

Brucella abortus histidine auxotrophs are copper sensitive

Charline Focant,¹ Agnès Roba,¹ Elisabeth Wanlin,¹ Katy Poncin,¹ Xavier De Bolle¹

AUTHOR AFFILIATION See affiliation list on p. 17.

ABSTRACT Despite decades of investigation into bacterial pathogens, the conditions met by intracellular bacteria are still unclear. These conditions can include access to nutrients, such as amino acids, and exposure to toxic compounds, like copper. To investigate the ability of *Brucella abortus*, a facultative intracellular pathogen responsible for a major zoonosis, to cope with copper, we performed a Tn-seq analysis to identify copper-sensitive mutants. Unexpectedly, we realized that classical copper resistance systems (involving CopA and CueO homologs) do not appear to be robustly needed, while histidine and purine biosynthesis pathways are crucial to cope with copper. We show that *hisA*, *hisB*, *hisC*, and *hisD* mutants are auxotrophic for histidine and sensitive to copper. This suggests that the reported attenuation of *his* mutants in macrophages could be based on auxotrophy and/or copper sensitivity. Therefore, we generated suppressor strains with a restored resistance to copper for *hisC*, but still auxotrophs for histidine. Our data suggest that this suppression is due to the overproduction of a homolog of OppA, a periplasmic oligopeptide-binding protein. Analysis of these suppressors shows that the absence of histidine biosynthesis capacity, and not copper sensitivity, is required for optimal growth of *B. abortus* in macrophages.

IMPORTANCE Investigating conditions in which intracellular bacteria grow inside host cells is challenging and often involves the characterization of attenuated bacterial mutants obtained by screening. But a single mutant can display two different phenotypes related to intracellular conditions. It was the case for histidine auxotrophs of *Brucella abortus*, an important zoonotic pathogen. These histidine auxotrophs are attenuated in a macrophage cell line, and they are also sensitive to copper stress. Using a suppressor strain still auxotroph for histidine but with an improved resistance to copper, we show that histidine auxotrophy, and not sensitivity to copper excess, is the main cause of attenuation in the conditions tested here.

KEYWORDS intracellular pathogen, Tn-seq, copper, histidine, *Brucella*

Brucella spp. are gram-negative bacteria that belong to the Alphaproteobacteria order, which is part of the alpha-proteobacteria class (1, 2). Bacteria from the genus *Brucella* are intracellular, facultatively extracellular pathogens responsible for brucellosis (3). This worldwide zoonosis disease affects a wide range of hosts, including domestic animals and humans, illustrating the complexity of *Brucella* spp. (4). The tropism of the bacteria for the reproductive systems correlates with abortion and sterility as main symptoms in animals, while humans present undulant fever symptoms (3, 5, 6). *Brucella* employs sophisticated strategies to enter, survive, and proliferate within host cells—encompassing both professional phagocytes and non-phagocytic cells—thereby effectively ensuring its pathogenicity (3, 6). Once within the cell, *Brucella* is found enclosed in a membrane-bound compartment, the *Brucella*-containing vacuole (BCV), which traffics along the endocytic pathway and undergoes an acidification of the compartment, becoming the endocytic BCV (eBCV) (7, 8). The vacuole acidification is crucial to produce the *Brucella* VirB Type IV secretion system (7, 9), which secretes

Editor Patricia A. Champion, University of Notre Dame, Notre Dame, Indiana, USA

Address correspondence to Xavier De Bolle, xavier.debolle@unamur.be.

The authors declare no conflict of interest.

See the funding table on p. 17.

Received 29 October 2025

Accepted 9 December 2025

Published 4 February 2026

Copyright © 2026 Focant et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

effector proteins inside the cell (10, 11). The establishment of the replicative niche (rBCV) inside the endoplasmic reticulum (ER) is dependent on the VirB function (7, 8). As a last step, the rBCVs are converted into autophagic BCVs (aBCVs) by getting associated to atypical autophagic membranes (12). Inside the aBCVs, *Brucella* can egress from the cell, being therefore able to reinfect the surrounding cells (12). During its trafficking, *Brucella* probably encounters many stresses, most of which are unknown or indirectly deduced from attenuated mutants.

In the arms race of host-pathogen co-evolution, host cells use several strategies, one of which is the sequestration of key metal ions for the pathogenic bacteria (13). Iron deprivation by host cells is well established. Most iron ions are predominantly sequestered, for example, bound by transferrin and hemoglobin, rendering them unavailable to bacteria (14, 15). Neutrophils recruited to the infection site release calprotectin, which sequesters manganese and zinc (16, 17). Manganese and iron can also be depleted from the phagosomes through the natural resistance-associated macrophage protein 1 (NRAMP1) in macrophages and neutrophils (13, 18). Despite the well-established role of metal starvation as a defense mechanism against pathogens, it is becoming increasingly evident that metal intoxication also occurs during infection to fight invading pathogens (16, 19). Host cells exploit copper toxicity to poison intrusive pathogens (20). In response to interferon- γ (IFN- γ) stimulation, activated macrophages accumulate copper within phagolysosomes (21). IFN- γ induces the expression of the copper transporter CTR1, allowing copper import into the cytosol (21, 22), where it is delivered by the ATOX1 chaperone to the ATP7A transporter associated with the phagolysosomal membrane (21, 23–25) and able to pump copper into the lumen of the phagosome. ATP7A expression is also upregulated by IFN- γ (24). Copper resistance has been linked to the virulence of intravacuolar pathogens, such as *Mycobacterium tuberculosis* (25, 26) and *Salmonella enterica* (27).

Several defensive mechanisms are used by bacteria to combat toxic concentrations of metals. A simple mechanism of averting metal toxicity is to sequester metal ions within the periplasm or the cytoplasm, resulting in fewer than one copper atom being available per cell (15, 28, 29). When metal ions accumulate to toxic levels, bacteria induce the expression of specific adapted genes. This defense response includes the induction of efflux systems as well as proteins involved in metal sequestration and storage (16). The bacterial response to copper could involve a core *cue* regulon, which is relatively well conserved among proteobacteria (28). The *cue* regulon includes CueR, a copper-responsive metalloregulatory protein that upregulates *cueO* and *copA* expression upon increased intracellular copper concentration (30). CueO, the periplasmic multicopper oxidase, oxidizes cuprous ion to cupric ion, which is less harmful (31). CopA is a copper efflux P-type ATPase (32). A cuprochaperone, CopZ, is also part of the Cue efflux system (33). Most *S. enterica* serotypes possess a duplication of the *cue* regulon, known as the *gol* regulon (34, 35). The expanded anti-copper arsenal of *S. enterica* is presumably an adaptation for survival within macrophages (28, 34). A second copper efflux system, the Cus system, is also present in *Escherichia coli* and *S. enterica*. This second regulon is controlled by the two-component system CusRS (28), which activates the expression of the *cusCFBA* operon in response to elevated copper levels (31). The *cusCBA* genes encode the CusCBA complex, a resistance, nodulation, and division (RND) proton-cation antiporter (28, 31), while *cusF* encodes a periplasmic cuprochaperone (28).

The specific environmental conditions encountered by intracellular bacteria remain poorly characterized. These conditions may involve limited access to nutrients, such as amino acids, and exposure to compounds like copper. To explore how *B. abortus* responds to copper stress, we conducted a transposon sequencing (Tn-seq) analysis to identify mutants with increased copper sensitivity. Although the classical CueO and CopA homologs do not appear to be required for *B. abortus* resistance to copper, the histidine biosynthesis pathway was found to be necessary for growth in the presence of copper. Since it is known (and confirmed here) that *his* mutants are attenuated in macrophages, this phenotype could be due to auxotrophy for histidine and/or to

sensitivity to copper. By generating suppressors with a restored copper resistance, we show that the attenuation of the *his* mutants in macrophages is probably not related to copper sensitivity.

RESULTS

Identification of genes involved in copper resistance in *B. abortus*

The mechanism by which *Brucella* copes with copper toxicity remains unknown. To identify genes required to face copper stress, a Tn-seq was performed on *B. abortus* 544 using a mini-Tn5 (36). To select the appropriate copper concentration for the Tn-seq, *B. abortus* and *E. coli* mini-Tn5 carrier strains were first mated. After incubation and optical density (OD) normalization, serial 10-fold dilutions were spotted on TSB agar plates supplemented with an increasing CuSO₄ concentration, and CFUs were then counted to identify the optimal copper concentration. A final concentration range of 1.6–2 mM CuSO₄ was chosen for the selection corresponding to the concentration where wild type (WT) was not impacted. Once the copper concentration to use for the Tn-seq was determined, the mating was repeated, and the *Brucella* library was grown on rich medium for the control condition and 2 mM CuSO₄-supplemented media for the test condition. From the library, 4.8×10^6 random mutants were recovered and sequenced using a deep sequencing method. We identified 1,684,839 and 1,743,143 unique insertion sites for control and copper conditions, respectively, illustrating genome saturation with a unique insertion every 1.95 bp for control and every 1.89 bp for the stress condition, on average. The raw data were analyzed by an automatic process called TnBox, following a previously described method (37). The transposon insertion frequency (TnIF) for each gene was computed according to the following definition: TnIF is defined as the per-gene average of $\log_{10}(r + 1)/l$, where r is the number of miniTn5 insertions at a given nucleotide and l is the coding sequence length. The calculation was performed considering only the central 80% of the coding sequence (37). A TnIF value below the genome-wide average indicates that disruption of the gene decreases bacterial fitness. Then, the Δ TnIF was determined as the difference between the TnIF under copper stress conditions and the TnIF under control conditions (Δ TnIF = TnIF_{copper} – TnIF_{control}). A gene will be annotated as required to grow in the presence of copper if the Δ TnIF is lower than two times the standard deviation of all Δ TnIF values (Δ TnIF > –0.357). Through the Tn-seq analysis, 38 genes were identified as essential for growth under copper-supplemented conditions (Table 1).

The genes coding for the Cue system, *copA*, *cueO*, and the regulator *cueR*, involved in copper resistance, appeared to be essential in the Tn-seq copper condition (Table 1), as expected compared to the data available from the work in *E. coli* (31). CopA is an efflux P-type ATPase of the *cue* regulon, while CueO is a periplasmic multi-copper oxidase (32). The expression of both *copA* and *cueO* is regulated by the copper-responsive transcriptional regulator CueR (30). The *cue* regulon also includes *copZ* (BAB1_0960 in *B. abortus* 2308 and BAB_v1_a0976 in *B. abortus* 544 [33]), which is not scored as required in the Tn-seq copper condition.

Genes coding for the whole histidine biosynthesis pathway (*hisA*, *hisB*, *hisC*, *hisD*, *hisE*, *hisF*, *hisG*, *hisH*, and *hisI*) are highlighted in our Tn-seq to be required to cope with copper stress. Each step of the pathway is catalyzed by a specific enzyme, where the five first steps lead to the production of imidazole glycerol phosphate (IGP) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate in purine biosynthesis (see Fig. S1). Histidine is produced from IGP after four other enzymatic reactions (see Fig. S1). While in *E. coli* and *S. enterica* Typhimurium, the *his* pathway is genetically organized in a single operon, it is distributed across both chromosomes in *Brucella* spp. (38).

Linked to the histidine biosynthesis pathway, the purine *de novo* synthesis pathway appeared to be also crucial to face copper stress. Indeed, most of the genes—*purH* (part I and II), *purN*, *purK*, *purD*, *purE*, *purF*, *purC*, *purQ*, and *purM*—required for inosine monophosphate (IMP) production were found to be required in the presence of copper.

TABLE 1 Attenuated *B. abortus* mutants on copper 2 mM supplemented TSB plates

Name ^a	Locus tag ^b		Product ^c	TnIF (CT) ^d	TnIF (Cu) ^d	ΔTnIF ^e
	544	2308				
Cue system						
<i>cueR</i>	BAB_v1_a0229	BAB1_0222	DNA-binding transcriptional dual regulator CueR	2.61	0.95	−1.66
<i>copA</i>	BAB_v1_a0228	BAB1_0221	Cu(+) exporting P-type ATPase	2.76	0.55	−2.22
<i>cueO</i>	BAB_v1_b0532	BAB2_0534	Blue copper oxidase CueO	2.75	0.39	−2.36
Histidine biosynthesis						
<i>hisD</i>	BAB_v1_a0293	BAB1_0285	Histidinol dehydrogenase	3.04	0.55	−2.49
<i>hisZ</i>	BAB_v1_b0179	BAB2_0182	ATP phosphoribosyltransferase regulatory subunit	2.57	0.41	−2.16
<i>hisG</i>	BAB_v1_b0180	BAB2_0183	ATP phosphoribosyltransferase	2.63	0.61	−2.02
<i>hisF</i>	BAB_v1_a2099	BAB1_2086	Imidazole glycerol phosphate synthase subunit HisF	2.31	0.58	−1.73
<i>hisI</i>	BAB_v1_a1109	BAB1_1098	Phosphoribosyl-AMP cyclohydrolase	2.92	1.22	−1.70
<i>hisH</i>	BAB_v1_a2097	BAB1_2084	Imidazole glycerol phosphate synthase subunit HisH	2.62	0.98	−1.65
<i>hisB</i>	BAB_v1_a2095	BAB1_2082	Imidazoleglycerol-phosphate dehydratase	2.45	0.87	−1.59
<i>hisE</i>	BAB_v1_a2100	BAB1_2087	Phosphoribosyl-ATP pyrophosphatase	3.10	1.53	−1.57
<i>hisC</i>	BAB_v1_a2005	BAB1_1988	Histidinol-phosphate aminotransferase	1.71	0.67	−1.04
<i>hisA</i>	BAB_v1_a2098	BAB1_2085	1-(5-phosphoribosyl)–5-[(5-phosphoribosylamino) methylideneamino] imidazole-4-carboxamide isomerase	2.49	0.38	−2.11
Purine biosynthesis						
<i>purH</i>	BAB_v1_a1839	BAB1_1824	Fragment of bifunctional AICAR transformylase/IMP cyclohydrolase (part 2)	1.74	0.73	−1.01
<i>purH</i>	BAB_v1_a1838	BAB1_1824	Fragment of bifunctional AICAR transformylase/IMP cyclohydrolase (part 1)	1.58	0.70	−0.87
<i>purN</i>	BAB_v1_a0751	BAB1_0730	Phosphoribosylglycinamide formyltransferase 1	1.93	1.32	−0.60
<i>purK</i>	BAB_v1_a1774	BAB1_1758	N5-carboxyaminoimidazole ribonucleotide synthase	1.91	1.32	−0.59
<i>purD</i>	BAB_v1_a0449	BAB1_0442	Phosphoribosylamine--glycine ligase	2.04	1.49	−0.55
<i>purE</i>	BAB_v1_a1773	BAB1_1757	N (5)-carboxyaminoimidazole ribonucleotide mutase	2.14	1.59	−0.54
<i>purF</i>	BAB_v1_a0480	BAB1_0472	Amidophosphoribosyltransferase	1.91	1.45	−0.46
<i>purC</i>	BAB_v1_a0881	BAB1_0862	Phosphoribosylaminoimidazole-succinocarboxamide synthase	1.74	1.29	−0.46
<i>purQ</i>	BAB_v1_a0879	BAB1_0860	Phosphoribosylformylglycinamidine synthase subunit PurQ	2.40	1.95	−0.45
<i>purM</i>	BAB_v1_a0752	BAB1_0731	Phosphoribosylformylglycinamide cyclo-ligase	2.00	1.62	−0.39
Pyridoxin biosynthesis						
<i>pdxJ</i>	BAB_v1_a1420	BAB1_1404	Pyridoxine 5′-phosphate synthase	2.54	0.80	−1.74
<i>pdxA</i>	BAB_v1_a0724	BAB1_0705	4-hydroxythreonine-4-phosphate dehydrogenase	2.26	0.63	−1.63
<i>pdxH</i>	BAB_v1_a0451	BAB1_0444	Pyridoxine/pyridoxamine 5′-phosphate oxidase	1.95	0.89	−1.06
Others						
<i>glcD</i>	BAB_v1_a0442	BAB1_0434	D-2-hydroxyglutarate dehydrogenase	2.57	0.85	−1.72
<i>gph</i>	BAB_v1_a0329	BAB1_0318	Phosphoglycolate phosphatase	2.47	1.07	−1.40
<i>metH</i>	BAB_v1_a0196	BAB1_0188	Cobalamin-dependent methionine synthase	2.35	1.57	−0.77
<i>tatC</i>	BAB_v1_a0920	BAB1_0903	Sec-independent protein translocase protein TatC	1.36	0.76	−0.61
<i>tatA</i>	BAB_v1_a0918	BAB1_0901	Sec-independent protein translocase protein TatA	1.65	1.21	−0.44
<i>wadA</i>	BAB_v1_a0661	BAB1_0639	LPSA protein	1.83	0.60	−1.23
<i>pgm</i>	BAB_v1_a0061	BAB1_0055	Phosphoglucomutase	1.31	0.77	−0.54
<i>fzIA</i>	BAB_v1_b0989	BAB2_1005	FtsZ-localized protein A	1.70	1.17	−0.53
<i>lysA</i>	BAB_v1_a2001	BAB1_1984	Diaminopimelate decarboxylase	2.04	1.60	−0.44
<i>xerC</i>	BAB_v1_a1939	BAB1_1916	Tyrosine recombinase XerC	2.02	1.58	−0.44
<i>ahcY</i>	BAB_v1_a2110	BAB1_2099	Adenosylhomocysteinase	1.85	1.42	−0.43

(Continued on next page)

TABLE 1 Attenuated *B. abortus* mutants on copper 2 mM supplemented TSB plates (Continued)

Name ^a	Locus tag ^b		Product ^c	TnIF (CT) ^d	TnIF (Cu) ^d	Δ TnIF ^e
	544	2308				
<i>bipA</i>	BAB_v1_b0358	BAB2_0361	Ribosome-dependent GTPase%2C ribosome assembly factor	1.95	1.58	−0.37

^aGene name. In bold, genes touched by a previous screening of mutant libraries (36).
^bThe coding sequences (ORFs) in *B. abortus* 544 and their correspondence in *B. abortus* 2308.
^cPredicted function for each gene.
^dThe TnIF for each gene was calculated, where TnIF is defined as the average per gene $\log_{10}(r + 1)/l$, where r is the read count at a given nucleotide and l is the gene length, considering only the central 80% of the coding sequence. CT; Control, Cu: copper.
^eThe Δ TnIF was determined as the difference between the TnIF under Cu stress conditions and the TnIF under control conditions (Δ TnIF = TnIFcopper – TnIFcontrol).

Next, three genes from the vitamin B6 biosynthesis (*pdxJ*, *pdxA*, and *pdxH*) also emerged from our analysis as being required. These genes code for enzymes allowing the biosynthesis of vitamin B6, in its active form, pyridoxal 5'-phosphate (39). Vitamin B6 is utilized as a cofactor by the HisC enzyme (40), which may explain why it is required to face copper stress.

Finally, there were 12 other genes that also presented a Δ TnIF lower than two times the standard deviation. Among them, *glcD*, *tatC*, *pgm*, and *fzIA* were already essential in the control condition, while *metH*, *tatA*, *wadA*, *lysA*, *xerC*, *ahcY*, and *bipA* had low fitness in the control condition. Only *gph* did not present any impact on growth in the absence of copper.

CopA and CueO are not required for copper resistance in *Brucella abortus*

To date, the mechanisms underlying copper resistance in *B. abortus* remain largely uncharacterized. Only a few homologs of genes involved in copper homeostasis are predicted from the *Brucella* genomes, including *copA*, *cueO*, and *cueR* (41). These three genes were identified as required under copper stress conditions in our Tn-seq screen (Table 1). Given their predicted function in copper transport and detoxification, we first focused on testing the role of *copA* and *cueO* in *B. abortus* copper resistance by constructing markerless single-gene deletion mutants called Δ *copA* and Δ *cueO*. The growth of Δ *copA* and Δ *cueO* was monitored during 48 h in liquid culture in rich medium in the presence of 2 mM CuSO₄ (Fig. 1B). Unexpectedly, both mutants did not present any sensitivity to copper stress, displaying growth curves similar to the WT strain. Δ *copA* presented a slight growth defect in the control condition (Fig. 1A) compared to the WT, which was also found in the copper condition (Fig. 1B). The minor effect of copper on Δ *cueO* was not confirmed when the copper concentration was increased.

We also conducted a soft agar well diffusion assay where the well was filled with 100 μ L of 200 mM CuSO₄. The inhibition zone was measured around the well and reported in millimeters, with the results presented as a bar graph. As with the growth curves, Δ *copA* and Δ *cueO* displayed the same inhibition zone as the WT strain around the copper source, confirming the absence of sensitivity of the two mutants in these conditions (Fig. 1C).

Taken together, these results demonstrate that the deletion of *copA* or *cueO* does not impact copper resistance in *B. abortus*, contrary to what has been shown in *E. coli*, and also in sharp contrast to our Tn-seq data (Table 1).

The *his* mutants are auxotrophic for histidine and sensitive to copper

In addition to our Tn-seq results, histidine biosynthesis genes have already been unveiled as required in *B. abortus* during RAW 264.7 macrophages infection (36), as well as previous studies with *B. suis* (42, 43) and Tn-seq with *B. melitensis* in mice (37). A series of deletion mutants in the histidine biosynthesis pathway has been constructed: *hisA*, *hisB* (previously reported [44]), *hisC*, and *hisD*. The auxotroph phenotypes of these four mutants have been confirmed by their inability to proliferate in minimal medium in the absence of amino acids. Their growth was rescued either when the gene was introduced in *trans* on a single-copy plasmid (Fig. 2) or if histidine was provided in the culture medium.

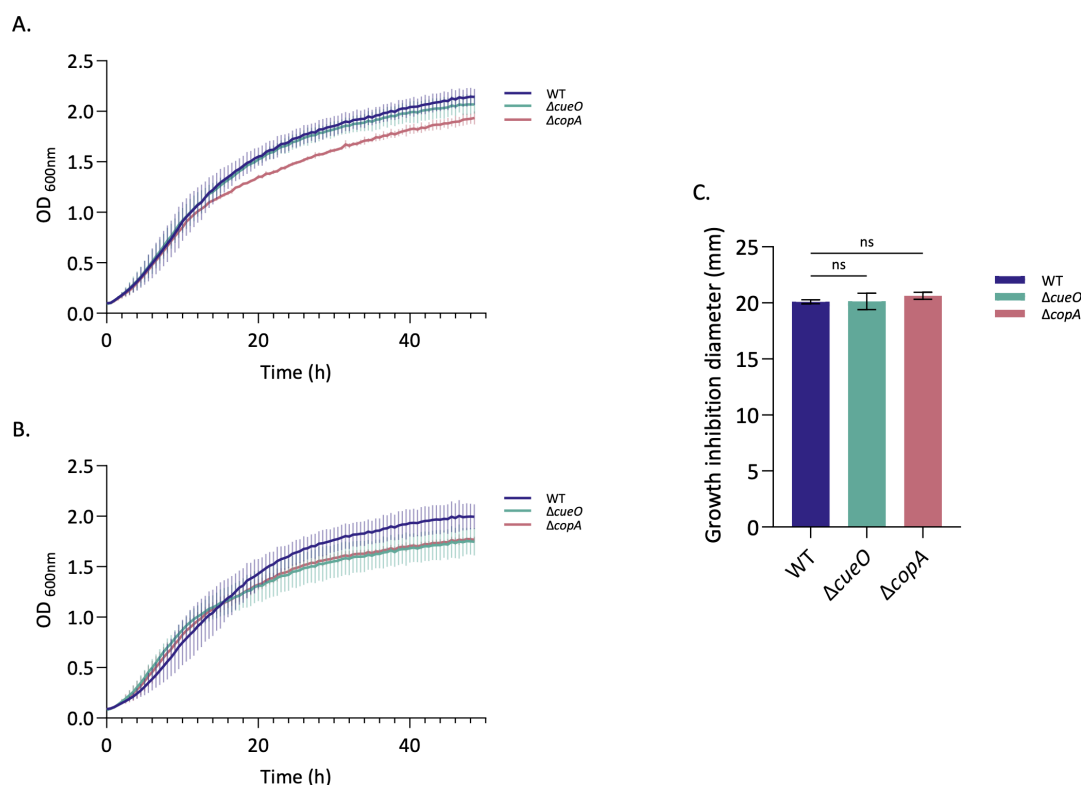


FIG 1 The *copA* and *cueO* genes are not required for copper resistance. WT, $\Delta copA$, and $\Delta cueO$ strains were tested for their sensitivity to copper toxicity for growth. (A) WT, $\Delta copA$, and $\Delta cueO$ strains were grown in liquid TSB rich medium. (B) WT, $\Delta copA$, and $\Delta cueO$ strains were grown in TSB rich medium containing 2 mM CuSO₄. (A and B) The OD at 600 nm was measured every 30 min for 48 h. The data represent three independent replicates. (C) Five hundred microliters of an overnight culture of each *B. abortus* strain, normalized to an OD of 0.1, was added to 4.5 mL of TSB soft agar (0.7% agar) and then plated on a TSB plate. In the middle, a well was dug and filled with 100 μ L of 200 mM CuSO₄. Inhibition zones around the agar well were measured after 3 days of incubation at 37°C, and the average diameter ($\pm$ standard deviation, SD) was reported in a histogram. The data represent the mean $\pm$ SD and were compiled from three independent replicates. Statistical analysis was carried out by a one-way ANOVA followed by Dunnett's multiple comparisons test (n.s., non-significant, $P > 0.05$).

Since the classical copper homeostasis actors (CopA, CueO) were not required for the growth of *B. abortus* in the presence of a copper stress, we decided to investigate the histidine biosynthesis pathway in the context of copper stress. The growth of the *his* mutants in the presence of copper was studied through diverse approaches. First, all strains were cultivated in liquid rich medium with a high copper concentration (1.6 mM), and the OD was measured over a period of 48 h (Fig. 3A), as done with $\Delta copA$ and $\Delta cueO$ strains. As suggested by the Tn-seq results, all auxotrophic mutants struggled to grow in the presence of the metal compared to the WT strain, which did not demonstrate a growth defect. The *hisA* mutant was particularly affected. However, it already struggled in the control condition compared to the other strains (see Fig. S2). The same sensitivity phenotype for all the *his* mutants was observed with 2 mM CuCl₂, indicating that the growth defect was due to copper rather than sulfate. The copper sensitivity of the *his* auxotroph mutants was rescued upon genetic complementation (Fig. 3B). Similar observations were made with the same strains cultivated with a concentration of 2 mM of CuSO₄, but with an incomplete complementation (see Fig. S3). These preliminary results suggested that impairment of the histidine biosynthesis pathway leads to copper sensitivity. The sensitivity of *his* mutants to copper was confirmed by a soft agar well diffusion assay in which the inhibition zone around the copper-containing well was measured. As with growth curves, the copper sensitivity was rescued when the strains were complemented with the missing genes (Fig. 3C). The growth of the four *his* auxotroph mutants was tested on solid TSB medium supplemented with 1.6 or 2 mM CuSO₄. Results showed a growth defect for each *his* mutant, in contrast to the WT strain,

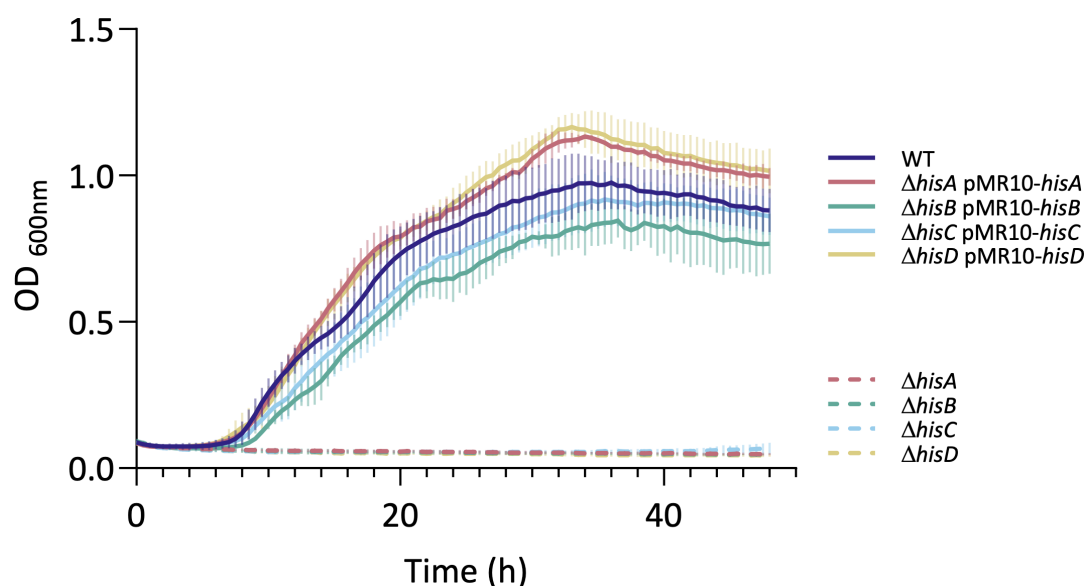


FIG 2 *B. abortus* $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, and $\Delta hisD$ are auxotrophic for histidine. WT and *his* mutant strains were tested for their histidine auxotrophy. Strains were grown in liquid PE minimal medium. OD_{600 nm} was measured every 30 min for 48 h. The pMR10 is a low-copy plasmid. The data represent three independent replicates.

which grew normally (Fig. 4). On these plates, a few colonies among the mutants were observed. The presence of mutations in these colonies could potentially enable them to withstand the presence of copper. In this case, these colonies could be suppressor clones, that is, clones in which a second mutation cancels out the phenotypic effect of the first mutation ($\Delta hisA$, $\Delta hisB$, $\Delta hisC$, or $\Delta hisD$). Identifying these secondary mutations could therefore provide valuable insight to understand how these strains can cope with copper.

Most suppressor mutations affect a pair of periplasmic proteins

To verify the suppressor phenotype of the colonies isolated from the copper-supplemented TSB plates, it was necessary to streak each of them onto a new plate with and without copper. Of the 29 potential suppressors isolated, all mutant backgrounds combined, eight were unable to retain the ability to grow on copper-supplemented media. The remaining potential suppressors were able to grow at 2 mM CuSO₄. The genomic DNA of the remaining 21 copper-resistant clones was extracted and analyzed by whole-genome sequencing to identify the mutations that could confer copper resistance in these suppressors. As a control, the genomes of the WT and the parental $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, and $\Delta hisD$ strains were also sequenced. To investigate mutations that were strictly present in the potential suppressor, only the mutations present in the suppressor and not in the parental strain were retained. Strikingly, of the 22 unique mutations identified, 15 appeared within the same operon, the *opp* operon (Table 2). Opp is homologous to an oligopeptide transport system, and it is encoded by five genes. The first two genes, *oppA1* and *oppA2*, encode periplasmic proteins. The next two genes, *oppB* and *oppC*, encode permeases, while the final gene, *oppD/F*, encodes an ATP-binding protein. Interestingly, only the first two genes were touched by a mutation in the suppressors (Table 2).

The suppressive mutation helps to face copper stress *in vitro*

In this study, we opted to exclude suppressors derived from the *hisB* mutant. Indeed, *hisB* is located downstream of the intersection between the histidine synthesis pathway and purine metabolism, via the production of AICAR (Fig. 1). Moreover, in the laboratory,

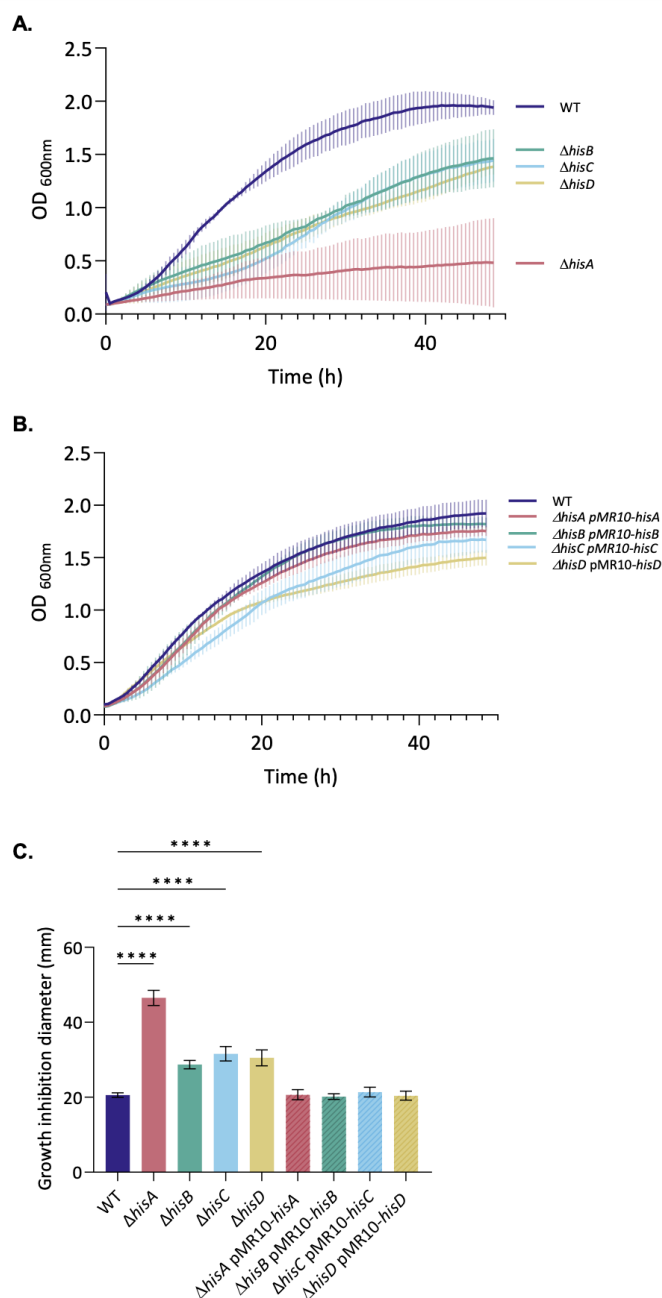


FIG 3 Histidine auxotrophic mutants are copper sensitive. WT, $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, $\Delta hisD$, $\Delta hisA$ pMR10-*hisA*, $\Delta hisB$ pMR10-*hisB*, $\Delta hisC$ pMR10-*hisC*, and $\Delta hisD$ pMR10-*hisD* strains were tested for their sensitivity to copper toxicity for growth. (A) WT, Δhis , and (B) complemented strains were grown in liquid TSB rich medium containing 1.6 mM of CuSO_4 . OD_{600 nm} was measured every 30 min for 48 h. The data represent three independent replicates. (C) Experiment performed as in Fig. 1C. Data represent the mean $\pm$ SD and were compiled from three independent replicates. Statistical analysis was carried out by a one-way ANOVA followed by Dunnett's multiple comparisons test (**** $P < 0.0001$).

hisB mutant had been studied and demonstrated to exhibit a morphological abnormality by the formation of chains, which is associated with a deficiency in cell division (44). It is also noteworthy that suppressors exhibiting a unique mutation within *hisA* or *hisD* background did not affect the *opp* operon (see Table S1). Out of all mutations in the *hisC* mutant background, one stood out as particularly interesting. It is located in the

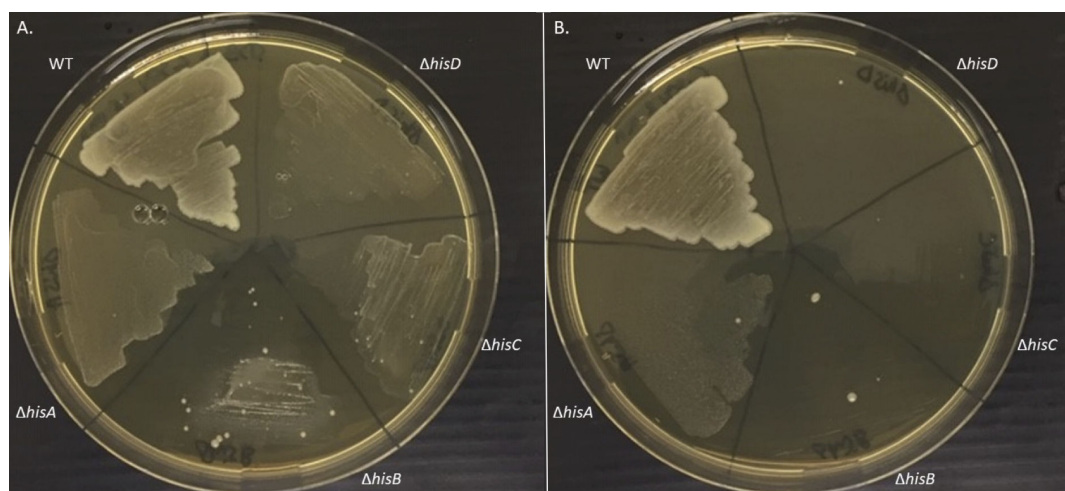


FIG 4 *B. abortus* WT and mutant strains are copper sensitive on plates. WT and $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, and $\Delta hisD$ mutant strains (in this counterclockwise order) were tested for their copper sensitivity on TSB medium containing 1.6 mM (A) or 2 mM (B) of $CuSO_4$. Both plates were divided into five parts where 20 μL of each culture was deposited and spread after their $OD_{600\text{ nm}}$ was normalized to 0.1. Pictures were taken after a 4-day incubation at 37°C. The data represent three independent replicates. It is interesting to note the presence of individual colonies for $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, and $\Delta hisD$, suggesting that suppressor mutants could be obtained for this phenotype.

suppressor named C6 (the sixth suppressor isolated within the *hisC* mutant), 70 pb upstream of the *oppA1* ATG, in a potential distal promoter region (Table 2). The mutation was investigated as it could influence the expression of the first two coding sequences of the operon.

For the purpose of confirming that the suppressor phenotype is attributable to the identified mutation, it was introduced back into the *hisC* mutant. The potential distal promoter region where the SNP occurred was amplified from C6 genomic DNA and subsequently inserted into $\Delta hisC$ strain by allelic exchange. The new strain, including the C6 mutation, was named $\Delta hisC$ SupPA1 (standing for Suppressor Promoter OppA1). To investigate the hypothesis that the suppressive mutation in the potential promoter enhances the abundance of OppA1 and OppA2 substrate proteins, an overexpression vector for each of the genes was constructed in the pBBR-MCS2 medium-copy plasmid and introduced into the $\Delta hisC$ mutant, generating $\Delta hisC$ pBBR-*oppA1* and $\Delta hisC$ pBBR-*oppA2* strains, respectively. The histidine auxotroph phenotype was first evaluated by measuring the OD in a minimal medium, as previously performed with the *his* mutants. All strains retained the auxotrophic phenotype of the mutant parental strain. Indeed, the growth was rescued when the minimal medium was supplemented with 1 mM of histidine (See Fig. S4).

The suppression phenotype was then studied to confirm its role in the rescue process in the presence of copper stress. For this purpose, the same experiments as for histidine auxotrophic mutants were carried out. The growth of the strains WT, $\Delta hisC$, C6, $\Delta hisC$ SupPA1, $\Delta hisC$ pBBR-*oppA1*, and $\Delta hisC$ pBBR-*oppA2* was observed in the presence of 2 mM of copper by measuring the OD during 48 h (Fig. 5A). Confirming the previous results, $\Delta hisC$ had a growth defect. The impaired growth due to copper sensitivity was rescued in C6, $\Delta hisC$ SupPA1, and $\Delta hisC$ pBBR-*oppA2* strains. These results confirmed the suppressor phenotype in C6 and the role of the mutation identified in this strain to cope with copper stress. Indeed, when reinserted in the $\Delta hisC$ parent strain, $\Delta hisC$ SupPA1, the mutation resolved the growth defect observed due to copper sensitivity. Having *oppA2* on a medium-copy vector allowed bacteria to grow as the WT strain in the presence of copper (Fig. 5A).

The soft agar assay was repeated with these strains, and the inhibition zone around the copper well was measured (Fig. 5B). As was evidenced in the case of the liquid growth culture, the C6, $\Delta hisC$ SupPA1, and $\Delta hisC$ pBBR-*oppA2* strains exhibited an

TABLE 2 Suppressor mutations

Mutant ^a	Suppressor ^b	Position ^c	Type ^d	Ref. ^e	Alt. ^g	Locus_tag ^h		Name ⁱ
<i>ΔhisB</i>	B1	chr2. 695058	Del	* ^f	C	BAB_v1_b0690	BAB2_0699	<i>oppA1</i>
	B9	chr2. 695451	SNP	G	C	BAB_v1_b0691	BAB2_0700	<i>oppA2</i>
		chr2. 695457	Complex	TCG	ACC			
		chr2. 695468	Complex	CATC	TCTG			
		chr2. 695483	SNP	G	A			
		chr2. 695489	SNP	A	T			
	B10	chr2. 694185	SNP	C	T	BAB_v1_b0690	BAB2_0699	<i>oppA1</i>
		chr2. 694191	Complex	GAAA	AAAG			
		chr2. 694200	Complex	ACAG	GGAT			
		chr2. 694219	Complex	CCG	TCT			
<i>ΔhisC</i>	C6	chr2. 693379	SNP	G	T	Potential promoter region operon <i>opp</i>		
	C8	chr2. 693942	SNP	C	T	BAB_v1_b0690	BAB2_0699	<i>oppA1</i>
		chr2. 693957	SNP	C	G			
		chr2. 693966	SNP	A	C			
		chr2. 695612	Complex	AAG	CTC	BAB_v1_b0691	BAB2_0700	<i>oppA2</i>

^aParental mutant strain.^bSuppressor number.^cPosition of mutation on chromosome (chr) 1 or 2.^dType of mutation: Del = deletion, SNP = single-nucleotide polymorphism and Complex = multiple nucleotide modification.^eReference genome nucleotide.^f*=CGCGGCCCTTGTCTTCGGGGGCCGAAGGGCCGCTTTTGACAAGCATTTCCAAGA.^gSuppressor alternative nucleotide.^hThe coding sequences (ORFs) in *B. abortus* 544 (left) and their correspondence in *B. abortus* 2308 (right).ⁱGene name.

inhibition zone, akin to that observed in the WT. In contrast, *ΔhisC* and *ΔhisC* pBBR-*oppA1* both displayed a copper sensitivity.

As an intracellular pathogen, *B. abortus* is subject to various stresses within the cell, including possibly copper intoxication induced by the host cell. We examined the ability of the *ΔhisC* strain and its different derivatives to replicate intracellularly. This was assessed by counting the number of CFUs after J774A.1 macrophage infection. The intracellular trafficking of the different strains was monitored at two early time points (2 and 5 h PI) and two late time points (24 and 48 h PI) following replication (Fig. 5C). The WT strain replicated after 24 h PI (positive control). In contrast, the mutant strain deleted for the entire *virB* operon was used as a negative control, since it is known to remain blocked in eBCVs (7, 8) and therefore was unable to replicate in macrophages. At 24 h PI, the *hisC* mutant appeared to have a weaker replication capacity than the WT. However, by 48 h PI, the CFUs increased and approached the WT level. The C6 suppressor had a replication profile that was slightly higher than that of the *hisC* mutant. Meanwhile, *ΔhisC* pBBR-*oppA1* and *ΔhisC* pBBR-*oppA2* replicated similarly to *ΔhisC*. The strain in which the suppressor mutation was reintroduced, *ΔhisCSupPA1*, appeared to be more affected than the *hisC* mutant itself, particularly at 48 h. Compared to the *virB* mutant, the *hisC* mutant and its derivatives could replicate within the cell but to a lesser extent than the WT (Fig. 5C).

DISCUSSION

Copper is an essential transition metal for microorganisms, yet it becomes rapidly toxic at slightly elevated concentrations (45, 46). In *E. coli*, the total intracellular copper pool (free, labile, and sequestered) is around 10⁴ atoms (~10 μM), where the free cuprous ion amount is at zeptomolar level, thus less than one copper atom per cell (15, 28). This implies strong buffering systems, likely mediated by cysteine-rich proteins (15, 20, 45). Metal ions are critical in numerous mechanisms, and their controlled availability is therefore important in the host-pathogen interplay as well. During intracellular pathogenic infection, host cells both deprive pathogens of essential metals such as iron, zinc, or manganese and also exploit copper toxicity as antimicrobial defense, a strategy

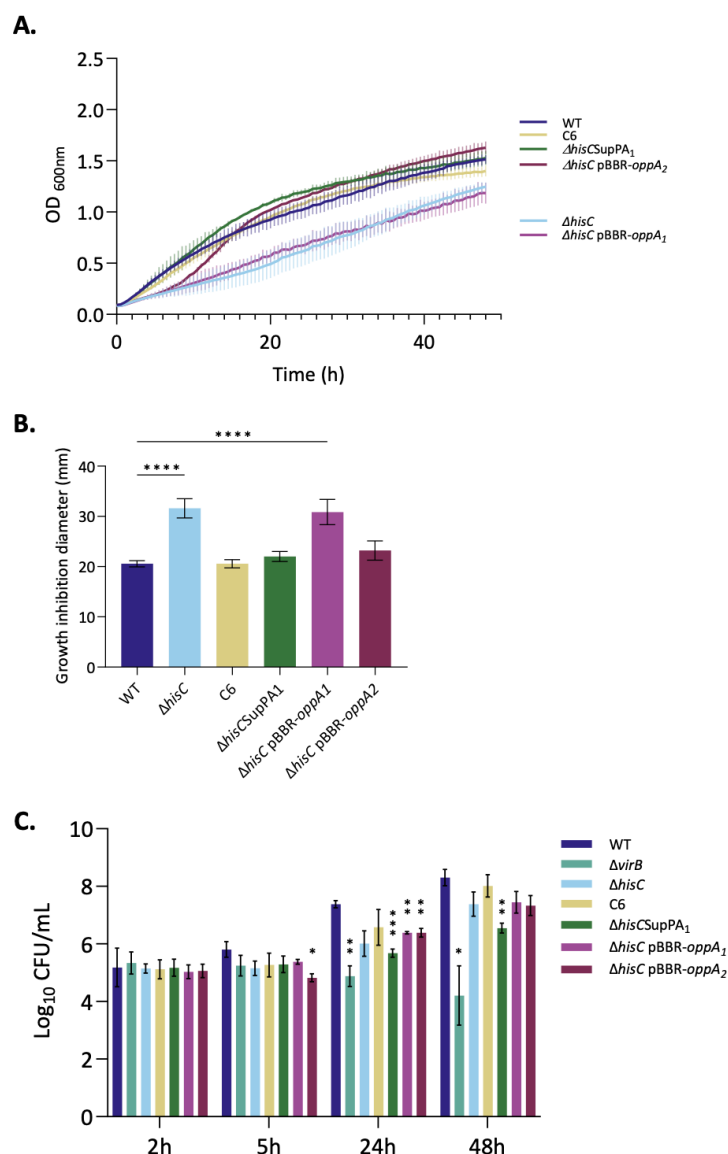


FIG 5 Suppressive mutation saves the sensitivity phenotype to copper stress. WT, $\Delta hisC$, $\Delta hisC$, C6, $\Delta hisC SupPA_1$, pBBR-*oppA1*, and $\Delta hisC$ pBBR-*oppA2* strains were tested for their sensitivity to copper toxicity for growth and their intracellular replication capacity. (A) WT, $\Delta hisC$ strains, and derivatives were grown in liquid TSB rich medium containing 2 mM of CuSO₄. OD_{600 nm} was measured every 30 min for 48 h. Data represent three independent replicates. (B) Experiment performed as in Fig. 1C. The data represent the mean $\pm$ SD and were compiled from three independent replicates. Statistical analysis was carried out by a one-way ANOVA followed by Dunnett's multiple comparisons test (*****P* < 0.0001). (C) Intracellular replication of WT, $\Delta hisC$ mutant, and derivatives was assessed by CFU at 2, 5, 24, and 48 h post-infection (PI) of J774.A1 macrophages. Data represent the mean $\pm$ SD and were compiled from three independent replicates. Statistical analysis was carried out by a two-way ANOVA followed by Dunnett's multiple comparisons test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

known as nutritional immunity (13, 14, 16, 17). Notably, IFN- γ -activated macrophages upregulate the copper transporter ATP7A and relocalize it into the phagolysosome membrane to poison invading microbes by pumping copper into the pathogen-containing vacuole (13, 24). Resistance to copper stress is a determinant for virulence in intravacuolar bacteria such as *M. tuberculosis* (25, 26) and *S. enterica* (27). Understanding how *Brucella* holds copper homeostasis may be a key to a better understanding of its mechanisms of pathogenicity. In the absence of knowledge regarding the way *Brucella*

cope with copper stress, the objective of the present study was to identify potential key actors involved in copper homeostasis through a transposon insertion analysis.

Genes involved in histidine biosynthesis emerged as critical for copper resistance. In this work, the role of this synthesis pathway was therefore investigated through the construction of *hisA*, *hisB*, *hisC*, and *hisD* deletion mutants. *Brucella* became auxotrophic for histidine and markedly sensitive to copper, as demonstrated in growth curves and soft agar assays with the loss of one of these genes (Fig. 2). Given the ability of histidine to coordinate metal ions through its imidazole ring, this observation suggests that histidine, free or ligated to a tRNA, might play a role of buffer during copper stress. Our findings, therefore, revealed an unexpected link between histidine biosynthesis and copper detoxification in *B. abortus*. The copper sensitivity phenotype does not result from a defect in histidine uptake, as supplementation of the minimal medium with histidine restores prototrophy, indicating that the bacterium can import histidine from the extracellular environment (Fig. S4). It has been demonstrated that, among a variety of organisms, histidine performs a role in metal resistance or as a buffer. In *Caenorhabditis elegans*, disruption of the histidine ammonia lyase (HALY-1) or dietary histidine supplementation results in an increase in zinc and nickel resistance, possibly through histidine's capacity to chelate zinc and nickel (47). In *Aspergillus fumigatus*, *hisB* deletion decreases resistance to several metals, which can be rescued by histidine supplementation. During iron starvation, an increase in histidine content was observed in this pathogenic fungus (48). Likewise, in *Acinetobacter baumannii*, Huth, the HALY, increases zinc availability by degrading histidine, which under normal conditions stores zinc in a labile histidine-zinc complex. This finding provides direct evidence for histidine-based buffering mechanisms (49). To support this connection between histidine biosynthesis and metal resistance, a histidine-enrichment analysis of the *Brucella* predicted proteome was performed, and the results indicated that the majority of proteins exhibiting a histidine percentage exceeding 5% are proteins implicated in metal homeostasis or using metals as cofactors (see Table S2). From this enrichment list, only CueO, the multicopper oxidase, was identified in the Tn-seq analysis performed in the presence of copper, suggesting that histidine-rich proteins cannot buffer copper in a non-redundant manner.

Whole-genome sequencing of the suppressor mutants revealed that, among the suppressors, 15 unique mutations were localized to the *opp* operon that encodes an ATP-binding cassette transporter, and more specifically, the first two genes encoding the periplasmic substrate-binding proteins OppA1 and OppA2. It is striking to observe that no mutations appeared in the permeases *oppB* and *oppC* or the ATPase components, *oppD/F*. At least eight *opp* operons are encoded in *B. abortus* genomes, including seven on the second chromosome. Among all of them, only one was affected by suppressive mutations within an impaired histidine biosynthesis background, highlighting its specificity in rescuing from copper resistance when histidine biosynthesis is lacking. In this study, we demonstrated that when histidine biosynthesis is impaired, and the bacteria have to face a copper stress, mutating the two substrate-binding protein genes, *oppA1* and *oppA2*, generates an efficient compensatory mechanism. The mutation in the C6 suppressor is of particular interest due to its location within the promoter region of *oppA1*. The C6 suppressor and Δ *hisC*SupPA1 strain, in which the mutation was reintroduced into the Δ *hisC* background, were confirmed to display a suppressor phenotype by rescuing growth in the presence of copper in comparison to the parent mutant (Fig. 5A and B). One non-exclusive hypothesis to account for this rescue is that OppA1 and/or OppA2 could facilitate increased histidine uptake, either as free amino acid or within short peptides, thereby enhancing the capacity of the bacterial cytosol to buffer copper ions. In this scenario, the C6 suppressive mutation may lead to *oppA* overexpression. This is consistent with the results obtained with the *oppA1* or *oppA2* overexpression strains, where increasing the copy number of the second *oppA2* gene rescued the copper sensitivity (Fig. 5A and B). Increasing the copy number of *oppA1* did not generate the same suppression, maybe because the overexpression of *oppA1*

is not achieved in this strain. Nevertheless, the suppressive mutations did not resolve histidine auxotrophy, which makes sense in the absence of histidine or histidine-containing peptides in the medium, if the uptake hypothesis proposed above is true (see Fig. S4). Since the attenuation of *hisC* mutants in infection can be either due to copper sensitivity or histidine auxotrophy, the suppressor strains offer a way to discriminate between these two hypotheses, since the suppressors are copper resistant but still histidine auxotrophic. As indicated in Fig. 5C, the suppressor mutation in the C6 strain does solve the intracellular replication problem of the *hisC* mutant, while the suppressive mutation inserted in the *hisC* mutant background does not. These results thus demonstrated that within the cell, the replication of *his* mutants is impacted due to a histidine biosynthesis dysfunction rather than a copper stress.

Amino acid starvation is a common feature of host-pathogen interactions. It can be imposed by the host as a nutritional immunity strategy to restrict pathogen growth, or alternatively, it may result from the infection by the pathogen. The source of amino acids for *B. abortus* in host cells is unknown. It may involve extracellular proteases or the hijacking of host proteases to gain small peptides that could fit into the pore of the major porin of *B. abortus*. To the best of our knowledge, such proteases have not been identified so far, and the availability of free amino acids in the rBCV of macrophages is unknown. Such lines of research would deserve an in-depth investigation in the future. Therefore, why is histidine the only amino acid whose biosynthesis would be required in macrophage infection? It is notorious that histidine biosynthesis is a costly amino acid to synthesize in terms of the ATP equivalents needed (50). It is therefore possible that the available quantities of histidine in the host cells are a limiting factor for growth. It has been demonstrated that *M. tuberculosis* depends on *de novo* histidine biosynthesis to overcome host-imposed histidine deprivation. IFN- γ -mediated upregulation of histidine catabolizing enzymes (HAL, HDC) reduces free histidine in infected tissues, creating a nutritional stress that *M. tuberculosis* Δ *hisD* mutant cannot withstand. These results highlight the histidine biosynthetic pathway as a critical bacterial strategy in *Mycobacterium* to evade host nutritional immunity and sustain intracellular survival (51). In macrophages, *S. Typhimurium* also relies on histidine biosynthesis genes upregulation to counter free histidine limitation in host cells (52). These studies demonstrate that histidine production is an important adaptation for survival under host-imposed nutrient restriction.

As expected, the results highlighted three genes within the *cue* operon: *copA*, *cueO*, and *cueR* as important for copper resistance *in vitro*. In addition, the *copA* transposon mutant was attenuated in macrophages in a previous Tn-seq from our lab (36). Unexpectedly, however, targeted deletions of *copA* or *cueO* did not confer a sensitivity phenotype to copper in our conditions in *B. abortus* (Fig. 1). These results remained consistent when copper concentration was increased. These findings are in striking contrast with observations in *B. melitensis*, where the *bmcO* (*Brucella* multicopper oxidase) mutant displays a copper sensitivity on minimal medium (53), which could be explained by differences related to the bacterial strain context and culture conditions. Also, in *E. coli* and *S. Typhimurium*, the *cue* system is the primary defense line to cope with copper toxicity (28). Indeed, the deletion of *copA* or *cueO* increased copper sensitivity, even stronger under anaerobic conditions for Δ *copA* (31). In *S. Typhimurium*, a similar phenotype has been reported where the loss of *copA* (34) or *cueO* (27) also gives a copper susceptibility. Likewise, the *cueO* deletion sensitivity phenotype is exacerbated in the absence of oxygen (27). A homolog of *cueO* called *mmcO* also plays a role in copper resistance in *M. tuberculosis* (54). *E. coli* and *S. Typhimurium* have other defense systems, including *cus* and *gol* systems, respectively, expressed under anaerobic conditions or higher copper concentrations (28, 31). Additional copper homeostasis systems are absent in the *Brucella* genome, according to our current knowledge. The role of CopA and CueO was also investigated in *S. Typhimurium* replication capacity within the host. While CopA is described as having a role in macrophage replication (34), CueO was more important in the mouse model (27). This demonstrated the importance of

copper homeostasis in *S. Typhimurium* for its virulence. Despite the lack of sensitivity to copper *in vitro*, it would be interesting to test the two mutants constructed in *B. abortus* during macrophage or mice infection to define their role in a more complex virulence model. Even more so, as *copA* was identified as required for survival in macrophages (36). It would also be interesting to repeat our experiments in different conditions, such as minimal media. Nevertheless, despite the indications of Tn-seq experiments, the *cue* system does not appear to be the primary strategy used by *Brucella*, at least under the conditions tested in this work. This could also demonstrate the limitations of Tn-seq analysis in predicting deletion mutant phenotypes.

Our study reveals an intriguing new aspect of copper homeostasis in *B. abortus*. While key copper-associated proteins such as CopA and CueO are critical in other bacteria, they appear surprisingly dispensable in *B. abortus*. Instead, histidine biosynthesis emerges as a possible frontline defense against copper stress. Indeed, we found that disruption of histidine synthesis leads to copper sensitivity in *B. abortus*, highlighting the importance of this pathway. Interestingly, the attenuation of *hisC* mutant in macrophages stems from histidine auxotrophy rather than copper sensitivity. The mechanism underlying nutrient uptake within the rBCV remains poorly understood, representing a promising avenue for future research into how *B. abortus* acquires amino acids in its intracellular replicative niche.

MATERIALS AND METHODS

Bacterial strains and media

Escherichia coli DH10B (Invitrogen) and S17-1 (55) strains were grown in Luria-Bertani (LB Lennox) medium at 37°C. *E. coli* MFDpir pXMCS2-Tn5 (36) was grown in LB medium supplemented with 300 µM *meso*-2,6-diaminopimelic acid. *Brucella abortus* 544 Nal^R (referred to as the WT in this work; J.-M. Verger, INRA, Tours) and its derivative strains were grown in 3% Bacto tryptic soy broth rich medium (TSB; Difco ref. 211825) at 37°C. For auxotrophy experiments, the defined medium Plommet erythritol (PE) (Plommet 1991) was used, composed of 7 or 9.2 g/L K₂HPO₄, 3 g/L KH₂PO₄, 0.1 g/L Na₂S₂O₃, 5 g/L NaCl, 0.2 mg/L nicotinic acid, 0.2 mg/L thiamine, 0.04 mg/L pantothenic acid, 0.01 g/L MgSO₄, 0.01 mg/L MnSO₄, 0.1 mg/L FeSO₄, 0.1 µg/L biotin, and 2 g/L erythritol. All the strains used in this study are listed in Table S5.

When it was necessary, the culture medium was supplemented with the appropriate antibiotics at the following concentrations: kanamycin (Kan, 10 or 50 µg/mL for chromosomal locus or plasmid selection, respectively) and nalidixic acid (Nal, 25 µg/mL).

Strain construction

For deletion mutants, the whole gene was removed by homologous recombination. Joined PCR was applied. Two regions (the upstream and downstream regions of the target gene) of more than 500 base pairs were amplified from purified *B. abortus* 544 gDNA using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and primer pairs (F1/R1 for the upstream region and F2/R2 for the downstream region). The two amplified fragments were fused together through a complementary region designed in the primers used, and the resulting fragment was amplified by PCR using F1 and R2 primers. To Δ *hisC*SupPA1 construction, the complementary region primer pair contained the desired SNP. The amplicon was purified and inserted into an *EcoRV* (New England Biolabs)-linearized pNPTS138 plasmid through an overnight ligation (T4 DNA ligase, Promega) at 20°C. The ligation product was transformed in DH10B *E. coli*, and clones positive in blue-white screening were screened by PCR using GoTaq DNA polymerase (Promega). The selected plasmid was purified and checked by sequencing. The plasmid was inserted into S17-1 *E. coli* to allow conjugation to *B. abortus* 544 Nal^R by mating. Allelic exchange on the chromosome occurs via the non-replicative plasmid, pNPTS138 (56). The gene deletion was then verified by PCR using GoTaq DNA

polymerase (Promega). $\Delta hisA$, $\Delta hisB$, $\Delta hisC$, and $\Delta hisD$ deletion strains were complemented with pMR10 carrying the deleted gene. Complementation plasmids were obtained by amplification of the genes with a region of 400 base pairs upstream of the coding sequence by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs). The PCR product was digested as well as the pMR10 plasmid with BamHI and XbaI enzymes (New England Biolabs). The insert was inserted in the linearized plasmid through ligation in the same direction as the *E. coli lac* promoter of the vector.

To construct the overexpression strains, the genes were amplified by regular PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs). The designed primers included restriction sites for KpnI and SacI restriction enzymes (New England Biolabs). PCR product was purified and restricted by KpnI and SacI as well as the multicopy-plasmid pBBR-MCS2 (56). The gene expression is under the control of the *E. coli lac* promoter.

All the primers and plasmids used in this study are listed in Table S3 and S4, respectively.

Transposon sequencing assay

One milliliter of an overnight culture of *B. abortus* 544 Nal^R and 50 μ L of an overnight culture of *E. coli* MFDpir pXMC52-Tn5 Kan^R were mixed. After recovery in 1 mL of TSB supplemented with *meso*-2,6-diaminopimelic acid (300 μ M), OD of the cultures was measured and normalized to an OD of 1. Using a 96-well plate, serial 10-fold dilutions were carried out. Fifteen microliters of these dilutions were plated on TSB plates supplemented with CuSO₄ concentrations ranging from 0.5 mM to 3 mM. CFUs were counted to determine the optimal concentration of copper. A CuSO₄ concentration of 2 mM was selected. The mating was repeated as described above, and plates were incubated overnight at RT. To ensure complete coverage of the genome, 8 *B. abortus* and *E. coli* matings were done in parallel. The matings were recovered in 1 mL of TSB from which 50 μ L were diluted in 450 μ L of TSB. One hundred microliters of the dilution were spread on TSB agar plates supplemented with kanamycin or kanamycin and CuSO₄ to allow the selection of the resulting *B. abortus* mini-Tn5 libraries. After 4 days of incubation, the *B. abortus* mutants were recovered in 2 mL of TSB. The bacterial suspension was centrifuged for 7 min at 7,000 RPM. The pellet was resuspended in 300 μ L of 2% SDS. Bacteria were inactivated for at least 1 h at 80°C. All the samples were pooled together following the two conditions. gDNA was extracted using the Nucleobond AXG 500 kit (Macherey-Nagel). This gDNA was sequenced via Illumina sequencing. The raw data were analyzed by an automatic process, TnBox (<https://github.com/fxstubbbe/TnBox>), following a previously described method (37). TnBox uses the BWA method (57) to map the raw reads from Illumina sequencing on the genome of *B. abortus* 544 and the SAMtools suite (58) to calculate read counts. The libraries underwent normalization based on read depth. For a detailed protocol, see reference (37). From the library, 4.8×10^6 random mutants were recovered and sequenced using a deep sequencing method. We identified 1,684,839 and 1,743,143 unique insertion sites for control and copper conditions, respectively, illustrating genome saturation with a unique insertion every 1.95 bp for control and every 1.89 bp for the stress condition, on average. To evaluate the contribution of each gene to *Brucella* fitness under copper stress, a TnIF was calculated. The TnIF was defined as the average $\log_{10}(r + 1)/l$, where r is the number of miniTn5 insertions at a given nucleotide and l is the coding sequence length in base pairs. To minimize insertional bias, the calculation was performed considering only the central 80% of the coding sequence. For each gene, the differential TnIF (Δ TnIF) between the interest and control conditions was calculated as Δ TnIF = TnIF_{copper} – TnIF_{control}. A negative Δ TnIF indicates that the gene is required in the copper condition, as the number of reads associated is lower than in the control condition.

Growth curves

Growth was assessed by using BioTek Epoch2 Microplate Reader by measuring OD_{600 nm} every 30 min for 48 h at 37°C with agitation. Culture of *B. abortus* 544 and derivative strains was grown in TSB overnight to reach the exponential phase (OD_{600 nm} 0.3–0.8) and normalized at an OD_{600 nm} of 0.1 final the following day. For the growth measurement in the presence of copper, TSB was supplemented with 1.6 or 2 mM of CuSO₄. For the growth measurements in PE, overnight bacterial cultures were washed twice in PBS and diluted to an OD_{600 nm} of 0.1 in PE. When necessary, PE was supplemented with 1 mM of histidine (Sigma).

Suppressor assays

Culture of *B. abortus* 544 and derivative strains were grown in TSB overnight to reach the exponential phase (OD_{600 nm} 0.3–0.8) and normalized at an OD_{600 nm} of 0.1. Twenty microliters of each strain was spread on a TSB agar plate containing 1.6 or 2 mM CuSO₄. Plates were incubated at 37°C for 4–5 days.

For all four Δhis mutants, 29 isolated colonies formed on CuSO₄ plates were streaked on new TSB agar 1.6 and 2 mM CuSO₄ plates to confirm the phenotype of the suppressors. Among the 29 clones, 21 were still resistant to copper on the new plates and were grown overnight and stored. To extract genomic DNA, bacterial cultures of around 7.5 mL were centrifuged at $8,200 \times g$ for 5 min. Supernatants were discarded, and bacterial pellets were resuspended in 300 μ L of PBS. Then, bacteria were inactivated at 80°C for at least 1 h. Afterward, 100 μ L of SDS 10% was added. The gDNA extraction was achieved following the manufacturer's instructions of the Macherey-Nagel NucleoSpin Tissue Kit. Bacterial SNP sequencing was performed by BIO, part of the Pathology and Genetics Institute. The analysis of sequencing reads was conducted utilizing the Snippy tool, which is available on the Galaxy.org website (<https://usegalaxy.org/>). Each parental mutant and suppressor genome was aligned to the reference *B. abortus* 544 genome.

Soft agar well diffusion assays

Culture of *B. abortus* 544 and derivative strains were grown in TSB overnight to reach the exponential phase (OD_{600 nm} 0.3–0.8) and normalized at an OD_{600 nm} of 0.1. Five hundred microliters was added to 4.5 mL of TSB soft agar (0.7% agar) and spread on a 20 mL TSB agar plate. After the culture-soft agar mix had dried, a well was dug in the upper part of a pipette tip and removed. The well was filled with 100 μ L of 200 mM CuSO₄. The plates were incubated for 3–4 days at 37°C upward-facing lid on the first day before being flipped upside down. After incubation, the inhibition zone around the well was measured in three different directions, and the mean was reported in a graph in millimeters.

J774A.1 macrophage culture and infection

J774A.1 macrophages (ATCC) were cultivated at 37°C in a 5% CO₂ atmosphere in GlutaMAX-supplemented Dulbecco's Modified Eagle Medium (DMEM, Gibco) in which 10% of heat-inactivated fetal bovine serum (Gibco) was added. The day before infection, J774A.1 macrophages were seeded in a 24-well plate at a concentration of 1.10^5 cells/mL. On the day of the infection, overnight cultures of *Brucella* strains at exponential phase (0.3–0.8) were washed twice in PBS and diluted in GlutaMAX-supplemented DMEM medium at a multiplicity of infection of 50. Bacterial suspensions were added to the cells, and the 24-well plates were centrifuged at $169 \times g$ for 10 min at RT. Infected cells were incubated at 37°C in a 5% CO₂ atmosphere for 1 h. Cell medium was refreshed and supplemented with 50 μ g/mL gentamycin to kill extracellular bacteria. Cells were incubated one extra hour before the media was again refreshed and supplemented with 10 μ g/mL gentamycin, to kill bacteria egressed and avoid a re-infection event, for the 46 h left. For CFU counts, at the interested time points (2, 5, 24, or 48 h after infection), cells

were washed twice with PBS and incubated with 0.1% Triton X-100 PBS for 10 min at RT before being scratched to be detached. Macrophage lysates were 10-fold diluted in PBS, and specific dilutions (20 μ L) were spread onto TSB agar plates. Plates were incubated at 37°C for 5 days, and CFUs were counted. The CFU number (log₁₀) was calculated per mL of lysate and reported in a graph.

Statistical analysis

Statistical analyses via one- or two-way ANOVA with Dunnett's test were carried out provided by the GraphPad Prism software. Values of $P < 0.05$ were considered to represent a significant difference.

ACKNOWLEDGMENTS

We thank the technical team of the URBM for their support, as well as the University of Namur for access to the BL3 platform and logistic and financial support. We thank Damien Devos (Pasteur Lille) for the analysis of histidine content in the predicted proteome of *B. abortus* and François Jacob-Dubuisson (Pasteur Lille) and Hilde De Reuse (Pasteur Paris) for stimulating discussions.

C.F. was supported by an FRIA (FNRS) PhD fellowship. A.R. was supported by an Aspirant (FNRS) PhD fellowship. K.P. was supported by a Chargé de Recherche grant fellowship from FNRS. This publication is supported by the Walloon Region as part of the funding for the FRFS-WELBIO strategic axis (X.1512.24). The work was also supported by PDR grants T.0058.20 and T.0068.24 from FRS-FNRS, as well as Concerted Research Action 17/22-087 and 22/27-128 from the Fédération Wallonie-Bruxelles.

AUTHOR AFFILIATION

¹URBM, Department of Biology, NARILIS, University of Namur, Namur, Belgium

AUTHOR ORCIDs

Charline Focant  <http://orcid.org/0000-0003-0282-2369>

Xavier De Bolle  <http://orcid.org/0000-0002-3845-3043>

FUNDING

Funder	Grant(s)	Author(s)
Fédération Wallonie-Bruxelles	17/22-087	Xavier De Bolle
Fédération Wallonie-Bruxelles	22/27-128	Xavier De Bolle
FRFS-Welbio	Advanced X.1512.24	Xavier De Bolle
FRS-FNRS	PDR T.0058.20	Xavier De Bolle
FRS-FNRS	PDR T.0068.24	Xavier De Bolle

AUTHOR CONTRIBUTIONS

Charline Focant, Conceptualization, Investigation | Agnès Roba, Conceptualization, Investigation | Elisabeth Wanlin, Investigation | Katy Poncin, Investigation | Xavier De Bolle, Conceptualization, Project administration, Supervision

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental tables and figures (JB00492-25-S0001.pdf). Tables S1 to S5 and Figures S1 to S4.

REFERENCES

- Hördt A, López MG, Meier-Kolthoff JP, Schleuning M, Weinhold L-M, Tindall BJ, Gronow S, Kypides NC, Woyke T, Göker M. 2020. Analysis of 1,000+ type-strain genomes substantially improves taxonomic classification of *Alphaproteobacteria*. *Front Microbiol* 11:468. <https://doi.org/10.3389/fmicb.2020.00468>
- González-Espinoza G, Arce-Gorvel V, Mémet S, Gorvel JP. 2021. *Brucella*: reservoirs and niches in animals and humans. *Pathogens* 10:186. <https://doi.org/10.3390/pathogens10020186>
- Corbel MJ. 1997. Brucellosis: an overview. *Emerg Infect Dis* 3:213–221. <https://doi.org/10.3201/eid0302.970219>
- Suárez-Esquivel M, Chaves-Olarte E, Moreno E, Guzmán-Verri C. 2020. *Brucella* genomics: macro and micro evolution. *IJMS* 21:7749. <https://doi.org/10.3390/ijms21077749>
- Moreno E, Moriyón I. 2006. The genus *Brucella*, p 315–456. In Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (ed), *The prokaryotes*. Springer, New York.
- von Bargen K, Gorvel J-P, Salcedo SP. 2012. Internal affairs: investigating the *Brucella* intracellular lifestyle. *FEMS Microbiol Rev* 36:533–562. <https://doi.org/10.1111/j.1574-6976.2012.00334.x>
- Starr T, Ng TW, Wehrly TD, Knodler LA, Celli J. 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic* 9:678–694. <https://doi.org/10.1111/j.1600-0854.2008.00718.x>
- Celli J, de Chastellier C, Franchini D-M, Pizarro-Cerda J, Moreno E, Gorvel J-P. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. *J Exp Med* 198:545–556. <https://doi.org/10.1084/jem.20030088>
- Boschiroli ML, Ouahrani-Bettache S, Foulongne V, Michaux-Charachon S, Bourg G, Allardet-Servent A, Cazeville C, Liautard JP, Ramuz M, O'Callaghan D. 2002. The *Brucella suis virB* operon is induced intracellularly in macrophages. *Proc Natl Acad Sci USA* 99:1544–1549. <https://doi.org/10.1073/pnas.032514299>
- Myeni S, Child R, Ng TW, Kupko JJ 3rd, Wehrly TD, Porcella SF, Knodler LA, Celli J. 2013. *Brucella* modulates secretory trafficking via multiple type IV secretion effector proteins. Edited by R. H. Valdivia. *PLoS Pathog* 9:e1003556. <https://doi.org/10.1371/journal.ppat.1003556>
- Celli J. 2019. The intracellular life cycle of *Brucella* spp. *Microbiol Spectr* 7:1–11. <https://doi.org/10.1128/microbiolspec.BAI-0006-2019>
- Starr T, Child R, Wehrly TD, Hansen B, Hwang S, López-Otin C, Virgin HW, Celli J. 2012. Selective subversion of autophagy complexes facilitates completion of the *Brucella* intracellular cycle. *Cell Host Microbe* 11:33–45. <https://doi.org/10.1016/j.chom.2011.12.002>
- Botella H, Stadthagen G, Lugo-Villarino G, de Chastellier C, Neyrolles O. 2012. Metallobiology of host-pathogen interactions: an intoxicating new insight. *Trends Microbiol* 20:106–112. <https://doi.org/10.1016/j.tim.2012.01.005>
- Palmer LD, Skaar EP. 2016. Transition metals and virulence in bacteria. *Annu Rev Genet* 50:67–91. <https://doi.org/10.1146/annurev-genet-120215-035146>
- Helmann JD. 2025. Metals in motion: understanding labile metal pools in bacteria. *Biochemistry* 64:329–345. <https://doi.org/10.1021/acs.biochem.4c00726>
- Chandrangsu P, Rensing C, Helmman JD. 2017. Metal homeostasis and resistance in bacteria. *Nat Rev Microbiol* 15:338–350. <https://doi.org/10.1038/nrmicro.2017.15>
- Kehl-Fie TE, Skaar EP. 2010. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr Opin Chem Biol* 14:218–224. <https://doi.org/10.1016/j.cbpa.2009.11.008>
- Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388:482–488. <https://doi.org/10.1038/41343>
- Djoko KY, Ong CY, Walker MJ, McEwan AG. 2015. The role of copper and zinc toxicity in innate immune defense against bacterial pathogens. *J Biol Chem* 290:18954–18961. <https://doi.org/10.1074/jbc.R115.647099>
- Helmman JD. 2025. Microbial metal physiology: ions to ecosystems. *Nat Rev Microbiol* 23:805–819. <https://doi.org/10.1038/s41579-025-01213-7>
- Hood MI, Skaar EP. 2012. Nutritional immunity: transition metals at the pathogen-host interface. *Nat Rev Microbiol* 10:525–537. <https://doi.org/10.1038/nrmicro2836>
- Kim BE, Nevitt T, Thiele DJ. 2008. Mechanisms for copper acquisition, distribution and regulation. *Nat Chem Biol* 4:176–185. <https://doi.org/10.1038/nchembio.72>
- Kim HW, Chan Q, Afton SE, Caruso JA, Lai B, Weintraub NL, Qin Z. 2012. Human macrophage ATP7A is localized in the trans-Golgi apparatus, controls intracellular copper levels, and mediates macrophage responses to dermal wounds. *Inflammation* 35:167–175. <https://doi.org/10.1007/s10753-011-9302-z>
- White C, Lee J, Kambe T, Fritsche K, Petris MJ. 2009. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J Biol Chem* 284:33949–33956. <https://doi.org/10.1074/jbc.M109.070201>
- Wolschendorf F, Ackart D, Shrestha TB, Hascall-Dove L, Nolan S, Lamichane G, Wang Y, Bossmann SH, Basaraba RJ, Niederweis M. 2011. Copper resistance is essential for virulence of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 108:1621–1626. <https://doi.org/10.1073/pnas.1009261108>
- Ward SK, Abomoelak B, Hoyer EA, Steinberg H, Talaat AM. 2010. CtpV: a putative copper exporter required for full virulence of *Mycobacterium tuberculosis*. *Mol Microbiol* 77:1096–1110. <https://doi.org/10.1111/j.1365-2958.2010.07273.x>
- Achard MES, Tree JJ, Holden JA, Simpfendorfer KR, Wijburg OLC, Strugnell RA, Schembri MA, Sweet MJ, Jennings MP, McEwan AG. 2010. The multi-copper-ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. *Infect Immun* 78:2312–2319. <https://doi.org/10.1128/IAI.01208-09>
- Giachino A, Waldron KJ. 2020. Copper tolerance in bacteria requires the activation of multiple accessory pathways. *Mol Microbiol* 114:377–390. <https://doi.org/10.1111/mmi.14522>
- Ma Z, Chandrangsu P, Helmman JC, Romsang A, Gaballa A, Helmman JD. 2014. Bacillithiol is a major buffer of the labile zinc pool in *Bacillus subtilis*. *Mol Microbiol* 94:756–770. <https://doi.org/10.1111/mmi.12794>
- Outten FW, Outten CE, Hale J, O'Halloran TV. 2000. Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, cueR. *J Biol Chem* 275:31024–31029. <https://doi.org/10.1074/jbc.M006508200>
- Outten FW, Huffman DL, Hale JA, O'Halloran TV. 2001. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* 276:30670–30677. <https://doi.org/10.1074/jbc.M104122200>
- Rensing C, Fan B, Sharma R, Mitra B, Rosen BP. 2000. CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. *Proc Natl Acad Sci USA* 97:652–656. <https://doi.org/10.1073/pnas.97.2.652>
- Meydan S, Klepacki D, Karthikeyan S, Margus T, Thomas P, Jones JE, Khan Y, Briggs J, Dinman JD, Vázquez-Laslop N, Mankin AS. 2017. Programmed ribosomal frameshifting generates a copper transporter and a copper chaperone from the same gene. *Mol Cell* 65:207–219. <https://doi.org/10.1016/j.molcel.2016.12.008>
- Osman D, Waldron KJ, Denton H, Taylor CM, Grant AJ, Mastroeni P, Robinson NJ, Cavet JS. 2010. Copper homeostasis in *Salmonella* is atypical and copper-CueP is a major periplasmic metal complex. *J Biol Chem* 285:25259–25268. <https://doi.org/10.1074/jbc.M110.145953>
- Osman D, Patterson CJ, Bailey K, Fisher K, Robinson NJ, Rigby SEJ, Cavet JS. 2013. The copper supply pathway to a *Salmonella* Cu,Zn-superoxide dismutase (SodCII) involves P(1B)-type ATPase copper efflux and periplasmic CueP. *Mol Microbiol* 87:466–477. <https://doi.org/10.1111/mmi.12107>
- Sternon J-F, Godessart P, Gonçalves de Freitas R, Van der Henst M, Poncin K, Francis N, Willemart K, Christen M, Christen B, Letesson J-J, De Bolle X. 2018. Transposon sequencing of *Brucella abortus* uncovers essential genes for growth *in vitro* and inside macrophages. Edited by A.

- J. Bäumler. Infect Immun 86:e00312-18. <https://doi.org/10.1128/IAI.00312-18>
37. Potemberg G, Demars A, Barbieux E, Reboul A, Stubbe F-X, Galia M, Lagneaux M, Comein A, Denis O, Pérez-Morga D, Vanderwinden J-M, De Bolle X, Muraille E. 2022 Genome-wide analysis of *Brucella melitensis* genes required throughout intranasal infection in mice. Edited by J. Mecsas. PLoS Pathog 18:e1010621. <https://doi.org/10.1371/journal.ppat.1010621>
 38. Alifano P, Fani R, Liò P, Lazcano A, Bazzicalupo M, Carlomagno MS, Bruni CB. 1996. Histidine biosynthetic pathway and genes: structure, regulation, and evolution. Microbiol Rev 60:44–69. <https://doi.org/10.1128/mr.60.1.44-69.1996>
 39. Fitzpatrick TB, Amrhein N, Kappes B, Macheroux P, Tews I, Raschle T. 2007. Two independent routes of *de novo* vitamin B6 biosynthesis: not that different after all. Biochem J 407:1–13. <https://doi.org/10.1042/BJ20070765>
 40. Sivaraman J, Li Y, Larocque R, Schrag JD, Cygler M, Matte A. 2001. Crystal structure of histidinol phosphate aminotransferase (HisC) from *Escherichia coli*, and its covalent complex with pyridoxal-5'-phosphate and L-histidinol phosphate. J Mol Biol 311:761–776. <https://doi.org/10.1006/jmbi.2001.4882>
 41. Roop II RM, Baumgartner JE, Pitzer JE, Martin DW. 2017. Chapter 6, Magnesium, Copper and Cobalt. In Roop II RM, Caswell CC (ed), Metals and the biology and virulence of *Brucella*. Springer International Publishing.
 42. Kohler S, Foulongne V, Ouahrani-Bettache S, Bourg G, Teyssier J, Ramuz M, Liautard J-P. 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. Proc Natl Acad Sci USA 99:15711–15716. <https://doi.org/10.1073/pnas.232454299>
 43. Joseph P, Abdo MR, Boigegrain RA, Montero JL, Winum JY, Köhler S. 2007. Targeting of the *Brucella suis* virulence factor histidinol dehydrogenase by histidinol analogues results in inhibition of intramacrophagic multiplication of the pathogen. Antimicrob Agents Chemother 51:3752–3755. <https://doi.org/10.1128/AAC.00572-07>
 44. Roba A, Carlier E, Godessart P, Naili C, De Bolle X. 2022. Histidine auxotroph mutant is defective for cell separation and allows the identification of crucial factors for cell division in *Brucella abortus*. Mol Microbiol 118:145–154. <https://doi.org/10.1111/mmi.14956>
 45. Solioz M. 2018. Copper and bacteria: evolution, homeostasis and toxicity. Springer International Publishing.
 46. Lemire JA, Harrison JJ, Turner RJ. 2013. Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Microbiol 11:371–384. <https://doi.org/10.1038/nrmicro3028>
 47. Murphy JT, Bruinsma JJ, Schneider DL, Collier S, Guthrie J, Chinwalla A, Robertson JD, Mardis ER, Kornfeld K. 2011. Histidine protects against zinc and nickel toxicity in *Caenorhabditis elegans*. Edited by K. Ashrafi. PLoS Genet 7:e1002013. <https://doi.org/10.1371/journal.pgen.1002013>
 48. Dietl A-M, Amich J, Leal S, Beckmann N, Binder U, Beilhack A, Pearlman E, Haas H. 2016. Histidine biosynthesis plays a crucial role in metal homeostasis and virulence of *Aspergillus fumigatus*. Virulence 7:465–476. <https://doi.org/10.1080/21505594.2016.1146848>
 49. Nairn BL, Lonergan ZR, Wang J, Braymer JJ, Zhang Y, Calcutt MW, Lisher JP, Gilston BA, Chazin WJ, de Crécy-Lagard V, Giedroc DP, Skaar EP. 2016. The response of *Acinetobacter baumannii* to zinc starvation. Cell Host Microbe 19:826–836. <https://doi.org/10.1016/j.chom.2016.05.007>
 50. Brenner M, Ames BN. 1971. The histidine operon and its regulation:349–387. <https://doi.org/10.1016/B978-0-12-299255-1.50018-3>
 51. Dwivedy A, Ashraf A, Jha B, Kumar D, Agarwal N, Biswal BK. 2021. *De novo* histidine biosynthesis protects *Mycobacterium tuberculosis* from host IFN- γ mediated histidine starvation. Commun Biol 4:410. <https://doi.org/10.1038/s42003-021-01926-4>
 52. Li Z, Liu Y, Fu J, Zhang B, Cheng S, Wu M, Wang Z, Jiang J, Chang C, Liu X. 2019. *Salmonella* proteomic profiling during infection distinguishes the intracellular environment of host cells. Edited by I. M. Cristea. mSystems 4:314–318. <https://doi.org/10.1128/mSystems.00314-18>
 53. Wu T, Wang S, Wang Z, Peng X, Lu Y, Wu Q. 2015. A multicopper oxidase contributes to the copper tolerance of *Brucella melitensis* 16M. FEMS Microbiol Lett 362:fnv078. <https://doi.org/10.1093/femsle/fnv078>
 54. Rowland JL, Niederweis M. 2013. A multicopper oxidase is required for copper resistance in *Mycobacterium tuberculosis*. J Bacteriol 195:3724–3733. <https://doi.org/10.1128/JB.00546-13>
 55. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1:784–791. <https://doi.org/10.1038/nbt1183-784>
 56. Deghelt M, Mullier C, Sternon J-F, Francis N, Laloux G, Dotreppe D, Van der Henst C, Jacobs-Wagner C, Letesson J-J, De Bolle X. 2014. G1-arrested newborn cells are the predominant infectious form of the pathogen *Brucella abortus*. Nat Commun 5:4366. <https://doi.org/10.1038/ncomms5366>
 57. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-wheeler transform. Bioinformatics 25:1754–1760. <https://doi.org/10.1093/bioinformatics/btp324>
 58. Li Heng, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>