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.

<u>AUTHOR SUMMARY</u>

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.

<u>INTRODUCTION</u>

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. *bo₃*-

type, *aa₃*-type, and *cbb₃*-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 $O_2 \cong 200nM$) [3], which are best suited for

atmospheric levels of oxygen (~21%), or high-affinity, which can function in microoxic conditions

(K_m for O_2 = 3-8 nM) [4,5]. Increased affinity for oxygen comes with a trade-off; high-affinity

cytochrome oxidases generate less proton motive force per electron transferred than low-affinity

cytochrome oxidases [6].

Respiration as a whole, and cytochrome oxidases in particular, have been shown to be

required for infection in many pathogenic species, including gastrointestinal pathogens

Escherichia coli [7,8]*, Vibrio cholerae* [9]*, Listeria monocytogenes* [10,11], *Shigella flexneri*

[12,13], and *Salmonella enterica* Serovar Typhimurium [14–16]; the oral pathogen

Aggregatibacter actinomycetemcomitans [17]; the respiratory pathogen *Mycobacterium*

tuberculosis [18,19]; and pathogens that can cause many types of infection including

bacteremia and sepsis like *Staphylococcus aureus* [20] and group B *Streptococcus* [21,22].

However, the majority of pathogens assessed have been facultative anaerobes, which do not

require cytochrome oxidases for survival. Additionally, many of these pathogens colonize

microenvironments that are expected to have limited levels of oxygen.

The classical *Bordetella* species (*B. pertussis, B. parapertussis$_{Hu}$,* and *B. bronchisceptica*)

are extremely closely related bacteria that colonize the upper and lower respiratory tracts of

humans (in the case of *B. pertussis* and *B. parapertussis$_{Hu}$*) and other mammals (in the case of

B. bronchiseptica). They are obligate aerobes that use only oxygen as their terminal electron

acceptor for electron transport-coupled oxidative phosphorylation [23,24]. Therefore, they

depend solely on components of the ETC, like cytochrome oxidases, to generate energy.

Previous work in *B. pertussis* has shown that cytochrome *c* is not required for survival *in vitro*

[25]. Little is known, however, about the role of cytochrome oxidases in the classical *Bordetella*

species.

In this study, we leveraged the evolution of classical bordetellae to investigate which

cytochrome oxidases are important for respiration in the mammalian respiratory tract, using *B.*

bronchiseptica as our model organism. *B. bronchiseptica*, which occupies multiple

environmental niches, has the potential to produce eight distinct cytochrome oxidases. By

contrast, *B. pertussis*, which survives in the environment only during transmission between

hosts, has undergone significant genome reduction as it evolved from a *B. bronchiseptica*-like

ancestor [24]. Genomic comparisons between *B. bronchiseptica* and *B. pertussis* found that *B.*

pertussis strains have only broadly conserved three intact cytochrome oxidase-encoding gene

loci. We hypothesized that the three cytochrome oxidases conserved in *B. pertussis* represent

the minimum number and class of cytochrome oxidases required for bordetellae to survive

within, and briefly between, hosts. We genetically manipulated our bacteria to determine the

necessity and sufficiency of different cytochrome oxidases, first for growth *in vitro* under ambient

air and different atmospheric conditions, then during infection, using two different mouse models

of respiratory infection. Our results demonstrate that *B. bronchiseptica* is able to survive with as

few as one cytochrome oxidase, even during infection and when challenged with low oxygen

conditions. These findings also inform our understanding of the murine respiratory tract,

indicating that bacteria may have more access to oxygen while attached to the ciliated

epithelium than previously supposed.

RESULTS

Bordetella bronchiseptica has eight cytochrome oxidase-encoding gene loci

The *B. bronchiseptica* RB50 genome has eight cytochrome oxidase-encoding gene loci,

annotated computationally based on predicted amino acid homology **(Table 1)**. Three of the loci

are predicted to encode high-affinity cytochrome oxidases. *ccoNOQP* (abbreviated *cco*)

encodes a *cbb₃-type* cytochrome oxidase. In *cbb₃*-type cytochrome oxidases, CcoN contains the

active site that facilitates the reduction of molecular oxygen to water and thus functions as the

catalytic subunit [26,27]. Two loci encode *bd*-type cytochrome oxidases (*cydAB1* and *cydAB3*,

abbreviated *cyd1* and *cyd3*). The *cyd1* operon of *B. bronchiseptica* also includes a third subunit,

cydX, which is seen in some, but not all, *bd*-type cytochrome oxidases and may be important for

complex function. The variation across different species in number of subunits, structural

arrangement of coordinated hemes, and positioning of the quinol binding site makes it difficult to

draw definitive conclusions about *bd*-type cytochrome oxidases [28]. CydA is the subunit that

coordinates the three hemes required by this cytochrome oxidase, making it the catalytic

subunit; however, *bd-type* cytochrome oxidases require both CydA and CydB for catalytic

activity, as evidenced by the loss of *d*-type heme when the genes encoding either subunit are

deleted [29,30].

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]
cbb3	*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%
aa3	*ctaCDFGE1*	BB4831-4827	Low	100%	100%	100%	100%
	ctaD2	BB4674	Low	100%	7.1%	100%	100%
bo3	*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.

Five of the eight cytochrome oxidase-encoding loci are predicted to encode low-affinity

cytochrome oxidases. These include one complete *aa3*-type cytochrome oxidase (*ctaCDFGE1,*

abbreviated *cta1*) and one lone *aa3*-type subunit (*ctaD2*). The *cta1* operon contains two

additional genes: a putative membrane protein (BB4829, here named *ctaF1*) and a putative

DUF2970-containing membrane protein (BB4828, here named *ctaG1*). This operon structure is

also found in *Achromobacter* species, which are closely related to *Bordetella*. *ctaF1* shares no

homology to the gene of the same name found in *Bacillus* species, which encodes part of a

caa3-type cytochrome oxidase [31]. CtaD is the catalytic subunit in *aa3*-type cytochrome

oxidases [32]. While CtaD2 cannot function within the electron transport chain on its own, it

could be forming heterocomplexes with CtaC1 and CtaE1, as CcoN orphan subunits have been

shown to do in *Pseudomonas aeruginosa* [33]. Two loci encode bo_3-type cytochrome oxidases

(*cyoABCD1* and *cyoABCD2*, abbreviated *cyo1* and *cyo2*). CyoB is the catalytic subunit for bo_3-

type cytochrome oxidases [34,35]. Finally, the *cioAB* locus (abbreviated *cio*) encodes a cyanide-

insensitive oxidase (CIO). CIOs are a subclass of *bd*-type cytochrome oxidases that are low-

affinity and were first characterized in *P. aeruginosa,* where CioAB confers resistance to cyanide

[36]. CIOs require both subunits to be functional, like other *bd*-type cytochrome oxidases.

Bordetella pertussis has only three broadly conserved cytochrome oxidase-encoding

gene loci

Genomic comparisons of the classical bordetellae have found that the three subspecies

can be divided into four complexes [37]. Complex I, which includes RB50, is most similar to the

last common ancestor shared by the classical bordetellae and contains *B. bronchiseptica*

strains, primarily those isolated from non-human mammals. Complex II contains *B. pertussis*

and Complex III contains *B. parapertussis*$_{Hu}$. Complex IV, which phylogenetically lies between

Complex I and II, contains *B. bronchiseptica* strains, primarily those isolated from humans. A

recent genotyping study identified a novel *B. bronchiseptica* branch, named lineage II, that is

distinct from the classical *Bordetella* [38]. This branch primarily contains strains that were

formerly classed as Complex IV and had been identified by other groups as being divergent

from other classical bordetellae [39,40].

B. pertussis and *B. parapertussis*$_{Hu}$ have shrunken their genomes since evolving from a

B. bronchiseptica-like ancestor, with genomes approximately 77% and 89% the size of the *B.*

bronchiseptica genome, respectively [24]. Additionally, *B. pertussis* and *B. parapertussis*$_{Hu}$ have

specialized, infecting only humans instead of maintaining the wide mammalian host range and

environmental survival capabilities of *B. bronchiseptica*. We investigated which cytochrome

oxidase-encoding genes have been conserved across the classical bordetellae, hypothesizing

that the human-adapted *B. pertussis* and *B. parapertussis*$_{Hu}$ have lost cytochrome oxidases that

are not required for survival within, and briefly between, human respiratory tracts. We chose 28

Bordetella strains for comparison, including both laboratory strains and clinical isolates: 6

Complex I, 14 Complex II, 4 Complex III, and 4 Complex IV (2 of which could be classified as

lineage II) **(S1 Table).** We found that almost all the strains in Complex I, III, and IV have

maintained intact copies of all eight cytochrome oxidase-encoding gene loci **(Table 1).** Only one

strain, the environmental isolate HT200, differed, having acquired frameshift mutations leading

to premature stop codons in *ccoN*, *cioB*, *cyoB1,* and *cyoB2* which most likely would prevent the

complexes encoded by each of these cytochrome oxidase-encoded loci (here notated as Cco,

Cio, Cyo1, and Cyo2) from functioning in this strain **(S2 Table).** This result is intriguing, as it

indicates that not all cytochrome oxidases are required to survive in the environment, or at least

within the thermal spring in which HT200 was isolated [40]. This strain is also a lineage II strain,

and thus phylogenetically distant from the classical *Bordetella* strains [38] **(S1 Figure)**.

B. pertussis strains have significantly diverged from the other classical bordetellae in

terms of their cytochrome oxidase-encoding genes. All 14 examined strains contained

premature stop codons within *ccoN* and *cyoA2* **(S2 Table)**. These mutations would most likely

prevent Cco and Cyo2 from functioning. Interestingly, the point mutations that led to the

premature stops were identical across all 14 strains, indicating that these mutations were

acquired by the ancestral strain that became *B. pertussis.* Additionally, all 14 strains have lost

cio and 13 strains have lost *ctaD2*. The identical changes across diverse *B. pertussis* strains

indicate that functional copies of Cco, Cio, Cyo2, and CtaD2 were lost during the evolution from

the last common ancestor shared by *B. pertussis* and *B. bronchiseptica.*

There are mutations in cytochrome oxidase-encoding genes that differ between *B.*

pertussis strains. Strain 18323, a common laboratory strain, had the most differences. 18323

has been shown by many typing methods to differ from most *B. pertussis* strains, lacking some

genes usually seen in *B. pertussis* while having copies of genes normally found in *B.*

bronchiseptica and *B. parapertussis_Hu* but not *B. pertussis* [41–44]. These genomic differences

are reflected in the phylogenetic tree, where 18323 is the closest *B. pertussis* strain to Complex

IV **(S1 Figure)**. 18323 was the only *B. pertussis* strain containing *ctaD2*. 18323 also contained

unique mutations, resulting in premature stop codons in *ccoP, cydB3, cyoB1,* and *cyoC1*, as

well as losing *cyoA1* and *cyoD1* **(S2 Table).** Interestingly, 18323 was not the only *B. pertussis*

strain that lost the ability to make Cyd3. Six of the 13 remaining Complex II strains contain a

frameshift mutation in the same position as 18323 but are shifted into a different reading frame.

The fact that these 6 strains all have identical mutations indicates that they may have evolved

from a common ancestor with this mutation and that this mutation occurred after *B. pertussis*

became its own subspecies.

Despite the differences within *B. pertussis* strains, a pattern emerges; excluding 18323,

all these strains have maintained functional loci encoding Cyd1, Cta1, and Cyo1. Therefore, we

hypothesize that these three cytochrome oxidases are necessary and sufficient for *Bordetella*

infection within the mammalian respiratory tract, the environment in which *B. pertussis* has

specialized, and the brief time spent between hosts during transmission.

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

To assess the necessity of each cytochrome oxidase for survival under various

conditions, we generated *B. bronchiseptica* strains with in-frame gene loci deletions using allelic

exchange. Starting with our wild-type strain RB50 (WT), we were able to generate strains

lacking *cco* (Δ*cco*), *cyd1* (Δ*cyd1*), *cyd3* (Δ*cyd3*), *cio* (Δ*cio*), *cta1* (Δ*cta1*), *ctaD2* (Δ*ctaD2*), or *cyo1*

(Δ*cyo1*) **(S2 Figure)**. We were unable to delete the *cyo2* locus in its entirety; instead, we deleted

cyoABC2 and left *cyoD2* intact. The resulting strain lacks a functional complex generated by the

proteins encoded by *cyo2* and is therefore called Δ*cyo2* hereafter.

Based on genome comparisons between *B. bronchiseptica* and *B. pertussis,* we aimed

to generate strains that allowed us to test the necessity and sufficiency of the three cytochrome

oxidase-encoding loci broadly conserved in *B. pertussis* (*cyd1, cta1,* and *cyo1*). We tried to

make a strain that lacks *cyd1, cyo1*, and *cta1* but were unable to generate it using our normal

growth conditions; all co-integrants reverted to WT at the last step of the allelic exchange

procedure, suggesting that at least one of the *Bp*-conserved cytochrome oxidases is required

for growth of *B. bronchiseptica* under standard laboratory conditions, i.e. in SS broth, in ambient

air. We were able to make a strain with only the three conserved cytochrome oxidases by

deleting the other five cytochrome oxidase-encoding loci (*cyd1⁺ cta1⁺ cyo1⁺,* abbreviated *Bp*-

conserved). We then used this strain to generate strains for assessing the necessity of each of

the cytochrome oxidases conserved in *B. pertussis* when only these three are present. We were

able to create all three iterations; a strain with only *cta1* and *cyo1* (*cta1⁺ cyo1⁺*), a strain with

only *cyd1* and *cyo1* (*cyd1⁺ cyo1⁺*), and a strain with only *cyd1* and *cta1* (*cyd1⁺ cta1⁺*)*.*

To assess the sufficiency of each cytochrome oxidase for growth under various

conditions, we generated strains that contain in-frame deletions of seven of the eight

cytochrome oxidase-encoding loci. We were able to generate three of the eight possible strains

with only one cytochrome oxidase-encoding gene loci: a strain with only *cyd1* (*cyd1⁺*), a strain

with only *cta1* (*cta1⁺*), and a strain with only *cyo1* (*cyo1⁺*). We were unable to generate the five

other possible strains with only one cytochrome oxidase-encoding gene locus remaining under

our normal growth conditions; indeed, we were only able to generate strains lacking six of the

eight oxidase-encoding gene loci if one of the two remaining loci was *cyd1, cta1,* or *cyo1* **(S2**

Figure). This result, combined with the fact that we were unable to generate a strain missing all

three cytochrome oxidases conserved in *B. pertussis,* indicates that Cyd1, Cta1, and Cyo1 are

the primary cytochrome oxidases used under normal growth conditions in ambient air and that

at least one is required under this condition.

All of the strains we constructed are able to respire

To determine how well our strains were able to respire after losing one or more

cytochrome oxidase(s), we grew our strains on medium containing triphenyl tetrazolium chloride

(TTC). TTC accepts electrons from the electron transport chain. When TTC is reduced in the

presence of cytochrome oxidases in a sufficiently dense culture, it undergoes an irreversible

color shift from colorless to red [45]. We examined the color of colony biofilms, normalized by

starting optical density (OD_{600}), after 24 hours of growth on media containing TTC. All strains

were able to respire, as evidenced by the red color of the colonies **(S3 Figure).** All strains

except *cta1$^+$* had a similar level of redness, indicating that the mutant strains had a similar level

of respiration as WT. *cta1$^+$* was less red overall but had spots with a higher density of red

pigment than WT. This color variation could reflect heterogeneity in the mutant population.

cta1$^+$ has other phenotypes that differ from WT. This strain generated smaller colonies

than WT on Bordet-Gengou (BG) blood agar plates, our standard culturing medium, requiring 72

hours to produce visible colonies as opposed to the 48 hours required by WT. Moreover, *cta1$^+$*

can't grow in our normal liquid culturing medium, Stainer-Scholte (SS) broth, when taken from

BG agar. However, colonies from BG agar are able to grow on SS agar, and these colonies can

then be grown in SS broth. *cta1$^+$* cultures grown in SS broth or on SS agar can grow on BG

agar. This phenotype was not due to second-site mutations, as whole-genome sequencing

revealed no unique mutations compared to WT, *cyd1$^+$,* or *cyo1$^+$*. The reason for this unusual

phenotype is not known, and we did not pursue it further.

No cytochrome oxidase is necessary for growth in ambient air, but Cyd1, Cta1, and Cyo1

are each sufficient.

To determine if individual cytochrome oxidases are required for normal growth, we grew

strains with deletions of individual cytochrome oxidase-encoding loci (i.e. Δ*cco,* Δ*cyd1,* Δ*cyd3,*

Δ*cta1,* Δ*ctaD2,* Δ*cio,* Δ*cyo1,* and Δ*cyo2*) in SS medium in ambient air conditions and measured

growth over time via OD_{600} and number of viable bacteria (CFU/mL). WT reached stationary

phase between 12 and 18 hours **(Figure 1A)**. While the OD_{600} of WT stayed consistent after the

culture entered stationary phase, the CFU/mL began to decline after 30 hours. All eight strains

lacking a single cytochrome oxidase phenocopied WT by both OD_{600} and CFU/mL **(Figure 1A).**

This result indicates that no single cytochrome oxidase is required for growth under standard

laboratory conditions.

To determine if the cytochrome oxidases broadly conserved in *B. pertussis* are sufficient

for growth under the same conditions, we assessed growth of *Bp*-conserved. *Bp*-conserved

phenocopied WT over time **(Figure 1B)**, indicating that the combination of Cyd1, Cta1, and

Cyo1 is sufficient under these conditions. Given that the combination of the three *Bp*-conserved

cytochrome oxidases was sufficient, we then assessed their individual sufficiency. To do so, we

used strains with only a single cytochrome oxidase-encoding locus remaining (i.e. *cyd1*$^+$*, cta1*$^+$*,

and *cyo1*$^+$). Overall, these strains behaved similarly to WT, reaching stationary phase at the

same time and having a similar growth rate **(Figure 1C).** *cyd1*$^+$ consistently had a slightly lower,

but not significantly different, OD_{600} and CFU/mL than WT. After 30 hours, when WT began to

decrease in CFU/mL, *cta1*$^+$ decreased in CFU/mL more slowly than WT, while *cyo1*$^+$ decreased

in CFU/mL more quickly than WT, especially between 36 and 48 hours. These data indicate that

each of the cytochrome oxidases conserved in *B. pertussis* is sufficient for WT levels of growth

in liquid media in ambient air.

None of the cytochrome oxidases conserved in *B. pertussis* are critical for growth in low

oxygen or in high carbon dioxide

Under normal laboratory conditions, bacteria have access to ambient air, which contains

approximately 21% O_2. When growing within the mammalian respiratory tract, however, bacteria

encounter a different atmospheric environment. Upon inhalation, alveoli inflate and oxygen in

the air is exchanged with carbon dioxide (CO_2) produced by cellular respiration; upon

exhalation, this new gas mixture, which contains approximately 16% O_2 and 5% CO_2, is

released back into the environment (reviewed in [46]). The classical bordetellae are likely further

restricted from access to oxygen since the ciliated epithelial cells to which the bacteria adhere

are coated in a layer of secreted mucus designed to protect the respiratory tract from

environmental pathogens [47].

To investigate how different cytochrome oxidases contribute to the ability of *B.*

bronchiseptica to grow in the different atmospheric conditions it may encounter in the

mammalian respiratory tract, we grew strains within an incubator that allowed us to manipulate

the atmospheric concentrations of oxygen, nitrogen, and CO_2. We collected endpoint samples to

keep the gas concentrations consistent for the duration of the experiment since taking samples

required us to open the incubator, thus exposing the cultures to ambient air. Since we

hypothesize that the three *Bp*-conserved cytochrome oxidases are sufficient for growth within

the mammalian respiratory tract, we focused on assessing the growth of *Bp*-conserved, *cyd1$^+$,*

cta1$^+$, and *cyo1$^+$* relative to WT*.*

First, we assessed growth in low oxygen. Previous studies in *E. coli* have shown that

atmospheric oxygen concentrations at or below 5% are sufficient to shift expression from low-

affinity to high-affinity cytochrome oxidases in liquid cultures [48].We therefore grew our cultures

under 5% O_2 with constant agitation to maximize gas exchange. WT grew slower in 5% O_2 than

in ambient air; cultures grown for 48 hours at 5% O_2 contained less than half the bacteria found

at 24 hours in cultures grown in ambient air **(Figure 2A vs 2B).** Surprisingly, *Bp*-conserved,

cyd1$^+$, cyo1$^+$, and *cta1$^+$* were all able to grow to WT levels in 5% $O_{2,}$ with no strain having a

significant defect relative to WT in both OD_{600} and CFU/mL **(Figure 2B).** Since no cytochrome

oxidase was critical for growth at 5% O_2, we lowered the level of oxygen to 2% O_2. Under 2%

$O_{2,}$ WT struggled to grow; cultures grown for 72 hours at 2% O_2 contained approximately 13% of

the bacteria found at 24 hours in cultures grown in ambient air **(Figure 2A vs 2C).** Despite low

oxygen availability, *cyo1$^+$* and *cta1$^+$* were able to grow to similar levels as WT and *Bp*-conserved

and $cyd1^+$ only had a slight growth defect **(Figure 2C)**. Together, these data indicate that none

of these cytochrome oxidases is required for growth under low oxygen conditions. Conversely,

these data indicate that Cyd1, Cta1, or Cyo1 is sufficient for WT-levels of growth under low

oxygen conditions.

Since bacteria in the respiratory tract also encounter increased carbon dioxide, we next

grew our strains in 5% CO_2 to determine if growth is impacted by this change. WT grew similarly

in 5% CO_2 as in ambient air **(Figure 2A vs 2D).** *Bp*-conserved, *cyd1$^+$, cyo1$^+$,* and *cta1$^+$,*

however, were all defective relative to WT by both OD_{600} and CFU/mL **(Figure 2D)**. This result is

surprising, as it suggests that the three cytochrome oxidases conserved in *B. pertussis* are not

sufficient for efficient growth in the presence of 5% CO_2. Indeed, it suggests that at least one of

the cytochrome oxidases deleted in *Bp*-conserved contributes to growth in 5% CO_2.

Given that the respiratory environment can simultaneously contain higher levels of

carbon dioxide and lower levels of oxygen than ambient air, we tested how well our strains are

able to grow in 5% O_2 5% CO_2. Under this condition, WT grew similarly to how it grew in 5% O_2

alone **(Figure 2B vs 2E).** After 48 hours of growth, *Bp*-conserved, *cyd1$^+$, cyo1$^+$,* and *cta1$^+$* were

all able to grow to WT levels, with no strain having a significant defect relative to WT by both

OD_{600} and CFU/mL **(Figure 2E)**.

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

To determine the role of individual cytochrome oxidases during infection, we inoculated

mice intranasally with bacteria using our high-dose, large-volume protocol, which allows us to

assess how well different strains can persist once introduced in the respiratory tract. We first

used strains containing deletions of individual cytochrome oxidase-encoding loci to determine

which cytochrome oxidases, if any, are necessary for persistence. We then collected the right

lung, the trachea, and nasal cavity tissue at 3 hours, 1 day, 3 days, and 7 days post-inoculation

and determined the bacterial burden in each organ. Due to the number of strains involved, we

could not test all eight strains simultaneously. Instead, we tested them in batches, with each

including WT, then compiled the data. All strains caused the same bacterial burden in mice as

WT in the nasal cavity, trachea, and lung **(Figure 3)**. These data indicate that no individual

cytochrome oxidase is required for persistence in the murine respiratory tract.

The cytochrome oxidases conserved in *B. pertussis* are sufficient for persistence during

murine infection

We next assessed the role of the three cytochrome oxidases conserved in *B. pertussis*

for persistence during infection. We infected mice with either *Bp*-conserved or WT, and

determined the bacterial burden in the nasal cavity, trachea, and lung over the course of

infection. The *Bp*-conserved strain was statistically indistinguishable from WT in all tissues at all

time points tested **(Figure 4),** indicating that the three cytochrome oxidases conserved in *B.*

pertussis are sufficient for persistence in mice.

Cyo1 is sufficient for persistence during infection, while strains with only Cyd1 or Cta1

are defective for persistence relative to WT

The three cytochrome oxidases conserved in *B. pertussis* are predicted to have different

characteristics **(Table 1).** Cyd1 is predicted to be a high-affinity *bd*-type ubiquinol oxidase. Cta1

is predicted to be a low-affinity HCO cytochrome *c* oxidase. Cyo1 is predicted to be a low-affinity

HCO ubiquinol oxidase. We hypothesize that each of these cytochrome oxidases fulfills a

different function during infection due to their different biochemical properties. To test this

hypothesis, we infected mice using strains with only a single cytochrome oxidase-encoding

gene locus remaining: *cyd1⁺, cta1⁺,* and *cyo1⁺*. *cyd1⁺* had a slight defect relative to WT in the

nasal cavity, causing a lower bacterial burden at day 1 that was able to reach WT levels by day

3 **(Figure 5A, top)**. *cyd1⁺* was consistently defective in the lower respiratory tract relative to WT,

causing a significantly lower bacterial burden in the trachea and the lung out to 14 days **(Figure**

5A, middle and bottom). *cta1⁺* was defective relative to WT in all three sampled tissues

(Figure 5B). However, this strain was not cleared from the lung faster than WT, as seen by the

statistically similar burdens recovered on day 14 when the burden of WT decreased relative to

earlier time points **(Figure 5B, bottom)**. Most surprisingly, *cyo1⁺* was recovered at levels similar

to WT in all three sampled tissues across the course of infection **(Figure 5C).** These data

indicate that Cyo1 is sufficient for WT-levels of growth in this model of mouse infection, despite

being predicted to be optimized for ambient air levels of oxygen, while Cyd1 and Cta1 are not

sufficient. Additionally, all three strains are able to persist to some degree throughout the

respiratory tract once introduced, indicating that any of these cytochrome oxidases is sufficient

for survival within a mouse.

The *Bp*-conserved strain is indistinguishable from WT in its ability to establish

respiratory colonization

We and others have used high-dose, large-volume and low-dose, small-volume mouse

protocols to investigate respiratory tract persistence and establishment of infection, respectively.

In our lab, we have previously used the low-dose, small-volume protocol only in rats, where we

showed that doses of 20 CFU are sufficient for infection in rats [49]. To adapt this approach to

mice, we performed preliminary experiments using WT to determine a viable volume and dose

for intranasal inoculation in wild-type mice. We found that 4μL was sufficient to introduce

bacteria to the nasal cavity but not the trachea, as we were able to recover CFU from the nasal

cavity but not the trachea at this volume on the same day as we inoculated the mice **(S4A**

Figure). We also found that 100 CFU per inoculum was sufficient to ensure robust colonization

of the nasal cavity, as well as consistently recoverable burden from the trachea by day 7 post-

inoculation **(S4A Figure).** In this model, bacteria were not consistently recovered from the lungs.

We next assessed whether the three *Bp*-conserved cytochrome oxidases are sufficient

for infection. *Bp*-conserved phenocopied WT in the high-dose, large-volume model **(Figure 4),**

demonstrating that the three cytochrome oxidases found in *B. pertussis* are sufficient for

persistence. We performed a preliminary experiment to determine if these three cytochrome

oxidases are also sufficient for establishing infection. Mice infected with *Bp*-conserved had a

similar bacterial burden in the nasal cavity 3 days post-inoculation as mice infected with WT,

indicating that the combination of Cyd1, Cyo1, and Cta1 is sufficient for establishing infection

(S3B Figure).

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

To assess the individual roles of Cyd1, Cyo1, and Cta1 during the establishment of

infection, we first tested whether any of these three cytochrome oxidases is required. We used

the derivatives of *Bp-conserved* that have two remaining cytochrome oxidases (i.e. $cta1^+$ $cyo1^+$,

$cyd1^+$ $cyo1^+$, and $cyd1^+$ $cta1^+$). These strains phenocopied WT growth in ambient air **(S5A**

Figure). We infected mice with these strains using our low-dose, small-volume protocol and

determined the nasal cavity bacterial burdens 3 and 7 days post-inoculation. We found that

mice infected with the strains with two cytochrome oxidases had similar bacterial burdens to

mice infected with *Bp*-conserved **(S5B Figure)**. One strain, $cyd1^+$ $cta1^+$, trended towards

causing a lower bacterial burden than *Bp*-conserved, but this difference was not significant.

Based on these data, no single cytochrome oxidase is required to establish infection.

To determine if any of the three *Bp*-conserved cytochrome oxidases is sufficient to

establish infection, we infected mice with the strains with only a single cytochrome oxidase-

encoding locus remaining (i.e. $cyd1^+$, $cta1^+$, and $cyo1^+$) and determined the bacterial burden as

above. Overall, the trends in the low-dose, small-volume experiments mirrored those seen in the

high-dose, large-volume experiments. Mice infected with $cyd1^+$ had a lower bacterial burden 3

days post-inoculation than mice infected with *Bp*-conserved but reached the same burden by

day 7 **(Figure 6).** Mice infected with $cta1^+$ had a significantly lower bacterial burden than mice

infected with *Bp*-conserved at both day 3 and 7 **(Figure 6)**. Despite causing less than one

percent of the bacterial burden of *Bp*-conserved strain at day 7 in mice, $cta1^+$ nonetheless was

able to establish infection and persist for the duration of the experiment. Mice infected with

$cyo1^+$ had the same bacterial burden as mice infected with *Bp*-conserved at both timepoints

(Figure 6). Together, these data indicate that while all three cytochrome oxidases are sufficient

to enable the establishment of infection, as seen by recoverable bacteria following infection with

all three strains, only Cyo1 is sufficient to achieve the level of colonization reached when all

three cytochrome oxidases conserved in *B. pertussis* are present.

Regulation of cytochrome oxidase-encoding genes broadly follows the predicted affinity

of the cytochrome oxidases they encode

Given that we were unable to construct a strain that lacks all three of the cytochrome

oxidase-encoding gene loci conserved in *B. pertussis* **(S2 Figure)***,* we investigated how different

cytochrome oxidase-encoding genes are regulated, hypothesizing that some loci may not be

expressed under laboratory growth conditions. We diluted overnight cultures to the same

density, grew them for 4 hours in either ambient air, 5% O_2, 2% O_2, or 5% CO_2, extracted RNA,

and sent it for sequence analysis.

First, we examined the level of transcripts in ambient air to investigate the expression of

cytochrome oxidase-encoding genes under our normal growth conditions. Raw normalized read

counts were converted to counts per million for comparison. All eight cytochrome oxidase-

encoding gene loci had detectable transcripts **(Figure 7A)**. However, when compared to *fhaB*

and *flaA,* two well-characterized and highly-regulated genes, we found that transcript levels for

most of the non-conserved cytochrome oxidase-encoding gene loci (*cyd3, cio, ctaD2,* and *cyo2*)

were similar to that of *flaA,* the protein product of which cannot be detected under these growth

conditions [50]. Therefore, transcription of *cyd3, cio, ctaD2,* and *cyo2* may be insufficient to

produce enough functional enzyme complexes to support respiration. By contrast, transcript

abundance for *ccoN* was the highest of all of the loci, with high expression of the rest of the *cco*

locus as well, yet we were unable to make a strain with only *cco*. This result suggests a lack of

sufficient translation, complex assembly, or cofactors required for Cco. The three *B. pertussis*-

conserved cytochrome oxidase-encoding loci had high levels of expression, though not as high

as *fhaB,* which encodes a highly abundant secreted protein.

We next examined the fold change in transcript abundance in bacteria grown in our

conditions of interest (5% O_2, 2% O_2, and 5% CO_2) and ambient air. In 5% O_2, *cydA1* and *cydB1*

had ~3 times more transcripts than in ambient air **(Figure 7B).** This result is consistent with the

prediction that Cyd1 functions as a high-affinity cytochrome oxidase and is therefore adapted for

use in low oxygen environments. No other gene had a significant difference in transcript

abundance in 5% O_2 versus ambient air.

In 2% O_2, differences in transcript abundance were greater **(Figure 7C)**. *cydA1* had

significantly more transcripts in 2% O_2 than in ambient air but fewer transcripts than in 5% O_2.

Genes within *cyo1* (*cyoC1* and *cyoD1*) had significantly fewer transcripts in 2% O_2 relative to

ambient air, which matches the prediction that these genes encode components of a low-affinity

cytochrome oxidase. *ctaC1* and *ctaD1* have less expression in 2% O_2 relative to ambient air,

with *ctaD1* having a larger relative decrease in transcripts. Interestingly, *ctaD2* had higher

expression in 2% O_2 relative to ambient air, with ~2.6 times more transcripts in ambient air than

in 2% O_2. Since the genes encoding other components of Cta1 did not significantly decrease in

expression in 2% O_2, CtaD2 could be interacting with CtaC1 and CtaE1 (and potentially CtaF

and CtaG1) to form a heterocomplex (i.e. CtaC1<u>D2</u>F1G1E1). Unexpectedly, *ccoQ* had

significantly lower levels of transcripts in 2% O_2 relative to ambient air, despite being predicted

to encode part of a high-affinity cytochrome oxidase, and *cyoC2* and *cyoD2* had significantly

higher levels of transcripts, despite being predicted to encode parts of a low-affinity cytochrome

oxidase.

In 5% CO_2, no gene had significant changes in expression relative to growth in ambient

air **(Figure 7D).** This was surprising, as 5% CO_2 was the only growth condition tested that

resulted in a large growth defect in *Bp*-conserved relative to WT **(Figure 2)**, leading us to

hypothesize that a non-*Bp*-conserved cytochrome oxidase that is not required for growth in

ambient air must be important for growth in 5% CO_2. While this hypothesis could still be true,

these results indicate that differences in growth in ambient air and 5% CO_2 are not due to

differences in cytochrome oxidase-encoding gene expression.

<u>DISCUSSION</u>

Our goal was to understand the importance of cytochrome oxidases for bacterial

pathogens that infect the mammalian respiratory tract. *B. bronchiseptica* contains eight

cytochrome oxidase-encoding gene loci, while *B. pertussis,* which is strictly a human pathogen

that survives only briefly in the environment during transmission between hosts, contains only

three cytochrome oxidase-encoding gene loci that are broadly conserved across strains.

Perhaps unsurprisingly, the three *B. pertussis*-conserved cytochrome oxidases were sufficient

for *B. bronchiseptica* to both establish infection and persist in the respiratory tracts of mice.

Somewhat surprisingly, however, *B. bronchiseptica* producing only a single low-affinity

cytochrome oxidase (encoded by *cyoABCD1,* abbreviated Cyo1) was indistinguishable from WT

in both infection models.

While it is well known that we inhale ambient air containing ~21% O_2 and low levels of

CO_2 and exhale air containing ~16% O_2 and ~5% CO_2, the levels of oxygen within specific

microenvironments in the respiratory tract are unknown. The classical bordetellae bind to the

surface of ciliated epithelial cells lining the respiratory tract, which do not continue into the

smallest bronchioles or the alveoli. The ciliated epithelium is covered in a layer of mucus

secreted by neighboring goblet cells and tethered mucins produced by the ciliated cells

themselves. These viscous layers protect the respiratory tract from environmental pathogens

but impede the diffusion of molecules, including oxygen, to the epithelium. We therefore

anticipated that a high-affinity cytochrome oxidase would be important in this environment, and

hence were surprised that *cyo1$^+$*, a strain producing only a predicted low-affinity cytochrome

oxidase, was able to infect mice with the same efficiency as WT. If the Cyo1 enzyme is, in fact,

low-affinity as predicted, then this result indicates that the surface of the ciliated epithelium has

access to more oxygen than previously thought.

The strain producing only Cyd1*,* by contrast, was attenuated relative to WT in both

animal models. The level of attenuation depended on the site of the respiratory tract examined.

In the nasal cavity, the *cyd1*+ strain caused lower bacterial burdens than WT early in infection,

but was able to reach WT-levels later in infection, indicating a growth delay in this environment.

In the lower respiratory tract, however, the *cyd1*+ strain was consistently defective relative to WT

throughout the course of infection. If the surface of the ciliated epithelium is sufficiently

oxygenated, as the results with the *cyo1*+ strain suggest, this result could reflect the lower

efficiency of high-affinity cytochrome oxidases, which pump fewer protons, and therefore

generate less proton motive force, per electron transferred [6]. *bd*-type cytochrome oxidases

have been linked to virulence in other pathogens not only for their oxygen affinity but also for

their unique resiliency in the face of immune system attacks. *bd*-type cytochrome oxidases are

more tolerant of oxidative and nitrosative stress than HCOs and have been shown to detoxify

hydrogen peroxide and peroxynitrite produced by neutrophils and macrophages [51]. This role

for *bd*-type cytochrome oxidases has been shown in *M. tuberculosis*, where the *bd*-type

cytochrome oxidase is dispensable for the establishment of infection but is necessary after the

initiation of adaptive immunity [18]. In this way, Cyd1 may play a larger role during in *B.*

pertussis, which causes an acute infection that is combated by a strong, and ultimately

sterilizing, mucosal immune response, compared to *B. bronchiseptica,* which typically causes

chronic and, in the case of our mouse models, asymptomatic infection (reviewed in [52,53]).

Cta1 was the least effective during infection, with the strain producing only this

cytochrome oxidase being recovered at lower levels than WT in all tissues at all timepoints in

both models of infection. Cta1 could, however, play a role that was not assessed by our models.

Indeed, previous microarray data indicated that *ctaC1, ctaD1, ctaG1,* and *ctaE1* are repressed

by BvgAS, the two-component system that positively regulates all known protein virulence

factors in *Bordetella* [54]. Given that the Bvg+ mode is both necessary and sufficient for

infection, Cta1 may play a role outside the host during transmission [55].

Importantly, the mouse respiratory tract is not identical to the respiratory tract of humans;

it is more similar to the human distal airways, both in terms of surface area and mucus

composition. In humans, proximal airways are coated in mucus primarily made of MUC5AC

mucins and distal airways have mucus made of MUC5B, whereas in mice, Muc5b is

constitutively produced throughout the airway and almost no Muc5ac is produced [56]. The

organization of the mucus is also different, with humans and other large mammals producing

long thick bundles of mucins such that the proximal airway is coated in a layer of mucus, while

in mice (and most likely, the distal airways of humans), the mucus is unevenly distributed,

forming a patchy covering [57,58]. This patchy covering creates areas with thinner mucus

layers, which would make oxygen diffusion easier. Therefore, the bacteria attached to the

mouse ciliated epithelium may have access to more oxygen than they would on the human

ciliated epithelium.

It is perhaps unsurprising that *B. bronchiseptica* can grow in low oxygen given its many

cytochrome oxidase-encoding loci, three of which are predicted to encode high-affinity

cytochrome oxidases. WT was able to grow in both 5% and 2% O_2 (albeit at a far slower rate

than in ambient air). It was surprising to us, however, that the *Bp*-conserved, $cyd1^+$, $cta1^+$, and

$cyo1^+$ strains were all able to grow similarly under low oxygen conditions. Indeed, the $cta1^+$ and

$cyo1^+$ strains, which encode only low-affinity cytochrome oxidases, grew as well as WT in 2%

O_2. This result indicates that all of the *B. pertussis*-conserved cytochrome oxidases all function

equally well under low oxygen in laboratory conditions, despite their predicted affinities.

Additionally, *B. bronchiseptica* is resilient when challenged with low oxygen. It is able to persist

in static PBS, where oxygen diffusion is limited, with viable CFU being recovered even 24

weeks post-inoculation [59]. In this aspect, *B. bronchiseptica* is comparable to *M. tuberculosis,*

another obligate aerobe that can persist in low oxygen conditions for long periods of time [60].

Our analysis primarily focused on the three cytochrome oxidase-encoding loci conserved

in *B. pertussis.* However, we also examined the expression of the other five loci to see if they

played a role in any of our experimental conditions. We found that *cyd3, cio, ctaD2,* and *cyo2*

had fewer transcripts per million than *flaA*, a BvgAS-repressed gene whose protein product is

not detected in our growth condition (SS medium) [50]. We were also unable to generate strains

with only *cco, cyd3, cio, ctaD2,* or *cyo2* intact; at least one of the three *B. pertussis*-conserved

loci had to be intact to generate a strain. Although the specific environments that *B.*

bronchiseptica occupies outside the mammalian respiratory tract are unknown, our results

suggests these environments are complex and varied, requiring different cytochrome oxidases

than those utilized in lab culture.

In our RNAseq analyses, we only examined expression in wild-type *B. bronchiseptica*

and therefore did not assess the effect that altering cell homeostasis by deleting cytochrome

oxidase-encoding genes could have on global gene expression. In the *cyd1$^+$, cta1$^+$,* and *cyo1$^+$*

strains*,* it is likely that the remaining cytochrome oxidase-encoding locus is more highly

expressed to compensate for the loss of the other two loci, which are normally expressed in our

growth conditions. Expression changes could also explain the unusual plate-based growth

phenotypes we observed for the *cta1$^+$* strain*,* which could not be explained by secondary

mutations. Upregulation of the remaining cytochrome oxidase-encoding loci in the strains with

only a single locus could be masking synergy that occurs when the cytochrome oxidases are

present at their endogenous levels.

Two additional trends emerged from the RNAseq. First, many highly BvgAS-activated

genes had lower expression in low oxygen than in ambient air, including *bvgA*, *bvgS*, *fhaB*, and

cyaA **(S3,4 Table).** By contrast, the common reference genes *rpoD* and *recA* were not

differentially expressed in low oxygen as compared to ambient air, indicating that the differences

seen for BvgAS-activated genes are not due to global changes in gene expression. *In vitro*

studies using purified solubilized BvgS have shown that BvgS activity is sensitive to the redox

state of ubiquinone through its PAS domain [61]. *In vivo* studies also support that BvgS is

sensitive to redox potential via its PAS domain [62]. Thus, decreased expression of BvgAS-

activated genes could be directly related to decreased respiration affecting BvgS activity in low

oxygen environments.

Second, we found that expression of the *ptx* genes (*ptxABCDE*), which encode pertussis

toxin, was increased in *B. bronchiseptica* in low oxygen **(S3,4 Table)**. This result is exciting,

because *ptx* gene expression have never been detected in *B. bronchiseptica in vitro* or *in vivo,*

and only a weak luminescent signal was detected when the *B. bronchiseptica ptx* promoter was

used to drive the expression of luciferase-encoding genes [63]. Pertussis toxin is an important

virulence factor during human infection, and in *B. pertussis,* the *ptx* genes are activated by

BvgAS (reviewed in [64]). In Complex I *B. bronchiseptica* strains, from which the other classical

bordetellae evolved, the *ptx* promoter region is not BvgAS-regulated but the *ptx* genes (as well

as the *ptl* genes that encode the export system for pertussis toxin) are present and intact

[65,66], suggesting that in *B. bronchiseptica*, pertussis toxin plays a different role than

contributing to mammalian infection. By contrast, many Complex IV *B. bronchiseptica* strains

have lost their *ptx* genes [37]. These strains also don't survive as well as Complex I strains

when grown in environmental conditions, instead being more adapted to the human hosts from

which they were isolated. These results combined, together with the fact that the substrate for

pertussis toxin is a eukaryotic G protein, suggest that pertussis toxin was used by ancestral *B.*

bronchiseptica strains to manipulate a non-mammalian eukaryotic host or predator in the

environment. As strains evolved to infect humans and lost their ability to survive in the

environment, those that became *B. pertussis* acquired mutations in the *ptx* promoter that

allowed for activation by BvgAS (and presumably not the regulator(s) that control *ptx* expression

in *B. bronchiseptica*). Our findings suggest that one feature of the environment in which the *ptx*

genes are expressed in *B. bronchiseptica* is low levels of accessible oxygen.

Previous genomic and experimental comparisons between the classical bordetellae

have focused on virulence-associated genes like *fhaB, cyaA,* and the *ptx* genes. By comparing

genes encoding proteins important for bacterial physiology, like cytochrome oxidases, we can

also learn about which bacterial processes and pathways are important for survival within the

host. Comparing *B. pertussis* and *B. bronchiseptica* led to our hypothesis that the three

cytochrome-oxidase encoding gene loci that are conserved in *B. pertussis* would be sufficient

during infection, which was supported by our experimental findings. Conversely, we can also

learn about what is important for surviving in the environment. HT200, a *B. bronchiseptica*

environmental isolate, differs from the other examined *B. bronchiseptica* strains in that HT200

does not have intact copies of all eight cytochrome-oxidase encoding gene loci. In particular,

this strain has premature stop codons in both *cyoB1* and *cyoB2*, meaning it likely has no

functional bo_3-type cytochrome oxidases (Table S2). This result, especially in contrast with the

broad conservation of *cyo1* within *B. pertussis* strains, indicates that while Cyo1 is important for

survival within mammalian hosts, it is not important for survival within aqueous environments.

This result also supports the hypothesis that the many different cytochrome oxidases encoded

within the *B. bronchiseptica* genome all contribute to the ability of this species to be a generalist,

surviving in many different environments. As strains specialize towards life within specific

environments, the bacteria no longer require all their cytochrome oxidases and the genes that

are no longer required can accrue mutations.

While the role of cytochrome oxidases has been investigated in other pathogens,

including *V. cholerae, M. tuberculosis,* and *S. aureus*, the majority of species studied have been

facultative anaerobes [9,18–20]. Additionally, most either infect regions like the gut that are

expected to have low levels of oxygen, or, like *M. tuberculosis,* have evolved to be able to

survive and grow as intracellular pathogens. In these pathogens, individual high-affinity

cytochrome oxidases are critical for normal infection. In the respiratory pathogen and obligate

aerobe *B. bronchiseptica,* this is not the case. Not only is no individual cytochrome oxidase

required for infection, but a predicted low-affinity cytochrome oxidase is sufficient in mice for

both the establishment of infection and persistence once bacteria are introduced. Therefore,

targeting *bd*-type cytochrome oxidases, which is being explored as a treatment for *M.*

tuberculosis among others*,* would not be effective in *B. bronchiseptica* [51]. This result

emphasizes the need to study not only virulence factors but also the basic bacterial physiology

to effectively combat bacterial pathogens. By expanding our understanding of the cytochrome

oxidases of other pneumonia-causing bacteria, like *Neisseria meningitidis* and *Haemophilus*

influenzae, we may find that low-affinity cytochrome oxidases are better drug targets to clear

infections within the respiratory tract.

<u>MATERIALS AND METHODS</u>

Bacterial strains, plasmids, and growth conditions. *B. bronchiseptica* strains were grown on

Bordet-Gengou (BG) agar plates supplemented with 6% defibrinated sheep blood (Hemostat,

catalog no. DSB1) or in Stainer-Scholte (SS) broth supplemented with SS supplement at 37°C

([67], updated in [68]). One strain (*cta1*[+]) had growth defects on BG agar plates and was

therefore plated on SS agar plates when possible. As needed, media were supplemented with

streptomycin (Sm, 20 μg/mL), gentamicin (Gm, 30 μg/mL), or sucrose (15% w/v). *E. coli* strains

were grown in Lysogeny Broth (LB) or on LB agar plates supplemented with diaminopimelic acid

(DAP, 300 μg/mL), ampicillin (Ap, 100 μg/mL), and gentamicin (Gm, 30 μg/mL) as needed. All

cultures were started from individual colonies from a clonal population.

Construction of plasmids and strains. The strains and plasmids used in this study are listed

in **Table 3** and **Table 4**, respectively, which are based on mutations of the cytochrome oxidase-

encoding loci as outlined in **Table 2**. In-frame deletions were generated using allelic exchange

via derivatives of the pEG7s vector. We used the DH5α *E. coli* strain for plasmid construction

and propagation. We used the Rho3 *E. coli* strain for transforming *B. bronchiseptica*. Any

mutations made within *B. bronchiseptica* strains were confirmed by PCR and/or whole-genome

sequencing. The order in which deletions were generated in strains with deletions in multiple

loci is described in **S2 Figure**.

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	ΔcyoABCD2	Deletion of 3641bp: from nucleotide 6 of cyoA2 (BB1310) to nucleotide 632 of cyoC2 (BB1308); cyoD2 is left intact
7	ΔctaCDFE1	Deletion of 4165bp: from nucleotide 9 of ctaC1 (BB4831) to nucleotide 493 of ctaE1 (BB4827)
8	ΔctaD2	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-ΔccoNOQP	pEG7s containing ~500bp upstream and downstream of mutation 1	This study
pMAB19	pEG7s containing ~500bp upstream and downstream of mutation 2	This study
pEG7s-ΔcydAB3	pEG7s containing ~500bp upstream and downstream of mutation 3	This study
pEG7s-ΔcioAB	pEG7s containing ~500bp upstream and downstream of mutation 4	This study
pEG7s-Δcyo1	pEG7s containing ~500bp upstream and downstream of mutation 5	This study
pEG7s-Δcyo2	pEG7s containing ~500bp upstream and downstream of mutation 6	This study
PEG7s-Δcta1	pEG7s containing ~500bp upstream and downstream of mutation 7	This study
pEG7s-ΔctaD2	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]
Δcco	RB50 containing mutation 1; Sm^R	This study
Δcyd1	RB50 containing mutation 2; Sm^R	This study
Δcyd3	RB50 containing mutation 3; Sm^R	This study
Δcio	RB50 containing mutation 4; Sm^R	This study
Δcyo1	RB50 containing mutation 5; Sm^R	This study
Δcyo2	RB50 containing mutation 6; Sm^R	This study
Δcta1	RB50 containing mutation 7; Sm^R	This study
ΔctaD2	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; *Δasd ΔaphA*, DAP auxotroph	[69]

TTC reduction assay. Cultures of *B. bronchiseptica* were grown overnight at 37°C in SS media

supplemented with SS supplement and Sm. Each culture was normalized to 1 OD_{600}/mL. Five

microliters were spotted onto tryptic soy agar (TSA) plates (Millipore 22091) containing 0.001%

(w/v) TTC (2,3,5-triphenyl-tetrazolium chloride) (Sigma-Aldrich T8877) and incubated in the dark

for 24 hours at 37°C. Plates were photographed to capture the irreversible color shift from

colorless to red that occurs following TTC reduction.

Liquid culture growth assays. Cultures of *B. bronchiseptica* were grown for 18 hours at 37°C

in SS media supplemented with SS supplement and Sm. Each culture was normalized to 0.1

OD_{600}/mL and grown on an orbital shaker (VWR Catalog # 89032-096) at 225 rpm at 37°C.

Samples were collected at 0, 3, 6, 9, 12, 18, 24, 36, and 48 hours after the start of the

experiments. We measured the OD_{600} of these samples and determined the CFU/mL by serially

diluting the samples and plating on BG blood agar plates. The number of colonies were

enumerated after at least 48 hours growth at 37°C, with *cta1⁺* requiring at least 72 hours growth

at 37°C.

Growth under different atmospheric conditions. Cultures for outgrowth were prepared in the

same manner as for growth curves. Samples were then grown on an orbital shaker at 225 rpm

at 37 °C within a trigas incubator connected to N_2 and CO_2 gas sources (HERACELL VIOS 160i;

Fisher Scientific 13998258). To reduce O_2 levels, pure N_2 was introduced. We took endpoint

samples to ensure that the experimental conditions remained constant throughout and were not

disrupted by the reintroduction of atmospheric oxygen at each sampling point. Samples were

collected at 24 hours when grown in 5% CO_2, 48 hours when grown in 5% O_2 and in 5% O_2 5%

CO_2, and 72 hours when grown in 2% O_2. We measured the OD_{600} of these samples and

determined the CFU/mL by serially diluting the samples and plating on BG blood agar plates.

The number of colonies were enumerated after at least 48 hours growth at 37°C, with $cta1^+$

requiring at least 72 hours growth at 37°C.

Bacterial infection of the mouse respiratory tract using the persistence model. Six-week-

old female BALB/c mice from Charles River Laboratories (catalog no. BALB/cAnNCrl) were

inoculated intranasally with 7.5×10^4 CFU *B. bronchiseptica* in 50 µL of Dulbecco PBS (DPBS).

Samples were collected at three hours, one day, three days, seven days, and fourteen days

post-infection. At each indicated time point, right lung lobes, trachea, and nasal cavity tissue

were harvested from each mouse and the tissues were homogenized in DPBS using a mini-

beadbeater with 0.1 mm zirconia beads (Biospec catalog no. 11079110zx). The number of CFU

was determined by plating dilutions of tissue homogenates on BG Sm blood agar and

enumerating the number of colonies per tissue after at least 48 hours growth at 37°C, with $cta1^+$

requiring at least 72 hours growth at 37°C.

Bacterial colonization of the mouse respiratory tract. Six-week-old female BALB/c mice

from Charles River Laboratories (catalog no. BALB/cAnNCrl) were inoculated intranasally with

100 CFU *B. bronchiseptica* in 4 µL of Dulbecco PBS (DPBS), divided equally between the two

nares. Samples were collected at three days and seven days post-infection. At each indicated

time point, nasal cavity tissue was harvested and homogenized in DPBS using a mini-

beadbeater with 0.1 mm zirconia beads (Biospec catalog no. 11079110zx). The number of CFU

was determined by plating dilutions of tissue homogenates on BG Sm blood agar and

enumerating the number of colonies per tissue after at least 48 hours growth at 37°C, with $cta1^+$

requiring at least 72 hours growth at 37°C.

RNA isolation and sequencing. Cultures of *B. bronchiseptica* were grown overnight at 37°C in

SS media supplemented with SS supplement and Sm. Each culture was normalized to 1

OD_{600}/mL and grown on an orbital shaker at 225 rpm at 37 ºC for 4 hours under different

atmospheric conditions as above. 3 biological replicates were generated for each condition.

Bacteria were pelleted, resuspended in RNAlater (Invitrogen AM7020), then stored at -80°C until

extraction. RNA was isolated using Trizol/chloroform extraction, followed by centrifugation in

Phasemaker Tubes (Invitrogen 12183555). The aqueous phase was precipitated in isopropanol,

washed in ethanol, and solubilized in RNase-free water. Samples were then cleaned using

RNAeasy column purification (Quiagen 74104). RNA sequencing was performed by SeqCenter

(Pittsburgh, PA; 12M Paired End Reads rRNA depletion sequencing package). Library

preparation was performed using Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero

Plus kit and 10bp unique dual indices. Sequencing was performed using a NovaSeq X Plus.

RNA analysis. RNA analysis was performed by SeqCenter (Pittsburgh, PA; intermediate RNA

analysis package). Read mapping was performed using HISAT2 [70] and read quantification

was performed using featureCounts [71]. Read counts were loaded into R, then normalized

using edgeR's Trimmed Mean of M values (TMM) algorithm [72]. These values were then

converted to counts per million. Differential expression analysis between our different conditions

(5% O_2 vs ambient air, 2% O_2 vs ambient air, and 5% CO_2 vs ambient air) was performed using

edgeR's glmQLFTest.

Ethics statement. All animal studies followed the guidelines in the *Guide for the Care and Use*

of Laboratory Animals of the National Institute of Health. Our protocols were approved by the

University of North Carolina Institutional Animal Care and Use Committee (Protocol ID: 22-140).

All animals were anesthetized for inoculations, monitored daily, and euthanized properly and

humanely. All efforts were made to minimize suffering.

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<u>SUPPORTING INFORMATION</u>

S1 Table. Genomes of classical bordetellae strains compared

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

compared to RB50

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

either in 5% O_2 or ambient air.

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

either in 2% O_2 or ambient air.

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

either in 5% CO_2 or ambient air.

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

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

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

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

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

Life (iTOL) v5 [73].

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

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

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

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

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

revertants; at least 16 clones were screened for each.

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

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

of 5 biological replicates per strain.

S4 Figure. The three cytochrome oxidases conserved in *B. pertussis* are sufficient for

colonization in mice. (A) Bacterial burden over time within the nasal cavity (upper), trachea

(middle), and right lung (lower) of mice infected with wild-type bacteria (WT, grey circles). Open

circles represent tissue samples with no detectable bacteria. n=2 for day 0, n=6 for all other

timepoints. (B) Bacterial burden over time within the nasal cavity of mice infected with 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=2 for day 0, n=4 for day 3. Each

point represents a single mouse. Dashed line represents the limit of detection. Statistical

significance was determined using unpaired Student's t-test; p-values are indicated when

$p<0.05$.

S5 Figure. No single cytochrome oxidase is required to establish infection. (A) Growth

over time, measured via optical density (left) or CFU/mL (right), for strains with two cytochrome

oxidase-encoding gene loci. (B) Bacterial burden over time within the nasal cavity of mice

infected with a strain with only the cytochrome oxidase-encoding gene loci conserved in *B.*

pertussis (*Bp*-conserved, maroon hexagon), a strain with only *ctaCDFGE1* and *cyoABCD1*

(*cta1$^{+.}$ cyo1^{+}*, orange upright triangle), a strain with only *cydAB1* and *cyoABCD1* (*cyd1^{+} cyo1^{+}*,

blue diamond), or a strain with only *cydAB1* and *ctaCDFGE1* (*cyd1^{+} cta1^{+}*, green upside-down

triangle). Samples from 5 mice were collected at each timepoint for each strain. However, due to

the natural microbiota of the nasal cavity, *B. bronchiseptica* could not always be innumerated

due to contamination. Therefore, n=4 for *cta1^{+}* day 3. Each point represents a single mouse.

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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<u>FIGURES</u>

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 $3.5x10^8$ 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 $3.5x10^8$ 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; ****, $p < 0.0001$.

Figure 3. No single cytochrome oxidase is required during murine infection. Bacterial

burden over time within the nasal cavity (upper), trachea (middle), and right lung (lower) of mice

infected with either RB50 (WT) or strains lacking a single cytochrome oxidase-encoding gene

loci. Different symbols represent different batches of inoculations, which always include WT for

comparison: circles (Δ*cco,* Δ*cyd1,* Δ*cyd3*, and Δ*cio*), upright triangles (Δ*cio,* Δ*cyo1,* Δ*cyo2,*

$\Delta cta1$, and $\Delta ctaD2$), upside-down triangles (Δcco, $\Delta cyd1$, and $\Delta cyd3$), and diamonds ($\Delta cyo1$,

$\Delta cyo2$, $\Delta cta1$, and $\Delta ctaD2$). Each point represents data from one mouse. n=4 for day 0, n=6 for

all other timepoints, from two independent experiments, for all mutant strains. Each point

represents a single mouse. Dashed line represents the limit of detection. Statistical significance

was determined using unpaired Student's t-test; p-values are indicated when $p < 0.01$.

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 $p < 0.05$.

Figure 5. Cyo1 is sufficient for WT-levels of persistence within the mammalian respiratory

tract, while Cyd1 and Cta1 are not. Bacterial burden over time within the nasal cavity (upper),

trachea (middle), and right lung (lower) of mice infected with wild-type bacteria (WT, grey

circles), a strain with only *cydAB1* (*cyd1*$^+$, orange upright triangles), a strain with only

ctaCDFGE1 (*cta1*$^+$, blue diamonds), or a strain with only *cyoABCD1* (*cyo1*$^+$, green upside-down

triangles). These strains were tested in the same experiment but separated into separate

graphs for analysis; thus, the mutant strains are compared to the same WT data. 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 $p < 0.05$.

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

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