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Stimulatory Effects of Bacitracin on Sporulation and Mycelial Growth in Thiostrepton-Producing *Streptomyces cyaneus* ATCC 14921 and *Streptomyces laurentii* ATCC 31255

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In a screening program for substances with morphogenic effects on the thiostrepton-producing *Streptomyces cyaneus* subsp. *azureus* and *S. laurentii*, bacitracin (BC) was found to have a remarkable growth-stimulating effect. The stimulatory effect on spore formation was so significant with about 5-fold increment in the number of spores and with striking elongation of the aerial mycelia in *S. c. azureus* and *S. laurentii* at 50 and 80 μg/ml, respectively. Also, BC markedly stimulated submerged mycelial growth after a long inhibitory lag period, accompanied by an increase in wet mass of the both species. These peculiar activities of BC on both streptomycetes were compared with its well-known biological and chemical activities. We speculated that the BC-induced stimulation of growth and sporulation might be due to its chelating and antimicrobial activities.

INTRODUCTION

Streptomyces spp. are gram-positive mycelial bacteria with high morphological life cycle that involves differentiation and sporulation, and produce many secondary metabolites containing important antibiotics. In the typical differentiation on agar media, the substrate mycelium develops and later gives rise to an aerial mycelium, which is finally converted, in part, into chains of spores. Most industrial productions take place in submerged cultures, in which almost all *Streptomyces* spp. do not differentiate into aerial mycelia and spores but generally exhibit two morphological shapes, dispersed mycelia and pellets (a spherical dense collection of mycelia) depending on the respective culture conditions and characteristics of the strains. An abundant production of spores is necessary for the cultivation of industrial streptomycetes, because they are generally used as the first inoculum (starter). However, spore formation of *S. cyaneus* subsp. *azureus* was markedly decreased in strains carrying the conjugative and pock-forming plasmid pSA1 (Doi *et al.*, 1998; Ogata, 1991; 1995; Ogata *et al.*, 1981; 1989). Great interests has been focused on the screening of exogenous and endogenous factors or substances that affect morphological differentiation, especially spore formation, and also on their mode of action (Chater, 1989; Coleman and Ensign, 1982; Hopwood, 1988; Karandikar *et al.*, 1996; 1997; Kondo *et al.*, 1988; Ogata, 1991; Ogata *et al.*, 1985; Schüz and Zähner, 1993; Ueda *et al.*, 1997; Vargha and Szabo, 1984). Much interests are also focused on the inhibition of spore formation by conjugative and pock-forming plasmids in

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several *Streptomyces* species. (Bibb *et al.*, 1977; Doi *et al.*, 1998; Hopwood *et al.*, 1973; 1984; Kieser *et al.*, 1982; Ogata, 1991; 1995).

BC is an antibiotic polypeptide complex produced by *Bacillus subtilis* and *Bacillus licheniformis*, and is effective against gram-positive bacteria. BC is widely used for growth promotion of animals (Froyshov, 1984). The antibacterial activity of BC has been attributed to two kinds of mechanisms. (i) Cell wall peptidoglycan production is inhibited as a result of the formation of a ternary complex constituted by BC, a divalent cation, and polyprenyl pp_i in such a way polyprenyl pp_i phosphatase cannot dephosphorylate its substrate (Siewert and Strominger, 1967; Stone and Strominger, 1971). (ii) The other mechanism is alteration of membrane permeability, which would be secondary to the formation of the ternary complex (Snoke and Cornell, 1965; Storm and Strominger, 1974). It has been reported that BC increases the size of parasporal crystals and spores in *Bacillus thuringensis* (Garcia-patrone, 1985) and it also has a stimulatory effect on kanamycin synthesis with slight inhibition of cellular growth in *S. kanamyceticus* (Basak and Majumdar, 1976)

In this paper, the effects of BC are focused on spore formation, aerial mycelia formation and submerged mycelia growth in the thiostrepton-producing *Streptomyces cyaneus* subsp. *azureus* and *S. laurentii*.

MATERIALS AND METHODS

Bacterial strains and Media

Sterptomyces cyaneus subsp. *azureus* ATCC 14921 (wild-type strain PK0) and its derivative strain PK100C and *S. laurentii* ATCC 31255 (wild-type strain P0) were used in this work. The wild-type strains PK0 and P0 carried pock-forming plasmid pSA1 and pSLS, respectively (Kinoshita-Iramina *et al.*, 1995, Ogata, 1995). Strain PK100C is a plasmid pSA1-free strain (Miyoshi *et al.*, 1986). The following *Streptomyces* spp. were also used: *S. hygroscopicus* JCM 4213, *S. griseus* NRRL B-2926, *S. coelicolor* A3(2) and its thiostrepton-resistant strain harboring plasmid pIJ702, on which was located a thiostrepton resistant gene *tsr*) (Hopwood *et al.*, 1985), and *S. lividans* 3131 (thiostrepton-resistant strain harboring plasmid pIJ702) and *S. lividans* TK21 (harboring no pIJ702). The latter three strains and plasmid pIJ702 were supplied by Prof. D. A. Hopwood. These strains were grown on agar plates containing rye flakes agar medium (pH 7.2), which consisted of rye flakes, 1%; glucose, 0.2%; yeast extract, 0.1%; $CaCO_3$, 0.3%; and agar, 1.5%, and Bennett medium (pH 7.2), which contains glucose, 1%; meat extract (Kyokuto Ltd., Tokyo), 0.1%; yeast extract (Nacalai Tesque, Inc., Kyoto), 0.1%; and NZ-amine type A (Wako Pure Chemical Industries, Ltd., Osaka), 0.2%. (Ogata *et al.*, 1981).

Agar dilution method

Bennett agar plates with bacitracin (BC) (Sigma Chemical Co., St. Louis, MO, USA) at various concentrations were inoculated with a suspension of spores and incubated for 5 to 7 days at 28°C. The lowest concentration of BC which yielded no growth was recorded as the minimal inhibitory concentration (MIC).

Measurement of length of aerial mycelium and number of spores

The length of aerial mycelium was measured by the method of Schauer *et al.*, (1988). A lawn of mycelium grown at 28 °C for a certain period (3 days for *S. laurentii*; 5 days for *S. c. azureus*) on petri plates of rye flakes agar was excised and sliced from top to bottom, and then placed sideways on a cover slip. The cover slip was placed on a microscope and the length of the mycelium was microscopically measured.

The number of spores scraped from slant cultures of rye flakes agar was estimated by microscopic counts of ten random haemocytometer fields (Ogata *et al.*, 1981).

Measurement of turbidity and wet cell mass

Following heat shock for 10 min at 45 °C, the stocked spores were inoculated at a final count of 10^7 spores in 250 ml Erlenmeyer flasks containing 50 ml Bennett medium supplemented with different concentrations of Mg^{2+} or EDTA. BC was also added to the autoclaved medium after filter-sterilization (by Millipore, 0.22 μm pore size) to give 80 μg/ml (or 10 to 120 μg/ml). The flasks were incubated at 28 °C on a reciprocal shaker at 250 rpm. Optical density (OD) of the Bennett culture was measured at 660 nm with a spectrophotometer (TAITEC Co., Saitama) using appropriate medium blanks.

Mycelia from 60 h-growing cultures of *S. c. azureus* and from 25 h-growing cultures of *S. laurentii* were harvested by centrifugation at 3,000 $\times$ g for 10 min, and washed twice with physiological saline solution and once with distilled water. The washed mycelia were collected and the cell amount was weighed (wet weight).

Endogenous metals in Bennett medium

Endogenous metals in meat and yeast extracts and NZ-amine were measured using an IRIS plasma spectrometer (Thermo Jarrell Ash Co., USA) and analyzed by the Thermo SPEC/CID software ver. 1.09 according to the manufacturer's manual.

RESULTS

Minimal inhibitory concentration (MIC) of bacitracin

Table 1 shows the effect of bacitracin on the growth of several typical streptomycetes. *S. c. azureus* and *S. laurentii* were resistant to bacitracin, showing MIC of 300 μg/ml and 700 μg/ml, respectively. The growth of the other strains used was completely inhibited at concentrations below 80 μg/ml, and growth stimulation was not observed: the stimulation was not seen even on the 300 μg/ml BC resistant mutants of these strains, except *S. hygroscopicus*, of which the resistant mutants has not been obtained. This result also showed that bacitracin resistibility would be uncorrelated with thiostrepton resistibility, because the thiostrepton-resistant transformants of *S. coelicolor* and *S. lividans* could not resist bacitracin to the same level as the normal strains.

Effect of bacitracin on spore formation

The wild-type strain PK0 of *S. c. azureus* which harbored a plasmid, pSA1, was inoculated onto the surface of rye flakes agar in petri plates as an indicator strain. Paper disks (8 mm in diameter) containing different concentrations of BC or EDTA were placed on

Table 1. Minimal inhibitory concentration (MIC) of bacitracin on the plate cultures of several *Streptomyces* species.

Strains	MIC of Bacitracin (μg/ml)
S. cyaneus azureus ATCC 14921[a]	300[d]
S. laurentii ATCC 31255	700[c]
S. hygroscopicus JCM 4213[b]	10
S. griseus NRRL B-2926[c]	50
S. coelicolor A3 (2)	50
S. coelicolor A3 (2) (pIJ702)	50
S. lividans TK21	80
S. lividans 3131 (pIJ702)	80

[a] ATCC, American Type Culture Collection.
[b] JCM, Japan collection of Microorganisms.
[c] NRRL, Northern Regional Research Laboratory, Peoria, Illinois, USA.
[d] Growth was initially inhibited by treatment with bacitracin, but normal growth resumed later.

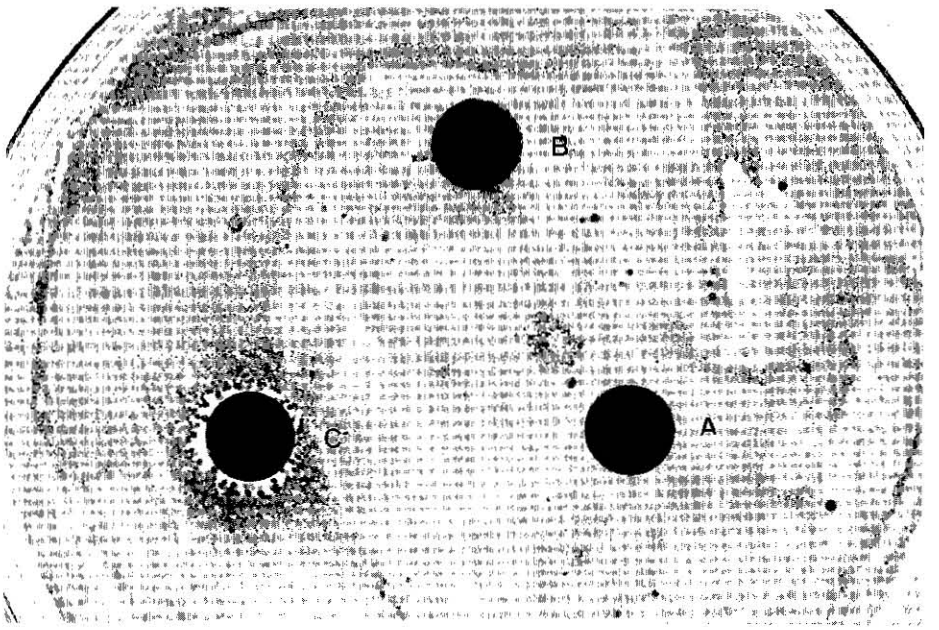

Fig. 1. Effect of bacitracin and EDTA on sporulation of strain PK0 of *S. cyaneus azureus*. Strain PK0 was inoculated on the surface of rye flakes agar and paper discs (8 mm in diameter) containing (A) Untreated control, (B) 1 mM EDTA and (C) 50 μg/ml BC were placed on the agar surface at the time of inoculation. Cultures were incubated at 28 °C and the photograph was taken at 7 days after inoculation. The coloring (bluish green) around the disk (C) of BC show spore production and stimulation of maturation age of spores.

the plates. As shown in Fig. 1, 50 μg/ml BC induced a faint bluish green color (indicating spore formation) around the paper disk, which was detected after 5–7 days. In contrast, no spore formation was induced by EDTA at any concentrations tested. Also, as shown in Fig. 2, 50 μg/ml BC showed spore production and stimulation of maturation age of spores in strain PK100C of *S. c. azureus* after 4 days. The effect of bacitracin on spore

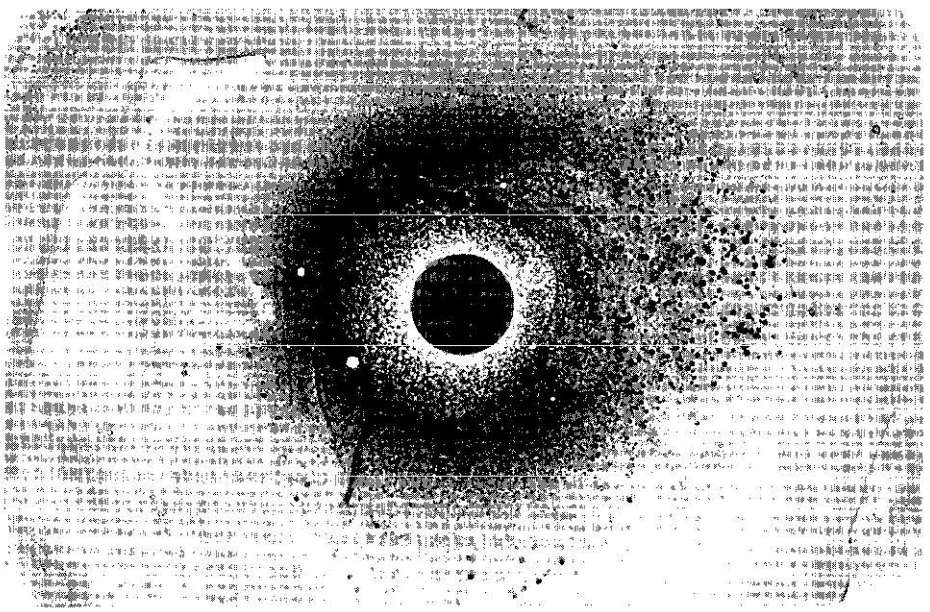

Fig. 2. Effect of bacitracin on sporulation of strain PK100C of *S. cyaneus azureus*. The coloring (bluish green) around the paper disk (containing 50 μg/ml BC) show spore production and stimulation of maturation age of spores after 4 days.

Table 2. Effect of bacitracin on the formation of spores in strains PK0 and PK100C of *S. cyaneus azureus* and strain P0 of *S. laurentii*.

Bacitracin (μg/ml)	Strain		
	PK0	PK100C (spores/slant)	P0
No addition	1.8×10^6	8.5×10^5	1.5×10^6
10	2.5×10^8	1.0×10^9	1.8×10^8
20	3.2×10^8	1.3×10^9	2.8×10^8
50	8.6×10^8	2.0×10^9	4.1×10^8
80	8.2×10^8	1.8×10^9	6.6×10^8
100	2.1×10^8	1.3×10^9	7.5×10^7

PK0, wild-type and plasmid pSA1-carrying strain; PK100C, plasmid-free strain; P0, wild-type and plasmid pSLS-carrying strain.
Each value is the average of five slants.

formation of *S. c. azureus* and *S. laurentii* were tested. The maximum sporulation efficiency of both strains was obtained at 7 days and 5 days of cultivation, respectively. The number of spores of *S. c. azureus* was increased 1.2 to 4.8-fold by bacitracin compared with that of the control without addition, as shown in Table 2. The maximum effect was obtained at 50 μg/ml. It was also effective on spore formation of *S. laurentii*, with 1.2 to 4.5-fold increment in the number of spores. The maximum effect was obtained at 80 μg/ml. It also advanced the age of coloring (maturation age) of spores or spore mass of *S. c. azureus* (bluish green color) and *S. laurentii* (yellowish orange color).

Effect of bacitracin on aerial mycelium

The stimulatory effect of bacitracin on the growth of the aerial mycelium was also observed. The length of the aerial mycelium estimated is shown in Table 3. The effect of bacitracin was more marked on *S. c. azureus* than on *S. laurentii*, with about 3.5-fold maximum stimulation. The growth of the aerial mycelium was so obvious as to be recognized by a glance at the colonies or the lawn of plate culture. It also showed a significant effect on *S. laurentii* with about 1.3-fold increase at the optimum concentration of 80 μg/ml. These results suggested that the stimulation of the aerial mycelium formation was closely correlated with the stimulation of the spore formation.

Effect of bacitracin on submerged mycelial growth

Figure 3 shows the effects of various concentrations of Mg^{2+} with or without BC on the submerged mycelial growth of *S. c. azureus* strain PK100C. The desired growth was obtained in the cultures with 0 (no supplement of Mg^{2-}) and 0.02 mM Mg^{2-}. Distinct suppression of growth was noted with the addition of 0.2 mM Mg^{2-}. The suppressive effect of Mg^{2+} increased with increasing concentration. BC at 80 μg/ml (Fig. 5) completely inhibited growth in cultures with 0 and 0.02 mM Mg^{2-}. As shown in Table 4, the content of endogenous divalent cations in Bennett medium, in which Mg^{2+} and Ca^{2+} are present in abundance. These endogenous divalent metal ions probably contributed to

Table 3. Effect of bacitracin on the length of aerial mycelia in the plate cultures of strains PK0 and PK100C of *S. cyaneus azureus* and strain P0 of *S. laurentii*.

Bacitracin (μg/ml)	Strain		
	PK0	PK100C (μm)	P0
No addition	69	80	110
10	124	92	115
20	153	107	120
50	243	123	137
80	198	119	145
100	130	114	118

Explanations of PK0, PK100C and P0 are the same as those described in Table 2.
Each value is the average of about 15 specimens which were prepared from five plates.

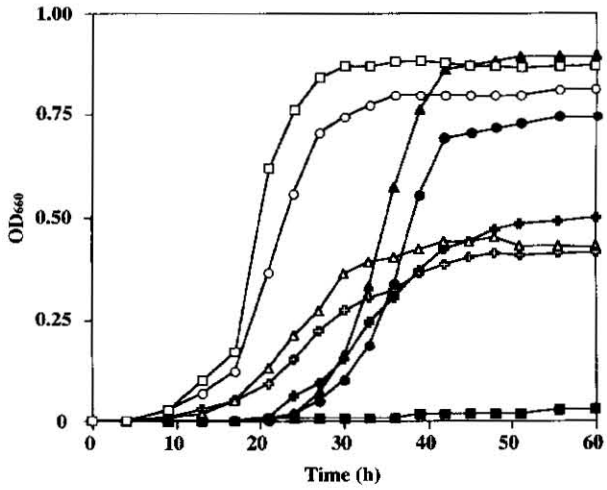

Fig. 3. Effect of Mg^{2+} with or without bacitracin on mycelial growth of strain PK100C of *S. cyaneus azureus*.
Symbols: □■, Mg^{2+} concentration at 0.0 or 0.02 mM; ○●, 0.2 mM; △▲, 2 mM; ⊕+, 10 mM. Open and filled symbols denote without or with bacitracin, respectively. Bennett medium contains 0.17 mM endogenous Mg^{2+}, as shown in Table 4.

the inhibitory action of BC, because cell growth was completely inhibited in the culture without Mg^{2+} supplementation. However, higher concentrations of Mg^{2+} resisted the inhibitory action of BC on mycelial growth. The peculiar effect of Mg^{2+} on BC activity was observed in cultures with 0.2, 2 and 10 mM Mg^{2+}: maximum effect was observed at 2 mM, in which mycelial growth was markedly suppressed during the first 25 h of incubation, but after this long lag period, rapid growth was observed. The growth pattern and OD_{660} of the cultures with 0 and 0.02 mM Mg^{2+} and without BC were very similar to those of the cultures with 2 mM Mg^{2+} and BC, but an essential difference was the occurrence of the long lag period in the latter culture.

As shown in Fig. 4 and Table 5, bacitracin showed a growth-stimulating effect on the submerged mycelial mass of *S. c. azureus* strain PK0 and *S. laurentii* strain P0. Their growth curves with bacitracin showed a clear long lag phase at an early period. After a lag period, the OD_{660} of the cultures increased like those of the control cultures without BC, and soon exceeded that of the control cultures. The maximal increase in the amount of the mycelial mass in *S. c. azureus* and *S. laurentii* was about 1.4-fold and 1.3-fold in comparison with that of the controls at the optimal concentration, respectively.

Excess Mg^{2+} in the liquid Bennett medium promoted pellet formation of *S. c. azureus* (Okba *et al.*, 1998). BC, similar to EDTA chelated with excess Mg^{2+}, leading to the inhibition of pellet formation.

Table 4. Content of various metals in meat and yeast extracts and NZ-amine in Bennett medium.

Metal	Mg	Ca	Mn	Zn	Cu	Fe	Co
Metal conc.[a] (μM)	171	33	0.1	5.2	0.1	5.7	0.05

[a] Values were calculated as μM in 1 L Bennett medium.

Table 5. Effect of bacitracin on the growth of mycelium in the liquid cultures of strains PK0 and PK100C of *S. cyaneus azureus* and strain P0 of *S. laurentii*.

Bacitracin (μg/ml)	Strain		
	PK0	PK100C (w. w. mg/ml)	P0
No addition	30.8	18	23.8
10	31.0	19	24.1
20	32.5	20	24.6
50	36.5	21.5	27.6
80	38.4	24.5	29.5
100	36.7	18.3	24.7

Explanations of PK0, PK100C and P0 are the same as those described in Table 2.
Each value is the average of five cultivations.
w. w. mg/ml, wet weigh of mycelia in mg per ml of cultures.

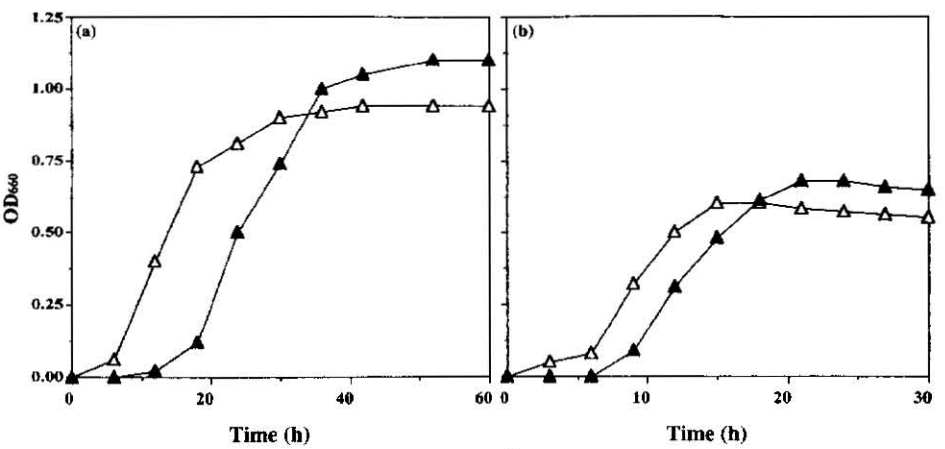

Fig. 4. Effect of bacitracin on mycelial growth of (a) strain PK0 of *S. cyaneus azureus* and (b) strain P0 of *S. laurentii*. Open and filled symbols denote without or with bacitracin, respectively. Bennett medium was supplemented with 80 μg/ml BC and 2 mM Mg^{2+}.

Effect of EDTA on submerged mycelial growth

BC is a chelating agent that combines easily with divalent metal ions (Garbutt *et al.*, 1961; Craig *et al.*, 1969). Therefore, experiments were performed to compare the action of BC and a typical metal chelator, EDTA, on the mycelial growth of *S. c. azureus*. Figures 5a and 5b show the effects of EDTA and BC on the cell mass in liquid cultures with 2 mM Mg^{2+} at 60 h. BC showed a growth-stimulating effect accompanied by an increase in submerged mycelial mass. The increase in cell mass at the optimum concentration of BC (80 μg/ml) was about 1.4-fold that of the control. In contrast, no growth stimulation was observed in the cultures with EDTA at any concentration tested. In solid cultures, BC stimulated spore formation and aerial mycelial growth, but EDTA did not. Therefore, BC-induced growth stimulation was not only due to the inhibition of pellet formation of mycelia (in another word, the dispersion of mycelia). From these results, BC-induced stimulation of growth and sporulation might be due to its chelating and antimicrobial activities.

DISCUSSION

In screening for substances with inducing activity on aerial mycelium formation and spore formation of various *Streptomyces* species, including *S. c. azureus* and *S. laurentii*, we found that BC acted as a streptomycete differentiation effector. It stimulated spourlation and growth of aerial and submerged mycelia of the thiostrepton-producing *Streptomyces, S. c. azureus* and *S. laurentii*.

Excess Mg^{2+} in the liquid Bennett medium suppressed the growth inhibitory action of BC. Similar effects of Mg^{2+} and Ca^{2+} have been reported by (Haavik, 1976; Podlesek and

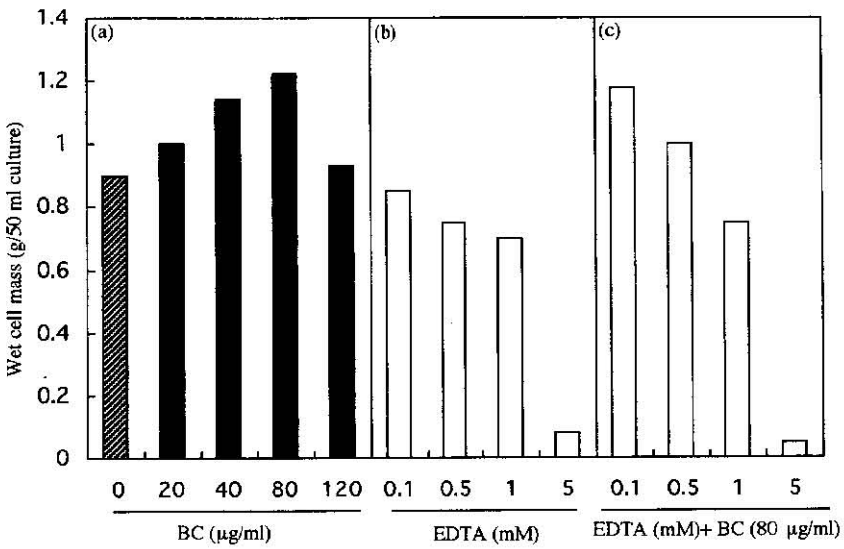

Fig. 5. Effect of bacitracin and EDTA on wet cell mass of strain PK100C of *S. cyaneus azureus*. (a) Bacitracin, (b) EDTA and (c) EDTA combined with bacitracin. Bennett medium was supplemented with 2 mM Mg^{2+}.

Comino, 1994). They have also reported that the growth inhibitory effect of non-toxic amounts of Mn^{2+} and Zn^{2+} in combination with BC could be antagonized by excess Mg^{2+} (and Ca^{2+}) in the medium. According to those reports, BC might be competitively displaced by Mg^{2+} (and Ca^{2+}) from the negatively charged phosphate groups on the membrane lipids. Mg^{2+} (and Ca^{2+}) effectively served as a bridge for the interaction of BC and the lipid carrier. Similarly, high concentrations of these ions might prevent access of BC to the lipid carrier that is buried inside the membrane. These explanations of the effects of Mg^{2+} (and Ca^{2+}) on BC were based on an analogy with polymyxin (Storm *et al.*, 1977). The behavior of BC, Mg^{2+} and Ca^{2+} in the present work could also be explained by the same argument.

There are some early reports that EDTA abolished the antimicrobial activity of BC (Adler and Snoke, 1962; Stone and Strominger, 1971). In view of the chelating properties of EDTA, this effect was due to its binding of metal ions. It has been suggested that Mg^{2+} prevented the access of BC to the lipid carrier, whereas EDTA with its chelating properties deprived BC of metal ions that it needed for binding to the lipid carrier (Podlesek and Comino, 1994). In our previous paper, we showed that Mg^{2+} (and Ca^{2+}) and EDTA antagonized the growth inhibitory action of BC (Okba *et al.*, 1998).

Garcia-Patrone found that the growth of *B. subtilis and B. thuringiensis* was initially inhibited by BC, but normal growth resumed later. This observation was very similar to the BC-induced growth stimulation of *S. c. azureus* and *S. laurentii*. He suggested that BC-induced proteins (BIP) antagonized the antimicrobial inhibitory action through a mechanism that required divalent cations as cofactors in a similar way that they mediated antibacterial activity of BC (Garcia-Patrone, 1990).

It has been suggested that the changes of membrane composition might correspond to the ability of *Streptomyces* mycelium to differentiate (Barabas *et al.*, 1994; Kalakoutskii and Agre, 1976; Ensign, 1978; Gräfe *et al.*, 1982). We suppose that the membrane is a key to understanding the initiation of *Streptomyces* differentiation (specific function of the cytoplasmic membrane may be necessary even in the late stages of differentiation). So, bacitracin may enhance or change the cell membrane function, and then stimulate mycelial growth or spore formation in the three strains used. Pamamycin-607 antibiotic stimulated aerial mycelium formation in an aerial mycelium-less mutant of *S. alboniger* (Kondo *et al.*, 1988). It has been reported that pamamycin-607 needed Ca^{2+} for its activity (Natsume and Marumo, 1992), bound tightly to bacterial membranes (Chou and Pogell, 1981), and might act on an ion-translocating mechanism in the cell membrane (Natsume *et al.*, 1995).

We are in the process of clarifying the role of BC and excess Mg^{2+} in the growth stimulation of *S. c. azureus*, and of finding some BC-induced membrane proteins. We assume that these membrane proteins will participate in the stimulation of BC-induced growth and sporulation. Properties and functions of the membrane proteins will be reported in greater detail in the future.

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