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Responses of Diverse Heterotrophic Bacteria to Elevated Copper Concentrations

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Responses of diverse heterotrophic bacteria to elevated copper concentrations

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The influence of copper on the growth of *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis, Vibrio parahaemolyticus, Vibrio alginolyticus* (three strains), and an unidentified *Vibrio* sp. was examined in batch cultures. The effects of copper at micromolar concentrations varied from undetectable to complete growth inhibition. Each strain was able to recover from a growth lag observed after copper addition at a characteristic concentration. Copper concentrations that allowed recovery ranged from 25 to 150 μM. Extracellular proteins in the medium of cultures that had recovered from copper stress were compared with those from control cultures. Protein profiles were analyzed for the presence of proteins similar to extracellular copper-binding proteins (CuBP) previously reported in *V. alginolyticus*. CuBP-like proteins were found in each *Vibrio* sp. examined. A protein of similar molecular mass was also detected in copper-stressed cultures of *P. aeruginosa* and not in control cultures. *Escherichia coli* and *Bacillus* spp. did not produce CuBP-like proteins. The data show that CuBP-like proteins are not produced by all bacteria in response to copper stress and indicate that such proteins are common in marine *Vibrio* spp.

Key words: copper toxicity, heterotrophic bacteria, extracellular protein.

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Nous avons étudié l'influence de la présence de cuivre dans les cultures en batch d'*Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis, Vibrio parahaemolyticus, Vibrio alginolyticus* (trois souches) et d'une espèce non-identifiée de *Vibrio* sp. L'effet de concentrations micromolaires de cuivre allait de non-détectable à une inhibition complète de la croissance. Chaque souche était cependant capable de redémarrer après une période de latence conséquente à l'addition de cuivre à une concentration précise. Les concentrations de cuivre permettant cette récupération allaient de 25 à 150 μM. Les protéines extracellulaires retrouvées dans le milieu de culture des souches qui avaient récupéré du stress par le cuivre ont été comparées à celles des cultures témoins. Parmi ces protéines, nous avons recherché la présence de protéines semblables aux protéines extracellulaires liant le cuivre (CuBP) connues chez *V. alginolyticus*. Des protéines de type CuBP ont été retrouvées chez chaque *Vibrio* sp. Une protéine de même masse moléculaire a aussi été détectée dans des cultures de *P. aeruginosa* stressées par le cuivre mais pas dans les cultures témoins. *Escherichia coli* et *Bacillus* spp. ne produisaient pas de protéines CuBP. Les résultats démontrent que les bactéries ne produisent pas toutes des protéines de type CuBP en réponse au stress par le cuivre et que ces protéines sont fréquentes chez les *Vibrio* spp. marins.

Mots clés : toxicité du cuivre, bactérie hétérotrophe, protéine extracellulaire.

[Traduit par la Rédaction]

The marine bacterium *Vibrio alginolyticus* ATCC 51160 has been shown to respond to copper stress by producing extracellular copper binding proteins (CuBP) that complex and detoxify copper in the growth medium (Harwood-Sears and Gordon 1990; Gordon et al. 1993; Schreiber et al. 1990). While diverse mechanisms for copper detoxification exist in bacteria, this novel mechanism of copper detoxification has, to date, only been reported in this strain of *V. alginolyticus*. If widespread among bacteria, production of such extracellular metal complexing proteins could profoundly influence metal speciation and thereby affect bioavailability in habitats where this process occurs (Capone and Bauer 1992).

Several classes of metal binding proteins or peptides exist that exhibit highly conserved structural attributes including characteristic amino acid sequences and sizes. A notable example is metallothionein, which has been reported in many divergent phyla (Kagi and Nordberg 1979; Hamer 1986; Shaw et al. 1992). Phytochelatins are also widely distributed in plants and microorganisms (Hayasi and Winge 1992). Whether such widespread distribution also occurs for CuBP-like proteins is unknown.

CuBP is identified on the basis of its induction by copper, presence in culture supernatants, apparent molecular weight, and retention volume in both immobilized copper ion affinity chromatography (Cu-IMAC) and reverse-phase high-performance liquid chromatography(HPLC) (Gordon et al. 1993). It is therefore possible to examine supernatants of bacterial cultures in the presence and absence of an elevated copper concentration to determine if CuBP-like proteins are produced by bacteria other than *V. alginolyticus* ATCC 51160.

In this study we examined copper sensitivity and extracellular proteins in control and copper-stressed cultures of two additional strains of *V. alginolyticus*, two other *Vibrio* species, and members of three additional bacterial genera from diverse habitats to determine whether bacteria other than *V. alginolyticus* (ATCC 51160) produce CuBP-like proteins when copper stressed.

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from American Type Culture Collection. *Bacillus cereus* 15-4872 was obtained from Carolina Biological Supply and *Bacillus subtilis* NSC 8/82 from the Old

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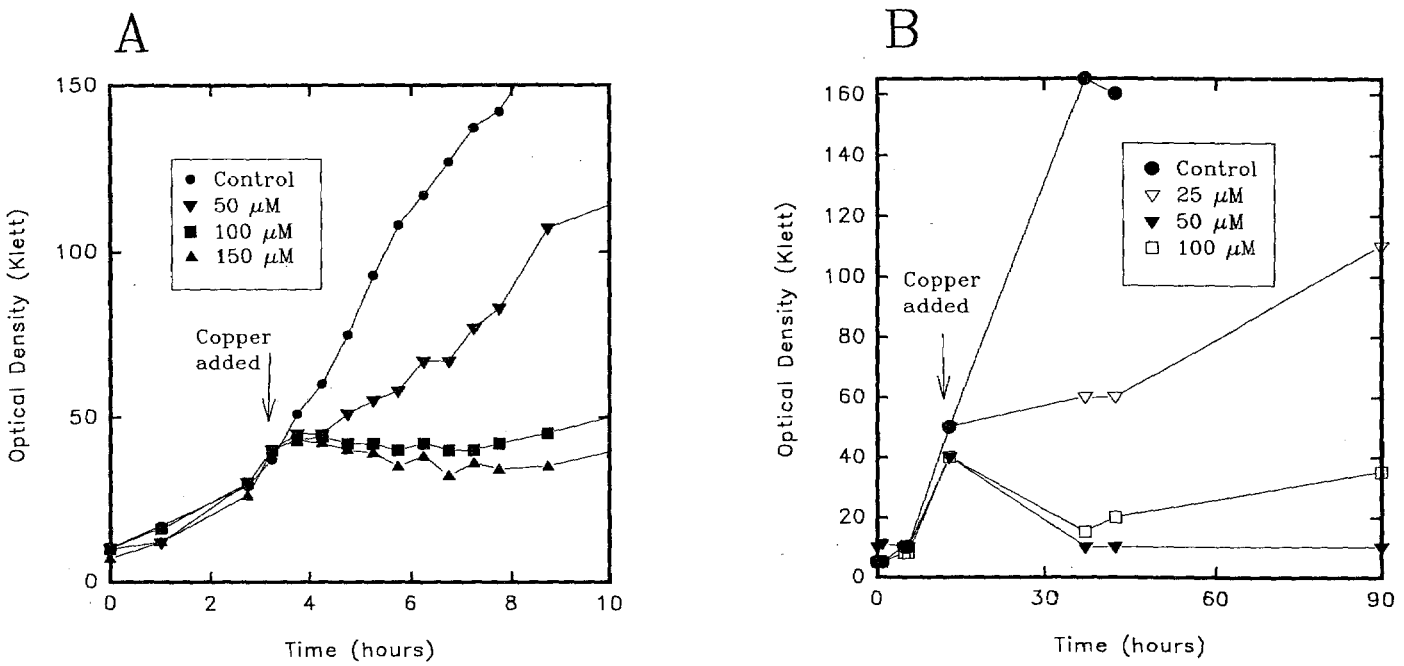

FIG. 1. Comparison of the responses of *Escherichia coli* (A) and *Bacillus cereus* (B) to copper at various concentrations. Copper was added to growing cultures that had reached equivalent cell densities.

Dominion University culture collection. *Vibrio* strains were as follows: *V. alginolyticus* ATCC 51160; *V. alginolyticus* isolated from Chesapeake Bay water in the vicinity of the Chesapeake light tower, coastal Virginia; *V. alginolyticus* isolated from sediments collected near Little Creek Amphibious Base, Virginia Beach, Virginia; *Vibrio parahaemolyticus* BB22, obtained from Dr. Robert Belas; and an unidentified *Vibrio* sp. (S141) obtained from Dr. J. Ostling. Original isolates were identified with API 20E test kits, using marine salts as a diluent (MacDonell et al. 1982).

Vibrio spp. were cultivated in minimal medium as described previously for *V. alginolyticus* (Harwood-Sears and Gordon 1990). The medium was modified to accommodate growth requirements of the other bacteria. The NaCl concentration was reduced to 8.6 mM for *Bacillus* spp., *E. coli,* and *P. aeruginosa* strains and the glucose concentration was increased to 28 mM for *Bacillus* spp.

Copper ($CuSO_4$) was added to exponentially growing cultures in mid-log phase (40 Klett units) as previously described (Harwood-Sears and Gordon 1990). Cell densities were determined as absorbance measured in a Klett-Summerson colorimeter (Klett units) before and after copper addition. Growth rate (k) was calculated from optical density (X) versus time (t) ($k = 3.3(\log X - \log X_0)/t$) from growth curves both before and after copper addition. Data points for calculation of growth rates after copper addition were taken after growth had resumed in cases in which a growth lag occurred after copper addition. Growth curves were used to determine a copper concentration for each bacterial strain that inhibited growth but allowed the cultures to attain a density of at least 100 Klett units (ca. 5×10^8 cells/mL) after a 24–48 h period. Supernatant protein profiles in control and copper-challenged cultures were analyzed by SDS-PAGE as previously described (Harwood-Sears and Gordon 1990).

Supernatants of *V. parahaemolyticus* were separated by Cu-IMAC and reverse-phase HPLC as described by Gordon et al. (1993) for comparison of the chromatographic properties of CuBP-like proteins from *V. parahaemolyticus* with those of CuBP from *V. alginolyticus*.

Growth curves were established for each bacterial strain over a range of copper concentrations. Examples of such growth curves for *E. coli* and *B. cereus* are shown in Fig. 1. In each case, copper addition resulted in an inhibition of growth that was overcome with time at concentrations in the micromolar range (Fig. 1). The most sensitive species was *B. subtilis,* which recovered from a 10 μM but not a 20 μM copper challenge, and the least sensitive was *P. aeruginosa,* which was minimally affected by copper additions up to 150 μM. The growth curves of the species that exhibited the capacity to recover from a 50 μM copper challenge were compared (Fig. 2). The growth response to copper at this concentration ranged from little change in growth rate after copper addition (*P. aeruginosa*) to temporary cessation of growth (*V. alginolyticus*). Slight differences were observed between the different strains of *V. alginolyticus,* with the sediment isolate exhibiting more rapid recovery after addition of copper to the culture.

The growth rates of the cultures after recovery from the initial inhibition by copper were reduced in comparison to the rates before copper addition (Table 1; Fig. 2).

Examination of SDS-PAGE gels of concentrated supernatant proteins in the region corresponding to a molecular mass of 15–25 kDa revealed CuBP-like proteins in each copper-stressed *Vibrio* culture (Table 2). These proteins were absent in control cultures. Similar proteins were not observed in copper-stressed, recovered cultures of *E. coli*, *B. cereus*, or *B. subtilis*. A protein with a molecular mass similar to those observed in the *Vibrio* cultures was detected in copper-stressed, recovered cultures of *P. aeruginosa* (Table 2).

Supernatant proteins from copper-challenged (100 μM) *V. parahaemolyticus* were separated by Cu-IMAC and reverse-phase HPLC to determine whether these proteins exhibited chromatographic properties similar to those of CuBP. Cu-IMAC

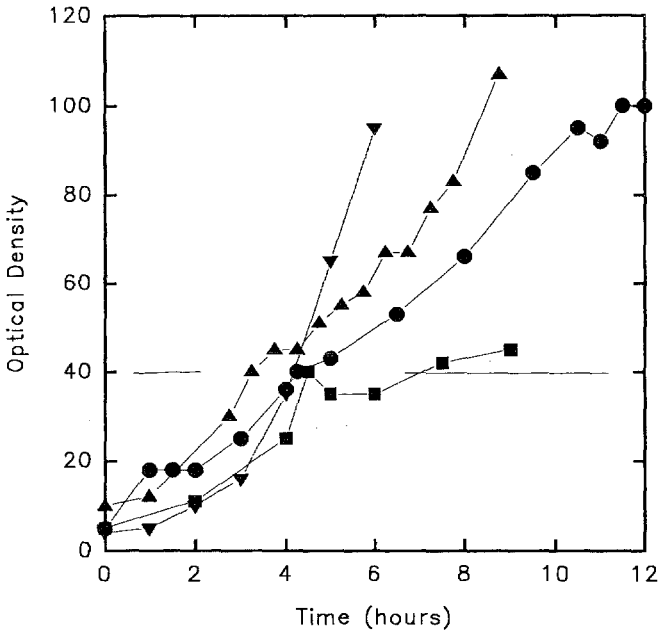

FIG. 2. Influence of 50 μM copper on growth of four species of Gram-negative bacteria. Copper ($CuSO_4$) was added to growing cultures when they had reached equal optical density (40 Klett units; horizontal line). ▼, *P. aeruginosa*; ▲, *E. coli*; ●, *V. parahaemolyticus*; ■, *V. alginolyticus*.

TABLE 1. Comparison of growth rates (generations/h) before copper addition and after growth resumed following copper addition in cultures of bacterial strains shown in Fig. 2

	Growth before Cu	Growth after recovery
E. coli	0.7	0.3
P. aeruginosa	0.7	0.5
V. parahaemolyticus	0.4	0.2
V. alginolyticus	0.6	0.1

NOTE: Copper was added to each culture at a final concentration of 50 μM when cells were in log phase and at equal cell concentrations.

TABLE 2. Comparison of extracellular proteins from culture supernatants of a variety of bacteria with CuBP from *Vibrio alginolyticus* ATCC 51160

	Molecular mass (kDa)	Cu induction
V. alginolyticus ATCC 51160	22.7	Yes
V. alginolyticus (CLT)	22.6	Yes
V. alginolyticus (LC)	22.8	Yes
Vibrio sp. strain S141	22.5	Yes
V. parahaemolyticus	22.6	Yes
E. coli	20.4	No
P. aeruginosa	23.9	Yes
B. cereus	nd	nd
B. subtilis	nd	nd

NOTE: Proteins with molecular mass similar to CuBP (in the range of 15–25 kDa) were examined on SDS-PAGE gels. Copper induction was determined by comparing protein profiles in control and copper-stressed, recovered cultures of each bacterium. CLT, Chesapeake light tower; LC, Little Creek; nd, none detected.

separations contained one major peak with the same retention volume as CuBP from *V. alginolyticus* (12–13 mL after initiation of 10 mM glycine elution; Gordon et al. 1993). SDS-PAGE of aliquots of fractions corresponding to this major peak contained

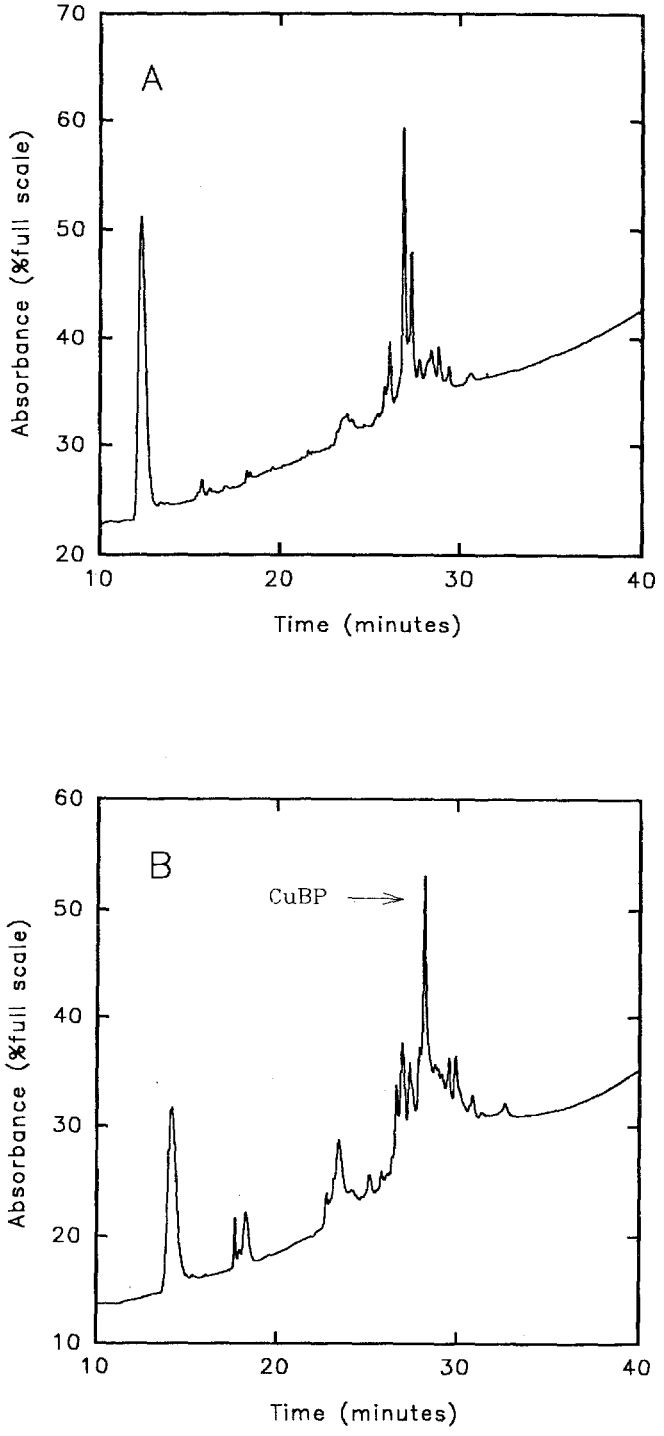

FIG. 3. Reverse-phase HPLC chromatograms of proteins contained in Cu-IMAC fractions derived from supernatants of copper-stressed cultures of *Vibrio parahaemolyticus* (A) and *Vibrio alginolyticus* (B). The position of the peak containing CuBP from *V. alginolyticus* is indicated by the arrow.

the 22.6-kDa protein as the predominant component. In a further separation of Cu-IMAC fractions by reverse-phase HPLC the major peak observed had the same retention time reported for CuBP from *V. alginolyticus* (26–28 min; Gordon et al. 1993) (Fig. 3A). Reference supernatant samples from *V. alginolyticus* cultures analyzed at the same time exhibited the expected reverse-phase HPLC chromatogram (Fig. 3B).

The data from this study demonstrate that the ability to recover from copper toxicity is common in heterotrophic bacteria. The response to copper added to exponentially growing cultures at micromolar levels is typified by a temporary inhibition of growth followed by recovery into exponential growth. The growth rate in the second exponential phase after recovery from copper toxicity is generally retarded. Therefore, copper inhibition is not entirely overcome.

Each of the *Vibrio* spp. examined demonstrated an extracellular protein of the same electrophoretic mobility as CuBP from *V. alginolyticus* ATCC 51160 in copper-challenged cultures and not in control cultures. This observation suggests that CuBP-like proteins are commonly produced by *Vibrio* spp. Further examination of supernatants of *V. parahaemolyticus* by liquid chromatography supports this suggestion. Only one of the other four species examined produced supernatant protein in the 20-kDa range in response to copper challenge. This protein, however, is distinguishable from the CuBP-like proteins of *Vibrio* spp. by its higher molecular mass. Although the data suggest that the *Vibrio* spp. examined to date produce a CuBP-like protein, additional evidence, including immunological cross-reactivity, amino acid composition, and sequence determination, is required to establish the degree of structural similarity that exists between these proteins.

The absence of CuBP-like proteins in other bacterial species does not rule out the possibility that copper-inducible extracellular proteins contribute to copper detoxification in cultures of these organisms. Proteins serving the same function as CuBP (i.e., complexing of free copper ions) but of different molecular mass could certainly exist. However, on the basis of the data from this study, the widespread distribution of copper-inducible, copper-complexing proteins structurally similar to CuBP in heterotrophic bacteria can be ruled out.

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