APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1993, p. 60–66
0099-2240/93/010060-07$02.00/0

Vol. 59, No. 1

Growth, Copper-Tolerant Cells, and Extracellular Protein Production in Copper-Stressed Chemostat Cultures of *Vibrio alginolyticus*

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Received 16 July 1992/Accepted 5 October 1992

The influence of elevated copper concentrations on cell numbers and extracellular protein production was investigated in chemostat cultures of *Vibrio alginolyticus*. High (20 μM) copper in the medium reservoir resulted in a dramatic drop in cell numbers which was overcome with time. The copper-stressed cultures established a new equilibrium cell concentration slightly (ca. 20%) lower than control cultures. Copper-stressed chemostat populations contained an increased number of copper-resistant cells, but these averaged only 26% of the copper-adapted population. Previously copper-stressed populations exhibited resistance to a second challenge with copper. Proteins with properties identical to those of copper-induced, copper-binding proteins (CuBPs) observed in batch cultures of *V. alginolyticus* were observed in the supernatants of copper-stressed chemostat cultures and not in controls. CuBPs from batch and chemostat cultures were identical in terms of their induction by copper, molecular weight, and retention volumes on both immobilized copper ion-affinity chromatography and reverse-phase high-performance liquid chromatography columns. The concentration of CuBP in the chemostat was dependent on copper concentration in the medium reservoir. Either one or two forms of CuBP were observed in various analyses from both batch and chemostat cultures. Gel-to-gel variability was implicated as a factor determining whether one or two forms were resolved in a given analysis. The data demonstrate that *V. alginolyticus* can recover from challenge by high copper levels in a chemostat when copper is continuously added, that resistance to copper is maintained by the population when copper stress is removed for many generations, and that recovery is accompanied by the production of the extracellular, CuBPs which have been described previously in batch cultures. That all individual cells surviving copper challenge do not exhibit increased copper tolerance indicates that some cooperative phenomenon, such as medium conditioning, is responsible for copper detoxification by the bacterial population.

Copper metabolism requires homeostatic physiological mechanisms which assure sufficient metal availability while regulating toxicity. When microorganisms encounter supraoptimal copper concentrations, detoxification is mediated by various mechanisms. These mechanisms include energy-dependent efflux (12), intracellular sequestration (3, 5), and extracellular complexation (9, 14). Of these mechanisms, the production of extracellular chelators is the most direct way to affect regulation of copper ion activity, which, in turn, determines the balance between toxicity and availability (for a recent review of the influence of metal speciation on microbial growth, see reference 11). Production of extracellular, organic copper chelators, however, is an energy-requiring process. Depending on their composition, chelator production may require a significant amount of the cellular energy budget. Presumably, this energy would be lost to the cell through chelator excretion. Thus, it is not clear whether detoxification mechanisms involving production of extracellular chelators would be effective in an "open" system, such as aquatic environments.

Vibrio alginolyticus, a marine bacterium, exhibits a copper concentration-dependent lag in growth when copper is added to exponentially growing batch cultures (6, 13). At copper concentrations up to at least 100 μM in minimal medium, this growth lag is temporary and the culture eventually resumes growth. The time required for recovery is dependent on the concentration of copper added (6). Schreiber et al. (13) demonstrated that copper-organic complexes (Cu-EDTA and Cu-nitrilotriacetic acid) are not toxic to *V. alginolyticus*. In another study (14) they showed that, as *V. alginolyticus* recovers from copper toxicity in seawater medium, copper in solution becomes organically complexed and is thereby detoxified. Copper was found to be complexed with organic material, presumably proteins, with molecular weights of ca. 28,000 as estimated by size exclusion chromatography (14). Label incorporation from [^{14}C]glucose demonstrated that the proteins were actively produced by the cells during recovery from copper toxicity (14). Harwood-Sears and Gordon (9) examined supernatant proteins in copper-challenged and control cultures of *V. alginolyticus* by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They demonstrated the production of 21- and 19-kDa extracellular proteins (copper-binding proteins [CuBPs]) in response to copper stress in batch cultures. The affinity of CuBPs for copper was demonstrated by potentiometric titration and immobilized copper ion-affinity chromatography (IMAC). These studies indicate that a ca. 20-kDa extracellular protein(s) produced in response to copper stress serves as a copper chelator, mediating recovery of *V. alginolyticus* from copper toxicity in batch cultures. CuBP from batch cultures has subsequently been characterized in terms of retention volume on reverse-phase high-performance liquid chromatography (RPHPLC) and IMAC columns (7, 8–10).

In the present study, we investigated the effect of copper on growth of *V. alginolyticus*, abundance of copper-resistant cells, and extracellular protein production in continuous

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culture. The experiments were designed to address the questions of whether *V. alginolyticus* is able to recover from copper toxicity in a situation in which copper is continuously added and whether CuBPs observed in batch cultures are also produced in copper-stressed continuous culture. The abundance of copper-resistant cells was examined to determine whether all surviving cells in a copper-adapted culture display increased copper resistance compared with cells in control cultures. This information can provide insights into the mechanisms by which the culture becomes copper resistant. In addition, an estimate of the energy cost of copper detoxification may be obtained by comparing equilibrium cell biomass in control and copper-stressed chemostat cultures providing that glucose is limiting in both cases.

MATERIALS AND METHODS

Chemostat culture. The effect of added copper on chemostat cultures of *V. alginolyticus* in artificial seawater medium was observed by monitoring the cell density in cultures with and without addition of micromolar levels of copper to the medium reservoir. *V. alginolyticus* was grown in a 1-liter chemostat (Bio-Flo III; New Brunswick Scientific) in artificial seawater medium composed of 20 g of Instant Ocean (Aquarium Systems, Mentor, Ohio) per liter, 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, 16, 20, or 32 μM. The chemostat pH was regulated at 7.5 with 0.2 N NaOH. The air flow was set at 1 liter/min, agitation was set at 200 rpm, and the temperature was 25°C. Experiments were performed at two dilution rates (D) in both control and copper-stressed cultures: 0.113/h (generation time [T_{gen}] = 6.1 h) or 0.253/h (T_{gen} = 2.7 h). Optical density was measured with a Klett-Summerson colorimeter. Absorbance on the meter was calibrated to cell numbers by acridine orange direct counts. The culture purity was monitored daily by streaking on tryptic soy agar and marine agar 2216 plates (Difco Laboratories). Both short-term (3 days) and long-term (up to 45 days) equilibration experiments were performed after copper addition to the medium reservoir. Cell suspensions for extracellular protein analysis were collected (0°C) overnight (ca. 2 liters) and separated from the medium by tangential flow filtration (0.2-μm-pore-size GVLP Millipore Pellicon cassette).

Batch culture. *V. alginolyticus* was grown in SWM9 medium (9) at room temperature on a shaker (100 rpm). Copper ($CuSO_4$) was added to exponentially growing cultures which had reached an optical density of 40 Klett units or approximately 2×10^8 cells per ml. Control cultures were harvested after 24 to 30 h, and copper-challenged cultures were harvested after they had recovered from the copper-induced growth lag (24 to 54 h). Cells and supernatant were separated by either tangential flow filtration or centrifugation followed by filtration (0.2-μm pore size; Gelman). In some experiments, the supernatant was subjected to molecular filtration (Amicon PM30) in a 250-ml stirred cell.

Supernatant protein analysis. Total supernatant proteins were examined in concentrates from batch and chemostat cultures by SDS-PAGE as described previously (9). In addition, supernatant proteins with affinity for copper were selectively concentrated from chemostat and batch cultures by IMAC and then further separated by RPHPLC, using methods shown previously to purify CuBP (7, 8–10) as follows: 175 ml of either the cell-free supernatant or the supernatant subjected to molecular filtration (Amicon PM30) was loaded (2 ml/min) onto an HR 10/2 chelating Superose column charged with 3 ml of 10 mM $CuSO_4$ operated on a fast protein liquid chromatography system (Pharmacia). The buffer system was (A) 0.05 M $NaCOOCH_3$–0.1 M NaCl, (pH 7.0) or (B) 10 mM glycine–0.1 M NaCl (pH 9.0). Supernatant proteins retained by the IMAC column were eluted with 100% buffer B.

Pooled fractions (10 ml) from replicate IMAC separations were injected onto either a Macrosphere C4 (Alltech 150 by 4.6 mm) or a ProRPC (Pharmacia HR 5/10) column on an ISCO HPLC after addition of 0.1% trifluoroacetic acid. The solvent system for RPHPLC was (i) 0.1% trifluoroacetic acid or (ii) 0.1% trifluoroacetic acid in CH_3CN. Elution was carried out by a 0 to 100% linear gradient of buffer B over 37 min at 1.0 ml/min for the Macrosphere column. The ProRPC column was run at 0.2 ml/min over 128 min. RPHPLC fractions from the Macrosphere column were collected (1.0 ml), frozen at −80°C, and lyophilized. Proteins were resuspended in phosphate buffer (5 g of NaCl, 2 g of $Na_2HPO_4 \cdot 7H_2O$ per liter, pH 7.5) for analysis by SDS-PAGE. Fractions (0.2 ml) from the ProRPC column were collected, stored at −20°C, purged with a stream of N_2 to remove acetonitrile, and then analyzed by SDS-PAGE. SDS-PAGE gels were run as described previously (9) or in a minigel system (Bio-Rad Mini Protean II) following the manufacturer's instructions for a discontinuous SDS–12% polyacrylamide gel.

Glucose determination in chemostat cultures. Glucose in the chemostat culture vessel was analyzed by removing cells from a subsample by filtration (0.2-μm pore size), followed by analysis of residual glucose in the supernatant with a glucose test kit based on glucose oxidase methodology (Glucose Liquicolor; Stanbio Laboratory). The instructions included with the kit were followed except that 0.1 ml of standard or unknown was added rather than the recommended 0.01 ml to increase the sensitivity of the assay.

Analysis of copper-resistant cells in chemostat cultures. Copper-resistant cells in the chemostat were isolated on artificial seawater medium buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma Chemical Co.) and supplemented with 20 μM $CuSO_4$ (HASW). The composition of the medium was as follows: Instant Ocean salts, 8 g/liter; agar (Difco Bacto-Agar), 15 g/liter; glucose, 28 mM; HEPES, 25 mM; NH_4Cl, 19 mM; Na_2HPO_4, 0.15 mM; pH 7.5.

Samples were collected aseptically from the chemostat with and without added copper. Serial dilutions of the samples were plated on HASW with 20 μM copper. A culturable cell count was obtained by plating the same sample on marine agar (Difco 2216). HASW plates with copper were counted after 5 to 7 days, and marine agar plates were counted after 24 and 48 h. Only colonies larger than 1 mm in 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 formed on marine agar.

RESULTS

Short-term copper exposure. When the medium reservoir was changed to copper-containing medium, cell numbers decreased (Fig. 1). In this experiment, cell numbers dropped

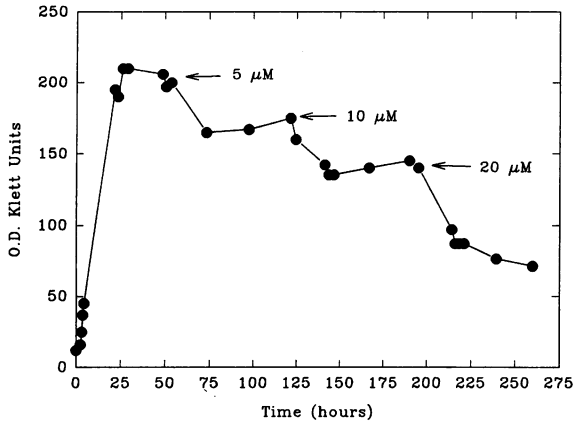

FIG. 1. Influence of copper concentration in the medium reservoir on bacterial concentration in the chemostat. In this experiment copper concentration was increased incrementally with short-term (ca. 3-day) equilibration time between increments. O.D., optical density.

from 9×10^8 to 7.5×10^8 cells per ml when 5 μM $CuSO_4$ was added to the reservoir (Fig. 1). After 2 additional days of exposure to 5 μM copper, the cell numbers had increased slightly. When a final concentration of 10 μM $CuSO_4$ was added to the reservoir, cell numbers dropped further, recovering slightly over the next 2 days. Increasing the total copper concentration to 20 μM caused cell numbers to decrease again.

Long-term copper exposure. In long-term experiments, 20 μM $CuSO_4$ was added to the reservoir of a chemostat culture which had been equilibrated previously in the absence of added copper (Fig. 2). Cell numbers dropped to 5.5×10^7 cells per ml over a period of 3 days and then began to recover. In replicate experiments at 20 μM copper (D = 0.113/h), the extent of the initial drop in cell numbers and the recovery time varied considerably. In one experiment recovery was not observed and washout occurred. In replicate experiments under these conditions, the equilibrium cell concentration in the absence of added copper was 7.7 ×

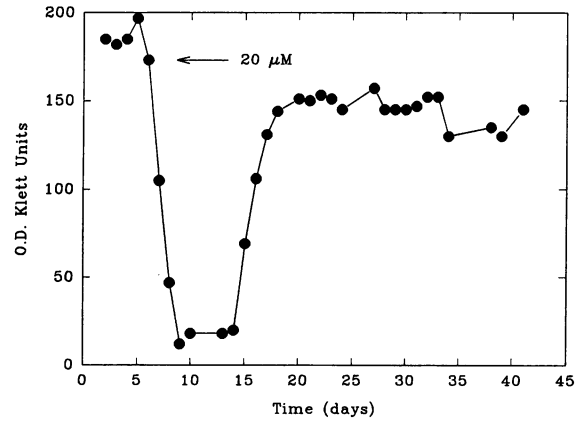

FIG. 2. Influence of copper concentration in the medium reservoir on bacterial concentration in the chemostat. In this experiment cells growing in the control medium were exposed to a high (20 μM) copper concentration and allowed to equilibrate for approximately 1 month. O.D., optical density.

TABLE 1. Influence of elevated copper concentration in the medium reservoir on cell concentration in the chemostat

Cell concn (10^8 ml^{-1})		[Cu] (μM)[a]	D (h^{-1})	T_{gen} (h)	n	% Reduction in cell concn
Control	Copper					
7.7 ± 0.6	6.2 ± 0.4	20	0.113	6.1	2	19
7.8 ± 0.1	6.5 ± 0.6	20	0.253	2.7	2	17
7.7	5.9	32	0.253	2.7	1	24

[a] Copper concentration in the reservoir under copper stress.

10^8/ml, and with 20 μM copper it was 6.2×10^8/ml (Table 1). At a higher dilution rate (D = 0.253/h), the response to copper was similar (Table 1). When copper concentration was increased to 32 μM, the number of cells dropped to 5.9×10^8/ml (Table 1).

Recovery time after 20 μM copper was added to the medium reservoir averaged 8 days at a dilution rate of 0.113/h. Recovery time was reduced to an average of 4 days if the flow was stopped after the cell density had dropped substantially following exposure to copper. In this case, when the flow was resumed after the cell concentration in the culture had returned to near-equilibrium values, cell numbers did not decrease significantly. When reservoirs were changed sequentially from control (days 1 to 7) to copper-containing medium (days 8 to 21) back to control (days 22 to 28) and then to copper-containing medium (days 29 to 32), no significant effect on cell density was observed on the second copper challenge.

Glucose concentration. Glucose concentration in the culture vessel was below the detection limit for the analysis used (>99% utilized) at equilibrium cell concentration both with and without 20 μM copper in the reservoir.

Characteristics of copper-stressed populations. A significant percentage of colonies on marine agar plates from equilibrated, copper-stressed cultures exhibited atypical colony morphology. These variants did not swarm readily and the colonies appeared more opaque. These variants were indistinguishable from the original *V. alginolyticus* strain by Gram stain, on tryptic soy agar plates, or by the API 20E identification system. When transferred repeatedly onto marine agar, some of these variants regained their ability to swarm. Direct observation of acridine orange-stained cells from equilibrated copper-stressed cultures revealed no significant difference in cell morphology or size in comparison to control cultures.

Equilibrated, copper-stressed populations in the chemostat had higher numbers of copper-resistant cells than control cultures. In the control, the ratio of colonies formed on marine agar to those formed on HASW with 20 μM copper was approximately 7×10^4:1 (0.0014%). After equilibration with 20 μM copper, copper-resistant cells averaged 26% of the total culturable count. The percentage of copper-resistant cells was quite variable in separate copper-challenged cultures, ranging from 0.03 to 58% of the total culturable count.

Supernatant protein production. (i) Analysis of whole supernatants. Concentrates of unfractionated supernatants from chemostat cultures prepared by the method of Harwood-Sears and Gordon (9) showed protein bands of 21 and 19 kDa in copper-challenged but not in control cultures (Fig. 5B, lanes labeled Cu and Con). When additional analyses of batch cultures were performed by using the same methods, either one or two predominant copper-induced bands in the 20-kDa range were observed when different cultures were

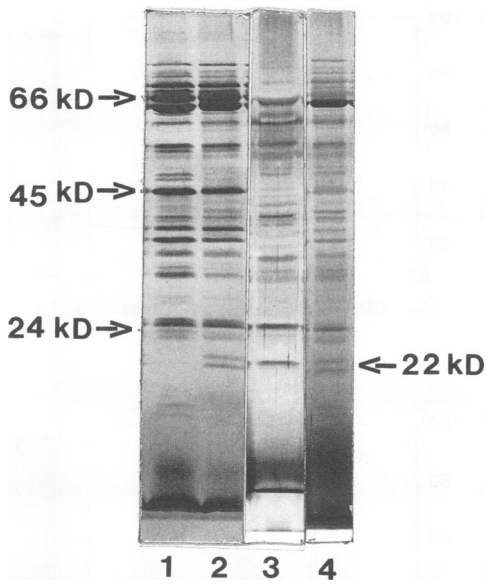

FIG. 3. Comparison of unfractionated supernatants from batch cultures illustrating how either one or two distinct ca. 20-kDa protein bands are resolved in copper-challenged (50 μM) cultures when cultures are analyzed on separate gels. Lanes: 1, control supernatant; 2 and 3, culture supernatant from separate cultures which had recovered after addition of 50 μM copper; 4, same supernatant as in lane 3 analyzed on a different gel.

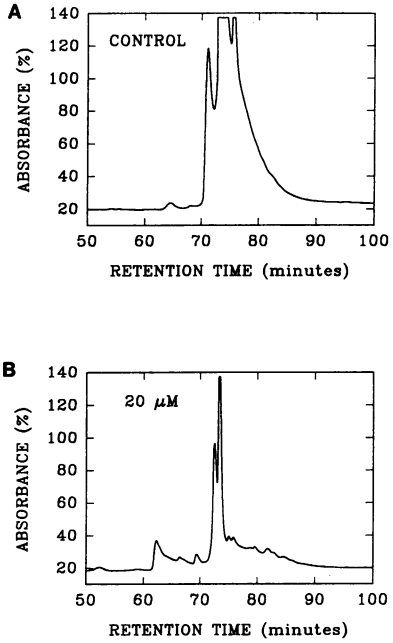

FIG. 4. Comparison of RPHPLC chromatograms of IMAC-concentrated proteins from control (A) and 20 μM copper-challenged (B) chemostat cultures (T_{gen} = 6.1 h). RPHPLC was performed with the ProRPC column as described in the text. Detector sensitivity was 1.0 absorbance unit full scale (215 nm).

analyzed or when the same sample was repeatedly analyzed on different gels (Fig. 3). The molecular weights of the ca. 20-kDa proteins (Fig. 3) calculated on the basis of the standards run on their respective gels are 22,000 (single band; lane 3) or 22,400 and 21,700 (two bands, lanes 2 and 4).

(ii) Analysis of supernatant proteins with affinity for immobilized copper. Analysis of IMAC-concentrated supernatant proteins from control and copper-stressed chemostat (D = 0.113/h; Cu = 20 μM) cultures using the ProRPC column showed a large amount of poorly resolved protein in the control culture (Fig. 4A). Total peak area was reduced in the copper-stressed culture (Fig. 4B). Two partially resolved peaks of retention time of 73 to 76 min were the major peaks observed (Fig. 4B). Fractions corresponding to peaks from these chromatograms were collected and analyzed by SDS-PAGE (Fig. 5). The dominant proteins in the HPLC fraction from the copper-stressed chemostat had molecular weights of 21,000 and 19,000 (Fig. 5B, lanes 73 to 76). Bands of this molecular weight were not observed in corresponding fractions from the control chromatogram (Fig. 5A). The major proteins observed had molecular weights of 56,000 and 52,000 (Fig. 5A, lanes 74 to 75).

The modified analytical scheme for copper-binding supernatant proteins (IMAC fractions) from the chemostat using the Macrosphere column showed a single, major protein peak in copper-stressed cultures with a retention time of ca. 26.5 min and a molecular weight of 22,600 as estimated by SDS-PAGE (Fig. 6B). Again, relatively large peaks with retention times similar to those from copper-stressed cultures were observed in control cultures (Fig. 6A). Analysis of fractions from control chromatograms by SDS-PAGE showed no detectable protein in the 20-kDa range. The major bands detected had molecular weights of 60,000 and 55,000 (Fig. 6A).

When the dilution rate of the culture was increased to 0.253/h, a 22.6-kDa protein was observed in copper-stressed cultures at 16 or 32 μM copper (Fig. 7B and C). The area of the peak which contained the protein was eightfold larger when the medium reservoir contained 32 μM copper than when it contained 16 μM copper. At the higher dilution rate, much less protein was detected in the control than in the control from the lower dilution rate as judged by either area of the chromatographic peaks or SDS-PAGE of the fractions (Fig. 7A and 6A).

Each analytical scheme involving IMAC and either RPHPLC column showed the ca. 20-kDa supernatant proteins with affinity for immobilized copper in batch and chemostat cultures to be indistinguishable (Table 2). Two CuBP forms were not detected when we used the alternate analytical protocol. However, the same results were obtained with batch cultures. Somewhat better separation between the 21- to 23- and 19- to 22-kDa proteins in batch cultures compared with chemostat cultures was observed with the ProRPC column. This is because of the decreased resolution of the ProRPC column with extended use under these conditions.

DISCUSSION

These experiments clearly demonstrate that *V. alginolyticus* can recover from copper toxicity in continuous culture and that extracellular CuBPs similar to those observed in batch cultures are produced in the chemostat during copper stress. When cultures recovered from 20 μM copper challenge, the equilibrium cell concentration was approximately 20% less than that of the control. Since cell size was not significantly altered in the presence of copper, the culture biomass is proportional to the cell number. Since the culture was glucose limited (glucose concentration below detection)

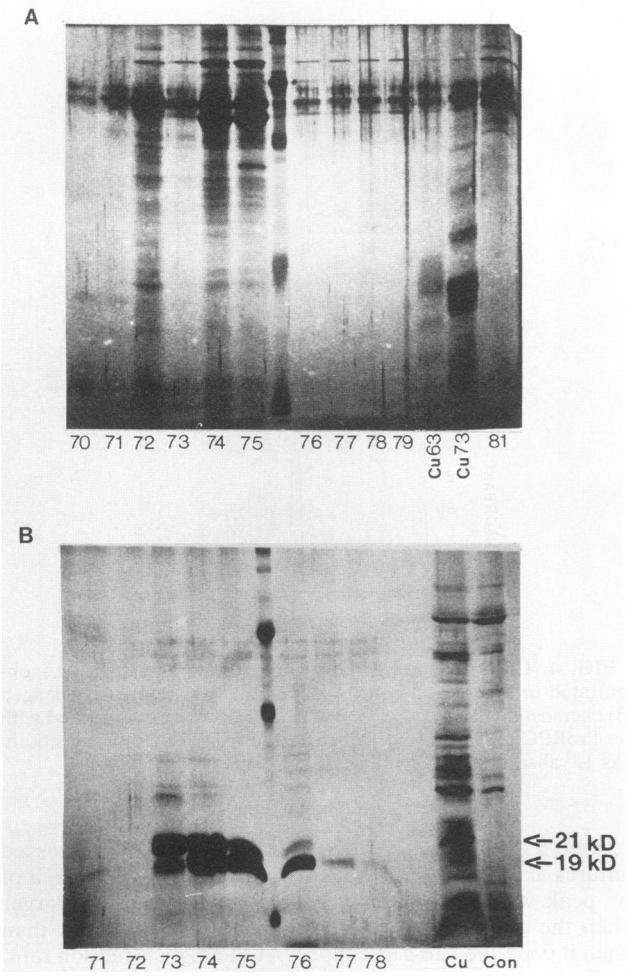

FIG. 5. SDS-PAGE of fractions from the chromatograms shown in Fig. 4. (A) Lanes 70 to 79 and 81 are from the control chromatogram (Fig. 4A); Cu63 and Cu73 are fractions from the chromatogram of the copper-challenged culture supernatant (Fig. 4B). (B) Lanes 71 to 78 are fractions from the chromatogram of the copper-challenged culture; Cu and Con are unfractionated supernatants of copper-challenged and control chemostat cultures, respectively, shown for comparison. Lane numbers correspond to the approximate retention time of the fractions as collected from the chromatograms shown in Fig. 4.

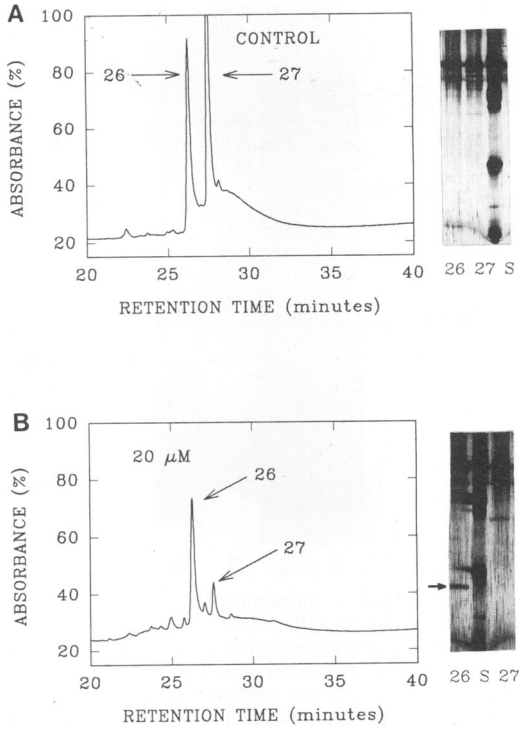

FIG. 6. Supernatant proteins from control (A) and copper-stressed (B) chemostat cultures at a slow (T_{gen} = 6.1 h) growth rate. The RPHPLC protocol utilizing the Macrosphere column was used (see text for details). Both RPHPLC chromatograms and lanes from electrophoretic gels of fractions corresponding to peaks in the chromatograms are shown. Numbers at the arrows and at the bottom of the gel lanes refer to corresponding fraction numbers and approximate retention times. Lanes labeled S contain protein standards. The arrow by the gels in panel B shows the location of the 22.6-kDa, copper-induced protein. Detector sensitivity was 1.0 absorbance unit full scale (215 nm).

in both cases, the growth yield (Y) equals culture biomass divided by reservoir substrate concentration (4). Thus, growth yield is reduced in the presence of 20 μM copper in the reservoir by approximately 20%. This difference in growth yield suggests that 20% of the cellular energy budget is directed toward physiological processes required for copper detoxification. At higher copper concentrations, this cost increases, as demonstrated by a lowered equilibrium cell concentration in the chemostat with 32 μM copper.

The characteristics of the bacterial cells in copper-stressed cultures at equilibrium were changed in comparison to those in the chemostat before copper addition. The reversible loss of swarming motility may be related to copper interference with induction of lateral flagella, which are required for swarming motility in *Vibrio* spp. (2, 15).

Adaptation of the population to copper was evidenced by an increase in numbers of colonies formed on copper-containing plates. These copper-resistant variants, however, made up only a fraction of the population even in copper-adapted cultures. Adaptation of the populations to copper was also apparent from results of experiments in which cultures were challenged a second time with copper after either recovery in batch mode or interchange of medium reservoirs with and without copper. Alleviation of copper stress would have occurred because of dilution of copper when the copper-containing reservoir was replaced with control medium. In this case, when copper was again added, the lack of a drop in cell numbers could have been due to an abundance of copper-resistant cells in the culture, continued accumulation of extracellular chelators in the absence of copper stress, or both. If chelator production continued, these would have been available to complex and detoxify copper when it was reintroduced to the system. In experiments in which the flow was stopped and cells recovered from copper stress in a batch mode, complexation of copper in the culture supernatant by accumulated extracellular proteins would alleviate copper toxicity (13). However, unless the cells had become resistant or excess complexation capacity was present, cell numbers would have dropped when the flow of the copper-containing medium was resumed. Therefore, the observed selection of copper-resistant cells during long-term exposure to copper in the chemo-

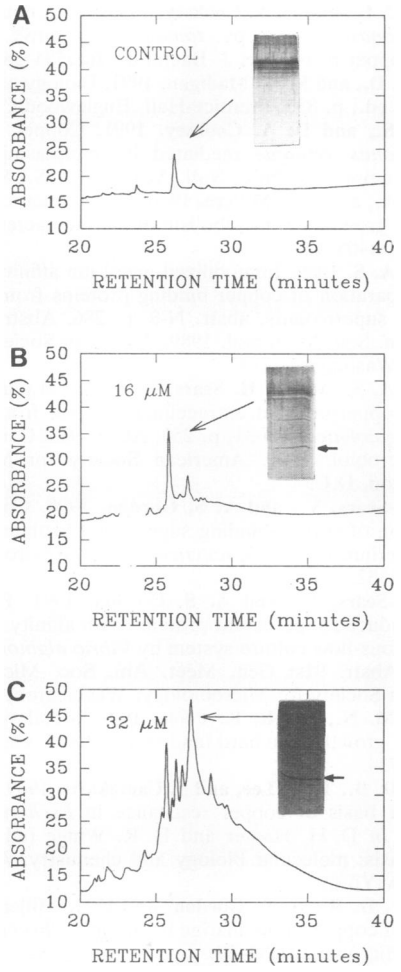

FIG. 7. Supernatant proteins from control and copper-stressed chemostat cultures at a fast (T_{gen} = 2.7 h) growth rate. Both RPHPLC chromatograms (Macrosphere column) and lanes from electrophoretic gels of fractions corresponding to the major peak in the chromatograms are shown. Long arrows indicate the peaks from which material on the gel originated. Short arrows show the location of the 22.6-kDa, copper-induced protein. Detector sensitivity was 0.5 absorbance unit full scale (215 nm).

stat and/or continued production of copper chelators after copper stress had been removed from populations of normal cells may be responsible for the copper resistance developed by *V. alginolyticus* populations in the chemostat. Since only a fraction of the population was found to be copper resistant even after long-term copper exposure, it is likely that both of these mechanisms come into play.

The percentage of copper-resistant cells observed in separate chemostat cultures was quite variable. This would be expected if a mutation were required for a cell to become copper resistant. In this case the number of copper-resistant cells would be a function of the number of generations since the mutation occurred and the relative growth rate of copper-resistant and normal cells in the chemostat.

Examination of extracellular proteins in copper-challenged chemostat supernatants showed accumulation of 21- and 19-kDa proteins which were not detectable in control chemostat supernatants when supernatants were prepared for SDS-PAGE by methods identical to those used by

TABLE 2. Comparison of ca. 20-kDa copper-induced supernatant protein concentrated by IMAC from copper-challenged chemostat and batch cultures of *V. alginolyticus* by two different analytical protocols

Analytical protocol	Culture condition	Retention time (min)		Mol wt (10^3)
		IMAC	RPHPLC	
A[a]	Batch	12–13	73–75; 78–80	21–23; 19–22
	Chemostat	12–13	73–75; 73–76	21–23; 19–22
B[b]	Batch	12–13	26–28	22–23
	Chemostat	12–13	26–28	22–23

[a] Samples were analyzed by using the ProRPC column. They were subjected to molecular filtration (Amicon PM30) and concentrated by IMAC before being injected. Acetonitrile was removed from the fractions with a stream of N_2, and the samples were stored at −20°C until analysis by SDS-PAGE for determination of the molecular weights of proteins in the HPLC fractions. Two CuBP forms were generally observed (see also Fig. 5B).

[b] Samples were analyzed by using the Macrosphere column. They were not subjected to molecular filtration before being concentrated by IMAC. Fractions were frozen at −80°C immediately after collection. Frozen fractions were lyophilized and resuspended in phosphate buffer for analysis by SDS-PAGE. Only one form of CuBP was detected (see also Fig. 6 and 7).

Harwood-Sears and Gordon (9). In that study, 21- and 19-kDa proteins (CuBP1 and CuBP2) were induced in batch culture supernatants of *V. alginolyticus* after exposure to toxic levels of copper. By using modified analytical protocols, only one copper-induced, copper-binding supernatant protein was observed. On further investigation of batch cultures, it was found that whether one or two CuBP forms were resolved is a function of gel-to-gel variability since the same sample produced either one or two ca. 20-kDa bands when analyzed repeatedly on separate gels. It is also possible that the modified analytical protocol using the Macrosphere column, although preferable in terms of column lifetime and time required for the analysis, does not purify the second form.

By either analytical scheme, the ca. 20-kDa, copper-induced proteins observed in batch versus chemostat culture were indistinguishable on the basis of their molecular weights and chromatographic properties, including their affinity for copper during IMAC. Whether one or two CuBP forms are observed in a given analytical scheme, it is clear that *V. alginolyticus* produces copper-induced, copper-binding supernatant proteins in chemostat culture similar to those produced in batch culture.

Supernatant proteins isolated from control cultures varied with dilution rate. At the slow growth rate (T_{gen} = 6.1 h), a large amount of material was isolated by the IMAC column from the supernatants as judged from the size of the peaks on HPLC. SDS-PAGE gels of these fractions revealed some material of relatively high molecular weight; other components may not have been resolved on the 12% gels. At the faster growth rate, the peak area in the control was greatly reduced. Again, only high-molecular-weight material was resolved on the gels. These data suggest a shift toward extracellular protein production at low growth rates. It has been reported that *Vibrio* spp. produce proteases in response to starvation (1) and that *Bacillus* spp. produce proteases in response to amino acid deprivation (17). It is possible, then, that at low growth rates the cells induce synthesis and excretion of extracellular proteases in response to depleted intracellular pools of amino acids.

On the basis of analysis of IMAC fractions, there was an apparent shift from production of the high-molecular-weight

proteins observed in control cultures to production of the ca. 20-kDa proteins in copper-stressed chemostat cultures. It has been reported that thermal stress can reduce production of extracellular proteases in *Bacillus* spp., presumably due to increased intracellular amino acid pools resulting from recycling of damaged protein (16). If the high-molecular-weight proteins observed in IMAC fractions from control cultures are, indeed, proteases, a similar mechanism may explain their decreased levels observed in IMAC fractions from copper-stressed cultures. Alternatively, the absence of these high-molecular-weight proteins in IMAC fractions from copper-stressed cultures may be due to competition for available binding sites on the IMAC column between CuBP (not present in control cultures) and other proteins present in both control and copper-challenged cultures. In this case CuBP, having a higher affinity for the immobilized copper, would displace proteins which bind to the column from the control supernatants.

The production of extracellular, copper-stress-related protein in both batch and continuous cultures supports the hypothesis that the proteins are an essential component of the physiological mechanisms mediating recovery from copper toxicity. The occurrence of the proteins in continuous cultures, which arguably mimic conditions that a bacterium might encounter in the environment better than batch culture, suggests that production of extracellular protein may occur in response to copper stress in marine environments where elevated copper concentrations occur. However, the cell concentrations and copper concentrations used in these experiments are clearly not representative of marine habitats.

The observation that the number of copper-resistant cells made up only a fraction of the population in copper-stressed chemostat cultures at equilibrium indicates that survival of the cells under these conditions was not accompanied by increased inherent copper tolerance in all surviving cells. This is consistent with the hypothesis that medium conditioning through production of extracellular chelators is a primary mechanism of copper detoxification in *V. alginolyticus* cultures.

ACKNOWLEDGMENTS

This work was supported by the Thomas F. and Kate Miller Jeffress Memorial Trust and by the National Institutes of Health (1R15 GM44101-01A1).

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