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Regulation of Extracellular Copper-Binding Proteins in Copper-Resistant and Copper-Sensitive Mutants of *Vibrio alginolyticus*

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Extracellular proteins of wild-type *Vibrio alginolyticus* were compared with those of copper-resistant and copper-sensitive mutants. One copper-resistant mutant (Cu40B3) constitutively produced an extracellular protein with the same apparent molecular mass (21 kDa) and chromatographic behavior as copper-binding protein (CuBP), a copper-induced supernatant protein which has been implicated in copper detoxification in wild-type *V. alginolyticus*. Copper-sensitive *V. alginolyticus* mutants displayed a range of alterations in supernatant protein profiles. CuBP was not detected in supernatants of one copper-sensitive mutant after cultures had been stressed with 50 μM copper. Increased resistance to copper was not induced by preincubation with subinhibitory levels of copper in the wild type or in the copper-resistant mutant Cu40B3. Copper-resistant mutants maintained the ability to grow on copper-amended agar after 10 or more subcultures on nonselective agar, demonstrating the stability of the phenotype. A derivative of Cu40B3 with wild-type sensitivity to copper which no longer constitutively expressed CuBP was isolated. The simultaneous loss of both constitutive CuBP production and copper resistance in Cu40B3 indicates that constitutive CuBP production is necessary for copper resistance in this mutant. These data support the hypothesis that the extracellular, ca. 20-kDa protein(s) of *V. alginolyticus* is an important factor in survival and growth of the organism at elevated copper concentrations. The range of phenotypes observed in copper-resistant and copper-sensitive *V. alginolyticus* indicate that altered sensitivity to copper was mediated by a variety of physiological changes.

Copper, a heavy metal whose anthropogenic input into natural waters has been increasing in recent years (10), is a highly toxic, relatively available element (4). The basis for copper toxicity lies in the reactivity of copper ions with cellular macromolecules and inorganic compounds, as it can act as an oxidant and also forms chelates with organic molecules, including DNA and proteins (14). The most thoroughly characterized bacterial copper detoxification systems are plasmid encoded. The *pco* genes encode the copper efflux system of *Escherichia coli* (11), and proteins encoded by the *cop* operon mediate extracellular sequestration of copper by extracellular and periplasmic proteins in *Pseudomonas syringae* (3). Copper-induced proteins play critical roles in both systems.

The evidence accumulated thus far for the mechanism of copper detoxification in *Vibrio alginolyticus* supports the hypothesis that an extracellular protein or proteins complex copper in culture supernatants of the organism (12). Copper-induced proteins with molecular masses of approximately 20 kDa (copper-binding proteins [CuBP]) are produced during the copper-induced lag phase in batch cultures, and they accumulate as cells resume growth in the presence of copper (7, 12). CuBP is currently identified on the basis of apparent molecular weight by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), induction by copper, affinity for copper (determined by immobilized metal ion affinity chromatography [IMAC]), and chromatographic behavior (determined by IMAC followed by reverse-phase high-performance liquid chromatography [HPLC]) (6). CuBP expression is also induced by copper in chemostat cultures of *V. alginolyticus* (6).

These results are consistent with a model for copper detoxification which includes involvement of a specific extracellular protein or proteins; however, they do not provide 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 detoxification system of *V. alginolyticus* would be strengthened by the demonstration of a correlation between CuBP production and growth or survival of the organism when copper levels are elevated. To that end, mutant *V. alginolyticus* strains with altered sensitivity to copper were isolated and 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.

MATERIALS AND METHODS

Bacterial strains. All mutants in this study were derived from *V. alginolyticus* ATCC 51160 (5), which was designated wild-type *V. alginolyticus*. The copper-resistant mutants Cu40B3 and Cu40A1 were isolated from a copper-stressed continuous culture (6). All other mutants were isolated during this study.

Culture conditions. Overnight broth cultures grown in M9 broth supplemented with 21 g of NaCl liter^{-1} (SWM9) and 2.5 μg of $FeCl_3$ liter^{-1} were used to inoculate 3-ml broth cultures in the same medium without iron. Cultures were incubated at 25°C on a shaker (100 rpm). Growth was monitored by measuring optical density at 595 nm. Copper ($CuSO_4$) was added to broth cultures in mid-log phase. Control (no-copper) cultures were harvested after 24 h. Copper-stressed cultures were harvested after 24 or 48 h.

During this study it was noted that CuBP was expressed at low levels in control cultures when the medium was prepared with deionized water but not when it was prepared with type 1 (Milli-Q; Millipore) water. Medium used in all subsequent studies was made with Type 1 water.

Electrophoresis. SDS-PAGE was carried out on 12% acryl-

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amide gels (8). Supernatants were concentrated to the same extent, i.e., 2 ml to 450 μl, and the same volume of each sample was loaded on gels. Proteins were visualized by silver stain and quantitated by densitometry (LKB Ultroscan XL laser densitometer). Molecular mass standards were bovine serum albumin, 66 kDa; egg albumin, 44 kDa; trypsinogen, 24 kDa; and lysozyme, 14.4 kDa.

Copper-resistant isolates. Spontaneous copper-resistant mutants of *V. alginolyticus* were isolated by plating overnight broth cultures on SWM9 or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-artificial seawater (HASW) plates amended with 20 to 40 μM $CuSO_4$. HASW was prepared with 8 g of Instant Ocean (Aquarium Systems, Mentor, Ohio), 13 g of NaCl, and 0.65 g of HEPES (pH 7.5) liter^{-1}, and the mixture was filtered through a 0.2-μm-pore-size filter to remove particulates. To prepare solid media, 15 g of Difco Bacto agar liter^{-1} was added to HASW, and the mixture was brought to a boil and autoclaved. Sterile glucose (28 mM), inorganic nutrients (Na_2HPO_4, 0.15 mM; NH_4Cl, 19 mM), and copper solutions were added after the medium was cooled. Glucose and inorganic nutrient stock solutions were sterilized by autoclaving, and copper was filter sterilized.

The stability of copper resistance in Cu40A1 and Cu40B3 was demonstrated by comparing the growth on copper-amended plates of recently copper-stressed cultures to those cultured repeatedly in the absence of copper. Broth cultures were inoculated from plates with or without 40 μM $CuSO_4$. Bacteria growing without copper had been subcultured 10 times on marine agar 2216. The Cu40A1 overnight culture was grown in SWM9 plus 50 μM Cu, while Cu40B3 was grown in unamended SWM9. Broth cultures for both treatments were incubated at room temperature on a shaker, serially diluted, and plated on HASW agar with and without 40 μM Cu. CFU were counted at room temperature after 4 days (no Cu) or 1 week (40 μM Cu).

CuBP expression in replicate control cultures of the wild type and three copper-resistant mutants (Cu20A6, Cu40A1, and Cu40B3) was analyzed in order to determine whether any of the mutants constitutively expressed CuBP. Three 3-ml broth cultures of each isolate were grown without copper as detailed above. The supernatant from each culture was collected, concentrated (see below), and analyzed by SDS-PAGE. Gels were silver stained and quantitated by densitometry. Paired *t* tests were used to compare the mean band densities of CuBP between isolates (values for which P was <0.05 were considered significantly different).

Inducibility of copper resistance. Copper resistance in wild-type *V. alginolyticus* and Cu40B3 was assessed on copper gradient plates by the method of Williams et al. (16). The strains were cultured in subinhibitory levels of copper (0, 0.5, 1.0, or 2.5 μM) in SWM9 medium by diluting 1 ml of overnight culture in 50 ml of SWM9 and then incubating with shaking for 7 h at room temperature. Cultures were sampled with a 3-mm-diameter inoculating loop, drawn in a straight line down the plate. Copper gradient plates were made by pouring 10 ml of HASW agar plus 40 μM $CuSO_4$ in an 87-mm-diameter petri dish. The agar was allowed to harden while inclined at an angle of approximately 5%. Ten milliliters of HASW agar amended with 10 μg of bromcresol purple ml^{-1} (BPHASW) was poured on top of the first layer while plates were level. Plates were inoculated within 2 h after they were poured and were incubated for 5 days at room temperature. Copper resistance was quantitated by measuring growth along the streak (in centimeters). This measurement was facilitated by the bromcresol purple-containing top layer of agar, which turned yellow where bacteria were growing.

Sample concentration and protein quantitation. Each culture was filtered through a 0.45-μm-pore-size filter, and the supernatant was retained. Supernatant proteins were concentrated approximately fivefold by centrifugation in microconcentrators (Centricon 3; Amicon) at 5,520 × g for 2 h. Supernatant protein was measured by the bicinchoninic acid assay (Pierce, Rockford, Ill.). CuBP concentrations (in micrograms per milliliter) were estimated by multiplying their contribution to total supernatant protein (obtained by densitometry) by the concentration of supernatant protein (in micrograms per milliliter).

Cells were collected for protein analysis by filtering 0.5 ml of culture onto a pretreated, 0.2-μm HT-200 filter (25-mm diameter; Gelman). The filters were pretreated by incubation at 90°C for 30 min in 1 N NaOH and were washed three times with distilled water. Samples were stored at −80°C in scintillation vials until analyzed.

Cells were digested by submerging filtered samples in 1 ml of 1 N NaOH and incubating them at 90°C for 30 min. The bicinchoninic acid assay was modified to quantitate protein in the digested samples. Ten microliters of concentrated HCl was added to each 100-μl sample and the blank to neutralize NaOH. Bovine serum albumin standards (25 to 150 μg · ml^{-1}) were diluted with an approximately equimolar solution of NaOH and HCl (10 ml of 1 N NaOH–1 ml of concentrated HCl). The level of background absorbance contributed by the digested filters was determined by measuring the absorbance of the assay blank from a digested filter preparation.

Chromatography. Supernatants of wild-type *V. alginolyticus* and Cu40B3 cultures were prepared by tangential flow filtration using a Pellicon cassette system (Millipore) with a 0.2-μm GVLP filter. A 175-ml portion of supernatant from 50 μM copper-stressed wild-type *V. alginolyticus* cultures or control Cu40B3 cultures was loaded onto an HR10/2 chelating Superose column charged with $CuSO_4$ for IMAC on a fast protein liquid chromatography system (6). IMAC fractions from the major peak were pooled and separated by reverse-phase HPLC using a Macrosphere C4 (150 by 4.6 mm; Alltech) column as previously described (6). HPLC fractions were analyzed on SDS–12% polyacrylamide gels.

Chemical mutagenesis. Nitrosoguanidine (NTG) mutagenesis in batch cultures of *V. alginolyticus* was performed essentially by the method of Adelberg et al. (1). Cultures (20 ml each) were grown in LB15 broth at 25°C on a shaker (100 rpm) to mid-log phase. A 10-ml portion of culture was filtered onto a 0.45-μm filter. The filter was washed twice with 10 ml of Tris-maleic acid buffer (1), and cells were resuspended in 20 ml of Tris-maleic acid buffer. NTG was added to a final concentration of 100 μg · ml^{-1}, and the culture was incubated for 30 min at room temperature on a shaker. Cells were filtered and washed twice with 10 ml of LB15 broth and incubated with shaking for 3 h in LB15 broth to allow phenotypic expression. Serial dilutions of the culture were spread on noninhibitory HASW plates and incubated overnight at room temperature.

Isolation of copper-sensitive mutants. Mutagenized cultures were screened for copper-sensitive mutants by using a filter transfer technique. HASW plates with 10 to 40 colonies from NTG-treated cultures were filter transferred to BPHASW-plus-15 μM $CuSO_4$ plates with 0.45-μm-pore-size, 85-mm-diameter nitrocellulose transfer membranes (NitroPlus; Micron Separations, Inc.). Plates were incubated at room temperature overnight. Putative Cu^s colonies remained purple because of decreased metabolic acid production and were transferred to marine agar plates for further study.

After one nonselective subculture, the copper sensitivity of the NTG isolates was confirmed on 15 μM copper-amended

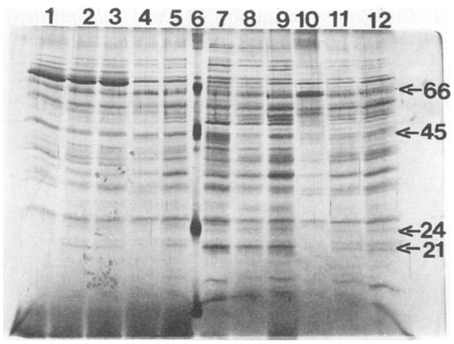

FIG. 1. Copper-resistant mutants. Shown are results of SDS-PAGE of supernatant proteins from control (no-copper) and 50 μM copper-challenged cultures harvested after 24 or 48 h. Lanes: 1, control wild-type *V. alginolyticus* (24 h); 2, copper-challenged *V. alginolyticus* (24 h); 3, copper-challenged *V. alginolyticus* (48 h); 4, control Cu20A6 (24 h); 5, copper-challenged Cu20A6 (48 h); 6, molecular weight standards; 7, control Cu40B3 (24 h); 8, copper-challenged Cu40B3 (24 h); 9, copper-challenged Cu40B3 (48 h); 10, control Cu40A1 (24 h); 11, copper-challenged Cu40A1 (24 h); 12, copper-challenged Cu40A1 (48 h). The apparent electrophoretic mobility of 21 kDa is marked.

plates. Isolates with significantly fewer and smaller colonies than the wild type after 4 days were designated Cu^s.

Supernatant protein profiles of Cu^s and Cu^r isolates. SDS-PAGE was used to analyze supernatant proteins from broth cultures which were filtered and concentrated as described above. SDS-PAGE was carried out on 12% acrylamide gels, which were silver stained (Rapid Ag Stain; ICN Radiochemicals). Proteins were quantitated by densitometry.

Phenotypic characterization. The following tests were carried out on cultures from marine agar 2216 plates: Gram stain, oxidase test (Pathotec cytochrome oxidase test strips; Organon Teknika Corp.), and API20E biochemical profile (Analytab). Colonies were suspended in 20 g of Instant Ocean $liter^{-1}$ for the API test (9) and incubated for 24 h at room temperature.

RESULTS

Copper-resistant isolates. No changes in the phenotypes of Cu^r isolates were detected by biochemical tests, with the exception of the mutation of Cu40A1 from an oxidase-positive to an oxidase-negative phenotype. Both Cu40A1 and Cu40B3 exhibited attenuated swarming motility. The frequency of spontaneous occurrence of colonies resistant to 20 μM copper was 1.2×10^{-7} in wild-type *V. alginolyticus*. The copper-resistant strain Cu20A6 was isolated as a spontaneous mutant.

The stability of the Cu^r phenotype in Cu40B3 and Cu40A1 was demonstrated by their ability to form colonies on 40 μM copper-amended plates after 10 passages on nonselective agar. Over half of the Cu40A1 cells could form colonies on copper-amended plates, whether they were cultured from copper-supplemented agar (frequency of Cu^r isolates, 8.6×10^{-1}) or after 10 passages on nonselective agar (5.4×10^{-1}). In contrast, the frequency of Cu^r Cu40B3 cells was several orders of magnitude lower than that of Cu^r Cu40A1 cells, whether cultures had recently been exposed to copper (2.4×10^{-4}) or not (4.7×10^{-4}). In replicate experiments, the mean frequency of resistance to 40 μM copper in Cu40B3 was 2.0×10^{-2} (n = 7), while that in the wild type was 6.9×10^{-9} (n = 5).

Supernatant proteins in control (no-copper) and 50 μM copper-stressed cultures of Cu^r mutants and the wild type were analyzed by SDS-PAGE (Fig. 1). While Cu20A6 (Fig. 1, lanes 4 and 5) and Cu40A1 (lanes 10 through 12) supernatant protein profiles were similar to those of the wild type, the control Cu40B3 supernatant (lane 7) contained a protein with the same relative electrophoretic mobility as CuBP, as did copper-challenged Cu40B3 supernatants (lanes 8 and 9).

TABLE 1. Measurements of supernatant protein in replicate batch cultures (no added copper) of wild-type *V. alginolyticus* and copper-resistant variants

Isolate	Total supernatant protein concn ($\mu g \cdot ml^{-1}$)	% CuBP[a]	CuBP concn ($\mu g \cdot ml^{-1}$)
Wild type	28.3	1.1	0.3
Cu40B3	24.1	14.7	3.5
Cu40A1	19.0	2.1	0.4
Cu20A6	30.7	1.4	0.4

[a] Calculated by densitometry of SDS-PAGE gels.

Constitutive expression of the 21-kDa protein in Cu40B3 was confirmed in replicate control cultures. The level of CuBP expression in Cu40B3 supernatants was significantly greater than in supernatants from the wild type or any of the other Cu^r mutants (Table 1). The 21-kDa protein represented 14.7% of the supernatant protein in Cu40B3 cultures but only 1.1% of supernatant protein in control wild-type cultures. In repeated experiments with replicate unchallenged cultures of Cu40B3, the 21-kDa protein made up a mean of 9.2% of the total supernatant protein. The total supernatant protein concentration was 24.1 $\mu g \cdot ml^{-1}$ in Cu40B3 cultures, compared with 28.3 $\mu g \cdot ml^{-1}$ in the wild type (Table 1); therefore, a generally increased supernatant protein concentration was not responsible for the relative copper resistance of Cu40B3.

The identity of the 21-kDa supernatant protein of Cu40B3 was established by IMAC followed by reverse-phase HPLC, as previously described (6). The chromatographic behavior of the CuBP-like protein purified from control Cu40B3 cultures was indistinguishable from that of CuBP purified from copper-stressed cultures of wild-type *V. alginolyticus*. The IMAC retention time for both was 11 to 13 min, with reverse-phase HPLC retention times of 26 to 28 min for the wild type (6) and 27.5 min for Cu40B3.

Induction of CuBP as a function of copper concentration was investigated by adding various concentrations of copper to wild-type and Cu40B3 cultures (Fig. 2; Table 2). CuBP was not detectable by densitometry in control wild-type cultures (Table

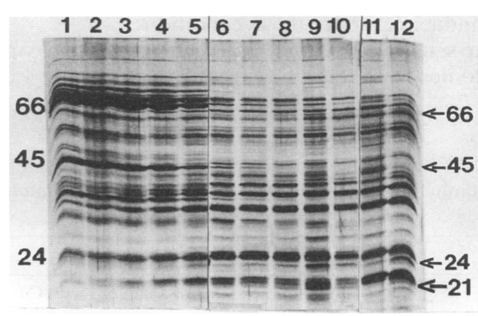

FIG. 2. Supernatant proteins from cultures of wild-type *V. alginolyticus* and the copper-resistant mutant Cu40B3 with increasing levels of copper. Lanes 1 through 5, wild-type *V. alginolyticus* with 0 (control), 1.0, 2.5, 5.0, and 10.0 μM Cu, respectively; lanes 6 through 12, Cu40B3 with 0, 1.0, 2.5, 5.0, 10.0, 0, and 50.0 μM Cu, respectively (lanes 6 and 11 contain supernatants from replicate control cultures).

TABLE 2. Comparison of [CuBP] and [Cu] in supernatants from broth cultures of wild-type *V. alginolyticus* and Cu40B3

V. alginolyticus culture and [Cu] (μM)	% CuBP[a]	[CuBP] (μg · ml⁻¹)	Normalized [CuBP][b]
Wild type			
0	ND[c]	ND	ND
1.0	1.2	0.3	1.5
2.5	1.3	0.4	1.7
5.0	1.9	0.6	2.4
10.0	3.0	1.0	3.7
50.0	5.0	1.7	10.3
Cu40B3			
0	4.5	0.9	3.6
1.0	4.2	0.7	3.3
2.5	3.8	0.8	4.7
5.0	9.0	2.2	14.1
10.0	5.7	1.1	6.6
50.0	5.1	0.8	5.5

[a] Contribution to total supernatant protein, calculated by densitometry of SDS-PAGE gels.
[b] Micrograms of CuBP per milligram of cell protein.
[c] ND, not detected by densitometry.

TABLE 4. CuBP in supernatants of Cu[s] *V. alginolyticus* mutants and wild-type *V. alginolyticus*

V. alginolyticus isolate	[Cu] (μM)	Concn of supernatant protein (μg · ml⁻¹)	% CuBP	[CuBP] (μg · ml⁻¹)
Wild type	0	26.5	ND[a]	ND
	15	24.4	0.7	0.2
	50	27.2	5.1	1.7
VA15S8	0	22.4	ND	ND
	50	21.4	2.2	0.5
VA15S9	0	15.2	ND	ND
	15	18.4	ND	ND
VA15S10	0	23.0	ND	ND
	15	23.6	1.6	0.3
	50	32.5	4.8	1.6
VA15S11	0	21.4	0.5	0.1
	15	21.2	0.3	0.1
VA15S12	0	16.6	ND	ND
	15	19.9	1.0	0.2
	50	23.8	ND	ND

[a] ND, not detected by densitometry.

2). CuBP expression was induced by 1 μM $CuSO_4$, the lowest concentration tested, and increased with increasing copper concentrations (Table 2). In contrast, control Cu40B3 supernatants contained CuBP levels comparable to those found in copper-stressed wild-type supernatants, with no clear trend as copper concentrations increased (Fig. 2; Table 2).

Loss of copper resistance in Cu40B3. A revertant to wild-type levels of copper sensitivity was isolated from a Cu40B3 culture after 15 nonselective subcultures. The revertant, Cu40B3(SW), also regained the ability to swarm on Marine agar. The frequency of Cu40B3(SW) cells able to form colonies on HASW plus 40 μM copper was 2.1×10^{-8} ($n = 4$).

CuBP was not detectable in control Cu40B3(SW) supernatants. Cu40B3(SW) supernatants from 50 μM copper-challenged cultures contained 3.4% CuBP, which is comparable to the percentage measured in wild-type *V. alginolyticus* (Table 2). Thus, Cu40B3(SW) appears to be a revertant, having changed from the observed Cu[r] nonswarming phenotype of Cu40B3 to a swarming phenotype with wild-type copper sensitivity.

Inducibility of copper resistance. Exposure of wild-type *V. alginolyticus* to subinhibitory levels of copper did not increase its resistance to the metal (Table 3). Cu40B3 displayed greater copper resistance than the wild type, and this resistance was also noninducible under these conditions.

Copper-sensitive mutants. Thirty-three putative copper-sensitive colonies were identified from approximately 1,000 colonies in the preliminary screening. Copper sensitivity in 6 of the 33 isolates, designated VA15S7 through VA15S12, was confirmed. Thus, 0.5% of the colonies recoverable after NTG treatments were relatively more sensitive to copper than the wild type.

TABLE 3. Lack of induction of copper resistance by preexposure to subinhibitory levels of copper in wild-type *V. alginolyticus* and copper-resistant Cu40B3

Strain	Resistance[a] in: Noninduced culture (0 μM Cu)	Cultures induced with Cu: 0.5 μM	1.0 μM	2.5 μM
Wild type	5.03 ± 0.14 (7)	4.90 ± 0.10 (3)	4.90 ± 0.14 (5)	4.90 ± 0.12 (6)
Cu40B3	6.03 ± 0.36 (7)	5.33 ± 0.06 (3)	6.04 ± 0.21 (7)	6.03 ± 0.28 (4)

[a] Resistance is expressed as growth (in centimeters) along a copper gradient. Numbers of replicates are in parentheses.

Typical cell yields varied between isolates; therefore, the possible contribution of total supernatant protein to copper resistance and sensitivity was assessed by normalizing the level of supernatant protein to the level of cell protein in control and copper-stressed cultures of copper-sensitive isolates, Cu40B3, and the wild-type. There was no significant difference between isolates in the supernatant protein/cell protein ratio in unstressed cultures and in copper-stressed cultures ($P < 0.05$; data not shown). Similarly, there was no significant difference in total supernatant protein concentration between copper-stressed mutant cultures and copper-stressed wild-type cultures (Table 4).

Results from SDS-PAGE of supernatant proteins from copper-sensitive mutants are presented in Table 4. Supernatants from VA15S7 (data not shown) and VA15S10 (Table 4) cultures were indistinguishable from those from the wild type. CuBP levels inVA15S8 were lower than those in the wild type but made up a significant percentage of total supernatant protein. No protein in the 20-kDa molecular mass range was detected in 15 μM copper-stressed supernatants of VA15S9. Although measured supernatant protein concentrations in this isolate were not significantly different from those obtained for the wild type, very little protein was resolved on gels. VA15S11 control supernatants contained a protein of the same molecular weight as CuBP which decreased in concentration with added copper. VA15S12 supernatants from cultures stressed with 15 μM copper contained approximately the same concentration of putative CuBP as the wild type, but when 50 μM copper was added, no ca. 20-kDa protein was detected in supernatants.

DISCUSSION

Copper-resistant and copper-sensitive mutants of *V. alginolyticus* were characterized with respect to CuBP production.

The copper-resistant mutant Cu40B3 was identified as a constitutive producer of CuBP. CuBP was always expressed in control Cu40B3 cultures; however, its concentration varied from culture to culture, as did the percentage of copper-resistant colonies per culture. The lowest CuBP concentrations measured in control Cu40B3 cultures were comparable to CuBP concentrations in 50 μM copper-stressed wild-type cultures.

The expression of CuBP was induced by as little as 1 μM $CuSO_4$ in wild-type *V. alginolyticus*. Copper concentrations higher than 2.5 μM inhibited growth in broth cultures, and cells could not form colonies on plates with 15 μM copper or more. While copper levels in this study were high compared with the nanomolar copper concentrations measured in offshore ocean waters (2), the sensitivity of *V. alginolyticus* to low micromolar copper concentrations and its lack of plasmids (unpublished data) are among the obvious differences between this system and previously described mechanisms of bacterial resistance to copper, which were plasmid mediated and allowed bacteria to tolerate millimolar copper concentrations (3, 11, 16).

The observation that preexposure to subinhibitory levels of copper fails to induce copper resistance in wild-type *V. alginolyticus* is consistent with the dose-dependent expression of CuBP and its extracellular location. Subinhibitory copper levels (1.0 and 2.5 μM) induced low levels of CuBP, which were two to nine times lower than CuBP levels in 50 μM copper-challenged-wild-type supernatants and in control Cu40 B3 supernatants. Substantially greater CuBP expression than that elicited by low micromolar copper levels is needed before increased resistance can be detected under the experimental conditions used here.

The supernatant protein profiles of some copper-sensitive mutants demonstrated altered levels of CuBP compared with that of the wild-type; however, none were unambiguously devoid of CuBP. VA15S12 cultures challenged with 15 μM copper were able to recover, and CuBP was expressed; however, no CuBP was detected in cultures challenged with 50 μM copper. Studies of the regulation of CuBP expression in this mutant will be undertaken.

Reversion of Cu40B3, a nonswarming, constitutive producer of CuBP, to the wild-type phenotype with respect to both copper-inducible CuBP expression and copper sensitivity [Cu40B3(SW)] indicates that constitutive CuBP production is required for the copper-resistant phenotype of Cu40B3. The correlation between swarming motility and reversion to wild-type regulation of CuBP has no obvious explanation. Pleiotropic alterations in phenotype, including changes in adhesiveness and outer membrane composition, were noted in nonluminescent variants of *Vibrio harveyi*, and reversible phenotypic switching from bright to dim is characteristic of the *V. harveyi* luminescence system (13). An investigation of the mechanism of phenotypic switching in these closely related *Vibrio* species could explain the link between swarming and constitutive CuBP expression.

Constitutive transcription of the metallothionein gene in *Saccharomyces cerevisiae* resulted in cadmium and copper resistance (15), indicating that constitutive production of metal binding proteins can increase resistance to copper. The existence of a copper-resistant mutant that constitutively expresses CuBP supports the hypothesis that this supernatant protein is an important factor in the response of *V. alginolyticus* to elevated copper levels. Studies in progress involve defining the CuBP-encoding genetic locus and associated regulatory region (*cbp*), which will further our understanding of the homeostatic regulation of copper in bacteria.

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