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NOTES

Enhancement of Copper Toxicity by Siderophores in *Bacillus megaterium*

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Chelation of copper by siderophores enhanced the toxicity of copper for *Bacillus megaterium*. Although this antibacterial activity appeared to be rapidly bactericidal, it could be partly reversed by addition of deferrisiderophore, or of ferrisiderophore at high concentration, immediately after exposure of cells to the cupric-siderophore complex.

Iron uptake in many microorganisms involves production of ferric chelating siderophores (5). The externally formed ferrisiderophore delivers iron to metabolism through a system of specific external receptors and cell-associated reductase enzymes that remove the iron from the chelate (2–5). Siderophore synthesis has been implicated as a virulence factor in some pathogenic organisms (13), and it is likely that these organisms maintain functional ferrisiderophore uptake systems during an infection.

Although siderophores have highest affinity for ferric iron, some other metals can form stable complexes with siderophores (1, 9). The chromium and gallium complexes of the siderophore ferrichrome are transported by the ferrisiderophore uptake systems of various microorganisms (6–8, 10), and the scandium and indium complexes of the siderophore enterochelin (enterobactin) have antibacterial activity for *Klebsiella pneumoniae* and *Escherichia coli* (11, 12). The bacteriostatic action of the scandium-enterochelin complex is reversed by ferrienterochelin; therefore, it was concluded that the scandium analog interferes with transport of ferrienterochelin.

Because siderophores have only weak affinity for ferrous iron, the metal can be readily released from ferrisiderophores by cellular ferrisiderophore reductase enzymes (2). The cytoplasmic ferrisiderophore reductase system of *Bacillus megaterium* also reductively removes copper from the cupric complexes of both the siderophores schizokinen and ferrioxamine B (J. E. L. Arceneaux, M. E. Boutwell, and B. R. Byers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K25 p. 141). *B. megaterium* produces schizokinen and utilizes ferrioxamine B; therefore, we examined growth of *B. megaterium* to determine if it was inhibited by the copper complexes of these two siderophores. The present paper describes the results; a preliminary report has appeared (J. E. L. Arceneaux, M. E. Boutwell, and B. R. Byers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K185, p. 207).

The cupric and ferric complexes of schizokinen and ferrioxamine B were prepared immediately before use by mixing the deferrisiderophore with either copper sulfate or ferric chloride at a ratio of 1 mol of metal per mol of siderophore. *B. megaterium* ATCC 19213 was grown in a Chelex-100-treated sucrose-mineral salts medium supplemented with magnesium and manganese as described previously (4); growth of cultures in Nephalo flasks at 37°C was monitored by periodic turbidity measurements with a Klett-Summerson colorimeter with a green filter.

At the inoculum level (10^6 CFU/ml) employed for growth studies in liquid medium, the cultures reached maximal growth without supplementation of the medium with iron (data not shown). This probably was due to carry-over of iron in the cells as the inoculum was previously grown in medium containing 18 μM iron. Nearly identical growth curves were obtained when iron (final concentration, 25 μM) was added as either ferric chloride or ferrischizokinen (Fig. 1). In cultures that contained no iron supplement, addition of 25 μM copper as copper sulfate lowered the total growth achieved; however, the toxicity of copper was significantly enhanced by addition of 25 μM copper as the cupric-schizokinen complex (Fig. 1). Similar results were obtained with the cupric complex of ferrioxamine B (data not shown).

An agar plate disk diffusion technique was used to determine if the growth toxicity of cupric-siderophores for *B. megaterium* could be reversed by iron, ferrisiderophores, or deferrisiderophores. To ensure a minimal level of iron in the culture medium, agar that was used to solidify the sucrose-mineral salts medium was treated with 0.025 M EDTA and then was rinsed extensively with water (purified by passage through a Millipore reverse osmosis-deionizer column system). Paper disks (1/4 in [0.635 cm]; Difco Laboratories) were treated with 0.025 M EDTA, washed with high-purity water, dried, and sterilized by autoclaving. Sterile disks impregnated with sterile test solutions were dried for 30 to 60 min before use. Molten agar medium containing 25 μM cupric-ferrioxamine B was seeded with *B. megaterium* (10^3 CFU/ml) and dispensed into petri dishes. Test disks were placed immediately on the solidified agar surface, and after incubation at 37°C for 24 h, zones of growth around the disks indicated reversal of cupric-ferrioxamine B inhibition. Ionic iron at concentrations up to 0.2 μmol per disk did not overcome inhibition (Table 1). All concentrations of deferriferrioxamine B that were tested reversed inhibition in a dose-dependent manner as evidenced by increased diameter of growth zones at higher concentrations (Table 1). Ferriferrioxamine B reversed inhibition only at the highest concentration (0.2 μmol) tested (Table 1).

It was noted that a delay of several hours before placement of the impregnated disks on the seeded, cupric-ferrioxamine B-containing medium resulted in no reversal of

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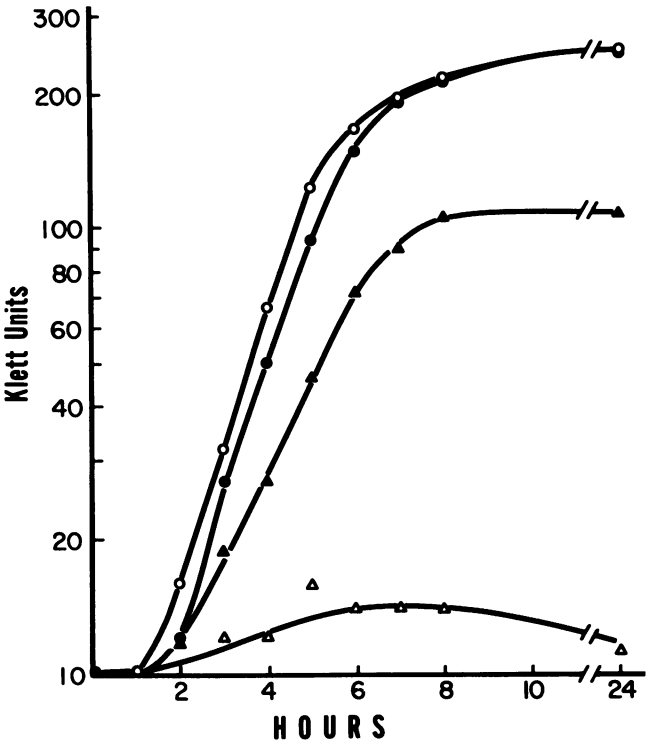

FIG. 1. Growth of *B. megaterium* in liquid medium supplemented with 25 μM iron or copper added as $FeCl_3$ (●), ferrischizokinen (○), $CuSO_4$ (▲); or cupric-schizokinen (△).

growth inhibition. The relationship between time of addition of deferriferrioxamine B and reversal of inhibition was examined. Sucrose-mineral salts medium containing 25 μM cupric-ferrioxamine B was inoculated with *B. megaterium* (10^3 CFU/ml), and at timed intervals three 10-ml samples were removed. At each interval, one of the samples was added directly to molten agar and plated; cells in the second sample were washed (by filtration) to remove cupric-ferrioxamine B, resuspended, added to molten agar, and plated; and to the third sample deferriferrioxamine B was added to give a final concentration of 25 μM, and this sample was added to molten agar and plated. Plates were incubated at 37°C for 24 h. At zero time, both washing of cells and addition of deferriferrioxamine B reversed inhibition, producing nearly confluent growth; little growth was evident in the sample that was plated without washing or addition of the reversing agent (Fig. 2). By 15 min, the removal of cupric-ferrioxamine B by washing permitted some growth, but the apparent number of viable organisms was significantly reduced; addition of deferriferrioxamine B at this time

TABLE 1. Reversal of cupric-ferrioxamine B inhibition of *B. megaterium*[a]

Concn per disk (μmol)	Compound and diam (mm) of growth		
	$FeCl_3$	Ferriferri-oxamine B	Deferri-ferrioxamine B
0.025	0	0	28
0.05	0	0	32
0.1	0	0	36
0.2	0	24	40

[a] Paper disks impregnated with the test compound were placed on the surface of agar medium that was seeded with *B. megaterium* and contained 25 μM cupric-ferrioxamine B.

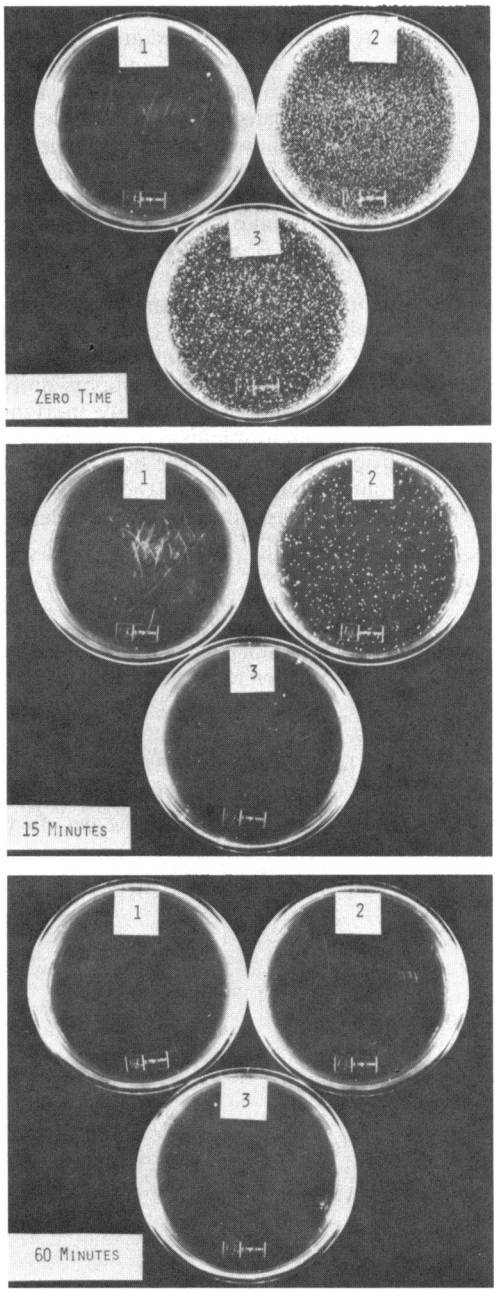

FIG. 2. Bactericidal activity of cupric-ferrioxamine B for *B. megaterium*. At indicated times, cells exposed to cupric-ferrioxamine B (25 μM) were plated directly (plates 1), were washed and plated (plates 2), or were mixed with 25 μM deferriferrioxamine B and plated (plates 3).

resulted in less than 10% cell survival (Fig. 2). By 60 min of exposure to cupric-ferrioxamine B, neither technique achieved reversal of toxicity (Fig. 2). The cupric-siderophore complex appeared to be rapidly bactericidal for *B. megaterium* under the experimental conditions employed.

The data suggest that the enhancement of copper toxicity by chelation of the metal to a siderophore may be due to cellular accumulation of bactericidal levels of copper. The cupric-siderophores may be taken up by the ferrisiderophore transport system; subsequent reductive release of copper from the chelate by the ferrisiderophore reductase system may insert the cuprous ion into various molecules that are

sensitive to copper inactivation. This antibacterial activity could be partly reversed by immediate addition of the deferrisiderophore and to a lesser extent by the ferrisiderophore. If reversal of growth inhibition was due to competition for either a specific transport receptor or a site on an enzyme of the ferrisiderophore reductase system (or both), then the affinity of these cellular sites may be highest for the deferri molecule. We have previously noted apparent affinity of the ferrisiderophore uptake system and the ferrisiderophore reductase process of *B. megaterium* for deferrisiderophore (3, 4).

Cupric-siderophores may have potential as antimicrobial agents for industrial, agricultural, or chemotherapeutic applications. Delivery of toxic concentrations of copper to microbial cells may make cupric-siderophores more useful for these applications than the kinetically inert nonferric complexes. As a given microorganism is capable of utilizing only certain siderophores (5), it might be possible to selectively inhibit certain organisms of a mixed microbial population by application of the appropriate cupric-siderophore.

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LITERATURE CITED

1. **Andregg, G., F. L'Eplattenier, and G. Schwarzenbach.** 1963. Hydroxamatcomplexe II. Die Anwendung der pH-methode. Helv. Chim. Acta **46**:1400–1408.
2. **Arceneaux, J. E. L.** 1983. Ferrisiderophore reductase and iron assimilation, p. 288–292. *In* D. Schlessinger (ed.), Microbiology—1983. American Society for Microbiology, Washington, D.C.
3. **Arceneaux, J. E. L., and B. R. Byers.** 1976. Ferric hydroxamate transport without subsequent iron utilization in *Bacillus megaterium*. J. Bacteriol. **127**:1324–1330.
4. **Arceneaux, J. E. L., and B. R. Byers.** 1980. Ferrisiderophore reductase activity in *Bacillus megaterium*. J. Bacteriol. **141**:715–721.
5. **Byers, B. R., and J. E. L. Arceneaux.** 1977. Microbial transport and utilization of iron, p. 215–249. *In* E. D. Weinberg (ed.), Microorganisms and minerals. Marcel Dekker, Inc., New York.
6. **Ecker, D. J., C. W. Passavant, and T. Emery.** 1982. Role of two siderophores in *Ustilago sphaerogena*: regulation of biosynthesis and uptake mechanisms. Biochim. Biophys. Acta **720**:242–249.
7. **Emery, T., and P. B. Hoffer.** 1980. Siderophore-mediated mechanism of gallium uptake demonstrated in the microorganism *Ustilago sphaerogena*. J. Nucl. Med. **21**:935–939.
8. **Leong, J., and J. B. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823–830.
9. **Raymond, K. N.** 1977. Kinetically inert complexes of the siderophores in studies of microbial iron transport. Adv. Chem. Ser. **162**:3–32.
10. **Raymond, K. N., and C. J. Carrano.** 1979. Coordination chemistry and microbial iron transport. Acc. Chem. Res. **12**:183–190.
11. **Rogers, H. J., C. Synge, and V. E. Woods.** 1980. Antibacterial effect of scandium and indium complexes of enterochelin on *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **18**:63–68.
12. **Rogers, H. J., V. E. Woods, and C. Synge.** 1982. Antibacterial effect of scandium and indium complexes of enterochelin on *Escherichia coli*. J. Gen. Microbiol. **128**:2389–2394.
13. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.