A role for copper in protozoan grazing - two billion years selecting for bacterial copper resistance

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Running Title

Role of copper in protozoan predation

Key words

Protozoan predation; copper resistance selection

Summary

The Great Oxidation Event (GOE) resulted in integration of soft metals in a wide range of biochemical processes including, in our opinion, killing of bacteria by protozoa. Compared to pressure from anthropologic copper contamination, little is known on impacts of protozoan predation on maintenance of copper resistance determinants in bacteria. To evaluate the role of copper and other soft metals in predatory mechanisms of protozoa, we examined survival of bacteria mutated in different transition metal efflux or uptake systems in the social amoeba *Dictyostelium discoideum*. Our data demonstrated a strong correlation between the presence of copper/zinc efflux as well as iron/manganese uptake, and bacterial survival in amoebae. The growth of protozoa, in turn, was dependent on bacterial copper sensitivity. The phagocytosis of bacteria induced upregulation of *Dictyostelium* genes encoding the copper uptake transporter p80 and a triad of Cu(I)-translocating P_{IB}-type ATPases. Accumulated Cu(I) in *Dictyostelium* was monitored using a copper biosensor bacterial strain. Altogether, our data demonstrate that Cu(I) is ultimately involved in protozoan predation of bacteria, supporting our hypothesis that protozoan grazing selected for the presence of copper resistance determinants for about two billion years.

Introduction

With the increasing bioavailability of copper during the Great Oxidation Event (GOE), natural selection favored the use of copper for the reduction of molecular oxygen (O_2) and simultaneously instigated the development of regulatory systems controlling copper homeostasis. Cu(I), when not protected by a stabilizing ligand, can give rise to Fenton-like reactions that produce toxic hydroxyl and hydroperoxyl radicals which lead to lipid peroxidation and oxidative reactions involving proteins and nucleic acids (Dupont *et al.*, 2011, Lemire *et al.*, 2013). In addition, Cu(I) is a soft metal with a high affinity for thiolates. Thus, soluble Cu(I) can attack the Achilles' heel of any cell: the reductive, anaerobic environment of the cytoplasm with multiple enzymes containing iron-sulfur clusters (Macomber & Imlay, 2009). There is evidence that this mechanism is employed by human macrophages to kill bacteria as part of the innate immune response (Hodgkinson & Petris, 2012).

Since the early evolution of eukaryotes accompanied the increased bioavailability of copper, it appears reasonable to suggest that this mechanism evolved in unicellular bacterial-feeding eukaryotes and that extant protozoa may use copper as an effective way to poison and kill their prey. Such evolutionary pressure, would likely have prompted the development of detoxification strategies by bacteria e.g. Cu resistance (Cu^r) systems and repair systems for Fe-S clusters. Currently, anthropologic factor is thought to be a fundamental reason for emerging of copper resistance in bacteria. However, copper resistance determinants are ubiquitous and conserved in microbes existing in various environments such as soil, water, and even in the deep ocean where copper concentrations are very low and these determinants seem to be redundant. Hence, apparently even environments with low levels of copper promote the appearance of copper resistance determinants. Therefore, we propose that appearance of copper resistance in bacteria is triggered to a significant extent by the predatory behavior of protozoa. Bacteria and protozoa share a long common evolutionary history and we hypothesize that copper mediated bacterial killing mechanisms first evolved in protozoa long before multicellular life arose, and that the

accumulation of Cu(I) in phagosomes is one of the strategies to poison and kill bacteria in protozoa (German *et al.*, 2013, Hao *et al.*, 2015). Such predatory mechanisms would create selective pressure on acquisition and maintenance of copper resistance determinants in bacteria. Here, we designed a study to investigate the possible role of Cu(I) for bacterial killing by protozoa. We performed a series of experiments using the social amoeba *Dictyostelium discoideum* as a model organism, supplemented with a few experiments with the distantly related flagellate *Paracercomonas crassicauda*.

Results

Transition metal exporters/importers protect bacterial survival in Dictyostelium

To investigate whether copper-sensitivity of bacteria affects their susceptibility to phagocytosis, we examined the number of different *Escherichia coli* and *Pseudomonas aeruginosa* strains (mutated in genes encoding various metal transporters) within *Dictyostelium discoideum*. We found that deletions in genes encoding Cu(I)-translocating P_{1B}-type ATPases led to decreased number of bacteria in *D. discoideum*. The number of *E. coli* mutant 258 (Δ*copA*) cells inside amoebal cells was reduced by a factor of ≥ 2.6 compared to the wild-type strain *E. coli* W3110 at a multiplicity of infection (MOI) of 1 (**Fig. 1A**). Deletions in *cus* operon and *cueO* in *E. coli* 75 also diminished the number of bacterial cells recovered from *Dictyostelium* by about 30%, but the impact was not as strong as in mutants with a *copA* deletion. In addition, the reduced number of intracellular bacteria was also found in bacterial strains with deletions in iron uptake systems, i.e., *E. coli* 417 (Δ*entC*) and 420 (Δ*feoAB*). Manganese is needed for the activity of Mn superoxide dismutase (MnSOD), an enzyme that responds to environmental stresses (Fee, 1991). A deletion in the manganese uptake-related gene *mntH* in *E. coli* 247 displayed the highest sensitivity to *Dictyostelium* digestion. Similar trends were also observed with increase of MOI to 5 (**Fig. 1B**), indicating an important role of genes encoding copper efflux, iron and manganese uptake systems in bacterial protection from amoebal grazing.

To determine the effects of further copper addition on the number of intracellular

bacteria, 10 ppm copper, which has no adverse effect on both *Dictyostelium* and bacteria, was added into the co-cultures (**Fig. 1C**). Compared to the wild-type strain, there was a significant reduction in the number of mutants 258 (Δ*copA*), 417 (Δ*entC*) and 420 (Δ*feoAB*) recovered from *Dictyostelium* ($p<0.01$). However, addition of 10 ppm copper led to an increase in the number of intracellular bacterial for both wild-type and mutant strains (**Fig. 1C**), when compared to co-culture without extra copper addition (**Fig. 1B)**.

Similarly, a copper sensitive mutant *P. aeruginosa* (Δ*cueA*) with a deletion in the gene encoding the main Cu(I)-translocating P-type ATPases was more susceptible to *Dictyostelium* killing when compared to the wild-type strain PAO1 at MOI of 5 at 6 h of co-culture (**Fig. 1D**). However, no significant difference in the number of intracellular bacteria was noted between the wild-type strain PAO1 and a mutant with *pcoA* deletion encoding a multicopper oxidase ($p<0.01$), meaning the role of *pcoA* is less important than *cueA* in bacterial protection against amoebal grazing. We observed the ingestion of PAO1 by *Dictyostelium* through transmission electron microscope (TEM) (**Fig. S1**).

Copper resistance determinants play crucial roles in bacterial resistance to Dictyostelium predation

The effects of Cu^r determinants on bacterial abilities to resist predation were assessed by depositing *Dictyostelium* on a lawn formed by either the wild-type strain (i.e., *E. coli* W3110 and *P. aeruginosa* PAO1) or their isogenic copper sensitive mutants. A dilution series of HL5/DB (Dilution Buffer) agar (2.5%-100%) was first applied to determine the appropriate concentration at which *Dictyostelium* did not grow on top of the wild type bacterial strain but instead formed phagocytic plaques on the different bacterial mutant strains. 2.5% and 5% HL5/DB agar was used to *E. coli* and *P. aeruginosa* cultures respectively to distinguish the difference between the wild-type strains and mutants (**Fig. 2A & B**). The phagocytic plaques formed on both *E. coli* 258 (Δ*copA*) and *E. coli* 342 (Δ*copA*Δ*cueO*Δ*cusCFBA*) lawns were much bigger than plaques formed on lawns of the wild-type strain W3110 after Day 3 (**Fig. 2A**). For *P. aeruginosa*, a loss of protozoan evasion was observed after Day 1 with the

greatest loss in the order 3920 (Δ*cueA*) > 2065 (Δ*pcoA*) ≥ PAO1 (**Fig. 2B**). Phagocytic plaques on PAO1 and 2065 (Δ*pcoA*) were attenuated and fully disappeared after Day 2, indicating the strong capacity of *P. aeruginosa* to resist *Dictyostelium* predation. However, the *cueA* mutant deficient in a Cu(I)-translocating P_{1B}-type ATPase in *P. aeruginosa* 3920 exhibited a stable and dramatic loss of protozoan evasion.

Bacterial copper resistance determinants influence the growth of protozoa

We speculated that correlation between copper tolerance in bacteria and their use as a food source for protozoa is stipulated by the use of copper to kill bacteria in protozoan phagosomes. If this is true, it should be more difficult for protozoa to kill and digest copper resistant bacterial strains than non-resistant strains. To validate this hypothesis, we monitored the growth of *D. discoideum* in AS (Amoeba Saline) medium supplemented with bacteria exhibiting different levels of copper sensitivity, where protozoan growth was entirely dependent on grazing. We found that *D. discoideum* could grow by grazing upon either *E. coli* wild-type strain W3110 or on copper sensitive mutants 258 (Δ*copA*) and 75 (Δ*cueO*Δ*cusCFBA*). While there was little growth in the control with no bacteria supplemented into AS medium (**Fig. 3A**). However, the number of *D. discoideum* fed with either 258 (Δ*copA*) or 75 (Δ*cueO*Δ*cusCFBA*) was significantly higher than the number of *D. discoideum* fed with the wild-type *E. coli* W3110 from ~ 5.5 to ~ 29.5 h after co-occulation ($p<0.05$). A similar assay was carried out to determine the growth of the distantly related flagellate *Paracercomonas crassicauda* (**Fig. 3B**). The number of *Paracercomonas* fed with either 258 (Δ*copA*) or 75 (Δ*cueO*Δ*cusCFBA*) was slightly higher than the number of *Paracercomonas* fed with the wild-type *E. coli* W3110 after 72.5 h. However, the differences were not as significant as the case of *Dictyostelium* ($p<0.05$).

Phagocytosis of bacteria by Dictyostelium triggered elevated expression of protozoan genes involved in metal trafficking

D. discoideum harbors one *p80* gene encoding a Ctr-type copper permease and three genes encoding putative Cu(I)-translocating P-type ATPases. These genes are

thought to be involved in copper acquisition, trafficking, or efflux due to their homology to copper transporters in macrophages (German *et al.*, 2013, Burlando *et al.*, 2002). We monitored expression of the following genes - *ATP1* (gi|66822199), *ATP7A* (gi|66809993), *ATP3* (gi|111218599), *p80* (gi|111226414), *NRAMP1* (gi|66818629) and *NRAMP2* (gi|66819499) (**Fig. 4**) - as a function of (1) added copper salts; (2) with ingested bacteria; or (3) with ingested bacteria and added copper salts. *ATP1* alone was induced dramatically (by a factor of 20) by added copper salts, which suggested that *ATP1* was in part responsible for the high copper tolerance of *D. discoideum*. When compared to the control, the expression of *ATP1* was also up-regulated by a factor of 10 when bacteria were ingested and highest when both copper was added and bacteria ingested. *ATP7A* and *ATP3* showed an elevated expression level in response to bacterial ingestion, but the expression of both genes decreased in *Dictyostelium* when both bacteria and copper were added, indicating their importance in copper trafficking during the predatory response. *p80* was only up-regulated when bacteria alone were ingested, indicating that *Dictyostelium* needs copper to successfully kill and digest bacterial prey. Consequently, the expression of *p80* decreased when both copper and bacteria were added, confirming p80 as a copper transporter. In addition, the expression of protozoan *NRAMP2* doubled in the presence of bacteria, but no significant change was observed in the presence of both copper and bacteria ($p<0.05$). Detailed information on analysis of the potential domains of ATP1, ATP7A and ATP3 could be found in supplementary materials (**Fig. S2 & S3**).

Elevated levels of copper and reactive oxygen species could be detected in Dictyostelium phagosomes

To better understand the role of copper in predation, a copper bioreporter strain *Pseudomonas putida* (pCuS), which contains the pPROBE-NT plasmid (Miller *et al.*, 2000) with a CueR-regulated sensor element upstreams of a promoterless *GFP* gene (Li *et al.*, 2014) was used. The expression of *GFP* was induced upon exposure of *P. putida* (pCuS) to copper or silver salts (Li *et al.*, 2014). **Fig. 5** shows the expression of GFP only when *P. putida* (pCuS) is inside the amoebal cells, while no fluorescence is detected when bacteria are outside of the amoeba. This evidence strongly suggests

that *D. discoideum* has elevated concentrations of copper in their phagosomes. A bioreporter *P. putida* (pKatA) expressing GFP under control of the catalase promoter regulated by OxyR (Svenningsen *et al.*, 2015) also showed increased GFP mediated fluorescence inside of phagosomes (**Fig. S4**), where OxyR mainly responds to H_2O_2.

Interactions of environmental isolates with Dictyostelium

To investigate the interaction between *Dictyostelium* and environmental isolates selected from copper exposure in pig feedlots, three copper resistant *E. coli* isolates (77-3009-5, 77-30013-2, and 77-30253-3) were used to feed *D. discoideum* in AS medium. These *E. coli* isolates were isolated from pigs supplemented with copper salts as a growth promoter, and contain either genes responsible for synthesis of yersiniabactin or a 19 gene mobile element conferring them additional copper resistance (Luthje *et al.*, 2014). We found that *Dictyostelium* was able to grow when fed with *E. coli* isolates, but with very low growth efficiency (**Fig. 6A**). Except *P. crassicauda* fed with *E. coli* 77-30253-3(Ybt), almost no growth was observed when feeding *P. crassicauda* with the other two isolates (**Fig. 6B**). To further explore the potential pathogenicity of the three *E. coli* isolates, we monitored cell viability of *D. discoideum* co-cultured with these isolates in HL5:SorC 1:4 medium (**Fig. 6C**). Under these conditions, *Dictyostelium* could get nutrients from HL5/SorC medium and would not aggregate due to starvation. The growth of the bacteria would also be promoted in HL5/SorC medium. Compared to the control without addition of bacteria, an increased number of dead amoeboid cells were detected with the order being 77-3009-5 > 77-30013-2 > 77-30253-3 after 12 h of co-culture at a MOI of 5 or 18 h at a MOI of 1. Bacteria at MOI 5 had greater effects on the viability of *Dictyostelium* than bacteria at MOI 1. Almost all *Dictyostelium* cells were dead after 24 h co-culture with any of the three isolates at both a MOI of 1 and 5.

Discussion

Free-living protozoa, as was observed by Sir van Leeuwenhoek via “more than ordinary magnifying glass” and confirmed by many scientists thereafter, are ubiquitous and abundant in various natural and man-made ecosystems including

terrestrial, aquatic, abiotic and biotic habitats (Vaerewijck *et al.*, 2014, Leeuwenhoek, 1700). If copper is used by protozoa to kill phagocytosed bacteria, protozoan predation could have a significant impact on the presence of bacterial Cu^r determinants because copper resistant strains are more resistant to predation. To verify this hypothesis, we first monitored the number of cells of the *E. coli*/ *P. aeruginosa* wild-type strain and their copper-sensitive mutants recovered and monitored as CFU from the social amoeba *D. discoideum* (**Fig. 1**). Cu(I)-translocating P_{1B}-type ATPases are a central component of the detoxification system transporting Cu(I) from cytoplasm to periplasm. We observed that deletions of Cu(I)-translocating P_{1B}-type ATPases, such as *copA* in *E. coli* and *cueA* in *P. aeruginosa*, significantly impaired the number of intracellular bacteria. Our phagocytosis plaque assay also demonstrated that deletions of the genes encoding the Cu(I)-translocating P_{1B}-type ATPases in either *E. coli* 258 (Δ*copA*) or *P. aeruginosa* (Δ*cueA*) led to a dramatic loss of bacterial resistance against *Dictyostelium* predation, resulting in formation of significantly bigger plaques (**Fig. 2**). All these results highlight the role of Cu(I)-translocating P_{1B}-type ATPases in the bacterial defense against protozoan phagocytosis. In *E. coli*, the *cueO* and *cus* operon together with *copA* constitute the main chromosomal copper efflux systems. CueO, a periplasmic multicopper oxidase, oxidizes Cu(I) to the less toxic Cu(II), while CusCFBA exports periplasmic Cu(I) to the extracellular space. Both the *cus* operon and *cueO* in *E. coli* handle periplasmic copper homeostasis in *E. coli* (Grass & Rensing, 2001). In the current study, deletions of both the *cus* operon and *cueO* also led to a reduction of the number of intracellular bacterial, for both MOI 1 and 5. All these results confirmed a role for copper resistance in assisting intracellular bacteria from protozoan copper poisoning and killing in protozoan predation. In addition, mutants with deficiency in either iron or manganese uptake systems, such as mutants *E. coli* 247 (Δ*mntH*) and 420 (Δ*feoAB*), showed a lower number of recovered bacteria from *D. discoideum* than the wild-type strain. Previously, transcriptome analysis of an *E. coli* strain in symbiosis with *D. discoideum* showed that the expression of bacterial genes encoding iron and sulfur acquisition was up-regulated under co-culture conditions (Kihara *et al.*, 2009). The

authors attribute the uptake of iron and sulfur to the possibility that iron is needed in the development of intracellular bacterial communities. This may be true, but one of the ultimate reasons bacteria need iron and sulfur is to repair iron-sulfur clusters destroyed by Cu(I) and reactive oxygen species. Interesting, we observed a higher number of intracellular bacteria under conditions of 10 ppm copper addition (Fig. 1C vs. Fig. 1B). As reported in previous studies, addition of copper could stimulate phagocytosis process in protozoa (Nilsson, 1981), increase the uptake of bacteria and copper adapted cells (Sharan *et al.*, 2011), and in turn, could induce more resistance to copper/ROS stress than in normal cells.

According to our hypothesis, if copper is used in protozoan phagosomes to kill bacteria, it would be much easier for protozoa to kill and digest copper sensitive strains than copper resistant strains. As predicted, the growth of *Dictyostelium* fed with copper sensitive mutants (258 and 75) was much better than the growth of *Dictyostelium* fed with the wild-type strain (**Fig. 3A**). The flagellate also showed lower growth on the wild-type strain than on mutant 258 (Δ*copA*) (**Fig. 3B**), although the difference was not distinct when compared to *Dictyostelium*. The two unicellular eukaryotes used in our experiments are not closely related and belong to two different lineages. One, *D. discoideum*, is a member of the dictyostelids (often referred to as cellular slime molds) which forms part of the "Amoebozoa", whereas the genus *Paracercomonas* is assigned to Cercozoa- one of the main groups in "Rhizaria" (Pawlowski & Burki, 2009). Interestingly, our data suggest similar mechanism of copper utilization by these organisms, despite such early divergence.

Copper addition in the livestock industry or other environments with copper exposure could select potentially dangerous pathogenic isolates (Yazdankhah *et al.*, 2014). In this study, three independent copper resistant *E. coli* strains isolated from pig feedlots showed different degrees of resistance to predation by both *Dictyostelium* or *Paracercomonos* in AS medium (**Fig. 6A&B**). In the viability assay, we observed a dramatic reduction in the viability of *Dictyostelium* in the presence of either one of the three copper resistant *E. coli* strains (**Fig. 6C**). As reported previously, these strains possessed either mobile Cu^r islands or the yersiniabactin-synthesis cluster as a

"copper pathogenicity island", which appears to be prevalent among various pathogens (Hao *et al.*, 2015). Therefore, isolates selected under copper stress could not only contain copper resistance determinants, but also contain other pathogenicity factors. However, the reason why these copper resistant isolates led to the death of *Dictyostelium* is unknown. It could be the result of the joint action of both increased copper resistance and other pathogenicity factors. Additionally, an increased number of bacteria in the viability assay could possibly result in oxygen deprivation or production of toxins and hereby lead to death of *Dictyostlium* (Adiba *et al.*, 2010).

Amoebae are known to share several features with human macrophages (Greub & Raoult, 2004, Tosetti *et al.*, 2014, Adiba *et al.*, 2010). Those features also include use of transition metals in bacterial killing as part of the innate immune response (Prohaska & Lukasewycz, 1981). The utilization of copper in macrophages follows the typical pattern for eukaryotic cells (Huffman & O'Halloran, 2001, Robinson & Winge, 2010): first Cu(I) is taken up into macrophages through the high-affinity plasma membrane Cu(I) permease Ctr1, and then shuttled to the P-type ATPase ATP7A within the Golgi network and endoplasmic reticulum by the copper chaperone Atox1. Cu(I) is then transported in and out of vesicles through Ctr2, where bacteria are killed by labile copper complexes that help to generate high concentrations of reactive oxygen and nitrogen species (Dupont *et al.*, 2011, Achard *et al.*, 2012, Kim *et al.*, 2008, Hodgkinson & Petris, 2012), along with other reactive transition metal complexes. However, macrophages focus more on the destruction of antigens, rather than gathering nutrients for survival, hence the sophistication of the copper acquisition and copper delivery pathway may not approach that of protozoa, although specialized macrophages are found at potential breaches - such as alveolar macrophages. As a unicellular organism, *Dictyostelium* must carry out all the life functions for the whole organism and therefore must acquire all of the necessary nutrients through phagocytic activity.

According to previous studies, several proteins homologous to the human copper transporters have been identified in protozoa, some of which were assumed to play roles in copper trafficking similar to the one in macrophages (German *et al.*, 2013).

Human macrophage harbors two Cu(I)-translocating P-type ATPases: hATP7A (NP_000043.4) and hATP7B (NP_000044.2), which are responsible for regulation and maintenance of copper homeostasis. hATP7a has been found in phagosomes (within macrophages) during the innate immune response in humans (White *et al.*, 2009). *D. discoideum* contains three homologous genes encoding putative Cu(I)-translocating P-type ATPases (XP_644454.1, XP_638720.1 and XP_646096.2). According to a previous study, at least one P-type ATPase was predicted to be responsible for copper resistance in *D. discoideum* (EC_{50}=469±30 μM) by efflux, while one or more P_{1B}-type ATPases were predicted to be involved in copper trafficking in vacuolar structures (Burlando *et al.*, 2002). Here, we monitored expressions of genes involved in metal trafficking (**Fig. 4**). We found that out of the three P-type ATPases genes, only *ATP1* was induced significantly by copper addition. This result would strongly suggest *ATP1* is induced in response to copper stress and encodes a Cu(I)-translocating P_{IB}-type ATPase responsible for copper efflux and resistance. Interestingly, all three genes encoding putative Cu(I)-translocating P_{IB}-type ATPases were highly induced during ingestion of bacteria, suggesting that during the process of phagocytosis in *D. discoideum* both copper uptake and transport into organelles such as the food vacuole are activated. These results have confirmed involvement of ATP7A and ATP3 in the copper trafficking pathway within the *D. discoideum* cell, and are consistent with a previous data locating P_{1B}-ATPases in cytoplasmic membrane and in vacuolar structures of *D. discoideum* (Burlando *et al.*, 2002). Similarly, the *p80* gene encoding a copper transporter, homologous to Ctr1 in macrophages, was induced by the presence of ingested bacteria. Simultaneous addition of copper salts and bacteria did not resulte in the expression of this copper uptake system, indicating that under normal conditions copper is taken up by p80 (Ctr1) via a mechanism similar to Ctr1 in macrophages with possible involvement of copper chaperone to further deliver Cu(I) to ATP1 or ATP7A. By taking into account localization of p80 at the cell surface and endocytic compartments reported previously (Ravanel *et al.*, 2001), one can suggest direct intake of Cu(I) into the intracellular vacuoles by p80 (Ctr1) or indirect one *via* a copper chaperone. This process could

closely resemble the copper trafficking by hATP7A in macrophages from the trans-Golgi network to late endosomal vacuoles or the plasma membrane (Petris *et al.*, 1996). Possibly, Cu(I) is taken up by p80 and delivered to ATP1 and ATP7A, where they could either pump Cu(I) directly into the phagosome or into membrane vesicles that then fuse with the phagosome. We observed elevated copper levels in *D. discoideum* after ingestion of bacteria expressing a *GFP* reporter gene under the control of a copper-responsive promoter (**Fig. 5**). These results further support our overall hypothesis for a central role of copper in protozoan killing of bacteria.

The roles of NRAMP proteins (NRAMP1 and NRAMP2) (a Natural Resistance-Associated Membrane Protein) on ion homeostasis and resistance to bacterial infection have been well studied in *Dictyostelium* (Peracino *et al.*, 2013, Bozzaro *et al.*, 2013). In this study, we looked at the expression levels of NRAMP transporters previously characterized in *D. discoideum* and responsible for iron and manganese efflux from the phagosome (Peracino *et al.*, 2006). The expression of *NRAMP2* in *Dictyostelium* was significantly induced when ingesting *E. coli* as prey ($p<0.05$), while the expression of *NRAMP1* was not affected, probably because its expression is time and dose dependent (Govoni *et al.*, 1997). Recently, it was shown that both NRAMP1 and NRAMP2 do not transport copper (Buracco *et al.*, 2015), which is consistent with the apparent insensitivity of both NRAMP1 and NRAMP2 to copper. Overall, genes involved in copper trafficking were more responsive to bacterial ingestion than *NRAMP* genes. Iron and manganese deprivation are complementary to poisoning with copper to kill bacteria.

In conclusion, our results verified that copper poisoning is employed by protozoa to first inactivate and then kill bacteria as in human macrophages. To avoid copper poisoning, bacteria, in turn, have evolved a number of copper detoxification strategies. This ongoing evolution, driven by protozoan predation, should be considered in the appearance of Cu^r determinants and pathogenicity islands in bacteria.

Experimental procedures

Strains and culture conditions

The social amoeba *D. discoideum* AX4 (provided by the Dicty Stock Center, Northwestern University, Chicago, IL, USA) was grown in modified HL5 medium (14 g/L Tryptone, 7 g/L yeast extract, 0.35 g/L Na_2HPO_4, 1.2 g/L KH_2PO_4, and 62.5 ml 224 g/L glucose) supplemented with 0.05 g/L dihydrostreptomycin-sulfate for axenic culture, or HL5 medium (Pukatzki *et al.*, 2002) with heat killed *E. coli* wild-type strain W3110 at 22°C for 3-4 days prior to experiments. Dihydrostreptomycin-sulfate was not added in HL5 medium unless specified. For some experiments we also used a cercomonad flagellate (ATCC 50334). This strain was originally derived from the strain ATCC 50316 which has recently been re-described as *Paracercomonas crassicauda* (Bass *et al.*, 2009). Hence we use this species designation here. *P. crassicauda* was maintained in HL5 medium with heat killed *E. coli* wild-type strain W3110 as food. Trypan blue at final concentration of 0.04% was used to access the cell viability of *D. discoideum* (Lambrecht *et al.*, 2013). For pre-culture, wild-type strains of *E. coli* and *P. aeruginosa*, and their copper sensitive mutants were grown in Luria-Bertani (LB) or Tryptic Soy Broth (BD Bacto™ 211825) at 37°C overnight before co-cultured with *Dictyostelium*. Detailed information on the wild-type strains and mutants are listed in Supplementary Table (**Table S1**).

Bacteria survival in Dictyostelium

To determine whether copper resistance is a prerequisite for bacterial survival in amoeba, gentamicin (Gm) protection assay was carried out to test intracellular survival of bacteria co-cultured with *Dictyostelium*. *D. discoideum* cells from mid-log cultures were collected (500×g; 4 min), washed and diluted in 1:4 solution of HL5:SorC (2.0 g KH_2PO_4, 0.29 g Na_2HPO_4, pH 6.0 ± 0.1, add 1 ml 50 mM $CaCl_2$ to 1 liters SorC solution, and autoclave) to a final concentration of 1×10^6 cell ml^{-1}. Overnight bacteria were harvested (12,000×g; 2 min), washed with HL5:SorC 1:4 solution and adjusted to OD_{600} of 0.1 (around 10^8 CFU ml^{-1}). 900 µl 1×10^6 cell $ml^{-1}$of *D. discoideum* was seeded in 24-well polystyrene plates and mixed with bacteria to MOI of 1 or 5. *D. discoideum* (900 µl, 1×10^6 cell ml^{-1}) with HL5:SorC 1:4 solution (100 µl) instead of bacteria, and HL5:SorC 1:4 solution (900 µl) instead of

Dictyostelium with 100 µl corresponding bacteria, were set as control. After 6 h of co-culture, the mixture was centrifuged at 400 ×g for 2 min and washed twice with 1 ml SorC buffer. 1 ml SorC with 400 µg ml^{-1} Gm was added and incubated at room temprature for 1 h. *Dictyostelium* cells were washed twice and lysed with 1 ml SorC containing 0.4% Triton X-100. 100 µl of the lysates were taken and plated on LB agar plates. After incubation at 37°C for 16 h, the colony-forming units were counted. All assays were performed in at least 6 replicates.

Grazing resistant capacities of bacteria against Dictyostelium

The capacities of bacteria to resist *D. discoideum* predation was measured on SM or HL5/DB plates as described previously (Froquet *et al.*, 2009). Briefly, bacterial overnight cultures were washed with DB and concentrated 4 times. 50 µl of concentrated bacterial cultures were added into different concentrations of 24-well HL5/DB-plates (2.5%-100% HL5, vol/vol) to form bacterial lawn. 5 µl of *Dictyostelium* cell suspension (0.2×10^6 cell ml^{-1}, 1,000 cells) was added to the center of a bacterial lawn. Three replicates were prepared in different 24-wells plates. Plates were incubated at 24°C and screened daily to check the phagocytic plaques. Photos from the same sample over 6 days were combined together using Adobe Photoshop CS6.

Protozoan growth experiment

Both bacteria and protozoa were centrifuged and had the media exchanged with AS medium (Rowbotham, 1983). The bacterial suspensions were diluted to OD_{600} 0.2 ($\sim2\times10^8$ CFU ml^{-1}). The suspensions of *D. discoideum* and *P. crassicauda* were diluted to 1000 and 100 cells ml^{-1}, respectively. For each bacterial strain, 50 µl bacterial suspension and 100 µl of one of the protozoan suspensions were added to three replicate wells of a 96-well plate. A control with 50 µl AS instead of bacteria was also made. The plates for *D. discoideum* and *P. crassicauda* were incubated at 22°C and at 15°C, respectively. Protozoan growth was monitored by counting the number of cells at the bottom of the well using an inverted microscope with a grid in the eyepiece (Lekfeldt & Ronn, 2008). The experiments were stopped when *D. discoideum* started aggregating, or when *P. crassicauda* became so dense that they no

longer lay in one layer on the well bottom.

Expression of Cu(I) trafficking genes in Dictyostelium

D. discoideum was co-cultured with or without *E. coli* W3110 at a MOI of 5 in the 1:4 solution of HL5:SorC supplemented with and without 5 μM $CuSO_4$•$5H_2O$ for 12 h. *D. discoideum* cells were harvest (400×g, 2 min), washed twice with SorC buffer and centrifuged at 10,000 ×g to break the cells. Total RNA of *D. discoideum* was extracted using aRNeasy mini kit (Qiagen). Total RNA of each sample was treated with DNaseI (Thermo Scientific) and reverse-transcribed using a RevertAid First Strand cDNA Synthesis Kit with Oligo $(dT)_{18}$ primer (Thermo Scientific) according to the manufacturer's instructions. qPCR was performed by LightCycler® 480II (Roche) using SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (Takara, Japan) with the following program: 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 10 s. The expression of target genes was normalized by the *rnlA* gene (encoding a mitochondrial large subunit rRNA) with the $2^{-\Delta\Delta Ct}$ method (Kuwayama & Kubohara, 2009). The primers used for qPCR were listed in Supplementary Table (**Table S2**).

Copper biosensor, GFP imaging and transmission electron microscope (TEM)

To monitor whether copper is involved in *D. discoideum* predation, a copper bioreporter strain *P. putida* (pCuS) which was kindly provided by Dr. Hu-Chun Tao of Peking University Shenzhen Graduate School (China), was used in this study. *D. discoideum* was cultured in glass bottom dishes (WillCo Wells) in 1ml 1/300 TSB with *P. putida* (pCuS) for 24 h at room temperature. Pictures were taken with a confocal microscope (Leica SP5-X) and a 63x water lens. The GFP was excited by a 405 nm pulsed diode laser and emission was monitored at 500-530 nm with a HyD detector. Image processing was done with Image-J.

P. aeruginosa was incubated with *Dictyostelium* in the 1:4 solution of HL5:SorC at a MOI of 1 for 12 h. *Dictyostelium* cells were harvested, washed by 0.1 M PB buffer (pH 7.4), and fixed with 0.8%, 1.25%, and 2.5% glutaraldehyde in 0.1 M PB buffer (pH 7.4). After fixation, cells were rinsed three times for 20 min in 0.1 M PB buffer, centrifuged at 400×g for 5 min, and fixed in 2% agar for post-fixation (1% osmium

tetroxide in 0.1 M PB buffer for 1 h). Post-fixed cells were rinsed twice in dH_2O. Dehydration process was carried out in a graded ethanol series as follows: 30% (20 min) - 50% (20 min) - 70% (20 min) - 90% (20 min) - 100% (15 min, twice), followed by embedding, semi-thin section and ultrathin sections. The cells were imaged using a transmission electron microscope (TEM) (H-7650, Hitachi).

Identification of the potential domains of ATP1, ATP7A and ATP3

The accession numbers of the three putative copper-transporting ATPases are ATP1 (XP_644454.1), ATP7A (XP_638720.1) and ATP3 (XP_646096.2). The programs Jpred4, HHpred and GlobPlot2 were used to determine the structured regions of each protein sequence. Jpred4 uses the JNet algorithm to predict the secondary structure of a protein (Cuff & Barton, 2000, Cuff *et al.*, 1998). HHpred is a homology detection and structure prediction tool, while GlobPlot2 predicts the tendency of a protein for disorder and globularity. Sequences identified as structured portions of each ATPase were then used to predict the three dimensional structure of potential domains of the protein using Phyre2. The predicted N-terminal metal binding domain (colored orange in **Fig. S2A**) and metallothionein-type domains $[CX_3CC]_5$ and $[CX_3CC]_4$ were modeled individually since Phyre2 could not model the entire ATP3 as one continuous structure. $[CX_3CC]_5$ and $[CX_3CC]_4$ are presented as globes. Phyre2 was used to model and analyze the secondary and tertiary structure of a protein (Kelley *et al.*, 2015). Phyre2 model structures were further processed using PyMOL. Sequences of homologous proteins with similar structure and functions (CopA from *Legionella pneumophila* and Human ATP7A also called the Menkes disease protein) were aligned using ClustalW2.

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Christensen (Center for Advanced Bioimaging, CAB) and Birger Brodin Larsen (Department of Pharmacy, University of Copenhagen) for help with confocal laser microscopy.

Author contributions

Nadezhda A. German, Yong-Guan Zhu and Christopher Rensing proposed the project. Regin Rønn and Hend A. Alwathnani helped to design the experiments. Xiuli Hao, Freja Lüthje, and Fuyi Huang performed the experiments. David Huffman and Javan Kisaka analyzed the three Cu(I)-translocating P-type ATPases. Xiuli Hao and Freja Lüthje did data analysis. Xiuli Hao, Freja Lüthje, Regin Rønn, Javan Kisaka, David Huffman and Christopher Rensing participated in the manuscript writing. Xuanji Li contributed during the revision process.

Competing Financial Interests statement

The authors declare no competing financial interests.

Supplementary Information

Fig. S1 to S4

Table S1 to S2

Supplementary References

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Figure Legends

Fig. 1 Copper resistance determinants enhance the number of bacteria recovered from *Dictyostelium*. **(A)** *D. discoideum* was inoculated with *E. coli* strains at a multiplicity of infection (MOI) of 1 for 6 h. The viability of intracellular bacteria was evaluated by colony-forming unit (CFU) method with at least six independent replicates as described in methods. *E. coli* with deleted Cu(I)-translocating P_{1B}-type ATPase (*copA*), iron uptake transproters (*feoAB* and *entC*) and manganese uptake transporter (*mntH*) were more sensitive to *D. discoideum* predation than their wild type parental strain W3110 ($p<0.01$). **(B)** *D. discoideum* was inoculated with *E. coli* strains at a MOI of 5 for 6 h. The number of intracellular bacteria was determined as described in Fig. 1A. **(C)** *D. discoideum* was inoculated with *E. coli* strains in the presence of 10 ppm copper at a MOI of 5 for 6 h. **(D)** *D. discoideum* was inoculated with *P. aeruginosa* wild-type strain PAO1, copper sensitive mutants 2065 (Δ*pcoA*) and 3920 (Δ*cueA*) at a MOI of 5 for 6 h. Mutant with deleted Cu(I)-translocating P_{1B}-type ATPase (*cueA*) showed a dramatic reduced number of bacteria recovered from *Dictyostelium* ($p<0.01$). All data are presented as the mean ± SEM of at least six independent replicates. The significance of difference between wild-type and mutant strains was indicated by *** $p<0.01$ or * $p<0.05$ (one-way ANOVA, followed by Duncan's test).

Fig. 2 Assessment of bacterial grazing resistance to *Dictyostelium*. **(A)** *E. coli* W3110 and mutant strains deficient in different copper export systems including 258 (Δ*copA*), 75 (Δ*cusCFBA*Δ*cueO*), and 342 (Δ*copA*Δ*cusCFBA*Δ*cueO*) were applied on 2.5% HL5-agar to form a bacterial lawn (black). 1,000 *Dictyostelium* cells were dropped onto the center of bacterial lawn and culture at 24 ^{o}C for 6 days. The differences in grazing resistance between the wild-type strain W3110 and copper sensitive mutants were evaluated through the formation of phagocytosis plaques (white). **(B)** The grazing resistant capacities of *P. aeruginosa* wild-type strain PAO1, copper sensitive mutants 2065 (Δ*pcoA*) and 3920 (Δ*cueA*) to *Dictyostelium* was

assessed by depositing 1,000 *Dictyostelium* on bacterial lawn grown on 5% HL5-agar at 24 °C for 5 days.

Fig. 3 Effects of bacterial copper resistance on the growth of protozoa. **(A)** 100 µl of *D. discoideum* (1,000 cells ml^{-1}) was co-cultured with 50 µl of bacteria (2×10^8 CFU ml^{-1}) at 22°C in AS medium, where the growth of protozoa was fully dependent on predation. Bacteria used as the only food source were *E. coli* wild type strain W3110 (–■–) and mutant strains with interruption of different Cu^r determinants including 258 (Δ*copA*) (–▲–) and 75 (Δ*cusCFBA*Δ*cueO*) (–▼–). AS medium was used instead of bacteria in "No bacterial" control (–☆–). Counting of protozoan numbers was carried out at interval time with an inverted microscope before *D. discoideum* starts aggregating. **(B)** 100 µl of *P. crassicauda* (100 cells ml^{-1}) was co-cultured with 50 µl of bacteria (2×10^8 CFU ml^{-1}) at 15°C in AS medium until forming a monolayer on the bottom of 96-well plate. Bacteria used for *P. crassicauda* feeding were the same as described in Fig. 3A. All data are presented as the mean ± SEM from at least three replicate wells. Blue and red asterisks indicate the significance of difference between wild type strain W3110 and 258 (Δ*copA*) and between W3110 and 75 (Δ*cusCFBA*Δ*cueO*), respectively (*** $p<0.01$; * $p<0.05$; Student's t test).

Fig. 4 Bacteria infection triggers copper trafficking in *D. discoideum* predation. Relative expressions of *D. discoideum* genes involved in copper trafficking treated with copper or/and *E. coli* W3110 infection. *D. discoideum* cells suspended in HL5:SorC solution (1:4) were treated with 5 µM $CuSO_4•5H_2O$ (A+Cu), W3110 infection at a MOI of 5 (AB), induction of both copper and W3110 (AB+Cu), or were left untreated (A) at 22 °C for 12 h. Expressions of *APT1* **(A)**, *ATP7A* **(B)** and *ATP3* **(C)** encoding three putative P-type ATPases, *p80* **(D)** encoding a copper transporter, as well as *NRAMP1* **(E)** and *NRAMP2* **(F)** encoding iron or manganese transporter were calculated relative to *rnlA*. Results were compared with untreated control and expressed as $2^{-\Delta\Delta CT}$. All data are presented as the mean ± SEM. The significance between untreated cells and bacteria or/and Cu treatments was indicated by ***

p<0.01 or * p<0.05 (one-way ANOVA, followed by Duncan's test).

Fig. 5 Bacteria infection triggers elevated copper level in *D. discoideum*. *D. discoideum* was incubated with the copper biosensor *P. putida* (pCuS-*GFP*) for 24 h in glass bottom dishes (WillCo Wells), and were imaged using a confocal microcope (Leica SP5-x). The expression of GFP in *P. putida* can be induced by elevated copper in phagosomes of *Dictyostelium*.

Fig. 6 Growth of protozoa fed with copper resistant *E. coli* strains isolated from pigs supplemented with copper salts as a growth promoter. *D. discoideum* **(A)** and *P. crassicauda* **(B)** were co-cultured with three copper resistant *E. coli* isolates including 77-3009-5 (copper island) (), 77-30013-2 (copper island) (), and 77-30253-3 (yersiniabactin) () in AS medium, respectively. AS medium was used instead of bacteria in "No bacterial" control (). The methods used for co-culture and protozoan counting were the same as described in Fig. 3. Red, blue and dark yellow asterisks indicate the significance of difference when compared 77-3009-5 (copper island), 77-30013-2 (copper island) and 77-30253-3 (yersiniabactin) with "No bacterial" control, respectively (*** p<0.01; * p<0.05; Student's t test). **(C)** *D. discoideum* viability was determined when co-cultured with three copper resistant *E. coli* isolates (A09: 77-3009-5, A13: 77-30013-2, and A253: 77-30253-3) in HL5/SorC (1:4) medium at a MOI of 1 and 5, respectively (orange: dead; blue: live). *D. discoideum* cells without bacteria (A) were set as control. All treatments were cultured at 22°C and counted using hemacytometer. Before counting, the culture was mixed with trypan blue to a final concentration at 0.04% to evaluate the viability of *D. discoideum*. All data are presented as the mean ± SEM from at least three replicate wells.

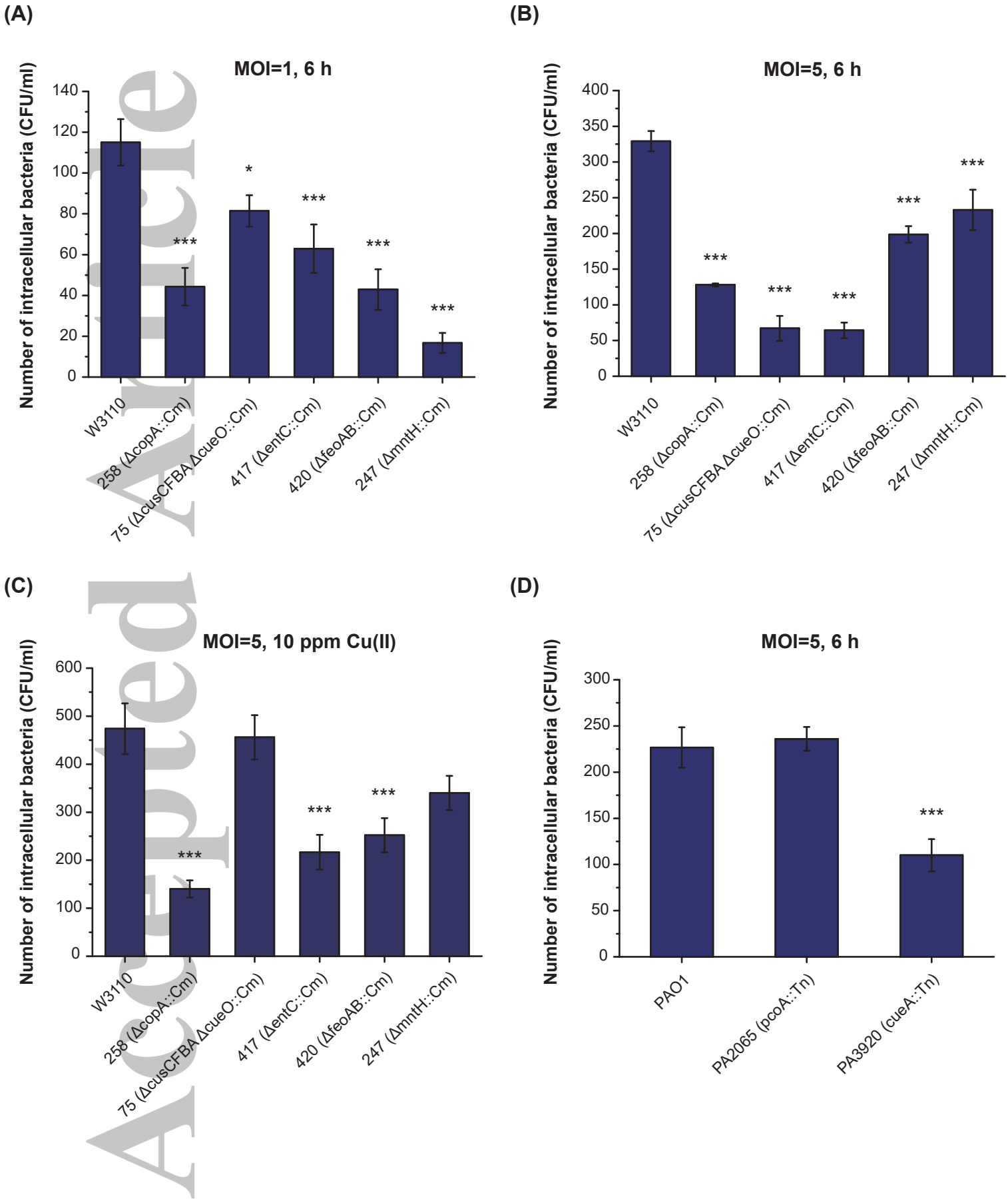
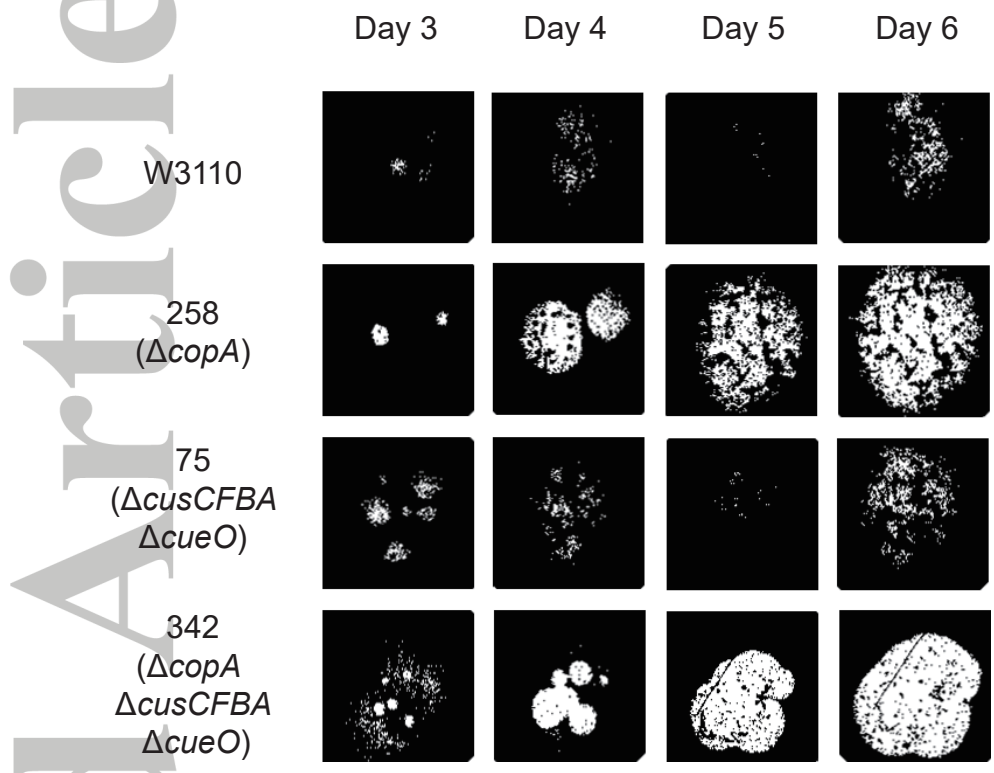


Figure 1

(A)



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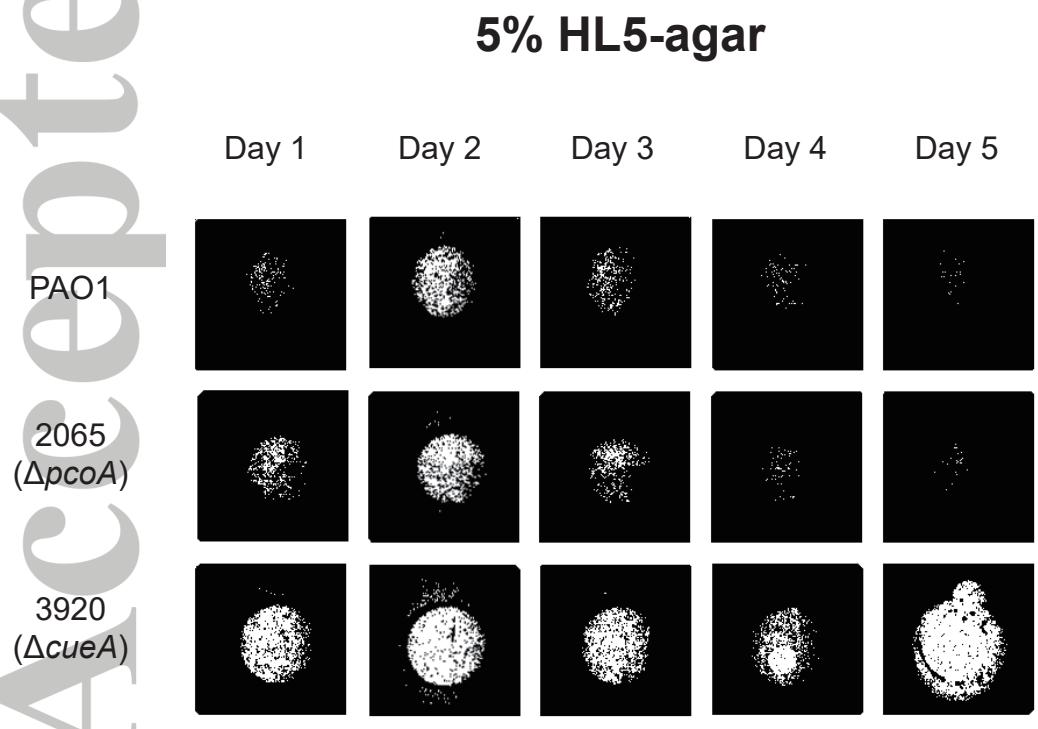
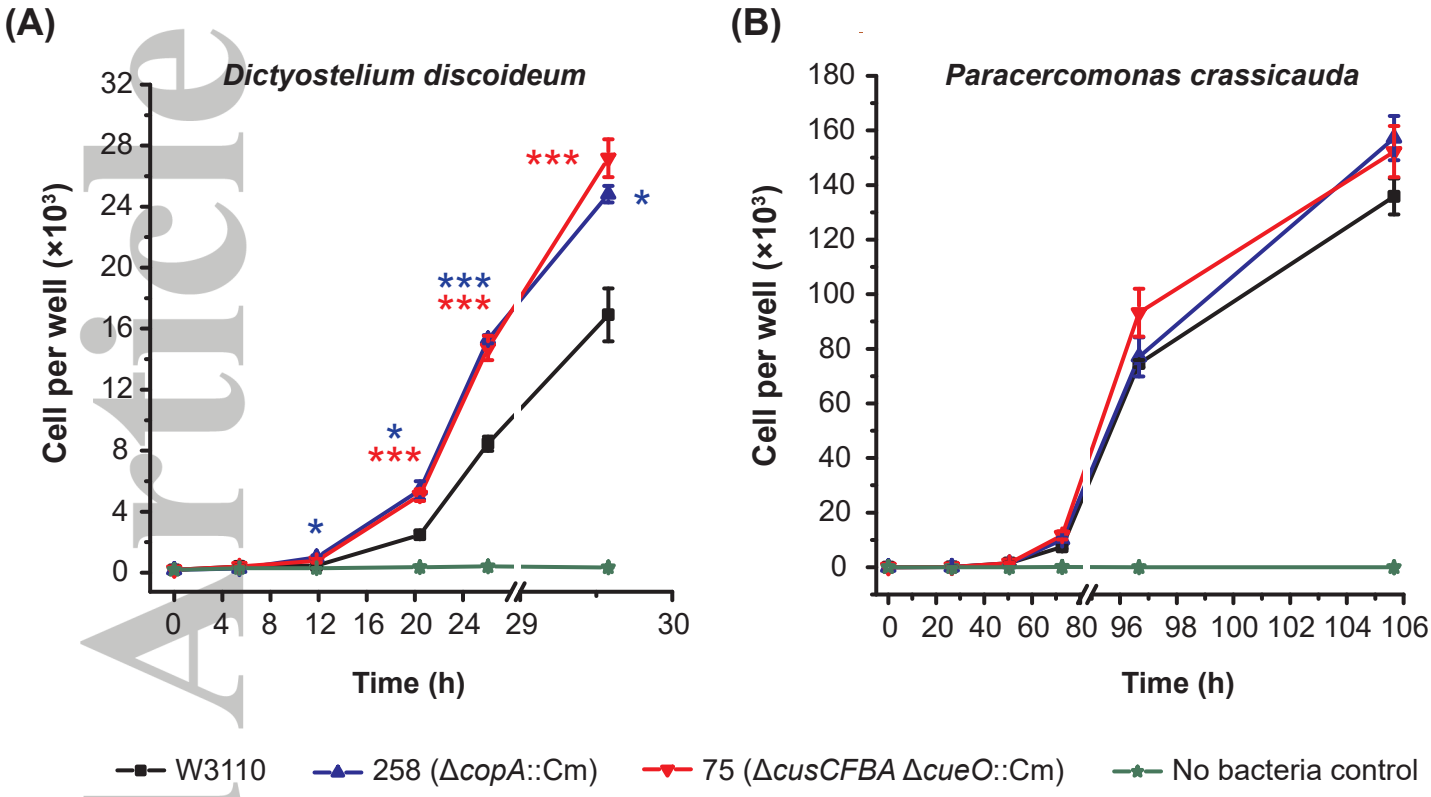
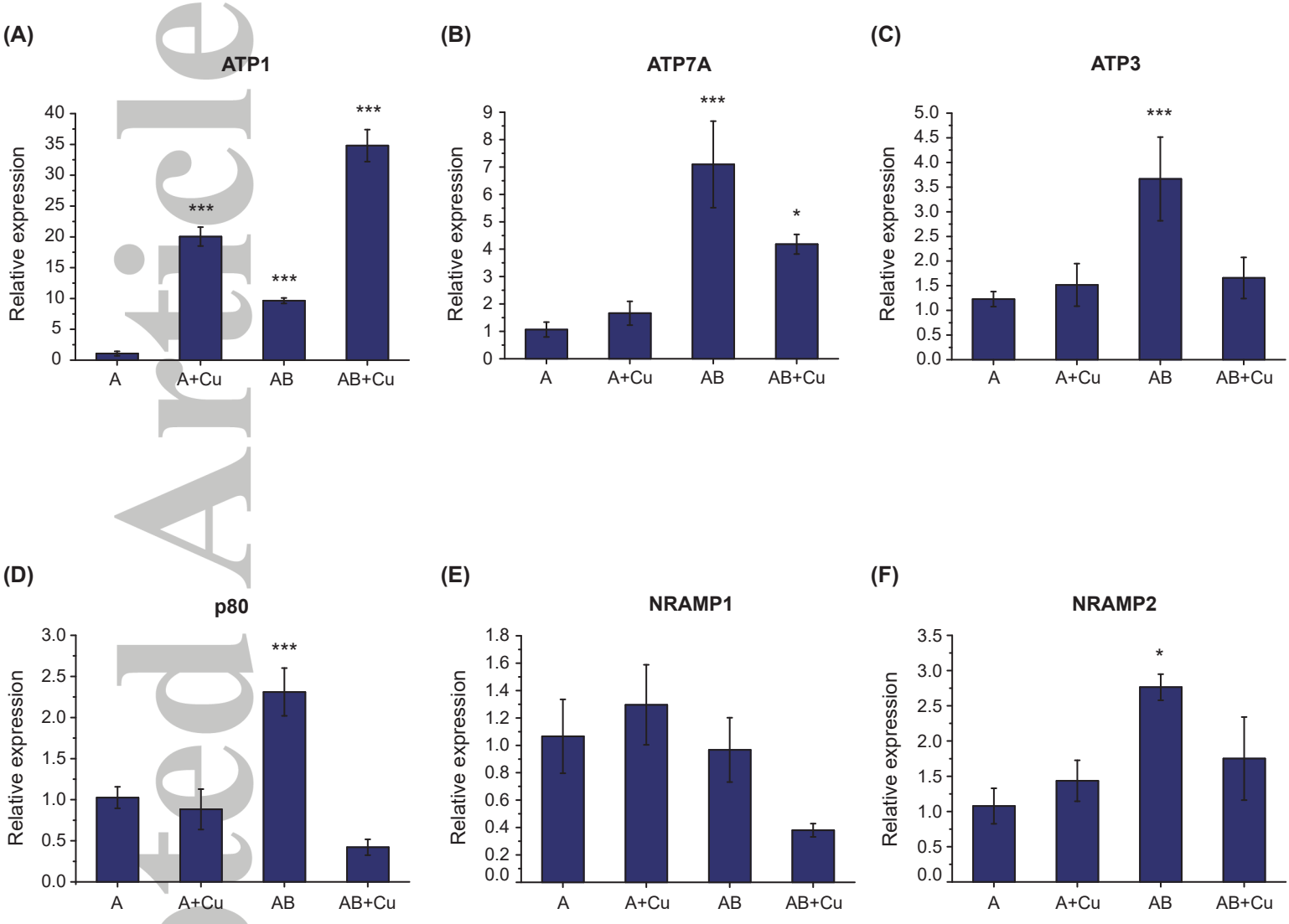
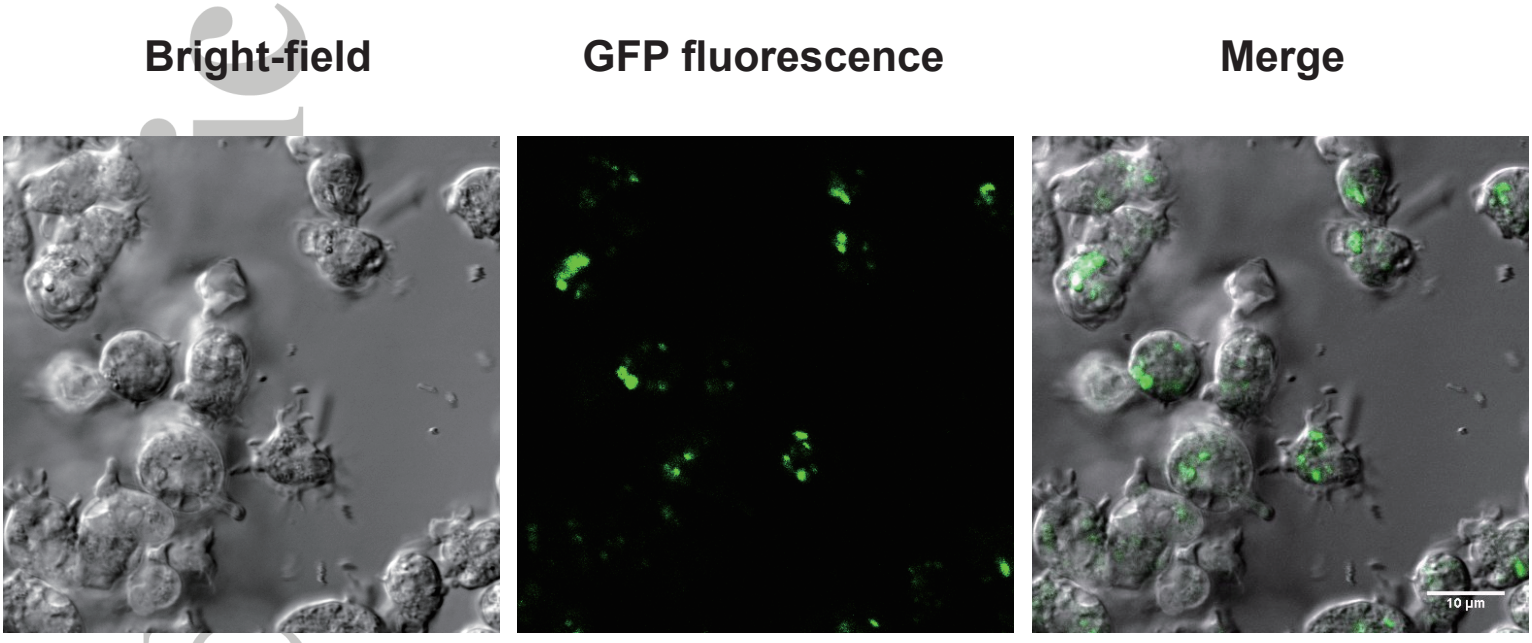
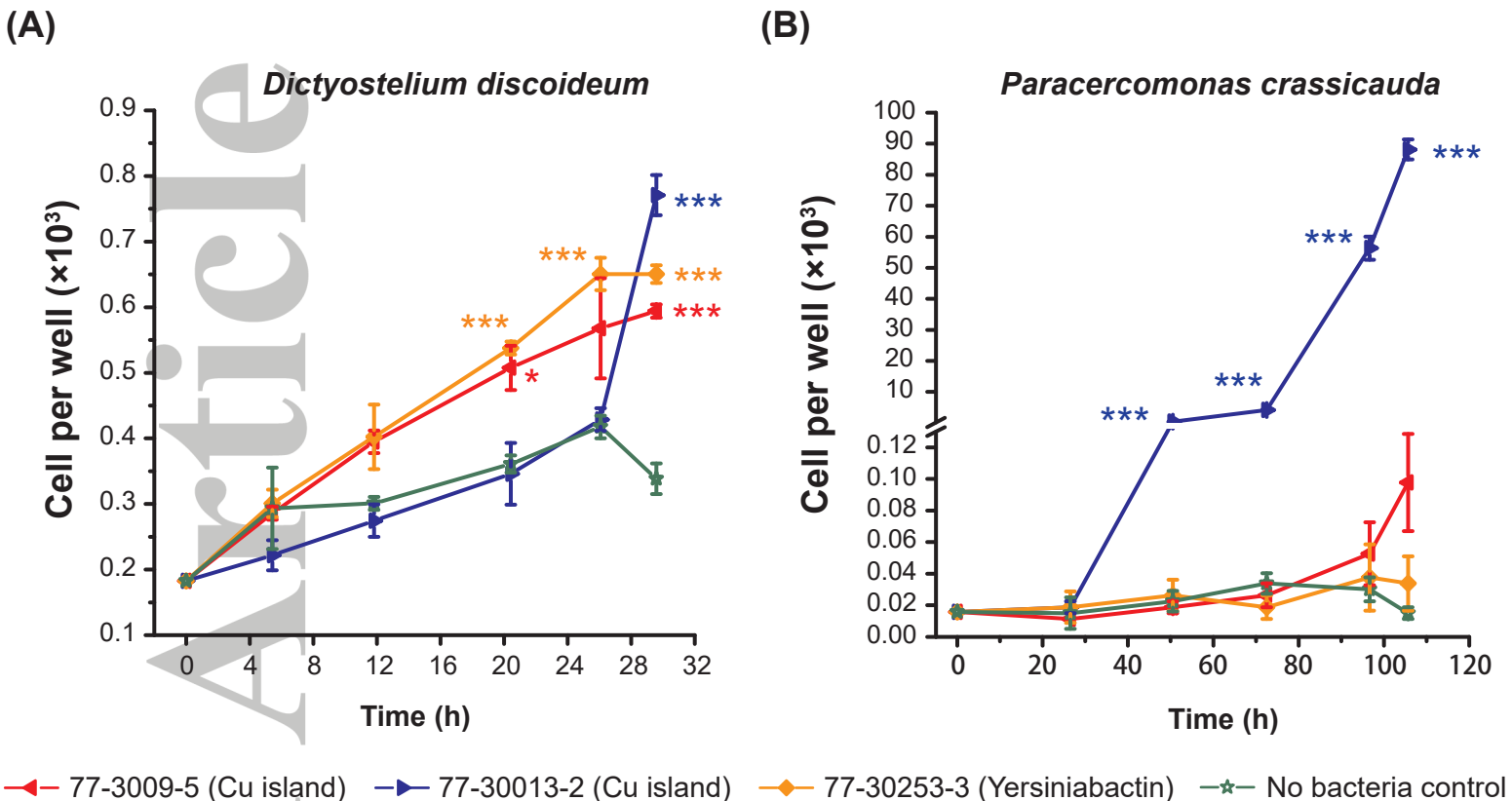
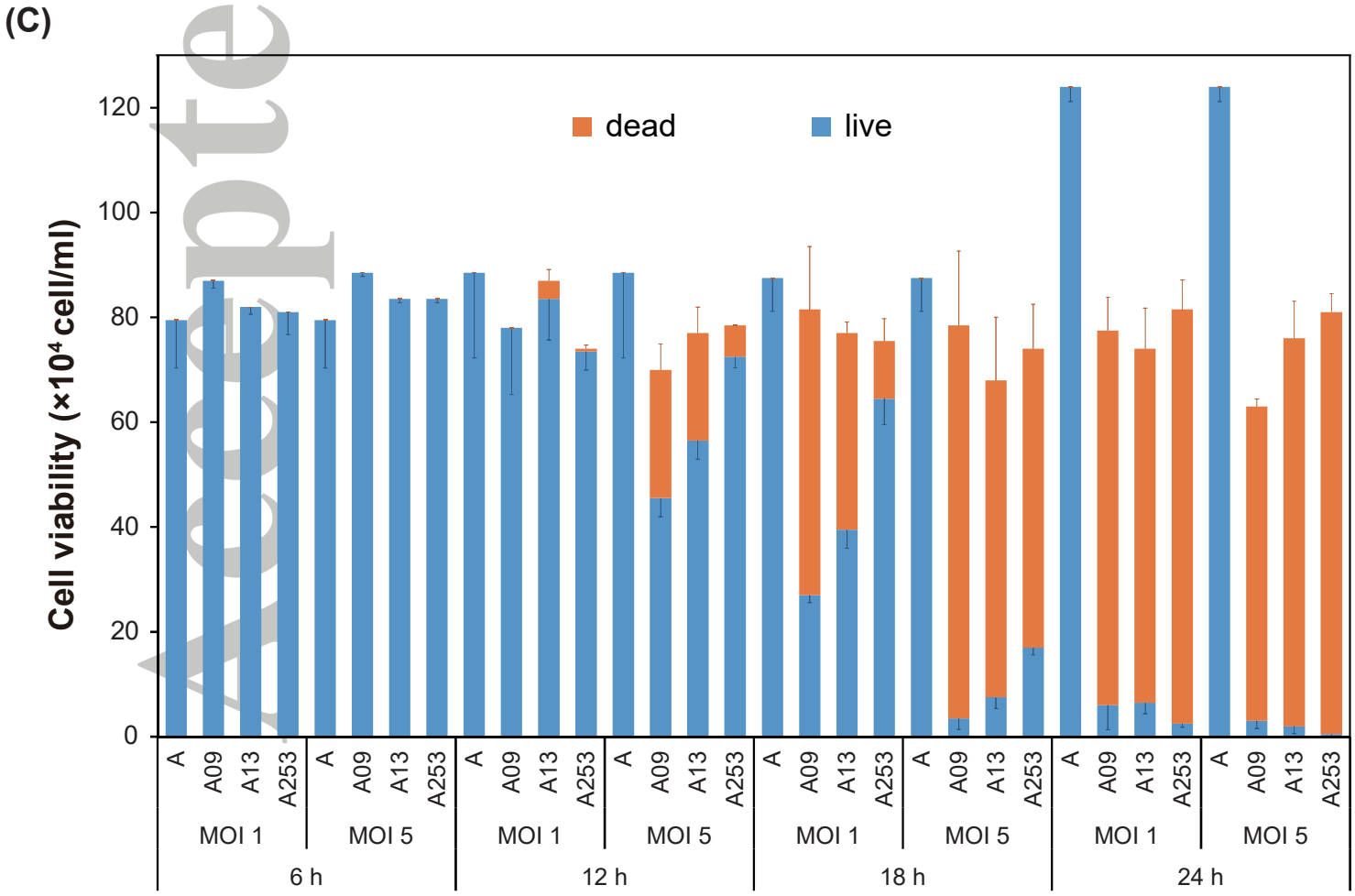


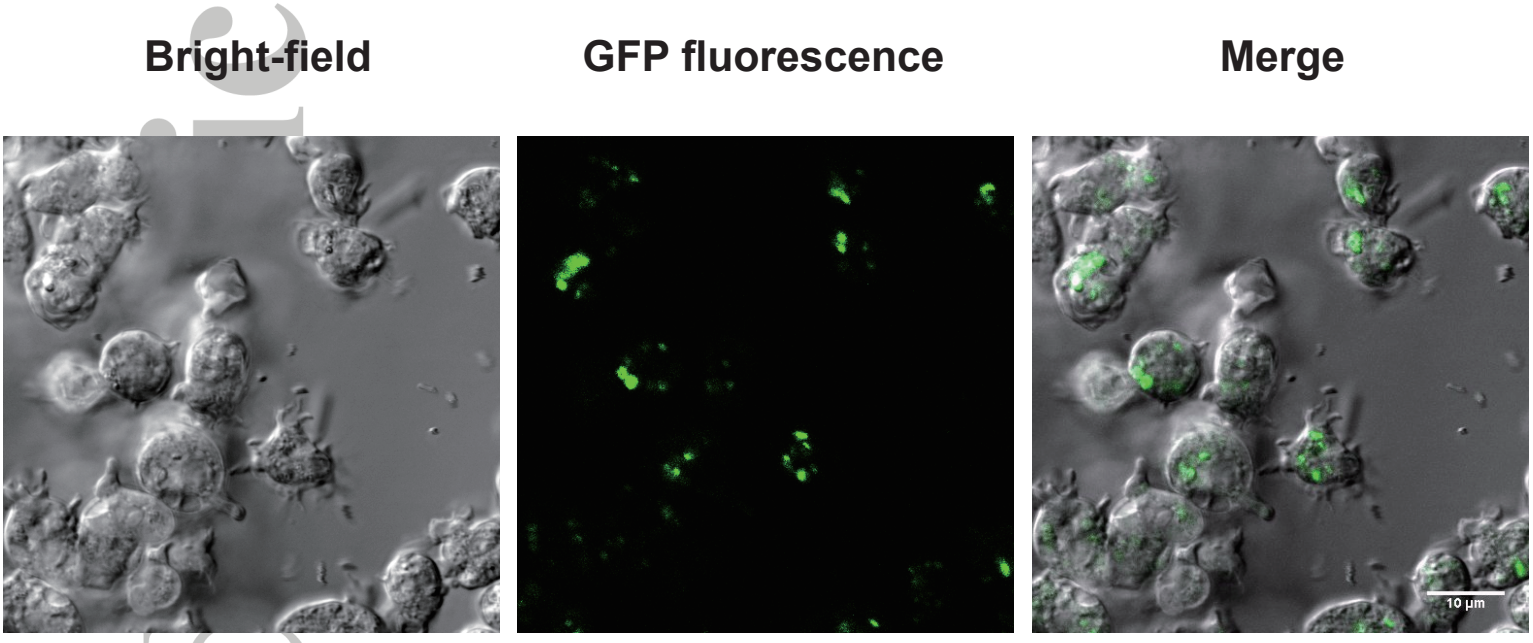






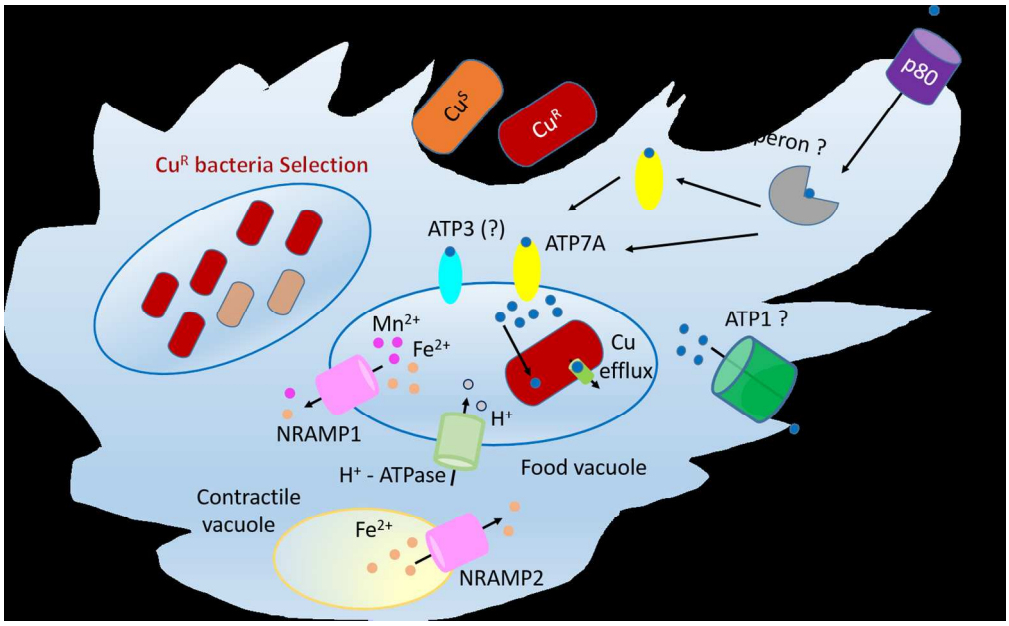






Abstract summary

Protozoans are ubiquitous bacterial predators and pathogen reservoirs. We have characterized the protozoan use of copper as an efficient weapon during predation. Thus copper poisoning imposes a selective pressure upon copper resistance determinants in bacteria.



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