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A Genetic and Physiological Study of the Role of Extracellular Copper-Binding Proteins in Copper Detoxification by the Marine Bacterium *Vibrio alginolyticus*

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A GENETIC AND PHYSIOLOGICAL STUDY OF THE ROLE OF EXTRACELLULAR COPPER-BINDING PROTEINS IN COPPER DETOXIFICATION BY THE MARINE BACTERIUM *Vibrio alginolyticus*

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ABSTRACT

Supernatant proteins in *Vibrio alginolyticus* batch cultures were analyzed by SDS-PAGE before copper was added, 24 and 48 hours after the addition of copper, and in 24 hour control (no Cu) cultures. Two proteins, one 21 kilodalton (kDa) and one 19 kDa, were found to be copper-induced, and were designated copper-binding protein 1 (CuBP1) and CuBP2. CuBP1 and CuBP2 became detectable in supernatants during the Cu-induced lag phase, and increased in concentration over the following 48 hours. Chloramphenicol inhibited production of these proteins. Gel-to-gel variability was implicated as the dominant factor determining whether one or two Cu-induced proteins were detected in *Vibrio alginolyticus* supernatants, and ca. 20 kDa Cu-induced proteins were quantitated together in subsequent analyses.

Experiments in continuous (chemostat) cultures of *Vibrio alginolyticus* demonstrated that the bacteria could survive copper stress in an open system. Cell numbers dropped at first, then re-equilibrated as copper concentrations were increased. Copper-resistant (Cu^r) mutants were isolated from continuous cultures undergoing long-term copper stress (32 and 20 μM Cu). Copper stress reversibly inhibited swarming in most colonies from long-term copper-stressed cultures, and permanent inhibition of swarming was observed in some isolates. Mutation to an oxidase negative phenotype, which was not reversible, occured at high frequency in copper-stessed continuous cultures.

The stability of two Cu^r mutants isolated from continuous culture was

demonstrated by subculturing each isolate ten times on nonselective marine agar (10° MA), and comparing plate counts on unamended and 40μM Cu-amended agar to corresponding plate counts of isolates freshly passed on Cu-amended agar. The oxidase negative mutant, Cu40A1, contained a majority of Cu^r cells after the nonselective subcultures, while Cu40B3 cultures contained $<1\%$ Cu^r cells whether it had been recently copper stressed or subcultured from 10° MA.

Supernatants of copper-resistant and copper-sensitive mutants of *Vibrio alginolyticus* were analyzed by SDS-PAGE for the presence and quantity of CuBP. One Cu^r isolate, Cu40B3, constitutively produced a ca. 21 kDa protein which displayed the same chromatographic behavior (immobilized metal ion affinity chromatography followed by reverse phase high performance liquid chromatography) as CuBP. After fifteen nonselective subcultures, a revertant Cu^s derivative of Cu40B3 [Cu40B3(SW)] was isolated. Cu40B3(SW) lost the mutation to constitutive CuBP production and copper resistance simultaneously, indicating that constitutive CuBP production in Cu40B3 is necessary for maintenance of its copper-resistant phenotype.

Copper-sensitive *Vibrio alginolyticus* mutants displayed a range of alterations in supernatant protein profiles, and two of the seven mutants were indistinguishable from the wild-type in terms of supernatant proteins with and without copper stress. One Cu^s mutant was isolated which contained no CuBP in supernatants from 50 μM copper-stressed cultures.

These data support the hypothesis that the extracellular, ca. 20 kDa protein(s) of *Vibrio alginolyticus* are an important factor in survival and growth of the organism

at elevated copper concentrations. The range of mutations observed in Cu^r and Cu^s *Vibrio alginolyticus* indicate that altered sensitivity to copper can be caused by a variety of physiological changes. The production of extracellular metal chelators by marine bacteria has implications for the speciation and biogeochemical cycling of metals in the world oceans, and therefore merits complete study.

DEDICATION

This Dissertation is dedicated to my mother,

Jewell Meek Harwood

who made everything possible,

and to

Jim Watson,

who always understood.

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STATEMENT OF THE PROBLEM

Reports of extracellular copper complexation and detoxification by microbial exudates have been appearing in the literature for over twenty years (Barber and Ryther, 1969; Hardstedt-Romeo and Gnassia-Barelli, 1980; Jardim and Pearson, 1984; Mittleman and Geesey, 1985). While these reports are intriguing, they are generally descriptive in nature and have not attempted to identify the class of compound responsible for metal detoxification. Metallothioneins (MT), cysteine-containing, metal-induced *intracellular* proteins, have become established as the paradigm of biological metal detoxification. Thus, the emphasis of much of the research on metal detoxification by prokaryotes and eukaryotes has focused on mechanisms mediated by intracellular proteins.

Microorganisms have the ability to produce extracellular compounds that contribute to their survival in unfavorable environments, i.e. iron-scavenging siderophores, bacterocins and antibiotics for killing competing microorganisms, and extracellular enzymes such as proteases, chitinase, and DNAase which break down complex biomolecules into substrates that can be transported into the cell. Excretion of metal-binding compounds, or intracellular complexation followed by excretion of the chelator-metal complex is, therefore, theoretically a viable alternative to intracellular sequestration of toxic metals.

The results of many studies indicate that the microbiota play a role in

determining the distribution and speciation of many metals, including copper, in natural waters (Bruland *et al.*, 1991; Coale and Bruland, 1988; Smith *et al.*, 1982; Wangersky, 1986; Zirino *et al.*, 1983). It is not known whether the contribution of the microbiota to metal speciation and partitioning in marine waters is dominated by passively mediated mechanisms, such as adhesion to microbial cell walls, or whether active, inducible processes play a major role. Our understanding of the biogeochemical cycling of metals will be insufficient to predict and model the effects of anthropogenic inputs of metals into marine waters until our knowledge of microbial-metal interactions in the oceans is significantly expanded. Thus, the reasonable and compelling hypothesis that marine microbes are capable of responding to altered concentrations of copper and other metals using physiological tools that affect metal speciation in the ocean merits investigation.

INTRODUCTION

Copper poses a physiological dilemma in biological systems. It is a component of metalloenzymes, including cytochrome oxidase, some forms of superoxide dismutase, and ceruloplasmin, an iron-storage protein (Sorenson, 1987; Wackett *et al.*, 1989). Copper is an important trace nutrient metal whose abundance in biological systems is exceeded only by that of iron and zinc (Cotton and Wilkinson, 1980). Copper, however, is toxic to bacteria at concentrations ranging from nanomolar to millimolar (Anderson and Morel, 1978; Brand *et al.*, 1986; Rouche *et al.*,1989; Schreiber *et al.*,1985), reflecting a diversity of physiological responses to the metal. The dual

nutrient/toxicant role played by copper in bacterial physiology mandates cellular regulatory systems that can ensure copper availability while controlling its toxicity.

Copper acts as a required nutrient and as a toxicant in multicellular animals (Grant *et al.*, 1989), including mammals (Auer *et al.*, 1989), as well as in bacteria (Bitton and Freihofer, 1978; Schreiber *et al.*, 1985; Zevenhuisen *et al.*, 1979). Copper, like mercury and lead, is classified as a *very toxic*, relatively available element. (Forstner and Wittmann, 1981).

Copper Distribution and Speciation. Copper is one of the dominant heavy metal water pollutants, and worldwide concern is increasingly focused on the danger of the accumulation of toxic metals in the food chain (Abel, 1989; Nriagu and Pacyna, 1988). Major sources of copper emission to the atmosphere include mining, coal and oil combustion by electric utilities, and incineration of sewage sludge (Nriagu and Pacyna, 1988). In aquatic ecosystems, sources of copper pollution include domestic wastewater and sewage effluents, mining, manufacturing processes and atmospheric fallout. Agricultural applications are also a significant source of copper pollution (Abel, 1989), and contributions of copper from anti-biofouling applications in heavily populated coastal or estuarine environments could be significant (Wright and Zamuda, 1991). Of the trace metals, i.e. As, Cd, Cu, Hg, Mo, Ni, Pb, Sb, Se, V, Zn, the yearly anthropogenic input of copper is highest save for that of zinc (2150 vs. 2340 thousand tonnes $yr.^{-1}$; Nriagu and Pacyna, 1988).

Total copper in unpolluted ecosystems is generally low, and the majority of the metal exists as organically complexed material (Bruland *et al.*, 1991; Sunda and

Hanson, 1987). In offshore ocean waters, where total copper is at nanomolar levels (Bruland and Franks, 1983; Chester and Stoner, 1974), organically complexed copper comprises greater than 90% of the dissolved copper in surface waters (Coale and Bruland, 1988; Sunda and Ferguson, 1983; Sunda and Hanson, 1987; Van den Berg, 1982, 1984;). The average pCu (-log [Cu^{2+}]) of surface ocean waters is estimated at 12 (Brand *et al.*, 1986; Sunda and Ferguson, 1983). The concentration of free copper ions in solution is the major determinant of copper toxicity in aquatic environments (Schreiber *et al.*, 1985; Sunda and Guillard, 1976; Zevenhuizen *et al.*, 1979), therefore the pCu, rather than the total copper concentration, is biologically relevant.

Copper concentrations are not constant throughout the surface waters of the oceans, varying spatially and temporally, and are highest in the particulate-rich subsurface microlayer (Bruland *et al.*, 1991; Wangersky, 1986). In the upper 150 m of northeast Pacific waters, nearly 100% of copper was organically complexed (pCu 13) (Coale and Bruland, 1990). The percent of organically bound copper decreased with depth, so that pCu was 10 at 500 m. Upwelling ocean waters, which are rich in inorganic nutrients but are organically depleted (and thus, enriched in copper ions), did not support phytoplankton growth until artificial (EDTA) or microbially derived metal chelators were added (Barber and Ryther, 1969; Smith *et al.*, 1982). Copper has been implicated as a major source of the toxicity of upwelling ocean waters (Smith *et al.*, 1982). Laboratory results combined with field studies indicate that, over the range of free copper ions concentrations in the world ocean, copper may act as both a biolimiting nutrient and a toxicant (Bruland *et al.*, 1991).

The toxicity of copper to microbial species is determined by its chemical speciation, the dominant factor affecting its bioavailability (Morel and Morel-Laurens, 1983). Since copper forms relatively stable coordination complexes with organic material (Mantoura, 1981), copper speciation in natural waters is determined by a complex interaction of many environmental parameters. The abiotic components of the environment, i.e. organic and inorganic ligands, and the biota (Boyle and Edmond, 1975; Morel and Morel-Laurens, 1983; Leckie and Davis, 1979) contribute to the complexing capacity of the ecosystem. Chemical parameters such as the pH and ionic strength of the solution also affect copper speciation (Leckie and Davis, 1979).

Mechanisms of Copper Toxicity. The basis for copper toxicity lies in the reactivity of copper ions with cellular macromolecules and inorganic molecules, as it can act as an oxidant and also forms chelating complexes with organic and inorganic molecules (Thurman and Gerba, 1989). The physiological consequence of increased intracellular copper ion concentrations is damage to DNA, proteins and lipids (Tappel, 1973; Thurman and Gerba, 1989). Excess copper has been shown to cause lipid peroxidation (Tappel, 1973). Alterations in enzyme structure and function may occur as copper binds inappropriately to amino acids. Cysteine, histidine and methionine have particularly high affinities for copper (Rogers *et al.*, 1991), and form the Cu ion binding site in several metalloenzymes, including plastocyanin (Colman *et al.*, 1978). Cu^{2+} may also replace the normal metals of enzyme prosthetic groups (Sterritt and Lester, 1980), altering or ablating their function. Enzymes of the

electron transport chain may be particularly vulnerable to damage by copper (Domek *et al.*, 1984).

Chelation of phosphate groups by Cu^{2+} has been proposed as a mechanism for cupric ion interaction with nucleic acids (Thurman and Gerba, 1989). Interaction of DNA with copper may cause strands to become disordered, broken, stabilized, or destabilized (Ueda *et al.*, 1980), thus disrupting DNA transcription and replication. In addition to direct Cu-DNA interaction, Cu^{2+} can act as an oxidant and Cu^{+} as a reductant, generating oxygen radicals such as the hydroxyl radical ($^{\cdot}OH$). Oxidation of cellular biomolecules by the highly reactive hydroxyl radical is a major mechanism of Cu-induced damage (Hanna and Mason, 1992). The reaction is analogous to the Haber-Weiss cycle, which is catalyzed by iron.

$$Cu(II) + H_2O_2 \rightarrow Cu(I) + HO_2^{\cdot} + H^+$$

$$2HO_2^{\cdot} \rightarrow 2O_2^{-} + 2H+ \rightarrow H_2O_2 + O_2$$

$$Cu(II) + O_2^{\cdot-} \rightarrow Cu(I) + (O_2)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\cdot}OH + {}^{\cdot}OH$$

(Hanna and Mason, 1992)

Copper can act as the primary effector of DNA damage, or it can exacerbate the effects of a second mutagenic agent. Copper has a comutagenic effect on *E. coli* subjected to UV irradiation, causing multiple strand breaks and increasing the rate of mutagenesis to three times that of UV light alone (Rossman, 1989; Rossman *et al.*, 1989). Interaction of thiols with Cu^{2+} caused single-strand nicks in supercoiled

plasmid DNA (Reed and Douglas, 1989).

The combination of copper and hydrogen peroxide generates hydroxyl and other oxygen radicals, which damage eukaryotic and prokaryotic DNA (Aruoma *et al.*, 1991; Dizdaroglu *et al.*, 1991; Sagripanti and Kraemer, 1989; Yamamoto and Kawanishi, 1989) and proteins (Simpson *et al.*, 1988). Copper is a required trace metal, and hydrogen peroxide is a normal byproduct of aerobically respiring cells. The interaction of copper and H_2O_2 is therefore likely to have a deleterious impact on cellular physiology, particularly when intracellular Cu^{2+} concentrations rise above normal.

Although the effects of excess copper are generally considered to be cytotoxic (Freedman *et al.*, 1989), evidence is accumulating that copper ions can act as mutagenic agents (Aruoma *et al.*, 1991; Dizdaroglu *et al.*, 1991; Rossman and Kneip, 1989; Rossman, 1989; Sagripanti and Kraemer, 1989; Tkeshelashvili *et al.*, 1991; Ulitzer and Barak, 1988; Yamamoto and Kawanishi, 1989). Evidence exists for mechanisms of metal-mediated mutagenesis of DNA including metal-base interaction, which impairs base pairing specificity, metal-DNA polymerase interaction, which interferes with DNA replication, metal-deoxynucleotide triphosphate interaction, and chelation of phosphate groups (Tkeshelashvili *et al.*, 1991). Copper was found to be a potent mutagen by a bacterial bioluminescence test (Ulitzur and Barak, 1988), although copper activity in systems designed to measure mutagenic potential had not been previously reported. In this study a solid agar diffusion method was used to overcome the disadvantages posed by the toxicity of copper to the reporter bacteria.

Mercury ($HgCl_2$) and silver ($AgNO_3$), which are also highly toxic but previously considered nonmutagenic, also displayed mutagenic activity in this test.

Several studies indicate that some DNA damage by copper is site-specific. The most common mutation in ϕX174 viral DNA incubated with copper was C → T transition. Mutation sites were clustered, rather than randomly spaced, suggesting interaction of specific DNA sequences with copper ions (Tkeshelashvili *et al.*, 1991). An *in vitro* study demonstrated preferential strand breakage at polyguanosine sequences by copper plus H_2O_2 (Sagripanti and Kraemer, 1989). Histones protected mammalian DNA from base modifications in the presence of H_2O_2 and copper (Dizdaroglu *et al.*, 1991). The frequency of cleavage at cytosine residues of cloned mammalian DNA was site-specific (Yamamoto and Kawanishi, 1989). Thus, a model is emerging in which copper ions bind to DNA sequences with some specificity. The copper ion then catalyzes an oxygen radical-producing reaction, whose product (currently thought to be primarily the hydroxyl radical) causes base modification and strand breakage.

Physiological Strategies for Metal Resistance. Copper, as a required trace metal, cannot be entirely excluded by living organisms, yet the toxicity and relative abundance of copper mandate that effective systems of intracellular copper management and, in some cases, resistance to elevated copper ion levels exist. The wide range of microbial copper tolerance indicates that some microbes have evolved strategies for copper detoxification.

Metal-inducible systems for metal detoxification have been identified in

microbes and in higher organisms. Transcription of metallothioneins (MT), low molecular weight proteins present in organisms ranging from yeasts to humans (Kagi and Nordberg, 1979), is induced by a variety of metals, including copper (Butt *et al.*, 1984; Furst and Hamer, 1989). In the case of the yeast *Saccaromyces cerevisise*, copper ions bind cooperatively to the cysteine-rich DNA binding domain of ACE1 transcription factor (Furst and Hamer, 1989). The resultant increase in production of MT mRNA insures a large increase in cellular copper complexing capacity in response to a small increase in the concentration of copper ions. This response is highly conserved in eukaryotes (Kagi and Norberg, 1979), indicating that the presence of potentially toxic metals in ecosystems exerts considerable selective pressure toward retention of an effective means of metal detoxification.

Several studies have suggested that MT-like systems for metal detoxication may exist in some prokaryotic species. Intracellular cadmium-induced proteins whose estimated molecular weights are similar to metallothioneins have been demonstrated in a *Pseudomonas putida* strain that accumulates the metal (Higham *et al.*, 1984, 1985). Cadmium also induced synthesis of an MT-like protein in the cyanobacterium *Synechoccus* (Olafson, 1986; Olafson *et al.*, 1988.).

Mechanisms for bacterial resistance to most metals, including copper, have been described. Cadmium-resistant *Staphylococcus aureus* detoxifies cadmium via a plasmid-mediated, energy-dependent efflux mechanism (Tynecka *et al.*,1981a, 1981b). The genetic determinant is termed *cadA*, which also confers resistance to Zn^{2+} (Silver and Misra, 1988). At least five other bacterial cadmium resistance systems

have been reported (see Silver and Misra, 1988 for review). Arsenate resistance in *S. aureus* and *E. coli* results from reduced accumulation mediated by an inducible efflux system (Silver and Misra, 1988). Bacterial mercury resistance carried on plasmid R100 (from a *Shigella* species) has been dissected at the genetic level to reveal an operon controlled by the *merR* product (a trans-acting inducer-repressor), an operator region for MerR binding, and five structural genes. The *merT* gene encodes the Hg^{2+} transport system, a transmembrane protein that delivers Hg^{2+} to the mercuric reductase dimer (*merA*). MerP is thought to be a periplasmic binding protein whose function is delivery of Hg^{2+} to the transport system (Silver and Misra, 1988). Thus, Hg^{2+} is tightly bound in order to prevent cell damage until it can be delivered to the mercuric reductase enzyme, where Hg^{2+} is reduced to Hg^{0}. Cadmium resistance in a soil bacterium was reported to be mediated by a 42,600 dalton protein that bound cadmium in an insoluble precipitate (Kurek *et al.*, 1991).

There have been reports of nonspecifically produced microbial compounds that fortuitously mediate copper detoxification. Siderophores, which protect cyanobacteria from copper toxicity, were not induced by copper (Clarke *et al.*, 1987). Constitutively produced polysaccharide capsule material (Bitton and Freihofer, 1978), and uncharacterized organic exudates from algae (Hardstedt-Romeo and Gnassia-Barelli, 1980) and bacteria (Mittelman and Geesey, 1985) decreased the toxic effects of copper.

Copper-inducible, plasmid-mediated systems of copper resistance have been described in terrestrial bacteria (Mellano and Cooksey, 1988; Rouche *et al.*, 1989).

Plasmid pRJ1004 carries the *pco* copper resistance determinant, which is composed of at least 4 genes (*pcoA,B,C* and *R*), whose induction is controlled at the transcriptional level. The model describing the mechanism of copper detoxification in *E. coli* pRJ1004 includes intracellular binding of copper by an inducible 26 kilodalton (kDa) protein, followed by enhanced efflux and chemical modification of copper so that it cannot reenter the cell (Rouche *et al.*, 1989).

Studies on copper maintenance and transport in *E. coli* have demonstrated that the *cut* element is involved in regulating intracellular copper homeostasis (Rogers *et al.*, 1991). CutE mutants are both copper sensitive and copper dependent. CutE contains the putative copper-binding region His-X-X-Met-X-X-Met, which has been implicated in copper resistance in *Pseudomonas syringae* (Mellano and Cooksey, 1988).

Copper resistance is plasmid mediated in a number of other systems, including the plant pathogen *Pseudomonas syringae* (Bender and Cooksey, 1986; 1987). A copper-binding motif of the general structure Asp-His-X-X-Met-X-X-Met with homology to the copper-containing enzymes azurin and plastocyanin was identified (Mellano and Cooksey, 1988). The four open reading frames (ORFs) of this element have been designated *copA, B, C* and *D* (Cha and Cooksey, 1991). CopA and CopC are located in the periplasm, between the inner and outer membranes, and CopB is an outer membrane protein (Cha and Cooksey, 1991). CopA, a protein high in histidine residues, bound 11 copper atoms per protein molecule (Cha and Cooksey, 1991). A putative copper-binding motif, Met-X-X-Met-X-His-X-X-Met common to

CopA and CopC (which bound one Cu/protein molecule) was identified. The model proposed to explain the mechanism of resistance is sequestration of copper outside the cytoplasm by the Cop proteins (Cha and Cooksey, 1991). The plasmid-mediated copper resistance described in *Mycobacterium scrofulaceum* involves precipitation of copper as copper sulfide outside the cell (Eradi *et al.*, 1987).

Current studies on copper detoxification in marine microorganisms have concentrated on extracellular complexation by phytoplankton (Clarke *et al.*, 1987; Wangersky, 1986; Zhou and Wangersky, 1985). Recently, conditional stability constants of 10^9 to 10^{10} were measured for extracellular ligands from cultures of marine fungi (Sunda and Gessner, 1989). Most of these studies were not designed to test inducibility of the complexing material by copper. Indeed, increased production of a copper complexing compound upon addition of copper was demonstrated in only one case (Jardim and Pearson, 1984). The cyanobacterial copper complexing compound was not identified in the study, however, further work with cyanobacteria suggested that the compound might be a siderophore (Clarke *et al.*, 1987).

COPPER DETOXIFICATION IN THE MARINE BACTERIUM *Vibrio alginolyticus*

Vibrio alginolyticus is a heterotrophic marine bacterium that is ubiquitously distributed throughout marine and estuarine ecosystems. The strain on which the following work is based was isolated from a stainless steel plate in Biscayne Bay, Miami during the course of a study on bacterial attachment (Gerchakov *et al.*, 1976).

Calorimetric studies of copper toxicity determined that heat production by *Vibrio alginolyticus* was inhibited by 50% (TC_{50}) at 6.4 μM $CuSO_4$ under aerobic conditions in M9 medium modified to contain 21 g/l NaCl (Schreiber *et al.*, 1985). When organic chelators were used to control free copper levels, toxicity was directly related to the concentration of Cu^{2+}, indicating that organically complexed copper is not toxic to *V. alginolyticus*.

Toxic levels of copper added to logarithmically growing cultures of *V. alginolyticus* cause a lag in growth that is proportional to the amount of copper added (Gordon and Millero, 1980; Howell *et al.*, 1992; Schreiber *et al.*, 1985). In experiments designed to measure the partitioning and speciation of copper in batch cultures of *V. alginolyticus* a significant amount of copper remained soluble through late stationary phase, however, growth of the culture was accompanied by a decrease in free copper ion levels (Schreiber *et al.*, 1990). As the cells entered stationary phase, no polarographically labile (ionic) copper was detectable, indicating that all soluble copper in the culture was complexed (Schreiber *et al.*, 1990). A copper-induced, ca. 30 kDa peak from supernatants fractionated by size exclusion HPLC (high performance liquid chromatography) contained ^{14}C if ^{14}C-glucose was added after copper was added, but not if ^{14}C-glucose was added before copper challenge. These results indicate that the material in the peak was the result of biosynthetic activity after copper addition, and was not the lytic product of dead cells. Fractions from the ca. 30 kDa peak contained ^{35}S-labeled material when ^{35}S-methionine was added to the culture after copper addition, suggesting that the biomolecules,

synthesized after copper addition, contained protein. Size exclusion HPLC with a different column separated two peaks from the void and inclusion volumes with relative molecular weights (M_r) of 28,000 and 26,000. These peaks were amplified 7.1-fold and 9.4-fold, respectively, in copper-challenged vs. control cultures (Schreiber *et al.*, 1990).

When copper-complexing activity was measured in gel permeation chromatography (GPC) fractions from control and 50 μM copper-challenged supernatants, a copper-induced peak was found in fractions corresponding to M_r 20,000 (Harwood-Sears and Gordon, 1990a). Because there was some evidence that the copper-complexing molecules might be proteins (including approximate molecular weights and incorporation of ^{35}S into copper-induced peak fractions), and because metallothioneins and many other agents of metal detoxification are proteins, we investigated the possibility that the extracellular copper-complexing activity of *V. alginolyticus* is mediated by a protein or proteins (Harwood-Sears and Gordon, 1990a). The research described in this Dissertation includes experiments designed to determine the biochemical nature of the copper-complexing compound(s), their induction kinetics, and distribution of the compound(s) in copper-stressed *V. alginolyticus* cultures (Harwood-Sears and Gordon, 1990a).

After the proteinaceous nature of the compound(s) was established, copper-resistant (Cu^r) and copper-sensitive (Cu^s) mutants of *V. alginolyticus* were isolated in order to solidify the link between the production of the copper-binding protein(s) (CuBP) and copper detoxification in this bacterium, and to further our general

understanding of copper toxicity, copper tolerance and copper detoxification in the organism. As little is known about metal-inducible systems of metal detoxification in marine bacteria, this work will further our understanding of microbial-metal interactions in the oceans, as well as contributing to elucidation of the systems that regulate copper ion homeostasis in bacteria.

CHAPTER 1: COPPER-INDUCED PRODUCTION OF COPPER-BINDING SUPERNATANT PROTEINS BY THE MARINE BACTERIUM VIBRIO ALGINOLYTICUS

MATERIALS AND METHODS

Bacteria and Medium. A strain of *Vibrio alginolyticus* originally isolated from the surface of a stainless steel plate immersed in Biscayne Bay, Miami (Gerchakov *et al.*, 1976) was used in this study. All cultures were incubated at room temperature on a shaker. Cultures were grown in M9 minimal medium with 8 mM glucose modified to contain 21 g/l NaCl (SWM9).

Copper Challenge. Except where noted, 0.5 ml of cells from an overnight culture of *V. alginolyticus* were used to inoculate 20 ml of medium. Copper was added to cultures when the optical density reached 40 Klett units in 20 ml cultures, or 0.08 absorbance units for 3 ml cultures whose optical density was measured in microtiter plates (see below). At this point in the growth curve, cells were doubling exponentially and cell numbers were approximately 1.8×10^8 cells $\cdot$ ml^{-1}. Unless noted otherwise, cultures were harvested after a total of 24 hours. In the experiment designed to measure the level of $CuSO_4$ necessary to induce CuBP, 3 ml batch cultures were started from 50 μl of overnight broth cultures in sterile, 12-well tissue culture dishes (Costar, Cambridge, MA). The optical density of the 3 ml cultures

was monitored by transferring 100 μl aliquots to a 96-well microassay plate, which were read at 595 nm on a microplate reader (Bio-Tek Instruments). Cultures that were treated with chloramphenicol received 200 μg/ml ten minutes before the addition of copper.

Sample concentration and preparation. Supernatants were collected by centrifuging cells from copper challenged and control cultures at 13,800 X g for ten minutes. The supernatant was filter sterilized with a 0.2 μm membrane filter. In cultures where cellular proteins were separated by SDS-PAGE, the cell pellet was resuspended to a tenfold concentrate with respect to the original volume in SWM9. The cells were frozen at -80° C, thawed at room temperature and sonicated for two 1-minute bursts. This procedure was repeated twice, and the sonicate was centrifuged at 13,800 X g for ten minutes. The cell lysate was then resuspended to the original volume in SWM9 and filter sterilized as above.

Supernatants were concentrated by lyophilization, by stirred cell molecular filtration, or by molecular filtration in microconcentrator tubes. Lyophilized samples were resuspended in distilled water to a tenfold concentration with respect to the original culture and dialyzed (Spectrapor, nominal molecular weight cutoff = 6,000-8,000) against a PO_4-NaCl buffer (NaCl, 85.5 mM; Na_2HPO_4 $7H_2O$, 7.5mM; pH 7.5). Samples intended for column chromatography were concentrated tenfold by molecular filtration in a stirred cell (300 ml) using an Amicon PM10 filter (nominal molecular weight cutoff = 10,000). In the [Cu] vs. CuBP induction experiment, 2 ml of supernatant obtained by filtering the culture through a 0.45 μm filter was loaded

in a microconcentrator tube with a 3 kDa nominal molecular weight cutoff (Amicon Centricon 3; Amicon, Beverly, MA). Tubes were centrifuged at 6500 X g for two hours to give a four to five fold concentration factor. In all cases where protein quantities in different samples were compared, (e.g. cellular vs. supernatant) values were corrected to reflect the concentration in the original culture.

Supernatant protein concentrations for samples concentrated in microconcentrator tubes were measured using a bicinchoninic acid assay (Pierce BCA, Rockford, IL). Standards were diluted in distilled water from a 200 $\mu g \cdot ml^{-1}$ stock in the range of 25 $\mu g \cdot ml^{-1}$ to 150 $\mu g \cdot ml^{-1}$. Samples were assayed following kit directions, and were incubated at 37° C for 30 min. Absorbance was read at 562 nm on a Shimadzu UV-160 spectrophotometer. CuBP concentrations were estimated by multiplying the densitometric calculation of the contribution of CuBP to total supernatant protein by the supernatant protein concentration. For example, if CuBP comprised 5% of the supernatant protein in a sample with 20 $\mu g \cdot ml^{-1}$ supernatant protein, the calculated estimate of CuBP concentration would be 1 $\mu g \cdot ml^{-1}$.

Estimates of standing stock. In order to normalize extracellular protein concentrations to cell numbers, or standing stock, cell numbers and/or cellular protein was estimated for cultures at the time of harvest. Cell numbers were estimated by using a previously calculated and reconfirmed relationship between optical density in a Klett-Sumerson colorimeter and acridine orange direct counts for *V. alginolyticus* (4.5 X 10^6 cells/Klett unit).

Cellular protein was measured by filtering 0.5 ml of culture onto a pre-treated,

0.2 μm HT-200 filter (25mm, Gelman, Ann Arbor, MI). The filters were pre-treated by incubation at 90° C for 30 minutes while submerged in 1 N NaOH. After pre-treatment the filters were washed several times and stored in sterile, distilled water at 4° C for up to several weeks. After cultures were filtered onto them, the filters were placed in scintillation vials and stored at -80° C until analyzed.

Cell digestion was accomplished by submerging the filtered samples in 1 ml of 1 N NaOH, and incubating the samples at 90° C for 30 minutes. A modification of the bicinchoninic acid assay (Pierce BCA, Rockford, IL) was used to assay the digested samples. Ten μl of concentrated HCl was added to each 100 μl sample (and the blank) in order to neutralize the NaOH. The BSA standard included with the BCA kit was diluted tenfold with sterile, distilled water to a final concentration of 200 $\mu g \cdot ml^{-1}$. In order to approximate the sample solvent composition, the stock BSA solution was diluted with an approximately 1:1 solution of NaOH:HCl (10 ml of 1 N NaOH:1 ml of concentrated HCl) to yield standards ranging from 25 $\mu g \cdot ml^{-1}$ to 150 $\mu g \cdot ml^{-1}$. In order to account for any background absorbance contributed by the filter, the assay blank was drawn from a pre-treated filter submerged in 1 ml of 1 N NaOH, and incubated at 90° C for 30 minutes. Samples and standards were prepared in duplicate, and incubated with 2 ml of the BCA reagent for 30 minutes at 37° C. Absorbance was read at 562 nm on a Shimadzu UV-160 spectrophotometer.

Gel Permeation Chromatography (GPC). Supernatants concentrated by molecular filtration were eluted from a Sephadex G50-150 column 50 cm long by

1.25 cm wide with HEPES-sodium nitrate buffer (HEPES, 1 mM; $NaNO_3$, 0.1 M; pH 7.1). 10 ml of supernatant was loaded on the column, and 10 ml fractions were collected at a flow rate of 3.25 ml/min. UV absorbance was monitored at 254 nm.

SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE was carried out in 1.5 mm thick slab gels with a total acrylamide concentration of 12% (Laemmli, 1970). The gels were run at 60 mA constant current at 4° C. 100 μl of sample was loaded into each well. Protein molecular weight markers (Sigma Chemical Co., St. Louis, Mo.) were lysozyme (MW 14,400), trypsinogen (24,000), egg albumin (45,000) and bovine serum albumin (66,000). Gels were silver stained (ICN Biomedicals, Cleveland) and bands were quantitated with an LKB Ultroscan XL laser densitometer.

RESULTS

Supernatants from cultures challenged with 50 μM copper were concentrated by molecular filtration and fractionated by GPC. When the fractions were run on an SDS-PAGE gel, a ca. 21 kDa protein (hereafter refered to as CuBP1, or Copper Binding Protein 1) appeared consistently in fraction 13 (Figure 1.1, lane 7). A ca. 19 kDa protein (CuBP2) was also visualized on SDS-PAGE gels from some GPC runs (data not shown). No proteins in the 18-21 kDa range were found in supernatant fractions from control cultures. The lower molecular weight bands resolved in Figure 1.1 (lanes 3 and 4) were not consistently present in copper-challenged samples (see, for example, Figure 1.4).

Figure 1.1. SDS-polyacrylamide gel electrophoresis of supernatants from *Vibrio alginolyticus* batch cultures. Lanes 1-4: induction kinetics of CuBPs.

Lane 1: before copper addition
Lane 2: 2 hours after copper addition
Lane 3: 24 hours after copper addition
Lane 4: 48 hours after copper addition
Lane 5: molecular weight standards
Lanes 6 and 7: GPC fraction 13 from supernatants -
Lane 6: no copper challenge
Lane 7: 50 μM copper challenged
Lane 8: control (no added copper) supernatant from a 24 hour old culture

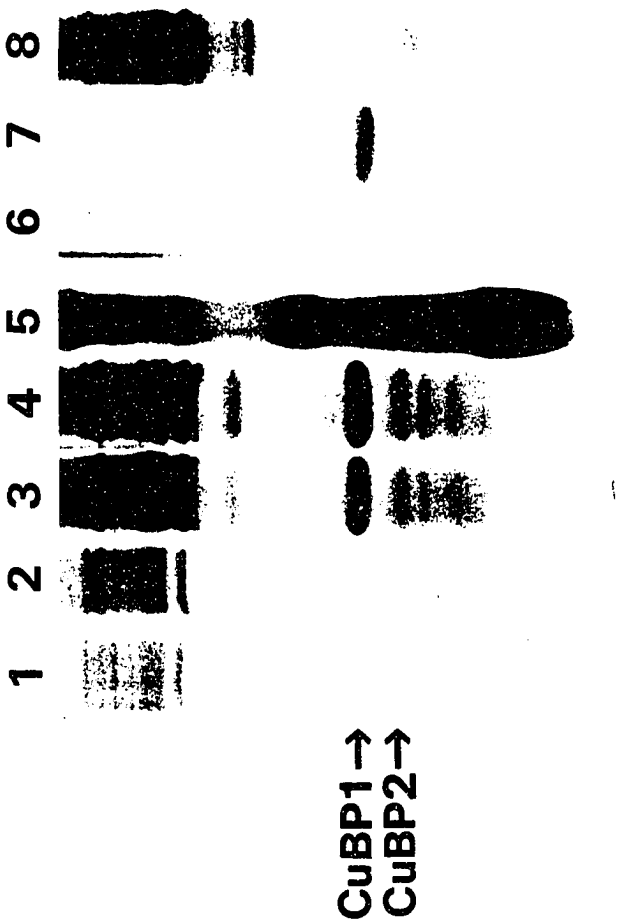


Figure 1.1

Comparison of SDS-PAGE gels of unfractionated supernatants from copper-challenged and control cultures (Figure 1.1, lanes 3 and 8) which were 24 hours old demonstrated prominent CuBP1 and CuBP2 bands in the copper challenged supernatants which were barely detectable in the control (Table 1). In terms of peak area, CuBP1 was 25 times more concentrated in 24 hour copper challenged supernatants than in comparable controls, while in copper-challenged supernatants CuBP2 was 46 times more concentrated than in controls. After these values were normalized to cell numbers at the time of harvest the difference was even greater: CuBP1 and CuBP2 were, respectively, 75 and 133 times more concentrated in copper challenged supernatants than in controls. CuBP1 and CuBP2 in copper challenged and control supernatants were also compared based on their contribution to total supernatant protein (estimated by quantitating total supernatant peak area in SDS-polyacrylamide gels by densitometry). CuBP1 in the 24 hour control was 0.9% of the area, and comprised 13.2% of the area in the 24 hour copper challenged supernatant, a 14.7 fold increase. The contribution of CuBP2 to percent area increased 26-fold from control to copper challenged cultures.

In an induction kinetics experiment, samples were taken from *Vibrio alginolyticus* cultures (1) before the addition of 50 μM copper, (2) two hours after the addition of copper, while cells were still in the lag phase, (3) 24 hours after copper addition, when the cells were well into recovery and (4) 48 hours after copper addition (Figure 1.1). Proteins in these samples were concentrated by lyophilization, separated by SDS-PAGE and quantitated by laser densitometry (Table 1.1). In the

sample taken before the addition of copper there were no detectable bands in the 21 kDa region. Two hours after the addition of copper CuBP1 was measured at 0.071 AU*mm by densitometry. At 24 hours CuBP1 had increased by sevenfold to 0.499 Au*mm and CuBP2 was measured at 0.229 Au*mm. After 48 hours the concentration of CuBP1 had increased to 0.699 Au*mm and CuBP2 to 0.359 Au*mm. There was a third band at 24 and 48 hours of 17.6 kDa which was not quantitated. This band is detectable in some copper challenged supernatants and not in others.

A corresponding pattern in the concentration of CuBP1 and CuBP2 was evident when peak area of the proteins was normalized to cell numbers or expressed as the percent of peak area in lanes from Figure 1.1 (Table 1.1). When expressed as peak area$\cdot$cell^{-1} CuBP1 increased fivefold from two to 24 hours after copper addition. CuBP2 increased from undetectable at two hours to 53 Au*mm$\cdot$cell^{-1} twenty-four hours after copper addition. The concentration of the CuBPs also increased relative to other supernatant proteins in each sample. CuBP1 was 4.7% of the peak area two hours after copper addition, 13.2% twenty-four hours after copper and 14.8% after 48 hours. CuBP2 was not detectable at two hours, was 6.0% of the peak area after 24 hours, and 7.6% after 48 hours.

Vibrio alginolyticus from an overnight culture was inoculated into SWM9 amended with copper concentrations ranging from 0 - 50 μM $CuSO_4$ in order to determine the concentration of copper necessary to induce CuBP expression. CuBP

Table 1.1. Quantitation of CuBP1 and CuBP2 in supernatants from 50 μM copper-challenged *Vibrio alginolyticus* cultures. Band density is expressed as peak area (Au*mm).

	CuBP1			CuBP2		
Supernatant	Peak Area	Area $\cdot$ Cell^{-1} [b]	Percent Area[c]	Peak Area	Area $\cdot$ Cell^{-1} [b]	Percent Area[c]
Pre Cu	ND[a]	ND	ND	ND	ND	ND
2 Hours	0.071	23	4.7	ND	ND	ND
24 Hours	0.499	120	13.2	0.229	53	6.0
48 Hours	0.699	130	14.8	0.359	65	7.6
24 Hour Control	0.020	1.6	0.9	0.005	0.4	0.23

[a] ND denotes not detectable by SDS-PAGE and laser densitometry.

[b] Area$\cdot$cell^{-1} is band density normalized to cell numbers in cultures at the time supernatants were harvested (X 10^{11}).

[c] Percent of integrated area in respective lanes (Fig. 2, lanes 1-4 and 8).

was not detectable by densitometry in the control culture, and was barely visible in the gel (Figure 1.2, lane 1). CuBP expression was induced at the lowest copper concentration, 1 μM. As the concentration of copper increased from 1 - 50 μM, so did CuBP in terms of the absolute amount (Figure 1.2). When expressed as the percentage of supernatant protein, CuBP increased from 1.2 % in 1 μM Cu-challenged cultures to 5% in 50 μM Cu-challenged cultures, approximately a 400% increase (Table 1.2). The concentration of supernatant CuBP was normalized to standing stock using both optical density and cellular protein as estimates of cell concentrations (Table 1.2). In each case, the concentration of CuBP increased incrementally as a function of added copper.

In cultures where chloramphenicol was added ten minutes before copper addition CuBP1 and CuBP2 were not detectable in the supernatant (Figure 1.3, lane 3). Shown for comparison are a 50 μM copper-challenged culture and a culture to which chloramphenicol and 50 μM copper were added.

The quantity of protein in the 19-25 kDa molecular weight range was compared in the supernatants and cell pellets of *Vibrio alginolyticus* cultures challenged with 25 μM $CuSO_4$ (Table 1.3). Analysis of SDS-PAGE gels by densitometry showed that CuBP1 was more concentrated in the supernatant than in the cellular fraction of these cultures. The proteins quantitated were 23.5, 21 and 19 kDa. The 23.5 kDa protein appears in some copper-challenged supernatants in varying density, and is not isolated by the IMAC column. The density of this protein in supernatants increased from 0.157 AU*mm at 24 hours to 0.324 AU*mm at 48

Figure 1.2. Supernatants from *Vibrio alginolyticus* batch cultures challenged with 0 - 50 μM $CuSO_4$.

Lane 1: 0 μM Cu
Lane 2: 1.0 μM Cu
Lane 3: 2.5 μM Cu
Lane 4: 5.0 μM Cu
Lane 5: 10.0 μM Cu
Lane 7: 50.0 μM Cu

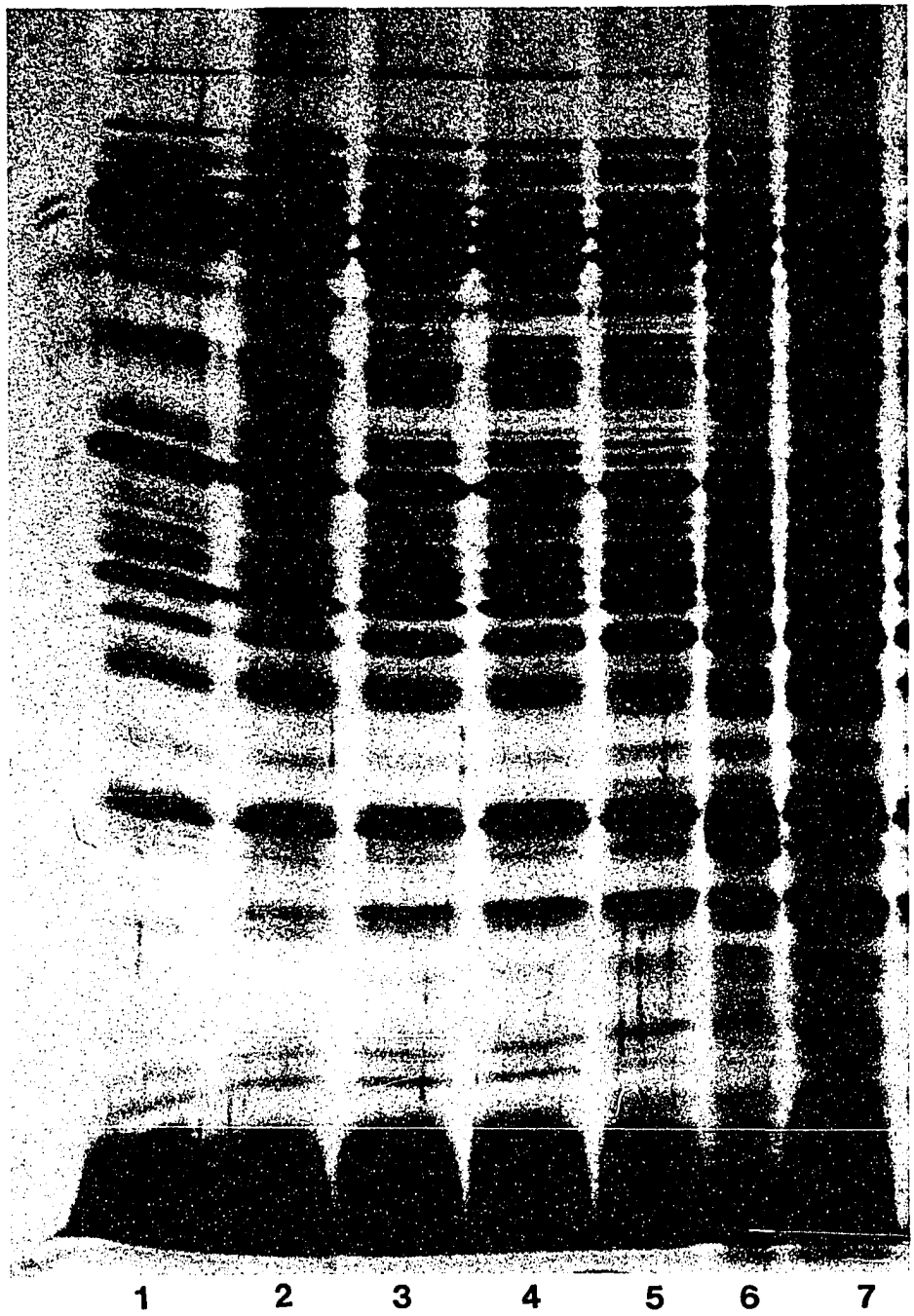


Figure 1.2

Figure 1.3. Effect of chloramphenicol on CuBP production. SDS-PAGE of *V. alginolyticus* supernatants:

Lane 1: 50 μM copper-challenged without chloramphenicol
Lane 2: chloramphenicol-treated only
Lane 3: 50 μM copper-challenged with chloramphenicol

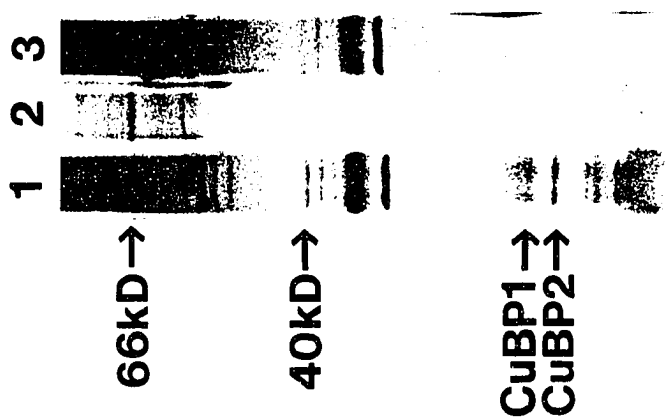


Figure 1.3

Table 1.2. Percent contribution of CuBP to total supernatant protein and [CuBP] vs. [Cu] in supernatants from broth cultures of *Vibrio alginolyticus*.

[Cu](μM)	% CuBP[1]	[CuBP]/OD[2]	[CuBP]/Protein[3] (X 10^3)
0	ND[4]		
1.0	1.2	1.08	1.47
2.5	1.3	1.30	1.71
5.0	1.9	2.01	2.39
10.0	3.0	3.97	3.76
50.0	5.0	10.88	10.28

[1] The percentage of supernatant protein comprised by CuBP was calculated by laser densitometry of SDS-PAGE gels.

[2] [CuBP] is expressed in $\mu g \cdot ml^{-1}$. OD (optical density) measured at 595 nm on a microtiter plate reader.

[3] Protein in this equation is cellular protein ($\mu g \cdot ml^{-1}$).

[4] Not detectable by densitometry of SDS-PAGE gels.

Table 1.3. Comparison of supernatant and cellular proteins[a] in the 19 to 25 kDa range from copper-challenged cultures of *Vibrio alginolyticus*. The 23.5 kDa protein, which was not consistently present in copper-challenged supernatants, included for comparison with the CuBPs.

	Band Density[a] (Au·mm)		
Protein	Supernatant 24 Hrs	Supernatant 48 Hrs	Cellular 48 Hrs
23.5 kDa	0.157	0.324	1.125
CuBP1	0.279	0.327	0.208
CuBP2	0.042	0.110	0.360

[a] Band density (Au • mm) was measured by laser densitometry of SDS-PAGE gels.

hours, while at 48 hours the pellet concentration was 1.125 AU*mm. CuBP1 increased from 0.279 AU*mm at 24 hours to 0.327 AU*mm at 48 hours, and was less concentrated at 48 hours in the cell pellet (0.208 AU*mm) than in the supernatant. CuBP2 also increased from 24 to 48 hours in the supernatant (from 0.042 AU*mmto 0.110 AU*mm), and was more concentrated in the pellet (0.360 AU*mm) than in the supernatant.

Since gel-to-gel variation can be a problem when comparing proteins separated by SDS-PAGE, an average molecular weight for CuBP1 was estimated from 21 samples on 14 different gels. The mean molecular weight calculated was 20.9 kDa with a standard deviation of .57 kDa. The molecular weight of CuBP2 was estimated at 19.1 kDa from 13 samples on 9 different gels.

At times CuBP1 and CuBP2 were resolved as two bands by SDS-PAGE, and at other times one band of approximately 20 kDa was resolved. In order to determine whether sample processing techniques were causing protein breakdown, supernatant samples dialyzed for 16 h or 3 days were subjected to freeze-thaw cycles and compared to others stored at -80° C. Protein profiles for all treatments were indistinguishable (Figure 1.4). The calculated molecular weight of CuBP1 from this gel was 22.4 kDa, and CuBP2 was 21.7 kDa. However, when the same samples were analyzed on replicate gels, samples from all treatments contained one band with a calculated molecular weight of 22.0 kDa (data not shown; Gordon *et al.*, 1993).

Figure 1.4. SDS-PAGE of unfractionated control and 50 μM copper-challenged *Vibrio alginolyticus* supernatants subjected to different dialysis times and storage temperatures.

Lane 1: Control, 16 h dialysis, -20° C storage, 3 ml culture
Lane 2: 50 μM Cu, 16 h dialysis, -20° C storage, 3 ml culture
Lane 3: Control, 3 day dialysis, -20° C storage, 3 ml culture
Lane 4: 50 μM Cu, 3 day dialysis, -20° C storage, 3 ml culture
Lane 5: Control, 16 h dialysis, -80° C storage, 3 ml culture
Lane 6: Molecular weight standards
Lane 7: 50 μM Cu, 16 h dialysis, -80° C storage, 3 ml culture
Lane 8: Control, 3 day dialysis, -80° C storage, 3 ml culture
Lane 9: 50 μM Cu, 3 day dialysis, -80° C storage, 3 ml culture
Lane 10: Control, 16 h dialysis, -80° C storage, 20 ml culture[1]
Lane 11: 50 μM Cu, 16 h dialysis, -80°C storage, 20 ml culture[1]

[1]Samples were frozen and thawed five times before being analyzed.

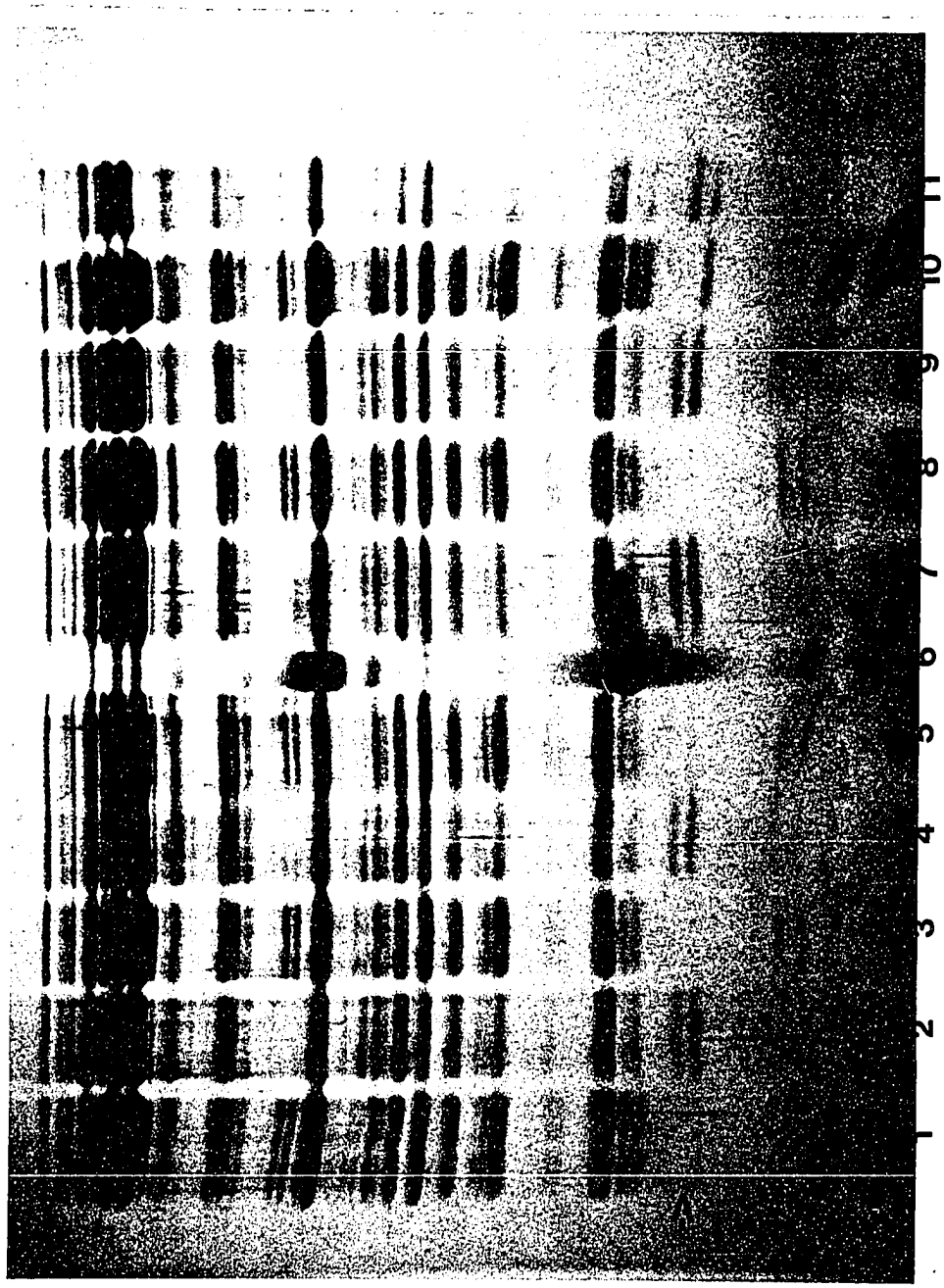


Figure 1.4

DISCUSSION

The presence of extracellular copper binding material in copper challenged *Vibrio alginolyticus* cultures was demonstrated by Schreiber *et al.* (1990). Coelution of copper-binding activity and a 21 kDa, copper-induced band (identified by SDS-PAGE) in GPC fractions were consistent with a model of copper complexation by extracellular protein(s) (Harwood-Sears and Gordon, 1990a). Supernatants from cadmium-stressed batch cultures contained ca. 20 kDa proteins that were not present in the control, suggesting that cadmium may induce a similar response (Gordon and Harwood-Sears, 1988). In this study, further evidence that the compound(s) responsible for extracellular copper binding are proteins was obtained, and their induction was studied as a function of time and of copper concentration.

In order to identify the copper-induced supernatant proteins of *Vibrio alginolyticus* with affinity for copper, gel permeation chromatography fractions were analyzed by SDS-PAGE. CuBP1 coeluted with copper-induced, copper binding activity in GPC fractions (Harwood-Sears and Gordon, 1990a), and CuBP2 was also sometimes detectable. Neither the CuBPs nor a peak in copper binding were detectable in GPC fractions from control supernatants (Harwood-Sears and Gordon, 1990a). When supernatants from 24 hour control cultures were compared to supernatants from 24 hour copper challenged cultures it was apparent that CuBP1 and CuBP2 are present at very low concentrations in controls. The dilution effect inherent in gel permeation chromatography coupled with the low concentration of CuBPs in control supernatants explains the absence of CuBPs in control GPC

fractions.

CuBP1 was detectable in 50 μM copper challenged supernatants as soon as two hours after the addition of copper. The concentrations of both CuBP1 and CuBP2 (detectable by 24 hours) increased most dramatically within 24 hours, and continued to increase from 24 to 48 hours. In a direct comparison of CuBP concentration in 24 hour control vs copper challenged supernatants, CuBP1 was 25 times more concentrated and CuBP2 was 46 times more concentrated in copper challenged supernatants. These proteins were not detectable in supernatants from copper challenged, chloramphenicol-treated cultures, supporting both the contention that they are the product of *de novo* synthesis and that they are proteinaceous.

CuBP expression (CuBP1 + CuBP2) was induced by copper concentrations as low as 1 μM, and increased steadily with increasing copper concentrations. Estimated CuBP concentrations were normalized to the standing stock in the cultures, since cell numbers in batch and chemostat (Gordon *et al.*, 1993) cultures decrease as a function of copper levels. In each case, whether the concentration of CuBP was normalized to optical density (tenfold increase from 1 - 50 μM Cu) or to cellular protein (tenfold increase), or was expressed as the percentage of supernatant protein (fourfold increase), the concentration of CuBP increased as a function of copper concentration.

Three ca. 20 kDa proteins (19 - 23.5 kDa) were quantitated and compared in the supernatants and cell pellets of 25 μM copper challenged cultures. Only CuBP1 was present at higher extracellular than intracellular concentrations. While this

observation supports the hypothesis that CuBP1 is an exported protein, it does not rule out the possibility that CuBP2 is also exported. CuBP2 was about three times more concentrated in the cell than in the supernatant, while there was seven times as much of the 23.5 kDa protein in the cell as outside the cell. The 23.5 kDa protein appeared erratically in supernatants from copper-challenged cultures, at varying concentrations or not at all.

The affinity of CuBP1 and CuBP2 for copper has been confirmed by immobilized metal ion affinity chromatography (IMAC) (Harwood-Sears and Gordon, 1990a). A copper-charged IMAC column on a Fast Protein Liquid Chromatography (FPLC) system was used to concentrate and partially purify the two copper-induced proteins. The 23.5 kDa protein mentioned above had relatively little affinity for the IMAC column, and is therefore not likely to be a protein with high affinity for copper. Likewise, a 17.6 kDa protein was detectable in some copper challenged cultures and not in others, but was always at very low concentration. This protein had some affinity for the IMAC column under the loading conditions described, but was one of the minor bands eluted from the column when it was detected (Harwood-Sears and Gordon, 1990a).

The model for copper detoxification by *Vibrio alginolyticus* suggested by all the data obtained to this point implicates an extracellular, proteinaceous copper-binding compound(s). The protein(s) are produced at an increased rate during the copper-induced lag phase and during regrowth of the cultures, resulting in a substantial concentration of soluble, nontoxic copper (Schreiber *et al.*, 1990). All proteins

complex copper to some extent and thus, any expression of extracellular protein would partially ameliorate the effects of copper. CuBP is a candidate for a specific copper-complexing protein in view of its inducibility by copper, its affinity for copper, and its increased relative contribution to supernatant protein with increasing copper concentrations. The possibility that these proteins complex copper intracellularly and are then exported in a manner analogous to the *E. coli pco* (Rouche *et al.*, 1989) system has not been ruled out.

CHAPTER II: CONTINUOUS CULTURE OF VIBRIO ALGINOLYTICUS IN THE PRESENCE OF COPPER: PHENOTYPIC AND GENETIC CHANGES

INTRODUCTION

Continuous culture of microorganisms is an alternative to conventional batch culture methods which can be advantageous in experiments designed to investigate the physiological effects of an environmental stress. During continuous culture, nutrient solution from a reservoir enters the system at flow rate f. Since the volume of the culture is finite, spent media containing cells must exit the system at the same value of f. The dilution rate, D, is determined by f/v, where v is the volume of the culture vessel. The rate of loss of cells (where x is the concentration of cells in the culture) can be expressed as:

$$\frac{dx}{dt} = \frac{f(x)}{v} = D(x)$$

In a continuous culture that has reached equilibrium μ, the specific growth rate, is equal to D, the dilution rate, so that $\mu x = Dx$. Thus, the contribution of new cell growth to the standing stock in the culture is equal to the number of cells exiting the culture. The maximum specific growth rate (μ_{max}) attainable in a given culture is determined by the minimum generation time (g) for the microorganism under the culture conditions, since $\mu = \ln 2/g$ (Brock and Madigan, 1991. p. 832). Among the

variables that contribute to μmax are the bacterium/strain being cultured, the composition of the medium, and temperature. If the flow rate in a continuous culture exceeds μmax, the quantity $\mu(x)$ will become less than D(x), and washout will occur.

The obvious advantage of continuous culture over batch culture in studies designed to measure an environmental effect on cell physiology is the continuous state of exponential growth the cells achieve in continuous culture. Heterotrophic bacteria enter a lag phase when first inoculated into fresh batch culture, followed by a period of exponential growth. After nutrients in the batch culture are exhausted, bacteria enter a stationary phase that is followed by cell death. The physiological state of the bacteria can be eliminated as a variable in continuous culture, allowing the effects of the environmental factor in question (in this case, added copper) to be more readily isolated.

The open nature of continuous culture systems is advantageous for studies of copper/microbial interaction, allowing continuous exposure of the organism to a calculated input of copper per unit time. Copper stress can be applied and maintained, or varied, over long periods of time (months) to a single culture. Dead cells and products of cell lysis are washed out of the system, minimizing any contribution they might make to supernatant protein profiles and/or copper complexation in batch culture.

Chemostat studies of the growth of *Vibrio alginolyticus* during continuously applied copper stress were initiated (1) to determine whether the bacteria could

survive in continuous culture during copper stress, (2) to test the hypothesis that cell numbers in copper-stressed cultures are significantly lower than their unstressed counterparts, which implies an energy-requiring mechanism of copper resistance, and (3) to determine whether CuBP is produced in continuous culture under copper-stressed conditions. We found that *V. alginolyticus* could survive long-term copper stress at levels of up to 33 μM with a generation time of 2.7 hours, that cell numbers decreased significantly in copper-stressed vs. unstressed cultures as a function of copper concentration, and that a protein of the same molecular weight as CuBP which displayed identical chromatographic behavior was expressed only in copper-stressed chemostat cultures (Gordon *et al.*, 1993).

This chapter describes the growth of *Vibrio alginolyticus* during short-term exposure (days) to increasing levels of copper in a continuous culture system. The possibility that the ca. 30 kilobase pair (kb) plasmid of *V. alginolyticus* is amplified during copper stress was explored by comparing plasmid DNA extracted from batch and chemostat cultures grown with and without copper stress (Harwood-Sears and Gordon, 1990b). Copper-resistant (Cu^r) *V. alginolyticus* were isolated from a chemostat culture under long-term copper stress (Gordon *et al.*, 1993). Their frequency (Cu^r cells/total culturable cells) was quantitated at several copper levels. Phenotypic variations from the wild-type (WT) *V. alginolyticus* and their relationship to the Cu^r phenotype were studied.

MATERIALS AND METHODS

Continuous Culture. The effect of added copper on chemostat cultures of *Vibrio alginolyticus* in artificial seawater medium (ASWM) was followed by monitoring the cell density in cultures with and without addition of micromolar levels of copper to the medium reservoir. *Vibrio alginolyticus* was grown in a one liter chemostat (Bio-Flo III, New Brunswick Scientific) in ASWM composed of 20 g/l Instant Ocean (Aquarium Systems, Mentor, OH), 19 mM NH_4Cl, 0.15 mM Na_2HPO_4 and 4 mM glucose, pH 7.5. The medium was filtered through a glass fiber filter before autoclaving; nutrients and glucose solutions were autoclaved separately. Copper ($CuSO_4$) was filter sterilized and added to the reservoir in copper-stressed cultures to a final concentration of 5, 10, or 20 μM. Chemostat pH was regulated at 7.5 with 0.2 N NaOH. Air flow was set at one liter/min., agitation at 200 r.p.m. and temperature at 25°C. The dilution rate (D) was 0.113/h (generation time (T_{gen}) = 6.1 h). Optical density was measured with a Klett-Summerson colorimeter. Absorbance on the meter was calibrated to cell numbers by acridine orange direct counts. Culture purity was monitored daily by streaking on Tryptic Soy Agar and Marine Agar 2216 plates (Difco).

Plasmid DNA was extracted from a chemostat culture before and after the addition of 20 μM $CuSO_4$ to the medium reservoir. The culture had been equilibrated with copper for 14 days, and had recovered to within approximately 80% of its pre-copper cell concentrations when it was collected (Gordon *et al.*, 1993).

Batch cultures for plasmid DNA extraction. Batch cultures were grown in

SWM9 broth as previously described (Chapter I) except that the culture volume was 9 liters and cultures were aerated with a stainless steel air stone. The control culture was harvested after 24 hours. Copper (25 μM) was added to the second culture during exponential growth. The copper-stressed culture was harvested after 48 hours.

Concentration of cells for plasmid DNA extraction. Cells were concentrated from cultures by tangential flow filtration (TFF) with a Pellicon cassette system (Millipore). Cells from the chemostat culture (3 l) were collected on ice, concentrated with a 0.2 μm (GVLP) filter cassette, and resuspended to a 1000X concentration in TE buffer (Tris HCl, 10mM; EDTA, 1 mM; pH 7.4). Batch cultures (8.5 l) were concentrated approximately 400X by TFF. Cell pellets were stored at --20° C until plasmid DNA was extracted.

Plasmid DNA extraction and electrophoresis. Plasmid DNA was extracted by the alkaline lysis method (Ausubel,1987). Seven ml of concentrated cells from each culture were used for the plasmid preparations. Plasmid DNA was treated with DNAase-free RNAase for 2 hours at 37° C. Electrophoresis was carried out in a 0.7% agarose gel run on a Fotodyne system. The gels were 9.5 mm x 7 mm x 1 mm, and were run at 59 V for 2 hours at room temperature. 10 μl of each sample were loaded on gels with the exception of the control batch culture (2 μl load), which was excessively viscous. Gels were stained in ethidium bromide (5 μg/ml) for 25 minutes, destained in distilled water for 10 minutes, and photographed.

Analysis of copper-resistant cells in chemostat cultures. Copper-resistant cells in the chemostat were isolated on artificial seawater medium buffered with HEPES

(Sigma) and supplemented with 20 μM $CuSO_4$ (HASW). Instant Ocean (I.O.) solution with HEPES added was filtered through a 0.2 μm filter to remove fine particulates. The I.O./HEPES solution was adjusted to pH 7.5. Agar was added, the mixture was brought to a boil, and autoclaved. Sterile glucose, nutrient (Na_2HPO_4/NH_4Cl) and copper solutions were added after the media was cooled. Glucose was made as a 200 g $\cdot l^{-1}$ stock solution, then autoclaved. The nutrients were made as a 100X solution (Na_2HPO_4, 15 mM; NH_4Cl, 1.9 M), then autoclaved. Copper was added from a filter-sterilized, 0.1 M stock solution. The composition of the medium less copper was as follows: Instant Ocean salts, 8 g/l; agar (Difco Bacto-agar) 15 g/l; glucose, 28 mM; HEPES, 25mM; NH_4Cl, 19 mM; Na_2HPO_4, 0.15 mM; pH 7.5.

Quality control for HASW plates included a positive control for both unamended and 40 μM Cu-amended plates (WT *V. alginolyticus* and Cu40B3 respectively), and a negative control for Cu-amended plates (WT *V. alginolyticus*). Controls were streaked from fresh (less than one week old) cultures, and growth was scored after two days (unamended) or four days (40 μM Cu). The variability in culturable counts on HASW plates was assessed by calculating the coefficient of variation (standard deviation divided by the mean x 100; Khazanie, 1979) for replicate ($n \geq 9$) sets of plates. Only sample dilutions containing 20-300 colonies were counted. Results from plate counts on marine agar 2216 (Difco), a commercially prepared medium, were used for comparison.

Samples were collected aseptically from the chemostat with and without added

copper. Serial dilutions of the samples were plated in triplicate on HASW with 20 or 40 μM copper. A culturable cell count was obtained by plating the same sample in triplicate on marine agar (Difco 2216) or on HASW agar. HASW plates with copper were counted after 5 - 7 days, and marine agar plates were counted after 24 and 48 hr. Only colonies larger than 1 mm diameter were counted on copper-containing plates. The number of these colonies, corrected for dilution, was designated as the number of copper-resistant cells in the culture. The percent copper-resistant cells was calculated from the ratio of colonies formed on copper-containing plates to those on marine agar or on HASW agar.

In one experiment, the ability of Cu^r isolates (Cu40A1 and Cu40B3) to form colonies on HASW + 40 μM Cu plates after 10 successive passages on marine agar (10° MA) was compared to that of recently Cu-stressed cultures. The inoculum for 10° MA broth cultures for both mutants was obtained from the tenth successive subculture on a marine agar plate. 40 ml broth cultures of Cu40A1 and Cu40B3 were grown in unamended SWM9 for the 10° MA treatment. The inoculum for recently Cu-stressed broth cultures of Cu40A1 and Cu40B3 was obtained from cultures growing on HASW + 40 μM Cu. A Cu40A1 broth culture was grown overnight in SWM9 + 50 μM Cu, while Cu40B3 was grown in unamended SWM9 (40 ml each). Broth cultures for both treatments were grown at room temperature on a shaker, serially diluted and plated on HASW agar with and without 40 μM Cu. Plates were incubated at room temperature for four days (no Cu) or one week (40 μM Cu).

Phenotypic determinations. The oxidase and swarming characteristics of *V. alginolyticus* from copper-stressed chemostat populations were scored for random samples isolated on marine agar, and for Cu^r colonies isolated on HASW + 40 μM Cu plates. Cu^r colonies were subcultured to marine agar for phenotypic determinations. Pathotec cytochrome oxidase test strips (Organon Teknika, Durham, NC) were used for the oxidase test. Swarming was scored visually after two days and five days, as some isolates swarmed more slowly than the wild-type.

RESULTS

Copper-stressed chemostat. In a short-term copper stress experiment, the chemostat culture was allowed to equilibrate for five days without added copper. When the medium reservoir was changed to HASW + 5μM $CuSO_4$, cell numbers decreased from 9 x 10^8 cells/ml to 7.5 x 10^8 cells/ml (Figure 2.1). After two additional days of exposure to 5 μM copper, the cell numbers had increased slightly to 8.0 x 10^8 cells/ml. When a final concentration of 10 μM $CuSO_4$ was added to the reservoir, cell numbers dropped to a low of 6.1 x 10^8 cells/ml, recovering slightly over the next two days. Increasing the total copper concentration to 20 μM caused cell numbers to decrease to 3.2 x 10^8 cells/ml after five days.

Plasmid DNA. Control and copper-stressed batch and chemostat cultures of *V. alginolyticus* were screened for the presence of plasmid DNA. All of the treatments contained a plasmid DNA band of ca. 30 kb (Figure 2.2). The cultures were not at the same optical density when harvested, nor were concentration factors

Figure 2.1. Short-term copper challenge of *Vibrio alginolyticus* in continuous culture. Optical density (O.D.) measured in Klett units.

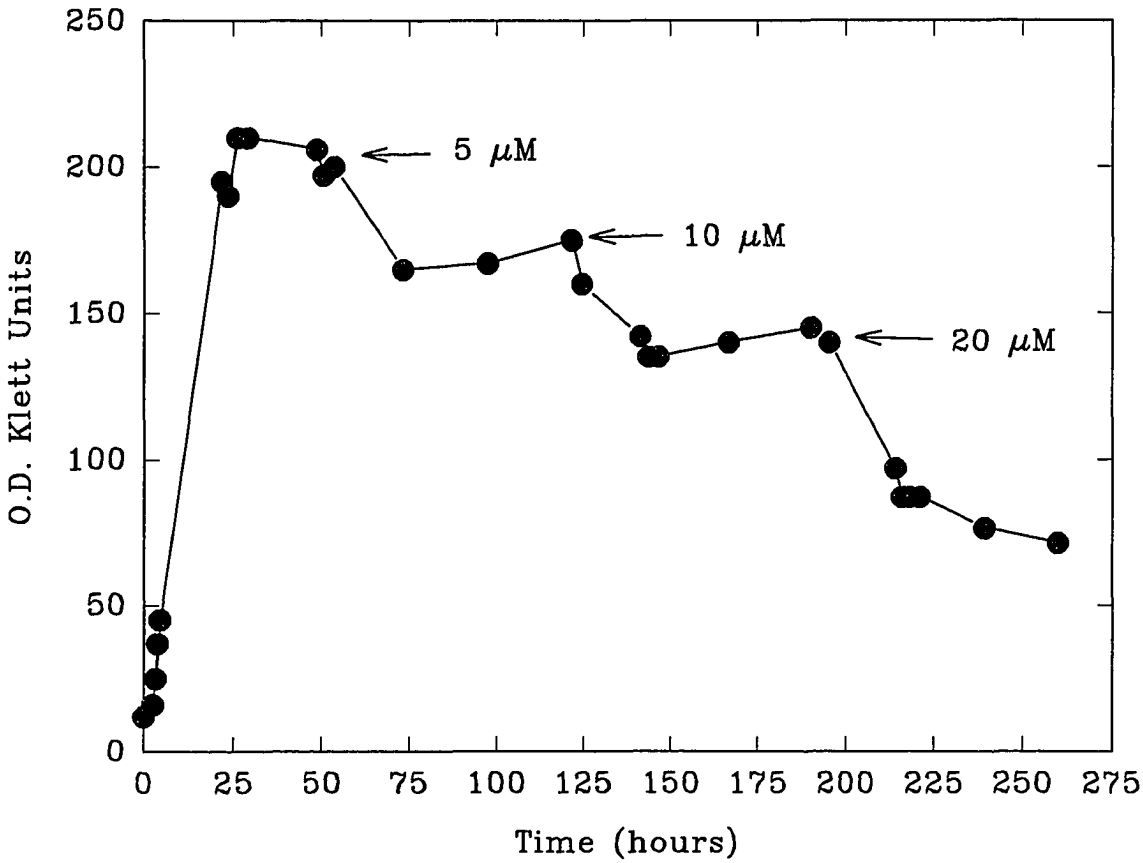


Figure 2.2. Plasmid DNA from control and copper-challenged *Vibrio alginolyticus* cultures.

Lane 1: Control batch culture
Lane 2: 50 μM copper-challenged batch culture
Lane 3: Molecular weight standards
Lane 4: control continuous culture
Lane 5: 20 μM copper-challenged continuous culture

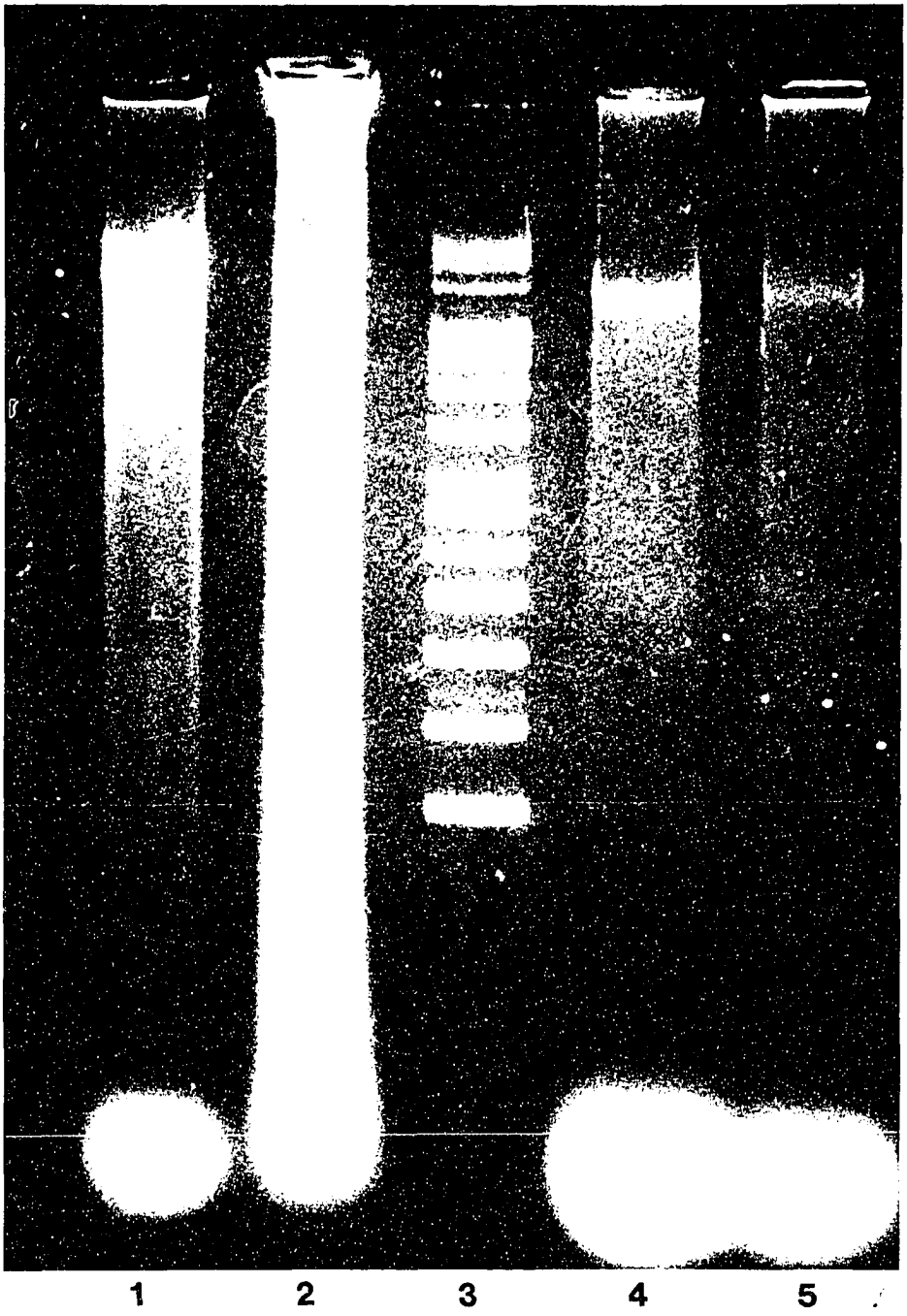


Figure 2.2

equal. These data are presented in Table 2.1. The quantity (cells/ml x CF) denotes the number of cells contributing plasmid DNA to the bands in Fig. 2.2.

Although the gel was not scanned, it is evident from visual inspection of Fig. 2.2 that the control batch culture sample contains the most plasmid DNA. Its copper-stressed counterpart has a faint plasmid band that is largely obscured by a smear which is probably chromosomal DNA fragments. The samples from the chemostat can be readily compared, as the plasmid bands are well resolved and plasmid DNA was extracted from similar cell numbers (Table 2.1). The plasmid band in the control chemostat sample is appreciably brighter than that from the copper-stressed chemostat culture, indicating that there was at least as much plasmid DNA/cell in the control chemostat culture as in its copper-stressed counterpart.

Copper-Resistant Mutants. Copper-resistant (Cu^r) mutants of *V. alginolyticus* were isolated from long-term, copper-stressed continuous cultures. Copper-resistant (Cu^r) mutants were selected by their ability to form colonies at least 1 mm in diameter on HASW agar plates supplemented with copper. Putative Cu^r isolates that could not form colonies 1 mm or greater when subcultured to copper-amended media were discounted. Cu^r variants of *V. alginolyticus* were indistinguishable from the wild type by gram stain and selected biochemical tests (API 20E), with the exception of the oxidase test (see below). All Cu^r *V. alginolyticus* isolates exhibited attenuated swarming on marine agar compared to the wild type, that is, they swarmed slowly or not at all. Copper concentrations in chemostat cultures and plates, and the frequency of colonies resistant to the stated concentrations of copper are given in Table 2.2.

Table 2.1. Cell numbers and concentration factors in cultures for plasmid extraction/electrophoresis.

Culture Conditions	Cells/ml	CF[1]	Cells x CF ($x10^{-11}$)
Control Batch	$1.3x10^{9}$	327X	4.3
25 μM Cu Batch	$4.3x10^{8}$	386X	1.7
Control Chemostat	$7.2x10^{8}$	160X	1.2
25 μM Cu Chemostat	$6.8x10^{8}$	150X	1.0

[1]CF = Concentration Factor

Table 2.2. Frequency of Cu^r mutants in copper-stressed continuous cultures.

Culture	Days[1]	[Cu] Chemostat	[Cu] Plates	Cells/ml No Cu	Cells/ml + Cu	F Cu^r [2]
A		0 μM	20 μM	$5.4x10^8$	$8.0x10^3$	$1.5x10^{-5}$
A	14	20 μM	20 μM	$2.4x10^8$	$1.4x10^8$	$5.8x10^{-1}$
A[3]	21	0 μM	20 μM	$5.6x10^8$	$7.5x10^5$	$1.3x10^{-3}$
B	27	20 μM	20 μM	$2.9x10^8$	$8.3x10^4$	$2.8x10^{-4}$
B	27	20 μM	30 μM	$2.9x10^8$	$5.5x10^3$	$2.0x10^{-5}$
C[4]	20	32 μM	40 μM	$1.9x10^7$	$4.2x10^6$	$2.2x10^{-1}$
C	30	20 μM	40 μM	$3.0x10^8$	$6.5x10^4$	$2.1x10^{-4}$
C[5]	34	20 μM	40 μM	$1.0x10^8$	$1.4x10^6$	$1.3x10^{-2}$
C	36	20 μM	40 μM	$5.4x10^8$	$1.0x10^5$	1.9×10^{-4}
C	36	20 μM	20 μM	$1.0x10^8$	0	$< 1.9x\ 10^{-4}$
C	42	20 μM	40 μM	$5.8x10^8$	$3.4x10^5$	5.9×10^{-4}
C	42	20μM	20 μM	$5.8x10^8$	$6.5x10^5$	$1.1x10^{-3}$
D[6]	18	20 μM	40 μM	$5.6x10^8$	$1.9x10^7$	$3.4x10^{-2}$

[1] Number of days of exposure to copper.

[2] Frequency Cu^r calculated by cell number on copper-amended media divided by cell number on nonselective media.

[3] Cu-containing medium was replaced with medium with no added copper 7 days before the sample was taken.

[4] Culture C had been exposed to 16 μM copper for 13 days before the copper concentration was raised to 32 μM. Time of exposure given is cumulative from beginning of exposure to 16 μM Cu.

[5] Cell numbers were dropping at the time this sample was taken. Later samples were collected after the culture had recovered.

[6] Culture D was exposed to 5, 10, 15, then 20 μM Cu in a stepwise manner. Time of exposure is cumulative over all Cu concentrations.

Coefficients of variation within triplicate plate counts for marine agar, HASW and HASW + Cu plates were calculated. The average coefficients of variation were: marine agar, 8.0 (n=9); HASW, 11.3 (13); HASW + Cu, 12.0 (9). The frequency of Cu^r (20 μM) cells in batch cultures averaged $4.7 \times 10^{-5} \pm 7.1 \times 10^{-5}$, ranging over several orders of magnitude between cultures.

The addition of copper to continuous cultures resulted in increased frequencies of Cu^r colonies. The frequency of cells resistant to 20 μM Cu [frequency Cu^r (20 μM)] in a control chemostat culture (Culture A, Table 2.2) was 1.5×10^{-5}. Fourteen days after the addition of 20 μM Cu, the same culture contained a Cu^r (20 μM) frequency of 5.8×10^{-1}, a 39,000 fold increase. When copper-amended media was replaced with unamended media, the frequency of cells resistant to 20 μM Cu decreased to 1.3×10^{-3}.

In a second continuous culture (Culture B, Table 2.2), the frequency of cells resistant to 20 μM Cu was 2.8×10^{-4} after 27 days of copper stress (20 μM). The same sample contained a frequency of 2.0×10^{-5} cells resistant to 30 μM Cu.

A third continuous culture (Culture C, Table 2.2) was stressed with 32 μM Cu. This culture contained 22% cells resistant to 40 μM Cu (frequency $= 2.2 \times 10^{-1}$). No control (0 μM Cu) data is available for this experiment, however, the frequency of cells resistant to 40 μM Cu from control batch cultures ranged from 6.7×10^{-8} to $< 3.1 \times 10^{-9}$ (Table 2.5). When copper in the reservoir was decreased to 20 μM, the frequency of Cu^r (40 μM) cells dropped to 2.1×10^{-4}. Cell densities dropped precipitously in the culture around day 33 (post Cu addition) due to

unknown factors. A sample taken while the density was dropping gave a frequency of 1.3×10^{-2} Cu^r (40 μM) cells. After the culture had recovered to its pre-crash density, the frequency Cu^r dropped to 1.9×10^{-4}. Cells from this sample were also spread on plates containing 20 μM Cu. No colonies grew on the lowest dilution plated (10^{-4}), resulting in a frequency Cu^r (20 μM) of $< 1.9 \times 10^{-4}$. Six days later, the frequency Cu^r (40 μM) was 5.9×10^{-4}, and the frequency Cu^r (20 μM) was 1.1×10^{-3}.

A fourth chemostat culture (D, Table 2.2) was stressed with incrementally increasing copper concentrations from 5 to 20 μM (unpublished data). The culture was sampled after 11 days of exposure to 20 μM Cu (18 days total exposure to elevated [Cu]). This culture was resistant to 40 μM Cu at a frequency of 3.4×10^{-2}.

Phenotypic changes in long-term copper-stressed continuous cultures. V. alginolyticus normally exhibits vigorous swarming motility on solid media containing relatively high Na levels, such as marine agar. Quality control for chemostat cultures in this lab includes streaking samples on marine agar (MA) and trypticase soy agar (TSA) to check culture purity. Over the course of long-term copper stress in chemostat cultures, nonswarming *V. alginolyticus* were consistently found among the swarming cells (Gordon *et al.*, 1993), and were sometimes the dominant phenotype found in extremely stressed cultures. This phenomenon occurred in cultures exposed to copper concentrations as low as 15 μM (Figure 2.3). In most cases, the nonswarming colonies swarmed normally

Figure 2.3. *Vibrio alginolyticus* colonies from a 20 μM copper-challenged continuous culture plated on marine agar. Colonies are a mixture of swarming (S) and nonswarming (NS) phenotypes.

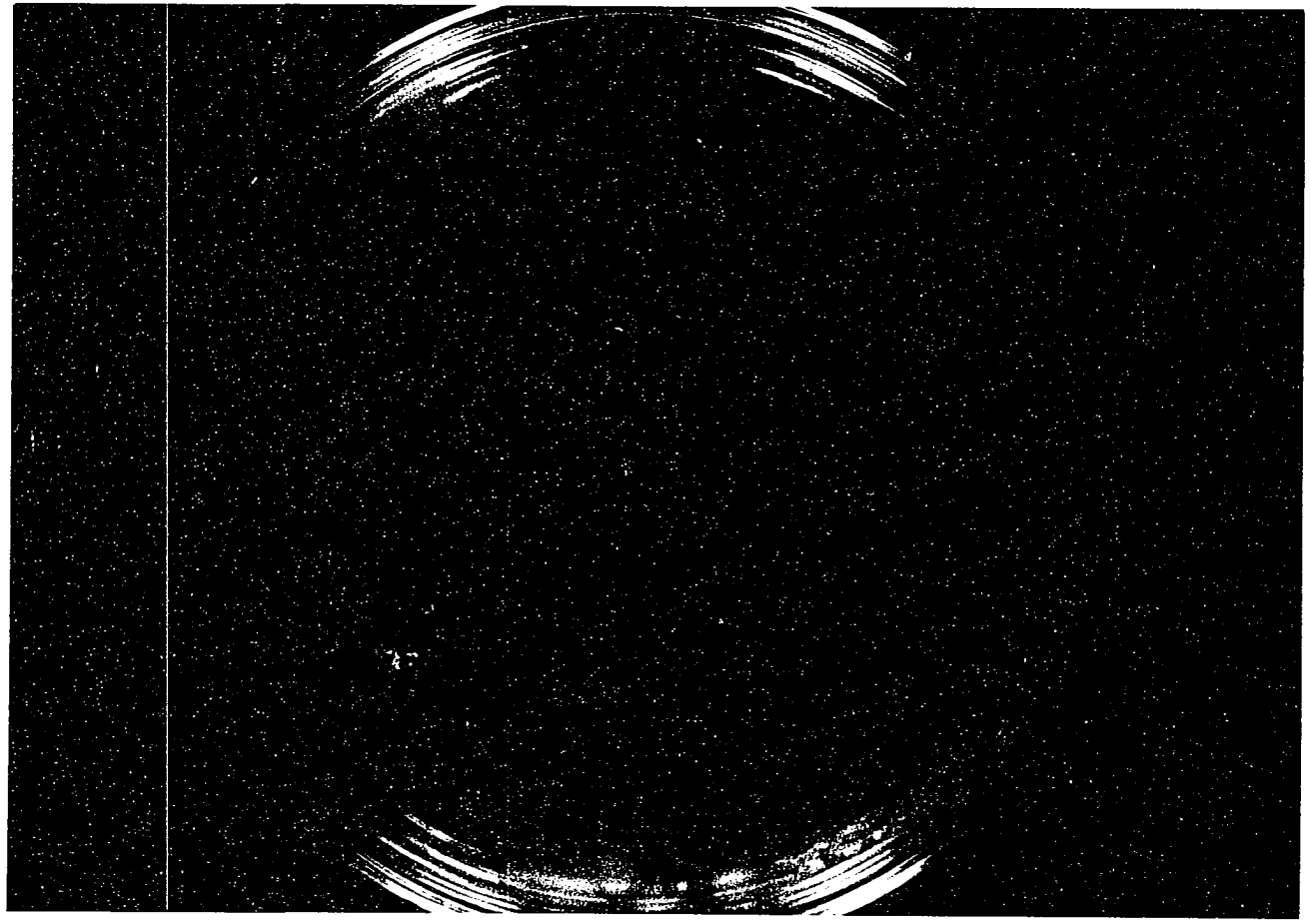


Figure 2.3

Figure 2.4. Wild-type *Vibrio alginolyticus* colonies plated on marine agar. All are displaying swarming motility.

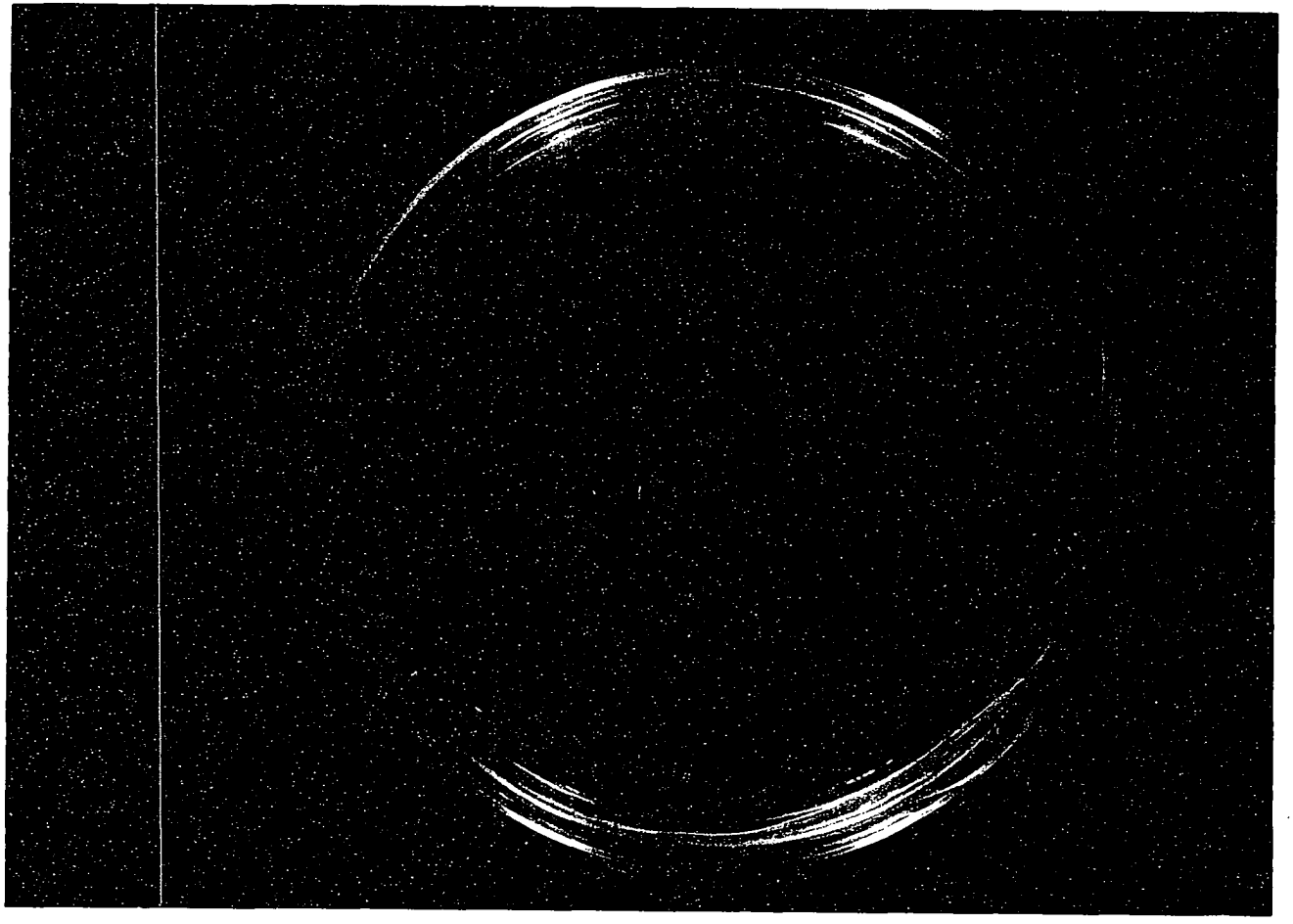


Figure 2.4

when subcultured to a fresh marine agar plate, however some isolates did not regain the swarming phenotype even after repeated subculture on nonselective agar.

A second phenotypic deviation noted after long-term copper stress was the mutation from an oxidase positive to oxidase negative phenotype. Unlike the nonswarming variants, which tend to readily revert to swarmers, the oxidase negative mutants retain this phenotype after repeated subculture on nonselective agar. A possible correlation between the oxidase negative phenotype and copper resistance was investigated after it became apparent that a high percentage of the copper-resistant isolates from a 32 μM copper-stressed culture (Culture C, Table 2.2) were oxidase negative.

At the time these experiments were carried out, the copper concentration in the reservoir had been reduced to 20 μM. Of ten colonies isolated on marine agar, none were oxidase negative (Table 2.3). The culture was sampled several days later, just after cell numbers had "crashed". The culture had become dimorphic, consisting of normal-sized colonies (2 - 3 mm diameter), and smaller colonies about 1 mm in diameter. None of the colonies exhibited swarming motility. None of the normal colonies tested were oxidase negative (Table 2.3), however 100% of the small colonies were oxidase negative. When the oxidase negative colonies were subcultured to HASW + 40 μM Cu, 7 of 15 isolates showed minimal growth on the plates. After further subculturing on Cu-amended agar, 2 of the 15 (13.3%) were found to be copper-resistant, i.e. showed

Figure 2.5. Growth of the copper-resistant mutant Cu40B3 plated on HASW + 35 μM Cu compared to that of wild-type *Vibrio alginolyticus*.

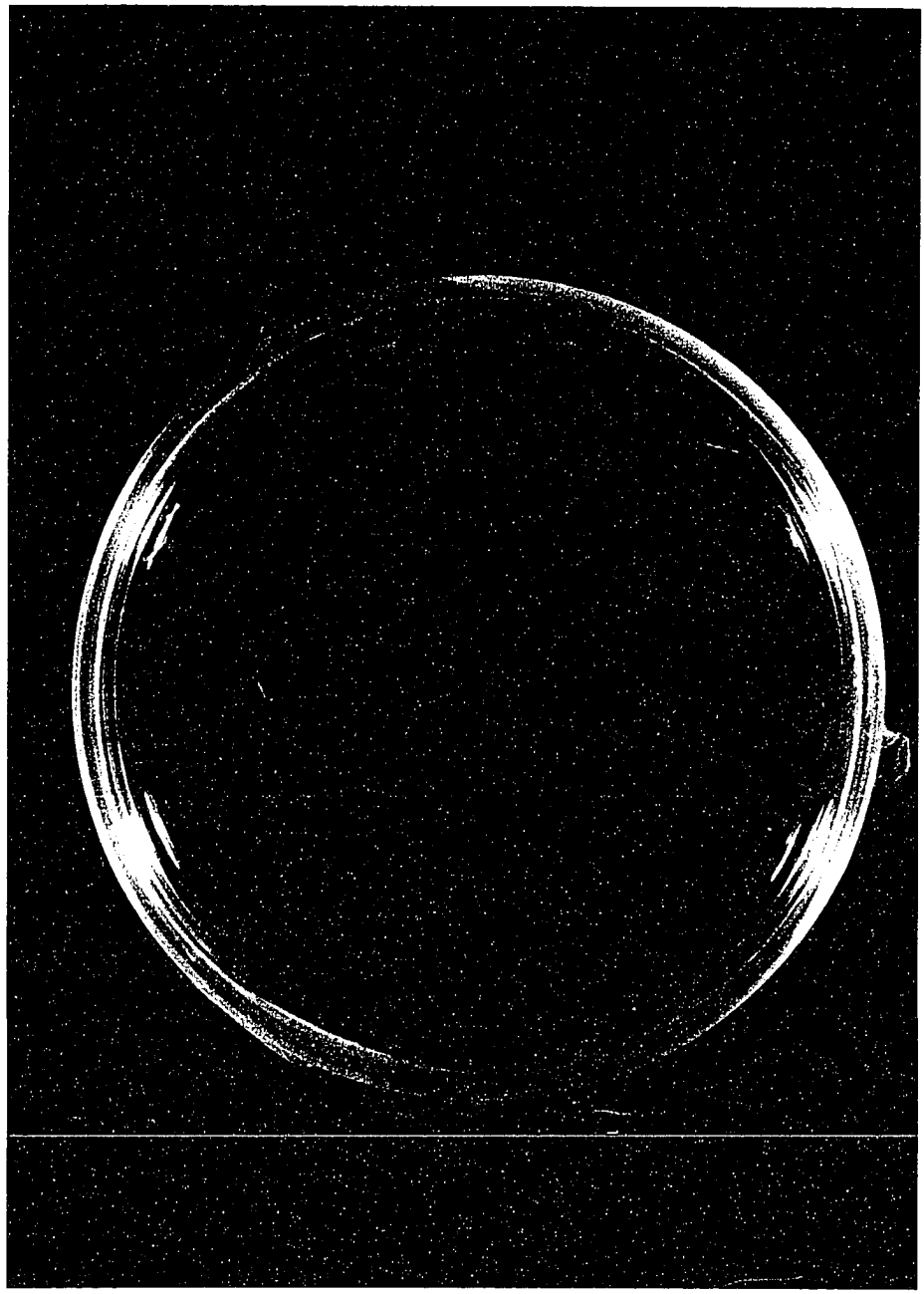


Figure 2.5

Table 2.3. Percent oxidase negative *Vibrio alginolyticus* in a copper-stressed chemostat culture.

Trial	Nonselective Isolates (n)[2]	40 μM Cu Isolates[3]
1	0% (10)	93% (14)
2 ($\geq$ 2 mm)[1]	0% (12)	
2 (1 mm)[1]	100% (15)	

[1] Colony diameter.

[2] Colonies isolated on Marine Agar 2216.

[3] Colonies isolated on HASW + 40 μM Cu, and subcultured to Marine Agar 2216 for oxidase test.

substantial growth on Cu-amended agar over several passages.

The occurence of a cytochrome oxidase negative phenotype was strikingly frequent among Cu^r *V. alginolyticus* isolates. Of 41 Cu^r isolates tested from batch and continuous cultures, 30 were oxidase negative (73%). When the oxidase test was carried out on copper-resistant isolates from continuous culture C, 93% were found to be oxidase negative. The sole oxidase positive, Cu^r isolate was designated Cu40B3. An oxidase negative isolate, isolated when the culture was stressed with 32 μM Cu, was designated Cu40A1. Both of these isolates were nonswarming after serial passages on marine agar.

The stability of the Cu^r phenotype was tested by plating Cu40A1 and Cu40B3 on HASW + 40 μM Cu after 10 successive passages on marine agar. Their ability to form colonies on copper-amended plates was compared to that of cultures recently exposed to copper (Table 2.4). Wild-type *V. alginolyticus* from an unchallenged broth culture, plated for comparison with the variants, were resistant to this level of copper at a frequency of 6.7×10^{-8}.

Eighty-six percent of the experienced (recently copper challenged) Cu40A1 cells could form colonies on Cu-amended plates. A slightly smaller proportion (54%) were resistant after ten nonselective passes. Only 0.02% (2.4×10^{-4}) of experienced Cu40B3 cells could grow on Cu-amended plates, and 0.05% (4.7×10^{-4}) could grow after ten nonselective passes. Thus, Cu40A1 and Cu40B3 populations contained essentially the same percentage of copper-resistant cells after extensive subculture on nonselective media as they did when recently

Table 2.4. Comparison of the frequency of copper-resistant colonies and stability of the phenotype after 10 successive nonselective passages for copper-resistant variants of *Vibrio alginolyticus*.

Isolate	Culture Conditions	Cell • ml^{-1} HASW (No Cu)	Cell • ml^{-1} HASW+40 μM Cu	Frequency Cu^r
Wild-Type	MA	1.0×10^9	67	6.6×10^{-8}
Cu40B3	10° MA[1]	1.5×10^9	7.2×10^5	4.7×10^{-4}
Cu40B3	Recent Cu[2]	1.2×10^9	3.0×10^5	2.4×10^{-4}
Cu40A1	10° MA[1]	5.2×10^8	2.8×10^8	5.4×10^{-1}
Cu40A1	Recent Cu[2]	3.5×10^8	3.0×10^8	8.6×10^{-1}

[1]Cu40B3 and Cu40A1 colonies were taken from HASW + 40 μM $CuSO_4$ plates, passed ten times on nonselective marine agar, and grown overnight in SWM9 broth. Stable Cu^r colonies were quantitated by spread plating the overnight culture on HASW + 40 μM Cu.

[2]Cu40B3 and Cu40A1 colonies were taken from HASW + 40 μM Cu plates, grown overnight in a broth culture (SWM9 for Cu40B3; SWM9 + 40 μM Cu for Cu40A1), and Cu^r colonies quantitated by spread plating on HASW + 40 μM Cu.

exposed to copper. Both mutants retained their oxidase phenotype through the nonselective passes.

In subsequent experiments, Cu^r individuals in Cu40B3 cultures comprised, at most, 34% of the total population (Table 2.5), and averaged 11.6%. In one experiment, Cu40B3 was grown in broth culture supplemented with 50 μM Cu, then plated on HASW + 40 μM Cu (Table 2.5). The frequency of Cu^r cells in this culture was 3.0×10^{-1} (30%) which, although high, was lower than the corresponding Cu40B3 culture grown in broth without Cu. When this experiment was performed twice with Cu40A1, 79% and 86% of cells grown in copper-amended broth were copper-resistant. The measured frequency of Cu^r cells in Cu40B3 cultures is thus both lower and more variable than that in Cu40A1 cultures.

After the fourteenth successive passage of Cu40B3 on marine agar, some colonies reverted to swarming motility. A concurrent decrease in the number of colonies on streak plates (HASW + 40 μM Cu) was noted. After several successive subcultures of swarming colonies on MA, a pure culture of swarming segregants was obtained (Cu40B3 SW). No discreet colonies grew on Cu-amended streak plates of Cu40B3 SW.

The loss of copper resistance in Cu40B3 SW was confirmed by comparing the frequency of Cu^r (40 μM) revertants against the frequency of Cu^r (40 μM) Cu40B3 (Table 2.5). In five experiments, the frequency of Cu^r revertants was never higher than 5.6×10^{-8}. No Cu^r colonies were isolated from revertant cultures in 3 of 5 experiments (Table 2.5). Nonswarming Cu40B3 (the phenotype originally isolated

Table 2.5. Comparison of the ability of Cu40B3 (NS) and its swarming derivative Cu40B3 (SW) to form colonies on 40 μM Cu-supplemented media.

Isolate	Culture Conditions	Date	Plate Count (0 μM Cu)	Plate Count 40 μM Cu (cells/ml)	Frequency Cu^r
WT V. alg.	MA→SWM9	4-30-92	1.0×10^9	66	6.7×10^{-8}
WT V. alg.	MA→SWM9	5-13-92	1.0×10^9	0	$<1.6 \times 10^{-9}$
WT V. alg.	MA→SWM9	7-22-92	1.6×10^9	0	$<2.0 \times 10^{-9}$
WT V. alg.	MA→SWM9	7-28-92	2.7×10^9	0	$<1.9 \times 10^{-9}$
WT V. alg.	MA→SWM9	8-11-92	3.2×10^9	0	$<3.1 \times 10^{-10}$
Cu40B3 (NS)	10° MA→ SWM9	4-30-92	1.5×10^9	7.2×10^5	4.7×10^{-4}
Cu40B3 (NS)	Cu→SWM9	4-30-92	1.2×10^9	3.0×10^5	2.4×10^{-4}
Cu40B3 (NS)	Cu→SWM9	6-4-92	4.1×10^7	1.7×10^6	4.2×10^{-2}
Cu40B3 (NS)	Cu→SWM9 +50 μM Cu	6-28-92	9.5×10^7	2.9×10^7	3.0×10^{-1}
Cu40B3 (NS)	Cu→SWM9	6-28-92	8.5×10^7	2.9×10^7	3.4×10^{-1}
Cu40B3 (NS)	Cu→SWM9	7-28-92	7.2×10^8	8.1×10^6	1.1×10^{-2}
Cu40B3 (NS)	Cu→SWM9	8-11-92	1.6×10^9	5.5×10^7	3.4×10^{-2}
Cu40B3 (NS)	Cu→SWM9 + 50μM Cu	9-16-92	4.0×10^7	3.0×10^7	7.5×10^{-1}
Cu40B3 (NS)	Cu→SWM9	9-16-92	4.1×10^8	1.4×10^7	3.4×10^{-2}
Cu40B3 (Sw)	MA→SWM9	6-4-92	1.4×10^9	0	$<3.6 \times 10^{-9}$
Cu40B3 (Sw)	MA→SWM9	7-12-92	8.3×10^8	0	$<6.1 \times 10^{-9}$
Cu40B3 (Sw)	MA→SWM9 + 50 μM Cu	7-12-92	9.5×10^6	0	$<5.3 \times 10^{-7}$
Cu40B3 (Sw)	MA→SWM9	7-22-92	1.4×10^9	17	1.2×10^{-8}
Cu40B3 (Sw)	MA→SWM9	7-28-92	1.7×10^9	95	5.6×10^{-8}

from continuous culture) yielded comparatively high frequencies of Cu^r colonies, ranging from 2.4×10^{-4} (10° MA) to 3.4×10^{-1} (Table 2.5). Cu^r (40 μM) colonies from WT *V. alginolyticus* cultures were isolated from only one experiment out of four, where the frequency Cu^r (40 μM) was 6.7×10^{-8}. Thus, the frequency of Cu^r cells in swarming revertants of Cu40B3 is close to that of the wild-type.

When Cu^r (40 μM) colonies were isolated from Cu40B3 SW cultures, they were subcultured on MA. Of seven isolates, two displayed wild-type swarming, and five were a mixture of swarming and nonswarming colonies. All formed discreet colonies when subcultured to copper-amended plates. When the secondary copper culture was subcultured to MA, all seven isolates contained a mixture of swarming and nonswarming colonies. The observed switch from swarming to nonswarming culture morphology in the Cu40B3 SW derivatives is consistent with the observations made for *V. alginolyticus* in copper-stressed continuous culture: exposure to toxic levels of copper inhibits swarming in some, but not all cells. In most isolates the phenomenon is readily reversible, but in others, i.e. Cu40B3 and Cu40A1, the ability to swarm is permanently or semi-permanently ablated.

DISCUSSION

The short-term copper stress experiment (Figure 2.1), in which cell densities dropped, then equilibrated at a lower level with increasing copper concentrations, suggested that *Vibrio alginolyticus* overcomes the toxicity of copper by an energy-requiring mechanism. The short-term copper-stressed chemostat results were also

consistent with observations of decreased cell densities with copper stress in batch cultures (Howell *et al.*, 1992). Studies of long-term copper stress in continuous cultures confirmed that, at equilibrium, the yield (Y = grams biomass/grams substrate) (Brock and Madigan, 1991) in copper-stressed cultures is significantly lower than in unstressed cultures, and is an inverse function of copper concentration (Gordon *et al.*, 1993). CuBP, which was purified from copper-stressed continuous cultures by IMAC and RPHPLC, was not detected in control cultures, and the concentration of CuBP was shown to be a function of copper concentration (Gordon *et al.*, 1993). When copper (20 μM) was withdrawn from a copper-stressed culture for several days, then reapplied, the culture did not experience the precipitous drop in cell densities consistently observed with the application of 20 μM Cu to a "naive" culture, indicating persistent copper resistance in the population in the absence of copper (Gordon *et al.*, 1993).

The presence of a ca. 30 kb plasmid in the wild-type *Vibrio alginolyticus* strain was confirmed by plasmid DNA extraction and agarose electrophoresis. Metal (Silver and Misra, 1988) and copper (Bender and Cooksey, 1986; Rouche *et al.*, 1989; Tetaz and Luke, 1983) resistance mechanisms are frequently plasmid-mediated, and metallothionein-mediated resistance to copper can be accomplished by gene amplification (Karin *et al.*, 1984) and plasmid amplification (Jeyaprakash *et al.*, 1991). Plasmid DNA content was compared in control and copper-challenged chemostat and batch cultures in order to determine whether plasmid amplification plays a role in copper detoxification in *V. alginolyticus*. Although the batch cultures were difficult

to compare due to the smearing of DNA fragments in the Cu-challenged culture, the chemostat preparations, which were extracted from similar cell numbers, argue against plasmid amplification as a mechanism for copper resistance.

The location of the *cbp* gene and its associated regulatory unit remains to be determined. The *V. alginolyticus* plasmid is cryptic, that is, no physiological or genetic function has been identified. Many members of the genus *Vibrio* carry plasmids, i.e. pJM1, which encodes anguibactin, a siderophore, in *Vibrio anguillarum* (Actis *et al.*, 1986). *V. cholerae* carries a conjugative P plasmid which is not associated with virulence factors (Silverman *et al.*, 1991), and *V. salmonicida* a plasmid whose function is thus far unknown (Valla *et al.*, 1992). We have found no reports in the literature of plasmids native to *V. alginolyticus*. The plasmid described in this dissertation may prove useful in genetic manipulations of *V. alginolyticus*.

Plasmid-mediated copper resistance systems often confer resistance to very high (millimolar) levels of copper (Bender and Cooksey, 1986; Tetaz and Luke, 1983). Extrachromosomal elements such as plasmids are also relatively easily lost from bacterial genomes (Atlas and Bartha, 1992), and generally do not carry genetic systems that are required under normal environmental stresses. Thus, chromosomal location of the genetic system responsible for Cu detoxification in *V. alginolyticus* would be consistent with the hypothesis that this marine bacterium has evolved under conditions of transient copper stress from environmental sources such as upwelling deep ocean waters, which have acted as a strong selective pressure for retention of the system.

Copper-resistant mutants, or variants of *V. alginolyticus*, could be isolated from long-term copper-stressed continuous cultures. The relatively high frequency of individuals resistant to 20 μM Cu in a noncopper-stressed chemostat (1.5×10^{-5}) is unrealistically high for a spontaneous mutation rate at a single locus, suggesting either (a) that many different mutations can confer resistance to 20 μM copper or (b) that a subpopulation of variant, relatively copper-tolerant individuals exists in each culture. The 10^{-5} frequency Cu^r (20 μM) falls within the range of estimates from batch cultures, thus it is not a phenomenon associated only with continuous cultures. Copper stress (20 μM) increased the number of resistant colonies to 5.8×10^{-1}, a 39,000 fold increase (Table 2.2). When copper stress was withdrawn from the system, the frequency of Cu^r colonies decreased almost 400 fold, although it was still 100 fold higher than in the control. At least two alternative explanations for the decrease in Cu^r colonies with relief of copper stress are possible: (a) some of the colonies from the copper-stressed culture that grew on Cu-amended plates may have benefitted from induction of a copper resistance mechanism, allowing them to form colonies only when "pre-induced" by growth in Cu-containing liquid media; or (b) some or all of the Cu^r variants had a slower growth rate than WT *V. alginolyticus* in the absence of copper, and so were slowly out-competed by the wild-type in the noncopper-stressed chemostat environment.

The second chemostat experiment at 20 μM Cu illustrates the great variability in the frequency of Cu^r mutants from one culture to the next. In this experiment, the frequency of cells resistant to 20 μM Cu was 2.8×10^{-4}. The frequency of cells

resistant to 30 μM Cu was 2.0×10^{-5}, an order of magnitude lower than the Cu^r (20 μM) frequency. Evidently, different mutation(s) are mediating resistance to 20 and 32 μM Cu. Thus, the culture-to-culture variability and the lower frequencies of individuals resistant to higher copper levels suggest that the mutations which give rise to copper-resistant populations can be different both within a culture, and from one culture to the next.

At 20 μM Cu in continuous culture, *Vibrio alginolyticus* are nearing their limit in terms of copper stress, and sometimes wash out of the system (Gordon *et al.*, 1993), i.e. the specific growth rate, μ, falls below D, the dilution rate, and cells exit the system faster than the rate of production of new cells. When a continuous culture was stressed at 32 μM Cu, a very high level for this system, the frequency of cells resistant to 40 μM Cu was 2.2×10^{-1}. Unlike the relatively high frequency of cells resistant to 20 μM Cu in control *V. alginolyticus* cultures, fewer than one in a billion (10^{-9}) are resistant to 40 μM Cu in HASW plates. The frequency Cu^r (40 μM) individuals dropped 1000 fold when copper in the chemostat reservoir was decreased to 20 μM; a change analogous to the decrease in frequency Cu^r (20 μM) when copper was withdrawn from the 20 μM Cu-stressed chemostat.

A noteable increase in the frequency Cu^r (40 μM) cells (from 2.1×10^{-4} to 1.3×10^{-2}) occurred when cell densities plunged while the continuous culture was stressed with 20 μM Cu. Although the cause of the "crash" was never determined, it may be speculated that, given the rise in frequency of Cu^r cells at that time, the culture may have been responding to a sudden influx of copper caused by unequal mixing in the

medium reservoir. Alternatively, if cell densities dropped due to factors not related to copper stress, fewer bacteria would be left to overcome the effects of a constant copper input (thus effectively raising the number of copper ions/bacterium). Copper is largely bacteriostatic, rather than bactericidal, to *V. alginolyticus* at low to mid micromolar levels, therefore the increase in Cu^{2+}/cell would probably depress the growth rate of the wild-type to the point where remaining mutants would regain their physiological advantage, outgrowing the wild-type until the level of Cu^{2+}/cell had equlibrated. Thus, the frequency of Cu^r cells in the culture would increase, at least transiently, in either case. The second scenario, in particular, argues that copper-resistant cells may grow significantly more slowly than the wild-type in media without copper, a characteristic which has been confirmed for Cu40B3 (Chapter 3).

Recovery of the culture was accompanied by a drop in the frequency of Cu^r (40 μM) cells, as would have been expected given previous results. The frequency of cells resistant to 20 μM Cu was higher than the frequency of cells resistant to 40 μM Cu in a split sample, once again suggesting a heterogeneous population of Cu^r mutants/variants.

The fourth continuous culture, Culture D, had never been exposed to copper concentrations higher than 20 μM, but contained a frequency of 3.4×10^{-2} cells resistant to 40 μM Cu. Cell numbers in this culture had suffered a "crash" six days before sampling, when the outflow system malfunctioned and became stuck in the "on" position, pumping approximately 25% of the culture volume out. The culture was brought back to original volume with 20 μM Cu-amended media, effectively

raising Cu^{2+}/cell. As predicted, cell numbers in the culture were seriously depleted, dropping to 20% of their pre-crash density. Data was not obtained earlier in the experiment, so no comparison of pre- and post-crash Cu^r frequencies can be made, however, the frequency Cu^r in this culture was very close to that of Culture C after its crash. Thus, the data from these continuous cultures supports the hypothesis that an effective rise in copper ions/cell will result in selection for a more copper-resistant organism, and suggest controlled studies of competition between copper-resistant and wild-type *V. alginolyticus* in continuous cultures.

The morphological variability in long-term copper-stressed *Vibrio alginolyticus* populations included attenuation or ablation of swarming motility. *V. alginolyticus* has a single, sheathed flagellum when grown in liquid culture (Baumann and Baumann, 1977). Swarming motility is accomplished by many unsheathed, peritrichous flagella whose synthesis is mediated by induction of the lateral flagellar genetic system (*laf* in *V. parahaemolyticus*; McCarter and Silverman, 1989) by surface contact. Marine agar streak plates from copper-stressed continuous cultures (15 μM or higher) consistently display high numbers of nonswarming or very slowly swarming colonies. Many of these colonies swarm like the wild-type when subcultured to new plates, however some are apparently "stuck" in a nonswarming mode. Although both of the copper-resistant mutants from continuous culture that were selected for further study are nonswarmers, the majority of nonswarming colonies were not copper-resistant, and swarming, Cu^r mutants of *V. alginolyticus* have been isolated (Chapter 3). Swarming Cu40B3 revertants lost copper resistance, and their Cu^r derivatives

displayed a mixed swarming/nonswarming phenotype, indicating that there is an inverse correlation between swarming motility and copper resistance in Cu40B3. Thus, although the nonswarming phenotype appears to be necessary for copper resistance in one mutant, it is neither required by nor exclusive to copper resistance in *V. alginolyticus* populations.

The two classes of nonswarming colonies isolated from copper-stressed chemostats, i.e. stable nonswarmers and those which readily revert to swarming motility, suggest that different mechanisms are mediating these phenomena. Copper may be acting directly or indirectly as an environmental stimulus in the case of reversible inhibition of swarming. Copper can be a competitive inhibitor of uptake of physiolgical cations, i.e. Mn^{2+} and Zn^{2+} (Bruland *et al.*, 1991). By creating a nutrient deficient environment in this or some other manner, copper may indirectly inhibit swarming. There are reports of interaction of copper and other metals with flagellar systems. Transcription of the *fliC* gene, which encodes the flagellin of the polar *Escherichia coli* flagellum, is induced by copper, aluminum, iron and nickel (Guzzo *et al.*, 1991). Transcription of the *lafA* gene of *V. parahaemolyticus*, which encodes the lateral flagellin subunit, is dependent on iron-limiting conditions (McCarter and Silverman, 1989). Thus, copper may interact directly with regulatory elements to block synthesis or function of lateral flagella, or may interact with sensory transduction pathways which monitor environmental stimuli such as metal ion levels.

The oxidase test is a relative measurement of cytochrome oxidase activity.

Cytochrome oxidase, which is the terminal oxidoreductase in mitochondrial and some bacterial electron transport systems, contains two copper ions and two heme groups. The enzyme catalyzes the transfer of electrons from cytochrome c to oxygen to yield H_2O (Stryer, 1988). Most *Vibrio* species, including *Vibrio alginolyticus*, are oxidase positive (Baumann and Baumann, 1977). The oxidase negative phenotypes noted in copper-stressed chemostat cultures were, apparently, due to mutations, as they were not reversible. Although not all oxidase negative mutants were copper-resistant, 73% of all copper-resistant *V. alginolyticus* mutants isolated to this date are oxidase negative. Additionally, 13.3% of oxidase negative colonies picked from nonselective agar proved to be copper-resistant in a sample in which 1.3% of all cells were copper-resistant. Thus, the oxidase negative colonies were resistant to 40 μM Cu at ten times the frequency of the total chemostat population.

The data presented here suggest that the oxidase negative phenotype may contribute to copper resistance in *V. alginolyticus*. The situation is complicated, however, by the fact that many of the isolates were obtained from the same copper-stressed chemostat. These isolates may all be "daughters" or clones of Cu40A1, the oxidase negative mutant that was isolated relatively early in the study. The oxidase negative phenotype of Cu40A1 may contribute to its resistance to copper, however, the resistance mechanism has not yet been determined. Lack of functional cytochrome oxidase could be advantageous to copper-stressed bacteria if, in the presence of increased intracellular copper, the enzyme catalyzes the production of oxygen radicals. O_2 is bound between the Fe^{2+} and Cu^{+} ions of cytochrome during

electron transfer (Stryer, 1988). If cytoplasmic Cu^{2+} ions interfered with proper binding and only one electron was transferred (instead of 4 e^- + 4 H^+), a superoxide anion would result. Alternatively (or additively), Cu^{2+} might interfere with the normal process of superoxide neutralization after superoxide dismutase (SOD) had catalyzed the reduction of superoxide to hydrogen peroxide (H_2O_2). Cu^{2+} could theoretically regenerate superoxide as follows (A.S. Gordon, personal communication):

$$3\ O_2^{\bullet} + 4\ H^+ \xrightarrow{SOD} 2\ H_2O_2 + 0_2$$

$$H_2O_2 + 2\ Cu^{2+} \rightarrow O_2^{\bullet} + 2\ H^+ + 2\ Cu^+$$

The stability of copper resistance in two chemostat-derived mutants, Cu40A1 (oxidase negative) and Cu40B3 (oxidase positive) was demonstrated by comparing the frequency of Cu^r cells after serial subculture on nonselective agar (fNS) to the frequency of Cu^r cells in cultures that had been recently exposed to copper (fCu). The frequency of Cu^r cells for both treatments was similar: fNS/fCu for Cu40A1 was 0.628, and was 2.5 for Cu40B3. Thus, for Cu40B3, a higher percentage of cells were Cu^r after ten nonselective passes than in a recently Cu-stressed culture. An intriguing aspect of Cu resistance in Cu40B3 cultures was that such a low percentage in the population was resistant (fCu = 2.4×10^{-4}; fNS = 4.7×10^{-4}), while most of the Cu40A1 individuals were resistant in both treatments. Subsequent experiments with Cu40B3 have yielded Cu^r (40 μM) frequencies ranging from 1.1×10^{-2} to 3.4×10^{-1} (Table 2.5), however the majority of individuals in all cultures tested were not

copper-resistant, leading to the hypothesis that the Cu^r individuals in this mutant strain somehow protect the nonresistant cells from the effects of copper.

This study of *V. alginolyticus* populations in long-term copper-stressed chemostat cultures showed that increased copper concentrations in the medium reservoir increase the frequency of copper-resistant cells, as measured by growth of colonies on Cu-amended agar plates. The data suggest that the level of copper ions\cell (Cu concentration normalized to cell numbers) may play a major role in determining the frequency and level of copper resistance in continuous culture. Relief of copper stress caused a decrease in the frequency of Cu^r cells, however the frequency of Cu^r cells remained elevated compared to frequencies in cultures that had never been exposed to copper. In fact, the remnant population of resistant cells and/or their excreted products was enough to protect the culture when copper was reapplied (Gordon *et al.*, 1993). The phenotypic variations noted, nonswarming motility and oxidase negativity, were not definitely correlated to copper resistance, however the oxidase negative phenotype may confer some advantage to cells under chronic copper stress. Copper resistance in two Cu^r mutants was retained under nonselective culture conditions. Most of the cells in Cu40A1 cultures proved to be copper-resistant, however the majority of cells in Cu40B3 cultures and in copper-stressed chemostat populations were not copper-resistant, suggesting that a minority of copper-resistant individuals may protect the remainder of the culture from the toxic effects of copper.

CHAPTER III: CHARACTERIZATION OF SUPERNATANT PROTEINS IN COPPER-RESISTANT AND COPPER-SENSITIVE *VIBRIO ALGINOLYTICUS* AND *VIBRIO PARAHAEMOLYTICUS*

INTRODUCTION

The evidence presented thus far for CuBP-mediated copper detoxification in *Vibrio alginolyticus* supports the hypothesis that it is an extracellular, copper-induced protein which is produced during the copper-induced lag phase, and which accumulates as cells resume growth in the presence of copper (Schreiber *et al.*, 1990; Harwood-Sears and Gordon, 1990a). CuBP expression in supernatants of copper-stressed chemostats is also copper-induced, and its concentration increases with increasing copper concentrations (Gordon *et al.*, 1993). Although the data presented in the preceeding chapters are consistent with our model of involvement of a specific extracellular protein in copper detoxification, showing a very strong correlation between copper stress and extracellular CuBP expression, we have no direct evidence that CuBP is necessary or advantageous to cells in the presence of excess copper. The argument that CuBP is an integral element in the copper management/detoxification system of *V. alginolyticus* would therefore benefit from the demonstration of a direct link between CuBP and alleviation of copper toxicity.

Mutagenesis experiments were designed with the aim of isolating mutant *V.*

alginolyticus with altered sensitivity to copper. These mutants were screened for altered CuBP production, with the rationale that some copper-resistant (Cu^r) mutants might overexpress CuBP, and some copper-sensitive (Cu^s) mutants might lack extracellular CuBP. Transposon insertion was selected as a preliminary mutagenic strategy. The advantages of mutagenesis with some commonly utilized transposons (miniMu, Tn5), i.e. the presence of antibiotic markers, known sequences amenable to probe techniques for gene localization, and no tendency to insert in "hot spots", combined with the proven utility of P1-vectored transposon systems in marine *Vibrio* species (Belas *et al.*, 1984), made this technique the best initial candidate for mutagenesis. The success of genetic exchange with transposons can vary widely from one species to the next, or within strains of the same species. When neither Tn5-132 nor miniMu could be transferred to WT *V. alginolyticus*, mutagenesis with a plasmid-vectored miniMu transposon (Ostling *et al.*, 1991) was attempted. Although this system had been used successfully with other marine species, including a *Vibrio* species in which the P1/miniMu system did not work, attempts to transfer the element to *V. alginolyticus* were unsuccessful. Chemical mutagenesis with nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine), an alkylating agent which causes point mutations and deletions (Brock and Madigan, 1991, p. 240) proved successful.

Vibrio parahaemolyticus BB22 was screened for production of copper-induced, extracellular proteins in the 20 kDa range. This organism can be mutagenized with the P1/Tn5-132 and P1/miniMu systems (Belas *et al.*, 1984). *V. parahaemolyticus* is

very closely related to *V. alginolyticus* (Dorsch *et al.*, 1992), therefore we reasoned that it would be useful for genetic analysis of *cbp* and its regulatory elements if it exhibited a similar response to copper.

Plasmids are frequently associated with bacterial copper resistance (Bender and Cooksey, 1986; Rouche *et al.*, 1989). The cryptic, ca. 30 kb plasmid isolated from WT *V. alginolyticus* may be involved in copper resistance, however it was not amplified during copper stress in batch and chemostat cultures (Chapter II). In order to assess the contribution of the plasmid to copper resistance, plasmid curing with acridine orange was attempted. Acridine orange, like other intercalating dyes such as ethidium bromide, has been used in sub-lethal concentrations to selectively interfere with plasmid replication (Blumenthal *et al.*, 1985; Brock and Madigan, 1991, p. 256).

MATERIALS AND METHODS

Bacterial strains. Vibrio alginolyticus (Gerchakov *et al.*, 1976) without mutations will be referred to as wild-type (WT) *V. alginolyticus*. *V. parahaemolyticus* BB22 was obtained from Dr. Robert Belas, Center for Marine Biotechnology, Baltimore, MD. *E. coli* C600 (P1 *clr*100CM::Tn5-132) and MC4100 [miniMu[Tet^r](P1 *clr* 100 CM)] were obtained from Dr. Michael Silverman, Agouron Inst., La Jolla CA. *Vibrio* S141, a streptomycin-resistant derivative of a marine *Vibrio*, was obtained from Dr. J. Ostling, University of Goteborg, Goteborg, Sweden. The copper-resistant mutants Cu40B3 and Cu40A1 were isolated from a chronically copper-stressed continuous

culture (Gordon *et al.*, 1993). All other mutants were isolated during this study (Table 3.1).

Copper-resistant isolates. Spontaneous copper-resistant mutants of *V. alginolyticus* were isolated from overnight broth cultures by plating serial dilutions on marine agar or HASW plates amended with inhibitory concentrations of copper (20 - 40 μM). Copper-resistant cultures of *V. parahaemolyticus* BB22 were obtained by plating broth cultures on HASW plates amended with 100 - 150 μM Cu. Plates were incubated at room temperature for up to two weeks, although most colonies appeared between four and seven days.

Except where noted otherwise, culture conditions were as follows: overnight broth cultures grown in SWM9 + 2.5 μg/ml $FeCl_3$ were used to inoculate 3 ml batch cultures in SWM9. Three ml cultures were grown in sterile, 12-well tissue culture dishes (Costar, Cambridge, MA). Generally, 50 μl of overnight culture served as the inoculum, however this volume was increased for isolates with a slower growth rate. For the experiment in which CuBP in replicate control cultures was compared, each culture was started from a separate overnight culture. Broth cultures were incubated at room temperature (approximately 25° C) on a shaker (100 rpm). The optical density of 3 ml cultures was monitored by transferring 100 μl aliquots to a 96-well microassay plate, which were read at 595 nm on a microplate reader (Bio-Tek Instruments).

Copper was added to broth cultures of Cu^s and Cu^r isolates in mid-log phase (approximately 0.080 AU) in order to compare their response to that of WT *V.*

alginolyticus. Control (no Cu) cultures were harvested after 24 h. Cu-stressed cultures were generally harvested after 24 h., although some were harvested after 48h. or 4 days where noted.

In an experiment designed to measure supernatant [CuBP] vs. [Cu] in the Cu^r mutant Cu40 B3, copper was added to 3 ml cultures growing in 12-well tissue culture dishes during mid-log phase. $CuSO_4$ was added from a 10 mM stock solution to a final concentration of 1.0, 2.5, 5.0, 10.0 and 50.0 μM. No copper was added to the control culture. Two ml of supernatant obtained by filtering the culture through a 0.45 μm filter was loaded in a microconcentrator tube with a 3 kDa nominal molecular weight cutoff (Centricon 3; Amicon, Beverly, MA). Tubes were centrifuged at 6500 X g for two hours to give a four to five fold concentration factor. Supernatant and cell protein was measured by the BCA assay (Chapter I). In all cases where protein quantities in different samples were compared, (e.g. cellular vs. supernatant) values were corrected to reflect the concentration in the original culture.

Copper distribution. Copper distribution in supernatants and pellets of *V. alginolyticus* and *V. parahaemolyticus* BB22 was compared by inductively coupled argon plasma spectroscopy (ICP). Forty ml cultures were grown in SWM9. Fifty μM Cu was added to *V. alginolyticus* and *V. parahaemolyticus* cultures during mid-log phase, and some *V. parahaemolyticus* cultures were stressed with 100 μM Cu. Cultures to which no copper was added were used as controls. Two replicate cultures for each treatment were analyzed, except that only one *V. parahaemolyticus*

+ 50 μM Cu supernatant was analyzed due to a laboratory accident. Supernatant and cellular fractions from each culture were analyzed in duplicate.

Control cultures were harvested after 24 h, and Cu-stressed cultures after 48 h by centrifugation (15,300 x g). The cell pellet was washed twice with 35 ml phosphate buffered saline (PBS), resuspended in PBS, and 1.5 ml aliquots were transferred to microcentrifuge tubes. Samples were centrifuged at maximum rpm in a microcentrifuge (Eppendorf 5415) for 5 minutes. In order to lyse cells, the pellets were resuspended in 400 μl lysozyme buffer (glucose, 50 mM; Tris base, 25 mM; EDTA, 10 mM; lysozyme, 4 mg/ml; pH 8.0) and incubated for 30 minutes at room temperature. 800 μl SDS solution (SDS, 1%; NaOH, 0.2 N) was added to each tube, which was held on ice for 5 minutes. At this point, the solution cleared. Pellets were resuspended to their original volume in SWM9. The pH was adjusted to $\leq$ 2 with concentrated nitric acid. Each supernatant was filtered through a 0.45 μm filter, divided into two 15 ml aliquots, and the pH was adjusted to $\leq$ 2 with concentrated nitric acid.

Total copper in supernatant and cell fractions was analyzed by ICP on an Applied Research Lab (Fision) 3410 instrument at a wavelength of 325 nm. ICP analysis was performed at the Applied Marine Research Lab (AMRL), Norfolk, VA. Sample Cu concentrations were corrected for background Cu in an SWM9 blank.

Supernatant proteins of Vibrio parahaemolyticus. V. parahaemolyticus BB22 cultures were grown in two liters of SWM9. The Cu-stressed culture received 100 μM $CuSO_4$ at mid-log phase. Supernatants from control cultures were harvested

after 24 h, and supernatants from Cu-stressed cultures after 48 h, by tangential flow filtration (TFF; see below). A 30 ml aliquot of each supernatant was lyophilized, dialyzed against a sodium phosphate/ NaCl buffer (Chapter 1), and analyzed by SDS-PAGE. The remaining supernatant was concentrated and partially purified by immobilized metal ion affinity chromatography (IMAC) followed by reverse phase high performance liquid chromatography (RPHPLC) (see below).

Chromatography. Culture supernatants were separated from cells by TFF using a Pellicon cassette system (Millipore) with a 0.2 μm (GVLP) filter. Twenty ml of *Vibrio parahaemolyticus* BB22 supernatant from copper-stressed cultures was loaded onto an H10/2 chelating Superose column charged with 3 ml of 10 mM $CuSO_4$ at 0.5 ml $\cdot$ min^{-1} for immobilized metal ion affinity chromatography. Due to the viscosity of the sample, only 14 ml of control supernatant could be loaded on the column. The column was coupled to a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). The column was washed with buffer A (0.05 M $NaCOOCH_3$. 0.1 M NaCl, pH 7.0) and bound proteins were eluted with 100% buffer B (10 mM glycine, 0.1 M NaCl, pH 9.0) at a flow rate of 1 ml $\cdot$ min^{-1}. Absorption was monitored at 280 nm.

Supernatants from two liter cultures of Cu40B3 were separated by TFF and concentrated by IMAC by the method above, except that 175 ml was loaded on the IMAC column. IMAC fractions from the major peak were pooled and frozen at -80° C for further analysis. Immediately before reverse phase high performance liquid chromatography (RPHPLC) was performed on the fraction, 0.1% trifluoracetic acid

(TFA) was added. IMAC fractions (10 ml) were injected onto a Macrosphere C4 column (Alltech 150 x 4.6 mm) on an ISCO HPLC. Solvent systems were: (A) 0.1% TFA; (B) 0.1% TFA in 100% acetonitrile (CH_3CN). A linear gradient from 0 to 100% buffer B was run over a 37 minute period at 1.0 $ml \cdot ml^{-1}$ to elute proteins. Absorbance was monitored at 280 nm.

Fractions from IMAC/RPHPLC chromatograpahy of *V. parahaemolyticus* BB22 and Cu40B3 supernatants were analyzed on 12% acrylamide SDS-polyacrylamide gels (Laemmli, 1970).

Transposon mutagenesis. Unsuccessful attempts were made to transfer Tn5-132 and miniMu to *V. alginolyticus* using bacteriophage P1 as the vector (Belas *et al.*, 1984). The procedure as detailed in Martin *et al.* (1989) was followed. Transfer of miniMu via an IncP-1 plasmid vector were also unsuccessful. This procedure (Ostling *et al.*, 1991) was performed as previously published. Spontaneous rifampicin-resistant (rif^r) mutants of *V. alginolyticus* were obtained by plating undiluted overnight cultures on LM plates (tryptone (Difco), 10 g/l; yeast extract (Difco) 5 g/l; NaCl, 20 g/l; agar (Difco) 15 g/l, pH 7.6) amended with 150 μg/ml rifampicin. Rif^r mutants were plated on LM agar containing 150 μg/ml rifampicin and 200 μg/ml streptomycin to select for rif^r $strep^r$ mutants for filter-mating with *E. coli*.

Chemical mutagenesis. Mutagenesis in batch cultures of *Vibrio alginolyticus* was accomplished with the chemical mutagen nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine). The procedure detailed by Adelberg *et al.* (1965) was followed with minor adjustments. Twenty ml cultures of *V. alginolyticus* were grown overnight

at room temperature in SWM9 broth supplemented with 2.5 μg/ml $FeCl_3$. Flasks (250 ml) were shaken at 100 rpm. The final optical density of the overnight cultures was approximately 300 KU. A flask containing 20 ml LB15 medium (tryptone (Difco) 10 g/l; yeast extract, 5 g/l; NaCl, 15 g/l; pH 7.6) was inoculated with 0.5 ml overnight culture, and was grown at room temperature on a shaker (100 rpm). Growth was monitored on a spectrophotometer set at 600 nm (Spectronic 21, Bausch and Lomb). When the culture reached mid-log phase (OD_{600} = 0.85, cuvette diameter 15 mm), 10 ml was filtered onto a 0.45 μm filter. The filter was washed twice with 10 ml of Tris-maleic acid (TM) buffer (Tris base (Sigma) 6.1 g/l; maleic acid, 5.8 g/l; NaCl, 23.4 g/l; $MgSO_4 \cdot 7\ H_2O$, 0.1 g/l; $(NH_4)_2SO_4$, 1.0 g/l; sodium citrate, 0.6 g/l; pH 6.0; sterilize by autoclaving) and cells were resuspended in 20 ml TM buffer. Two mg NTG was added to the cells as a solid (final concentration = 100 μg/ml. After swirling the flask to dissolve NTG, the culture was incubated for 30 minutes at room temperature on a shaker. Serial dilutions of the culture were spread on HASW plates, and incubated overnight at room temperature.

Selection for copper-sensitive mutants. Putative copper-sensitive mutants were identified on HASW plates amended with bromcresol purple (10 μg/ml) and 15 μM $CuSO_4$ (BPHASW + Cu). Spread plates with 10 - 40 colonies from NTG-treated cultures were chosen for filter transfer to BPHASW + Cu plates. Filter transfer was accomplished by laying a 0.45 μm, 85 mm nitrocellulose transfer membrane (NitroPlus, MSI, Westboro, MA) on the surface of a plate with colonies growing on it. The filter was left on the plate long enough to become wet (about 30 s.) and was

carefully transferred with sterile tweezers to a BPHASW + 15 μM Cu plate. The filter was oriented so that it was between the colonies and the agar (colonies on top). Plates were incubated at room temperature overnight. By the next day, most colonies and the agar beneath them had turned yellow. Colonies that remained purple were designated putative Cu^s and transferred to marine agar plates for further study.

The copper sensitivity of the NTG isolates was confirmed by streaking the isolates from marine agar to BPHASW + 15 μM Cu and to HASW + 15 μM Cu. The HASW plates were used to rule out the possibility that putative Cu^s isolates were sensitive to bromcresol purple rather than copper. Colony growth and acid production (yellowing of plates) were compared to that of WT *V. alginolyticus* inoculated at the same time after one, two and four days incubation. Isolates with significantly fewer and smaller colonies than WT *V. alginolyticus* after four days were designated Cu^s. Broth cultures of several isolates were diluted and plated on BPHASW and BPHASW + 15 μM Cu. The plate count on unamended and Cu-amended media were compared in order to confirm copper sensitivity.

The copper-sensitive *V. parahaemolyticus*, BBT50S3, was isolated by plating a TN5-132 mutagenized culture on HASW with tetracycline (12 $\mu g \cdot ml^{-1}$) and 50 μM Cu. Areas where no visible colonies were growing were swabbed onto an HASW + tetracycline (HASWT) plate. Copper sensitivity of these isolates was confirmed by plating on HASWT + 50 and 75 μM Cu, which is not inhibitory to *V. parahaemolyticus* BB22.

Plasmid curing. WT *V. alginolyticus* grown overnight in SWM9 was used as inoculum for three ml cultures grown in LM medium in 12-well tissue culture dishes. Inoculum was added in a 1:20 (150 μl) or 1:50 (60 μl) ratio. Acridine orange (AO) was diluted from a stock solution (1 mg/ml) to a final concentration of 5, 10, or 20 μg/ml in cultures, which were incubated overnight at room temperature on a shaker (100 rpm). The 10 μg/ml and 5 μg/ml AO-treated cultures were chosen for plating based on the limited amount of growth that had taken place overnight. Serial dilutions of these cultures were spread on HASW plates and incubated at room temperature for 48 h. Colonies from plates with 20 - 50 colonies (10^{-5} dilution) were filter-transferred to BPHASW + 15 μM Cu plates, which were incubated overnight at room temperature. The copper sensitivity of putative Cu^s colonies was confirmed as above.

One Cu^s isolate was confirmed from the acridine orange experiments, and designated VA15S13. WT *V. alginolyticus*,a non copper-sensitive, AO-treated isolate, and VA15S13 were grown overnight at room temperature in 50 ml LM broth cultures. Cells were concentrated by centrifugation at 10,000 rpm (15,300 x g) for 15 minutes. The supernatant was decanted off, and the entire culture (48 ml, or approximately 7.5×10^{10} cells) was used for plasmid DNA extraction by the alkaline lysis method (Ausubel *et al.*, 1987).

Plasmid DNA was stored at -20° C in TE buffer (10mM Tris•Cl; 1 mM EDTA; pH 7.5) until it was analyzed by agarose electrophoresis. Some samples were treated overnight with RNAase A (1.67 μg/ml) at 37° C. Sample and 10X loading

buffer (glycerol, 25%; bromphenol blue, 0.025%; SDS, 1%; H_2O, 73.975%) were mixed in a 9:1 ratio for WT *V. alginolyticus* and VA15S13, and a 4:1 ratio for the second AO-treated *V. alginolyticus*. The supercoiled plasmid ladder standard (0.19 $mg \cdot ml^{-1}$) was diluted 2:1 with loading buffer. 10 μl of each sample and 6 μl standard were loaded on a 1% agarose gel (SeaKem LE Agarose, FMC Bioproducts, Rockland ME) in a TAE buffer system (0.004 M Tris•acetate; 0.002 M EDTA; pH 8.5). Gels were run at low voltage (Fotodyne power supply; approximately 79 V) for 45 minutes, then at high voltage (approximately 138 V) for 25 minutes. Gels were stained in ethidium bromide (5 μg/ml) for 25 minutes, and destained in distilled H_2O for two hours. Plasmid DNA in agarose gels was visualized and photographed on a UV transilluminator (Foto/phoresis, Fotodyne, New Berlin, WI).

Supernatant protein in Cu^s and Cu^r Vibrio isolates. Cell-free supernatants for SDS-PAGE were obtained by filtering each culture through a 0.45 μm filter. Two ml of filtrate was centrifuged in a microconcentration tube with a 5 kDa nominal molecular weight limit (NMWL) (Ultrafree-CL PLCC, Millipore) or a 3 kDa NMWL (Centricon 3, Amicon). Centrifugation time was two hours at 4170 x g (5500 rpm; Ultrafree) or 5520 x g (6000 rpm; Centricon 3) in a JA14 rotor (Beckman), which concentrated the samples 3.3 - 5 fold. The concentrated supernatants were analyzed by SDS-PAGE on 12% acrylamide gels (see Chapter 1), visualized with silver stain (Rapid Ag Stain, ICN Radiochemicals) and quantitated by laser densitometry. Supernatant and cellular protein concentrations were determined by the bicinchoninic acid assay (BCA, Pierce) as detailed in Chapter I.

Phenotypic Characterization. The following tests were carried out on cultures on Marine Agar: gram stain, oxidase test (Pathotec cytochrome oxidase test strips, Organon Teknika Corp.), and API20E biochemical profile (Analytab). Colonies were suspended in an Instant Ocean solution (21 g/l) for the API test, and incubated for 24 h at room temperature.

RESULTS

Copper-resistant isolates. Spontaneous copper-resistant mutants of *Vibrio alginolyticus* were obtained from control broth cultures and Cu-stressed chemostat cultures. Isolates were chosen for further characterization based on the stability and magnitude of copper resistance exhibited by each isolate. Biochemical tests (API20E) were used to confirm their species and to determine whether other phenotypic changes had occured. Results were identical to the wild type with the exception of the oxidase test for Cu40A1. Cu20A6, which was isolated from a batch culture spread on 20 μM Cu-amended plates (Table 3.1), has exhibited resistance to copper concentrations as high as 100 μM. This isolate was passed repeatedly on nonselective agar without losing its Cu-resistant phenotype. Cu40A1 and Cu40B3 were isolated from a Cu-stressed continuous culture (Chapter II) on 40 μM Cu-amended plates. The stability of the Cu^r phenotype in these isolates was demonstrated by their ability to form colonies on 40 μM Cu-amended plates after ten passages on nonselective agar (Chapter II).

Copper-resistant strains were maintained on HASW or BPHASW agar + 40

Table 3.1. Bacterial Strains

Species/ Isolate	Cu Sensitivity	Oxidase Phenotype	Stability[1]	Source	Reference
V. alginolyticus					
Wild-type	Normal	+	N/A		Gerchakov 1976
Cu20A6	Resistant	+	+	Batch	This study
Cu40A1	Resistant	-	+	Chem[2]	Gordon 1993[4]
Cu40B3	Resistant	+	+	Chem[3]	Gordon 1993[4]
VA15S7	Sensitive	+	+	NTG[5]	This study
VA15S8	Sensitive	+	+	NTG	This study
VA15S9	Sensitive	+	+	NTG	This study
VA15S10	Sensitive	+	+	NTG	This study
VA15S11	Sensitive	+	+	NTG	This study
VA15S12	Sensitive	+	+	NTG	This study
VA15S13	Sensitive	+	+	AO[6]	This study
V. parahaem.					
BB22	Normal	+	N/A		Belas *et al.*, 1984
BBT50S3	Sensitive	+	+	Tn5	This study
BB100 series	Resistant	+	-	Batch	This study
BB150 series	Resistant	+	-	Batch	This study

[1]Isolates scored (+) retained their variant phenotype with respect to copper resistance when subcultured on nonselective media. Isolates scored (-) lost their resistance to copper when subcultured on nonselective media.

[2]Isolated from a continuous flow culture stressed with 32 μM $CuSO_4$.

[3]Isolated from the same continuous flow culture as Cu40A1 after the copper concentration had been lowered from 32 μM to 20 μM CuSO[4].

[4]Gordon *et al.*, 1993.

[5]Isolated from NTG-mutagenized cultures.

[6]Isolated from acridine-orange treated culture.

μM Cu. WT *V. alginolyticus* cannot form colonies on these media amended with 25 μM or higher Cu concentrations (see Figure 2.5). Cu40B3 plated on BPHASW + 40 μM Cu was subcultured from a Cu-amended plate (Figure 3.1A) and a marine agar plate (Figure 3.1B). The decreased number of colonies observed after the nonselective passage occurs fairly consistently, however the cultures on Cu-amended agar must be subcultured fairly frequently (one week) to maintain high viability.

CuBP expression in control and 50 μM Cu-stressed batch cultures of Cu^r mutants was analyzed by SDS-PAGE (Figure 3.2). WT *V. alginolyticus* supernatants were included for comparison (arrow at CuBP bands). While Cu20A6 and Cu40A1 supernatant protein profiles proved quite similar to those of the wild-type, the Cu40B3 control supernatant contained a substantial quantity of a protein or proteins of the same molecular weight as CuBP (Figure 3.2). A quantitative determination of the amount of CuBP (CuBP1 + CuBP2) in unchallenged culture supernatants of *V. alginolyticus* variants and wild-type was carried out in three replicate cultures of each isolate. From SDS-PAGE gels, the relatively high concentration of CuBP-like protein in Cu40B3 supernatants was obvious (Figure 3.3), with over ten times the amount found in unchallenged WT *V. alginolyticus* supernatants (Table 3.2). There was significantly more CuBP in Cu40B3 supernatants normalized to total supernatant protein (Figure 3.3; $p < 0\ 0.05$ by Student's t-test) than in the other isolates' supernatants.

Since supernatant protein normalized to total cellular protein was also greater in Cu40B3 than in the unchallenged cultures of the other isolates, the contribution

Figure 3.1. The copper-resistant mutant, Cu40B3, plated on HASW + 40 μM Cu. The plate was streaked from cultures growing on HASW + 40 μM Cu (A) and marine agar (B).

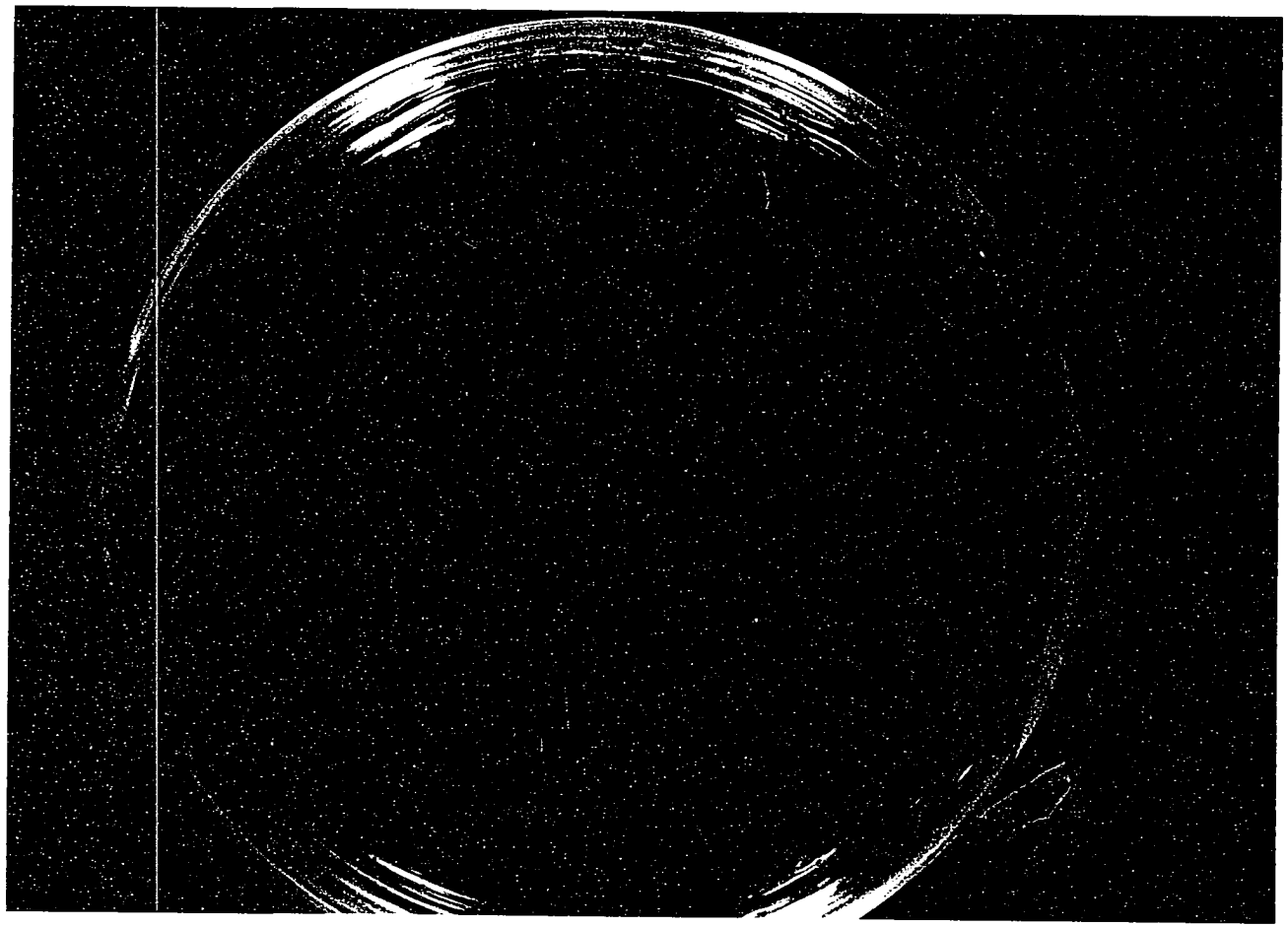


Figure 3.1

Figure 3.2. Supernatant proteins of unchallenged and 50 μM copper-challenged wild-type (WT) and variant *V. alginolyticus*.

Lane 1: Control WT *V. alginolyticus*
Lane 2: 50 μM Cu-challenged WT *V. alginolyticus*, 24 h
Lane 3: 50 μM Cu-challenged WT *V. alginolyticus*, 48 h
Lane 4: Control Cu20A6
Lane 5: 50 μM Cu-challenged Cu20A6, 48 h
Lane 6: Molecular weight standards
Lane 7: Control Cu40B3
Lane 8: 50 μM Cu-challenged Cu40B3, 24 h
Lane 9: 50 μMCu-challenged Cu40B3, 48 h
Lane 10: Control Cu40A1
Lane 11: 50 μM Cu-challenged Cu40A1, 24 h
Lane 12: 50 μM Cu-challenged Cu40A1, 48 h

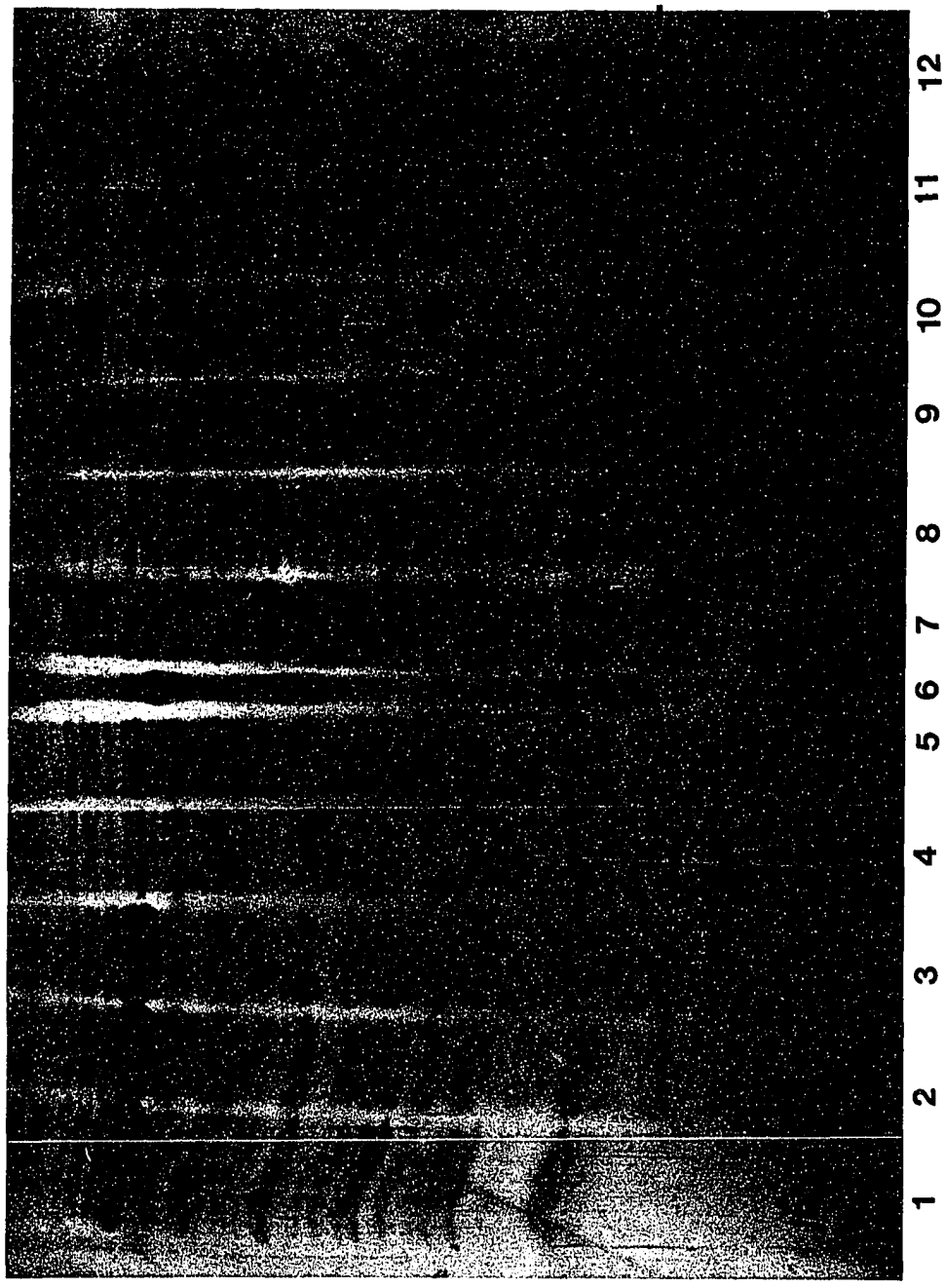


Figure 3.2

Figure 3.3. Replicate unchallenged supernatants from batch cultures of wild-type (WT) *V. alginolyticus* and Cu^r variants. A supernatant from a 50 μM Cu-challenged WT *V. alginolyticus* culture is included for comparison.

Lanes 1-3: Cu20A6
Lanes 4-6: Cu40B3
Lanes 7,9 & 10: Cu40A1
Lane 8: molecular weight standards
Lane 11: Cu-challenged WT *V. alginolyticus*
Lane 12-14: WT *V. alginolyticus*

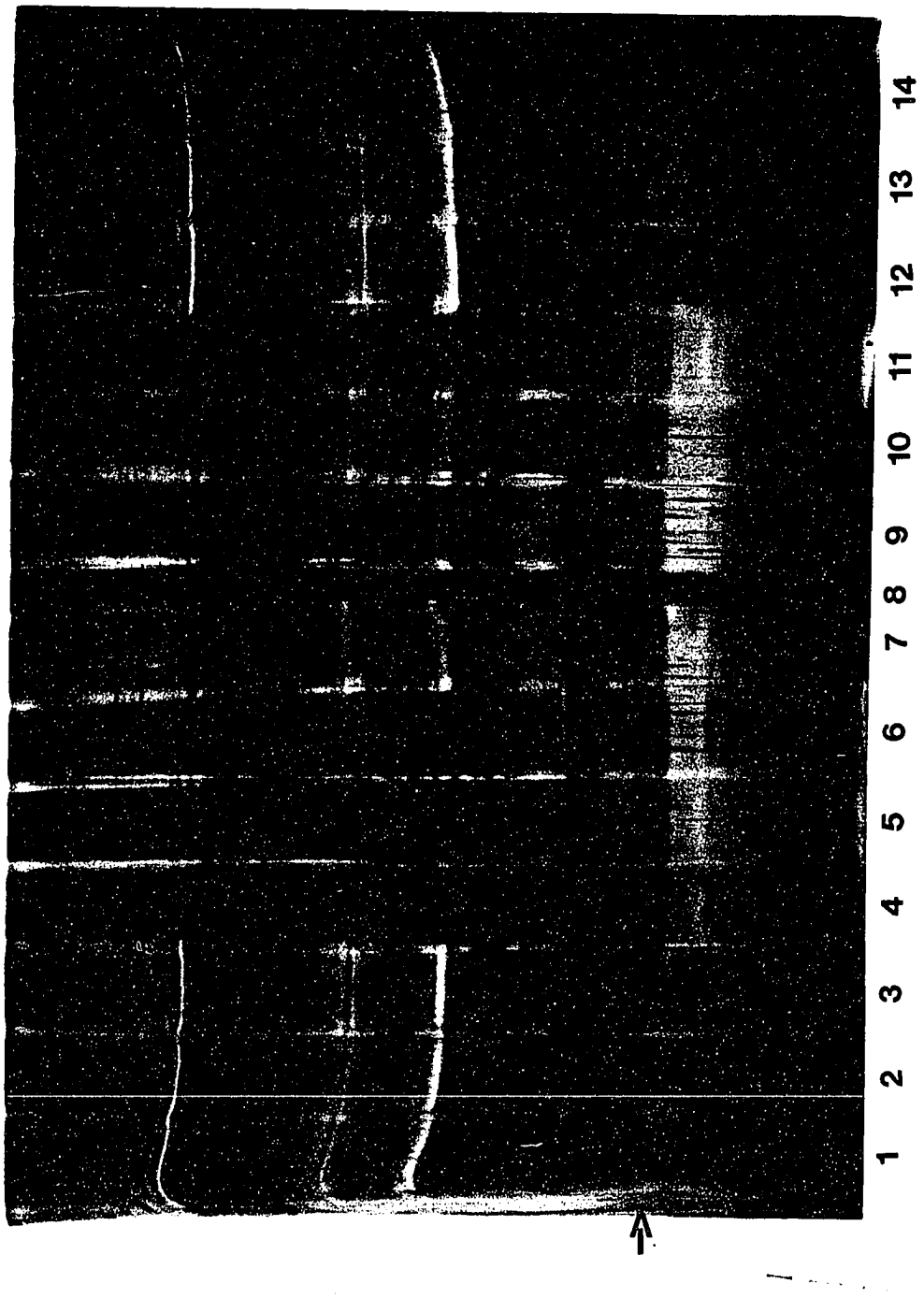


Figure 3.3

Table 3.2. Percent CuBP in supernatants from control batch cultures of wild-type *Vibrio alginolyticus* and copper-resistant variants.

Isolate	% CuBP	Supernatant Protein (μg)	CuBP (μg)	Supernatant Protein - CuBP
Wild-Type	1.14	28.34	0.32	28.02
Cu40B3	14.65	24.06	3.52	20.54
Cu40A1	2.09	18.96	0.40	18.56
Cu20A6	1.36	30.73	0.42	30.31

of CuBP to total supernatant protein was estimated (Table 3.2). CuBP made up 14.7% of the supernatant protein in Cu40B3 cultures, and only 1.1% in control WT *V. alginolyticus* cultures. After CuBP was subtracted from supernatant protein and the values were normalized to cellular protein, the calculated values of supernatant protein in cultures of the wild-type and Cu40B3 were nearly identical (Table 3.2).

In order to investigate the relationship between [Cu] and [CuBP] in Cu40B3 supernatants, copper was added to cultures in increments from 1 to 50 μM. Data from WT *V. alginolyticus* cultures, which received identical treatments, are included for comparison (Figure 3.4; discussed in Chapter I). Unlike the wild-type, percent CuBP in Cu40B3 supernatants did not increase incrementally from 0 to 50 μM Cu (Table 3.3). Control Cu40B3 supernatants were comprised of 4.5% CuBP, which changed little except for a spike in % CuBP and [CuBP] with the addition of 5 μM Cu. When [CuBP] was normalized to cell numbers (OD) or protein, a trend of increasing CuBP with copper concentration was more evident. Note that in the case of Cu40B3, the values for [CuBP]/OD do not closely reflect the values for CuBP/Protein, as in the case of WT *V. alginolyticus*. The discrepancy is probably due to the increased tendency of Cu40B3 to clump, or form aggregates, in Cu-amended broth culture, a characteristic which is seen in WT *V. alginolyticus* to a lesser degree.

In addition to its induction by copper and molecular weight, CuBP can be identified by its two-dimensional chromatographic behavior as it is separated by

Figure 3.4. Unfractionated supernatants from WT *Vibrio alginolyticus* and Cu40B3 batch cultures challenged with 0 - 50 μM $CuSO_4$.

Lane 1: Control WT *Vibrio alginolyticus*
Lane 2: 1 μM Cu-challenged *Vibrio alginolyticus*
Lane 3: 2.5 μM Cu-challenged *Vibrio alginolyticus*
Lane 4: 5 μM Cu-challenged *Vibrio alginolyticus*
Lane 5: 10 μM Cu-challenged *Vibrio alginolyticus*
Lane 6: N/A
Lane 7: 50 μM Cu-challenged *Vibrio alginolyticus*
Lane 8: Control Cu40B3
Lane 9: 1 μM Cu-challenged Cu40B3
Lane 10: 2.5 μM Cu-challenged Cu40B3
Lane 11: 5 μM Cu-challenged Cu40B3
Lane 12: 10 μM Cu-challenged Cu40B3
Lane 13: 50 μM Cu-challenged Cu40B3

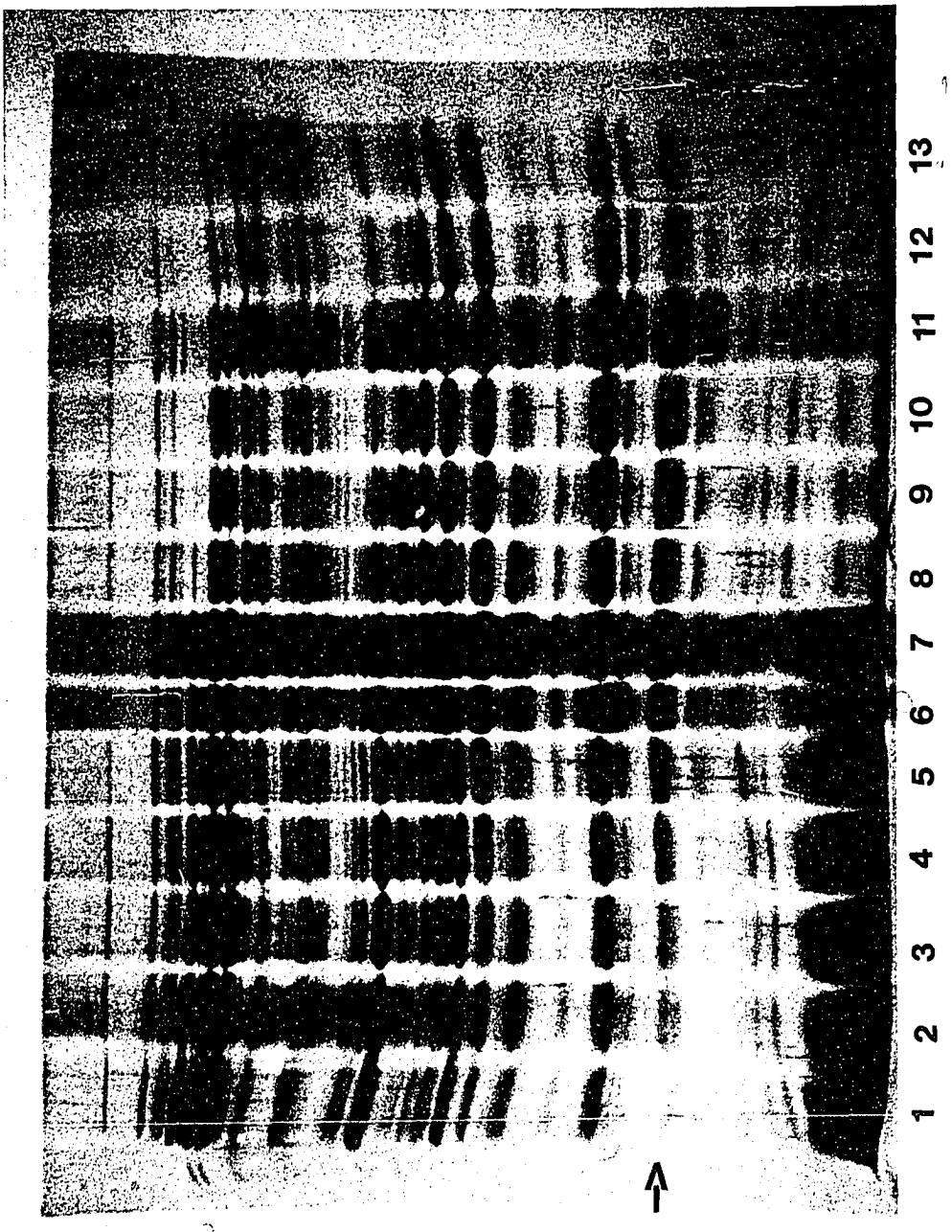


Figure 3.4

Table 3.3. [CuBP] vs. [Cu] in WT *V. alginolyticus* and Cu40B3.

[Cu](μM) WT *V. alginolyticus*	% CuBP[1]	[CuBP] (μg/ml)	[CuBP]/OD[2]	[CuBP]/Protein[3] (x 10^3)
0	ND[4]	ND	ND	ND
1.0	1.2	0.34	1.08	1.47
2.5	1.3	0.43	1.30	1.71
5.0	1.9	0.61	2.01	2.39
10.0	3.0	0.98	3.97	3.76
50.0	5.0	1.67	10.88	10.28

[Cu](μM) Cu40B3	% CuBP[1]	[CuBP] (μg/ml)	[CuBP]/OD[2]	[CuBP]/Protein[3] (x 10^3)
0	4.5	0.88	3.12	3.58
1.0	4.2	0.69	2.45	3.29
2.5	3.8	0.83	3.03	4.65
5.0	9.0	2.20	11.28	14.1
10.0	5.7	1.05	9.30	6.65
50.0	5.1	0.84	14.67	5.43

[1] % CuBP calculated by laser densitometry of SDS-PAGE gels.

[2] [CuBP] is expressed in $\mu g \cdot ml^{-1}$. OD (optical density) measured at 595 nm on a microtiter plate reader.

[3] Protein in this equation is cellular protein ($\mu g \cdot ml^{-1}$).

IMAC followed by RPHPLC (Gordon *et al.*, 1993). Supernatants from control CuBP cultures were concentrated and purified by IMAC followed by RPHPLC (Table 3.4). Their chromatographic behavior, confirmed by SDS-PAGE of fractions, was indistinguishable from that of CuBP purified from Cu-stressed chemostat cultures of WT *V. alginolyticus* (Gordon *et al.*, 1993).

The ability of Cu40B3 and WT *V. alginolyticus* to grow in SWM9 broth amended with 50 μM Cu was compared by calculating the ratio of cell protein in control vs. Cu-stressed cultures of each isolate. Two of the Cu40B3 cultures were harvested after 24 h and two were harvested after 48 h. The ratio of cell protein in Cu-stressed vs. control Cu40B3 cultures was 0.77 ± 0.21. Two of the WT *V. alginolyticus* cultures were harvested after 24 h and one was harvested after 48 h. The ratio of cell protein for the wild-type was 0.647 ± 0.03. The means were not significantly different (Student's t-test, $p < 0.05$).

Copper-resistant *Vibrio parahaemolyticus* BB22 isolates were obtained by replica plating colonies from nonselective plates to Cu-amended plates. Since BB22 proved inherently less copper-sensitive than *V. alginolyticus*, 100 and 150 μM Cu-amended plates were used to isolate Cu^r BB22 colonies. Unlike the Cu^r *V. alginolyticus* mutants (see Chapter II), none of the BB22 isolates were oxidase negative, and all lost the ability to grow on copper-amended plates after one passage on nonselective agar (Table 3.1).

Copper distribution. V. alginolyticus and *V. parahaemolyticus* BB22 cultures were grown in broth cultures with and without 50 μM Cu, divided into cellular

Table 3.4. Chromatographic behavior of CuBP for WT *V. alginolyticus* and Cu40B3.

Isolate	[Cu](μM)	Retention Time IMAC[1]	Retention Time RPHPLC[1]
WT *V. alg.*	20	11 - 13	26 - 28[2]
BB22	100	18	26.9
Cu40B3	0	11 - 13	27.5

[1] Retention time is reported in minutes.

[2] Gordon *et al.*, 1993

and supernatant fractions, and assayed for total copper by ICP spectroscopy. BB22 was also stressed with 100 μM Cu. The distribution of copper between cellular and supernatant fractions was similar for the two organisms (Figure 3.5). Copper in control samples was evenly distributed between fractions, comprising a total background copper concentration of approximately 5 μM. Supernatants from 50 μM Cu-challenged cultures of both organisms contained more copper than the corresponding cellular fractions. There was slightly more copper in 50 μMcopper-challenged supernatants of *V. alginolyticus* than BB22, and the difference was significant ($p \leq 0.05$; Student's t-test). There was no significant difference between copper concentrations in *V. alginolyticus* vs. BB22 cellular fractions. In BB22 cultures challenged with 100 μM Cu, more copper was found in the supernatant than in the cellular fraction, however this distribution was probably due to the fact (determined after these experiments were completed) that copper precipitates out of SWM9 above approximately 60 μM Cu.

Vibrio parahaemolyticus supernatant proteins. Protein profiles of whole supernatants and IMAC peak fractions from control and 100 μM Cu-challenged *V. parahaemolyticus* cultures were analyzed by SDS-PAGE. Control BB22 cultures contained relatively little supernatant protein (Figure 3.6). The 100 μM Cu-challenged supernatant contained a ca. 20 kDa protein (estimated molecular weight 23 kDa) that was absent from the control, as well as several high molecular weight proteins. Copper affinity chromatography purified primarily the 23 kDa protein (Figure 3.6), demonstrating its affinity for copper under these conditions. Its

Figure 3.5. Copper in supernatant and cellular fractions of control and copper-challenged *Vibrio alginolyticus* and *Vibrio parahaemolyticus* cultures.

Figure 3.5

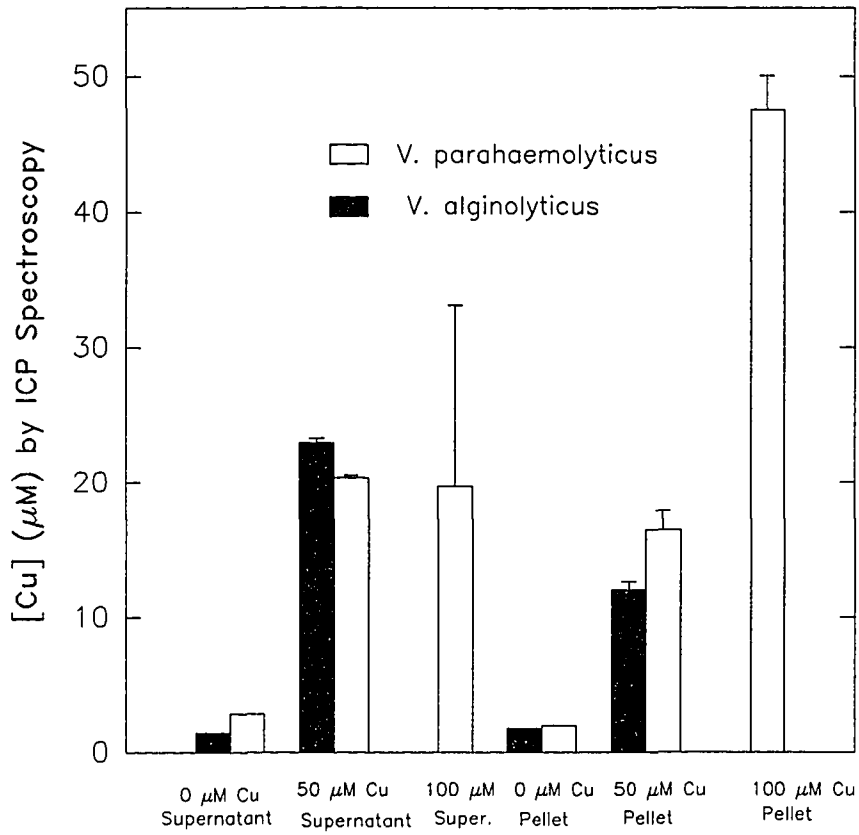


Figure 3.6. FPLC-IMAC fractions and unfractionated supernatants from control and 100 μM Cu-challenged *Vibrio parahaemolyticus* BB22 cultures.

Lanes 1 & 6: Control supernatants, unfractionated
Lanes 2 & 7: 100 μM Cu supernatants, unfractionated
Lanes 3 & 8: Control supernatants separated by IMAC
Lanes 4 & 9: 100 uM Cu supernatants separated by IMAC

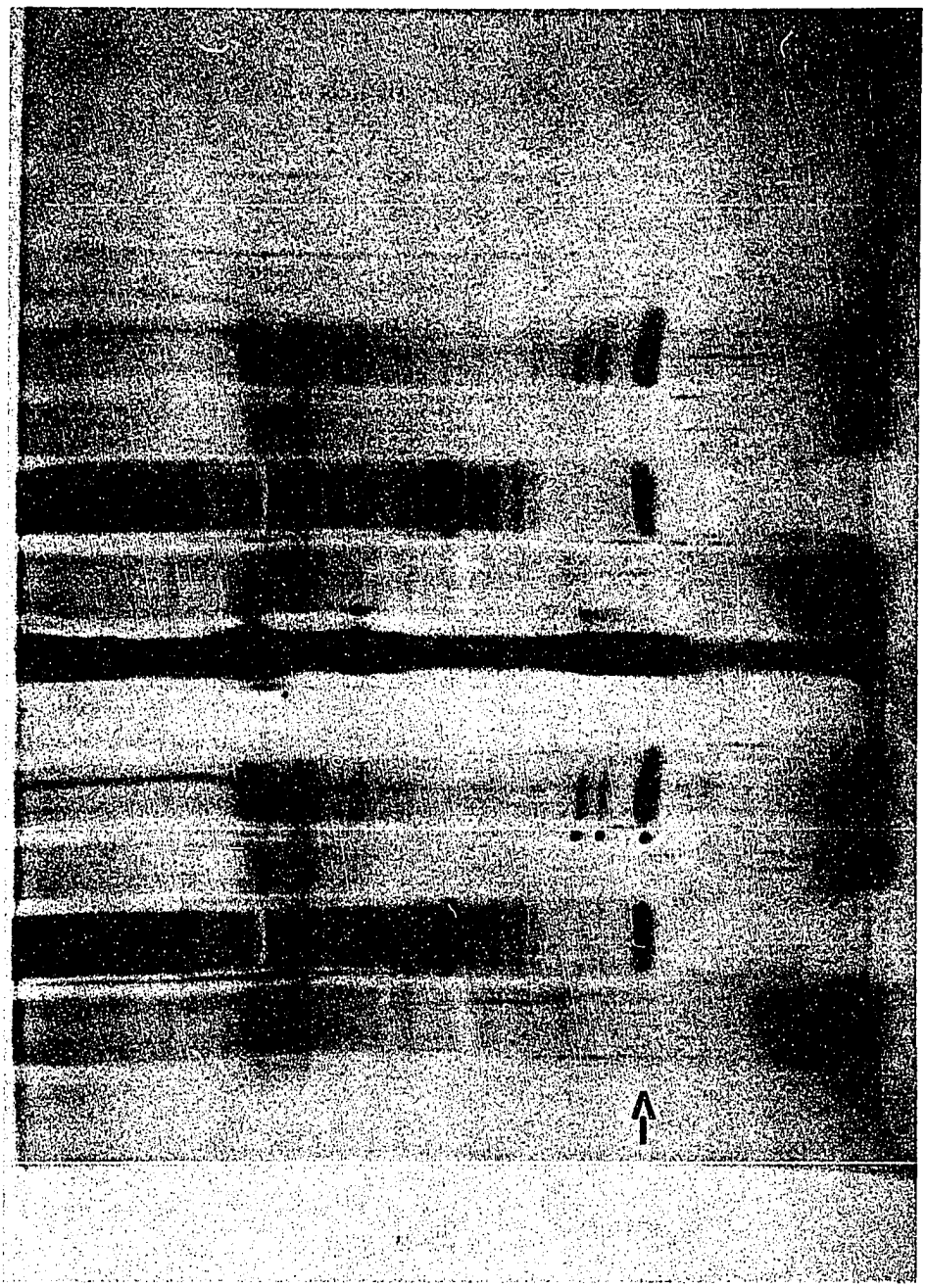


Figure 3.6

retention time on the IMAC column was 18 minutes, compared to an average 12-13 minutes for *V. alginolyticus* CuBP (Table 3.4). When concentration by IMAC was followed by an RPHPLC purification step, the 23 kDA protein of BB22 was eluted within the range of fractions observed for CuBP (Table 3.4).

Transposon mutagenesis. No tetracycline-resistant *V. alginolyticus* were isolated from repeated attempts to insert Tn5-132 and miniMu(Tet^r) into the genome, indicating that transposition was unsuccessful. *V. parahaemolyticus* BB22, which was used as a positive control, incorporated the P1-vectored Tn5-132 and miniMu(Tet^r) the first time the experiments were attempted. One copper-sensitive Tn5::BB22 isolate was isolated, and was denoted BBT50S3. BBT50S3 was unable to form colonies on 50 μM Cu-amended plates, unlike WT BB22, which can form colonies on 75 μM Cu-amended plates.

Supernatant protein profiles in 50 μM Cu-stressed cultures of BBT50S3, WT BB22 and WT *V. alginolyticus* were compared in order to determine whether copper sensitivity in BBT50S3 might be the result of a defect in CuBP production. Analysis of supernatant proteins by SDS-PAGE and densitometry indicated that the response of BBT50S3 to copper stress in terms of CuBP production was similar to that of wild-type *V. alginolyticus* and *V. parahaemolyticus* BB22 (Table 3.5).

Copper-sensitive Vibrio alginolyticus mutants. In order to screen large numbers of colonies from mutagenized bacterial cultures, a filter transfer technique using HASW plates amended with bromcresol purple and 15 μM Cu (BPHASW + 15 μM Cu) was developed. Colonies plated on HASW agar plates were filter-transferred

Table 3.5 Percent CuBP in supernatants of BBT50S3 (Cu^s) compared to WT BB22 and WT *V. alginolyticus*.

Isolate	[Cu] (μM)	Time[1] (h)	Percent CuBP
WT *V. alginolyticus*	0	24	2.1
WT *V. alginolyticus*	50	24	3.7
WT *V. alginolyticus*	50	96	7.8
WT BB22	50	24	3.1
WT BB22	50	96	5.5
BBT50S3	50	24	3.0
BBT50S3	50	96	5.5

[1]Total incubation time. Copper was added during mid-log phase, between four and six hours after the culture was started.

to BPHASW + 15 μM Cu, where colonies with normal sensitivity to Cu maintained sufficient metabolic activity (acid production) to lower the pH in the surrounding, bromcresol purple-amended medium and create a yellow zone. Colonies with heightened sensitivity to copper were metabolically inhibited, remaining purple, but could be retreived by subculture to HASW or marine agar after 24 h exposure to Cu.

Six Cu^s mutants were isolated from NTG-mutagenized batch cultures, and were designated VA15S7 - S12. Copper sensitivity of all the mutants was confirmed by comparing streaks of the mutants to WT *V. alginolyticus* on HASW + 15 μM Cu and BPHASW + 15 μM Cu. All confirmed Cu^s mutants formed fewer and smaller colonies on Cu-amended agar than the wild-type, and acid production, indicated by color change in BPHASW + Cu from purple to yellow, was delayed or did not occur. A comparison of WT *V. alginolyticus* (Figure 3.7), VA15S8 (Figure 3.8) and VA15S12 (Figure 3.9) on BPHASW + 15 μm Cu plates is shown.

Approximately 1000 colonies were screened to obtain the six Cu^s mutants. Thus, approximately 0.5% of the recoverable colonies from NTG treatments were Cu^s. Of 33 colonies identified as putative Cu^s isolates, six, or 18%, were confirmed Cu^s.

Broth cultures of several Cu^s isolates were diluted and plated on BPHASW + 15 μM Cu. Their ability to grow on these plates was compared with that of WT *V. alinolyticus* (Table 3.6). While 86.8% of WT *V. alginolyticus* were able to form colonies on 15 μM Cu-amended agar, the Cu^s isolates were unable to form colonies after six days incubation.

Figure 3.7. Wild-type *Vibrio alginolyticus* plated on BPHASW + 15 μM Cu.

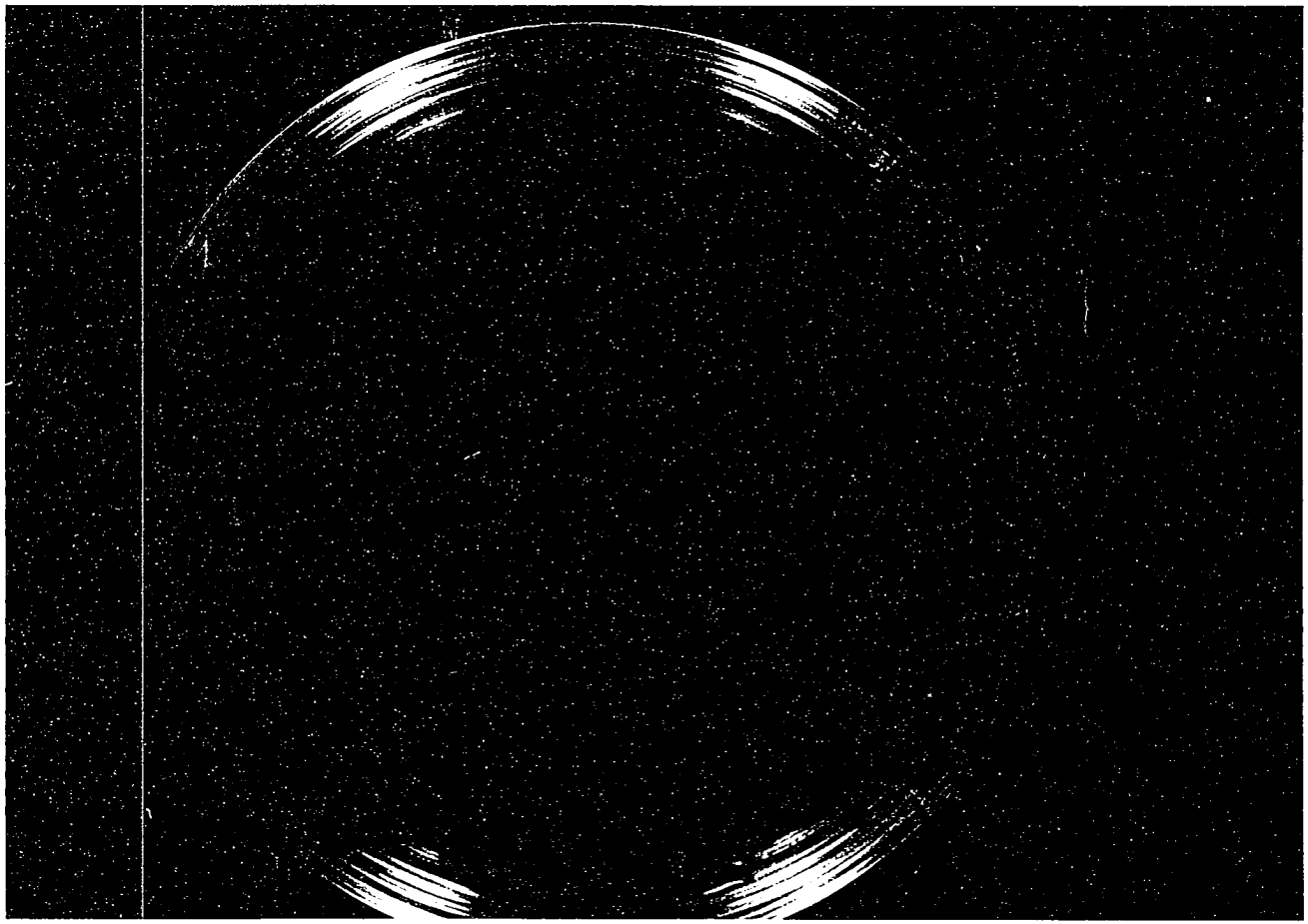


Figure 3.7

Figure 3.8. VA15S8 plated on BPHASW + 15 μM Cu.

Figure 3.8

Figure 3.9. VA15S12 plated on BPHASW + 15 μM Cu.

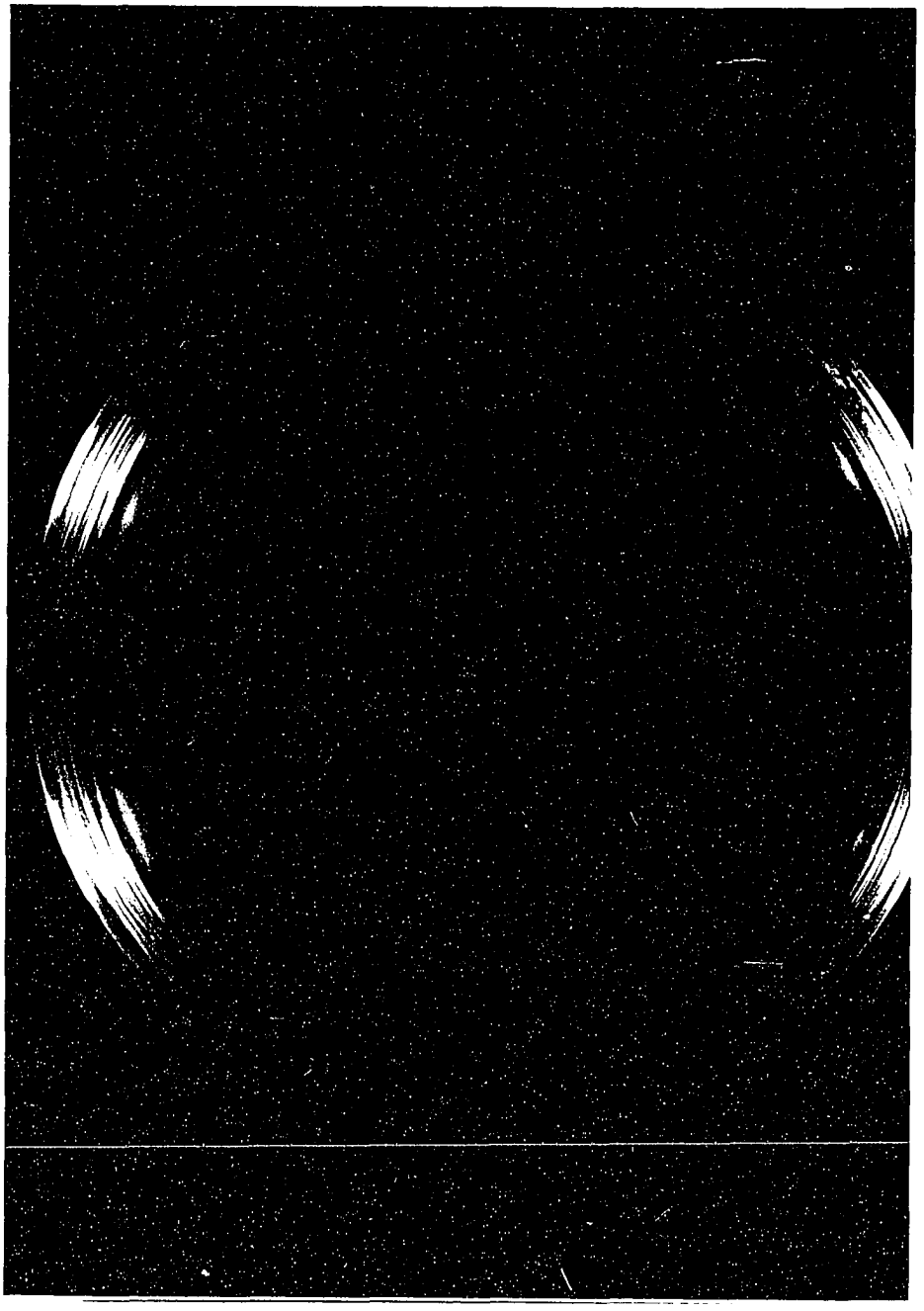


Figure 3.9

Table 3.6. Growth of Cu^s *V. alginolyticus* and WT *V. alginolyticus* on 15 μM Cu-amended plates.

Isolate	Colonies • ml^{-1} Nonselective	Colonies • ml^{-1} 15 μM Cu	% Growth (15μM)
WT *V. alginolyticus*	3.8×10^9	3.3×10^9	86.8
VA15S7	1.1×10^9	$< 1 \times 10^6$	<0.03%
VA15S8	2.46×10^9	$< 1 \times 10^4$	<0.0004
VA15S9	2.50×10^8	$< 1 \times 10^4$	<0.005
VA15S11	1.35×10^8	$< 1 \times 10^4$	<0.007

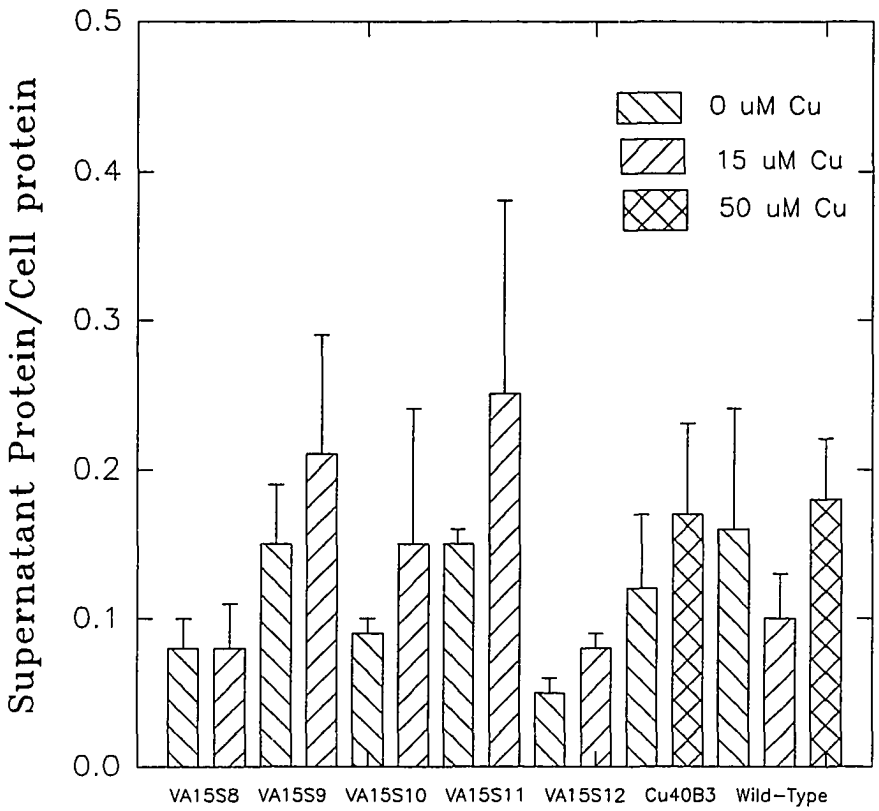
Total supernatant protein was measured in control and Cu-stressed cultures of Cu^s mutants (15 μM Cu), WT *V. alginolyticus* (15 and 50 μM) and Cu40B3 (50 μM Cu), then normalized to cellular protein (Figure 3.10). When the meansupernatant protein concentrations of control mutant cultures were compared to that of control WT *V. alginolyticus* by paired t-tests, there was no significant difference between any mutant (Cu^s or Cu^r) and the wild-type ($p < 0.05$). Similarly, there was no significant difference in supernatant protein between any Cu-stressed mutant and Cu-stressed wild-type cultures.

Supernatant proteins of Cu^s mutants were separated by SDS-PAGE in order to determine if any of them were deficient in extracellular CuBP. Supernatants from VA15S7 cultures stressed with 15 and 50 μM Cu were consistently indistinguishable from the wild-type (Figure 3.11). CuBP in the other Cu^s mutants was quantitated from SDS-polyacrylamide gels (Figures 3.12 and 3.13) in order to compare their CuBP production with that of WT *V. alginolyticus* (Table 3.7).

VA15S8, VA15S9, VA15S11 and VA15S12 stand out from the isolates in Table 3.7 as possibly aberrant in CuBP production, while CuBP expression in VA15S10 is very similar to WT *V. alginolyticus* (Figures 3.11,3.12,3.13). VA15S8was generally depressed in extracellular protein expression (Figure 3.11,3.12). No CuBP was detected in 15 μM Cu-stressed supernatants of VA15S9, however this isolate also appears have a general deficiency in extracellular protein production (Figures 3.11, 3.13). VA15S11 control supernatants contained a protein of the same molecular weight as CuBP which decreased in concentration with added Cu (Figure 3.11,3.13).

Figure 3.10. Supernatant protein normalized to cell protein for WT *Vibrio alginolyticus*, Cu40B3 (Cu^r) and Cu^s mutants.

Figure 3.10



Supernatant protein normalized to cell protein for Cu-resistant and Cu-sensitive mutants and wild-type V. alginolyticus.

Figure 3.11. Supernatant protein profiles of WT *Vibrio alginolyticus* and copper-sensitive mutants from control and Cu-challenged cultures.

Lane 1: Control WT *Vibrio alginolyticus*
Lane 2: 15 μM Cu-challenged *Vibrio alginolyticus*
Lane 3: Control VA15S11
Lane 4: 15 μM Cu-challenged VA15S11
Lane 5: N/A
Lane 6: molecular weight standards
Lanes 7 - 10: N/A
Lane 11: Control VA15S10
Lane 12: 15 μM Cu-challenged VA15S10
Lane 13: Control VA15S7
Lane 14: 15 μM Cu-challenged VA15S7
Lane 15: 50 μM Cu-challenged VA15S7

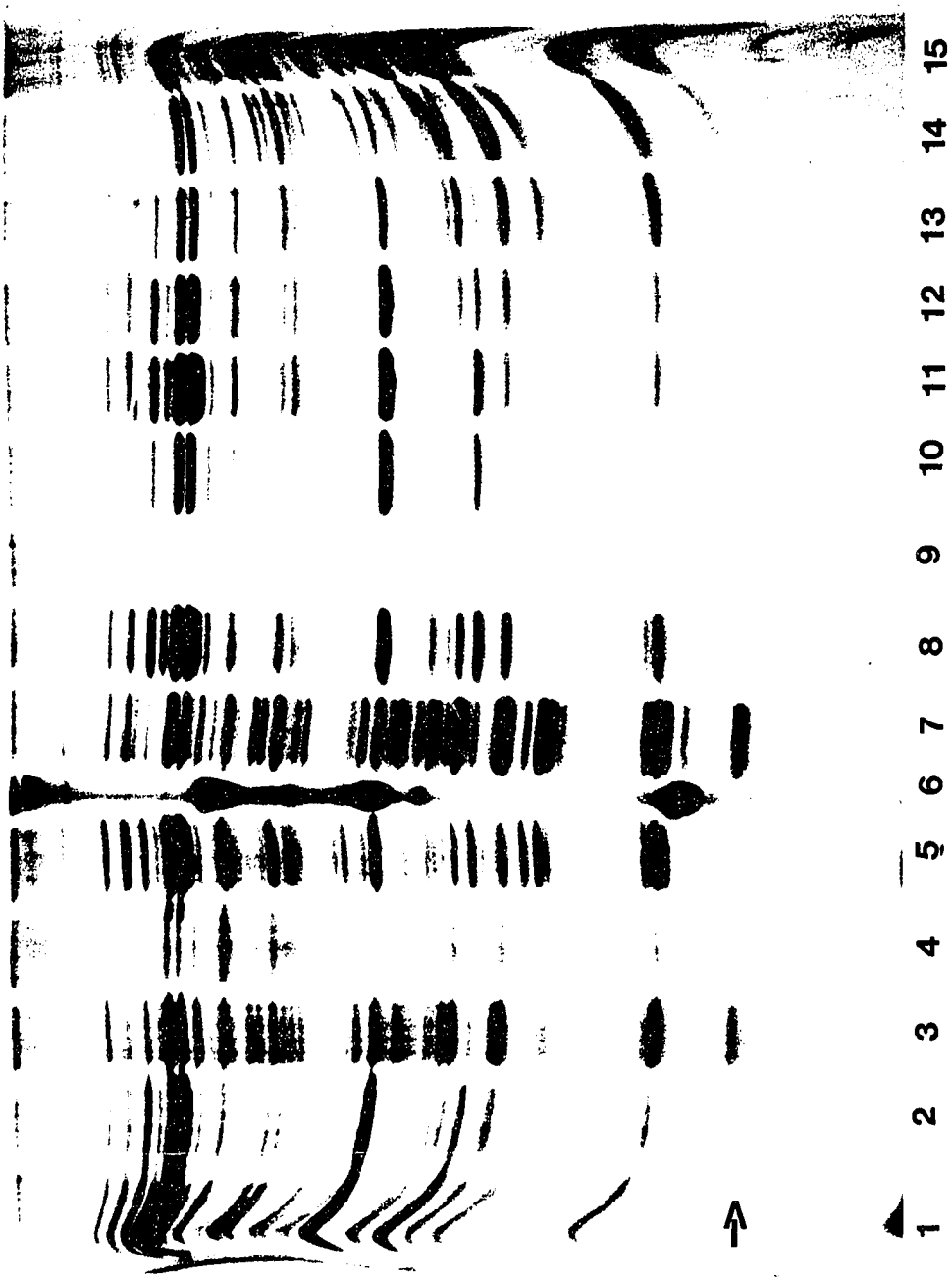


Figure 3.11

Figure 3.12. Supernatant protein profiles of WT *Vibrio alginolyticus* and copper-sensitive mutants from control and Cu-challenged cultures.

Lane 1: 15 μM Cu-challenged WT *Vibrio alginolyticus*
Lane 2: 50 μM Cu-challenged WT *Vibrio alginolyticus*
Lane 3: Control Cu40B3
Lane 4: 50 μM Cu-challenged Cu40B3
Lane 5: Control VA15S8
Lane 6: Molecular weight standards
Lane 7: 15 μM Cu-challenged VA15S8
Lane 8: 50 μM Cu-challenged VA15S8
Lane 9: Control VA15S10
Lane 10: 50 μM Cu-challenged VA15S10
Lane 11: Control VA15S12
Lane 12: 15 μM Cu-challenged VA15S12
Lane 13: 50 μM Cu-challenged VA15S12

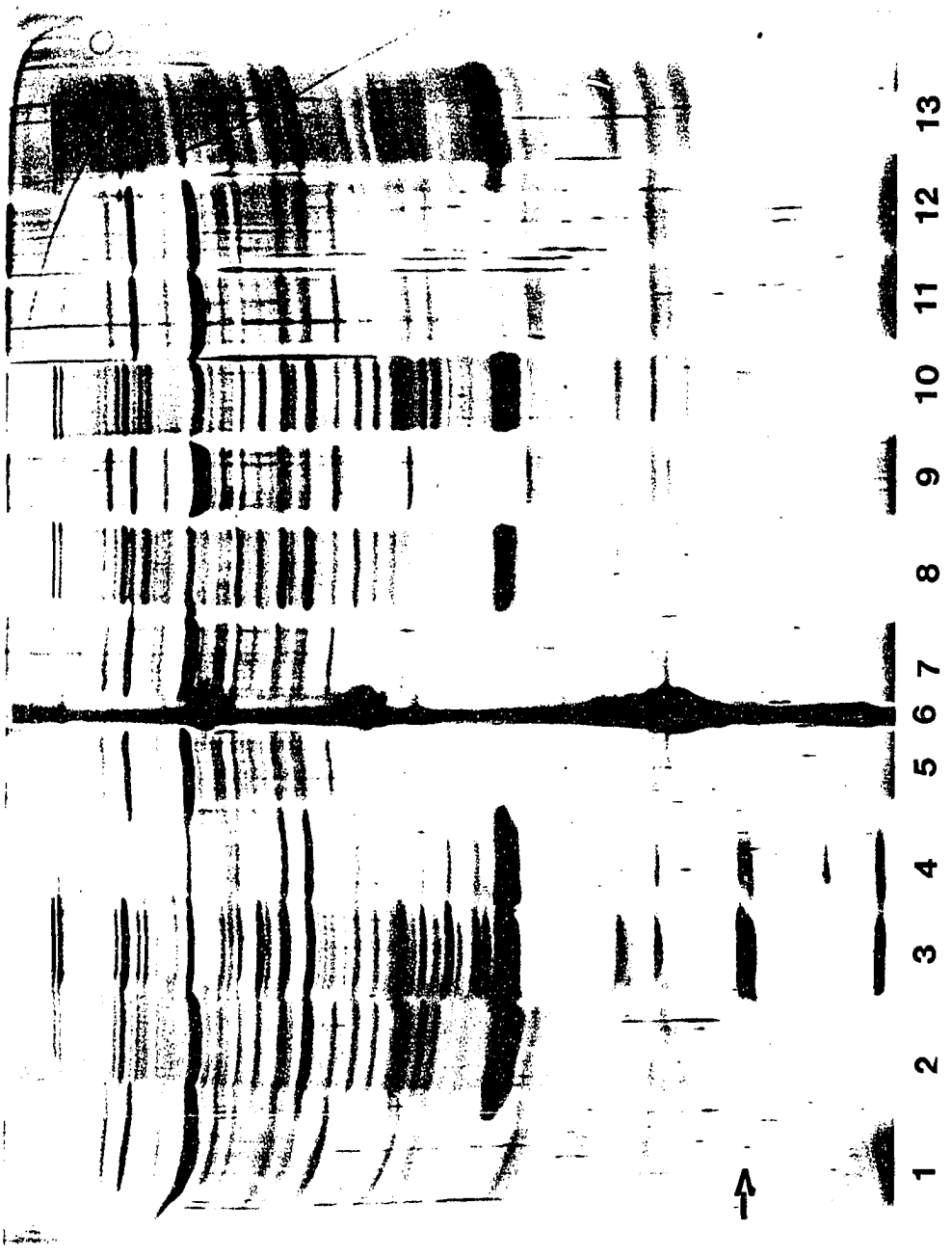


Figure 3.12

Figure 3.13. Supernatant protein profiles of Wt *Vibrio alginolyticus*, copper-sensitive mutants, Cu40B3 (Cu^r) and it copper-sensitive revertant, Cu40B3(SW).

Lane 1: Control WT *Vibrio alginolyticus*
Lane 2: 15 μM Cu-challenged WT *Vibrio alginolyticus*
Lane 3: Control VA15S9
Lane 4: 15 μM Cu-challenged VA15S9
Lane 5: Control VA15S10
Lane 6: 15 μM cu-challenged VA15S10
Lane 7: Molecular weight standards
Lane 8: Control VA15S11
Lane 9: 15 μM Cu-challenged VA15S11
Lane 10: Control VA15S12
Lane 11: 15 μM Cu-challenged VA15S12
Lane 12: Control Cu40B3 (Cu^r)
Lane 13: 50 μM Cu-challenged Cu40B3 (Cu^r)
Lane 14: Control Cu40B3(SW) (Cu^s)
Lane 15: 50 μM Cu-challenged Cu40B3(SW) (Cu^s)

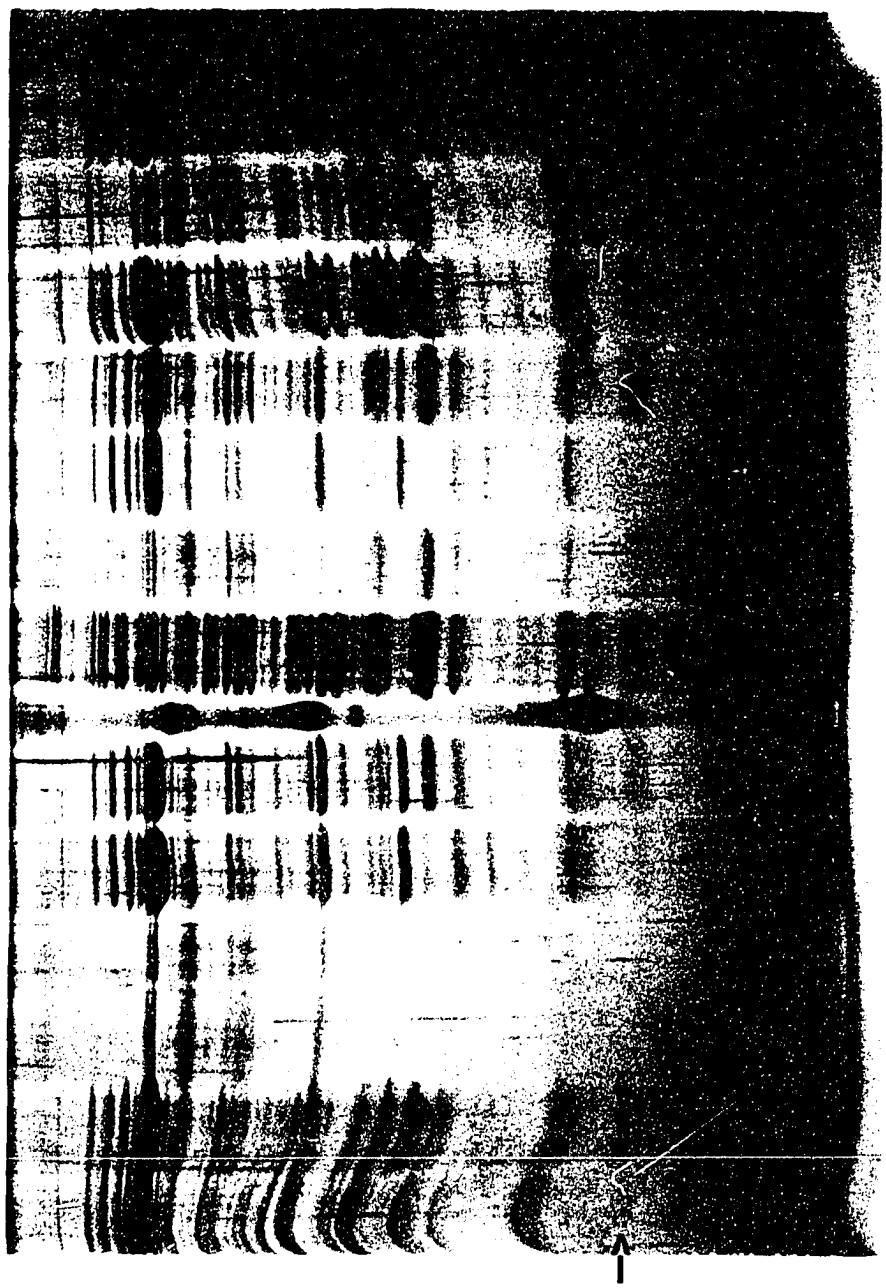


Figure 3.13

Table 3.7. CuBP in supernatants of Cu^s *V. alginolyticus* mutants and WT *V. alginolyticus*.

Isolate	[Cu](μM)	% CuBP	[CuBP] ($\mu g \cdot ml^{-1}$)
WT *V. alginolyticus*	0	ND[1]	ND
WT *V. alginolyticus*	15	0.68	0.16
WT *V. alginolyticus*	50	5.05	1.65
VA15S8	0	ND	ND
VA15S8	15	ND	ND
VA15S8	50	2.21	0.47
VA15S9	0	ND	ND
VA15S9	15	ND	ND
VA15S10	0	ND	ND
VA15S10	15	1.58	0.32
VA15S10	50	4.77	1.55
VA15S11	0	0.52	0.13
VA15S11	15	0.25	0.006
VA15S12	0	ND	ND
VA15S12	15	0.99	0.18
VA15S12	50	ND	ND

[1]Not detected by densitometry.

VA15S12 supernatants from cultures stressed with 15 μM Cu contained approximately the same of CuBP concentration as the wild-type, but when 50 μMCu was added no CuBP was detected in supernatants (Figure 3.12).

Plasmid curing. WT *V. alginolyticus* broth cultures were treated with acridine orange in an attempt to selectively inhibit plasmid replication, with the goal of assessing the contribution of the cryptic *V. alginolyticus* plasmid to copper tolerance. The strategy was to treat the cultures with acridine orange, isolate Cu^s colonies, by the filter transfer technique used above, and screen the Cu^s isolates for plasmid DNA. One Cu^s isolate, VA15S13, was obtained from the treatment. Plasmid DNA from this isolate, an AO-treated isolate with normal Cu sensitivity, and WT *V. alginolyticus* was extracted and purified on an agarose gel (Figure 3.14). All the isolates contained the ca. 30 kb plasmid previously observed in the wild-type, therefore acridine orange treatment failed to cure the plasmid.

Loss of copper resistance in Cu40B3. After the fifteenth passage on nonselective agar, Cu40B3, a nonswarming mutant, began to exhibit a high percentage of swarming colonies. A concommitant decrease in the number of cells from these plates able to form colonies on HASW + 40 μM Cu plates was noted, while Cu40B3 cultures that had recently been exposed to copper continued to grow well on the plates. In order to investigate the possible link between the regained ability to swarm, CuBP production in nonstressed cultures, and copper resistance, broth cultures of nonswarming, Cu^r Cu40B3 and the swarming derivative of Cu40B3 [Cu40B3(SW)] were plated on nonselective and 40 μM Cu-amended HASW agar.

Figure 3.14. Plasmid DNA from WT *Vibrio alginolyticus*, VA15S13 and an acridine orange (AO)-treated isolate with normal sensitivity to copper.

Lane 1: WT *Vibrio alginolyticus*
Lane 2: WT *Vibrio alginolyticus* treated with RNase
Lane 3: VA15S13 treated with RNase
Lane 4: Supercoiled molecular weight standards
Lane 5: AO-treated *Vibrio alginolyticus* treated with RNase

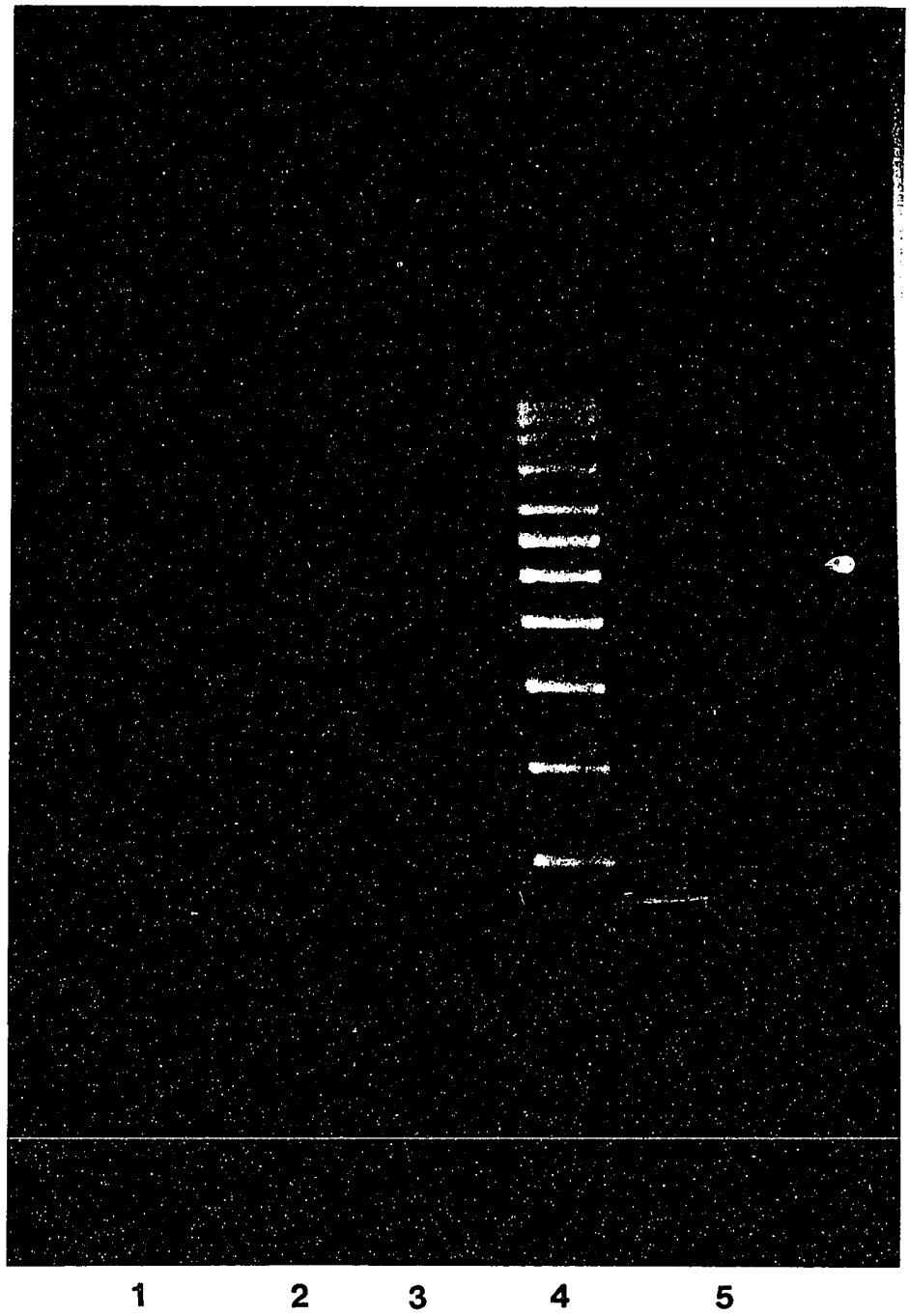


Figure 3.14

A high percentage of Cu40B3 cells formed colonies on Cu-amended plates, while Cu40B3(SW) cells were able to form very few colonies(Table 3.8).

The copper-resistant colonies isolated from Cu40B3(SW) cultures consistently contained swarming and nonswarming individuals when subcultured on MA. Figure 3.15 demonstrates the swarming phenotype of Cu40B3(SW), and Figure 3.16 the mixed swarming/nonswarming phenotype of Cu^r colonies obtained from Cu40B3(SW) cultures. One Cu^r, consistently swarming derivative of Cu40B3(SW) was isolated and designated CuSWA3.

Densitometric analysis of SDS-polyacrylamide gels (Figures 3.12 and 3.13) was used to quantitate CuBP concentrations in supernatants of Cu40B3(SW) and Cu40B3. CuBP was not detectable in control Cu40B3(SW) supernatants, but comprised an average 9.2% of Cu40B3 supernatant protein (Table 3.9). Fifty μM copper elicited CuBP expression in Cu40B3(SW) comparable to that observed in WT *V. alginolyticus*. Thus, Cu40B3(SW) appears to be a revertant from the observed Cu^r, nonswarming phenotype of Cu40B3 to a swarming phenotype with wild-type CuBP expression and sensitivity to copper.

Figure 3.15. Cu40B3(SW) plated on marine agar.

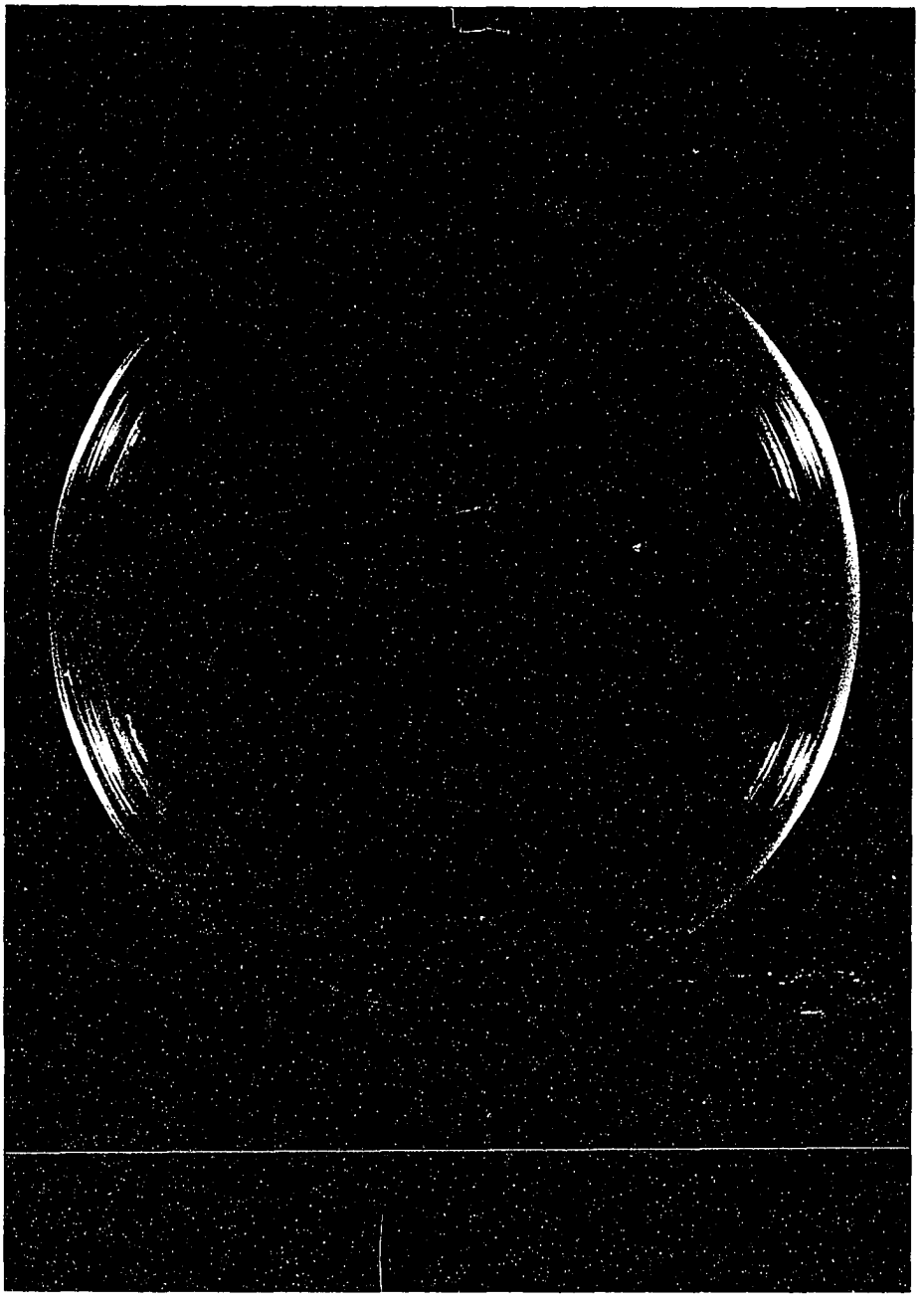


Figure 3.15

Figure 3.16. Mixed swarming and nonswarming colonies streaked from a copper-resistant colony derived from Cu40B3(SW).

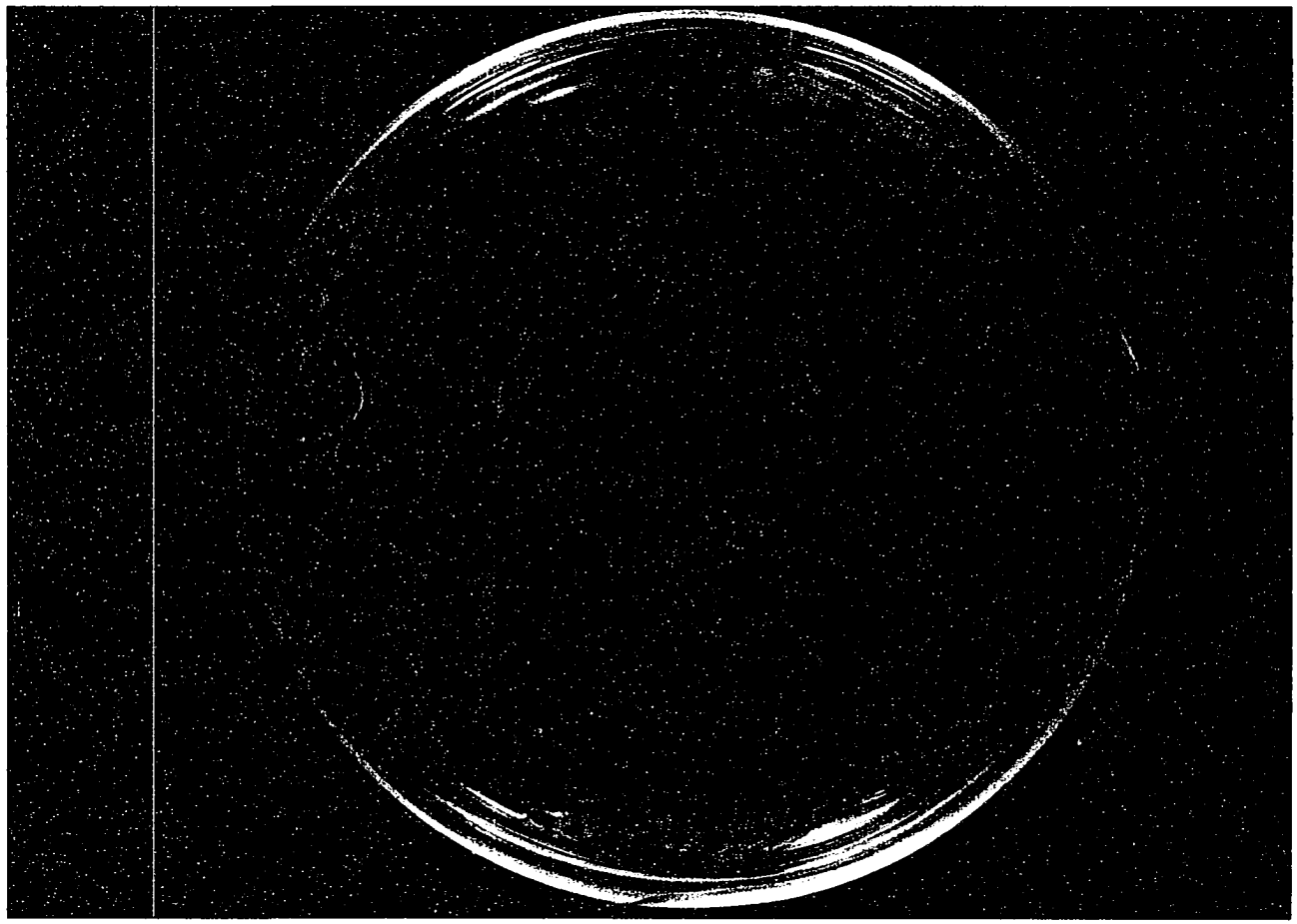


Figure 3.16

Table 3.8. Comparison of the ability of Cu40B3 (NS) and its swarming derivative Cu40B3 (SW) to form colonies on 40 μM Cu-supplemented media.

Isolate	Culture Conditions	Date	Plate Count (0 μM Cu)	Plate Count 40 μM Cu (cells/ml)	Frequency Cu^r
WT V. alg.	MA→SWM9	4-30-92	1.0×10^9	66	6.7×10^{-8}
WT V. alg.	MA→SWM9	5-13-92	1.0×10^9	0	$<1.6 \times 10^{-9}$
WT V. alg.	MA→SWM9	7-22-92	1.6×10^9	0	$<2.0 \times 10^{-9}$
WT V. alg.	MA→SWM9	7-28-92	2.7×10^9	0	$<1.9 \times 10^{-9}$
WT V. alg.	MA→SWM9	8-11-92	3.2×10^9	0	$<3.1 \times 10^{-10}$
Cu40B3 (NS)	10° MA→ SWM9	4-30-92	1.5×10^9	7.2×10^5	4.7×10^{-4}
Cu40B3 (NS)	Cu→SWM9	4-30-92	1.2×10^9	3.0×10^5	2.4×10^{-4}
Cu40B3 (NS)	Cu→SWM9	6-4-92	4.1×10^7	1.7×10^6	4.2×10^{-2}
Cu40B3 (NS)	Cu→SWM9 +50 μM Cu	6-28-92	9.5×10^7	2.9×10^7	3.0×10^{-1}
Cu40B3 (NS)	Cu→SWM9	6-28-92	8.5×10^7	2.9×10^7	3.4×10^{-1}
Cu40B3 (NS)	Cu→SWM9	7-28-92	7.2×10^8	8.1×10^6	1.1×10^{-2}
Cu40B3 (NS)	Cu→SWM9	8-11-92	1.6×10^9	5.5×10^7	3.4×10^{-2}
Cu40B3 (NS)	Cu→SWM9 + 50μM Cu	9-16-92	4.0×10^7	3.0×10^7	7.5×10^{-1}
Cu40B3 (NS)	Cu→SWM9	9-16-92	4.1×10^8	1.4×10^7	3.4×10^{-2}
Cu40B3 (Sw)	MA→SWM9	6-4-92	1.4×10^9	0	$<3.6 \times 10^{-9}$
Cu40B3 (Sw)	MA→SWM9	7-12-92	8.3×10^8	0	$<6.1 \times 10^{-9}$
Cu40B3 (Sw)	MA→SWM9 + 50 μM Cu	7-12-92	9.5×10^6	0	$<5.3 \times 10^{-7}$
Cu40B3 (Sw)	MA→SWM9	7-22-92	1.4×10^9	17	1.2×10^{-8}
Cu40B3 (Sw)	MA→SWM9	7-28-92	1.7×10^9	95	5.6×10^{-8}

Table 3.9. CuBP in supernatants of the copper-resistant mutant, Cu40B3 vs. CuBP in supernatants of its swarming, copper-sensitive derivative [Cu40B3 (SW)]. Results for WT *V. alginolyticus* are included for comparison.

Isolate	[Cu] (μM)	% CuBP	[CuBP] (μg/ml)
WT *V. alginolyticus*	0	ND	ND
WT *V. alginolyticus*	50	5.05	1.65
Cu40B3	0	9.25	2.16
Cu40B3	50	7.85	2.15
Cu40B3 (SW)	0	ND	ND
Cu40B3 (SW)	50	3.41	0.85

DISCUSSION

Cu^r isolates. The correlation between extracellular CuBP expression and exposure to/ recovery from copper stress (Harwood-Sears and Gordon, 1990; Gordon *et al.*, 1993) coupled with the data indicating that Cu^{2+} becomes complexed in supernatants of copper-stressed *V. alginolyticus* cultures by a copper-induced protein of ca. 20-30 kDa (Schreiber *et al.*, 1990; Harwood-Sears and Gordon, 1990), constitutes strong indirect evidence that CuBP is involved in copper detoxification by *V. alginolyticus*. Direct evidence of the link between CuBP expression and Cu tolerance was obtained by the classic microbial genetics technique of isolating mutants with altered sensitivity to copper and analyzing their supernatants for altered expression of extracellular proteins in the 19-22 kDa molecular weight range.

Spontaneous copper-resistant mutants could be isolated from batch cultures (Cu20A6) and Cu-stressed continuous cultures (Cu40A1, Cu40B3). The mechanism of copper resistance in the oxidase positive Cu20A6 is not evident from experiments conducted to date. Both the induction pattern and supernatant protein profile appear similar to that of the wild-type (Figure 3.2). SDS-PAGE gels of unchallenged vs. 50 μM Cu-challenged Cu20A6 suggest that this variant may have more CuBP and/or total supernatant protein in copper-challenged cultures than the wild-type, but this observation has not been confirmed by analysis of replicate cultures, and no statistically significant differences in total supernatant protein measurements between WT *V. alginolyticus* and its mutants (Cu^s and Cu^r) were found. Alternatively, the CuBP of Cu20A6 may have a higher affinity for copper than that of WT *V.*

alginolyticus, or some unrelated mechanism may be involved.

Clearly, Cu40B3 constitutively produces ca. 20 kDa supernatant proteins in cultures that have not been copper-stressed, and their level in control and Cu-stressed cultures, while somewhat variable, meets or exceeds the level of CuBP in Cu-stressed wild-type *Vibrio alginolyticus* cultures. Constitutive transcription of the metallothionein gene in *Saccaromyces cerevisiae* resulted in cadmium and copper resistance, indicating that constitutive production of metal binding proteins is one physiological avenue for metal resistance (Tohoyama *et al.*, 1992).

The greater frequency of Cu^r individuals in the oxidase negative Cu40A1 cultures compared to the CuBP-constitutive Cu40B3 cultures may well reflect a fundamental difference in their respective mechanisms of copper detoxification. The frequency of oxidase negative isolates among Cu^r *V. alginolyticus* suggests that decreased cytochrome oxidase activity could lead indirectly to greater copper tolerance, perhaps by reducing the level of oxygen free radicals with which intracellular Cu could interact. In this case, each mutant or variant cell would offer its neighbor no protection from copper, while a variant which overexpressed an extracellular, copper-complexing protein would decrease the free copper in the media, benifiting both itself and surrounding cells. Thus, not every cell in a Cu40B3 population on (or in) copper-containing media would have to overexpress CuBP to survive.

Decreased growth efficiency in the presence of copper was demonstrated in continuous cultures of *V. alginolyticus* (Gordon *et al.*, 1993). While Cu40B3 clearly

displays greater resistance to copper on solid media than WT *V. alginolyticus* (Chapter II) its resistance to copper in liquid culture is less obvious. Optical density is not an accurate estimate of Cu40B3 cell numbers in broth culture due to its increased tendency to form aggregates, therefore cell protein was used as an estimate of culture biomass. While the mean for the ratio of protein in Cu-stressed cultures/ protein in control cultures was higher for Cu40B3 than for WT *V. alginolyticus*, the difference was not significant. The slower growth rate of Cu40B3 complicates a time-based comparison, and Cu40B3 cultures harvested at 24 h had probably not yet reached stationary phase. Even after these complications are considered, the difference in efficiency between Cu40B3 and the wild-type is less than one might expect based on the plate count results.

A model for a possible explanation of the greater observed copper resistance of Cu40B3 on solid compared to broth media is shown in Figure 3.17. The mutant phenotype of Cu40B3 has two important characteristics which must be considered in a model: (1) constitutive CuBP production and (2) increased production of CuBP when challenged with copper. Constitutive CuBP production has been consistently observed, while overproduction in Cu-challenged cultures is more variable. Thus, while both attributes will be considered in the model, the lack of induction time necessary for CuBP production in Cu40B3 probably offers at least as great an advantage as overproduction of protein.

The major differences apparent between broth and solid media include cell density, diffusion of CuBP away from resistant individuals, and diffusion of copper

Figure 3.17. Cu40B3 in copper-stressed conditions in liquid and solid media: a model for the interaction of copper, CuBP and Cu^r and Cu^s bacteria in the cultures.

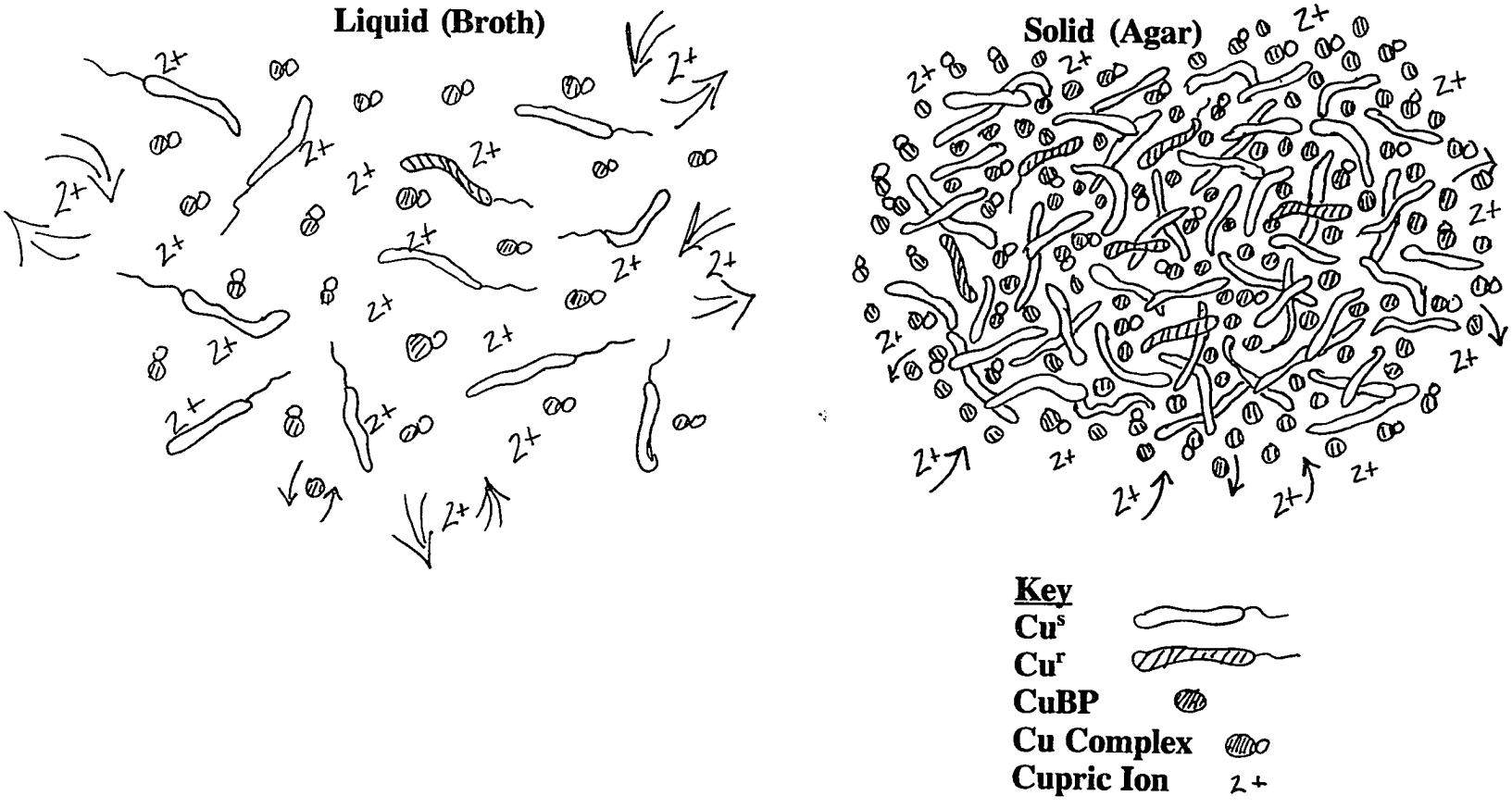


Figure 3.17

into proximity with the bacteria. Broth cultures contain fewer individuals per unit area than colonies growing on agar. Broth cultures are shaken, therefore mixing and diffusion of solute molecules is relatively free. Thus, in broth culture, rapid mixing and diffusion of CuBP away from resistant individuals, coupled with mixing and diffusion of Cu^{2+} among the cells, could decrease the advantage that bacteria which constitutively produce CuBP display over the wild-type on solid media.

Constitutive producers of CuBP which find themselves on solid media would have the immediate advantage of no lag in CuBP production, and mixing would not be a factor in increasing dissemination of CuBP away from the bacteria and of Cu^{2+} into proximity with the bacteria. After only ten divisions, there would be over 1,000 cells in a very small area, all of which would be producing CuBP and some of which would be overproducing CuBP. Excess CuBP would diffuse away from the culture, complexing Cu^{2+} diffusing toward the colony before it could contact the bacteria.

The cytotoxic effects of copper on *V. alginolyticus* depend partly on the time of exposure, as Cu^s *V. alginolyticus* and *V. parahaemolyticus* can be retreived from inhibitory agar if they have not been exposed for more than a day or two. Experience with these isolates and with WT *V. alginolyticus* plated on copper concentrations just above inhibitory levels has shown that what is initially a cytostatic effect will become cytotoxic, and colonies will become nonculturable much more quickly than if they were on minimal agar without copper. Cu40B3 viability on Cu-amended plates decreases dramatically after ten days, probably due to continued copper diffusion after colonies have ceased growing. Bacterial survival in the

presence of excess copper may thus be framed as a race between the damage inflicted by copper and bacterial growth/ protein production, a race where conditions should favor a constitutive producer of copper-binding protein.

Vibrio parahaemolyticus. The similarity in the response of *V. parahaemolyticus* and *V. alginolyticus* to copper first became apparent in growth curves (Howell *et al.*, 1992), which displayed the characteristic Cu-induced lag phase, albeit at a higher copper concentration. The majority of added copper was recovered from the supernatants of both organisms after they were grown in the presence of 50 μM Cu; the cellular fractions contained less than 50% of recovered copper. Production of an extracellular protein which has similar chromatographic behavior and the same molecular weight as CuBP suggests that copper detoxification is accomplished by a similar mechanism in *V. alginolyticus* and *V. parahaemolyticus*.

Because Cu^r variants of *V. alginolyticus* and *V. parahaemolyticus* BB22 arose at approximately the same frequency on plates amended with 30 μM and 150 μM copper, respectively (Harwood *et al.*, 1992), the possibility that their increased resistance to copper is mediated by the same mechanism(s) was investigated. The observation that increased copper resistance is not stable in BB22, i.e. one passage on nonselective media eliminated the ability of putative Cu^r isolates to grow on Cu-amended plates, argues against the possibility that a mutation was involved. Although the frequency of Cu^r individuals (defined as colonie forming units on HASW + 40 μM Cu) varied from culture to culture in mutants such as Cu40B3 (see Table 3.8), some individuals retained the copper-resistant phenotype after ten

nonselective subcultures, indicating that a genetic change had occured. Heightened copper resistance in BB22 may be mediated by an induced compound,rather than a genetic mechanism.

Cu^{s} *isolates.* Tn5-132 and miniMu(Tet^{r}) are genetically engineered transposons which encode tetracycline resistance (Belas *et al.*, 1984), providing a selectable marker which facilitates genetic studies. *V. alginolyticus*, like most naturally-occuring *Vibrio* species, is sensitive to tetracycline, which inhibits protein synthesis at the translational level (Ausubel *et al.*, 1987). Resistance to tetracycline was deliberately chosen for genetic work with *Vibrio* species, which very rarely mutate to a Tet^{r} phenotype (Belas *et al.*, 1984). P1, the bacteriophage vector used in this mutagenesis system, is a coliphage (originally from *E. coli*), and although it can insert DNA into a range of gram negative bacteria, it has has failed to transfer miniMu (Tet^{r}) to marine vibrios in other investigators' hands (Ostling *et al.*, 1991). In fact, many strains of *V. parahaemolyticus* were screened before a susceptible isolate (BB22) was found (Bob Belas, personal communication). Mutagenesis with lysates from *E. coli* C600 (Tn5-132) and *E. coli* MC4100 [miniMu(Tet^{r})] was unsuccessful after many attempts with *V. alginolyticus*. Transposition into BB22 was successful for both Tn5-132 and miniMu the first time it was attempted in this lab.

Since *V. alginolyticus* did not seem susceptible to DNA transfer by P1, transposition using a plasmid vector system was attempted. This system had been successfully applied to marine *Vibrio* species (Ostling *et al.*, 1991), although it was noted that the frequency of transposition was very low. In this lab, transposition in

Vibrio S141, the positive control, did not occur at detectable levels ($< 10^{10} \cdot ml^{-1}$). An additional problem occured as *E. coli* J100, which harbors pRK2013::miniMu(Tet^r), the plasmid carrying miniMu, mutated to a streptomycin resistant (Sm^r) phenotype at a frequency of 10^{-9}. Since streptomycin resistance is used to differentiate between *E.coli* and *Vibrio* S141 (or *V. alginolyticus*) after the filter mating, the Sm^r mutation rate for *E. coli* J100 was unacceptably high.

Chemical mutagenesis with NTG was employed to obtain Cu^s *V. alginolyticus* after transposon mutagenesis proved unsuccessful. While less elegant and less amenable to further genetic manipulation than transposon insertion, chemical mutagenesis has been successfully used to investigate the genetics of collagenase (an extracellular protein) production in *Vibrio alginolyticus* (Robbertse *et al.*, 1978).

The screening method utilizing bromcresol purple developed for isolating Cu^s mutants proved useful, and eliminated the need to pick individual colonies to replica plate arrays or to use velveteen replica plating devices, which frequently result in false negative scores and colony smearing. Although this method was limited to one transfer, i.e. from one nonselective plate to one selective plate, if the template was allowed to grow overnight after filter transfer a second transfer was possible.

Because of the limitation on serial transfers of the same template, the highest concentration of HASW + Cu on which the majority of wild-type *V. alginolyticus* could form colonies was determined and used as the selective medium. The filter transfer method proved more sensitive than a copper-impregnated filter disk assay developed at about the same time. The copper-disk assay could differentiate

between copper sensitivity in *V. alginolyticus* and *V. parahaemolyticus*, but not between *V. alginolyticus* and Cu40B3 or the other Cu^r and Cu^s mutants. The filter transfer technique can also be used to isolate Cu^r mutants by transferring colonies to plates containing copper concentrations inhibitory to the wild-type, and selecting yellow colonies for further testing. This technique was used to isolate relatively copper-resistant bacteria from Chesapeake Bay sediments (unpublished data).

Although there seemed to be a trend toward decreased total supernatant protein in Cu^s mutants, no significant difference was found between supernatant protein normalized to cell protein for any isolate, including Cu40B3 (Cu^r), compared to *V. alginolyticus*. SDS-PAGE of some isolates indicated very little protein (i.e. VA15S9) although normal amounts of protein were measured. A possible explanation for this phenomenon is that excessive protease activity in supernatants reduced the proteins to small fragments which could not be resolved on 12% polyacrylamide gels. CuBP, which was not detected in VA15S9 supernatants, would be expected to be destroyed by elevated protease activity along with other supernatant proteins.

VA15S7 and VA15S10 supernatants were indistinguishable from the wild-type in control and Cu-stressed (15 and 50 μM) cultures. VA15S11 expressed an abnormal amount of a protein of the same molecular weight as CuBP in control supernatants, but the concentration of this protein decreased with added Cu. If this protein is CuBP, the copper sensitivity of VA15S10 suggests that other functional elements, perhaps a regulatory region or an intracellular chaperone or membrane

protein, are involved in the copper detoxification system.

VA15S12 appears similar to WT *V. alginolyticus* in all respects, except that it does not produce CuBP during 50 μM copper stress. VA15S12 is one of the most copper sensitive mutants isolated during this study, and does not grow on HASW + 15 μM Cu, unlike some of the less sensitive isolates, i.e. VA15S10, whose growth is slowed but not completely inhibited by 15 μM Cu. Supernatant protein (absolute and normalized to cell numbers) was not significantly different from the wild-type. Why VA15S12 expresses a CuBP-like protein when stressed with 15 μM Cu and not with 50 μM Cu stress has not been determined. Perhaps there is a deficiency in the rate of CuBP production, which could be caused by mutations in the CuBP regulatory region, so that a successful response only to the lower concentration of copper is possible.

Plasmid DNA. Acridine orange did not cure the *V. alginolyticus* plasmid, therefore the possible contribution of this plasmid to copper resistance in the organism could not be determined. However, the micromolar levels of copper used in this study are low compared to levels of plasmid-mediated copper resistance reported in the literature (Bender and Cooksey, 1984; Rouche *et al.*, 1989). If the CuBP system is involved in copper ion homeostasis under normal levels as well as in copper detoxification at higher Cu levels, it is likely to be chromosomally encoded in order to ensure genomic stability. The development of a probe to the *cbp* gene is planned which will allow the location of the gene to be determined, obviating the need for plasmid curing.

Cu^{s} *revertants.* The gene(s) and regulatory elements of *cbp* have yet to be identified. CuBP expression may be regulated by a metallothionein-like mechanism, in which copper ions bind to and activate the transcriptional regulatory protein ACE1 (Furst and Hamer, 1989). Constitutive metallothionein production in yeast is thought to be due to a novel transcriptional factor (Tohoyama *et al.*, 1992), a mechanism which could mediate constitutive CuBP production in *V. alginolyticus*. Alternatively, CuBP induction may be mediated by a second messenger or by the physiological effects of copper toxicity in a manner analogous to induction of the heat shock response, which is not a response to heat *per se* but to the presence of damaged proteins (Anathan *et al.*, 1986; Craig and Gross, 1991). Heat shock transcription factor (σ^{32}) can activate *CUP1* (metallothionein) transcription (Silar *et al.*, 1991; Yang *et al.*, 1991).

Reversion of Cu40B3, a nonswarming, Cu^{r}, constitutive producer of CuBP, to a swarming, Cu^{s} phenotype in which CuBP expression must be induced by copper Cu40B3(SW), indicates that constitutive CuBP production is required for the copper-resistant phenotype of Cu40B3. The apparent correlation between swarming motility and reversion to copper sensitivity has no obvious explanation. Some swarming Cu^{r} mutants have been isolated, i.e. Cu20A6 and CuSWA3, therefore swarming motility does not preclude copper resistance. Copper resistance in Cu^{r} derivatives of Cu40B3(SW) was correlated with the nonswarming phenotype, suggesting that some physiological change(s) associated with swarming may inhibit constitutive expression of CuBP in Cu40B3.

Metals, including copper (Guzzo *et al.*, 1991) and iron (McCarter and Silverman, 1989) have been shown to have a regulatory effect on flagellar production in bacteria. If copper directly inhibits lateral flagella production by binding to a regulatory locus or transcription factor, thus causing the reversible inhibition of swarming observed in plated chemostat populations, it is possible that an altered regulatory locus or transcription factor in nonswarmers such as Cu40B3 binds copper with unusually high affinity. The altered regulatory element could then bind and hold copper ions even under normal intracellular copper levels, resulting in a mutant nonswarming phenotype. In this scenario the *laf* genes which encode lateral flagella would be negatively regulated by copper ions. The *cbp* system should be positively regulated by copper ions, therefore it is difficult to reconcile direct interaction (as in *CUP1* activation by σ^{32}) between one mutated regulatory element or transcription factor and the *laf* and *cbp* systems. If two mutations occured, one which negatively regulates the *laf* genes and another which positively (and constitutively) regulates *cbp*, back mutation of the mutated *laf* regulatory element might preclude constitutive expression of *cbp*.

Alternatively, copper may activate a second messenger system which inhibits swarming, and it may be component(s) of this system which have been altered in nonswarming mutants. If the same second messenger system that shuts down lateral flagellar production also acts to induce CuBP production, the nonswarming phenotype would be coupled to constitutive CuBP production.

The isolation of a Cu^r mutant that apparently has alterations in the *cbp*

regulatory locus not only supports the hypothesis that CuBP is an important factor in the response of *V. alginolyticus* to copper, but also provides opportunities for further genetic study. Thus, the defective regulatory locus could be cloned and identified by its ability to confer Cu resistance and constitutive CuBP expression upon *V. alginolyticus*. The Cu^s mutants also provide opportunities for genetic study, as restoration of the wild-type phenotype by clones from a genetic library could lead to indentification of *cbp* and other physiological systems that participate in the homeostatic regulation of copper in *V. alginolyticus*.

SYNOPSIS

Vibrio alginolyticus is the only marine bacterium whose response to toxic levels of heavy metals has been investigated at the biochemical and genetic level. The biota is a dominant component of the factors that control metal speciation, residence time and turnover in the oceans, therefore the investigation of microbial/metal interactions in the ocean is critical to our understanding of the complex, interacting physical, chemical and biological systems involved in determining the fate of metal ions once they enter the world Ocean.

The extracellular location of the copper-induced protein we have designated CuBP is unusual in light of the metal detoxification models common in the literature, i.e. intracellular metallothioneins and the intracellular/periplasmic *mer* system involved in mercury detoxification. However, there are reports in the literature of extracellular deposition of copper (Eradi *et al.*, 1987; Rouche *et al.*, 1989) and of the involvement of extracellular proteins in cadmium detoxification (Francis and Bollag, 1991).

The extracellular, ca. 20 kDa copper-binding protein(s) of *V. alginolyticus* can be resolved by SDS-PAGE as two bands differing in apparent molecular weight by approximately two kDa, or as one band of intermediate molecular weight, depending upon the quantity of protein (more protein = one band) and gel-to-gel variability. It is not known whether the two bands represent entirely different proteins, or

whether the larger band is an incompletely processed form of the smaller band, although the latter hypothesis seems more plausible. Work in progress on the amino acid composition of CuBP will contribute to the resolution of this question, as will the planned synthesis of oligonucleotide probes for the *cbp* gene and generation of antisera specific to CuBP.

At present, CuBP can be identified by electrophoretic mobility on SDS-polyacrylamide gels, two-dimensional chromatographic behavior and its induction by copper. On the basis of these criteria, CuBP has been shown to be present in supernatants from copper-stressed chemostats as well as batch cultures. It has been shown to be absent in supernatants from 50 μM copper-stressed cultures of a copper-sensitive mutant (VA15S12) which was otherwise similar to the wild-type in all measured characteristics. When CuBP expression was altered from copper-induced to constitutive, the resultant mutants (Cu40B3) were copper-resistant compared to WT *V. alginolyticus*. The revertant derivative of Cu40B3 [Cu40B3(SW)] was no longer copper-resistant, and CuBP expression reverted to a copper-inducible characteristic.

These results support the hypothesis that CuBP is an important component in the response of *V. alginolyticus* to increased copper concentrations. CuBP expression is induced by copper concentrations as low as 1 μM, and the lower limit of copper required to induce the response has not been determined. Although low compared to the millimolar metal concentrations at which many plasmid-mediated metal detoxification systems are capable of operating, one μM Cu is comparatively

high with respect to normal ocean/estuarine copper concentrations (total copper = nanomolar; see Introduction). The system may have evolved to operate at much higher than normal copper levels due to transient surges in metal concentrations encountered under conditions such as upwelling, metal-rich waters. CuBP expression and survival of *V. alginolyticus* at high copper concentrations may also be an artifact of laboratory culture conditions, where bacterial numbers are high and dilution of copper-binding material is limited. In marine waters, bacterial numbers are several orders of magnitude lower than in laboratory broth cultures, populations are heterogeneous, and dilution is practically infinite. Therefore, the effect of a very low concentration of free copper in the ocean would have a relatively large effect on an individual bacterium, which would need to produce more CuBP per copper ion in order to reduce $[Cu^{2+}]$ to nontoxic levels.

The copper-resistant (Cu20A6, Cu40A1) and copper-sensitive (VA15S7, VA15S10) mutants isolated during the course of this study in which supernatant protein production was not apparently different from WT *Vibrio alginolyticus* indicate than both increased sensitivity and increased resistance to copper can result from different mutations. Respiratory systems, cell walls and DNA are all potential targets of mutation which, if altered, could result in greater or lesser sensitivity to copper than the wild-type. Like all thoughtful research, this investigation has raised many more questions than it has answered. Answers to the questions of whether copper is acting as a mutagen or purely as a selective agent, definition of the regulatory elements of *cbp* and its possible associated proteins, elucidation of the frequency and

distribution of *cbp*-like systems in populations of marine bacteria, and the structure of CuBP and its copper-binding regions are fertile areas for future research.

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