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Inhibition of copper uptake in yeast reveals the copper transporter Ctr1p as a potential molecular target of saxitoxin

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Abstract

Saxitoxin is a secondary metabolite produced by several species of dinoflagellates and cyanobacteria which targets voltage-gated sodium and potassium channels in higher vertebrates. However, its molecular target in planktonic aquatic community members that co-occur with the toxin producers remains unknown. Previous microarray analysis with yeast identified copper and iron-homeostasis genes as being differentially regulated in response to saxitoxin. This study sought to identify the molecular target in microbial cells by comparing the transcriptional profiles of key copper and iron homeostasis genes (*CTR1*, *FRE1*, *FET3*, *CUP1*, *CRS5*) in cells exposed to saxitoxin, excess copper, excess iron, an extracellular Cu(I) chelator, or an intracellular Cu(I) chelator. Protein expression and localization of Ctr1p (copper transporter), Fet3p (multicopper oxidase involved in high-affinity iron uptake), and Aft1p (iron regulator) were also compared among treatments. Combined transcript and protein profiles suggested saxitoxin inhibited copper uptake. This hypothesis was confirmed by intracellular Cu(I) imaging with a selective fluorescent probe for labile copper. Based on the combined molecular and physiological results, a model is presented in which the copper transporter Ctr1p serves as a molecular target of saxitoxin and these observations couched in the context of the eco-evolutionary role this toxin may serve for species that produce it.

Keywords

saxitoxin; copper transporter; *FET3*; trace metal uptake; *Saccharomyces cerevisiae*

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Supporting Information Available

Details on total RNA extraction, gene expression calculations, a table listing primer and probe sequences of genes examined via qRT-PCR, and preliminary protein profiling experiments can be found in the Supplementary Information. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

Introduction

Over the past several decades, the occurrence of harmful algal blooms (HABs) has increased in both frequency and geographical distribution, such that every coastal nation is now affected [1]. Some HAB species produce secondary metabolites that, via bioaccumulation in filter-feeders and subsequent transfer through the food web, are detrimental to both human and ecosystem health. Saxitoxin is one such compound. The parent molecule of the Paralytic Shellfish Toxins, it is often referred to as a potent neurotoxin due to its ability to block the passage of nerve impulses in humans and other mammals [2].

The long-established molecular target of saxitoxin and its analogues is the voltage-gated sodium channel in nerve and muscle cells [3], to which saxitoxin binds with high affinity. More recently, saxitoxin has also been shown to target voltage-gated potassium and calcium ion channels [4, 5]. The alpha subunit of voltage-gated ion channels contains the central pore region, which defines channel selectivity and permeability, as well as the voltage sensor domains [6]. Within the sodium channel, extracellular loops between the fifth and sixth transmembrane helices of each domain back into the membrane to form the outer lining of the pore and the selectivity filter, within which are found two clusters of predominantly negatively charged residues that contribute to toxin sensitivity [7]. The positively-charged 7,8,9-guanidinium moiety of saxitoxin has been identified as the active group in channel binding [8]. As guanidinium is able to act as a cationic substitute for sodium ions in action potential generation, it has been proposed that the 7,8,9-guanidinium moiety enters the channel, while the remainder of the toxin blocks further ion passage into the channel [9]. Thus, saxitoxin is able to effectively block the inward flow of sodium ions into the cell, resulting in death due to respiratory paralysis.

While the ill effects of saxitoxin and its analogues are well-documented with regards to human health, its role in phytoplankton ecology is less clear. Many theories have been proposed as to saxitoxin's function, including: (1) its use as a nitrogen storage reserve due to the high number of nitrogen atoms found within its structure [10]; (2) its involvement in chromosome structural organization in a manner similar to that of polyamines and cations [11]; (3) its use as a chemical defense against zooplankton predators [12, 13]; and (4) its use as a pheromone [14]. While both bacterial and eukaryotic microbes possess voltage-gated sodium channels [15-17], these proteins, at least in bacteria, are not affected by saxitoxin or its derivatives [18] (no data currently exists as to saxitoxin's effects on the channels of eukaryotic microbes). Though it has been shown to affect sodium and potassium uptake and homeostasis in bacterial species [19], the molecular target(s) of saxitoxin in microbes remains unknown. Overall, limited molecular data exist on this topic, and elucidating the molecular target of saxitoxin in aquatic microbial systems can provide insights as to its role in the ecology of the phytoplankton that produce it; this information might then be extrapolated to aid in prediction and mitigation of HABs.

The yeast *Saccharomyces cerevisiae* provides a tractable model for elucidating molecular targets, as its genome has been sequenced and the molecular and biological functions of many of the genes determined. Additionally, the yeast system contains genes with homologs in many other organisms [20]. Prior microarray analysis with *S. cerevisiae* identified copper and iron homeostasis genes as significantly differentially expressed following saxitoxin exposure [21]. Copper and iron homeostasis are tightly linked in yeast due to the requirement of copper ions for a functional high-affinity iron uptake system (Fet3/Ftr1) [22]. The mechanisms of both systems have been well-characterized [23, 24] and are summarized in Figure 1. Though *S. cerevisiae* is not a common member of aquatic communities, numerous other yeast can be found in these systems [25-27] and thus baseline molecular studies with saxitoxin and *S. cerevisiae* are applicable to these and other aquatic microbes.

For example, genetic evidence suggests some diatoms and other algae contain homologues of the yeast ferric reductase, and transcript data suggest the presence of a high-affinity iron transport system in the diatom *Thalassiosira pseudonana* similar to that of yeast [28, 29]. Evidence for a copper-dependent high-affinity uptake system in phytoplankton is also supported by studies showing that some diatom species exhibit an increase in copper demands in response to iron limitation [30], or decreased iron uptake due to decreased copper [31].

The current study sought to determine the molecular target of saxitoxin in microbial eukaryotes using a combination of transcript and protein profiling in yeast cells exposed to saxitoxin, excess copper, excess iron, and the copper chelators bathocuproinedisulfonate and neocuproine. A selective Cu(I) fluorescent sensor was then used to compare copper uptake in saxitoxin-exposed and control (water-exposed) cells.

Methods and Materials

Strains and Culture Conditions

Saccharomyces cerevisiae S288C (ATTC #204508: MAT α *SUC2 mal mel gal2 CUP1 flo1 flo8-1 hap1*) was used for transcriptional profiling and intracellular imaging. Three strains derived from the haploid parent strain *S. cerevisiae* BY4741 (ATCC 201388: MAT α *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*), all containing a C-terminal chromosomal green fluorescent protein (GFP) tag, were used for protein profiling: Ctr1-GFP, Fet3-GFP, and Aft1-GFP [32]. All strains were maintained on synthetic complete (SC) plates (2% dextrose, 0.67% yeast nitrogen base without amino acids, and supplemented with amino acid mixture plus adenine (20 μ g mL⁻¹) and uracil (20 μ g mL⁻¹), 15 g L⁻¹ agar) [33]. Unless otherwise noted, individual colonies were inoculated into liquid SC (SC minus agar, pH 5.3-5.5) and grown overnight at 30 °C with agitation (200 rpm) to mid-exponential phase (OD₆₀₀=1.0) prior to use in all experiments.

Chemicals

Saxitoxin (MW 299.3) was purchased from National Research Council Canada (NRCC) (CRM-STX-e). Saxitoxin was lyophilized, concentrated, and re-suspended in sterile deionized water (pH 5.5) at a final concentration of 330 μ M. Copper (as cupric sulfate, CuSO₄), iron (as ferric sulfate, Fe₂SO₄³), bathocuproinedisulfonate (BCS), and neocuproine (as neocuproine hydrochloride monohydrate) were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions were prepared in deionized water, filter-sterilized through 0.2 μ M nitrocellulose filters, and stored in sterile, light-tight polycarbonate bottles. Working solutions were freshly prepared in sterile deionized water at concentrations that allowed for volumes similar to that of saxitoxin to be added to the cultures. Coppersensor-1 (CS1) was prepared and characterized according to previously reported procedures [34, 35].

Growth Curves

S. cerevisiae was grown overnight in SC broth and re-inoculated into fresh SC at a starting OD₆₀₀=0.06–0.08. Growth curves were conducted at 10 mL and 400 μ L volumes (the same volume as gene expression experiments) to ensure that growth was not affected by culture size. The OD₆₀₀ of 10 mL cultures was measured with a Beckman DU-600 Spectrophotometer; a Biophotometer was used to measure 400 μ L cultures. The following ranges of concentrations were used for each compound: 0–40 μ M copper, 0–40 μ M iron, 0–50 μ M BCS, and 0–10 mM neocuproine. Neocuproine growth curves were conducted over a 4 h time span rather than overnight.

Transcriptional Profiling

All experiments were conducted at compound concentrations that did not affect growth (data not shown). Four hundred μL from 10 mL overnight cultures of *S. cerevisiae* S288C were transferred to sterile 2 mL tubes and compounds added to the following final concentrations: saxitoxin, 16 μM ; copper sulfate, 8 μM ; iron sulfate, 40 μM ; BCS, 10-50 μM ; neocuproine, 5 mM. Controls consisted of filter-sterile deionized water. Upon addition of each compound, samples were mixed gently and incubated with moderate agitation at 30 °C for 45 min. Cells were harvested via sequential centrifugations that included a single washing with 300 μL nuclease-free water and pellets stored immediately in liquid nitrogen. Total RNA was extracted following a protocol described previously [21]. RNA concentration and purity were assessed with the Nanodrop-1000 (Nanodrop Technologies Inc., Wilmington, DE). Samples were stored at -80 °C until gene expression analysis.

Quantitative RT-PCR (qRT-PCR) based on Taqman® probe methodology was used to measure changes in expression of the following copper and iron-related genes: *CTR1*, *CUP1*, *CRS5*, *FET3*, and *FRE1*. Primers and probes were designed using Primer3 software to target an approximately 100 bp region each gene, with an annealing temperature of 60 °C. The primers and probe for each target in *S. cerevisiae* have been described previously [21] and are re-iterated in Table S1, along with the biological or molecular function of each gene. Oligonucleotide primers and (FAM)-BHQ probes were obtained from Biosearch Technologies (Novato, CA). Reactions were performed in triplicate in a DNA engine equipped with the Chromo4 detector (MJ Research Inc., Waltham, MA). The Quantitect Probe RT-PCR kit (Qiagen, Valencia, CA) was used for all assays. Reactions consisted of 12.5 μL 2x Quantitect Probe RT-PCR Master Mix, 600-800 nM primers, 200 nM probe, 0.25 μL QuantiTect RT Mix, 5 μL of template, and adjusted to a final volume of 25 μL with nuclease-free water. Template consisted of a 1:100 dilution of the total RNA extract. The following protocol was used for all assays: 50 °C for 30 minutes, 95 °C for 15 minutes, and 45 cycles of denaturing at 94 °C for 15 seconds and annealing/extension at 60 °C for 1 min.

Changes in gene expression were measured using absolute quantification as described previously [21]. Briefly, full-length transcripts of each gene were generated via *in vitro* transcription and used to create standard curves for each assay. Data were measured as transcript copies per ng total RNA for each functional gene followed by normalization to each sample's corresponding *ACT1* transcript copies per ng total RNA ("normalized transcript copies"). Data were analyzed for statistical significance (SPSS v.15, Chicago, IL) between exposed and control samples with the independent samples *t*-test if data passed the tests for equal variance (Levene's test) and normality (Shapiro-Wilkes). The Mann-Whitney U-test with Monte Carlo correction was applied if samples were not normally distributed.

Protein Profiling

Preliminary experiments were conducted at a range of time points to select the earliest time point at which fluorescence was observed under at least one of the conditions. Each strain was viewed at 2 h, 4 h, 6 h, and overnight incubation with compounds.

Based on preliminary experiments (presented in Supplementary Information), Fet3-GFP and Aft1-GFP cells were grown overnight to mid-exponential phase ($\text{OD}_{600}=1-1.2$). The next morning, 100 μL aliquots were distributed into sterile microcentrifuge tubes and incubated in the dark with each compound for 2 h. Ctr1-GFP cells were grown in SC for 5 hours to an $\text{OD}_{600}=0.2$, whereupon 100 μL aliquots were distributed into microcentrifuge tubes and incubated in the dark overnight. All strains were exposed to the following compounds: iron sulfate (40 μM), BCS (10 μM), saxitoxin (16 μM), copper sulfate (10 μM). Fet3p-GFP and Ctr1p-GFP were exposed to BCS (10 μM); Aft1p-GFP was exposed to neocuproine (5 mM).

The iron regulator Aft1p-GFP was exposed to neocuproine rather than BCS so as to avoid perturbations to Fre1p, as BCS would bind extracellular copper, likely repressing *FRE1* and thus affecting Aft1p expression. Water served as the control for all treatments. The final copper concentration was increased from 8 μM in the expression profiling experiments to 10 μM for visualization of protein localization, as previous studies showed that the amount of copper needed to observe degradation of Ctr1p is 10 μM [36]. Preliminary experiments conducted here at a range of copper concentrations (8-20 μM) confirmed this (data not shown).

Following exposures, cells were washed and re-suspended in 0.5 volumes 1x PBS under dim-light conditions and 4 μL aliquots spotted onto positively-charged slides (AmFrost Ultra+, AmLabs, Boulder, CO). Slides were examined immediately by epifluorescence microscopy (Nikon Eclipse Ti-E, Nikon Instruments Inc., Melville, NY) using a 60x objective lens (NA 1.49). Both differential interference contrast (DIC) and GFP-fluorescence images were captured. Display settings for fluorescence data were standardized for each GFP-fusion protein. Thus, images from different treatments of the same protein are directly comparable, but fluorescence intensities are not comparable between different GFP-fusion proteins. Protein localizations were assigned based on GFP fluorescence.

Visualization of Labile Intracellular Copper Levels

Coppersensor-1 (CS1) was synthesized and employed to compare labile intracellular copper levels in saxitoxin-exposed versus untreated (i.e. water-exposed) cells. CS1 is a membrane-permeable fluorescent dye comprised of a boron dipyrromethane (BODIPY) chromophore coupled to a thioether-rich receptor whose fluorescence increases ca. 10-fold upon binding to Cu(I) [34, 35]. The dye forms a stable and selective complex with Cu(I) over other biologically relevant metal cations in aqueous solutions [34, 35]. CS1 provides a qualitative readout for labile copper pools, and was used here to determine if saxitoxin inhibited copper uptake in yeast cells. To visualize intracellular labile copper levels, cultures were grown overnight in SC media as described above. Upon reaching $\text{OD}_{600}=1.0$, 50 μL aliquots were transferred to sterile microcentrifuge tubes, and incubated with saxitoxin (final concentration 16 μM), or sterile DI water (saxitoxin carrier) for 45 min (the same exposure time used for transcriptional profiling). Cells were then supplemented with 20 μM CuSO_4 and incubated an additional 15 min. Following incubation, cells were washed three times, re-suspended in an equal volume of PBS, and stained with 2.4 μM CS1 in the dark for 10 min at 30 °C. After incubation, cells were washed twice in PBS to remove excess dye, re-suspended in PBS and examined immediately by epifluorescence microscopy (ex/em 473-491/513-533 nm). Both DIC and CS1-fluorescence images were captured. Control experiments were performed with a cell-permeable copper chelator in order to confirm that observed fluorescence change was due to copper binding [35]. Cells were grown as described above, and incubated with 40 μM CuSO_4 for 45 min. The copper chelator *N*-ethyl-3,6,12,15-tetrathia-9-monoazaheptadecane [35] was then added to a final concentration of 200 μM , and cells incubated an additional 10 min followed by staining with CS1 as described above.

Results

Expression Profiling

The overall transcriptional profile of copper and iron homeostasis genes following 45 min saxitoxin exposure was identical to that of excess iron and nearly identical to that of excess copper, with the exception of *FET3* (Fig. 2A-E). *FET3* remained unchanged following exposure to 8 μM copper yet was repressed greater than 3.6-fold following saxitoxin exposure, prompting the molecular comparison between saxitoxin and excess iron. Consistent with its role in high-affinity iron uptake, *FET3* was repressed greater than 2-fold

following iron exposure (Figure 2B). However, multiple proteins, including Fet3p, require copper ions to form holoenzymes; thus the effects of copper limitation on *FET3* expression were also assessed via exposure to both extra- and intracellular copper chelators. Cells were exposed to the extracellular copper chelator BCS to examine the hypothesis that saxitoxin functions in a manner similar to an extracellular chelator, thereby reducing the amount of copper ions transported into the cell. However, exposure to BCS at concentrations of 10-50 μ M resulted in little (ca. 1.2-fold) to no repression of *FET3*. Though no reports exist in the literature as to *FET3* expression when intracellular Cu(I) is limiting, we reasoned that if this were the case, *FET3* would be repressed due to its holoenzyme requirement of four copper ions. To test the hypothesis that saxitoxin was blocking copper transport into the cells, creating a copper-limited intracellular environment, *FET3* expression was examined following exposure to the intracellular Cu(I) chelator neocuproine; however, *FET3* was induced 2.6-fold following this exposure.

CTR1, encoding the plasma membrane copper transporter that serves as the primary means of copper assimilation in *S. cerevisiae*, was repressed at similar levels following exposure to saxitoxin, excess copper, or excess iron (-2.1-, -2.25-, and -1.85-fold, respectively) (Fig 2A). As expected, *CTR1* was induced following exposure to BCS, with levels of induction reaching nearly 2-fold at a concentration of 50 μ M (Fig. 2A), providing evidence that, at the transcriptional level, saxitoxin does not bind to external copper ions. *FRE1*, encoding a Cu(II) and Fe(III) reductase situated in the plasma membrane, was repressed at similar levels (-1.5 – -1.7-fold) following saxitoxin, iron, or copper exposure (Fig. 2C). As with *CTR1*, *FRE1* was induced (1.2 – 1.5-fold) following BCS exposure at all concentrations.

Genes encoding the metallothioneins *CUP1* and *CRS5* were both induced upon exposure to saxitoxin, copper, and iron. However, the level of induction varied among exposures. This was more pronounced in *CUP1*, the primary metallothionein, which was induced greater than 6.5-fold under conditions of copper excess, in comparison to the 2- to 3-fold levels of induction seen in response to saxitoxin and iron, respectively (Figure 2D). *CRS5* expression mirrored that of *CUP1*, with a greater level of induction recorded upon exposure to copper (approximately two-fold) than saxitoxin or iron (Figure 2E). As expected, *CUP1* was mildly repressed following 10 μ M BCS exposure, while *CRS5* remained unchanged.

Protein Profiling

As patterns of transcriptional regulation were identical between saxitoxin- and iron-exposed cells, expression and localization of Ctr1p, Fet3p, and the iron regulator Aft1p were compared among saxitoxin and relevant compounds via GFP fusions. If the molecular response of the cells upon saxitoxin exposure was the same as for excess iron, expression and localization of Ctr1 and Fet3 would be expected to be similar between the two treatments. Fet3p expression and localization following saxitoxin exposure most closely resembled that of BCS-exposed cells (Fig. 3A). In both treatments, Fet3p-GFP displayed patterns consistent with localization to the vacuole, plasma membrane, and nuclear periphery. Unlike transcript profiles, Fet3p-GFP was not similar between saxitoxin and iron-exposed cells. Iron exposure resulted in reduced expression and Fet3p no longer localized to the vacuole or plasma membrane. Exposure to 10 μ M copper resulted in Fet3 images consistent with plasma membrane and vacuolar localization. Unlike saxitoxin exposure, Fet3p did not localize to the nuclear periphery. Control cells (i.e., water-exposed) exhibited low levels of GFP; when visible, localization remained intracellular, consistent that of the nuclear periphery and/or endoplasmic reticulum.

Expression of the copper transporter Ctr1p following saxitoxin exposure was most similar to that of iron-exposed cells, with little to no observable GFP in most cells (Fig. 3B). These data indicate that Ctr1p was not expressed, or had been expressed at an earlier point in time

and was since degraded. (2-6 hr exposures also resulted in similar GFP expression.) Ctr1p-GFP cells exposed to BCS displayed strong GFP expression. Localization appeared intracellular, as if being trafficked to the plasma membrane, consistent with induction of Ctr1p under conditions of copper deprivation (Fig. 3B). Exposure to 10 μ M copper resulted in overall diffuse GFP expression, likely cells in process of Ctr1p degradation.

The iron regulator Aft1p localizes to the cytoplasm under iron-replete conditions, and to the nucleus under iron-depleted conditions [37]. Based on the similarity of transcriptional profiles between saxitoxin and excess iron, we hypothesized that saxitoxin-exposed cells would exhibit an Aft1-GFP profile similar to excess iron. However, Aft1-GFP profiles were not similar between saxitoxin- and iron-exposed cells. After 2 h of 40 μ M iron exposure, Aft1-GFP expression was diffuse, indicative of degradation, and spread throughout cells, consistent with localization to the cytoplasm. In contrast, saxitoxin-exposed cells displayed GFP localization consistent with that of the nucleus and plasma membrane (Fig. 3C). Overall, Aft1-GFP in saxitoxin-exposed cells most closely resembled that of neocuproine-exposed cells (Fig. 3C). Copper-exposed cells did not produce any visible fluorescence (Fig. 3C).

Visualization of Intracellular Copper Following Saxitoxin Exposure

With the exception of *FET3*, the yeast transcriptional profile upon exposure to saxitoxin was identical to that of excess copper. *FET3* repression in saxitoxin-exposed cells, coupled with a transcriptional profile identical to excess copper among the remaining copper and iron homeostasis genes, led to the hypothesis that saxitoxin inhibited copper uptake. Thus, the Cu(I)-specific fluorescent dye CS1 was used to examine intracellular copper levels in cells incubated for 45 min with (a) water or (b) saxitoxin followed by 20 μ M copper supplementation for 15 min. Cells incubated with water displayed discrete areas of fluorescence, indicative of labile intracellular Cu(I) (Fig. 4A). In contrast, no fluorescence was observed in cells exposed to saxitoxin (Fig. 4B), suggesting a reduction in labile intracellular copper pools and providing strong evidence that saxitoxin inhibits copper uptake in yeast cells. Control experiments in which cells were incubated with copper followed by supplementation with a copper chelator prior to CS1 staining also displayed little to no fluorescence, confirming that fluorescence was due to copper binding (Fig. 4C).

Discussion

Previous microarray analysis [21] led to the current hypothesis that the response of yeast upon saxitoxin exposure was similar to that as would be seen for excess copper, prompting direct molecular and physiological comparisons between saxitoxin and copper-exposed cells. In this study, the 3.6-fold level of repression in *FET3* following saxitoxin exposure as compared to unchanging expression levels after copper exposure suggested Fet3p may not be receiving an adequate supply of Cu(I) ions, due to the requirement of four copper ions for its activity [38]. The source of the copper ions needed for proper Fet3p functioning comes from the copper transporter Ctr1p [22, 39, 40], which was repressed greater than 2-fold following saxitoxin exposure. As transcript profiles of saxitoxin-exposed cells were identical to iron-exposed cells, expression and localization patterns of the iron regulator Aft1p, along with Ctr1p and Fet3p, were then compared among treatments. However, protein profiles differed between saxitoxin and iron treatments and the lack of correlation between *FET3* transcript and Fet3-GFP profiles indicates that saxitoxin alters *FET3* expression by some unknown mechanism.

Examining the structural and mechanistic properties of Ctr proteins provides a working hypothesis as to how saxitoxin may be functioning with respect to both Ctr1p and Fet3p: saxitoxin binds to the selectivity filter of Ctr1p in a manner analogous to that of sodium

channels, thereby blocking Cu(I) transport into the cells and creating a Cu(I)-limited intracellular environment. A projection structure of the human copper transporter (hCTR1) reveals a structural design closely resembling that of ion channels rather than classic transporters [41]. Additionally, the presence of a particular glycine motif (GXXXG [GG4]) on transmembrane 3 suggests a functional model similar to that of the potassium ion channel GYG motif: the GG4 motif may contribute to selectivity and gating, with the state of the gate regulated by the occupancy of both the intra- and extracellular metal binding sites [41].

In examining the structural properties of the cumulative proteins for which saxitoxin is known to bind, the theme of metal binding sites emerges. Saxiphilin (a soluble protein isolated from bullfrog [42, 43]), though a member of the Fe^{3+} -binding transferrin family, does not contain an Fe^{3+} binding site [42-44]; rather, it appears the saxitoxin binding site within saxiphilin has evolved from the transferrin Fe^{3+} binding site and now utilizes some of the amino acid residues previously used to ligate the metal [as reviewed in 18]. Pioneering studies examining saxitoxin binding in sodium and potassium channels showed that the toxin acted at a metal cation binding site, with several monovalent cations able to compete reversibly with saxitoxin for the binding sites [45].

While many theories have been proposed as to the eco-evolutionary role of saxitoxin, the data collected here yield two additional theories. The first is that saxitoxin targets membrane proteins involved in trace metal assimilation in co-existing phytoplankton species. The results of this study indicate saxitoxin targets the copper transporter, a protein that is mechanistically and structurally similar to ion channels, known targets of saxitoxin. Interestingly, studies with fish which served as framework for the current biotic ligand model showed that copper specifically targeted the sodium ion transport site [46]. As a greater proportion of the toxin is retained intracellularly rather than actively secreted in healthy natural phytoplankton populations [47], one plausible scenario is the release of large amounts of the toxin during bloom termination (i.e., cell senescence and lysis), when nutrients are likely to be limiting. (However, it must be noted that the saxitoxin concentration used here was higher than that previously recorded for extracellular concentrations (i.e., $75 \mu\text{g L}^{-1}$ in lab cultures [48], the equivalent of approximately 240 nM; and $1 \mu\text{g L}^{-1}$ in the environment [49])). In nutrient-limited waters, preventing trace metal uptake in co-existing organisms may provide a competitive advantage to toxin producers. While molecular data on mechanisms of copper and iron transport are scant in marine eukaryotic microbes, recent work provides molecular evidence for cell-surface reductases and multicopper oxidases in some species of diatoms, with physiological data suggesting a high-affinity iron uptake system similar to that of yeast [28, 50]. Collectively, these findings implore an evaluation of the relationship between the structural evolution of phytoplankton metal transporters and algal toxins.

A second theory as to the function of saxitoxin is that it alleviates metal stress in the toxin-producing cells. Copper is an essential trace element required for several important processes in phytoplankton, including photosynthesis and respiration, oxidative stress repair, and high-affinity iron transport systems. It is also a common pollutant in aquatic systems due to its use in pesticides, fertilizers, and marine antifouling agents, and is commonly used as an algaecide to treat water bodies such as lakes and dams that serve as water supply reservoirs [51, 52]. Exposure to excess copper has been shown to result in an increase in lipid peroxidation and membrane permeability in *Scenedesmus* and *Alexandrium* spp [53, 54], resulting in leakage and eventual destruction of plasma membrane integrity [55]. However, copper-resistant mutants were shown to arise in the toxic cyanobacterium *Microcystis aeruginosa* [56].

Saxitoxin is a nitrogen-rich molecule. Nitrogen metabolism is central to the response of plants and some algae to metal stress [57], including synthesis of the polyamines spermine, spermidine, and putrescine, all of which, similar to saxitoxin, are positively-charged at cytoplasmic pH. As tolerance for trace metals has been shown to be a determining factor for shifts in phytoplankton species composition [58], an enhanced understanding of the influence of copper in coastal areas may necessitate a re-evaluation of currently accepted levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wang D-Z. Neurotoxins from marine dinoflagellates: a brief review. *Mar Drugs*. 2008; 6:349–371. [PubMed: 18728731]
2. Wiese M, D'Agostino PM, Mihali TK, Moffitt MC, Neilan BA. Neurotoxic alkaloids: saxitoxin and its analogs. *Mar Drugs*. 2010; 8:2185–2211. [PubMed: 20714432]
3. Catterall, WA. The voltage sensitive sodium channel: a receptor for multiple neurotoxins. In: Anderson, W.; Baden, D., editors. *Toxic Dinoflagellates*. Elsevier Science Publishing Co, Inc.; 1985. p. 329-342.
4. Wang JX, Salata JJ, Bennett PB. Saxitoxin is a gating modifier of hERG K⁺ channels. *J Gen Physiol*. 2003; 121(6):583–598. [PubMed: 12771193]
5. Su Z, Sheets M, Ishida H, Li FH, Barry WH. Saxitoxin blocks L-type/(Ca). *J Pharmacol Exp Ther*. 2004; 308(1):324–329. [PubMed: 14566004]
6. Charalambous K, Wallace BA. NaChBac: the long lost sodium channel ancestor. *Biochemistry*. 2011; 50:6742–6752. [PubMed: 21770445]
7. Terlau H, Heinemann SH, Stuhmer W, Pusch M, Conti F, Imoto K, Numa S. Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II. *FEBS Lett*. 1991; 293:93–96. [PubMed: 1660007]
8. Kao CY, Walker SE. Active groups of saxitoxin and tetrodotoxin as deduced from actions of saxitoxin analogs on frog muscle and squid axon. *J Physiol-London*. 1982 Feb.323:619–637. [PubMed: 6284918]
9. Baden, DG.; Trainer, VL. The mode and action of toxins and seafood poisoning. In: Falconer, IR., editor. *Algal Toxins in Seafood and Drinking Water*. Academic Press; San Diego: 1993. p. 49-74.
10. Loeblich, AR, III. Dinoflagellate physiology and biochemistry. In: Spector, DL., editor. *Dinoflagellates*. Academic Press; Orlando: 1984. p. 299-243.
11. Anderson DM, Cheng TP-O. Intracellular localization of saxitoxins in the dinoflagellate *Gonyaulax tamarensis*. *J Phycol*. 1988; 24(1):17–22.
12. Teegarden GJ. Copepod grazing selection and particle discrimination on the basis of PSP toxin content. *Mar Ecol Prog Ser*. 1999; 181:163–176.
13. Zimmer RK, Ferrer RP. Neuroecology, chemical defense, and the keystone species concept. *Biol Bull*. 2007; 213(3):208–225. [PubMed: 18083963]
14. Wyatt T, Jenkinson IR. Notes on *Alexandrium* population dynamics. *J Plankton Res*. 1997; 19(5): 551–575.

15. Shimomura T, Irie K, Nagura H, Imai T, Fujiyoshi Y. Arrangement and mobility of the voltage sensor domain in prokaryotic voltage-gated sodium channels. *J Biol Chem.* 2011; 286(9):7409–7417. [PubMed: 21177850]
16. Ren D, Navarro B, Xu H, Yue L, Shi Q, Clapham DE. A prokaryotic voltage-gated sodium channel. *Science.* 2001; 294(5550):2372–2375. [PubMed: 11743207]
17. Koishi R, Xu HX, Ren DJ, Navarro B, Spiller BW, Shi Q, Clapham DE. A superfamily of voltage-gated sodium channels in bacteria. *J Biol Chem.* 2004; 279(10):9532–9538. [PubMed: 14665618]
18. Llewellyn LE. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. *Nat Prod Rep.* 2006; 23:200–222. [PubMed: 16572228]
19. Pomati F, Rossetti C, Manarolla G, Burns BP, Neilan BA. Interactions between intracellular Na⁺ levels and saxitoxin production in *Cylindrospermopsis raciborskii* T3. *Microbiol-(UK).* 2004; 150:455–461.
20. Mustacchi R, Hohmann S, Nielsen J. Yeast systems biology to unravel the network of life. *Yeast.* 2006; 23(3):227–238. [PubMed: 16498699]
21. Cusick KD, Boyer GL, Wilhelm SW, Sayler GS. Transcriptional profiling of *Saccharomyces cerevisiae* upon exposure to saxitoxin. *Environ Sci Technol.* 2009; 43(15):6039–6045. [PubMed: 19731715]
22. Dancis A, Yuan DS, Haile D, Askwith C, Eide D, Moehle C, Kaplan J, Klausner RD. Molecular characterization of a copper transport protein in *S. cerevisiae*: An unexpected role for copper in iron transport. *Cell.* 1994; 76(2):393–402. [PubMed: 8293472]
23. Eide D. The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. *Annu Rev Nutr.* 1998; 18:441–469. [PubMed: 9706232]
24. Puig S, Thiele DJ. Molecular mechanisms of copper uptake and distribution. *Curr Opin Chem Biol.* 2002; 6(2):171–180. [PubMed: 12039001]
25. Statzell-Tallman A, Scorsetti G, Fell JW. *Candida spencermartinsiae* sp nov., *Candida taylorii* sp nov and *Pseudozyma abaconensis* sp nov., novel yeasts from mangrove and coral reef ecosystems. *Int J Syst Evol Microbiol.* 2010; 60:1978–1984. [PubMed: 19783617]
26. Burgaud G, Arzur D, Durand L, Cambon-Bonavita MA, Barbier G. Marine culturable yeasts in deep-sea hydrothermal vents: species richness and association with fauna. *FEMS Microbiol Ecol.* 2010; 73(1):121–133. [PubMed: 20455940]
27. Kutty SN, Philp R. Marine yeasts - a review. *Yeast.* 2008; 25(7):465–483. [PubMed: 18615863]
28. Kustka AB, Allen AE, Morel FMM. Sequence analysis and transcriptional regulation of iron acquisition genes in two marine diatoms. *J Phycol.* 2007; 43(4):715–729.
29. La Fontaine S, Quinn JM, Nakamoto SS, Page MD, Gohre V, Moseley J, Kropat J, Merchant S. Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Euk Cell.* 2002; 1:736–757.
30. Annett AL, Lapi S, Ruth TJ, Maldonado MT. The effects of Cu and Fe availability on the growth and Cu : C ratios of marine diatoms. *Limnol Oceanogr.* 2008; 53(6):2451–2461.
31. Peers G, Quesnel SA, Price NM. Copper requirements for iron acquisition and growth of coastal and oceanic diatoms. *Limnol Oceanogr.* 2005; 50:1149–1158.
32. Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK. Global analysis of protein localization in budding yeast. *Nature.* 2003; 425(6959):686–691. [PubMed: 14562095]
33. Sherman F. Getting started with yeast. *Methods Enzymol.* 2002; 350:3–41. [PubMed: 12073320]
34. Zeng L, Miller EW, Pralle A, Isacoff EY, Chang CJ. A selective turn-on fluorescent sensor for imaging copper in living cells. *J Am Chem Soc.* 2006; 128:10–11. [PubMed: 16390096]
35. Miller EW, Zeng L, Domaille DW, Chang CJ. Preparation and use of Coppersensor-1, a synthetic fluorophore for live-cell copper imaging. *Nat Protoc.* 2006; 1(2):824–827. [PubMed: 17406313]
36. Ooi CE, Rabinovich E, Dancis A, Bonifacino JS, Klausner RD. Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. *EMBO J.* 1996; 15:3515–3523. [PubMed: 8670854]

37. Yamaguchi-Iwai Y, Ueta R, Fukunaka A, Sasaki R. Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. *J Biol Chem*. 2002; 277:18914–18918. [PubMed: 11877447]
38. Taylor AB, Stoj CS, Ziegler L, Kosman DJ, Hart PJ. The copper-iron connection in biology: Structure of the metallo-oxidase Fet3p. *Proc Natl Acad Sci U S A*. 2005; 102(43):15459–15464. [PubMed: 16230618]
39. Dancis A, Haile D, Yuan DS, Klausner RD. The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J Biol Chem*. 1994; 269(41):25660–25667. [PubMed: 7929270]
40. Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM, Kaplan J. The FET3 gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell*. 1994; 76(2):403–410. [PubMed: 8293473]
41. Aller SG, Unger VM. Projection structure of the human copper transporter CTR1 at 6-Å resolution reveals a compact trimer with a novel channel-like architecture. *Proc Natl Acad Sci U S A*. 2006; 103(10):3627–3632. [PubMed: 16501047]
42. Mahar J, Lukacs GL, Li Y, Hall S, Moczydlowski E. Pharmacological and biochemical properties of saxiphilin, a soluble saxitoxin-binding protein from the bullfrog (*Rana catesbeiana*). *Toxicon*. 1991; 29(1):53–71. [PubMed: 1851343]
43. Morabito MA, Moczydlowski E. Molecular cloning of bullfrog saxiphilin - a unique relative of the transferrin family that binds saxitoxin. *Proc Natl Acad Sci U S A*. 1994; 91(7):2478–2482. [PubMed: 8146142]
44. Li Y, Moczydlowski E. Purification and partial sequencing of saxiphilin, a saxitoxin-binding protein from the bullfrog, reveals homology to transferrin. *J Biol Chem*. 1991; 266(23):15481–15487. [PubMed: 1869567]
45. Henderson R, Ritchie JM, Strichartz GR. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in sodium channels of nerve membrane. *Proc Natl Acad Sci U S A*. 1974; 71(10):3936–3940. [PubMed: 4530274]
46. Niyogi S, Wood CM. Biotic Ligand Model, a flexible tool for developing site-specific water quality guidelines for metals. *Environ Sci Technol*. 2004; 38(23):6177–6192. [PubMed: 15597870]
47. Lefebvre KA, Bill BD, Erickson A, Baugh KA, O'Rourke L, Costa PR, Nance S, Trainer VL. Characterization of intracellular and extracellular saxitoxin levels in both field and cultured *Alexandrium* spp. samples from Sequim Bay, Washington. *Mar Drugs*. 2008; 6:103–116. [PubMed: 18728762]
48. Velzeboer RMA, Baker PD, Rositano J. Saxitoxins associated with the growth of the cyanobacterium *Anabaena circinalis* (Nostocales, Cyanophyta) under varying sources and concentrations of nitrogen. *Phycologia*. 2001; 40(3):305–312.
49. Costa PR, Botelho MJ, Lefebvre KA. Characterization of paralytic shellfish toxins in seawater and sardines (*Sardina pilchardus*) during blooms of *Gymnodinium catenatum*. *Hydrobiologia*. 2010; 655(1):89–97.
50. Maldonado MT, Allen AE, Chong JS, Lin K, Leus D, Karpenko N, Harris SL. Copper-dependent iron transport in coastal and oceanic diatoms. *Limnol Oceanogr*. 2006; 51:1729–1743.
51. Baptista MS, Vasconcelos MT. Cyanobacteria metal interactions: requirements, toxicity, and ecological implications. *Crit Rev Microbiol*. 2006; 32:127–137. [PubMed: 16893750]
52. Li H, Miao J, Cui F, Li G. Surfactant promotion of the inhibitory effects of cupric glutamate on the dinoflagellate *Alexandrium*. *J Phycol*. 2008; 44:1364–1371.
53. Sabatini SE, Juárez ÁB, Eppis MR, Bianchi L, Luquet CM, Ríos de Molina MdC. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. *Ecotox Environ Safe*. 2009; 72(4):1200–1206.
54. Tripathi BN, Gaur JP. Relationship between copper- and zinc-induced oxidative stress and proline accumulation in *Scenedesmus* sp. *Planta*. 2004; 219(3):397–404. [PubMed: 15014994]
55. Li H, Miao JL, Cui FX, Li GY. Surfactant promotion of the inhibitory effects of cupric glutamate on the dinoflagellate *Alexandrium*. *J Phycol*. 2008; 44(6):1364–1371.

56. García-Villada L, Rico M, Altamirano M, Sánchez-Martín L, López-Rodas V, Costas E. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: characterisation and future implications in the use of copper sulphate as algicide. *Water Res.* 2004; 38(8):2207–2213. [PubMed: 15087203]
57. Sharma SS, Dietz K-J. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot.* 2006; 57(4):711–726. [PubMed: 16473893]
58. Graneli E, Persson H, Edler L. Connection between trace metals, chelators, and red tide blooms in the Laholm Bay, SE Kattegat - An experimental approach. *Mar Environ Res.* 1986; 18:61–78.
59. Dancis A, Roman DG, Anderson GJ, Hinnebusch AG, Klausner RD. Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc Natl Acad Sci U S A.* 1992; 89(9):3869–3873. [PubMed: 1570306]
60. Hassett R, Kosman DJ. Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J Biol Chem.* 1995; 270(1):128–134. [PubMed: 7814363]
61. Stearman R, Yuan DS, Yamaguchi-Iwai Y, Klausner RD, Dancis A. A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science.* 1996; 271(5255):1552–1557. [PubMed: 8599111]
62. Karin M, Najarian R, Haslinger A, Valenzuela P, Welch J, Fogel S. Primary structure and transcription of an amplified genetic locus: The CUP1 locus of yeast. *Proc Natl Acad Sci U S A.* 1984; 81(2):337–341. [PubMed: 6364141]
63. Culotta VC, Howard WR, Liu XF. CRS5 encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. *J Biol Chem.* 1994; 269(41):25295–25302. [PubMed: 7929222]

Figure 1. Copper and iron uptake in *S. cerevisiae*

Cu(II) and Fe(III) are reduced prior to transport across the cell membrane by the cupric/ferric reductase Fre1p [59, 60]. Cu(I) is transported across the plasma membrane by the high affinity copper transporter Ctr1p [22]. Once inside the cell, it is trafficked to varied destinations by multiple copper chaperones. One of the destinations for Cu(I) is the multicopper oxidase Fet3p, as posttranslational insertion of four copper ions is essential for its activity [38]. Upon coordination of the copper ions, Fet3p forms a complex with the iron permease Ftr1p [61]. Together, these enzymes cotranslocate to the plasma membrane, where they function in the cell-surface high-affinity iron uptake system. Conditions of copper excess induce the expression of Cup1p and Crs5p: small, cysteine-rich metallothioneins that sequester excess copper ions in the cytoplasm [62, 63] via thiol-mediated interactions.

Figure 2. Expression profiles of copper and iron homeostasis genes

(a) *CTR1*, (b) *FET3*, (c) *FRE1*, (d) *CUP1*, and (e) *CRS5* in wild-type *S. cerevisiae* (S288C) following 45 min exposure to 16 μ M saxitoxin, 40 μ M iron, 8 μ M copper, or a range of concentrations of the copper chelator BCS in comparison to water-only controls. *FET3* was also examined following exposure to the intracellular copper chelator neocuproine. For each functional gene, fold-change was determined by dividing the average normalized transcript copy number (as described in Methods) of exposed by the average normalized transcript copy number of control. Error bars represent standard deviation of three independent measurements from five biological replicates. stx = saxitoxin, BCS = bathocuproinedisulfonate (number represents μ M concentration), neo = neocuproine. * indicates significant difference ($p < 0.05$).

Figure 3. Expression and localization of copper and iron-associated proteins using GFP fusions (A) Fet3-GFP and (B) Ctr1-GFP expression and localization following exposure to stx, copper, iron, BCS, or water (control). (C) Aft1-GFP expression and localization following exposure to saxitoxin, copper, iron, the intracellular Cu(I) chelator neocuproine (neo), or water Scale bar = 10 μ M.

Figure 4. Imaging of intracellular labile Cu(I) levels with CS1

Wild-type *S. cerevisiae* cells were incubated at 30 °C for 45 min with (A) sterile water (saxitoxin control) or (B) 16 μM saxitoxin followed by supplementation with 20 μM CuSO_4 for 15 min. (C) Copper-supplemented cells treated with 200 μM of the competing copper chelator *N*-ethyl-3,6,12,15-tetrathia-9-monoazaheptadecane for 10 min. Cells were washed three times in 1x PBS, and incubated with 2.4 μM CS1 for 10 min at 30 °C.