TITLE

Cytochrome oxidase requirements in *Bordetella* reveal insights into evolution towards life in the mammalian respiratory tract

SHORT TITLE

Cytochrome oxidases and *Bordetella* infection

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ABSTRACT

Little is known about oxygen utilization during infection by bacterial respiratory pathogens. The classical *Bordetella* species, including *B. pertussis*, the causal agent of human whooping cough, and *B. bronchiseptica*, which infects nearly all mammals, are obligate aerobes that use only oxygen as the terminal electron acceptor for electron transport-coupled oxidative phosphorylation. *B. bronchiseptica*, which occupies many niches, has eight distinct cytochrome oxidase-encoding loci, while *B. pertussis*, which evolved from a *B. bronchiseptica*-like ancestor but now only survives only in and between human respiratory tracts, has only three functional cytochrome oxidase-encoding loci: *cydAB1, ctaCDFGE1*, and *cyoABCD1.* To test the hypothesis that the three cytochrome oxidases encoded within the *B. pertussis* genome represent the minimum number and class of cytochrome oxidase required for respiratory infection, we compared *B. bronchiseptica* strains lacking one or more of the eight possible cytochrome oxidases *in vitro* and *in vivo*. No individual cytochrome oxidase was required for growth in ambient air, and all three of the cytochrome oxidases conserved in *B. pertussis* were sufficient for growth in ambient air and low oxygen. Using a high-dose, large-volume persistence model and a low-dose, small-volume establishment of infection model, we found that *B. bronchiseptica* producing only the three *B. pertussis*-conserved cytochrome oxidases was indistinguishable from the wild-type strain for infection. We also showed that CyoABCD1 is sufficient to cause the same level of bacterial burden in mice as the wild-type strain and is thus the primary cytochrome oxidase required for murine infection, and that CydAB1 and CtaCDFGE1 fulfill auxiliary roles or are important for aspects of infection we have not assessed, such as transmission. Our results shed light on respiration requirements for bacteria that colonize the respiratory tract, the environment at the surface of the ciliated epithelium, and the evolution of virulence in bacterial pathogens.

AUTHOR SUMMARY

Cytochrome oxidases, critical components for aerobic respiration, have been shown to be vital for pathogenesis and tissue tropism in several bacterial species. However, the majority of the research has focused on facultative anaerobes and infections of microoxic to anaerobic host environments, like the gut. We sought to understand the role of cytochrome oxidases during respiratory infection by *Bordetella bronchiseptica*, an obligate aerobe, performing the first analysis of cytochrome oxidases in an extracellular respiratory pathogen that we know of. By comparing *B. bronchiseptica* to the closely related *B. pertussis*, a strictly human-specific pathogen and the causative agent of whooping cough, we found three cytochrome oxidases that are important for growth and survival within the mammalian respiratory tract. We also found that a bo_3-type cytochrome oxidase, predicted to have a low affinity for oxygen and therefore best suited to ambient air levels of oxygen, was sufficient for both the establishment of infection and persistence in the respiratory tract in mice. Our findings reveal the importance of low affinity cytochrome oxidases in respiratory pathogens, and emphasize the need to study the physiology of diverse pathogens.

INTRODUCTION

To be effective pathogens, bacteria must be able to survive within the host. Even pathogens that survive exclusively within a host must contend with diverse microenvironments, moving from the initial site of infection to colonization sites and eventually to a new host while avoiding or neutralizing host defenses. As bacteria encounter new microenvironments within the host, they must adjust their physiologies to continue to grow. Most of the research on bacterial pathogens has focused on identifying and characterizing virulence factors. However, there is now an increased appreciation for the role that metabolism and physiology play during infection. Without energy, bacteria cannot survive and therefore cannot cause disease.

Electron transport-coupled oxidative phosphorylation pairs the transfer of electrons from donors to acceptors with the transport of protons across a membrane, generating the proton motive force used to drive the synthesis of ATP from ADP. This process occurs in a step-wise manner via the electron transport chain (ETC), starting with electron donors like NADPH and ending with a terminal electron acceptor. Aerobic respiration uses oxygen as the terminal electron acceptor, but alternative terminal electron acceptors like nitrate or nitrite are used by some bacteria to grow anaerobically. Redox-active protein complexes with heme cofactors called cytochrome oxidases are responsible for the transfer of electrons to oxygen. Cytochrome oxidases are named based on the types of hemes (*a, b, c, d,* or *o*) that the proteins bind. bo_3-type, aa_3-type, and cbb_3-type cytochrome oxidases are all heme-copper oxidases (HCOs), so-named for the copper ions that form a bi-metallic center with hemes in their catalytic subunits, while *bd*-type cytochrome oxidases form a unique class of heme-only cytochrome oxidases found exclusively in bacteria and archaea with no sequence homology to HCOs [1,2]. Cytochrome oxidases can also be categorized by their electron donors. Quinol oxidases receive their electrons directly from reduced quinones, while cytochrome *c* oxidases receive their electrons from cytochrome *c*. Cytochrome oxidases can also be grouped according to their affinity for oxygen: either low-affinity (K_m for $O_2 \cong 200$nM) [3], which are best suited for

100 atmospheric levels of oxygen (~21%), or high-affinity, which can function in microoxic conditions
101 (K_m for O_2 = 3-8 nM) [4,5]. Increased affinity for oxygen comes with a trade-off; high-affinity
102 cytochrome oxidases generate less proton motive force per electron transferred than low-affinity
103 cytochrome oxidases [6].

104 Respiration as a whole, and cytochrome oxidases in particular, have been shown to be
105 required for infection in many pathogenic species, including gastrointestinal pathogens
106 *Escherichia coli* [7,8], *Vibrio cholerae* [9], *Listeria monocytogenes* [10,11], *Shigella flexneri*
107 [12,13], and *Salmonella enterica* Serovar Typhimurium [14–16]; the oral pathogen
108 *Aggregatibacter actinomycetemcomitans* [17]; the respiratory pathogen *Mycobacterium*
109 *tuberculosis* [18,19]; and pathogens that can cause many types of infection including
110 bacteremia and sepsis like *Staphylococcus aureus* [20] and group B *Streptococcus* [21,22].
111 However, the majority of pathogens assessed have been facultative anaerobes, which do not
112 require cytochrome oxidases for survival. Additionally, many of these pathogens colonize
113 microenvironments that are expected to have limited levels of oxygen.

114 The classical *Bordetella* species (*B. pertussis, B. parapertussis*$_{Hu}$, and *B. bronchisceptica*)
115 are extremely closely related bacteria that colonize the upper and lower respiratory tracts of
116 humans (in the case of *B. pertussis* and *B. parapertussis*$_{Hu}$) and other mammals (in the case of
117 *B. bronchiseptica*). They are obligate aerobes that use only oxygen as their terminal electron
118 acceptor for electron transport-coupled oxidative phosphorylation [23,24]. Therefore, they
119 depend solely on components of the ETC, like cytochrome oxidases, to generate energy.
120 Previous work in *B. pertussis* has shown that cytochrome *c* is not required for survival *in vitro*
121 [25]. Little is known, however, about the role of cytochrome oxidases in the classical *Bordetella*
122 species.

123 In this study, we leveraged the evolution of classical bordetellae to investigate which
124 cytochrome oxidases are important for respiration in the mammalian respiratory tract, using *B.*
125 *bronchiseptica* as our model organism. *B. bronchiseptica*, which occupies multiple

126 environmental niches, has the potential to produce eight distinct cytochrome oxidases. By
127 contrast, *B. pertussis*, which survives in the environment only during transmission between
128 hosts, has undergone significant genome reduction as it evolved from a *B. bronchiseptica*-like
129 ancestor [24]. Genomic comparisons between *B. bronchiseptica* and *B. pertussis* found that *B.*
130 *pertussis* strains have only broadly conserved three intact cytochrome oxidase-encoding gene
131 loci. We hypothesized that the three cytochrome oxidases conserved in *B. pertussis* represent
132 the minimum number and class of cytochrome oxidases required for bordetellae to survive
133 within, and briefly between, hosts. We genetically manipulated our bacteria to determine the
134 necessity and sufficiency of different cytochrome oxidases, first for growth *in vitro* under ambient
135 air and different atmospheric conditions, then during infection, using two different mouse models
136 of respiratory infection. Our results demonstrate that *B. bronchiseptica* is able to survive with as
137 few as one cytochrome oxidase, even during infection and when challenged with low oxygen
138 conditions. These findings also inform our understanding of the murine respiratory tract,
139 indicating that bacteria may have more access to oxygen while attached to the ciliated
140 epithelium than previously supposed.
141

142 RESULTS

143 ***Bordetella bronchiseptica* has eight cytochrome oxidase-encoding gene loci**

144 The *B. bronchiseptica* RB50 genome has eight cytochrome oxidase-encoding gene loci,
145 annotated computationally based on predicted amino acid homology **(Table 1)**. Three of the loci
146 are predicted to encode high-affinity cytochrome oxidases. *ccoNOQP* (abbreviated *cco*)
147 encodes a *cbb$_3$-type* cytochrome oxidase. In *cbb$_3$*-type cytochrome oxidases, CcoN contains the
148 active site that facilitates the reduction of molecular oxygen to water and thus functions as the
149 catalytic subunit [26,27]. Two loci encode *bd*-type cytochrome oxidases (*cydAB1* and *cydAB3*,
150 abbreviated *cyd1* and *cyd3*). The *cyd1* operon of *B. bronchiseptica* also includes a third subunit,
151 *cydX*, which is seen in some, but not all, *bd*-type cytochrome oxidases and may be important for

152 complex function. The variation across different species in number of subunits, structural
153 arrangement of coordinated hemes, and positioning of the quinol binding site makes it difficult to
154 draw definitive conclusions about *bd*-type cytochrome oxidases [28]. CydA is the subunit that
155 coordinates the three hemes required by this cytochrome oxidase, making it the catalytic
156 subunit; however, *bd-type* cytochrome oxidases require both CydA and CydB for catalytic
157 activity, as evidenced by the loss of *d*-type heme when the genes encoding either subunit are
158 deleted [29,30].

159 **Table 1.** Cytochrome oxidase-encoding gene loci in classical *Bordetella* species.

Class	Gene names	*Bb* locus (RB50)	Predicted oxygen affinity	Is the locus present/intact?[1]			
				Complex I (n=6)	Complex II (n=14)	Complex III (n=4)	Complex IV (n=4)[3]
cbb$_3$	*ccoNOQP*	BB3329-3326	High	100%	0%	100%	75%
bd	*cydAB1*	BB4498-4497	High	100%	100%	100%	100%
	cydAB3	BB4012-4011	High	100%	50%	100%	100%
	cioAB	BB1238-1239	Low[2]	100%	0%	100%	75%
aa$_3$	*ctaCDFGE1*	BB4831-4827	Low	100%	100%	100%	100%
	ctaD2	BB4674	Low	100%	7.1%	100%	100%
bo$_3$	*cyoABCD1*	BB1283-1286	Low	100%	92.9%	100%	75%
	cyoABCD2	BB1310-1307	Low	100%	0%	100%	75%

Shaded cells represent cytochrome oxidase-encoding loci that are broadly conserved in *B. pertussis* (Complex II)
[1] Further information about strains compared can be found in Supplemental Table 1; details about the differences from RB50 can be found in Supplemental Table 2.
[2] *cioAB*, formerly known as *cydAB2*, was previously predicted to encode a high-affinity cytochrome oxidase.
[3] One strain, the environmental isolate HT200, has gained mutations in 4 cytochrome oxidase-encoding gene loci; the other three strains have maintained all 8 loci.

160 Five of the eight cytochrome oxidase-encoding loci are predicted to encode low-affinity
161 cytochrome oxidases. These include one complete *aa*$_3$-type cytochrome oxidase (*ctaCDFGE1,*
162 abbreviated *cta1*) and one lone *aa*$_3$-type subunit (*ctaD2*). The *cta1* operon contains two
163 additional genes: a putative membrane protein (BB4829, here named *ctaF1*) and a putative
164 DUF2970-containing membrane protein (BB4828, here named *ctaG1*). This operon structure is
165 also found in *Achromobacter* species, which are closely related to *Bordetella*. *ctaF1* shares no
166 homology to the gene of the same name found in *Bacillus* species, which encodes part of a
167 *caa3*-type cytochrome oxidase [31]. CtaD is the catalytic subunit in *aa*$_3$-type cytochrome

168 oxidases [32]. While CtaD2 cannot function within the electron transport chain on its own, it
169 could be forming heterocomplexes with CtaC1 and CtaE1, as CcoN orphan subunits have been
170 shown to do in *Pseudomonas aeruginosa* [33]. Two loci encode bo_3-type cytochrome oxidases
171 (*cyoABCD1* and *cyoABCD2*, abbreviated *cyo1* and *cyo2*). CyoB is the catalytic subunit for bo_3-
172 type cytochrome oxidases [34,35]. Finally, the *cioAB* locus (abbreviated *cio*) encodes a cyanide-
173 insensitive oxidase (CIO). CIOs are a subclass of *bd*-type cytochrome oxidases that are low-
174 affinity and were first characterized in *P. aeruginosa,* where CioAB confers resistance to cyanide
175 [36]. CIOs require both subunits to be functional, like other *bd*-type cytochrome oxidases.

176 ***Bordetella pertussis* has only three broadly conserved cytochrome oxidase-encoding**
177 **gene loci**

178 Genomic comparisons of the classical bordetellae have found that the three subspecies
179 can be divided into four complexes [37]. Complex I, which includes RB50, is most similar to the
180 last common ancestor shared by the classical bordetellae and contains *B. bronchiseptica*
181 strains, primarily those isolated from non-human mammals. Complex II contains *B. pertussis*
182 and Complex III contains *B. parapertussis*$_{Hu}$. Complex IV, which phylogenetically lies between
183 Complex I and II, contains *B. bronchiseptica* strains, primarily those isolated from humans. A
184 recent genotyping study identified a novel *B. bronchiseptica* branch, named lineage II, that is
185 distinct from the classical *Bordetella* [38]. This branch primarily contains strains that were
186 formerly classed as Complex IV and had been identified by other groups as being divergent
187 from other classical bordetellae [39,40].

188 *B. pertussis* and *B. parapertussis*$_{Hu}$ have shrunken their genomes since evolving from a
189 *B. bronchiseptica*-like ancestor, with genomes approximately 77% and 89% the size of the *B.*
190 *bronchiseptica* genome, respectively [24]. Additionally, *B. pertussis* and *B. parapertussis*$_{Hu}$ have
191 specialized, infecting only humans instead of maintaining the wide mammalian host range and
192 environmental survival capabilities of *B. bronchiseptica*. We investigated which cytochrome

193 oxidase-encoding genes have been conserved across the classical bordetellae, hypothesizing
194 that the human-adapted *B. pertussis* and *B. parapertussis*$_{Hu}$ have lost cytochrome oxidases that
195 are not required for survival within, and briefly between, human respiratory tracts. We chose 28
196 *Bordetella* strains for comparison, including both laboratory strains and clinical isolates: 6
197 Complex I, 14 Complex II, 4 Complex III, and 4 Complex IV (2 of which could be classified as
198 lineage II) **(S1 Table).** We found that almost all the strains in Complex I, III, and IV have
199 maintained intact copies of all eight cytochrome oxidase-encoding gene loci **(Table 1).** Only one
200 strain, the environmental isolate HT200, differed, having acquired frameshift mutations leading
201 to premature stop codons in *ccoN*, *cioB*, *cyoB1,* and *cyoB2* which most likely would prevent the
202 complexes encoded by each of these cytochrome oxidase-encoded loci (here notated as Cco,
203 Cio, Cyo1, and Cyo2) from functioning in this strain **(S2 Table).** This result is intriguing, as it
204 indicates that not all cytochrome oxidases are required to survive in the environment, or at least
205 within the thermal spring in which HT200 was isolated [40]. This strain is also a lineage II strain,
206 and thus phylogenetically distant from the classical *Bordetella* strains [38] **(S1 Figure)**.
207 *B. pertussis* strains have significantly diverged from the other classical bordetellae in
208 terms of their cytochrome oxidase-encoding genes. All 14 examined strains contained
209 premature stop codons within *ccoN* and *cyoA2* **(S2 Table**). These mutations would most likely
210 prevent Cco and Cyo2 from functioning. Interestingly, the point mutations that led to the
211 premature stops were identical across all 14 strains, indicating that these mutations were
212 acquired by the ancestral strain that became *B. pertussis.* Additionally, all 14 strains have lost
213 *cio* and 13 strains have lost *ctaD2*. The identical changes across diverse *B. pertussis* strains
214 indicate that functional copies of Cco, Cio, Cyo2, and CtaD2 were lost during the evolution from
215 the last common ancestor shared by *B. pertussis* and *B. bronchiseptica.*
216 There are mutations in cytochrome oxidase-encoding genes that differ between *B.*
217 *pertussis* strains. Strain 18323, a common laboratory strain, had the most differences. 18323
218 has been shown by many typing methods to differ from most *B. pertussis* strains, lacking some

219 genes usually seen in *B. pertussis* while having copies of genes normally found in *B.*
220 *bronchiseptica* and *B. parapertussis*$_{Hu}$ but not *B. pertussis* [41–44]. These genomic differences
221 are reflected in the phylogenetic tree, where 18323 is the closest *B. pertussis* strain to Complex
222 IV **(S1 Figure)**. 18323 was the only *B. pertussis* strain containing *ctaD2*. 18323 also contained
223 unique mutations, resulting in premature stop codons in *ccoP, cydB3, cyoB1,* and *cyoC1*, as
224 well as losing *cyoA1* and *cyoD1* **(S2 Table).** Interestingly, 18323 was not the only *B. pertussis*
225 strain that lost the ability to make Cyd3. Six of the 13 remaining Complex II strains contain a
226 frameshift mutation in the same position as 18323 but are shifted into a different reading frame.
227 The fact that these 6 strains all have identical mutations indicates that they may have evolved
228 from a common ancestor with this mutation and that this mutation occurred after *B. pertussis*
229 became its own subspecies.

230 Despite the differences within *B. pertussis* strains, a pattern emerges; excluding 18323,
231 all these strains have maintained functional loci encoding Cyd1, Cta1, and Cyo1. Therefore, we
232 hypothesize that these three cytochrome oxidases are necessary and sufficient for *Bordetella*
233 infection within the mammalian respiratory tract, the environment in which *B. pertussis* has
234 specialized, and the brief time spent between hosts during transmission.

235 **Cyd1, Cta1, or Cyo1 is required for growth in ambient air**

236 To assess the necessity of each cytochrome oxidase for survival under various
237 conditions, we generated *B. bronchiseptica* strains with in-frame gene loci deletions using allelic
238 exchange. Starting with our wild-type strain RB50 (WT), we were able to generate strains
239 lacking *cco* (Δ*cco*), *cyd1* (Δ*cyd1*), *cyd3* (Δ*cyd3*), *cio* (Δ*cio*), *cta1* (Δ*cta1*), *ctaD2* (Δ*ctaD2*), or *cyo1*
240 (Δ*cyo1*) **(S2 Figure)**. We were unable to delete the *cyo2* locus in its entirety; instead, we deleted
241 *cyoABC2* and left *cyoD2* intact. The resulting strain lacks a functional complex generated by the
242 proteins encoded by *cyo2* and is therefore called Δ*cyo2* hereafter.

243 Based on genome comparisons between *B. bronchiseptica* and *B. pertussis,* we aimed
244 to generate strains that allowed us to test the necessity and sufficiency of the three cytochrome
245 oxidase-encoding loci broadly conserved in *B. pertussis* (*cyd1, cta1,* and *cyo1*). We tried to
246 make a strain that lacks *cyd1, cyo1*, and *cta1* but were unable to generate it using our normal
247 growth conditions; all co-integrants reverted to WT at the last step of the allelic exchange
248 procedure, suggesting that at least one of the *Bp*-conserved cytochrome oxidases is required
249 for growth of *B. bronchiseptica* under standard laboratory conditions, i.e. in SS broth, in ambient
250 air. We were able to make a strain with only the three conserved cytochrome oxidases by
251 deleting the other five cytochrome oxidase-encoding loci ($cyd1^+$ $cta1^+$ $cyo1^+$, abbreviated *Bp*-
252 conserved). We then used this strain to generate strains for assessing the necessity of each of
253 the cytochrome oxidases conserved in *B. pertussis* when only these three are present. We were
254 able to create all three iterations; a strain with only *cta1* and *cyo1* ($cta1^+$ $cyo1^+$), a strain with
255 only *cyd1* and *cyo1* ($cyd1^+$ $cyo1^+$), and a strain with only *cyd1* and *cta1* ($cyd1^+$ $cta1^+$).
256 To assess the sufficiency of each cytochrome oxidase for growth under various
257 conditions, we generated strains that contain in-frame deletions of seven of the eight
258 cytochrome oxidase-encoding loci. We were able to generate three of the eight possible strains
259 with only one cytochrome oxidase-encoding gene loci: a strain with only *cyd1* ($cyd1^+$), a strain
260 with only *cta1* ($cta1^+$), and a strain with only *cyo1* ($cyo1^+$). We were unable to generate the five
261 other possible strains with only one cytochrome oxidase-encoding gene locus remaining under
262 our normal growth conditions; indeed, we were only able to generate strains lacking six of the
263 eight oxidase-encoding gene loci if one of the two remaining loci was *cyd1, cta1,* or *cyo1* **(S2**
264 **Figure)**. This result, combined with the fact that we were unable to generate a strain missing all
265 three cytochrome oxidases conserved in *B. pertussis,* indicates that Cyd1, Cta1, and Cyo1 are
266 the primary cytochrome oxidases used under normal growth conditions in ambient air and that
267 at least one is required under this condition.

268 **All of the strains we constructed are able to respire**

269 To determine how well our strains were able to respire after losing one or more
270 cytochrome oxidase(s), we grew our strains on medium containing triphenyl tetrazolium chloride
271 (TTC). TTC accepts electrons from the electron transport chain. When TTC is reduced in the
272 presence of cytochrome oxidases in a sufficiently dense culture, it undergoes an irreversible
273 color shift from colorless to red [45]. We examined the color of colony biofilms, normalized by
274 starting optical density (OD_{600}), after 24 hours of growth on media containing TTC. All strains
275 were able to respire, as evidenced by the red color of the colonies **(S3 Figure).** All strains
276 except *cta1*$^+$ had a similar level of redness, indicating that the mutant strains had a similar level
277 of respiration as WT. *cta1*$^+$ was less red overall but had spots with a higher density of red
278 pigment than WT. This color variation could reflect heterogeneity in the mutant population.

279 *cta1*$^+$ has other phenotypes that differ from WT. This strain generated smaller colonies
280 than WT on Bordet-Gengou (BG) blood agar plates, our standard culturing medium, requiring 72
281 hours to produce visible colonies as opposed to the 48 hours required by WT. Moreover, *cta1*$^+$
282 can't grow in our normal liquid culturing medium, Stainer-Scholte (SS) broth, when taken from
283 BG agar. However, colonies from BG agar are able to grow on SS agar, and these colonies can
284 then be grown in SS broth. *cta1*$^+$ cultures grown in SS broth or on SS agar can grow on BG
285 agar. This phenotype was not due to second-site mutations, as whole-genome sequencing
286 revealed no unique mutations compared to WT, *cyd1*$^+$, or *cyo1*$^+$. The reason for this unusual
287 phenotype is not known, and we did not pursue it further.

288 **No cytochrome oxidase is necessary for growth in ambient air, but Cyd1, Cta1, and Cyo1**
289 **are each sufficient.**

290 To determine if individual cytochrome oxidases are required for normal growth, we grew
291 strains with deletions of individual cytochrome oxidase-encoding loci (i.e. Δ*cco,* Δ*cyd1,* Δ*cyd3,*
292 Δ*cta1,* Δ*ctaD2,* Δ*cio,* Δ*cyo1,* and Δ*cyo2*) in SS medium in ambient air conditions and measured

293 growth over time via OD_{600} and number of viable bacteria (CFU/mL). WT reached stationary
294 phase between 12 and 18 hours **(Figure 1A)**. While the OD_{600} of WT stayed consistent after the
295 culture entered stationary phase, the CFU/mL began to decline after 30 hours. All eight strains
296 lacking a single cytochrome oxidase phenocopied WT by both OD_{600} and CFU/mL **(Figure 1A).**
297 This result indicates that no single cytochrome oxidase is required for growth under standard
298 laboratory conditions.

299 To determine if the cytochrome oxidases broadly conserved in *B. pertussis* are sufficient
300 for growth under the same conditions, we assessed growth of *Bp*-conserved. *Bp*-conserved
301 phenocopied WT over time **(Figure 1B)**, indicating that the combination of Cyd1, Cta1, and
302 Cyo1 is sufficient under these conditions. Given that the combination of the three *Bp*-conserved
303 cytochrome oxidases was sufficient, we then assessed their individual sufficiency. To do so, we
304 used strains with only a single cytochrome oxidase-encoding locus remaining (i.e. *cyd1*$^+$*, cta1*$^+$*,*
305 and *cyo1*$^+$). Overall, these strains behaved similarly to WT, reaching stationary phase at the
306 same time and having a similar growth rate **(Figure 1C).** *cyd1*$^+$ consistently had a slightly lower,
307 but not significantly different, OD_{600} and CFU/mL than WT. After 30 hours, when WT began to
308 decrease in CFU/mL, *cta1*$^+$ decreased in CFU/mL more slowly than WT, while *cyo1*$^+$ decreased
309 in CFU/mL more quickly than WT, especially between 36 and 48 hours. These data indicate that
310 each of the cytochrome oxidases conserved in *B. pertussis* is sufficient for WT levels of growth
311 in liquid media in ambient air.

312 **None of the cytochrome oxidases conserved in *B. pertussis* are critical for growth in low**
313 **oxygen or in high carbon dioxide**

314 Under normal laboratory conditions, bacteria have access to ambient air, which contains
315 approximately 21% O_2. When growing within the mammalian respiratory tract, however, bacteria
316 encounter a different atmospheric environment. Upon inhalation, alveoli inflate and oxygen in
317 the air is exchanged with carbon dioxide (CO_2) produced by cellular respiration; upon

318 exhalation, this new gas mixture, which contains approximately 16% O_2 and 5% CO_2, is
319 released back into the environment (reviewed in [46]). The classical bordetellae are likely further
320 restricted from access to oxygen since the ciliated epithelial cells to which the bacteria adhere
321 are coated in a layer of secreted mucus designed to protect the respiratory tract from
322 environmental pathogens [47].

323 To investigate how different cytochrome oxidases contribute to the ability of *B.*
324 *bronchiseptica* to grow in the different atmospheric conditions it may encounter in the
325 mammalian respiratory tract, we grew strains within an incubator that allowed us to manipulate
326 the atmospheric concentrations of oxygen, nitrogen, and CO_2. We collected endpoint samples to
327 keep the gas concentrations consistent for the duration of the experiment since taking samples
328 required us to open the incubator, thus exposing the cultures to ambient air. Since we
329 hypothesize that the three *Bp*-conserved cytochrome oxidases are sufficient for growth within
330 the mammalian respiratory tract, we focused on assessing the growth of *Bp*-conserved, *cyd1*$^+$*,*
331 *cta1*$^+$*,* and *cyo1*$^+$ relative to WT*.*

332 First, we assessed growth in low oxygen. Previous studies in *E. coli* have shown that
333 atmospheric oxygen concentrations at or below 5% are sufficient to shift expression from low-
334 affinity to high-affinity cytochrome oxidases in liquid cultures [48].We therefore grew our cultures
335 under 5% O_2 with constant agitation to maximize gas exchange. WT grew slower in 5% O_2 than
336 in ambient air; cultures grown for 48 hours at 5% O_2 contained less than half the bacteria found
337 at 24 hours in cultures grown in ambient air **(Figure 2A vs 2B).** Surprisingly, *Bp*-conserved,
338 *cyd1*$^+$*, cyo1*$^+$*,* and *cta1*$^+$ were all able to grow to WT levels in 5% O_2, with no strain having a
339 significant defect relative to WT in both OD_{600} and CFU/mL **(Figure 2B).** Since no cytochrome
340 oxidase was critical for growth at 5% O_2, we lowered the level of oxygen to 2% O_2. Under 2%
341 O_2, WT struggled to grow; cultures grown for 72 hours at 2% O_2 contained approximately 13% of
342 the bacteria found at 24 hours in cultures grown in ambient air **(Figure 2A vs 2C).** Despite low
343 oxygen availability, *cyo1*$^+$ and *cta1*$^+$ were able to grow to similar levels as WT and *Bp*-conserved

344 and *cyd1*$^+$ only had a slight growth defect **(Figure 2C)**. Together, these data indicate that none
345 of these cytochrome oxidases is required for growth under low oxygen conditions. Conversely,
346 these data indicate that Cyd1, Cta1, or Cyo1 is sufficient for WT-levels of growth under low
347 oxygen conditions.

348 Since bacteria in the respiratory tract also encounter increased carbon dioxide, we next
349 grew our strains in 5% CO_2 to determine if growth is impacted by this change. WT grew similarly
350 in 5% CO_2 as in ambient air **(Figure 2A vs 2D).** *Bp*-conserved, *cyd1*$^+$*, cyo1*$^+$*,* and *cta1*$^+$*,*
351 however, were all defective relative to WT by both OD_{600} and CFU/mL **(Figure 2D)**. This result is
352 surprising, as it suggests that the three cytochrome oxidases conserved in *B. pertussis* are not
353 sufficient for efficient growth in the presence of 5% CO_2. Indeed, it suggests that at least one of
354 the cytochrome oxidases deleted in *Bp*-conserved contributes to growth in 5% CO_2.

355 Given that the respiratory environment can simultaneously contain higher levels of
356 carbon dioxide and lower levels of oxygen than ambient air, we tested how well our strains are
357 able to grow in 5% O_2 5% CO_2. Under this condition, WT grew similarly to how it grew in 5% O_2
358 alone **(Figure 2B vs 2E).** After 48 hours of growth, *Bp*-conserved, *cyd1*$^+$*, cyo1*$^+$*,* and *cta1*$^+$ were
359 all able to grow to WT levels, with no strain having a significant defect relative to WT by both
360 OD_{600} and CFU/mL **(Figure 2E)**.

361 **No single cytochrome oxidase is required for persistence in the murine respiratory tract.**

362 To determine the role of individual cytochrome oxidases during infection, we inoculated
363 mice intranasally with bacteria using our high-dose, large-volume protocol, which allows us to
364 assess how well different strains can persist once introduced in the respiratory tract. We first
365 used strains containing deletions of individual cytochrome oxidase-encoding loci to determine
366 which cytochrome oxidases, if any, are necessary for persistence. We then collected the right
367 lung, the trachea, and nasal cavity tissue at 3 hours, 1 day, 3 days, and 7 days post-inoculation
368 and determined the bacterial burden in each organ. Due to the number of strains involved, we

369 could not test all eight strains simultaneously. Instead, we tested them in batches, with each
370 including WT, then compiled the data. All strains caused the same bacterial burden in mice as
371 WT in the nasal cavity, trachea, and lung **(Figure 3)**. These data indicate that no individual
372 cytochrome oxidase is required for persistence in the murine respiratory tract.

373 **The cytochrome oxidases conserved in *B. pertussis* are sufficient for persistence during**
374 **murine infection**

375 We next assessed the role of the three cytochrome oxidases conserved in *B. pertussis*
376 for persistence during infection. We infected mice with either *Bp*-conserved or WT, and
377 determined the bacterial burden in the nasal cavity, trachea, and lung over the course of
378 infection. The *Bp*-conserved strain was statistically indistinguishable from WT in all tissues at all
379 time points tested **(Figure 4),** indicating that the three cytochrome oxidases conserved in *B.*
380 *pertussis* are sufficient for persistence in mice.

381 **Cyo1 is sufficient for persistence during infection, while strains with only Cyd1 or Cta1**
382 **are defective for persistence relative to WT**

383 The three cytochrome oxidases conserved in *B. pertussis* are predicted to have different
384 characteristics **(Table 1).** Cyd1 is predicted to be a high-affinity *bd*-type ubiquinol oxidase. Cta1
385 is predicted to be a low-affinity HCO cytochrome *c* oxidase. Cyo1 is predicted to be a low-affinity
386 HCO ubiquinol oxidase. We hypothesize that each of these cytochrome oxidases fulfills a
387 different function during infection due to their different biochemical properties. To test this
388 hypothesis, we infected mice using strains with only a single cytochrome oxidase-encoding
389 gene locus remaining: *cyd1*$^+$*, cta1*$^+$*,* and *cyo1*$^+$. *cyd1*$^+$ had a slight defect relative to WT in the
390 nasal cavity, causing a lower bacterial burden at day 1 that was able to reach WT levels by day
391 3 **(Figure 5A, top)**. *cyd1*$^+$ was consistently defective in the lower respiratory tract relative to WT,
392 causing a significantly lower bacterial burden in the trachea and the lung out to 14 days **(Figure**
393 **5A, middle and bottom)**. *cta1*$^+$ was defective relative to WT in all three sampled tissues

394 **(Figure 5B)**. However, this strain was not cleared from the lung faster than WT, as seen by the
395 statistically similar burdens recovered on day 14 when the burden of WT decreased relative to
396 earlier time points **(Figure 5B, bottom)**. Most surprisingly, *cyo1*$^{+}$ was recovered at levels similar
397 to WT in all three sampled tissues across the course of infection **(Figure 5C).** These data
398 indicate that Cyo1 is sufficient for WT-levels of growth in this model of mouse infection, despite
399 being predicted to be optimized for ambient air levels of oxygen, while Cyd1 and Cta1 are not
400 sufficient. Additionally, all three strains are able to persist to some degree throughout the
401 respiratory tract once introduced, indicating that any of these cytochrome oxidases is sufficient
402 for survival within a mouse.

403 **The *Bp*-conserved strain is indistinguishable from WT in its ability to establish**
404 **respiratory colonization**

405 We and others have used high-dose, large-volume and low-dose, small-volume mouse
406 protocols to investigate respiratory tract persistence and establishment of infection, respectively.
407 In our lab, we have previously used the low-dose, small-volume protocol only in rats, where we
408 showed that doses of 20 CFU are sufficient for infection in rats [49]. To adapt this approach to
409 mice, we performed preliminary experiments using WT to determine a viable volume and dose
410 for intranasal inoculation in wild-type mice. We found that 4μL was sufficient to introduce
411 bacteria to the nasal cavity but not the trachea, as we were able to recover CFU from the nasal
412 cavity but not the trachea at this volume on the same day as we inoculated the mice **(S4A**
413 **Figure).** We also found that 100 CFU per inoculum was sufficient to ensure robust colonization
414 of the nasal cavity, as well as consistently recoverable burden from the trachea by day 7 post-
415 inoculation **(S4A Figure).** In this model, bacteria were not consistently recovered from the lungs.

416 We next assessed whether the three *Bp*-conserved cytochrome oxidases are sufficient
417 for infection. *Bp*-conserved phenocopied WT in the high-dose, large-volume model **(Figure 4),**
418 demonstrating that the three cytochrome oxidases found in *B. pertussis* are sufficient for

419 persistence. We performed a preliminary experiment to determine if these three cytochrome
420 oxidases are also sufficient for establishing infection. Mice infected with *Bp*-conserved had a
421 similar bacterial burden in the nasal cavity 3 days post-inoculation as mice infected with WT,
422 indicating that the combination of Cyd1, Cyo1, and Cta1 is sufficient for establishing infection
423 **(S3B Figure).**

424 **Cyo1 is sufficient for efficient establishment of infection, while Cyd1 and Cta1 are not**

425 To assess the individual roles of Cyd1, Cyo1, and Cta1 during the establishment of
426 infection, we first tested whether any of these three cytochrome oxidases is required. We used
427 the derivatives of *Bp-conserved* that have two remaining cytochrome oxidases (i.e. *cta1*$^+$ *cyo1*$^+$*,*
428 *cyd1*$^+$ *cyo1*$^+$*,* and *cyd1*$^+$ *cta1*$^+$). These strains phenocopied WT growth in ambient air **(S5A**
429 **Figure)**. We infected mice with these strains using our low-dose, small-volume protocol and
430 determined the nasal cavity bacterial burdens 3 and 7 days post-inoculation. We found that
431 mice infected with the strains with two cytochrome oxidases had similar bacterial burdens to
432 mice infected with *Bp*-conserved **(S5B Figure)**. One strain, *cyd1*$^+$ *cta1*$^+$*,* trended towards
433 causing a lower bacterial burden than *Bp*-conserved, but this difference was not significant.
434 Based on these data, no single cytochrome oxidase is required to establish infection.

435 To determine if any of the three *Bp*-conserved cytochrome oxidases is sufficient to
436 establish infection, we infected mice with the strains with only a single cytochrome oxidase-
437 encoding locus remaining (i.e. *cyd1*$^+$*, cta1*$^+$*,* and *cyo1*$^+$) and determined the bacterial burden as
438 above. Overall, the trends in the low-dose, small-volume experiments mirrored those seen in the
439 high-dose, large-volume experiments. Mice infected with *cyd1*$^+$ had a lower bacterial burden 3
440 days post-inoculation than mice infected with *Bp*-conserved but reached the same burden by
441 day 7 **(Figure 6).** Mice infected with *cta1*$^+$ had a significantly lower bacterial burden than mice
442 infected with *Bp*-conserved at both day 3 and 7 **(Figure 6)**. Despite causing less than one
443 percent of the bacterial burden of *Bp*-conserved strain at day 7 in mice, *cta1*$^+$ nonetheless was

444 able to establish infection and persist for the duration of the experiment. Mice infected with
445 *cyo1*$^{+}$ had the same bacterial burden as mice infected with *Bp*-conserved at both timepoints
446 **(Figure 6)**. Together, these data indicate that while all three cytochrome oxidases are sufficient
447 to enable the establishment of infection, as seen by recoverable bacteria following infection with
448 all three strains, only Cyo1 is sufficient to achieve the level of colonization reached when all
449 three cytochrome oxidases conserved in *B. pertussis* are present.

450 **Regulation of cytochrome oxidase-encoding genes broadly follows the predicted affinity**
451 **of the cytochrome oxidases they encode**

452 Given that we were unable to construct a strain that lacks all three of the cytochrome
453 oxidase-encoding gene loci conserved in *B. pertussis* **(S2 Figure)***,* we investigated how different
454 cytochrome oxidase-encoding genes are regulated, hypothesizing that some loci may not be
455 expressed under laboratory growth conditions. We diluted overnight cultures to the same
456 density, grew them for 4 hours in either ambient air, 5% O_2, 2% O_2, or 5% CO_2, extracted RNA,
457 and sent it for sequence analysis.

458 First, we examined the level of transcripts in ambient air to investigate the expression of
459 cytochrome oxidase-encoding genes under our normal growth conditions. Raw normalized read
460 counts were converted to counts per million for comparison. All eight cytochrome oxidase-
461 encoding gene loci had detectable transcripts **(Figure 7A)**. However, when compared to *fhaB*
462 and *flaA,* two well-characterized and highly-regulated genes, we found that transcript levels for
463 most of the non-conserved cytochrome oxidase-encoding gene loci (*cyd3, cio, ctaD2,* and *cyo2*)
464 were similar to that of *flaA,* the protein product of which cannot be detected under these growth
465 conditions [50]. Therefore, transcription of *cyd3, cio, ctaD2,* and *cyo2* may be insufficient to
466 produce enough functional enzyme complexes to support respiration. By contrast, transcript
467 abundance for *ccoN* was the highest of all of the loci, with high expression of the rest of the *cco*
468 locus as well, yet we were unable to make a strain with only *cco*. This result suggests a lack of

469 sufficient translation, complex assembly, or cofactors required for Cco. The three *B. pertussis*-
470 conserved cytochrome oxidase-encoding loci had high levels of expression, though not as high
471 as *fhaB,* which encodes a highly abundant secreted protein.

472 We next examined the fold change in transcript abundance in bacteria grown in our
473 conditions of interest (5% O_2, 2% O_2, and 5% CO_2) and ambient air. In 5% O_2, *cydA1* and *cydB1*
474 had ~3 times more transcripts than in ambient air **(Figure 7B).** This result is consistent with the
475 prediction that Cyd1 functions as a high-affinity cytochrome oxidase and is therefore adapted for
476 use in low oxygen environments. No other gene had a significant difference in transcript
477 abundance in 5% O_2 versus ambient air.

478 In 2% O_2, differences in transcript abundance were greater **(Figure 7C)**. *cydA1* had
479 significantly more transcripts in 2% O_2 than in ambient air but fewer transcripts than in 5% O_2.
480 Genes within *cyo1* (*cyoC1* and *cyoD1*) had significantly fewer transcripts in 2% O_2 relative to
481 ambient air, which matches the prediction that these genes encode components of a low-affinity
482 cytochrome oxidase. *ctaC1* and *ctaD1* have less expression in 2% O_2 relative to ambient air,
483 with *ctaD1* having a larger relative decrease in transcripts. Interestingly, *ctaD2* had higher
484 expression in 2% O_2 relative to ambient air, with ~2.6 times more transcripts in ambient air than
485 in 2% O_2. Since the genes encoding other components of Cta1 did not significantly decrease in
486 expression in 2% O_2, CtaD2 could be interacting with CtaC1 and CtaE1 (and potentially CtaF
487 and CtaG1) to form a heterocomplex (i.e. CtaC1D2F1G1E1). Unexpectedly, *ccoQ* had
488 significantly lower levels of transcripts in 2% O_2 relative to ambient air, despite being predicted
489 to encode part of a high-affinity cytochrome oxidase, and *cyoC2* and *cyoD2* had significantly
490 higher levels of transcripts, despite being predicted to encode parts of a low-affinity cytochrome
491 oxidase.

492 In 5% CO_2, no gene had significant changes in expression relative to growth in ambient
493 air **(Figure 7D).** This was surprising, as 5% CO_2 was the only growth condition tested that
494 resulted in a large growth defect in *Bp*-conserved relative to WT **(Figure 2)**, leading us to

495 hypothesize that a non-*Bp*-conserved cytochrome oxidase that is not required for growth in
496 ambient air must be important for growth in 5% CO_2. While this hypothesis could still be true,
497 these results indicate that differences in growth in ambient air and 5% CO_2 are not due to
498 differences in cytochrome oxidase-encoding gene expression.
499

500 DISCUSSION

501 Our goal was to understand the importance of cytochrome oxidases for bacterial
502 pathogens that infect the mammalian respiratory tract. *B. bronchiseptica* contains eight
503 cytochrome oxidase-encoding gene loci, while *B. pertussis,* which is strictly a human pathogen
504 that survives only briefly in the environment during transmission between hosts, contains only
505 three cytochrome oxidase-encoding gene loci that are broadly conserved across strains.
506 Perhaps unsurprisingly, the three *B. pertussis*-conserved cytochrome oxidases were sufficient
507 for *B. bronchiseptica* to both establish infection and persist in the respiratory tracts of mice.
508 Somewhat surprisingly, however, *B. bronchiseptica* producing only a single low-affinity
509 cytochrome oxidase (encoded by *cyoABCD1,* abbreviated Cyo1) was indistinguishable from WT
510 in both infection models.

511 While it is well known that we inhale ambient air containing ~21% O_2 and low levels of
512 CO_2 and exhale air containing ~16% O_2 and ~5% CO_2, the levels of oxygen within specific
513 microenvironments in the respiratory tract are unknown. The classical bordetellae bind to the
514 surface of ciliated epithelial cells lining the respiratory tract, which do not continue into the
515 smallest bronchioles or the alveoli. The ciliated epithelium is covered in a layer of mucus
516 secreted by neighboring goblet cells and tethered mucins produced by the ciliated cells
517 themselves. These viscous layers protect the respiratory tract from environmental pathogens
518 but impede the diffusion of molecules, including oxygen, to the epithelium. We therefore
519 anticipated that a high-affinity cytochrome oxidase would be important in this environment, and
520 hence were surprised that *cyo1*$^+$, a strain producing only a predicted low-affinity cytochrome
521 oxidase, was able to infect mice with the same efficiency as WT. If the Cyo1 enzyme is, in fact,
522 low-affinity as predicted, then this result indicates that the surface of the ciliated epithelium has
523 access to more oxygen than previously thought.

524 The strain producing only Cyd1*,* by contrast, was attenuated relative to WT in both
525 animal models. The level of attenuation depended on the site of the respiratory tract examined.

526 In the nasal cavity, the *cyd1*$^+$ strain caused lower bacterial burdens than WT early in infection,
527 but was able to reach WT-levels later in infection, indicating a growth delay in this environment.
528 In the lower respiratory tract, however, the *cyd1*$^+$ strain was consistently defective relative to WT
529 throughout the course of infection. If the surface of the ciliated epithelium is sufficiently
530 oxygenated, as the results with the *cyo1*$^+$ strain suggest, this result could reflect the lower
531 efficiency of high-affinity cytochrome oxidases, which pump fewer protons, and therefore
532 generate less proton motive force, per electron transferred [6]. *bd*-type cytochrome oxidases
533 have been linked to virulence in other pathogens not only for their oxygen affinity but also for
534 their unique resiliency in the face of immune system attacks. *bd*-type cytochrome oxidases are
535 more tolerant of oxidative and nitrosative stress than HCOs and have been shown to detoxify
536 hydrogen peroxide and peroxynitrite produced by neutrophils and macrophages [51]. This role
537 for *bd*-type cytochrome oxidases has been shown in *M. tuberculosis*, where the *bd*-type
538 cytochrome oxidase is dispensable for the establishment of infection but is necessary after the
539 initiation of adaptive immunity [18]. In this way, Cyd1 may play a larger role during in *B.*
540 *pertussis,* which causes an acute infection that is combated by a strong, and ultimately
541 sterilizing, mucosal immune response, compared to *B. bronchiseptica,* which typically causes
542 chronic and, in the case of our mouse models, asymptomatic infection (reviewed in [52,53]).
543 Cta1 was the least effective during infection, with the strain producing only this
544 cytochrome oxidase being recovered at lower levels than WT in all tissues at all timepoints in
545 both models of infection. Cta1 could, however, play a role that was not assessed by our models.
546 Indeed, previous microarray data indicated that *ctaC1, ctaD1, ctaG1,* and *ctaE1* are repressed
547 by BvgAS, the two-component system that positively regulates all known protein virulence
548 factors in *Bordetella* [54]. Given that the Bvg$^+$ mode is both necessary and sufficient for
549 infection, Cta1 may play a role outside the host during transmission [55].
550 Importantly, the mouse respiratory tract is not identical to the respiratory tract of humans;
551 it is more similar to the human distal airways, both in terms of surface area and mucus

552 composition. In humans, proximal airways are coated in mucus primarily made of MUC5AC
553 mucins and distal airways have mucus made of MUC5B, whereas in mice, Muc5b is
554 constitutively produced throughout the airway and almost no Muc5ac is produced [56]. The
555 organization of the mucus is also different, with humans and other large mammals producing
556 long thick bundles of mucins such that the proximal airway is coated in a layer of mucus, while
557 in mice (and most likely, the distal airways of humans), the mucus is unevenly distributed,
558 forming a patchy covering [57,58]. This patchy covering creates areas with thinner mucus
559 layers, which would make oxygen diffusion easier. Therefore, the bacteria attached to the
560 mouse ciliated epithelium may have access to more oxygen than they would on the human
561 ciliated epithelium.

562 It is perhaps unsurprising that *B. bronchiseptica* can grow in low oxygen given its many
563 cytochrome oxidase-encoding loci, three of which are predicted to encode high-affinity
564 cytochrome oxidases. WT was able to grow in both 5% and 2% O_2 (albeit at a far slower rate
565 than in ambient air). It was surprising to us, however, that the *Bp*-conserved, *cyd1*$^+$*, cta1*$^+$*,* and
566 *cyo1*$^+$ strains were all able to grow similarly under low oxygen conditions. Indeed, the *cta1*$^+$ and
567 *cyo1*$^+$ strains*,* which encode only low-affinity cytochrome oxidases, grew as well as WT in 2%
568 O_2. This result indicates that all of the *B. pertussis*-conserved cytochrome oxidases all function
569 equally well under low oxygen in laboratory conditions, despite their predicted affinities.
570 Additionally, *B. bronchiseptica* is resilient when challenged with low oxygen. It is able to persist
571 in static PBS, where oxygen diffusion is limited, with viable CFU being recovered even 24
572 weeks post-inoculation [59]. In this aspect, *B. bronchiseptica* is comparable to *M. tuberculosis,*
573 another obligate aerobe that can persist in low oxygen conditions for long periods of time [60].

574 Our analysis primarily focused on the three cytochrome oxidase-encoding loci conserved
575 in *B. pertussis.* However, we also examined the expression of the other five loci to see if they
576 played a role in any of our experimental conditions. We found that *cyd3, cio, ctaD2,* and *cyo2*
577 had fewer transcripts per million than *flaA*, a BvgAS-repressed gene whose protein product is

578 not detected in our growth condition (SS medium) [50]. We were also unable to generate strains
579 with only *cco, cyd3, cio, ctaD2,* or *cyo2* intact; at least one of the three *B. pertussis*-conserved
580 loci had to be intact to generate a strain. Although the specific environments that *B.*
581 *bronchiseptica* occupies outside the mammalian respiratory tract are unknown, our results
582 suggests these environments are complex and varied, requiring different cytochrome oxidases
583 than those utilized in lab culture.

584 In our RNAseq analyses, we only examined expression in wild-type *B. bronchiseptica*
585 and therefore did not assess the effect that altering cell homeostasis by deleting cytochrome
586 oxidase-encoding genes could have on global gene expression. In the *cyd1*$^{+}$*, cta1*$^{+}$*,* and *cyo1*$^{+}$
587 strains*,* it is likely that the remaining cytochrome oxidase-encoding locus is more highly
588 expressed to compensate for the loss of the other two loci, which are normally expressed in our
589 growth conditions. Expression changes could also explain the unusual plate-based growth
590 phenotypes we observed for the *cta1*$^{+}$ strain*,* which could not be explained by secondary
591 mutations. Upregulation of the remaining cytochrome oxidase-encoding loci in the strains with
592 only a single locus could be masking synergy that occurs when the cytochrome oxidases are
593 present at their endogenous levels.

594 Two additional trends emerged from the RNAseq. First, many highly BvgAS-activated
595 genes had lower expression in low oxygen than in ambient air, including *bvgA*, *bvgS*, *fhaB*, and
596 *cyaA* **(S3,4 Table).** By contrast, the common reference genes *rpoD* and *recA* were not
597 differentially expressed in low oxygen as compared to ambient air, indicating that the differences
598 seen for BvgAS-activated genes are not due to global changes in gene expression. *In vitro*
599 studies using purified solubilized BvgS have shown that BvgS activity is sensitive to the redox
600 state of ubiquinone through its PAS domain [61]. *In vivo* studies also support that BvgS is
601 sensitive to redox potential via its PAS domain [62]. Thus, decreased expression of BvgAS-
602 activated genes could be directly related to decreased respiration affecting BvgS activity in low
603 oxygen environments.

604 Second, we found that expression of the *ptx* genes (*ptxABCDE*), which encode pertussis
605 toxin, was increased in *B. bronchiseptica* in low oxygen **(S3,4 Table)**. This result is exciting,
606 because *ptx* gene expression have never been detected in *B. bronchiseptica in vitro* or *in vivo,*
607 and only a weak luminescent signal was detected when the *B. bronchiseptica ptx* promoter was
608 used to drive the expression of luciferase-encoding genes [63]. Pertussis toxin is an important
609 virulence factor during human infection, and in *B. pertussis,* the *ptx* genes are activated by
610 BvgAS (reviewed in [64]). In Complex I *B. bronchiseptica* strains, from which the other classical
611 bordetellae evolved, the *ptx* promoter region is not BvgAS-regulated but the *ptx* genes (as well
612 as the *ptl* genes that encode the export system for pertussis toxin) are present and intact
613 [65,66], suggesting that in *B. bronchiseptica*, pertussis toxin plays a different role than
614 contributing to mammalian infection. By contrast, many Complex IV *B. bronchiseptica* strains
615 have lost their *ptx* genes [37]. These strains also don't survive as well as Complex I strains
616 when grown in environmental conditions, instead being more adapted to the human hosts from
617 which they were isolated. These results combined, together with the fact that the substrate for
618 pertussis toxin is a eukaryotic G protein, suggest that pertussis toxin was used by ancestral *B.*
619 *bronchiseptica* strains to manipulate a non-mammalian eukaryotic host or predator in the
620 environment. As strains evolved to infect humans and lost their ability to survive in the
621 environment, those that became *B. pertussis* acquired mutations in the *ptx* promoter that
622 allowed for activation by BvgAS (and presumably not the regulator(s) that control *ptx* expression
623 in *B. bronchiseptica*). Our findings suggest that one feature of the environment in which the *ptx*
624 genes are expressed in *B. bronchiseptica* is low levels of accessible oxygen.
625 Previous genomic and experimental comparisons between the classical bordetellae
626 have focused on virulence-associated genes like *fhaB, cyaA,* and the *ptx* genes. By comparing
627 genes encoding proteins important for bacterial physiology, like cytochrome oxidases, we can
628 also learn about which bacterial processes and pathways are important for survival within the
629 host. Comparing *B. pertussis* and *B. bronchiseptica* led to our hypothesis that the three

630 cytochrome-oxidase encoding gene loci that are conserved in *B. pertussis* would be sufficient
631 during infection, which was supported by our experimental findings. Conversely, we can also
632 learn about what is important for surviving in the environment. HT200, a *B. bronchiseptica*
633 environmental isolate, differs from the other examined *B. bronchiseptica* strains in that HT200
634 does not have intact copies of all eight cytochrome-oxidase encoding gene loci. In particular,
635 this strain has premature stop codons in both *cyoB1* and *cyoB2*, meaning it likely has no
636 functional bo_3-type cytochrome oxidases (Table S2). This result, especially in contrast with the
637 broad conservation of *cyo1* within *B. pertussis* strains, indicates that while Cyo1 is important for
638 survival within mammalian hosts, it is not important for survival within aqueous environments.
639 This result also supports the hypothesis that the many different cytochrome oxidases encoded
640 within the *B. bronchiseptica* genome all contribute to the ability of this species to be a generalist,
641 surviving in many different environments. As strains specialize towards life within specific
642 environments, the bacteria no longer require all their cytochrome oxidases and the genes that
643 are no longer required can accrue mutations.
644 While the role of cytochrome oxidases has been investigated in other pathogens,
645 including *V. cholerae, M. tuberculosis,* and *S. aureus*, the majority of species studied have been
646 facultative anaerobes [9,18–20]. Additionally, most either infect regions like the gut that are
647 expected to have low levels of oxygen, or, like *M. tuberculosis,* have evolved to be able to
648 survive and grow as intracellular pathogens. In these pathogens, individual high-affinity
649 cytochrome oxidases are critical for normal infection. In the respiratory pathogen and obligate
650 aerobe *B. bronchiseptica,* this is not the case. Not only is no individual cytochrome oxidase
651 required for infection, but a predicted low-affinity cytochrome oxidase is sufficient in mice for
652 both the establishment of infection and persistence once bacteria are introduced. Therefore,
653 targeting *bd*-type cytochrome oxidases, which is being explored as a treatment for *M.*
654 *tuberculosis* among others, would not be effective in *B. bronchiseptica* [51]. This result
655 emphasizes the need to study not only virulence factors but also the basic bacterial physiology

656 to effectively combat bacterial pathogens. By expanding our understanding of the cytochrome
657 oxidases of other pneumonia-causing bacteria, like *Neisseria meningitidis* and *Haemophilus*
658 *influenzae*, we may find that low-affinity cytochrome oxidases are better drug targets to clear
659 infections within the respiratory tract.
660

661 MATERIALS AND METHOODS

662 **Bacterial strains, plasmids, and growth conditions.** *B. bronchiseptica* strains were grown on
663 Bordet-Gengou (BG) agar plates supplemented with 6% defibrinated sheep blood (Hemostat,
664 catalog no. DSB1) or in Stainer-Scholte (SS) broth supplemented with SS supplement at 37°C
665 ([67], updated in [68]). One strain (*cta1*$^{+}$) had growth defects on BG agar plates and was
666 therefore plated on SS agar plates when possible. As needed, media were supplemented with
667 streptomycin (Sm, 20 µg/mL), gentamicin (Gm, 30 µg/mL), or sucrose (15% w/v). *E. coli* strains
668 were grown in Lysogeny Broth (LB) or on LB agar plates supplemented with diaminopimelic acid
669 (DAP, 300 µg/mL), ampicillin (Ap, 100 µg/mL), and gentamicin (Gm, 30 µg/mL) as needed. All
670 cultures were started from individual colonies from a clonal population.

671 **Construction of plasmids and strains.** The strains and plasmids used in this study are listed
672 in **Table 3** and **Table 4**, respectively, which are based on mutations of the cytochrome oxidase-
673 encoding loci as outlined in **Table 2**. In-frame deletions were generated using allelic exchange
674 via derivatives of the pEG7s vector. We used the DH5α *E. coli* strain for plasmid construction
675 and propagation. We used the Rho3 *E. coli* strain for transforming *B. bronchiseptica*. Any
676 mutations made within *B. bronchiseptica* strains were confirmed by PCR and/or whole-genome
677 sequencing. The order in which deletions were generated in strains with deletions in multiple
678 loci is described in **S2 Figure**.

679 **Table 2. Mutations generated in this study**

Deletion number	Deletion Name	Description
1	Δ*ccoNOQP*	Deletion of 3188bp: from nucleotide 7 of *ccoN* (BB3329) to nucleotide 2084 of *ccoP* (BB3326)
2	Δ*cydAB1*	Deletion of 2772bp: from nucleotide 9 of *cydA1* (BB4498) to nucleotide 1147 of *cydB1* (BB4497)
3	Δ*cydAB3*	Deletion of 2373bp: from 41 nucleotides upstream of *cydA3* (BB4012) to nucleotide 994 of *cydB3* (BB4011)
4	Δ*cioAB*	Deletion of 2380bp: from nucleotide 9 of *cioA* (BB1238) to nucleotide 1000 of *cioB* (BB1239)
5	Δ*cyoABCD1*	Deletion of 3842bp: from nucleotide 11 of *cyoA1* (BB1283) to nucleotide 339 of *cyoD1* (BB1286)

6	Δ*cyoABCD2*	Deletion of 3641bp: from nucleotide 6 of *cyoA2* (BB1310) to nucleotide 632 of *cyoC2* (BB1308); *cyoD2* is left intact
7	Δ*ctaCDFE1*	Deletion of 4165bp: from nucleotide 9 of *ctaC1* (BB4831) to nucleotide 493 of *ctaE1* (BB4827)
8	Δ*ctaD2*	Deletion of 1578bp: from nucleotide 10 to nucleotide 1587 of *ctaD2* (BB4674)

680 **Table 3. Plasmids used in this study**

Plasmid name	Description	Source
pEG7s	Suicide allelic exchange plasmid for *B. bronchiseptica* used to generate in-frame deletions; confers sucrose sensitivity through *sacB.* Gm^R Ap^R	[49]
pEG7s-Δ*ccoNOQP*	pEG7s containing ~500bp upstream and downstream of mutation 1	This study
pMAB19	pEG7s containing ~500bp upstream and downstream of mutation 2	This study
pEG7s-Δ*cydAB3*	pEG7s containing ~500bp upstream and downstream of mutation 3	This study
pEG7s-Δ*cioAB*	pEG7s containing ~500bp upstream and downstream of mutation 4	This study
pEG7s-Δ*cyo1*	pEG7s containing ~500bp upstream and downstream of mutation 5	This study
pEG7s-Δ*cyo2*	pEG7s containing ~500bp upstream and downstream of mutation 6	This study
PEG7s-Δ*cta1*	pEG7s containing ~500bp upstream and downstream of mutation 7	This study
pEG7s-Δ*ctaD2*	pEG7s containing ~500bp upstream and downstream of mutation 8	This study

681 **Table 4. Strains used in this study**

Strain	Description	Source
B. bronchiseptica		
RB50 (WT)	Wild-type *B. bronchiseptica* strain isolated from nares of a naturally infected rabbit; Sm^R	[55]
Δ*cco*	RB50 containing mutation 1; Sm^R	This study
Δ*cyd1*	RB50 containing mutation 2; Sm^R	This study
Δ*cyd3*	RB50 containing mutation 3; Sm^R	This study
Δ*cio*	RB50 containing mutation 4; Sm^R	This study
Δ*cyo1*	RB50 containing mutation 5; Sm^R	This study
Δ*cyo2*	RB50 containing mutation 6; Sm^R	This study
Δ*cta1*	RB50 containing mutation 7; Sm^R	This study
Δ*ctaD2*	RB50 containing mutation 8; Sm^R	This study
Bp-conserved	RB50 containing mutations 1, 3, 4, 6, and 8; Sm^R	This study
cyo1$^+$ *cta1*$^+$	RB50 containing mutations 1-4, 6, and 8; Sm^R	This study
cyd1$^+$ *cta1*$^+$	RB50 containing mutations 1, 3-6, and 8; Sm^R	This study

cyd1$^+$ *cyo1*$^+$	RB50 containing mutations 1, 3, 4, and 6-8; Sm^R	This study
cyd1$^+$	RB50 containing mutations 1 and 3-8; Sm^R	This study
cyo1$^+$	RB50 containing mutations 1-4 and 6-8; Sm^R	This study
cta1$^+$	RB50 containing mutations 1-6 and 8; Sm^R	This study
E. coli		
DH5α	Molecular cloning strain derived from K-12	Gibco-BRL
Rho3	Conjugation strain; *ΔasdΔaphA*, DAP auxotroph	[69]

682

683 **TTC reduction assay.** Cultures of *B. bronchiseptica* were grown overnight at 37°C in SS media
684 supplemented with SS supplement and Sm. Each culture was normalized to 1 OD_{600}/mL. Five
685 microliters were spotted onto tryptic soy agar (TSA) plates (Millipore 22091) containing 0.001%
686 (w/v) TTC (2,3,5-triphenyl-tetrazolium chloride) (Sigma-Aldrich T8877) and incubated in the dark
687 for 24 hours at 37°C. Plates were photographed to capture the irreversible color shift from
688 colorless to red that occurs following TTC reduction.

689 **Liquid culture growth assays.** Cultures of *B. bronchiseptica* were grown for 18 hours at 37ºC
690 in SS media supplemented with SS supplement and Sm. Each culture was normalized to 0.1
691 OD_{600}/mL and grown on an orbital shaker (VWR Catalog # 89032-096) at 225 rpm at 37ºC.
692 Samples were collected at 0, 3, 6, 9, 12, 18, 24, 36, and 48 hours after the start of the
693 experiments. We measured the OD_{600} of these samples and determined the CFU/mL by serially
694 diluting the samples and plating on BG blood agar plates. The number of colonies were
695 enumerated after at least 48 hours growth at 37°C, with *cta1*$^+$ requiring at least 72 hours growth
696 at 37°C.

697 **Growth under different atmospheric conditions.** Cultures for outgrowth were prepared in the
698 same manner as for growth curves. Samples were then grown on an orbital shaker at 225 rpm
699 at 37 ºC within a trigas incubator connected to N_2 and CO_2 gas sources (HERACELL VIOS 160i;
700 Fisher Scientific 13998258). To reduce O_2 levels, pure N_2 was introduced. We took endpoint

701 samples to ensure that the experimental conditions remained constant throughout and were not
702 disrupted by the reintroduction of atmospheric oxygen at each sampling point. Samples were
703 collected at 24 hours when grown in 5% CO_2, 48 hours when grown in 5% O_2 and in 5% O_2 5%
704 CO_2, and 72 hours when grown in 2% O_2. We measured the OD_{600} of these samples and
705 determined the CFU/mL by serially diluting the samples and plating on BG blood agar plates.
706 The number of colonies were enumerated after at least 48 hours growth at 37°C, with *cta1*$^+$
707 requiring at least 72 hours growth at 37°C.
708 **Bacterial infection of the mouse respiratory tract using the persistence model.** Six-week-
709 old female BALB/c mice from Charles River Laboratories (catalog no. BALB/cAnNCrl) were
710 inoculated intranasally with 7.5×10^4 CFU *B. bronchiseptica* in 50 µL of Dulbecco PBS (DPBS).
711 Samples were collected at three hours, one day, three days, seven days, and fourteen days
712 post-infection. At each indicated time point, right lung lobes, trachea, and nasal cavity tissue
713 were harvested from each mouse and the tissues were homogenized in DPBS using a mini-
714 beadbeater with 0.1 mm zirconia beads (Biospec catalog no. 11079110zx). The number of CFU
715 was determined by plating dilutions of tissue homogenates on BG Sm blood agar and
716 enumerating the number of colonies per tissue after at least 48 hours growth at 37°C, with *cta1*$^+$
717 requiring at least 72 hours growth at 37°C.
718 **Bacterial colonization of the mouse respiratory tract.** Six-week-old female BALB/c mice
719 from Charles River Laboratories (catalog no. BALB/cAnNCrl) were inoculated intranasally with
720 100 CFU *B. bronchiseptica* in 4 µL of Dulbecco PBS (DPBS), divided equally between the two
721 nares. Samples were collected at three days and seven days post-infection. At each indicated
722 time point, nasal cavity tissue was harvested and homogenized in DPBS using a mini-
723 beadbeater with 0.1 mm zirconia beads (Biospec catalog no. 11079110zx). The number of CFU
724 was determined by plating dilutions of tissue homogenates on BG Sm blood agar and
725 enumerating the number of colonies per tissue after at least 48 hours growth at 37°C, with *cta1*$^+$
726 requiring at least 72 hours growth at 37°C.

727 **RNA isolation and sequencing.** Cultures of *B. bronchiseptica* were grown overnight at 37°C in
728 SS media supplemented with SS supplement and Sm. Each culture was normalized to 1
729 OD_{600}/mL and grown on an orbital shaker at 225 rpm at 37 ºC for 4 hours under different
730 atmospheric conditions as above. 3 biological replicates were generated for each condition.
731 Bacteria were pelleted, resuspended in RNAlater (Invitrogen AM7020), then stored at -80°C until
732 extraction. RNA was isolated using Trizol/chloroform extraction, followed by centrifugation in
733 Phasemaker Tubes (Invitrogen 12183555). The aqueous phase was precipitated in isopropanol,
734 washed in ethanol, and solubilized in RNase-free water. Samples were then cleaned using
735 RNAeasy column purification (Quiagen 74104). RNA sequencing was performed by SeqCenter
736 (Pittsburgh, PA; 12M Paired End Reads rRNA depletion sequencing package). Library
737 preparation was performed using Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero
738 Plus kit and 10bp unique dual indices. Sequencing was performed using a NovaSeq X Plus.
739 **RNA analysis.** RNA analysis was performed by SeqCenter (Pittsburgh, PA; intermediate RNA
740 analysis package). Read mapping was performed using HISAT2 [70] and read quantification
741 was performed using featureCounts [71]. Read counts were loaded into R, then normalized
742 using edgeR's Trimmed Mean of M values (TMM) algorithm [72]. These values were then
743 converted to counts per million. Differential expression analysis between our different conditions
744 (5% O_2 vs ambient air, 2% O_2 vs ambient air, and 5% CO_2 vs ambient air) was performed using
745 edgeR's glmQLFTest.
746 **Ethics statement.** All animal studies followed the guidelines in the *Guide for the Care and Use*
747 *of Laboratory Animals* of the National Institute of Health. Our protocols were approved by the
748 University of North Carolina Institutional Animal Care and Use Committee (Protocol ID: 22-140).
749 All animals were anesthetized for inoculations, monitored daily, and euthanized properly and
750 humanely. All efforts were made to minimize suffering.
751

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992

993 SUPPORTING INFORMATION

994 **S1 Table. Genomes of classical bordetellae strains compared**

995 **S2 Table. Percent amino acid homology of cytochrome oxidases of strains of interest**

996 **compared to RB50**

997 **S3 Table. Fold change differences in transcript abundance of all genes of RB50 grown**

998 **either in 5% O_2 or ambient air.**

999 **S4 Table. Fold change differences in transcript abundance of all genes of RB50 grown**

1000 **either in 2% O_2 or ambient air.**

1001 **S5 Table. Fold change differences in transcript abundance of all genes of RB50 grown**

1002 **either in 5% CO_2 or ambient air.**

1003 **S1 Figure. Phylogenetic analysis of classical *Bordetella* strains.** This tree was generated

1004 by comparing the concatenated multiple sequence alignments in the *cgMLST_genus* scheme in

1005 the BIGSdb-Pasteur genomic platform for *Bordetella* [38]. The tree was rooted on the branch

1006 leading to *B. petrii*. Leaves are labeled with the strain name and Institut Pasteur Bordetella

1007 cgMLST id. More information can be seen in S1 Table. Tree created using Interactive Tree of

1008 Life (iTOL) v5 [73].

1009 **S2 Figure. Order of mutations added to generate strains.** A full list of the strains used in this

1010 study can be found in Table 4. Numbers represent the in-frame deletion mutation added and

1011 also correspond to the mutations listed in Table 2. The names of strains have been added where

1012 applicable. Black arrows represent the successful deletion of a cytochrome oxidase-encoding

1013 gene locus. Gray crossed lines represent an allelic exchange that resulted only in wild-type

1014 revertants; at least 16 clones were screened for each.

1015 **S3 Figure. All generated strains respire.** TTC reduction after 24 hours of growth. When

1016 reduced, TTC undergoes an irreversible color change to red. Shown are representative images

1017 of 5 biological replicates per strain.

1018 **S4 Figure. The three cytochrome oxidases conserved in *B. pertussis* are sufficient for**
1019 **colonization in mice.** (A) Bacterial burden over time within the nasal cavity (upper), trachea
1020 (middle), and right lung (lower) of mice infected with wild-type bacteria (WT, grey circles). Open
1021 circles represent tissue samples with no detectable bacteria. n=2 for day 0, n=6 for all other
1022 timepoints. (B) Bacterial burden over time within the nasal cavity of mice infected with wild-type
1023 bacteria (WT, grey circles) or a strain with only the cytochrome oxidase-encoding gene loci
1024 conserved in *B. pertussis* (*Bp*-conserved, maroon hexagon). n=2 for day 0, n=4 for day 3. Each
1025 point represents a single mouse. Dashed line represents the limit of detection. Statistical
1026 significance was determined using unpaired Student's t-test; p-values are indicated when
1027 $p<0.05$.
1028 **S5 Figure. No single cytochrome oxidase is required to establish infection.** (A) Growth
1029 over time, measured via optical density (left) or CFU/mL (right), for strains with two cytochrome
1030 oxidase-encoding gene loci. (B) Bacterial burden over time within the nasal cavity of mice
1031 infected with a strain with only the cytochrome oxidase-encoding gene loci conserved in *B.*
1032 *pertussis* (*Bp*-conserved, maroon hexagon), a strain with only *ctaCDFGE1* and *cyoABCD1*
1033 (*cta1*$^{+}$, *cyo1*$^{+}$, orange upright triangle), a strain with only *cydAB1* and *cyoABCD1* (*cyd1*$^{+}$ *cyo1*$^{+}$*,*
1034 blue diamond), or a strain with only *cydAB1* and *ctaCDFGE1* (*cyd1*$^{+}$ *cta1*$^{+}$*,* green upside-down
1035 triangle). Samples from 5 mice were collected at each timepoint for each strain. However, due to
1036 the natural microbiota of the nasal cavity, *B. bronchiseptica* could not always be innumerated
1037 due to contamination. Therefore, n=4 for *cta1*$^{+}$ day 3. Each point represents a single mouse.
1038 Dashed line represents the limit of detection. Statistical significance was determined using
1039 unpaired Student's t-test; p-values are indicated when $p < 0.05$.
1040

1041 FIGURES

1042 **Figure 1. No cytochrome oxidase is necessary for growth in ambient air, but Cyd1, Cta1,**
1043 **and Cyo1 are each sufficient.** Growth over time, measured via optical density (left) or CFU/mL
1044 (right), for strains lacking a single cytochrome oxidase-encoding gene loci (A), a strain with only
1045 the cytochrome oxidase-encoding gene loci conserved in *B. pertussis* (*Bp*-conserved) (B), or
1046 strains with only a single cytochrome oxidase-encoding gene loci (C). Cultures were started at
1047 0.1 OD_{600}, which contains approximately $3.5x10^8$ CFU/mL. Points represent the mean of at least
1048 3 biological replicates, with bars representing the standard deviation.

1049 **Figure 2. Growth under different atmospheric conditions relevant to the environment of**
1050 **the mammalian respiratory tract.** Growth, measured via optical density (left) or CFU/mL
1051 (right), for RB50 (WT, gray), a strain with only the cytochrome oxidase-encoding gene loci
1052 conserved in *B. pertussis* (*Bp*-conserved, maroon), a strain with only *cydAB1* (*cyd1*$^+$*,* orange), a
1053 strain with only *ctaCDFGE1* (*cta1*$^+$, blue), or a strain with only *cyoABCD1* (*cyo1*$^+$, green).
1054 Cultures were started at 0.1 OD_{600}, which contains approximately $3.5x10^8$ CFU/mL. Cultures
1055 were grown in either: ambient air for 24 hours (A), 5% O_2 for 48 hours (B), 2% O_2 for 72 hours
1056 (C), 5% CO_2 for 24 hours (D), or 5% O_2 5% CO_2 for 48 hours (E). The data from A are identical
1057 to the 24-hour timepoint of Figure 1A and are included for comparison. Bars represent the mean
1058 of at least 3 biological replicates, with error bars representing the standard deviation. Statistical
1059 significance was determined using unpaired Student's t-test. *, $p < 0.05$; **, $p < 0.005$; ***, $p <$
1060 0.0005; ****, $p < 0.0001$.

1061 **Figure 3. No single cytochrome oxidase is required during murine infection.** Bacterial
1062 burden over time within the nasal cavity (upper), trachea (middle), and right lung (lower) of mice
1063 infected with either RB50 (WT) or strains lacking a single cytochrome oxidase-encoding gene
1064 loci. Different symbols represent different batches of inoculations, which always include WT for
1065 comparison: circles (Δ*cco,* Δ*cyd1,* Δ*cyd3*, and Δ*cio*), upright triangles (Δ*cio,* Δ*cyo1,* Δ*cyo2,*

1066 Δ*cta1,* and Δ*ctaD2*), upside-down triangles (Δ*cco,* Δ*cyd1*, and Δ*cyd3*), and diamonds (Δ*cyo1*,
1067 Δ*cyo2,* Δ*cta1*, and Δ*ctaD2*). Each point represents data from one mouse. n=4 for day 0, n=6 for
1068 all other timepoints, from two independent experiments, for all mutant strains. Each point
1069 represents a single mouse. Dashed line represents the limit of detection. Statistical significance
1070 was determined using unpaired Student's t-test; p-values are indicated when $p<0.01$.

1071 **Figure 4. The three cytochrome oxidases conserved in *B. pertussis* are sufficient for**
1072 **persistence in the murine respiratory tract.** Bacterial burden over time within the nasal cavity
1073 (upper), trachea (middle), and right lung (lower) of mice infected with either wild-type bacteria
1074 (WT, grey circles) or a strain with only the cytochrome oxidase-encoding gene loci conserved in
1075 *B. pertussis* (*Bp*-conserved, maroon hexagon). n=4 for day 0, n=6 for all other timepoints, with
1076 each point representing a single mouse. Samples were collected from two independent
1077 experiments. Dashed line represents the limit of detection. Statistical significance was
1078 determined using unpaired Student's t-test; p-values are indicated when $p < 0.05$.

1079 **Figure 5. Cyo1 is sufficient for WT-levels of persistence within the mammalian respiratory**
1080 **tract, while Cyd1 and Cta1 are not.** Bacterial burden over time within the nasal cavity (upper),
1081 trachea (middle), and right lung (lower) of mice infected with wild-type bacteria (WT, grey
1082 circles), a strain with only *cydAB1* (*cyd1*$^{+}$*,* orange upright triangles), a strain with only
1083 *ctaCDFGE1* (*cta1*$^{+}$, blue diamonds), or a strain with only *cyoABCD1* (*cyo1*$^{+}$, green upside-down
1084 triangles). These strains were tested in the same experiment but separated into separate
1085 graphs for analysis; thus, the mutant strains are compared to the same WT data. n=4 for day 0,
1086 n=6 for all other timepoints, with each point representing a single mouse. Samples were
1087 collected from two independent experiments. Dashed line represents the limit of detection.
1088 Statistical significance was determined using unpaired Student's t-test; p-values are indicated
1089 when $p < 0.05$.

1090 **Figure 6. Cyo1 is sufficient for the efficient establishment of colonization, while Cyd1 and**
1091 **Cta1 are not.** Bacterial burden over time within the nasal cavity of mice infected with a strain
1092 with only the cytochrome oxidase-encoding gene loci conserved in *B. pertussis* (*Bp*-conserved,
1093 maroon hexagon), a strain with only *cydAB1* (*cyd1*$^{+}$, orange upright triangles), a strain with only
1094 *ctaCDFE1* (*cta1*$^{+}$, blue diamonds), or a strain with only *cyoABCD1* (*cyo1*$^{+}$, green upside-down
1095 triangles). Samples from 10 mice across two independent experiments were collected at each
1096 timepoint for each strain. However, due to the natural microbiota of the nasal cavity, *B.*
1097 *bronchiseptica* could not always be innumerated due to contamination. Therefore, n=7 for *cta1*$^{+}$
1098 day 3, n=9 for *cyd1*$^{+}$ day 7 and *cta1*$^{+}$ day 7. Each point represents a single mouse. Dashed line
1099 represents the limit of detection. Statistical significance was determined using unpaired
1100 Student's t-test; p-values are indicated when $p < 0.05$.

1101 **Figure 7. Regulation of cytochrome oxidase genes under different atmospheric**
1102 **conditions, measured using RNA sequencing.** (A) Transcript abundance, measured by
1103 counts per million, for cytochrome oxidase-encoding genes from *B. bronchiseptica* grown in
1104 ambient air. *fhaB* and *flaA*, two highly-regulated and well-characterized genes, are included for
1105 comparison. *flaA* transcription level (gray shading) are used as a reference, as *flaA* is negatively
1106 regulated under this condition and no protein product can be detected. Numbers in the bars
1107 represent the mean number of transcripts. (B-D) Comparative transcription, measured by fold
1108 change in transcript abundance, comparing 5% O_2 (B), 2% O_2 (C), or 5% CO_2 (D) to ambient air.
1109 Genes with $\log_2$ fold changes greater than 1 or less than -1 have filled-in bars. Differential
1110 expression analysis was performed using edgeR's glmQLFTest.

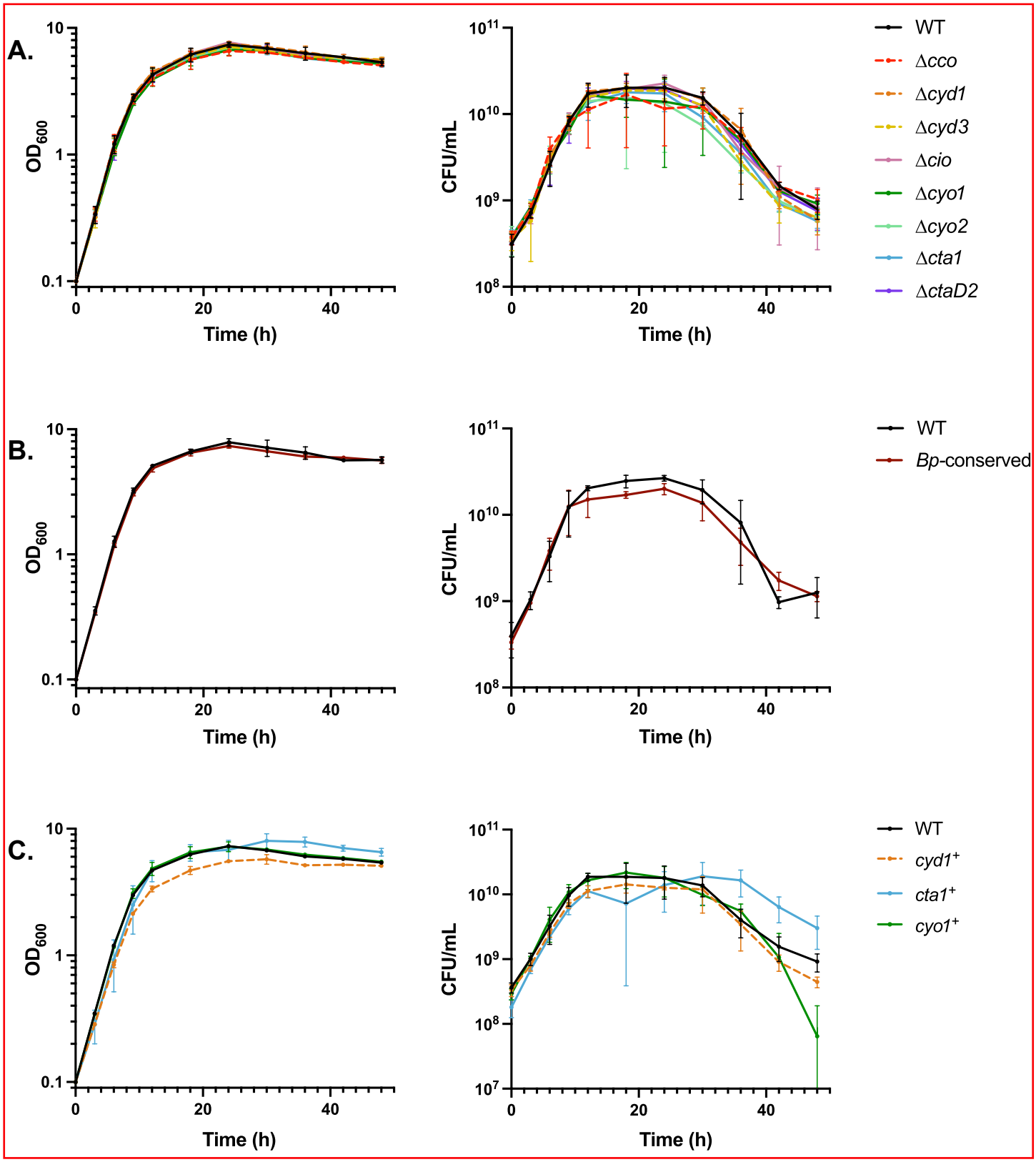


Figure 1. No cytochrome oxidase is necessary for growth in ambient air, but Cyd1, Cta1, and Cyo1 are each sufficient. Growth over time, measured via optical density (left) or CFU/mL (right), for strains lacking a single cytochrome oxidase-encoding gene loci (A), a strain with only the cytochrome oxidase-encoding gene loci conserved in *B. pertussis* (*Bp*-conserved) (B), or strains with only a single cytochrome oxidase-encoding gene loci (C). Cultures were started at 0.1 OD_{600}, which contains approximately 3.5×10^8 CFU/mL. Points represent the mean of at least 3 biological replicates, with bars representing the standard deviation.

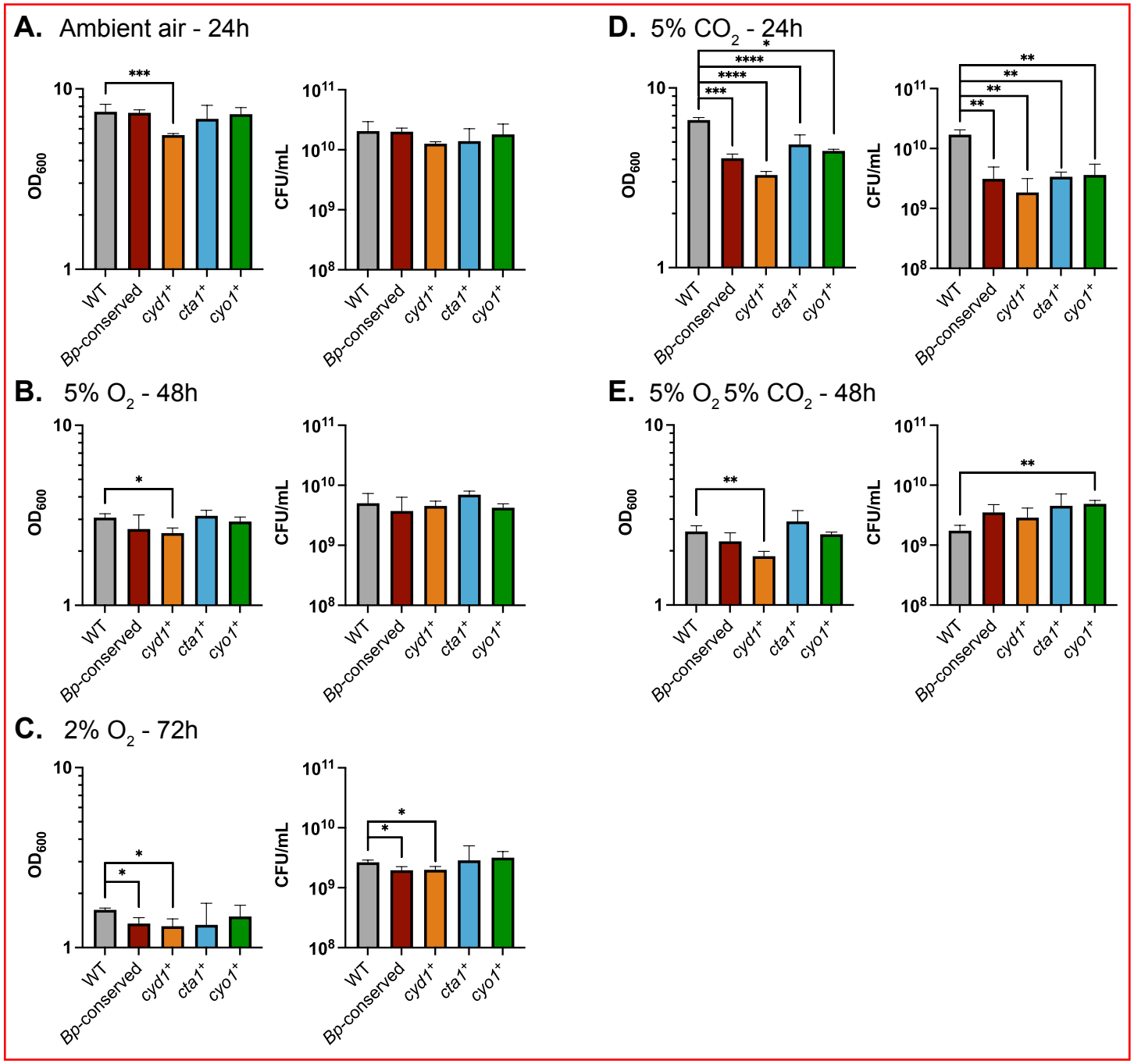
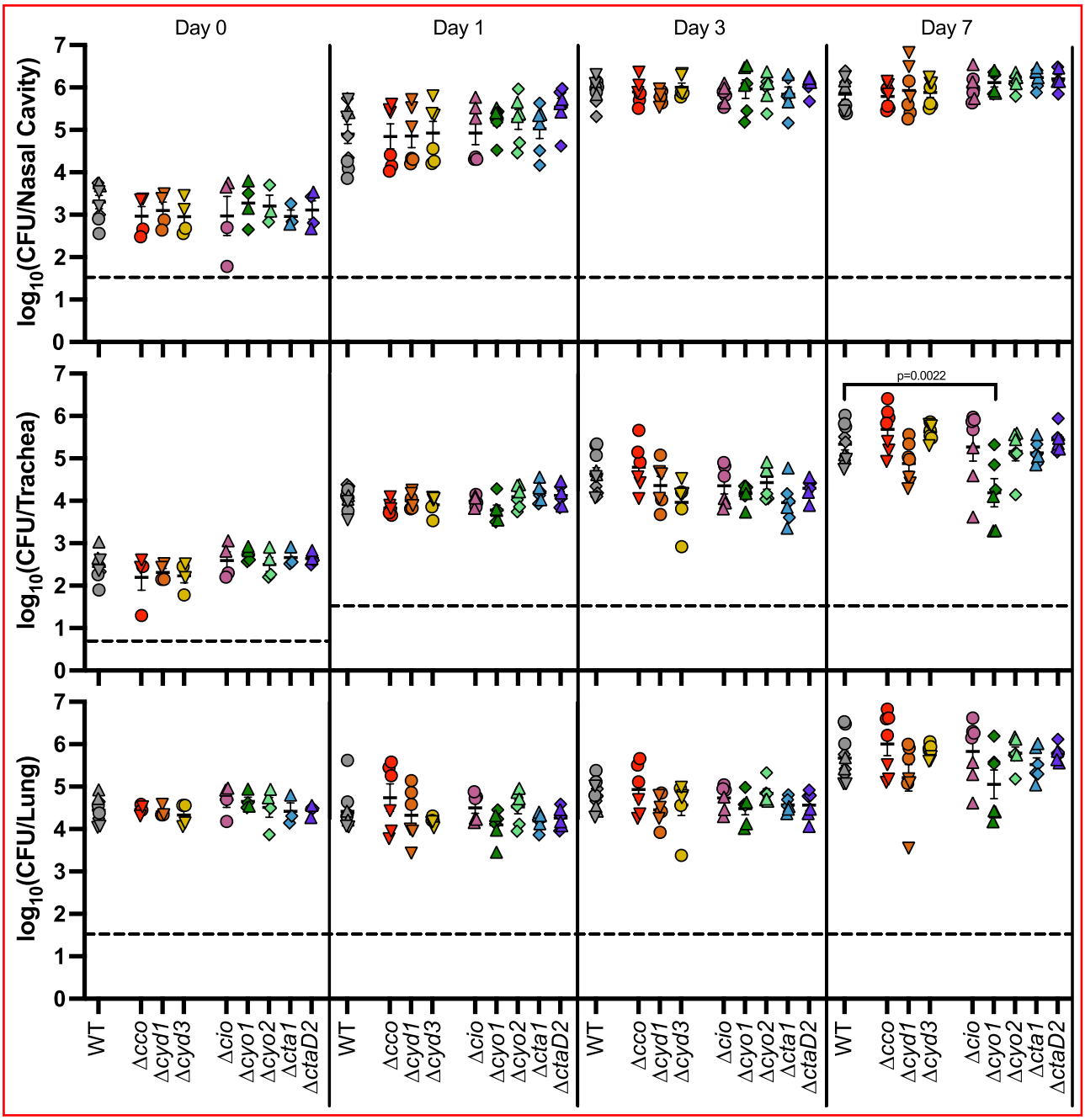


Figure 2. Growth under different atmospheric conditions relevant to the environment of the mammalian respiratory tract. Growth, measured via optical density (left) or CFU/mL (right), for RB50 (WT, gray), a strain with only the cytochrome oxidase-encoding gene loci conserved in *B. pertussis* (*Bp*-conserved, maroon), a strain with only *cydAB1* (*cyd1*$^+$, orange), a strain with only *ctaCDFGE1* (*cta1*$^+$, blue), or a strain with only *cyoABCD1* (*cyo1*$^+$, green). Cultures were started at 0.1 OD_{600}, which contains approximately 3.5×10^8 CFU/mL. Cultures were grown in either: ambient air for 24 hours (A), 5% O_2 for 48 hours (B), 2% O_2 for 72 hours (C), 5% CO_2 for 24 hours (D), or 5% O_2 5% CO_2 for 48 hours (E). The data from A are identical to the 24-hour timepoint of Figure 1A and are included for comparison. Bars represent the mean of at least 3 biological replicates, with error bars representing the standard deviation. Statistical significance was determined using unpaired Student's t-test. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$.



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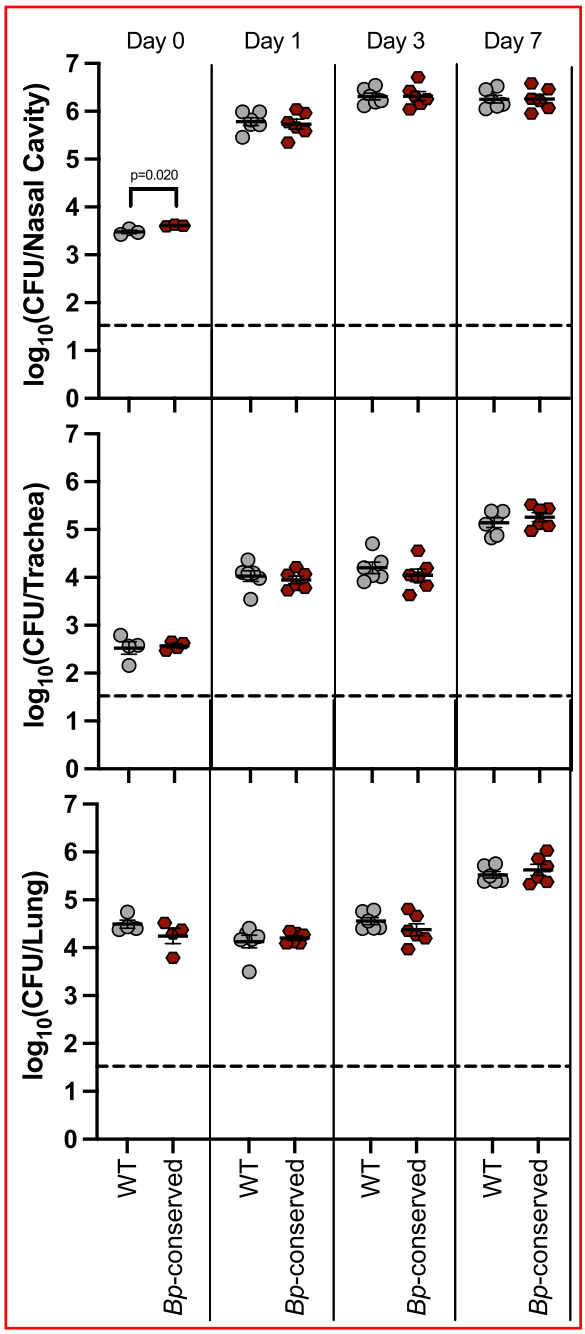
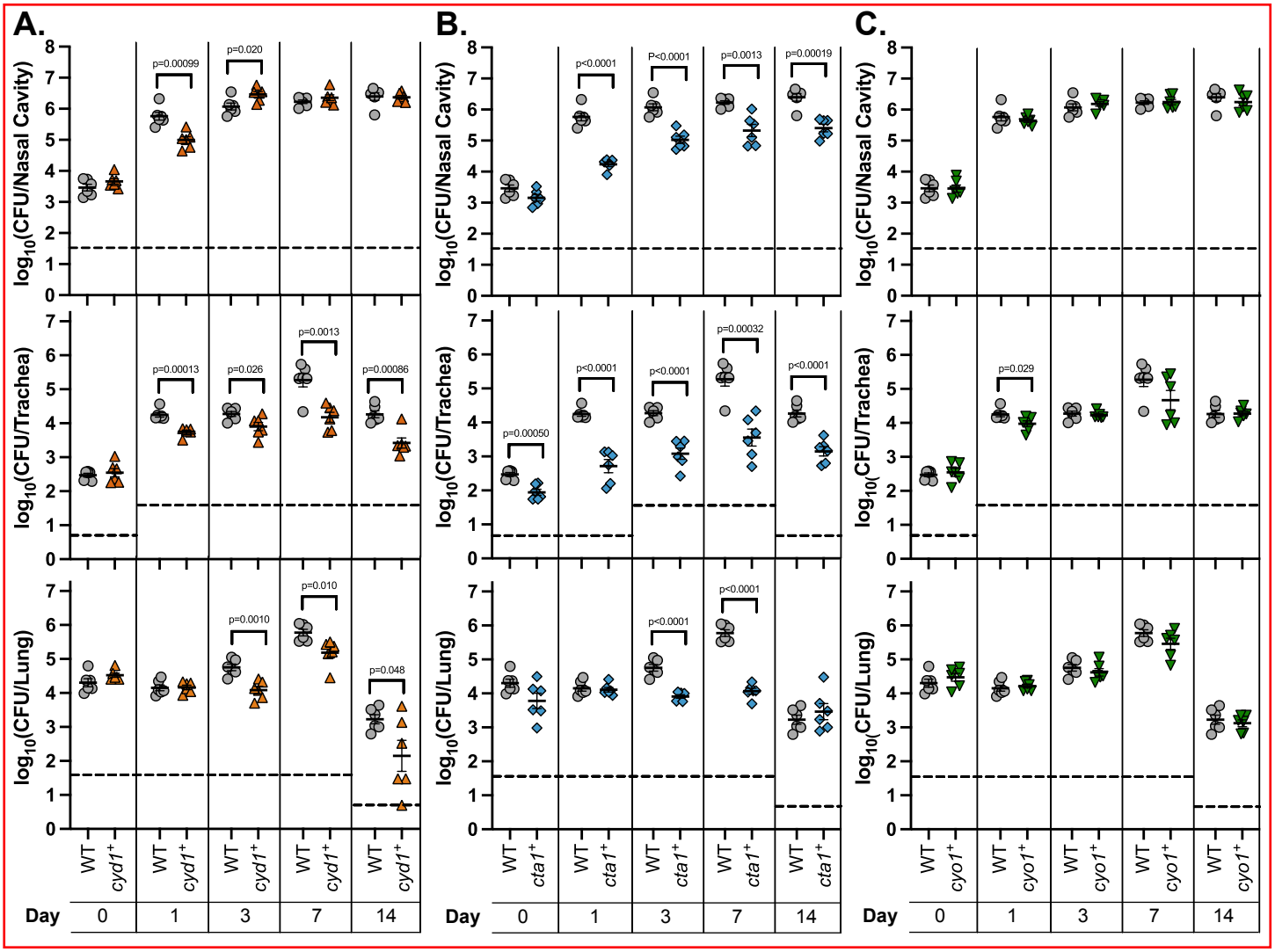


Figure 4. The three cytochrome oxidases conserved in *B. pertussis* are sufficient for persistence in the murine respiratory tract. Bacterial burden over time within the nasal cavity (upper), trachea (middle), and right lung (lower) of mice infected with either wild-type bacteria (WT, grey circles) or a strain with only the cytochrome oxidase-encoding gene loci conserved in *B. pertussis* (*Bp*-conserved, maroon hexagon). n=4 for day 0, n=6 for all other timepoints, with each point representing a single mouse. Samples were collected from two independent experiments. Dashed line represents the limit of detection. Statistical significance was determined using unpaired Student's t-test; p-values are indicated when $p < 0.05$.



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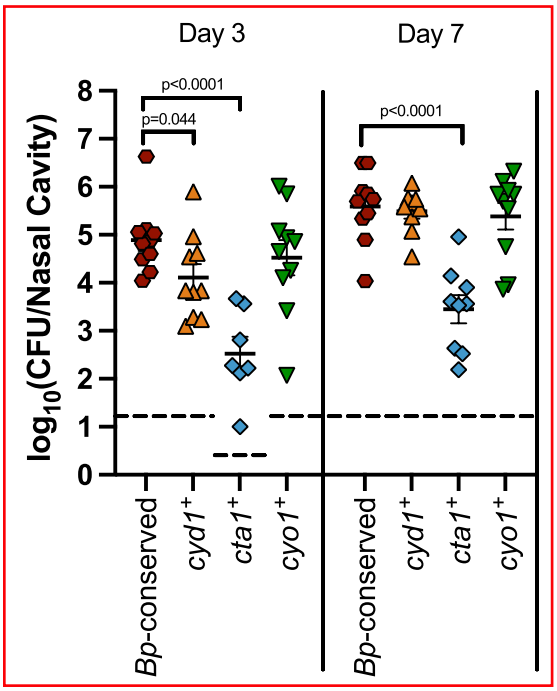

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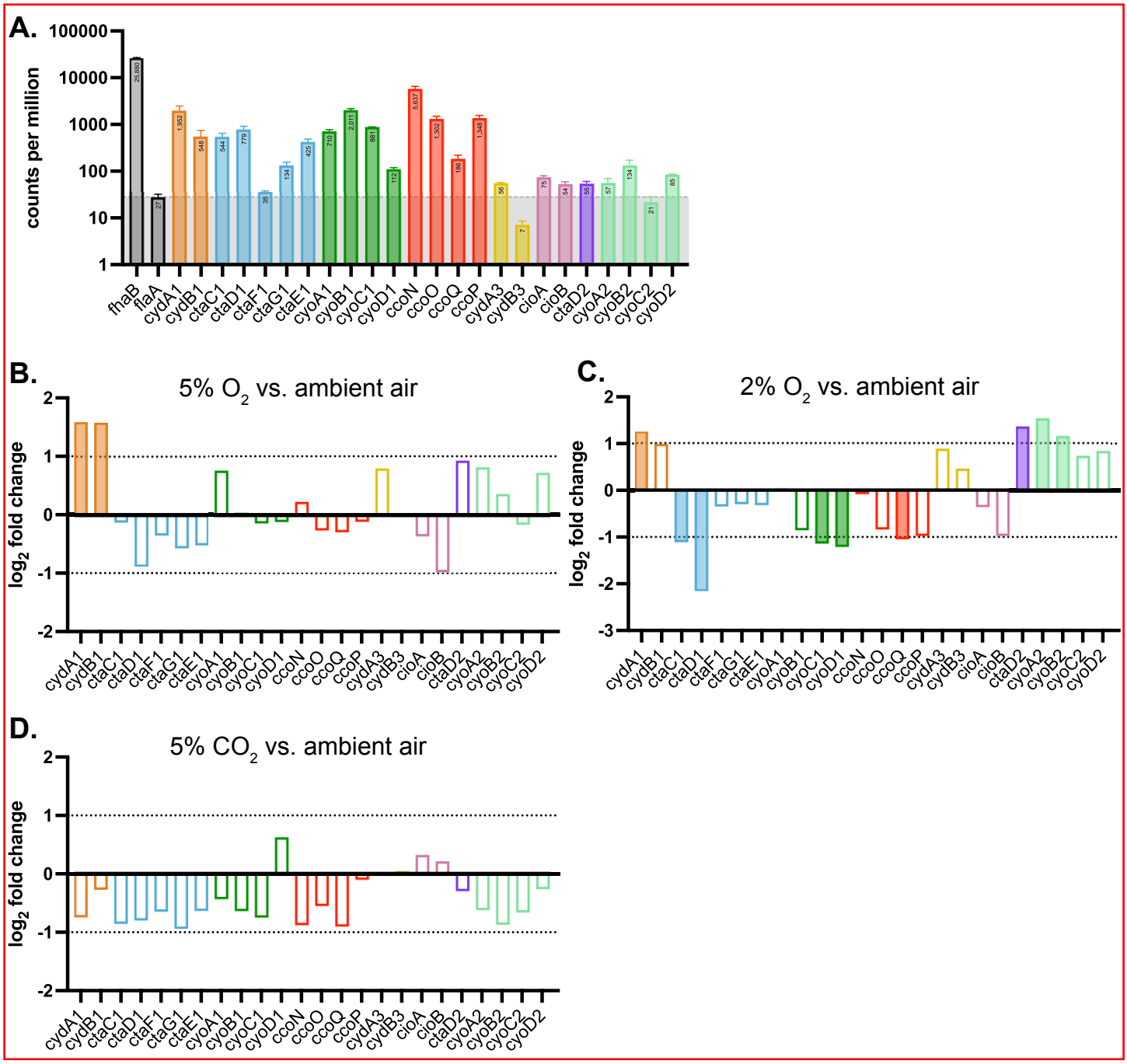

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