononuclear iron (181), or iron sulfur clusters (182) which can result in the loss or exchange of metals that are bound by the protein. Another feature of these oxidation-driven responses is that they are reversible by reduction, which allows a sensitive and transient response to the stress as the cell copes with oxidative stress (178).

We considered whether TlpD could use some of these concepts to respond to cytoplasmic ROS. The first option to consider was whether reversible oxidation at either cysteine residues drove repellent responses. TlpD possesses a number of cysteines at its N- and C-terminus that could potentially undergo oxidation. In addition, TlpD was previously shown by inductively coupled plasma mass spectrometry (ICP-MS) to interact with both iron and zinc, with zinc binding occurring at a domain in its C-terminus called the chemoreceptor zinc binding domain (CZB), which coordinates zinc through cysteine and histidine residues (51). This raised the possibility that oxidation involving or excluding bound metals could drive repellent responses.

The other feature apparent in other model ROS sensors was the transient nature of the given response during oxidative stress. Classical chemotactic responses, including other repellent responses, are dynamic and initiated either
attractant or repellent responses when their cognate ligands were available. The observation that cytoplasmic iron chelation by pretreatment with dipyridyl abrogated repellent responses to H\textsubscript{2}O\textsubscript{2} suggested that consequences of oxidative stress that were remedied by other bacteria upon clearance of oxidative stress could counter TlpD repellent responses (166).

Finally, we were interested in what role membrane association and other chemotaxis signaling proteins played in TlpD ROS repellent responses. TlpD has been previously observed in both soluble and membrane associated fractions previously. Immunofluorescence had also revealed that TlpD was capable of coordinating a chemotaxis complex at the pole in the absence of transmembrane chemoreceptors (166). These observations suggested that TlpD organized a chemotaxis complex in a membrane associated state, although it was unclear how the chemoreceptor established a chemotaxis signaling complex at the pole in the absence of transmembrane chemoreceptors. It seemed likely that TlpD physically interacted with certain chemotaxis signaling proteins, and that these interactions were important for membrane association based on fractionation and immunofluorescence. These proteins included transmembrane chemoreceptors, CheA, CheW and the alternative coupling protein CheV1 (166, 183). Despite these findings, it remained unclear how the chemoreceptor organized itself in a membrane associated state, and more specifically what role
individual chemotaxis signaling proteins played in chemotactic responses mediated by TlpD.

In order to gain an appreciation for how TlpD sensed and responded to ROS specifically, we examined some of the core principles that other model sensors employed to sense ROS. Specifically, we were interested if TlpD underwent oxidation during ROS exposure, and if so we could detect cysteine oxidation, metal loss or exchange and finally whether the elimination of ROS would counter TlpD-mediated repellent responses. Results presented below suggest that cysteine oxidation was not apparent, but zinc loss was detected following *H. pylori* treatment with ROS-producing treatments. These results may suggest that zinc loss, perhaps due to oxidation of coordinating zinc histidine residues contribute to toward cytoplasmic ROS sensing. Additionally TlpD repellent responses were sensitive to elimination of H₂O₂ by either exogenous catalase or reductant application. Taken together these results argue against TlpD responding to cytoplasmic ROS by oxidation at cysteines, but may involve zinc loss during transient repellent responses to ROS.

As it was unclear how TlpD established membrane association, we explored a family of intermediate filament like proteins called coiled coil rich proteins (CCRP) (184) which were proposed to operate similarly to other bacterial pole organizing factors. CCRPs were suspected to play a similar role to
the well studied cell pole organizing protein PopZ in *Caulobacter crescentis*, where a protein called PopZ polymerizes at the pole and coordinates activities including chromosome attachment during cell division as well as organizing other proteins at the pole (185). *H. pylori* encodes four CCRP proteins: ccrp 58, ccrp 59, ccrp 1142 and ccrp 1143. Mutation of ccrp genes leads to pleiotropic defects including alterations in cellular morphology (186), motility defects (186), and ion homeostasis or import and T4SS defects (187). To address the possible role of pole organization by CCRP proteins, we have assessed their role on TlpD membrane association and repellent responses mediated by the chemoreceptor. It appears that both membrane association, as monitored by membrane fractionation and western blotting, appear negatively affected by the loss of either ccrp 59 or ccrp 1143. ccrp 1143 mutants also do not mount repellent responses to elevated extracellular iron, but do mediate repellent responses to acid. Repellent responses to iron have been attributed to TlpD activity solely, while repellent responses to acid have recently been reported to be mediated by TlpD and the transmembrane chemoreceptor TlpA and TlpB (102, 165). Taken together these results suggest that Ccrp proteins may be involved in TlpD membrane association, and furthermore that specific members of this group, namely Ccrp 1143, may be more specifically involved in TlpD functionality.

Finally we assessed the contribution of individual chemotaxis signaling proteins on TlpD-mediated repellent responses. The contribution of individual
chemotaxis signaling proteins was assessed by monitoring mutant *H. pylori* for their ability to mount chemotactic repellent responses to elevated extracellular iron, a response that is attributed solely to TlpD. The results suggest that the loss of two CheV proteins, CheV1 and CheV3, and the CheY phosphatase CheZ show dampened or complete loss of repellent responses to extracellular iron.

**Results:**

TlpD cysteine oxidation is not apparent during *H. pylori* exposure to ROS

To examine whether cysteine oxidation occurred during TlpD repellent responses, we decided to monitor the oxidation state of cysteines after *H. pylori* were exposed to ROS. The oxidation state of cysteine was monitored through differential alkylation, a method which allows discrimination of reduced and reversibly oxidized (disulfide or sulfenic acid) cysteines (188). Reduced cysteines were labeled with either N-ethyl maleimide (NEM) or a PEG-derivatized NEM which imparted either small (\(~1\) kDa) or large (\(~10\) kDa) apparent mass shifts to proteins per labeled cysteine, respectively. Discrimination between reduced and oxidized cysteines is possible due to a two-stage labeling strategy in which a first label is used initially that forms stable adducts with reduced cysteines but is non-reactive with oxidized cysteines. Subsequently, unlabeled cysteines are reduced and a second alkylation agent is
applied to form adducts with newly reduced cysteines. To prevent cross contamination of labels, lysates are washed following each incubation and ensure that mass shifts can be attributed to either the primary or secondary label. Labeling was then assessed by comparing TlpD migration in SDS PAGE by western blotting.

To assess whether TlpD cysteines were oxidized during repellent responses, we compared the migration of TlpD by western between untreated and ROS treated *H. pylori*. The concentrations and durations of treatments used had been previously shown to provoke TlpD-dependent repellent previously (166). Both NEM and PEG-NEM were capable of labeling TlpD in lysates when applied as the primary label, suggesting that both formed stable adducts with cysteines in the chemoreceptor. In addition secondary labeling of cysteines was apparent in labeling experiments where improper care was taken and rampant oxidation occurred during labeling (Fig. 1A). In these cases three populations of TlpD were visible, the majority was labeled with a primary label, PEG-NEM, in this case a smaller population was labeled variably with NEM and a portion of TlpD remained unlabeled (Fig 1A). When proper care was taken to prevent oxidation during sample preparation however, only the primary label was detected based on migration changes in TlpD western blots (Fig 1B). The inability to observe secondary labeling following *H. pylori* treatment with ROS
suggested that cysteine oxidation was not a physiological event that occurred during oxidative stress events that provoked TlpD repellent responses.

TlpD iron and zinc content change during *H. pylori* exposure to paraquat and dipyridyl.

Metal exchange or loss can occur due to oxidation of either metal centers or amino acids including cysteine and histidine that coordinate the metals (142), which can activate certain ROS sensors (178). Prior studies indicated that TlpD interacts with zinc through a C-terminal domain that coordinates the metal through histidines and a cysteine, and additionally appears to interact with iron (51). Our prior results had suggested that chelating cytoplasmic iron abrogated repellent responses to H₂O₂, which lead to the possibility that TlpD-metal interactions could drive repellent responses (166).

To investigate this possibility, we immunoprecipitated TlpD and used inductively coupled plasma mass spectrometry (ICP-MS) to investigate its iron and zinc content. We isolated TlpD from a *H. pylori* strain that expressed TlpD as the sole chemoreceptor, which we will refer to as TlpD-only. Employing this strain was necessary as we lacked a TlpD-specific antibody. TlpD-only *H. pylori* were grown overnight in the presence of stable 57FeCl and 67ZnCl isotopes and subsequently split into cultures that remained untreated or were treated with
paraquat or dipyridyl for fifteen minutes, at a concentration and duration of treatment previously shown to trigger or counter TlpD-mediated repellent responses respectively (166). Cultures were subsequently concentrated by centrifugation, lysed, and TlpD was immunoprecipitated. Immunoprecipitated TlpD was submitted for ICP-MS to compare the iron and zinc content of the immunoprecipitate. Following ICP-MS, the concentration of TlpD within the immunoprecipitate was estimated by western blot with purified TlpD standards. We thus calculated the number of moles of Zn or Iron per TlpD, and compared this to the amount after paraquat treatment. Using these estimated concentrations we were able to compare the metal concentrations obtained from ICP-MS to the TlpD concentrations in the precipitate. TlpD isolated from untreated *H. pylori* showed zinc content, as judged from a prior report, where prior findings suggested a rough estimate of ~0.7 zinc/TlpD and our results found 0.74 zinc/TlpD. The iron content differed from this report however, with our findings were roughly double prior estimates of iron content, with a 0.24 iron/TlpD (51). Treatment with dipyridyl, which prevents TlpD repellent responses to *H₂O₂* (166), did not appear to impact the iron content of TlpD, with a slight shift to 0.21 iron/TlpD, but did show an increase in the proportion of zinc to 0.84 zinc/TlpD. This result suggests that dipyridyl treatment likely does not impact the iron bound by TlpD, but rather chelates cytoplasmic iron during treatment and dampens Fenton driven ROS formation. Paraquat treatment had an effect on both iron and zinc content, with iron dropping roughly half to 0.14
iron/TlpD and zinc to 0.25 zinc/TlpD. These effects are striking as they suggest that metal loss, particularly zinc, may occur during TlpD-driven repellent responses (Fig. 2). Other ROS sensors employ zinc exchange during sensing (189, 190), with concurrent oxidation of coordinating cysteine residues typically, although histidine oxidation has also been reported. It is also worth noting that iron and zinc appear to bound at less than full TlpD occupancy, which is to say that TlpD is not saturated by either metal in untreated or treated conditions. Iron and zinc may not occupy the same site in the chemoreceptor, as mutation of zinc-coordinating residues does not appear to impact zinc binding as measured previously (51), although this does not rule out the possibility that iron and zinc binding may preclude the other.

TlpD does not activate CheA autophosphorylation in response to iron or H$_2$O$_2$ in vitro

To explore the possibility that iron and zinc could compete for TlpD during repellent responses, we considered the possibility that TlpD could sense oxidative stress through binding either iron during oxidative stress, as the concentration of iron was shown previously to increase in H. pylori cultures grown at atmospheric oxygen tensions (141), which could coincide with zinc loss. It seemed possible then that TlpD could sense oxidative stress through binding iron as its concentration rose in the cytoplasm due to inactivation of
mononuclear iron containing proteins (191). To test this possibility we assessed whether iron addition impacted TlpD activation of CheA autophosphorylation activity \textit{in vitro}.

CheA autophosphorylation was assessed in complexes containing purified TlpD, CheA and CheW, by autoradiography, as done before (183). Autophosphorylation was assessed by initiating kinase activity in premixed protein complexes with the addition of radiolabeled ATP, and aliquots of the reaction were quenched in SDS sample buffer over a time course. The contribution of metals were tested by adding varying molar ratios of iron to reactions containing either CheA alone, or to an complex of CheA, CheW and TlpD. We first evaluated whether TlpD appeared functional, by examining TlpD-dependent CheA activation. TlpD plus CheW activated CheA autophosphorylation, suggesting that the chemoreceptor was able to interact with the kinase via CheW (Fig 3A). Iron addition showed varying effects; the addition of iron at 1x or 10x molar ratios resulted in no change in CheA autophosphorylation relative to control complexes. The addition of 100x iron affected CheA autophosphorylation activity, resulting in 20-fold greater phosphorylation levels at 10 seconds. The same response occurred in CheA+CheW and CheA+CheW+TlpD (Fig 3B). These results suggest TlpD did not respond to iron \textit{in vitro}, and furthermore suggests that CheA kinase activity is
responsive to the addition of iron. The significance of this regulation of CheA activity by iron as it relates to chemotactic responses in intact *H. pylori* is unclear.

To test whether hydrogen peroxide could be sensed directly *in vitro*, purified complexes were exposed to varying molar ratios of H$_2$O$_2$ (50x, 2500x, 5000x) during incubation prior to initiation of autophosphorylation activity. The effects of H$_2$O$_2$ appeared slightly activating for reactions containing solely CheA at 50x and 2500x, while complexes containing CheW and TlpD seemed unaffected at these concentrations. At the highest concentration of H$_2$O$_2$ tested however all complexes showed reduced autophosphorylation activity (Fig 3C). The observation that complexes containing TlpD were not stimulated at lower concentrations of H$_2$O$_2$, and were inactivated similarly to complexes lacking the chemoreceptor suggests that *in vitro* the addition of the oxidant does not seem to stimulate a repellent response that would be inferred from increased autophosphorylation activity. This finding suggests that that TlpD does not appear to sense H$_2$O$_2$ directly *in vitro* under these conditions.

TlpD repellent responses are transient

The logic of responding to a given input by sensors, including ROS, includes a cessation of the given response following the clearance of the stress. Other well characterized sensors of ROS respond transiently due to the eventual
reduction of oxidations that drive ROS sensing (178). A transient response is especially critical for chemoreceptors, as these responses are short and rapid. Despite an understanding that TlpD responded rapidly to ROS, it was unknown whether repellent responses mediated by the chemoreceptor would abate following clearance of the stress or given ROS. To follow TlpD repellent responses over time, we compared swimming direction change frequency, which was indicative of chemotactic responses, where elevation of the frequency represented a chemotactic repellent response (166). This direction change frequency was recorded by filming cultures and manually tracking direction changes for one hundred cells. The swimming behavior of WT SS1 H. pylori was recorded, prior to H₂O₂ exposure, immediately after treatment and at subsequent time points. WT SS1 showed a robust repellent response following treatment with 1 mM H₂O₂, with an elevated frequency of direction changes relative to untreated cells immediately after treatment. To gain an understanding of the duration of the repellent response we incubated the H₂O₂ for an additional twenty minutes. At this time point WT SS1 showed a persistent repellent response, similar to that which was observed immediately after application (Fig. 4A). This suggested that TlpD-mediated repellent responses persisted over this duration, and furthermore that the ROS was not cleared by the culture by twenty minutes. To test the possibility that clearance of ROS would impact the TlpD-mediated repellent response, we exposed WT SS1 to 1 mM H₂O₂, and immediately treated the culture with either the reductant
dithiothreitol (DTT) or exogenous catalase for twenty minutes before filming the culture again.

The addition of either catalase or DTT did appear to affect the sustained repellent response observed previously at twenty minutes post treatment, and cells showed a significantly lower direction change frequency that was observed in cultures prior to or immediately after addition of H$_2$O$_2$ (Fig 4B). This result suggested that a sustained repellent response observed was likely due to incomplete clearance of the initial treatment of H$_2$O$_2$. Furthermore these results suggest that the signal TlpD intercepts during oxidative stress is transient, and likely diminishes as ROS is cleared by detoxification systems.

The loss of CCRP proteins impacts TlpD polar localization and repellent responses.

To gain a better understanding of how Ccrp proteins impact motility we obtained G27 ccrp 59 and ccrp 1143 mutants provided by Nina Salama. We carried out two sets of experiments to analyze whether the CCRP proteins are needed for polar localization of chemotaxis proteins in *H. pylori*. We initially examined the localization of two chemotaxis proteins found at the pole in distinct complexes via immunofluorescence. CheA is found in complexes at the pole that contain chemoreceptors, the coupling protein CheW, and accessory
CheV coupling proteins. CheZ is also found at the pole in a distinct complex with the accessory chemotaxis protein ChePep. The localization of CheA and CheZ was examined by staining fixed ccrp59 and ccrp1143 cells with antibodies specific for either CheA or CheZ. Interestingly, aberrant CheA and CheZ localization was observed in both ccrp59 and ccrp1143 mutants although not through the entire population of cells in either case. Due to the incomplete penetrance of the phenotype, we did not follow up with further localization studies although the observation warrants further study.

To assess whether the loss of Ccrp 59 or Ccrp 1143 impacted TlpD-mediated chemotactic responses, we filmed and tracked the swimming behavior of liquid grown *H. pylori* to compare the frequency of direction changes observed when the strains were exposed to either excess iron or acid. Exposure to iron induces a repellent response characterized by an increased frequency of direction changes mediated by TlpD (166), while acid exposure induces a repellent response mediated by both TlpD, TlpA and TlpB (102, 192). WT, ccrp 59, and ccrp 1143 were grown overnight and assessed for a chemotactic response to iron by diluting the cultures in HAMS107 10% FBS containing 100 μM ferric chloride or 50 mM HCl. To assess the chemotactic response of these mutants to these stimuli, the frequency of direction changes over a three second time course was counted manually. The loss of Ccrp 59 and Ccrp 1143 appear to impact *H. pylori* chemotaxis differentially. Untreated ccrp 59 mutants showed a similar frequency
of direction changes to isogenic WT *H. pylori*, while ccrp 1143 mutants displayed a smooth-swimming phenotype that resembles either non chemotactic or tlpD deficient strains (Fig. 6). Further, the loss of either ccrp 59 and 1143 did not impact repellent responses following treatment with 50 mM HCl, with both strains showing repellent responses, albeit to lower degrees than WT *H. pylori*. Responses to elevated iron were not similar between the Ccrp mutants however, ccrp 1143 mutants did not respond to the presence of 1 mM iron chloride, while Ccrp 59 mutants retain a WT-like response (Figure 2). These results suggested that Ccrp proteins appeared to impact the repellent responses to acid generally, and showed a dampened response, while ccrp 1143 mutants showed more specific defects in TlpD mediated repellent responses to iron.

The loss of CheV1, CheV3 and CheZ abrogates TlpD repellent responses to iron.

In order to separate the contribution of membrane association and repellent responses we investigated whether the loss of individual chemotaxis signaling proteins negatively impacted TlpD-mediated repellent responses. In order to test this we filmed and tracked the swimming behavior of chemotaxis mutants and compared the direction change frequency the bacteria exhibited after supplementing media with iron.
The swimming behavior of WT, *tlpD*, cheV1, cheV2, cheV3, chePep and cheZ mutants were filmed and tracked prior to and after iron supplementation. As expected, WT *H. pylori* showed a repellent response to iron addition characterized by an increased direction change frequency (Fig. 7). *tlpD* mutants failed to mount a repellent response to iron as was observed previously (166). cheV1, cheV3 and cheZ *H. pylori* also did not display a significant increase in their direction change frequency following iron supplementation, while all other strains showed repellent responses following iron treatment (Fig 7). These results suggest that CheV1, CheV3 and CheZ are actively involved in TlpD repellent responses, while the loss of other chemotaxis signaling proteins appears dispensable.

**Conclusions:**

The recognition that TlpD responds to conditions that either promote the formation of ROS in its environment or in the cytoplasm begs the question of how the chemoreceptor senses and responds to these stimuli. Without prior data relating to a mechanism, we have considered other ROS sensors and their means of sensing ROS in the cytoplasm. These include oxidation of sensitive amino acids, metal exchange or loss during oxidative stress, iron sensing in vitro and finally whether repellent responses mediated by the chemoreceptor are transient following elimination of ROS.
Cysteine oxidation was assessed with differential alkylation, a method that allows discrimination between reduced and oxidized cysteines. We conducted a two stage labeling procedure which permits labeling of reduced cysteines with labels that impart variable apparent mass increases. Although it appeared that both labels employed in the study were capable of forming adducts with reduced cysteines, we did not detect adduct formation with secondary labels suggesting that TlpD cysteines did not undergo oxidation during exposure to ROS generating treatments. These results suggest that a model where TlpD cysteine oxidation serving as a ROS sensor in the chemoreceptor is unlikely. If however only a fraction of TlpD underwent oxidation, this method would be largely insensitive to detect mass shifts for a minority of the protein in the population. Alternative methods employing cysteine labeling by differential alkylation followed by identification of sites modified by mass spectrometry (193) could be a more sensitive way to detect a small, but possibly relevant minority population which could be relevant for TlpD responses to ROS.

The next possibility we tested involved metal loss or exchange during TlpD ROS sensing. Other ROS sensors can undergo oxidation at metal centers which ultimately drives activity relating to remedying oxidative stress (178). We tested this model by exposing TlpD-only *H. pylori* to paraquat and dipyridyl,
which have been shown previously to initiate, or prevent repellent responses by TlpD (166). Metal content of the chemoreceptor was monitored by ICP-MS, and metal concentrations were subsequently correlated to the concentration of TlpD present in immunoprecipitates used for the analysis. The results of this work indicate that iron and zinc content of TlpD is sensitive primarily to paraquat treatment, where the relative concentrations of both metals decreased. These results suggest that zinc loss in particular may be of importance during TlpD repellent responses, and would be worthwhile following up upon.

Cytoplasmic iron concentrations in *H. pylori* increase during oxidative stress inducing treatments, including growth at atmospheric oxygen tensions (141). One possible caveat of our determination of the metal content of TlpD was that oxidation occurred during lysis or preparation of samples, masking metal binding that would have normally occurred during oxidative stress. To test the possibility that TlpD serves as an iron sensor, we determined whether iron addition impacted TlpD activation of CheA autophosphorylation in vitro. TlpD showed activation of CheA kinase activity compared to reactions that lacked the chemoreceptor as noted previously (183), suggesting that TlpD physically associated with both CheW and CheA to activate its autophosphorylation. This result suggested that the purified chemoreceptor was functional. The addition of iron at equimolar or 10x molar excess appeared to have no significant effects on autophosphorylation activity in complexes containing or lacking TlpD. When
iron was added at 100x molar excess however CheA kinase activity showed a drastic increase, with roughly 20x elevated autophosphorylation measured by densitometry compared to untreated complexes (Fig 3B).

These results argue against TlpD serving as an iron sensor, but the activation of CheA autophosphorylation activity is striking regardless. CheA requires magnesium for its autophosphorylation activity and subsequent transfer of the phosphoryl group to CheY (194), although to the best of our knowledge there are no prior reports that suggest the kinase responds directly to iron. In addition, TlpD does not appear to respond to H₂O₂ directly in vitro, as CheA autophosphorylation in complexes containing the chemoreceptor were unaffected or dampened by high concentrations of the oxidant. Taken, together TlpD does not appear to be a direct iron or H₂O₂ sensor, although it is worth noting that we cannot fully rule out the possibility that our reaction conditions do not mimic changes that would occur in the cytoplasm during oxidative stress.

The methods use to monitor TlpD repellent responses previously did not provide information relating to whether the responses were transient. It seemed unlikely that TlpD responded to a long-lived signal generated by oxidative stress, although we and others had no data to refute this possibility. A more plausible possibility was that TlpD recognized a short-lived signal generated by oxidative stress. This would allow the chemoreceptor to initiate sensitive and transient
repellent responses while *H. pylori* was undergoing oxidative stress. To investigate this option we followed the swimming behavior of *H. pylori* prior to and immediately following treatment with H$_2$O$_2$, and after an incubation with either catalase or DTT which should eliminate extracellular H$_2$O$_2$. This was required due to the observation that repellent responses were sustained after H$_2$O$_2$ addition for at least twenty minutes. This suggests that the concentration of the oxidant provided was not cleared by endogenous systems at this point, and the bacteria were likely still experiencing oxidative stress. The results of these experiments suggest that TlpD does indeed show transient repellent responses while *H. pylori* was undergoing oxidative stress. Repellent responses that were evident following initial treatment with H$_2$O$_2$ were not present after incubations with either catalase or DTT, suggesting that the signal intercepted by the chemoreceptor was transient.

**CCRP proteins play an important but unclear role in *H. pylori* which includes morphology (186), motility defects (186), and ion homeostasis or import (187) and T4SS defects (187). In order to gain a greater understanding of their potential role in chemotaxis, we have analyzed whether the loss of either Ccrp 59 or Ccrp 1143 impacted chemotaxis by either disrupting chemotaxis complex localization, TlpD membrane-association or TlpD mediated chemotactic responses. The loss of either Ccrp 59 or Ccrp 1143 showed aberrant chemotaxis complex localization, although the phenotype was apparent in only a proportion**
of a population of cells, although the precise impact is unclear as it was not measured. Additionally the loss of Ccrp 1143 but not Ccrp 59 appeared to impact the TlpD-mediated repellent response to elevated extracellular iron. These results implicate these bacterial intermediate-filament like family in the membrane association of TlpD, however the contribution of individual members of the family, specifically Ccrp 1143 appear to have a more profound effect on TlpD-mediated repellent responses. As this family of proteins appears to have pleiotropic effects on H. pylori biology, it would be intriguing to probe further into the localization and contribution of individual members on chemotactic effects including those mediated by TlpD.

The contribution of chemotaxis signaling proteins on chemotactic responses mediated by TlpD were assessed next. TlpD-specific chemotaxis defects could be differentiated between membrane association defects and those specific to CheA activation following the initiation of repellent responses. Prior data had showed that mutants lacking transmembrane chemoreceptors, cheA, cheW and cheV1 showed reduced TlpD in membrane fractions (166). This data did not show however the contribution of other chemotaxis signaling proteins on TlpD-mediated repellent responses. Our analysis suggested that there was redundancy in part with defects in membrane association and repellent responses raised against extracellular iron. Specifically cheV1, cheV3 and cheZ mutants showed abrogated repellent responses to iron. The loss of CheV1 would
be expected to prevent chemoreceptor complex formation at the pole, as reported previously (183), however the role of CheV3 is less clear. Furthermore the loss of CheZ would be expected to impact chemotactic responses, as it dephosphorylates the response regulator CheY (107) and should therefore dampen the sensitivity of repellent responses.

Taken together these results have not provided a concrete model for how TlpD senses and responds to cytoplasmic ROS, but have eliminated models employed by other ROS sensors. The signal that TlpD responds to has remained elusive although it appears to be transient during oxidative stress. TlpD has been reported to respond to other stimuli that are not classically associated with oxidative stress including acid shock (165), which raises the possibility that the signals sensed by the chemoreceptor may not be restricted to oxidative stress. Further exploration of this possibility may provide clues to the identity of the ligand or conditions that TlpD senses to initiate repellent responses.

**Methods:**

Bacterial strains and culture conditions.

*Helicobacter pylori* strains mG27, and G27 used for experimental studies are listed in Table 1. *Helicobacter pylori* was cultured on either Columbia horse blood agar (CHBA), or 0.8 Ham's F-12 with 10% FBS (Ham's-10). CHBA consisted of Columbia agar (BD)
with 5% defibrinated horse blood (Hemostat Labs, Davis, CA), 50 g/ml cycloheximide, 10 g/ml vancomycin, 5 g/ml cefsulodin, 2.5 U/ml polymyxin B, and 0.2% (wt/vol) cyclodextrin.

Cysteine labeling with N-ethyl maleimidide derivatives

TlpD-only mG27 H. pylori (ΔtlpABC) (KO# 1016) (109) were grown overnight in HAMS-10 media and diluted to an OD = 0.6 and split. Split cultures remained untreated or received treatment with 10.5 μM paraquat (Sigma Aldrich) for 15 minutes at which cysteines were labeled following the protocol described (193). Briefly, cells were lysed in ice cold 20% (v/v) trichloroacetic acid (TCA) and incubated at 4ºC for 20 minutes to precipitate protein. Protein precipitates were collected by centrifugation and resuspended in 10% TCA and this was repeated in 5% TCA. Precipitates were resuspended in TUNES buffer (100 mM Tris pH 7.0, 8 M urea, 100 mM N-ethyl-maleimide (NEM) (Sigma), 10 mM EDTA, and 2% SDS, sonicated to allow access of cysteines in precipitated proteins and subsequently incubated at 50ºC for 30 minutes with shaking. Proteins were subsequently precipitated with 20% TCA, centrifuged to collect precipitated proteins and washed twice with 10% and then twice with 5% TCA. Protein was next resuspended in TUNES buffer containing 5.5 mM tris(2-carboxyethyl)phosphine (TCEP), sonicated and incubated at 50ºC for 30 minutes with shaking. Pellets were resuspended in 100 mM PEG-N-ethyl-maleimide (Sigma) and incubated at
50°C for 30 minutes. Proteins were subsequently stored at -80°C prior to separation on 15% SDS PAGE prior to western blotting.

**Western Blotting**

Samples run on SDS-PAGE gels were transferred to immunoblot polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Successful transfer and relative protein loading was visualized by staining the membrane with DB71 dye prior to antibody binding. Membranes were incubated with a 1:2000-1:5000 dilution of anti-glutathione transferase (GST)-TlpA22 (104) For visualization, the blots were incubated with the horseradish peroxidase conjugated secondary antibodies ‘goat anti-rabbit’ (Santa Cruz Biotech) at a dilution of 1:2000, followed by incubation with luminol, p-coumaric acid, and hydrogen peroxide. Luminescent blots were then exposed to a Biorad Chemidoc MP.

**Determination of TlpD metal content**

TlpD-only mG27 *H. pylori* were grown overnight in HAMS-F12 media (Sigma) supplemented with 10% fetal bovine serum and 1 uM 57FeCl₃ and 67ZnCl, which was generated by resuspending 57Fe²O₃ and 67ZnO (Isoflex) in dilute hydrochloric acid. Cultures were subsequently diluted to an OD = 0.6 and split into three cultures which remained untreated or received 50 μM dipyridyl or 10.5 μM paraquat and incubated for fifteen minutes microaerophilically.. TlpD was immunoprecipitated following the Dynabeads protein A manufacturer
protocol (Thermo scientific) and immunoprecipitates were concentrated with Centriplus concentrators (10-kDa cutoff), diluted 1:10 with MilliQ H2O and analyzed on a Thermofinnigan Neptune inductively coupled plasma (ICP) mass spectrometer by the University of California at Santa Cruz (UCSC) Keck Isotope Facility. Protein concentrations were estimated in immunoprecipitates by densitometry in ImageJ against a range of concentrations of purified TlpD whose concentration was measured using the Quick start Bradford Assay (Biorad).

Protein purification

TlpD, CheA and CheW were expressed as described previously in E. coli (51, 107, 183). TlpD and CheA were expressed in BL21 E. coli and CheW was expressed in Arctic Express DE3 E. coli. BL21 cells were grown at 37 °C and Arctic express cells were grown at 15 °C, and induced with 1 mM IPTG. GST fusion proteins (TlpD and CheW) were applied to a GST Prep column (GE Healthcare), and the GST tag was subsequently cleaved by treatment with Prescision protease treatment. His-tagged CheA was purified over a His Prep column (GE Healthcare). Proteins were subsequently dialyzed in storage buffer (50 mM HEPES, pH 7.6, 50 mM KCL, 20% glycerol (v/v)) and subsequently stored at -80 °C. Protein concentrations were estimated using the Quick start Bradford Assay (Biorad).

CheA autophosphorylation assays
Phosphorylation reactions were conducted in a reaction buffer containing 20 mM MgCl2, 50 mM KCl, 50 mM Tris/HCl pH 7.5, as utilized in previous reports (107, 183). Ninety pmole CheA, 90 pmole CheW and 90 pmole TlpD were incubated in 50 μl with or without varying molar ratios of iron chloride indicated (90, 9000, 90000 pmole) in reaction buffer for 30 minutes. Autophosphorylation was initiated by addition of an ATP solution containing 2 mM unlabeled ATP and 11 μM [γ-32P] ATP (10Ci/mMole) (PerkinElmer). Kinase activity was quenched at 10 and 30 seconds after ATP addition by mixing aliquots with 2x Laemmli sample buffer containing 10 mM EDTA. Proteins were subsequently separated on 10% SDS-PAGE gels, and dried thoroughly. exposing the gels after drying to a phosphorimager screen and quantified with Image J.

Chemotaxis analysis

Filming and tracking were performed as described previously (166). Briefly G27 H. pylori WT, cheV1, cheV2, cheV3, chePep, cheZ, tlpD, ccrp 1143 and ccrp 59H. pylori cells were grown overnight in HAMS-10 media and diluted to an OD600 = 0.12 in media and 80 μl of the diluted culture were filmed on hanging drop slides under a cover slip using phase-contrast microscopy with a Hamamatsu C4742-95 digital camera mounted on Nikon Eclipse E600 at 400 magnification. The diluted cultures were next treated with iron or H2O2 or HCl at 100 μM, 1 mM and 50 mM respectively and immediately filmed. Catalase and dithiothreitol treatments occurred immediately after H2O2 treatment at 100 units and 1 mM
respectively. Catalase and dithiothreitol treated cultures were incubated microaerophilically for twenty minutes and filmed as described for other treatments.

Videos were recorded at maximum acquisition speed, at five second durations. Files were exported as AVI files, and viewed in ImageJ. Direction changes were analyzed for individual cells in these films over three second durations. One hundred cells were tracked over this time period and the average and standard error of the mean was compared between strains and treatments by Student’s t-test.

Immunofluorescence

Immunofluorescence was performed as described in (166). Briefly, G27 H. pylori ccrp 1143 and ccrp 59 were grown overnight in HAMS-10 media to an OD600 of 0.6 and applied to poly-L-Lysine coated slides. Cells were fixed and permeabilized and preadsorbed anti-CheA or anti-CheZ antibodies were subsequently applied at 1:200 dilutions and incubated for thirty minutes. Slides were subsequently washed with blocking buffer (3% BSA, 0.1% Triton X-100 in PBS), and the secondary Goat anti-chicken antibody–Alexa Fluor 488 antibody (Abcam) was subsequently added at a 1:500 dilution and incubated in the dark for thirty minutes. Slides were washed again with blocking buffer three times, and a drop of Vectashield (Vector Laboratories) was applied to the slides prior to
sealing the slides with a cover slip using clear nail polish. Images were taken on a Zeiss LSM 5 Pascal confocal microscope. Images were taken separately in each channel and merged in Adobe Photoshop CS2.
### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>KO reference number</th>
<th>genotype</th>
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<tbody>
<tr>
<td>G27</td>
<td>379</td>
<td></td>
</tr>
<tr>
<td>G27 cheA</td>
<td>857</td>
<td>ΔcheA::cat</td>
</tr>
<tr>
<td>G27 tlpD</td>
<td>1165</td>
<td>rdxA::kan-sac, ΔtlpD::cat</td>
</tr>
<tr>
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<td>1277</td>
<td>ΔcheV1::cat</td>
</tr>
<tr>
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<td>1278</td>
<td>ΔcheV2::cat</td>
</tr>
<tr>
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<td>1279</td>
<td>ΔcheV3::cat</td>
</tr>
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<td>G27 MA Δchepep::cat</td>
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<tr>
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<td>1269</td>
<td>ΔcheZ::KanSac</td>
</tr>
<tr>
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<td>1619</td>
<td>ccrp59::cat</td>
</tr>
<tr>
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<td>ccrp1143::cat</td>
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<tr>
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<td>mG27 TlpD only</td>
<td>1016</td>
<td>ΔtlpA-21, ΔtlpB, ΔtlpC::km</td>
</tr>
</tbody>
</table>
**Figure 1A. TlpD is labeled by both N-ethyl maleimide and PEG-maleimide in the absence of reductant.**

Two split cultures of TlpD-only *H. pylori* were treated with either 1 mM H$_2$O$_2$ for five minutes, 10.5 μM paraquat for fifteen minutes or were untreated. Cysteines were labeled initially with PEG-Maleimide (P) which should impart a 10 kDa mass increase per cysteine and subsequently labeled with N-ethyl-maleimide (N) which should impart a 1 kDa mass increase per cysteine. Notably this treatment was performed in the absence of reductant during lysis. P = PEG-Maleimide labeled TlpD; N = NEM labeled TlpD; U = unlabeled TlpD. The
migration of untreated and paraquat treated TlpD-only *H. pylori* lysates were followed by western blot with a pan-chemoreceptor TlpA antibody. This blot is representative of three replicates.
Figure 1B. TlpD cysteine oxidation is not apparent during H. pylori exposure to paraquat.

Depicted are the steps of differential alkylation of a split TlpD-only H. pylori culture that was either untreated or exposed to two cultures exposed to 10.5 mM paraquat for fifteen minutes. N-ethyl maleimide was employed as the primary label, and PEG-maleimide was employed as the secondary label. The lefthand lane of control or paraquat represents TlpD migration in an unlabeled state, which runs around 48 kDa. Lanes labeled with the “+ P” represent labeling with
the primary N-ethyl maleimide label, which should impart 1 kDa apparent mass increases per reduced cysteine labeled. Lanes labeled with the “+ P” represent labeling with the secondary PEG-derivized N-ethyl maleimide label, which should impart 10 kDa apparent mass increases per reduced cysteine labeled. This blot is representative of five replicates.
Figure 2. Paraquat treatment leads to TlpD zinc loss while dipyridyl does not appear to impact TlpD metal content.

TlpD metal content was measured by ICP-MS and correlated to the concentration of protein in immunoprecipitates used in the analysis. Data represents analysis of a single split culture that was either untreated or received dipyridyl or paraquat treatment. Protein concentrations of immunoprecipitates used for ICP-MS were assessed by western blot with a purified TlpD reference. Data represents a single experiment using a split culture.
Figure 3. TlpD activates CheA kinase activity in vitro, but is not responsive to iron.

In vitro phosphorylation assay of CheA using reactions containing 90 pmole CheA, 90 pmole CheW and/or 90 pmole TlpD. Proteins were preincubated for 30 minutes to allow for complex formation and autophosphorylation was initiated by the addition of ATP. Aliquots were quenched in Laemmli sample buffer, electrophoresed on SDS-PAGE gels, exposed to a phosphorimager screen, and phosphorylated CheA was quantified by densitometry. (A) Temporal analysis of CheA phosphate (CheA-P) over time in isolation, with CheW, or with CheW and TlpD. (B) Complexes were prepared as in A in the presence or absence of equimolar (1x), 10x and 100x molar excess FeCl3 added during the 30 minutes pre-incubation step, before the addition of ATP. (C) Complexes were prepared as in A in the presence or absence of 50x, 2500x and 5000x molar excess FeCl3 added during the 30 minutes pre-incubation step, before the addition of ATP. After initiation of ATP, CheA-P was examined at seconds by densitometry and these values were compared to control reactions lacking iron. Error bars represent standard error of the mean of densitometry values of iron supplemented reactions divided by untreated controls for three replicates.
Figure 4. TlpD repellent responses to H2O2 are transient.

A) WT SS1 *H. pylori* grown overnight in BB10 media were diluted to an OD = 0.2, filmed prior to and following treatment with 1 mM H2O2. Cultures were incubated microaerophilically for twenty minutes and filmed again. B) WT SS1 *H. pylori* grown overnight in BB10 media were diluted to an OD = 0.2, filmed prior to and following treatment with 1 mM H2O2. Cultures were treated with 1 mM dithiothreitol following exposure to H2O2 and incubated for twenty minutes microaerophilically prior to another round of filming. C) WT SS1 *H. pylori* grown overnight in BB10 media were diluted to an OD = 0.2, filmed prior to and following treatment with 1 mM H2O2 and immediately treated with 1 mM dithiothreitol and incubated twenty minutes microaerophilically prior to another round of filming. Films taken of cultures were tracked manually for direction changes over a three second window. The error bars represent the standard error of the mean. *P < .05; **P < .01; ***P < .001; ****P < .0001 compared to the untreated culture using Student’s t test. Data is representative of three cultures with 100 cells tracked total.
Figure 5. The loss of Ccrp 1153 impacts the TlpD-driven repellent response to elevated extracellular iron.

Overnight cultures of *H. pylori* were diluted into fresh media and filmed in the presence or absence of either 1 mM iron chloride or 50 mM hydrochloric acid. Direction changes over a three second window were enumerated and averaged for one hundred cells per strain in each condition, averaged and the standard error of the mean is plotted above. *P < .05; **P < .01; ***P < .001; ****P < .0001. Data represents two independent cultures for all strains.
Figure 6. The loss of CheV1, CheV3 and CheZ abrogate TlpD repellent responses to iron.

The loss of CheV1, CheV3 and CheZ impacts repellent responses to extracellular iron. Overnight cultures of *H. pylori* were diluted into fresh media and filmed in the presence or absence of either 1 mM iron chloride. Direction changes over a three second window were enumerated and averaged for one hundred cells per strain in each condition, averaged and the standard error of the mean is plotted above where *P < .05. Data represents two independent trials.
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Future directions

This work collectively has shed some light on the signals that TlpD responds to during repellent responses and their relevance during host colonization. There are of course many open questions that remain concerning how the chemoreceptor senses ROS and the exact nature of TlpD control of tactic behaviors in the host.

The contribution of iron and zinc that is bound by TlpD on tactic responses is one open question that relates to the recognition of ROS by the chemoreceptor. TlpD binds zinc at the CZB domain through histidines and a cysteine, and it is unclear where iron binding occurs at the chemoreceptor (51). In other ROS sensing proteins, zinc coordinating cysteines can be poised for oxidation which can subsequently lead to metal loss and transient oxidation forming disulfides which drives protein activity (189, 190). Results presented suggest that zinc loss may occur during *H. pylori* exposure to paraquat, which would be consistent with these model ROS sensors. Experiments should be repeated to measure TlpD metal interactions during repellent responses to validate the results discussed in the prior chapter, as it could offer a model to explain ROS sensing by the chemoreceptor. Zinc loss and resulting reversible oxidation at the CZB domain could represent a mechanism
for TlpD ROS sensing and would be a step forward in understanding the role of the CZB domain in TlpD and other proteins containing the domain.

Colonization defects of tlpD mutants appear to be connected to gastric gland colonization and possibly the migration of H. pylori between glands during infection. Due to the method used to study gland colonization we cannot discern the position that infected glands originated in the tissue itself. Presumably H. pylori migrates to local neighboring glands through the combination of repellent responses that drive the bacteria out from glands and attractant responses that pull them back toward presumably uncolonized glands. Explorations of the dynamics of gastric gland colonization by H. pylori would be aided by microscopy techniques that would allow for analysis of intact tissue including glands. This would allow for colonization to be viewed as it occurs during infection, and ideally would allow for visualization of H. pylori within glands. Prior results suggest that H. pylori is driven increasingly into glands as infection proceeds (164), therefore it is tempting to speculate that these sites serve as a reservoir of bacteria that are able to persist into chronic infection. Investigating new methods to explore colonization of the gastric epithelium stands to pay dividends in allowing investigators to have a more clear view of the distribution of H. pylori during the course of infection.

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