

1 **Four chemoreceptors govern bidirectional pH taxis in *Bacillus subtilis***

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10 Running Head: pH taxis in *Bacillus subtilis*

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16 **ABSTRACT**

17 We investigated pH taxis in *Bacillus subtilis*. This bacterium was found to perform
18 bidirectional taxis in response to external pH gradients, enabling it to preferentially
19 migrate to neutral environments. We next investigated the chemoreceptors involved in
20 sensing pH gradients. We found that four chemoreceptors are involved in sensing pH:
21 McpA and TlpA for sensing acidic environments and McpB and TlpB for alkaline ones.
22 In addition, TlpA was found to also weakly sense alkaline environments. By analyzing
23 chimeras between McpA and TlpB, the principal acid and base-sensing
24 chemoreceptors, we identified four critical amino-acid residues – Thr¹⁹⁹, Gln²⁰⁰, His²⁷³,
25 and Glu²⁷⁴ on McpA and Lys¹⁹⁹, Glu²⁰⁰, Gln²⁷³, and Asp²⁷⁴ on TlpB – involved in sensing
26 pH. Swapping these four residues between McpA and TlpB converted the former into a
27 base receptor and the latter into an acid receptor. Based on the results, we propose that
28 disruption of hydrogen bonding between the adjacent residues upon pH changes
29 induces signaling. Collectively, our results further our understanding of chemotaxis in *B.*
30 *subtilis* and provide a new model for pH sensing in bacteria.

31

32 **IMPORTANCE**

33 Many bacteria can sense the pH in their environment and then use this information to
34 direct their movement towards more favorable locations. In this study, we investigated
35 the pH sensing mechanism in *Bacillus subtilis*. This bacterium preferentially migrates to
36 neutral environments. It employs four chemoreceptors to sense pH. Two are involved in
37 sensing acidic environments and two are involved in sensing alkaline ones. To identify
38 the mechanism for pH sensing, we constructed receptor chimeras of acid and base
39 sensing chemoreceptors. By analyzing the response of these chimeric receptors, we
40 were able to identify four critical amino-acid residues involved in pH sensing and
41 propose a model for the pH sensing mechanism in *B. subtilis*.

42

43 INTRODUCTION

44 *Bacillus subtilis* performs chemotaxis to a wide range of attractants and repellents
45 (1-3). As a brief background, *B. subtilis* employs ten chemoreceptors to sense these
46 compounds (4). The governing chemotaxis pathway functions differently than the better
47 understood chemotaxis pathway in *Escherichia coli*. The core signaling pathways
48 consists of a membrane-associated complex involving the chemoreceptors, CheA
49 histidine kinase, and CheW and CheV scaffold proteins (5) that preferentially form
50 clusters at the cell poles (6). The chemoreceptors sense attractants and repellents
51 either by binding them directly or through binding proteins associated with their uptake
52 (7). Attractants are known to increase the rate of CheA phosphorylation (8). The
53 phosphate group is then transferred to CheY, which in the phosphorylated form binds to
54 the cytoplasmic face of the flagellar motors and induces a motile response (9).

55 To sense chemical gradients, *B. subtilis* employs three adaptation systems (10-12) .
56 The primary system involves receptor methylation. Two enzymes, the CheR
57 methyltransferase and the CheB methylesterase, add and remove methyl groups on
58 conserved glutamate residues located on the cytoplasmic domain of the
59 chemoreceptors (13, 14). Depending on the specific glutamate residue, these
60 modifications can either increase or decrease the ability of the chemoreceptors to
61 activate the CheA kinase (5, 15). In addition, two other adaptation systems are involved
62 in sensing gradients. One involves the scaffold protein CheV, which contains a C-
63 terminal response regulator domain that is phosphorylated by CheA (16). Depending on
64 the methylation state of the chemoreceptors, phosphorylated CheV can either increase
65 or decrease chemoreceptor activity (5). The other system involves three proteins:

66 CheD, a chemoreceptor deamidase (17, 18) ; CheC, a phosphatase for phosphorylated
67 CheY (19); and CheY. In addition to being a deamidase, CheD binds the
68 chemoreceptors and increases their ability to activate CheA (5, 20). CheC can also bind
69 CheD and prevent it from binding the chemoreceptors. Phosphorylated CheY increases
70 the affinity between CheC and CheD, thus providing a feedback mechanism for
71 controlling CheA activity in response to phosphorylated CheY (21, 22).

72 Our understanding of *B. subtilis* chemotaxis is principally limited to amino-acid
73 chemotaxis. Aside from amino acids (7, 23), oxygen (24), and sugars transported by the
74 phosphoenolpyruvate-dependent phosphotransferase system (25), little is known about
75 the sensing mechanisms involved in *B. subtilis* chemotaxis. A number of reports have
76 shown that diverse bacteria migrate in response to pH gradients. This process is best
77 understood in *Escherichia coli*, *Salmonella enterica* and *Helicobacter pylori* (26-32). In
78 the case of *E. coli* and *S. enterica*, these bacteria preferentially migrate to neutral (pH
79 7.5) environments (33). The response is bidirectional, as the bacteria will migrate down
80 pH gradients when the ambient pH is too high or migrate up pH gradients with the
81 ambient pH is too low. The underlying mechanism involves the competitive response
82 between two chemoreceptors, one that induces cells to migrate down pH gradients and
83 the other that induces them to migrate up pH gradients. These two chemoreceptors
84 respond to both internal and external pH. While the sensing mechanism is still not well
85 understood, external pH is believed to be sensed by the extracellular domain of the
86 chemoreceptors (27) and internal pH by the linker region between the transmembrane
87 helices and cytoplasmic domain of the chemoreceptors (30). Swapping the entire linker

88 region or specific amino-acid residues within this linker region inverts the response of
89 these two chemoreceptors to changes in internal pH (30).

90 In this work, we investigated chemotaxis to external pH gradients in *B. subtilis*.
91 Similar to *E. coli* and *S. enterica*, *B. subtilis* exhibits bidirectional chemotaxis to pH
92 gradients. To sense these pH gradients, *B. subtilis* employs four chemoreceptors, two
93 for sensing acids and two for sensing base. By analyzing chimeras between acid and
94 base-sensing chemoreceptors, we identified four critical amino-acid residues involved in
95 sensing external pH. Swapping these four residues changed a base-sensing
96 chemoreceptor into an acid-sensing one, and vice versa. Based on these data, we
97 propose a model for pH sensing in *B. subtilis*.

98

99 **RESULTS AND DISCUSSION**

100 ***B. subtilis* exhibits bidirectional taxis to external pH gradients.** To determine
101 whether *B. subtilis* performs chemotaxis in response to external pH gradients, we
102 employed the capillary assay (26). Briefly, cells suspended in buffer at different pH's
103 (6.0-8.5) were incubated with capillaries filled with buffer at pH 7.0 and then the number
104 of cells that entered the capillaries after 1 h were counted. The resulting data show that
105 *B. subtilis* exhibits bidirectional taxis to pH gradients in manner similar to what is
106 observed in *E. coli* (**Figure 1A**). In particular, we found that *B. subtilis* preferentially
107 migrates to neutral (pH 7) environments when the cells were initially suspended in either
108 acidic or alkaline buffer (pH 6-8). Outside of this pH range, however, the cells were less
109 motile (data not shown) and, consequently, taxis was reduced.

110

111 **The methylation system is required for pH taxis.** *B. subtilis* employs three
112 adaptions systems for sensing chemical gradients (11). Of the three adaptation
113 systems, the methylation system is the dominant one for sensing amino-acid gradients.
114 To determine whether the methylation system is also required for pH taxis, we tested
115 whether a $\Delta cheR\Delta cheB$ mutant was capable of pH taxis (**Figure 1B**). This mutant lacks
116 the requisite methyltransferase (CheR) and methylesterase (CheB). It was unable to
117 perform pH taxis, indicating that the methylation system is necessary for sensing pH
118 gradients.

119

120 **Four chemoreceptors are involved in sensing pH gradients.** *B. subtilis*
121 possesses ten chemoreceptors (4). To determine which chemoreceptors are involved in

122 pH taxis, we tested mutants expressing just one chemoreceptor (**Figure 2A**). Of the
123 single chemoreceptor mutants, only the one expressing McpA as its sole
124 chemoreceptor was capable of acid sensing. In addition, this mutant exhibited a weak
125 repellent response to base, with fewer cells migrating into the capillary when the buffer
126 was at pH 6 as compared to pH 7 (33.7 ± 10.5 versus 54.7 ± 7.1 cells). This repellent
127 response is consistent with McpA being an acid sensor. Two single chemoreceptor
128 mutants were found to exhibit base sensing: the strains expressing McpB or TlpB as
129 their sole chemoreceptors. In particular, these strains exhibited taxis to increases in pH.
130 They also exhibited a weak repellent response to acid (McpB: 37.3 ± 8.6 versus $51.0 \pm$
131 15.1 cells; TlpB: 44.3 ± 2.5 versus 68.7 ± 15.8 cells). No significant responses to any
132 changes in pH were observed for the other mutants. The existence of chemoreceptors
133 sensing both acids and bases would explain how *B. subtilis* is capable of preferentially
134 swimming to neutral conditions, namely that it reflects competition between the acid and
135 base responses.

136 The genes encoding McpA, McpB, TlpA, and TlpB reside in a four-gene cluster (34).
137 Since these four chemoreceptors exhibit high (57-65%) amino-acid sequence identity,
138 we hypothesized that TlpA may also be involved even though a strain expressing it as
139 the sole chemoreceptor failed to exhibit a response to changes in pH. The reason may
140 be that TlpA is weakly expressed: the wild type expresses 2,000 copies of this
141 chemoreceptor as compared to 16,000 copies for McpA (35). Therefore, we tested
142 whether expressing TlpA from a stronger promoter would enable pH sensing. When *tlpA*
143 was expressed as the sole chemoreceptor using the *mcpA* promoter, we observed both
144 an acid and base response (**Figure 2A**, inset). The acid response, however, was

145 stronger than the base response. These results imply that TlpA alone, when over
146 expressed, can direct *B. subtilis* to neutral environments. This begs the question as to
147 why multiple chemoreceptors are employed for pH taxis when potentially one would
148 suffice. Unfortunately, we cannot answer this question at this time.

149 We next tested the effect of deleting these four chemoreceptors, both individual and
150 in combination, on pH sensing (**Figure 2B**). When *mcpA* was deleted in the wild type
151 ($\Delta mcpA$), we observed a reduction in acid sensing, consistent with this chemoreceptor
152 being involved in chemotaxis towards lower pH's. We also observed an increase in the
153 base response, perhaps reflecting the competition between the acid and base
154 responses. When *tlpA* was deleted in the wild type ($\Delta tlpA$), we observed no significant
155 change in pH sensing. However, when both of the chemoreceptors were deleted in the
156 wild type ($\Delta mcpA\Delta tlpA$), the acid response was almost completely eliminated and the
157 base response increased. These results suggest that McpA and TlpA are the primary
158 chemoreceptors involved in sensing decreases in pH. Additional chemoreceptors may
159 be involved; however, their contribution appears minor.

160 When either McpB or TlpB were deleted in the wild type ($\Delta mcpB$ or $\Delta tlpB$), the base
161 response was reduced. In addition, the acid response increased. When both
162 chemoreceptors were deleted ($\Delta mcpB\Delta tlpB$), the base response was completely
163 eliminated and the acid response further increased. These results suggest that McpB
164 and TlpB are the sole chemoreceptors involved in sensing increases in pH.

165

166 **Identification of the regions involved in sensing pH gradients.** All four
167 chemoreceptors involved in pH taxis employ the same double Cache domain

168 (dCache_1) for their extracellular sensing domain (36). This would suggest that specific
169 amino-acid residues are involved in pH. As a first step towards identifying these
170 residues, we constructed chimeras between McpA, involved in acid sensing, and TlpB,
171 involved in base sensing. We focused on these two chemoreceptors because they are
172 primary ones involved in pH taxis. One challenge with constructing these chimeras is
173 that they may not be functional. Indeed, many were not. Unfortunately, little was known
174 prior regarding any additional ligands for these chemoreceptors. As *B. subtilis* is known
175 to perform chemotaxis towards amino acids, we tested whether these chemoreceptors
176 were involved in sensing casamino acids. TlpB alone was able to support chemotaxis to
177 casamino acid; however, McpA alone did not. While we were not able to identify any
178 attractants for McpA, we did find that McpA governs the repellent response to indole.

179 We first constructed a series of chimeras where we fused the N-terminal region of
180 TlpB with the C-terminal region of McpA: *tlpB₃₆₂mcpA*, *tlpB₂₈₄mcpA*, *tlpB₂₆₀mcpA*,
181 *tlpB₂₃₈mcpA* and *tlpB₁₈₀mcpA* (**Figure 3**). We then tested the ability of strains
182 expressing these hybrids as their sole chemoreceptor to sense acid and base gradients
183 using the capillary assay. In addition, we employed casamino acids as a control. Strains
184 expressing *tlpB₃₆₂mcpA* or *tlpB₂₈₄mcpA* behaved the same as wild-type *tlpB* (**Figure 4**).
185 These results indicate that pH is not sensed by the cytoplasmic domain of the
186 chemoreceptor or by the HAMP domain. The strain expressing *tlpB₂₆₀mcpA* was able to
187 sense both increases and decreases in pH. As the base response was similar to the
188 strain expressing wild-type *tlpB*, these results would suggest that the region 260-284
189 from McpA is involved in acid sensing. The strain expressing *tlpB₂₃₈mcpA* was able to
190 sense both increases and decreases in pH, albeit at reduced levels. However, the

191 strains expressing *tlpB₁₈₀mcpA* were unable to sense base gradients and only
192 responded to acid gradients, indicating that the region spanning the residues 180-284 is
193 directly involved in pH sensing. In addition, strains expressing *tlpB₁₈₀mcpA* no longer
194 responded to casamino acids. Likely, this is due to disruption of the sensing domain. All
195 other N-terminal TlpB chimeras were able to sense casamino-acid gradients.

196 To further characterize the region spanning residues 180-284, we replaced this
197 region in McpA with the corresponding region from TlpB (*mcpA₁₈₀tlpB₂₈₄A*). Strains
198 expressing this chimera as their sole chemoreceptor only responded to increases in pH
199 (**Figure 4**). We then created two more chimeric strains, *mcpA₁₉₇tlpB₂₈₄A* and
200 *mcpA₂₂₂tlpB₂₈₄A*, to narrow down the regions responsible for pH sensing. The mutant
201 expressing *mcpA₁₉₇tlpB₂₈₄A* also responded only to increases in pH. However, the
202 mutant expressing *mcpA₂₂₂tlpB₂₈₄A* did not exhibit a significant response to pH (**Figure**
203 **4**). These results indicated that the sub-region spanning the residues 197-222 is also
204 involved in base sensing.

205 All of the strains expressing the McpA chimeras exhibited a repellent response to
206 indole. However, the response of these strains was significantly reduced as compared
207 to the strain expressing wild-type *mcpA* (average net accumulation within capillary:
208 219.7 ± 90.5 (*mcpA₁₈₀tlpB₂₈₄A*), 232.0 ± 183.1 (*mcpA₁₉₇tlpB₂₈₄A*), and 248.6 ± 109.5
209 (*mcpA₂₂₂tlpB₂₈₄A*) versus $1,286.1 \pm 467.6$ cells (wild type)). This would suggest that the
210 McpA chimeras are not fully functional, which may explain their reduced response to
211 increases in pH as compared to TlpB.

212

213 **Identification of residues involved in pH sensing.** To identify the specific amino-
214 acid residues involved in pH sensing, we aligned the amino-acid sequences spanning
215 residues 195-284 on the four chemoreceptors (**Figure 5A**). Charged amino acids are
216 more likely to be involved in pH sensing as their side-chains can be protonated and
217 deprotonated. In addition, the polar amino acids may play an indirect role in pH sensing
218 by impacting the local amino-acid pK_a values and/or forming hydrogen bonds with side-
219 chains of ionizable amino-acid residues. We first focused on the charged amino acids
220 that carried opposite signs on McpA and TlpB. Within the pH sensing sub-region
221 spanning residues 260-284, the potential candidate residues were the Gln²⁷³-Asp²⁷⁴ pair
222 for TlpB and the corresponding His²⁷³-Glu²⁷⁴ pair for McpA. Within the other pH sensing
223 sub-region spanning residues 197-222, the potential candidate residues were the Ile²¹⁸-
224 Lys²¹⁹ pair for TlpB and the corresponding Glu²¹⁸-Gln²¹⁹ pair for McpA.

225 The pH sensing sub-regions are separated by 38 amino-acid residues. However,
226 when the predicted tertiary structure of TlpB sensing domain was visualized, we found
227 the amino-acid pair Lys¹⁹⁹-Glu²⁰⁰ was in close proximity to the Gln²⁷³-Asp²⁷⁴ pair (**Figure**
228 **5B**). Similarly, we observed the mutual Thr¹⁹⁹-Gln²⁰⁰ pair was in close proximity to the
229 His²⁷³-Glu²⁷⁴ pair on McpA (not shown in Figure). Therefore, Lys¹⁹⁹-Glu²⁰⁰ on TlpB and
230 Thr¹⁹⁹-Gln²⁰⁰ on McpA were also potential candidate residues for mutational analysis as
231 local amino-acid interactions could affect pH sensing.

232 We examined the role of these candidate pairs by swapping them with their
233 counterparts on the opposite receptor, both individually and in combination. We first
234 replaced Gln²⁷³-Asp²⁷⁴ pair on TlpB with the counterpart His²⁷³-Glu²⁷⁴ pair from McpA.
235 Cells expressing *tlpB-Q273H/D274E* as their sole chemoreceptor exhibited a reduced

236 base response and an increased acid response (**Figure 5C**). When we performed the
237 reciprocal experiment, replacing the His²⁷³-Glu²⁷⁴ pair on McpA with the Gln²⁷³-Asp²⁷⁴
238 pair from TlpB, we observed a large decrease in the acid response and small increase
239 in the base response (**Figure 5D**). These results demonstrate that Gln²⁷³-Asp²⁷⁴ on
240 TlpB and His²⁷³-Glu²⁷⁴ on McpA are involved in pH sensing.

241 We next tested the effect of replacing the Lys¹⁹⁹-Glu²⁰⁰ pair on TlpB with the Thr¹⁹⁹-
242 Gln²⁰⁰ pair from McpA. This mutant (*tlpB-K199T/E200Q*) exhibited a weak response to
243 both acids and bases (**Figure 5C**). However, the reciprocal mutation (*mcpA-*
244 *T199K/Q200E*), where we replaced the Thr¹⁹⁹-Gln²⁰⁰ pair on McpA with the Lys¹⁹⁹-
245 Glu²⁰⁰ pair from TlpB, led to a small reduction in the acid response and a small increase
246 in the base response (**Figure 5D**). When the four residues on TlpB were swapped with
247 their counterpart residues for McpA, the base response was eliminated and the resulting
248 strain only exhibited an acid response, albeit at reduced levels (**Figure 5C**). Similarly,
249 when the four residues on McpA were swapped with their counterpart residues from
250 TlpB, the acid response was significantly reduced and the resulting strain exhibited a
251 base response, again at reduced levels. Collectively, these results imply that these four
252 amino-acid residues are sufficient to define the polarity of pH sensing for both McpA
253 and TlpB. Therefore, we did not examine Ile²¹⁸-Lys²¹⁹ pair on TlpB or Glu²¹⁸-Gln²¹⁹ on
254 McpA as these residues are located far away from the identified key residues (**Figure**
255 **5B**) and likely are not involved in pH sensing.

256 We note that these mutant chemoreceptors with swapped polarity exhibited a
257 reduced response to pH. Likely, the mutations are disrupting overall chemoreceptor

258 function. When we tested the chemoreceptors against casamino acids and indole, they
259 exhibited a weaker response than the wild type.

260

261 **Model for pH sensing.** The experiments described above identified four critical
262 amino-acid residues involved in pH sensing. The predicted structures for the
263 extracellular domains of TlpB and McpA reveal that these four amino-acid residues are
264 in close proximity of one another, suggesting that direct interactions between them may
265 govern the sensing mechanism (see **Fig. 5B**). Side-chains of ionizable amino-acid
266 residues can accept or donate protons as the local pH varies. It is generally thought that
267 changes in protonation state of ionizable amino-acid residues can lead to
268 conformational changes in the protein and possibly alter its activity. These
269 conformational changes are typically induced by the formation or disruption of hydrogen
270 bonds between two or more proximal residues. The protonation state of ionizable
271 amino-acid residues largely relies on their local pK_a values. The local pK_a of an amino-
272 acid residue in the folded protein depends on its interactions with neighbor residues. As
273 the result of such interactions – including charge-dipole, charge-charge, and ion-pairs –
274 pK_a values can be significantly different from the intrinsic pK_a (pK_{a-int}) values measured
275 in blocked pentapeptides (37). For example, the pK_a of a lysine residue can be as low
276 as 5.7 and the pK_a of an aspartate residue can be as high as 9.2 in folded proteins (37).

277 In the case of TlpB, increase in pH can directly affect three ionizable residues:
278 Asp²⁷⁴, Lys¹⁹⁹, and Glu²⁰⁰. The point mutation *tlpB-D274N* did not affect the responses
279 to pH ($1,334.7 \pm 352.4$ versus $1,037.2 \pm 76.9$ cell for wild-type *tlpB*), suggesting that the
280 protonation state of Asp²⁷⁴ remains intact or does not affect interactions with other

281 residues. However, the double mutant K199T/E200Q significantly reduced the response
282 to increase in pH (**Figure 5C**). This result implies increases in pH affects the protonation
283 state of Lys¹⁹⁹ or/and Glu²⁰⁰. Based on these results, we offer one potential model to
284 explain the response of TlpB to increases in pH (**Figure 6**). At lower pH (e.g. pH=6),
285 Lys¹⁹⁹ and/or Glu²⁰⁰ are protonated and form stable hydrogen bonds with Gln²⁷³ or/and
286 Asp²⁷⁴. As pH increases, Lys¹⁹⁹ and/or Glu²⁰⁰ likely become deprotonated, and the loss
287 of the hydrogen bonds destabilizes the local structure of lower region of the sensing
288 domain and the proximal transmembrane helix (TM2, **Fig. 3**). This structural transition is
289 then propagated through TM2 to the HAMP domain and subsequently to distal
290 cytoplasmic signaling, thus inducing the autophosphorylation of CheA histidine kinase
291 (**Figure 6A**).

292 In the case of McpA, the ionizable residues are His²⁷³ and Glu²⁷⁴. The other two key
293 residues Thr¹⁹⁹ and Gln²⁰⁰ contain polar side-chains that are insensitive to pH changes.
294 As expected, the double mutant H273Q/E274D significantly reduced the response to
295 decrease in pH (**Figure 5D**). Among the two residues, His²⁷³ seems to play a pivotal
296 role as the point mutation E274Q did not affect the response to pH gradients (1,176.0 ±
297 124.5 versus 910.4 ± 77.0 cells for wild-type *tlpB*). One possible model is that at high pH
298 (e.g. pH=8), His²⁷³ is in its neutral form. As pH decreases, His²⁷³ becomes protonated
299 and no longer forms hydrogen bonds with either Thr¹⁹⁹ and/or Gln²⁰⁰. This destabilizes
300 the local structure. Similar to the TlpB case, this conformational change induces a
301 structural transition in signaling module, which in turn promotes phosphorylation of the
302 CheA histidine kinase (**Figure 6B**).

303

304 **CONCLUSION**

305 We identified the chemoreceptors governing pH taxis in *B. subtilis*. McpA is the
306 primary acid chemoreceptor while McpB and TlpB are the base chemoreceptors. In
307 addition, TlpA alone functions both as an acid and base chemoreceptor, though its
308 primary role appears to be acid sensing. Using receptor chimeras, we identified four
309 critical amino-acid residues involved in pH sensing. Swapping these residues between
310 McpA and TlpB was able to convert the former into a base sensor and the latter into an
311 acid sensor. Based on our results, we were able to propose a model for pH sensing in
312 *B. subtilis*. Collectively, these results further our understanding of pH taxis and provide a
313 model for pH sensing.

314

315 **MATERIALS AND METHODS**

316 **Chemicals and growth media.** The following media were used for cell growth:
317 tryptone broth (TB: 1% tryptone and 0.5% NaCl); tryptose blood agar base (TBAB: 1%
318 tryptone, 0.3% beef extract, 0.5% NaCl, and 1.5% agar); and capillary assay minimal
319 medium (CAMM: 50 mM potassium phosphate buffer (pH 7.0), 1.2 mM MgCl₂, 0.14 mM
320 CaCl₂, 1 mM (NH₄)₂SO₄, 0.01 mM MnCl₂, and 4.2 μM ferric citrate). Chemotaxis buffer
321 consists of 10 mM potassium phosphate buffer (pH 7.0), 0.14 mM CaCl₂, 0.3 mM
322 (NH₄)₂SO₄, 0.1 mM EDTA, 5 mM sodium lactate, and 0.05% (v/v) glycerol.

323

324 **Bacterial strains and plasmids.** All *B. subtilis* strains were derived from the strain
325 Ol1085 (38). All cloning was performed using NEB® 5-alpha Competent *E. coli* (New
326 England Biolabs). Bacterial strains and plasmids used in this work are listed in **Tables 1**
327 and **2**, respectively. All oligonucleotides used in this study are provided in **Table S1**.

328 Gene deletions were constructed using plasmids derived from pJSpe. pJSpe was
329 derived from pJOE8999, which provides a CRISPR/Cas9-based, marker-free genome
330 editing system for *B. subtilis* (39). We found that the *Sfi*l restriction sites on the original
331 pJOE8999 were inefficient for subcloning homology templates. In addition, a 13-bp long
332 DNA scar remained on the chromosome after the targeted DNA fragment was deleted
333 using pJOE8999-derived vectors. Therefore, we created pJSpe for more efficient
334 assembly of homology templates based on Gibson assembly and scar-less deletion of
335 DNA fragments. Briefly, a 50-bp annealed complementary DNA oligonucleotides
336 containing a *Spe*l restriction site and optimized for Gibson assembly was inserted
337 between the *Sfi*l restriction sites on pJOE8999 to yield pJSpe. For construction of the

338 knockout plasmids, a 20-bp sgRNA target sequence for the targeted gene was
339 designed using CRISPy-web online tool (40). Annealed complementary oligonucleotides
340 were then subcloned into *BsaI* restriction sites on pJSpe as described in (39). Two PCR
341 fragments (~600-800 bp) flanking the targeted gene using overlapping primers were
342 subcloned into *SpeI*-linearized vector using Gibson assembly (41). The resultant vector
343 was then linearized using *XhoI* and ligated with T4 DNA ligase to create a long DNA
344 concatemer. The concatemer was then transformed into *B. subtilis* strain using the two-
345 step Spizizen method (42). Single colonies were isolated and twice streaked on fresh
346 plates. Plasmid curing and genotype verification were performed as previously
347 described (39).

348 Strains expressing a single wild-type chemoreceptor were constructed by integrating
349 the respective chemoreceptor expression cassette into the *amyE* locus. The region
350 containing the promoter, gene, and terminator was PCR amplified from genomic DNA
351 isolated from *B. subtilis* 168. The PCR fragment was then cloned into the plasmid
352 pAIN750 using the *EcoRI* and *BamHI* restriction sites. The plasmid was then
353 transformed in OI3545, which lacks all ten chemoreceptors, as described above.

354 Chemoreceptor chimeras were constructed using Gibson assembly (41). Briefly, two
355 opposing primers were designed to prime DNA synthesis outwards from the fusion point
356 of the chimeric gene using PCR with pAIN750*mcpA* as the DNA template. Then, a
357 second pair of primers with overlapping regions were designed to PCR-amplify the
358 desired fragment of *tlpB* gene from pAIN750*tlpB* plasmid. Following purification the PCR
359 products by gel extraction, the DNA fragments were assembled (41).

360 Point mutations were introduced into chemoreceptor genes using the inverse PCR
361 method with pAIN750mcpA and pAIN750tlpB as DNA templates. Briefly, plasmid was
362 PCR amplified using two opposing primers containing the desired mutations. Following
363 purification by gel extraction, the DNA fragment was phosphorylated with T4
364 polynucleotide kinase and then blunt-end ligated using T4 DNA ligase. Ligation product
365 was heat-inactivated and transformed into *E. coli*. The plasmid was then isolated from
366 *E. coli* and transformed into *B. subtilis* OI3545 as described above.

367

368 **Capillary assay for chemotaxis.** The capillary assay was performed as described
369 previously (1, 26). Briefly, cells were grown for 16 hours at 30 °C on TBAB plates. The
370 cells were then scraped from the plates and resuspended to $A_{600} = 0.03$ in 5-mL CAMM
371 supplemented with 50 µg/mL histidine, methionine, tryptophan and 20 mM sorbitol, and
372 2% TB. The cultures were grown to $A_{600} = 0.4-0.45$ at 37 °C and 250 rpm shaking. At
373 this point, 50 µl of GL solution (5% (v/v) glycerol, 0.5 M sodium lactate) was added to
374 culture and the cells were incubated for another 15 minutes (at 37 °C, 250 rpm
375 shaking). Cells were then washed twice with chemotaxis buffer (pH 7.0) and incubated
376 for additional 25 minutes (at 37 °C, 250 rpm shaking) to assure that the cells were
377 motile. Cells were diluted to $A_{600} = 0.001$ in chemotaxis buffer at desired pH values for
378 pH taxis experiments and in chemotaxis buffer (pH 7.0) for casamino-acid control
379 experiments. For indole control experiments, cells were diluted to $A_{600} = 0.005$ in
380 chemotaxis buffer containing indole (50 µM, pH 7.0). Prior to assay, cells were briefly
381 incubated in chemotaxis buffer at room temperature (shaking slowly) and then aliquoted
382 into 0.3-mL ponds on a slide warmer at 37 °C and closed-end capillary tubes filled with

383 chemotaxis buffer or casamino acid (3.16×10^{-5} g/mL) solutions prepared with
384 chemotaxis buffer (pH 7.0) were inserted. After a fixed time (30 minutes for casamino
385 acids and 1 hour for pH and indole), cells that migrated into the capillaries were
386 harvested and transferred to 3 mL of top agar (1% tryptone, 0.8% NaCl, 0.8% agar, 0.5
387 mM EDTA) and plated onto TB (1.5% agar) plates. These plates were incubated at 37
388 °C for 16 hours and colonies were counted. Experiments were performed in triplicate
389 each day and then repeated on three different days.

390

391 **Structural analysis.** Domain predictions were performed using the phmmmer search
392 engine on the HMMER web-server using the UniProt reference proteomes database
393 with default sequence E-values thresholds (43). Boundaries of both transmembrane
394 alpha helices were first predicted using TMHMM web-server v.2.0 (44) and then
395 manually adjusted using information from propensity analysis of amino acid distributions
396 around lipid/water interfaces (45). Pairwise amino-acid sequence alignments between
397 McpA and TlpB for chimeric-receptor analysis were performed using EMBOSS Water
398 (46) and multiple sequence alignment between McpA, McpB, TlpA, and TlpB for
399 mutational analysis were carried out in T-Coffee (47). Structures for the McpA and TlpB
400 sensing domains (residues 38-278) were predicted using the I-TASSER web-server
401 (48). The C-scores were 1.15 and 1.13 for McpA and TlpB, respectively. Both models
402 are structurally close to the ligand-binding domain of the PctA chemoreceptor from
403 *Pseudomonas aeruginosa* PAO1 (PDB: 5LTX) with TM-score of 0.955 for both McpA
404 and TlpB. Visualization of all structures was accomplished using the VMD software
405 package (v-1.9.3) (49).

407 **ACKNOWLEDGEMENTS**

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410

411

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542

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Table 1. Strains used in this study

Strain	Relevant genotype or description	Reference
5-alpha	<i>E. coli</i> cloning host	New England Biolabs
OI3269	<i>Bacillus subtilis</i> 168, <i>trpC2</i>	
OI1085	<i>trpF7 hisH2 metC che⁺</i>	(38)
OI3017	<i>cheB/cheR::cat</i>	(13)
PTS324	$\Delta mcpA$	This work
PTS325	$\Delta tlpA$	This work
PTS185	$\Delta mcpB$	This work
PTS186	$\Delta tlpB$	This work
PTS334	$\Delta mcpA \Delta tlpA$	This work
PTS187	$\Delta tlpB \Delta mcpB$	This work
OI3545	$\Delta 10mcp che^+$	(24)
OI3921	OI3545 <i>amyE5720::mcpA</i>	(17)
OI3605	OI3545 <i>amyE5720::mcpB</i>	(25)
OI3974	OI3545 <i>amyE5720::mcpC</i>	(17)
OI4474	OI3545 <i>amyE5720::tlpA</i>	This work
OI4475	OI3545 <i>amyE5720::tlpB</i>	This work
OI4483	OI3545 <i>amyE5720::tlpC</i>	This work
OI4476	OI3545 <i>amyE5720::yfmS</i>	This work
OI4477	OI3545 <i>amyE5720::yvaQ</i>	This work
OI4482	OI3545 <i>amyE5720::hemAT</i>	This work
OI4479	OI3545 <i>amyE5720::yoaH</i>	This work
PTS163	OI3545 <i>amyE5720::tlpB₂₈₄mcpA</i>	This work
PTS165	OI3545 <i>amyE5720::tlpB₃₆₂mcpA</i>	This work
PTS368	OI3545 <i>amyE5720::tlpB₂₆₁mcpA</i>	This work
PTS493	OI3545 <i>amyE5720::tlpB₂₃₈mcpA</i>	This work
PTS505	OI3545 <i>amyE5720::tlpB₁₈₀mcpA</i>	This work
PTS500	OI3545 <i>amyE5720::mcpA₁₈₀ tlpB₂₈₄ A</i>	This work
PTS507	OI3545 <i>amyE5720::mcpA₁₉₇ tlpB₂₈₄ A</i>	This work
PTS509	OI3545 <i>amyE5720::mcpA₂₂₂ tlpB₂₈₄ A</i>	This work
PTS441	OI3545 <i>amyE5720::tlpB[Q273H][D274E]</i>	This work
PTS421	OI3545 <i>amyE5720::tlpB[K199T][E200Q]</i>	This work
PTS464	OI3545 <i>amyE5720::tlpB[Q273H][D274E][K199T][E200Q]</i>	This work
PTS373	OI3545 <i>amyE5720::mcpA[H273Q][E274D]</i>	This work
PTS462	OI3545 <i>amyE5720::mcpA[T199K][Q200E]</i>	This work
PTS481	OI3545 <i>amyE5720::mcpA[H273Q][E274D][T199K][Q200E]</i>	This work
PTS251	OI3545 <i>amyE5720::tlpB[D274N]</i>	This work
PTS276	OI3545 <i>amyE5720::mcpA[E274Q]</i>	This work
GB041	OI3545 <i>amyE5720::P_{mcpA}::tlpA</i>	Unpublished

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546

Table 2. Plasmids used in this study

Plasmid	Description	Reference
pJOE8999	Shuttle vector for Cas9 expression and tracrRNA transcription; Kan ^R	(39)
pJSpe	Modified pJOE8999 optimized for Gibson assembly of homology templates; Kan ^R	This work
pPT058	pJSpe:: <i>mcpB</i> (<i>mcpB</i> knockout vector)	This work
pPT074	pJSpe:: <i>tlpB</i> (<i>tlpB</i> knockout vector)	This work
pPT116	pJSpe:: <i>mcpA</i> (<i>mcpA</i> knockout vector)	This work
pPT118	pJSpe:: <i>tlpA</i> (<i>tlpA</i> knockout vector)	This work
pPT141	pJSpe:: <i>mcpA</i> - <i>tlpA</i> (<i>mcpA</i> - <i>tlpA</i> knockout vector)	This work
pAIN750	<i>B. subtilis</i> <i>amyE</i> integration vector; Amp ^R , Spc ^R	(17)
pAIN750mcpA	pAIN750:: <i>mcpA</i>	(25)
pAIN750mcpB	pAIN750:: <i>mcpB</i>	(25)
pAIN750mcpC	pAIN750:: <i>mcpC</i>	(17)
pAIN750tlpA	pAIN750:: <i>tlpA</i>	This work
pAIN750tlpB	pAIN750:: <i>tlpB</i>	This work
pAIN750tlpC	pAIN750:: <i>tlpC</i>	This work
pAIN750yfmS	pAIN750:: <i>yfmS</i>	This work
pAIN750yvaQ	pAIN750:: <i>yvaQ</i>	This work
pAIN750hemAT	pAIN750:: <i>hemAT</i>	This work
pAIN750yoaH	pAIN750:: <i>yoaH</i>	This work
pPT065	pAIN750:: <i>tlpB</i> ₃₆₂ <i>mcpA</i>	This work
pPT063	pAIN750:: <i>tlpB</i> ₂₈₄ <i>mcpA</i>	This work
pPT143	pAIN750:: <i>tlpB</i> ₂₆₁ <i>mcpA</i>	This work
pPT224	pAIN750:: <i>tlpB</i> ₂₃₈ <i>mcpA</i>	This work
pPT234	pAIN750:: <i>tlpB</i> ₁₈₀ <i>mcpA</i>	This work
pPT233	pAIN750:: <i>mcpA</i> ₁₈₀ <i>tlpB</i> ₂₈₄ A	This work
pPT236	pAIN750:: <i>mcpA</i> ₁₉₇ <i>tlpB</i> ₂₈₄ A	This work
pPT237	pAIN750:: <i>mcpA</i> ₂₂₂ <i>tlpB</i> ₂₈₄ A	This work
pPT129	pAIN750:: <i>tlpB</i> [Q273H][D274E]	This work
pPT162	pAIN750:: <i>tlpB</i> [K199T][E200Q]	This work
pPT202	pAIN750:: <i>tlpB</i> [Q273H][D274E][K199T][E200Q]	This work
pPT196	pAIN750:: <i>mcpA</i> [H273Q][E274D]	This work
pPT163	pAIN750:: <i>mcpA</i> [T199K][Q200E]	This work
pPT222	pAIN750:: <i>mcpA</i> [H273Q][E274D][T199K][Q200E]	This work
pPT101	pAIN750:: <i>tlpB</i> [D274N]	This work
pPT107	pAIN750:: <i>mcpA</i> [E274Q]	This work
pGB045	pAIN750::P _{<i>mcpA</i>} :: <i>tlpA</i>	Unpublished

547

548 **FIGURE CAPTIONS**

549 **Figure 1.** *B. subtilis* exhibits bidirectional chemotaxis to pH gradients. **(A)** Response to
550 increasing and decreasing pH gradients. **(B)** Methylation is necessary for pH taxis.

551

552 **Figure 2.** McpA and TlpA are the principle chemoreceptors involved in the acid
553 response and McpB and TlpB are the sole chemoreceptors involved in the base
554 response. **(A)** Response of strains expressing just one chemoreceptor to pH gradients.
555 **Inset:** response of strain over-expressing *tlpA* as its sole chemoreceptor. **(B)** Response
556 of mutants lacking key receptors to pH gradients. Error bars denote the standard
557 deviation of three biological replicates performed on three separate days.

558

559 **Figure 3.** Construction of chimeric receptors to determine regions involved in pH
560 sensing. **(A)** Domain structure of McpA. Extracellular ligand-binding, dCache_1 domain
561 (orange), transmembrane transmembrane, TM1 and TM2 (gray), HAMP domain
562 (yellow), and cytoplasmic domain (green). **(B)** Cartoon structure of McpA. **(C)** Amino-
563 acid sequence alignment of McpA and TlpB around chimera junction points. The
564 numbers designate the fusion points between two chemoreceptors, and the local
565 sequences of the final chimeric chemoreceptors are highlighted in gray.

566

567 **Figure 4.** Response of strains expressing receptor chimeras involving different
568 fragments of McpA (red) and TlpB (blue) to pH and casamino-acid (CA) gradients. Error
569 bars denote the standard deviation of three biological replicates performed on three
570 separate days.

571

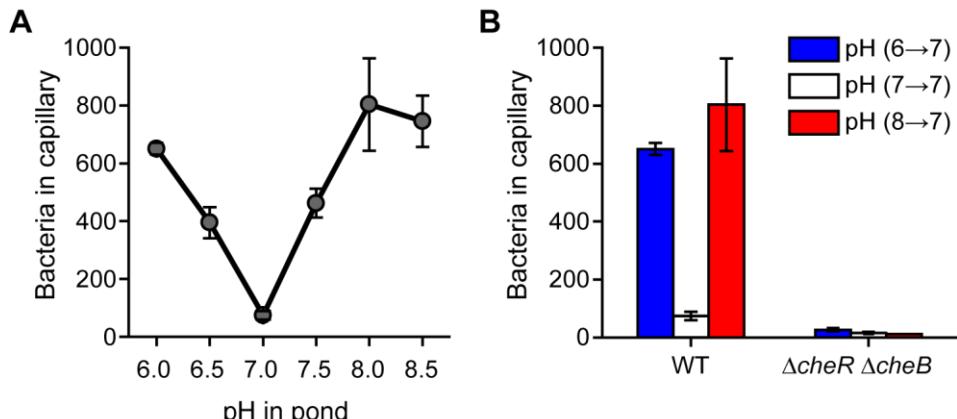
572 **Figure 5.** Identification of critical residues involved in sensing pH. **(A)** Amino-acid
573 sequence alignment of pH sensing regions spanning residues (195-284) for the four pH
574 chemoreceptors reveals candidate residues for mutational analysis. Candidate residues
575 are shown within the green and yellow dashed boxes. Amino-acid sequence for TlpA
576 are shown in purple because TlpA is sensitive to both acid and base. pH sensing sub-
577 regions identified from chimeric receptor analysis are highlighted in the orange boxes.
578 **(B)** Predicted structure of the TlpB ligand binding domain (LBD). Two pH sensing sub-
579 regions are shown in orange consistent with panel A. The candidate amino-acid
580 residues on the TlpB extracellular LBD are shown in green and yellow. **(C)** Response of
581 strains expressing *tplB* mutants to pH and casamino-acid (CA) gradients. **(D)** Response
582 of strains expressing *mcpA* mutants to pH and negative indole gradients. Error bars
583 denote the standard deviation of three biological replicates performed on three separate
584 days.

585

586 **Figure 6.** Model for pH sensing mechanism in *B. subtilis*. **(A)** At low pH, two ionizable
587 residues (solid green circle) on TlpB are in their protonated state and form hydrogen
588 bonds with two adjacent residues (white circle). Deprotonation of these residues upon
589 pH increase disrupts the local structure due to decreased hydrogen bonding and
590 induces signaling. **(B)** At high pH, the key histidine residue (lower white circle) within the
591 pH sensing region of McpA is in neutral state and forms hydrogen bonds with adjacent
592 residues (upper white circle). As pH decreases, the histidine residue becomes

593 protonated (solid green circle), leading to loss of hydrogen bonding. This disrupts the
594 local structure and induces signaling.

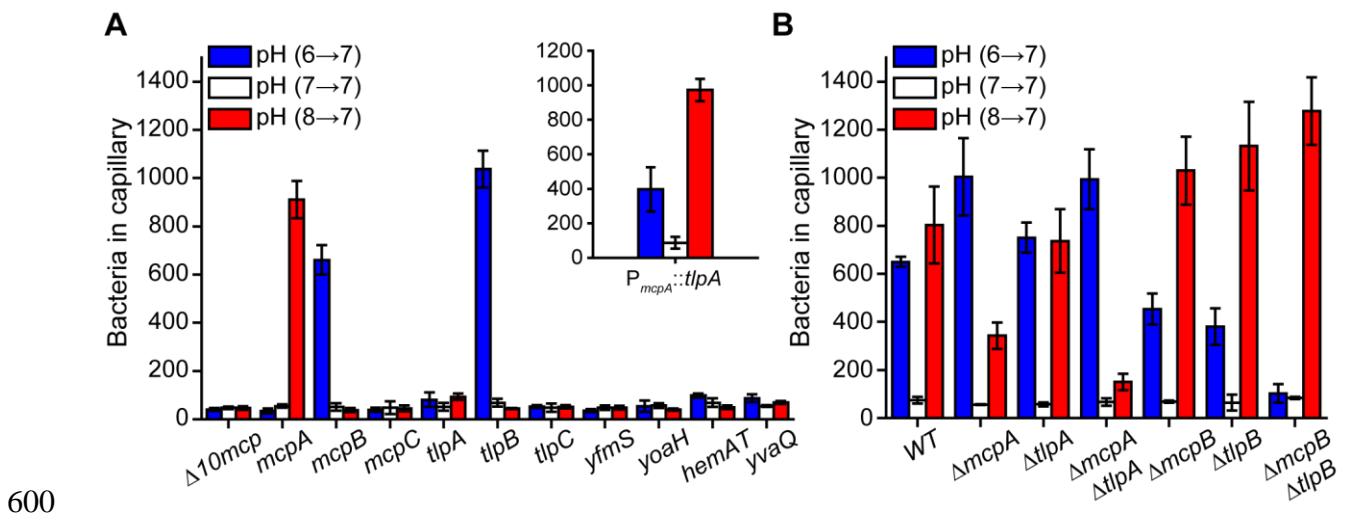
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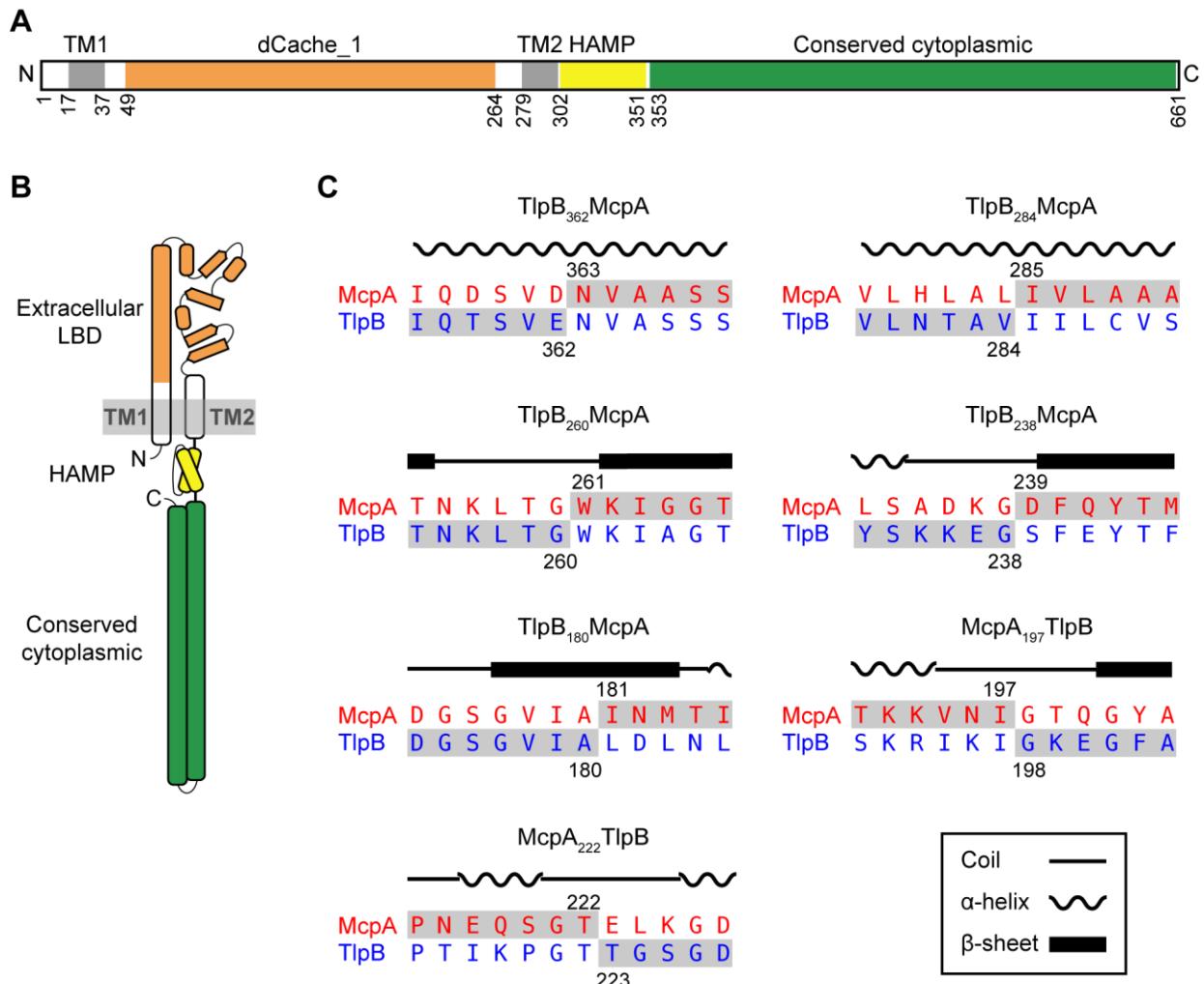
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598 increasing and decreasing pH gradients. **(B)** Methylation is necessary for pH taxis.

599

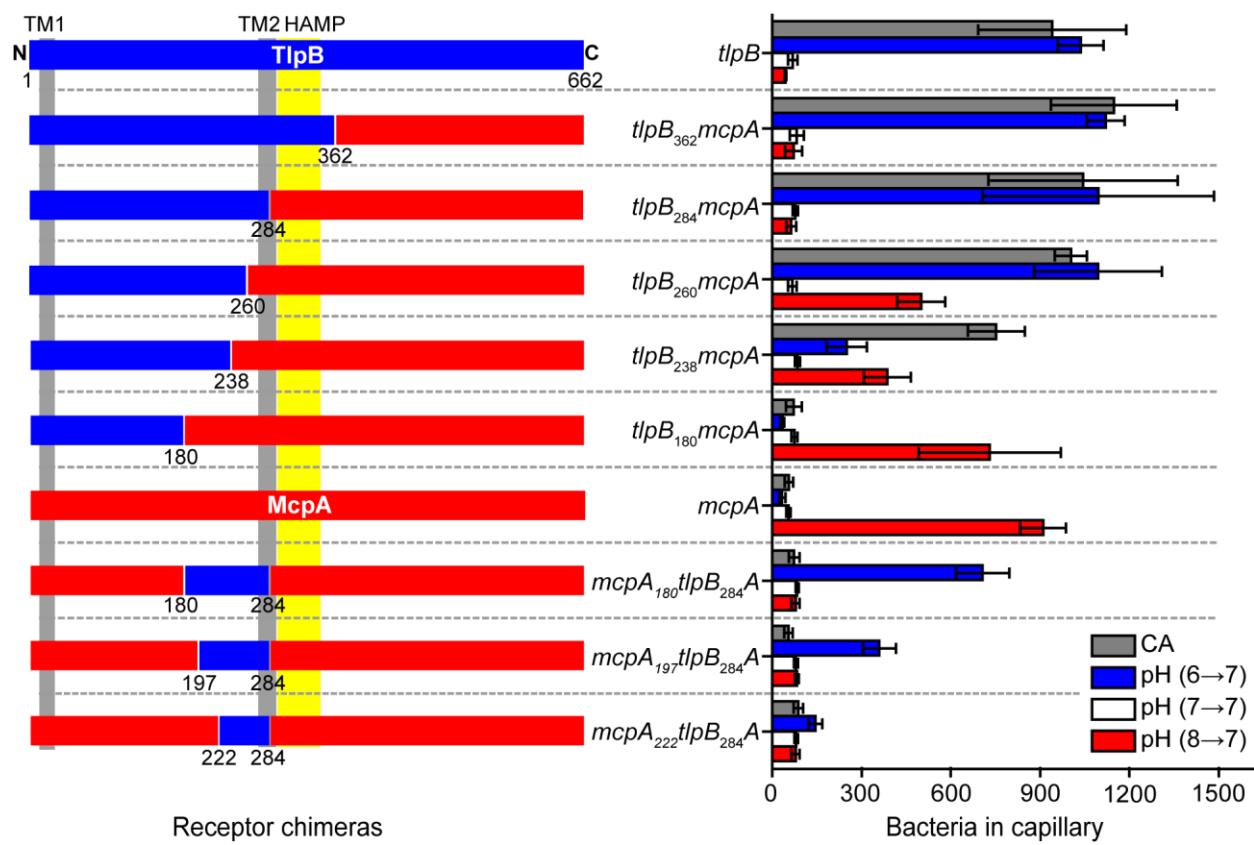


601 **Figure 2.** McpA and TlpA are the principle chemoreceptors involved in the acid
602 response and McpB and TlpB are the sole chemoreceptors involved in the base
603 response. **(A)** Response of strains expressing just one chemoreceptor to pH gradients.
604 **Inset:** response of strain over-expressing *tlpA* as its sole chemoreceptor. **(B)** Response
605 of mutants lacking key receptors to pH gradients. Error bars denote the standard
606 deviation of three biological replicates performed on three separate days.

607

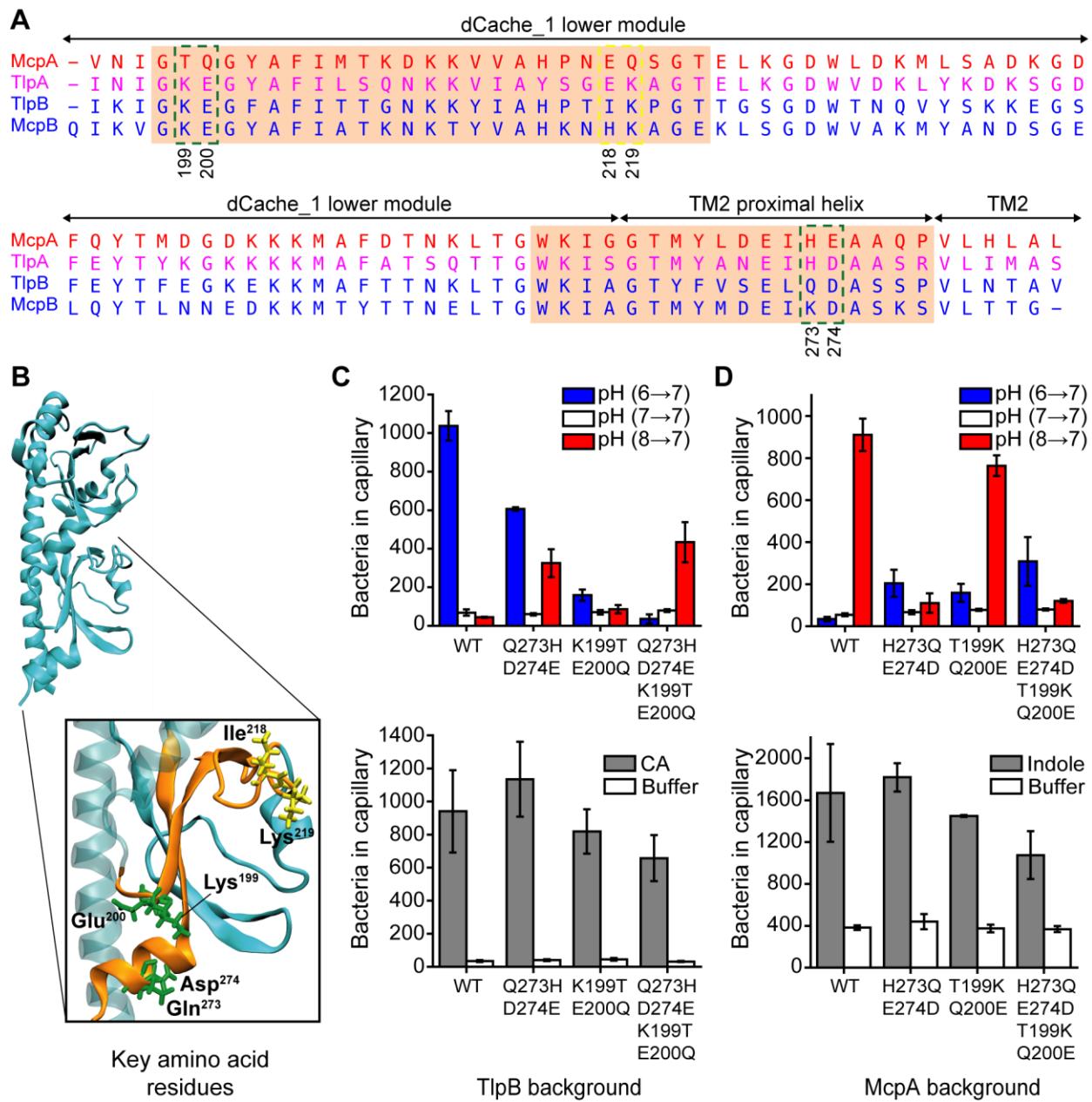


608 223
609 **Figure 3.** Construction of chimeric receptors to determine regions involved in pH
610 sensing. **(A)** Domain structure of McpA. Extracellular ligand-binding, dCache_1 domain
611 (orange), transmembrane transmembrane, TM1 and TM2 (gray), HAMP domain
612 (yellow), and cytoplasmic domain (green). **(B)** Cartoon structure of McpA. **(C)** Amino-
613 acid sequence alignment of McpA and TlpB around chimera junction points. The
614 numbers designate the fusion points between two chemoreceptors, and the local
615 sequences of the final chimeric chemoreceptors are highlighted in gray.



618 **Figure 4.** Response of strains expressing receptor chimeras involving different
619 fragments of McpA (red) and TlpB (blue) to pH and casamino-acid (CA) gradients. Error
620 bars denote the standard deviation of three biological replicates performed on three
621 separate days.

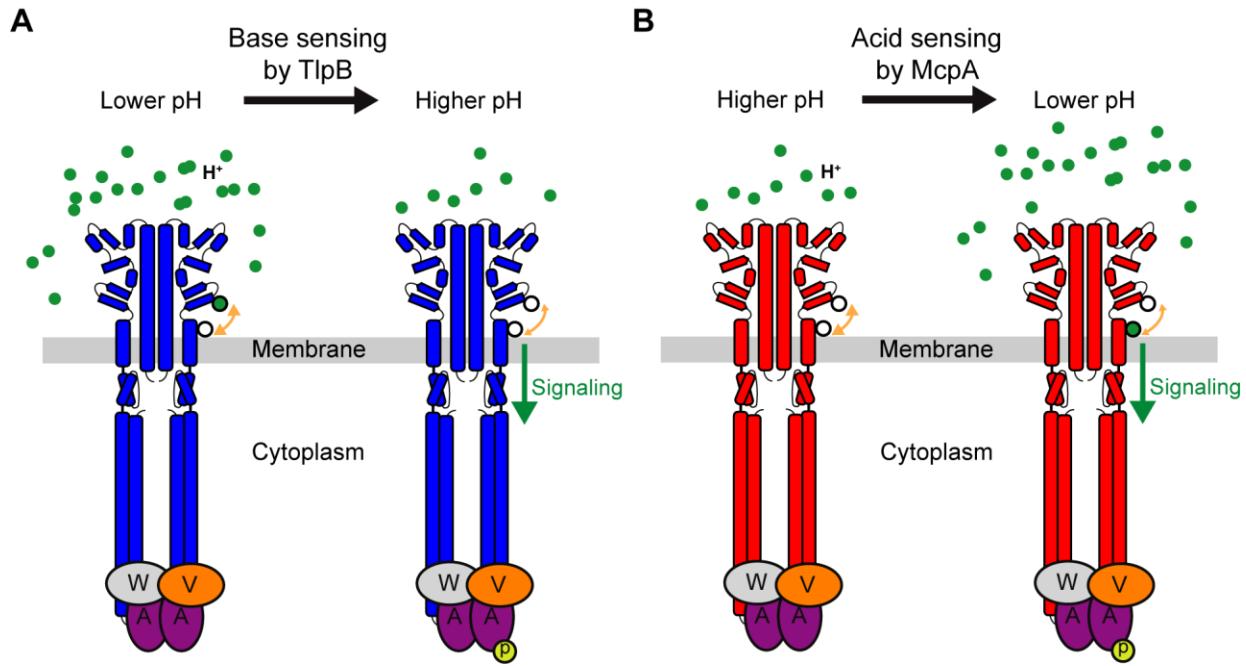
622



630 (B) Predicted structure of the TlpB ligand binding domain (LBD). Two pH sensing sub-
631 regions are shown in orange consistent with panel A. The candidate amino-acid
632 residues on the TlpB extracellular LBD are shown in green and yellow. (C) Response of
633 strains expressing *tlpB* mutants to pH and casamino-acid (CA) gradients. (D) Response
634 of strains expressing *mcpA* mutants to pH and negative indole gradients. Error bars
635 denote the standard deviation of three biological replicates performed on three separate
636 days.

637

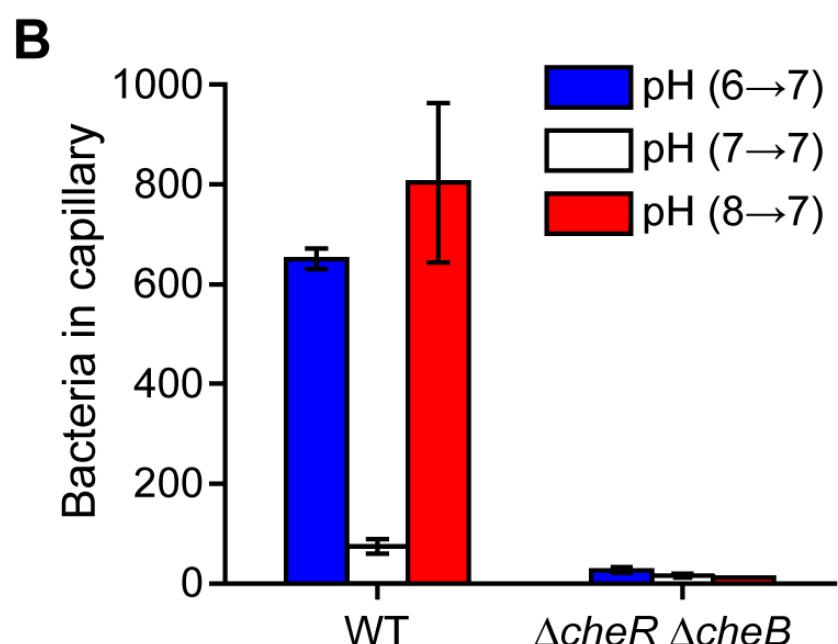
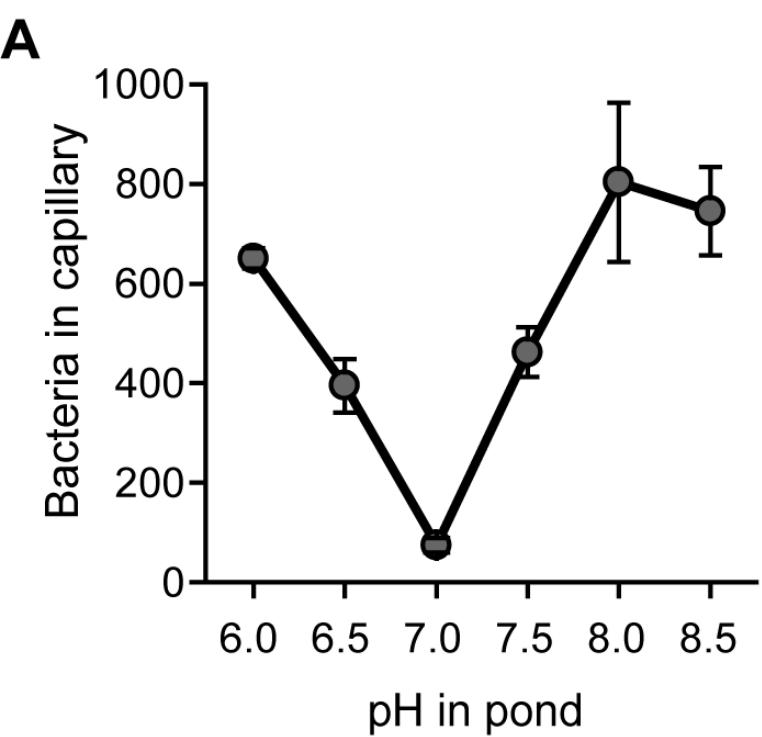
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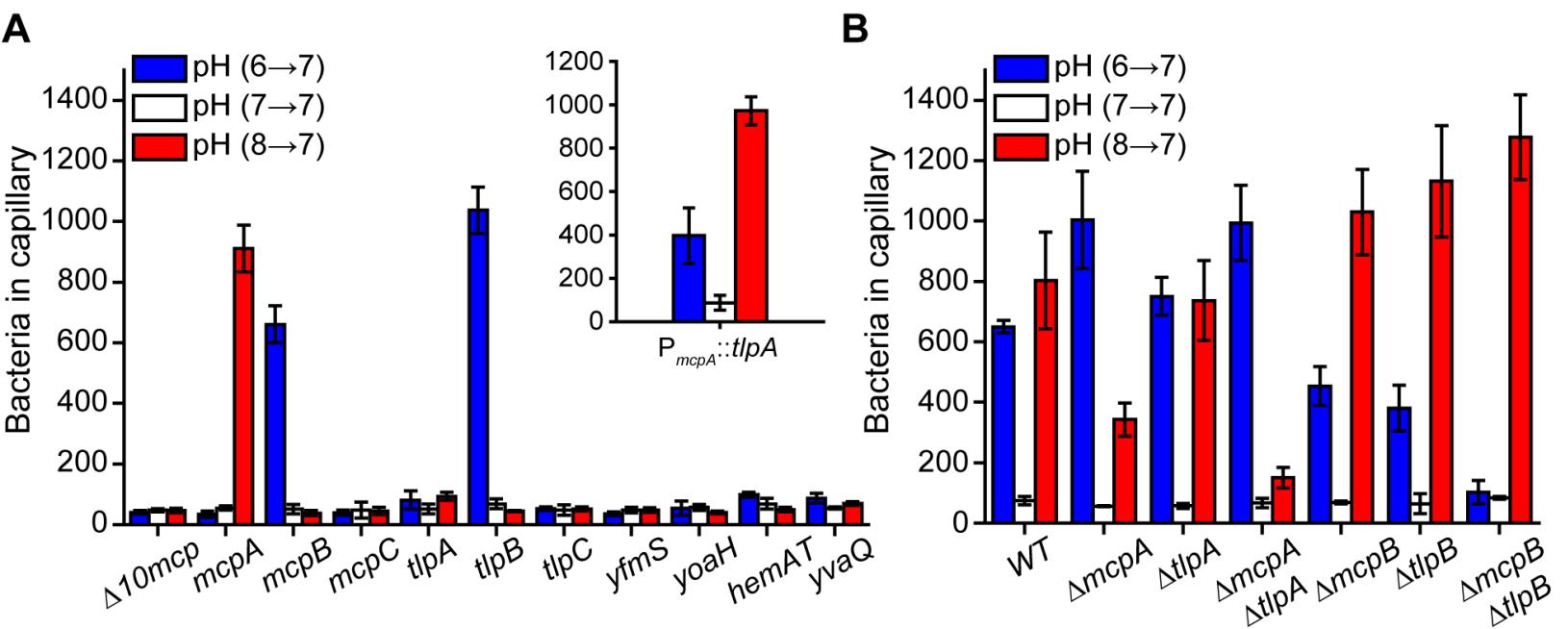


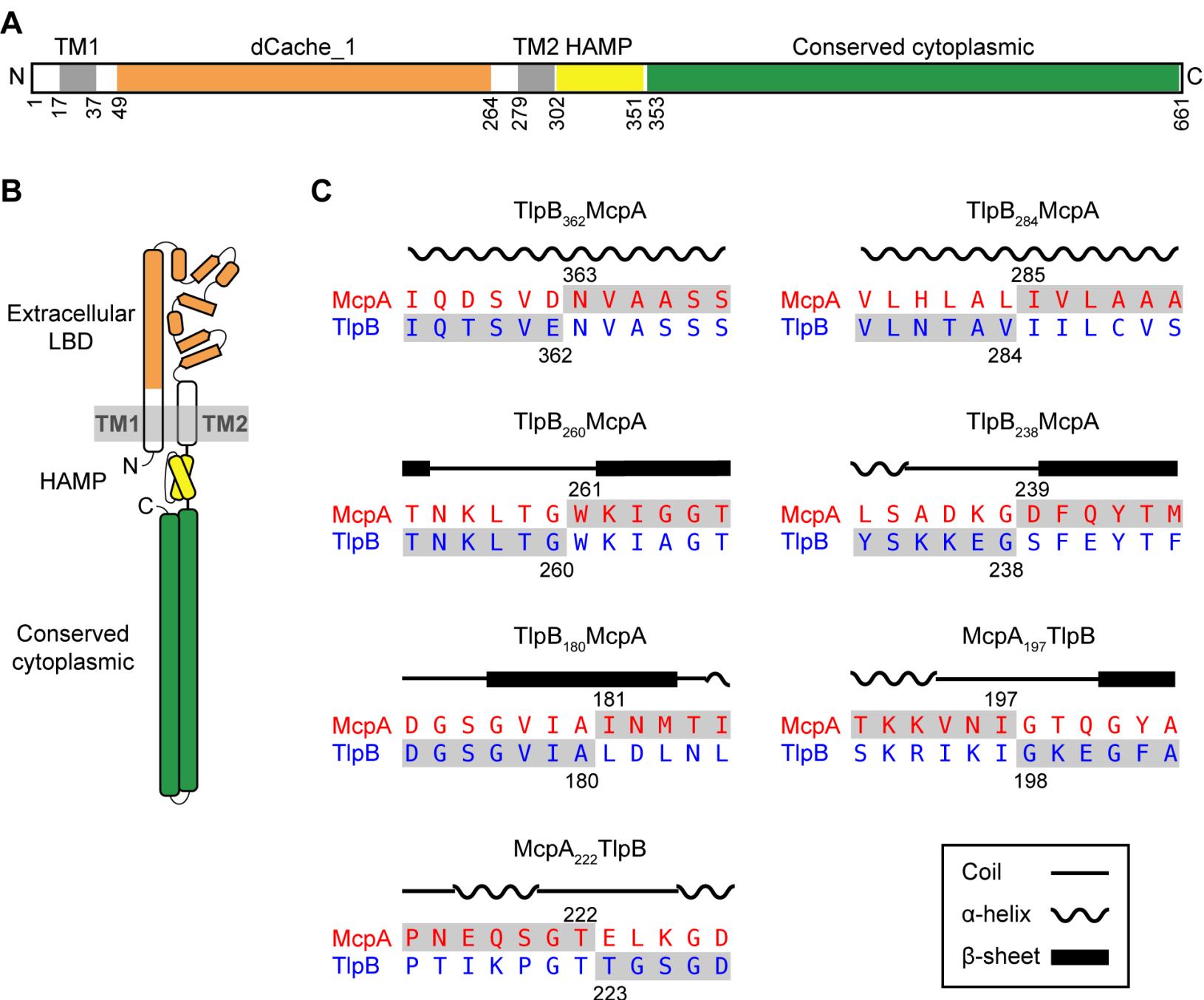
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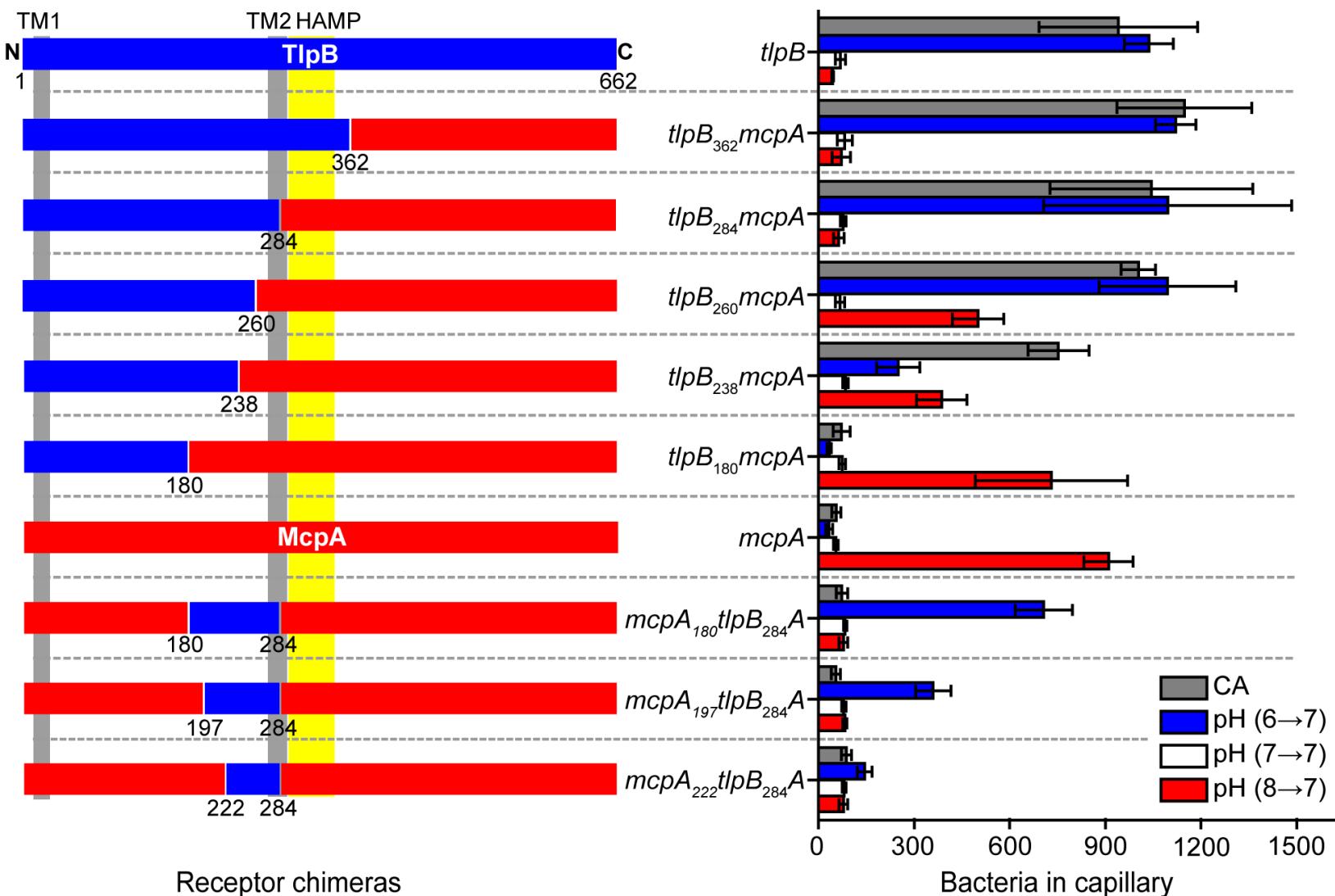
640 **Figure 6.** Model for pH sensing mechanism in *B. subtilis*. **(A)** At low pH, two ionizable
641 residues (solid green circle) on TlpB are in their protonated state and form hydrogen
642 bonds with two adjacent residues (white circle). Deprotonation of these residues upon
643 pH increase disrupts the local structure due to decreased hydrogen bonding and
644 induces signaling. **(B)** At high pH, the key histidine residue (lower white circle) within the
645 pH sensing region of McpA is in neutral state and forms hydrogen bonds with adjacent
646 residues (upper white circle). As pH decreases, the histidine residue becomes
647 protonated (solid green circle), leading to loss of hydrogen bonding. This disrupts the
648 local structure and induces signaling.

649

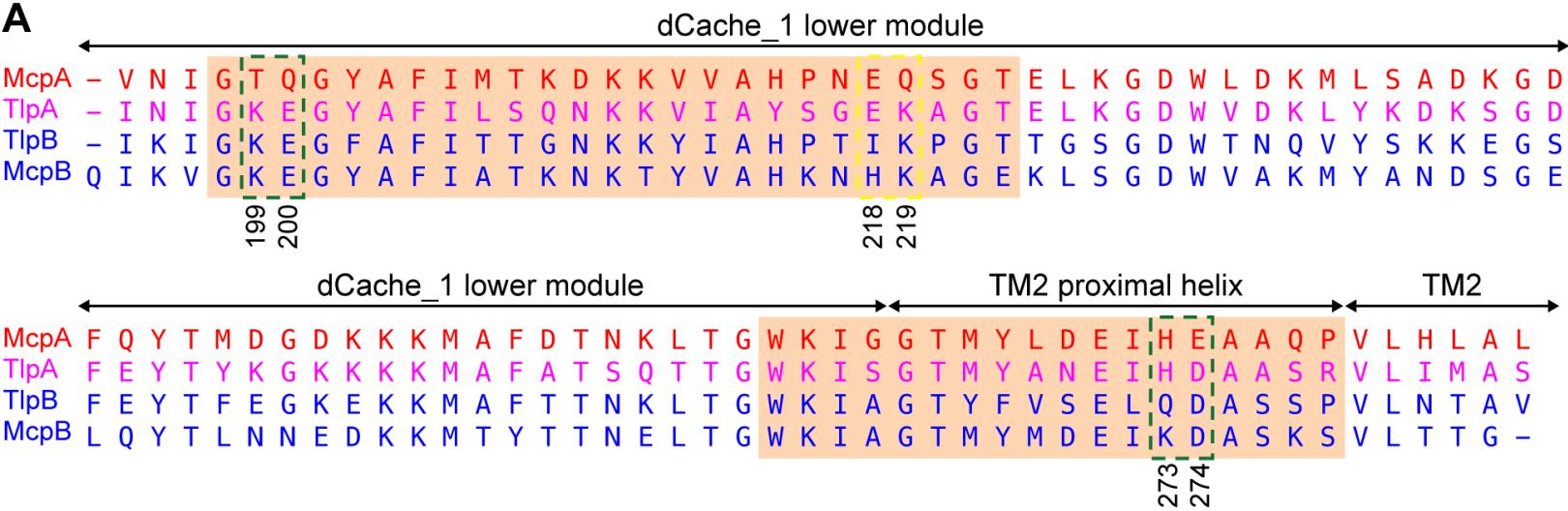




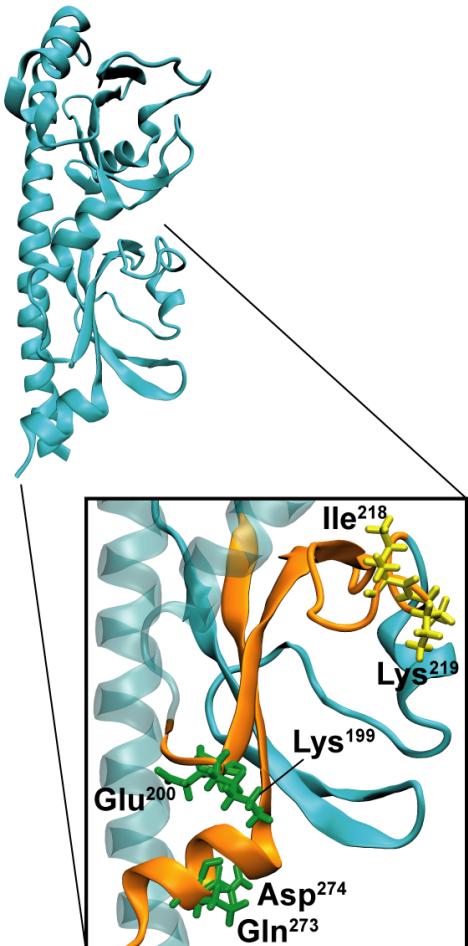




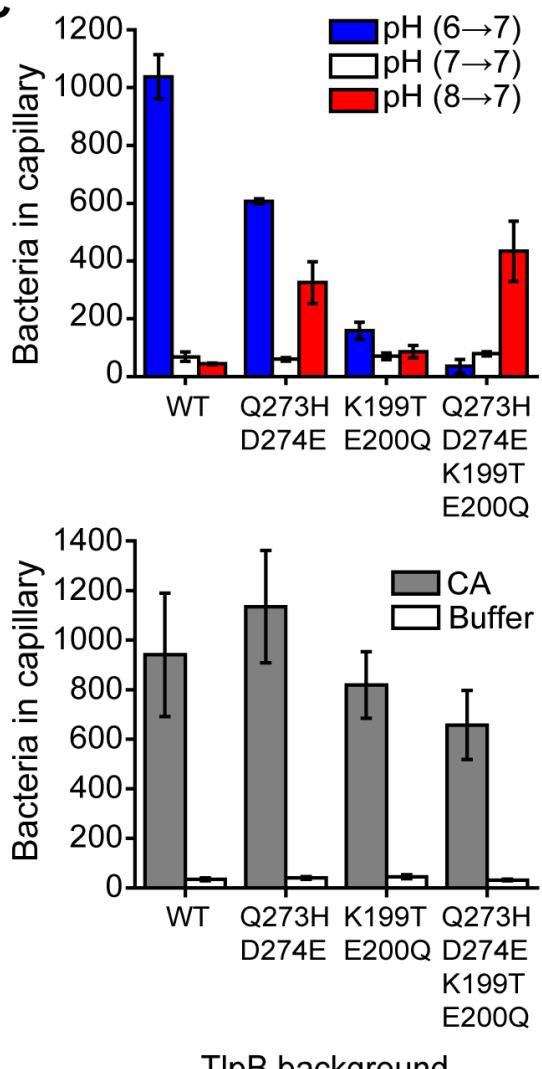
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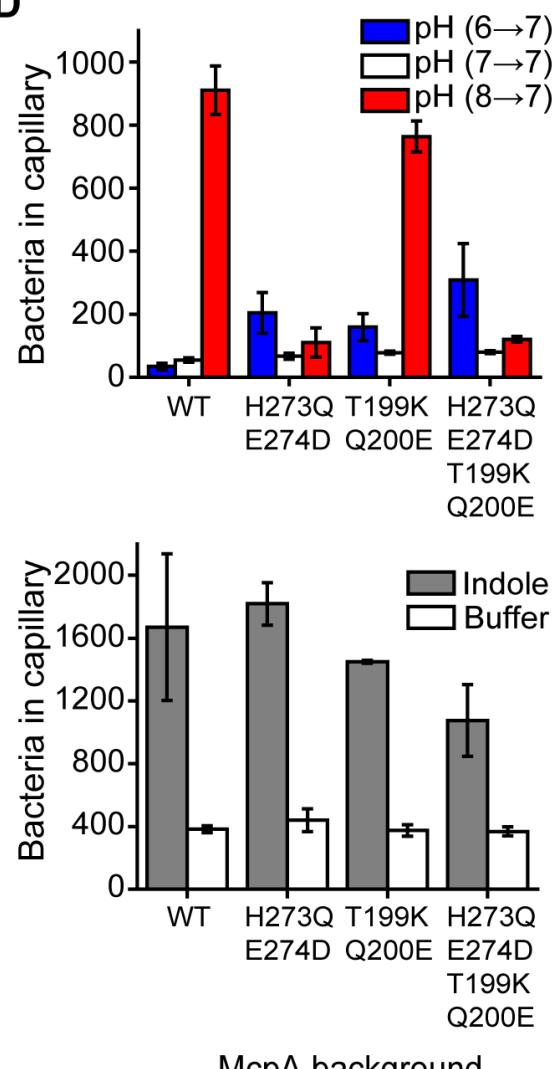
B



C



D



Key amino acid residues

TlpB background

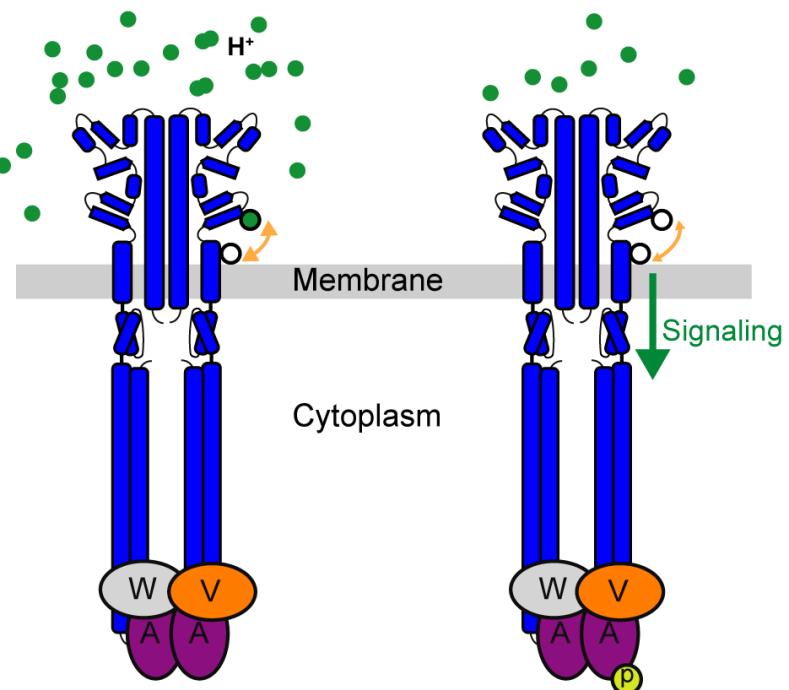
McpA background

A

Base sensing
by TlpB

Lower pH

Higher pH



B

Acid sensing
by McpA

Higher pH

Lower pH

