On the Role of Bacitracin Peptides in Trace Metal Transport by *Bacillus licheniformis*

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SUMMARY

Bacitracin markedly increased the toxic effect of several divalent metal ions towards growth of the producer strain *Bacillus licheniformis* ATCC14580. Magnesium ions antagonized the toxic effect of these divalent cations both in the presence and absence of bacitracin. It is suggested that bacitracin increases the uptake of several divalent metal ions. The function of the bacitracin peptides may be to extract essential divalent cations from 'waiting sites' on the surface of the cells and transfer the cations to the transport mechanisms in the cytoplasmic membrane.

INTRODUCTION

The peptide antibiotic bacitracin is synthesized by the protein thiotemplate mechanism present in *Bacillus* species (Laland & Zimmer, 1973; Frøyskov & Laland, 1974). It consists of a group of closely related dodecapeptides containing cyclic structures (Craig, Phillips & Burachik, 1969). Bacitracin-like peptides are probably produced by all *Bacillus* species (Sadoff, 1972, 1973).

Bacitracin is a potent antimicrobial agent toward many Gram-positive bacteria (Weinberg, 1967). Its antimicrobial effect on susceptible organisms may be due to a rupture of the membrane structure resulting from the interaction of bacitracin with the functional membrane lipid C₅₅-isoprenyl pyrophosphate (Storm & Strominger, 1974). Another interesting biological property of bacitracin is its ability to bind divalent metal ions (Garbutt, Morehouse & Hanson, 1961).

We have recently suggested that bacitracin may have a natural role during growth of the producer organisms (Haavik, 1974) and may promote the uptake of essential divalent cations (Haavik & Frøyskov, 1975; Haavik, 1975a). This has been shown for *B. licheniformis* ATCC10716 (Haavik, 1976). The aim of the present work was to investigate the effect of divalent metal ions and bacitracin on growth of the producer strain *Bacillus licheniformis* ATCC14580.

METHODS

Organism. The bacitracin-producing strain *Bacillus licheniformis* ATCC14580 was kept as a spore suspension at 4 °C.

Media and growth conditions. The complex medium for growth and bacitracin production (RMO medium) contained (g/l distilled water): Difco Bacto-soytone, 20.0; Difco Bacto-peptone, 10.0. The pH was adjusted to 7.0 with 1 M-HCl before autoclaving at 121 °C for 20 min. The inoculum was 0.05 ml of the spore suspension (Haavik, 1974). Bacteria were grown in 500 ml Erlenmeyer flasks (containing 50 ml medium) in a 37 °C water bath with

Fig. 1. Bacitracin production during growth of *B. licheniformis* ATCC14580 in complex medium. ●, Growth. ○, Bacitracin production.

shaking (120 rev. min⁻¹). Bacitracin, EDTA and salts were added to the medium as described previously (Haavik, 1976).

Growth. The extinction at 650 nm (E_{650}) was measured using a Spectronic 20 spectrophotometer.

Microbiological assay and detection of bacitracin. Bacitracin activity was determined by an agar diffusion method described previously (Haavik & Thomassen, 1973). The antibiotic activity was identified as bacitracin by thin-layer chromatography (t.l.c.) and overlaying with agar seeded with the test organism *Micrococcus flavus* (Haavik & Thomassen, 1973; Haavik, 1975*b*).

RESULTS

Growth and bacitracin production in complex medium

During active growth in the complex RMO medium, *B. licheniformis* ATCC14580 produced a bacitracin-like antibiotic which showed similar chromatographic behaviour to commercial bacitracin. Further characterization of this antibiotic is in progress.

Bacitracin production closely paralleled growth but ceased before growth was completed. During later stages of growth the amount of bacitracin in the culture fluid gradually decreased (Fig. 1).

Effect of bacitracin on growth

Bacitracin was not particularly toxic to growth of *B. licheniformis* ATCC14580 in the RMO medium. The addition of 10 i.u. bacitracin ml⁻¹ with the inoculum caused only a slight inhibition of growth, and 30 i.u. bacitracin ml⁻¹ had to be added to suppress growth during the first 6 h of incubation (Fig. 2). Growth of the assay organism *Micrococcus flavus* was inhibited by 0.1 i.u. bacitracin ml⁻¹ in the same medium.

The inhibition of *B. licheniformis* ATCC14580 by 30 i.u. bacitracin ml⁻¹ could be antagonized by Mg²⁺ (1 g l⁻¹) and partially by the metal chelator EDTA (100 mg l⁻¹). The addition

Fig. 2

Fig. 3

Fig. 2. Effect of bacitracin on growth of *B. licheniformis* ATCC14580. Bacitracin added (i.u. ml⁻¹): ●, none or 5; ○, 10; ◐, 15; ◑, 20; ◒, 25; △, 30.

Fig. 3. Effect of Mg²⁺ on the growth inhibitory effect of divalent metal ions in the presence of bacitracin (5 i.u. ml⁻¹). ●, No addition. ○, Added bacitracin plus metal ion (mg l⁻¹): Mn²⁺, 30; Co²⁺, 50; Ni²⁺, 120; Cu²⁺, 12; or Zn²⁺, 10. Added Mg²⁺ (1 g l⁻¹) plus the same concentrations of bacitracin and metal ion: ◐, Mn²⁺; △, Co²⁺; ▲, Ni²⁺; ▼, Cu²⁺; ■, Zn²⁺.

of several other divalent cations (Ca²⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Cd²⁺) had no such antagonistic effect.

Effect of bacitracin on the toxicity of divalent metal ions

Several divalent cations inhibited growth of *B. licheniformis* ATCC14580, Cd²⁺ being the most inhibitory and Fe²⁺ the least (Table 1). Mg²⁺ and Ca²⁺ were essentially non-inhibitory at concentrations up to 2 mg ml⁻¹. The addition of non-inhibitory amounts of bacitracin (5 i.u. ml⁻¹) markedly increased the toxic effect of several divalent metal ions, especially Mn²⁺ and Zn²⁺ (Table 1). Bacitracin also altered the relative effectiveness of the inhibitory metal ions but had no effect on growth with magnesium and calcium.

Antagonistic effect of Mg²⁺ on inhibition of growth by divalent metal ions

Inhibitory amounts of Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺ or Cu²⁺ could be effectively antagonized by the addition of Mg²⁺ (1 g l⁻¹) to the medium (Table 2). Toxic concentrations of Zn²⁺ or Cd²⁺ were less effectively antagonized. The growth inhibitory effect of non-toxic amounts of a divalent metal ion combined with 5 i.u. bacitracin ml⁻¹ could also be antagonized by excess Mg²⁺ in the medium (Fig. 3). This indicates that there is no essential difference between the growth inhibitory action of excess divalent metal ions alone or combined with bacitracin.

Catalytic effect of bacitracin

In the presence of relatively high but non-toxic amounts of Mn²⁺ or Co²⁺, small amounts of bacitracin significantly inhibited growth of *B. licheniformis* ATCC14580 (Table 3).

Table 1. *The influence of bacitracin (5 i.u. ml⁻¹) on the toxicity of divalent metal ions toward growth of B. licheniformis ATCC14580*

Metal ion added	Concn of metal ion required for 50 % inhibition of growth (mg l ⁻¹)	
	No bacitracin	With bacitracin
Cd ²⁺	9.0	8.4
Cu ²⁺	21.8	9.5
Co ²⁺	90.0	17.0
Zn ²⁺	125.0	4.0
Ni ²⁺	150.0	37.0
Mn ²⁺	320.0	8.0
Fe ²⁺	340.0	240.0

Table 2. *Effect of Mg²⁺ (1 g l⁻¹) and inhibitory concentrations of divalent metal ions on growth of B. licheniformis ATCC14580*

Metal ion added	Concn (mg l ⁻¹)	Growth (<i>E</i> ₆₅₀) after 6 h incubation	
		No Mg ²⁺	With Mg ²⁺
None	—	2.10	2.10
Mn ²⁺	400	0.15	1.98
Fe ²⁺	400	0.50	2.05
Co ²⁺	110	0.12	2.00
Ni ²⁺	200	0.10	2.08
Cu ²⁺	30	0.15	2.15
Zn ²⁺	150	0.30	0.76
Cd ²⁺	12	0.10	0.88

Table 3. *Effect of bacitracin on growth of B. licheniformis ATCC14580 in the presence of non-toxic amounts of Mn²⁺ (150 mg l⁻¹) or Co²⁺ (70 mg l⁻¹)*

Bacitracin added (i.u. ml ⁻¹)	Growth (<i>E</i> ₆₅₀) after 6 h incubation	
	With Mn ²⁺	With Co ²⁺
0	2.08	2.05
0.1	1.96	2.00
0.2	1.72	1.90
0.4	1.41	1.62
0.6	1.05	1.30
1.0	0.26	0.52
1.4	0.08	0.10

DISCUSSION

Bacillus licheniformis ATCC14580 produced a bacitracin-like antibiotic during the early period of growth in the complex RMO medium. A similar relationship between growth and antibiotic production has also been observed in a chemically defined medium (Haavik, 1975*b*). Bacitracin production by *B. licheniformis* ATCC10716 also paralleled growth in the defined medium, but continued throughout the growth period (Haavik, 1974). It is possible that *B. licheniformis* ATCC14580 firmly controls its bacitracin production and produces only the small amounts necessary during growth (Haavik, 1975*b*). Alternatively, *B. licheniformis* ATCC14580 and ATCC10716 may produce different bacitracin peptides, and that elaborated by *B. licheniformis* ATCC14580 may lose its antibacterial effect more easily.

Excess Mg²⁺ antagonized the inhibition by several divalent cations of growth of *B. licheniformis* ATCC14580. Abelson & Aldous (1950) showed that Mg²⁺ antagonizes the toxic effect

of Mn^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} and Cd^{2+} towards *E. coli* and suggested that the toxicity of these divalent metal ions may be due to an interference with the normal function of Mg^{2+} in cellular metabolism. The amounts of divalent cations within the cells increased with increasing concentrations of cations in the medium. Nelson & Kennedy (1971) have suggested that Co^{2+} must enter the cells to exert their toxicity, and that Mg^{2+} may protect at the level of uptake. It is possible that the divalent metal ions which are toxic to the growth of *B. licheniformis* ATCC14580 must also enter the cells in order to exert their toxicity. Another target for toxic metal ions might be the cytoplasmic membrane (Rothstein, 1959).

Bacitracin markedly increased the toxic effect of several divalent cations (particularly Mn^{2+} , Zn^{2+}) on the growth of *B. licheniformis* ATCC14580. Mg^{2+} could also antagonize this effect indicating similarity between the inhibitory effect of divalent metal ion alone or together with bacitracin. Garbutt *et al.* (1961) reported that bacitracin is able to complex with divalent metal ions. Thus only a complex between bacitracin and a divalent metal ion which is itself toxic when in excess may have a growth inhibitory action towards *B. licheniformis* ATCC14580. This indicates that the cation is the more important part of the bacitracin-cation complex, i.e. that bacitracin only promotes the effect of the cation. The increased toxicity of divalent metal ions in the presence of bacitracin may therefore be due to their increased uptake. This is consistent with our previous suggestion that the normal role of bacitracin during growth of the producer strains may be to promote the uptake of several divalent metal ions (Haavik & Frøyshov, 1975; Haavik, 1975a).

Bacitracin alone also inhibited growth of *B. licheniformis* ATCC14580, but relatively large amounts were needed. Small amounts of divalent cations present in the complex medium used may have been sufficient to inhibit growth under these conditions since EDTA could antagonize this effect.

The surface of a bacterium has many chelating groups which may bind metal ions (Cutinelli & Galdiero, 1967). Anionic polymers in the walls of *Bacillus* species, i.e. teichoic acid and teichuronic acids, may be involved in cation assimilation (Meers & Tempest, 1970) by scavenging the medium for small amounts of cation (Hurst *et al.*, 1975). The wall binds divalent and monovalent cations to a different degree but otherwise shows non-specific binding (Cutinelli & Galdiero, 1967). But the wall is a thick boundary (50 to 60 nm) compared with the size of the essential cations (about 0.1 nm). So how can a bacterium take up essential cations which may be present at low concentration, and, in addition, may bind to the wall distant from the transport mechanism in the cytoplasmic membrane?

The properties of bacitracin peptides could overcome these difficulties. Bacitracin is excreted from bacteria and binds metals well (Garbutt *et al.*, 1961); it is resistant to proteases and peptidases (Hickey, 1964) and it binds to membranes (Storm & Strominger, 1974). The role of bacitracin in the producer strains may therefore be to extract essential divalent cations from 'waiting sites' on the bacterial surface and transfer them to the transport mechanisms in the cytoplasmic membrane. Different bacitracin peptides may bind divalent metal ions to a different degree and bacitracin may even function catalytically (Haavik, 1975b). Bacitracin could also extract divalent cations from incidental metal complexes present in the environment.

Membrane teichoic acid between the wall and the surface of the cytoplasmic membrane could provide an integrated cation-exchange system between the exterior of the bacterium through the wall to the membrane itself. Thus membrane teichoic acids may mediate the interaction of bound cation with the membrane (Hughes, Hancock & Baddiley, 1973), and bacitracin peptides may then aid the transfer of cations from the teichoic acids to the membrane.

Several antibiotic-like peptides which may be part of metal-ion transport mechanisms in micro-organisms have been reported. Scribner *et al.* (1975) have suggested that an antibiotic-like peptide may be engaged in Mn^{2+} transport in *B. subtilis*. Furthermore, some cyclic peptide antibiotics (the siderochromes) participate in Fe^{3+} transport (Neilands, 1973). The peptide chelin, which is excreted by *B. thuringiensis*, is reported to react with molybdate ions in the medium and to have a transport function (Ketchum & Owens, 1975). The function of the peptide antibiotic edeine in the producer strain *B. brevis* vm4 may be similar to that of bacitracin (Kurylo-Borowska & Szer, 1976). Thus it is probable that many antibiotic-like peptides may be engaged in cation transport during the growth of micro-organisms.

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