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Regulation of biosynthesis of bacilysin by *Bacillus subtilis*

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SUMMARY

Production of the dipeptide antibiotic bacilysin by *Bacillus subtilis* 168 was growth associated and showed no evidence of repression by glucose or sucrose. Carbohydrates other than glucose and sucrose yielded lower specific titers of bacilysin. Bacilysin production in three such carbon sources (maltose, xylose, ribose) was delayed until growth slowed down. Ammonium salts were poor for bacilysin production when used as the sole nitrogen source. When added to the standard medium containing glutamate, they suppressed antibiotic production. Aspartate was slightly better than glutamate for antibiotic production as sole nitrogen source. No other nitrogen source tested, including inorganic, organic or complex, approached the activity of glutamate or aspartate. When added to glutamate, casamino acids, phenylalanine and alanine (a substrate of bacilysin synthetase) suppressed bacilysin production while stimulating growth. Phosphate provided for optimum growth and production at 7.5 mM and both processes were inhibited at higher concentrations. Ferric citrate stimulated growth and inhibited bacilysin production, the effects being due to both the iron and the citrate components. Elimination of ferric citrate stimulated production as did increasing the concentration of Mn to its optimum concentration of 6.6×10^{-4} M.

INTRODUCTION

The regulatory mechanisms operating in secondary metabolite formation are of great importance from the point of view of microbial physiology. Regulation by sources of carbon, nitrogen, phosphorus, trace metals, precursor and nonprecursor effectors has been studied for many secondary metabolite systems. Inter-relationships between primary and secondary metabolism have been discussed in a number of review articles [2,4,8].

Microbially produced peptides are characteristic secondary metabolites and most of them have antibiotic activity [7]. Bacilysin, which is produced by *Bacillus subtilis*, was first found by Abraham et al. [1] to cause partial lysis of growing cultures of *Staphylococcus aureus*. Its structure was found to be a dipeptide composed of L-alanine and L-anticapsin [12,16]. Recently, it was demonstrated that the aromatic amino acid biosynthetic pathway branches from prephenic acid to yield anticapsin [5]. It was also reported that the bacilysin synthetase gene is located between the *ctrA* and *sacA* loci, near 90% on the standard *B. subtilis* 168 chromosomal map [6]. We now report on the regulation of bacilysin biosynthesis in *B. subtilis* strain 168.

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MATERIALS AND METHODS

Organism. B. subtilis 168 was used in this study and maintained as a spore suspension at 4°C [15].

Media and growth conditions. The PA fermentation medium described by Perry and Abraham [10] was used for growth and bacilysin production. It contained (g/l in distilled water) sucrose, 10.1; Na glutamate · H_2O, 4.0; KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; ferric citrate, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.0001; $CaCl_2 \cdot 6H_2O$, 0.0001; $(NH_4)_6Mo_7O_{24}$, 0.0001; $MnCl_2 \cdot 4H_2O$, 0.001 and $CuSO_4 \cdot 5H_2O$, 0.00001. The initial pH of the medium was adjusted to 7.0. The medium contained 100 mM morpholinopropane sulphonic acid (MOPS) for pH control only when indicated.

Seed culture preparation was carried out as described by Sakajoh et al. [15]. The culture was harvested by centrifugation, and the cells were resuspended in 0.01 M potassium phosphate buffer, pH 7.0. One ml portions of this cell suspension (containing about 1×10^9 cells) were inoculated into 40 ml PA media in 250 ml Erlenmeyer

flasks. The cultures were incubated with shaking at 37°C (225 rpm).

Bioassay of bacilysin. Bacilysin in culture fluids was determined by the paper disc-agar diffusion assay using *S. aureus* ATCC 9144 as the assay organism. Since pure bacilysin was not available, relative antibiotic activity was estimated using erythromycin as a standard [15]. A solution of bacilysin that gives a zone of inhibition identical to that given by 1.25 μg erythromycin per ml (or 0.025 μg/disc) was arbitrarily assigned an activity of 1 unit per ml. A calibration curve was established accordingly.

Bacterial dry weight determination. Samples of culture were filtered by suction through Sartorius SM-11307 filter paper discs. After being washed twice with water, the papers were placed in Petri dishes and dried overnight at 105°C. Upon cooling in a desiccator, the papers were weighed.

Growth measurement. Bacterial growth was measured as absorbance at 620 nm using a Spectronic 20 spectrophotometer. The cultures were appropriately diluted with fresh media before measurement and growth was expressed as A_{620} multiplied by the dilution factor.

Spore count. For determination of spores, 1 ml samples were serially diluted in physiological saline, heated at 80°C for 20 min in a water bath, cooled in ice, plated on nutrient agar and counted after overnight incubation at 37°C.

Total sugar determination. Total sugar was determined by the dinitrosalicylic acid method [9] after acid hydrolysis. For hydrolysis, three drops of concentrated HCl were added to each 3 ml sample followed by 5 min in a boiling water bath. The sample was cooled, neutralized with 5 N NaOH and assayed for reducing sugar.

Thin layer chromatography. Bacilysin broth concentrates were prepared as described by Roscoe and Abraham [13]. Bacilysin was detected by thin layer chromatography on Silica gel G-F_{254} aluminum plates with butan-1-ol-acetic acid-water (4 : 1 : 4) as the solvent system and identified by bioautography using *S. aureus* as the test organism. Bioautography revealed that a single substance in the broth (Rf = 0.325) was active against *S. aureus*. Its identity as bacilysin was confirmed by its UV spectrum [11].

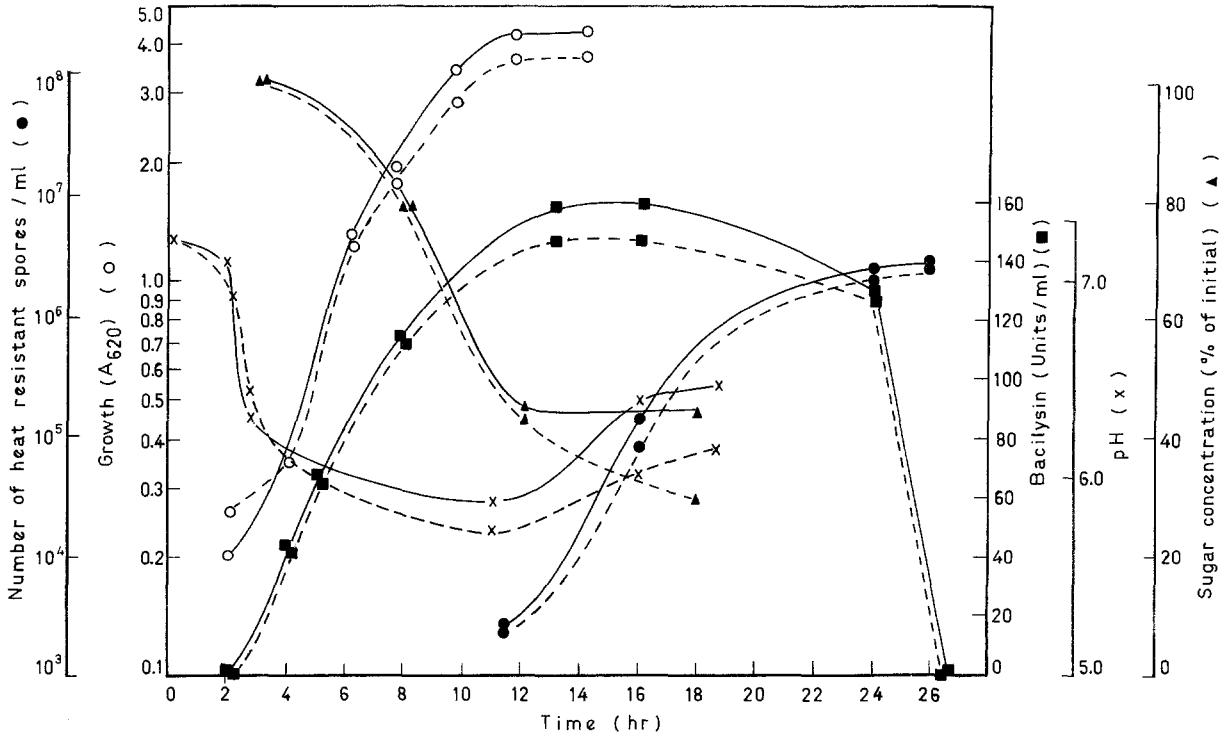

Fig. 1. Bacilysin production by *B. subtilis* during the course of fermentation in sucrose (———), or glucose (– – – –) as the sole carbon source.

RESULTS

Carbon source regulation

Bacilysin production in relation to growth, sporulation, pH change and sugar utilization in fermentation media containing sucrose or glucose as the sole sugar is shown in Fig. 1. Bacilysin formation began as the pH decreased during the first few hours and when the pH started to rise, bacilysin titer had reached its maximum value. Thus production occurred in a growth-associated manner and slowed down in the late stage of exponential growth. Antibacterial activity rapidly disappeared at around 24 h. About 50% of the sugar had been utilized at the time of peak bacilysin titer and sporulation started. No apparent repression by glucose of bacilysin biosynthesis was observed as compared to fermentation with sucrose.

The influence of varying concentrations (from 0.5 to 2.0%) of glucose and sucrose as the sole carbohydrate source on bacilysin production was determined. It was found that neither of the sugars had any negative effect on production at any of the concentrations tested. Similarly, when 1% glucose was added to 1% sucrose, it caused no depression of production. However, when the effects of other carbon compounds were examined, it was found that a number of carbohydrates suppress bacilysin formation while supporting a reasonable amount of growth (Fig. 2). Such carbon sources include ribose, glucosamine, glycerol, maltose, starch, inulin, dextrin and possibly xylose and arabinose. Galactose, lactose, pyruvate and acetate were not good carbon sources for growth; citrate failed to support any growth.

Bacilysin production in relation to growth on xylose, ribose and maltose was compared to that on sucrose (Fig. 3). In contrast to sucrose, production was delayed on these sugars until the late logarithmic phase.

Nitrogen source regulation

Replacement of glutamate by 10 mM ammonium sulfate severely interfered with bacilysin biosynthesis. The specific production obtained with ammonium as the sole nitrogen source was only 15% of that with glutamate. However, the pH dropped to 4.0 in this case, and thus, a pH effect was possible. When 0.1 M MOPS buffer was

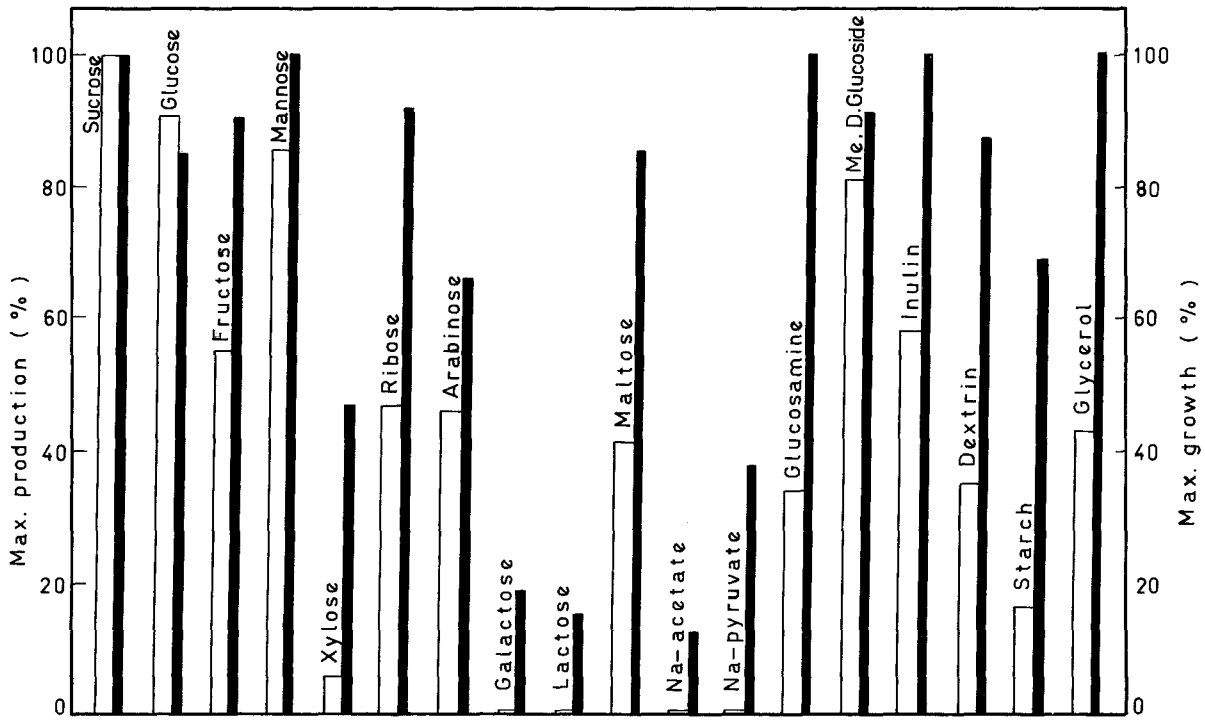

Fig. 2. Carbon source regulation of bacilysin production by various sole carbon sources. Compounds were provided at 1% concentration. MOPS was not present in the medium. Maximum production of bacilysin was 160 units per ml. □, bacilysin; ■, growth.

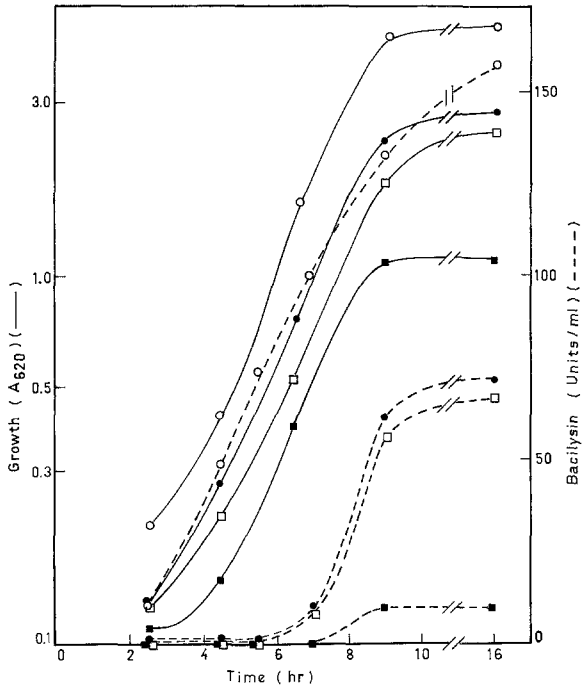

Fig. 3. Bacilysin production (----) in relation to growth (———) with sucrose (○), ribose (●), xylose (■) and maltose (□) each at 1% concentration in PA. MOPS was not present in the medium.

included in the control (glutamate) medium, the pH held at 7.0 with almost no effect on bacilysin production. In the case of ammonium sulfate with MOPS, the pH remained constant for 10 h and then dropped gradually to 6.2. Bacilysin production was markedly lower throughout the fermentation despite the presence of buffer. Of importance was the marked interference in bacilysin production even before the pH dropped. Production amounted to only 25% of the glutamate control (Fig. 4a). When ammonium sulfate was added to the glutamate (control) medium, it showed a major depressive effect despite almost no effect on growth or pH. The effect was much greater when the ammonium was added at zero time than when added at 6 h (Fig. 4b) as would be expected from a repression mechanism.

Table 1 shows a comparison of some nitrogenous compounds as sole nitrogen sources. Glutamate was the best in terms of both volumetric and specific production of bacilysin. Poor production with ammonium ion was observed using various salts. Nitrate was better than ammonium permitting almost half of the maximum production. None of the organic nitrogen sources tested was as good as glutamate. Similar findings were obtained when each inorganic nitrogen source was varied from 10 to 80 mM (data not shown). The organic nitrogenous compounds were tested again, but this time over a concentration range of 0.1 to 0.8% (Table 2). Again, glutamate was best, supporting optimum production at 0.4% Urea was rather good for growth but was very poor for bacilysin production.

In a comparison of additional amino acids as sole nitrogen sources at 0.4% concentrations, it was found that aspartate and glutamate were best in terms of specific

TABLE 1

Growth and bacilysin formation with various types of sole nitrogen sources[a]

Nitrogen source	Maximum DCW (mg/ml)	Maximum volumetric bacilysin production (units/ml)	Specific bacilysin production (units/mg DCW)
Glutamate	2.5	136	54
KNO_3	2.1	55.6	26
Soybean flour	–[b]	52.1	–[b]
$(NH_4)_2HPO_4$	2.9	52.1	18
$(NH_4)_2SO_4$	2.8	44.6	16
Peptone	2.8	44.6	16
NH_4CL	2.9	36.9	13
Urea	3.7	32.0	8.6

[a] The nitrogen sources were used at the following concentrations: organic sources, 0.2%; inorganic sources, 40 mM. MOPS was present in the medium.

[b] Maximum DCW not determined due to the fact that soybean flour did not completely dissolve. Glucosamine failed to support growth.

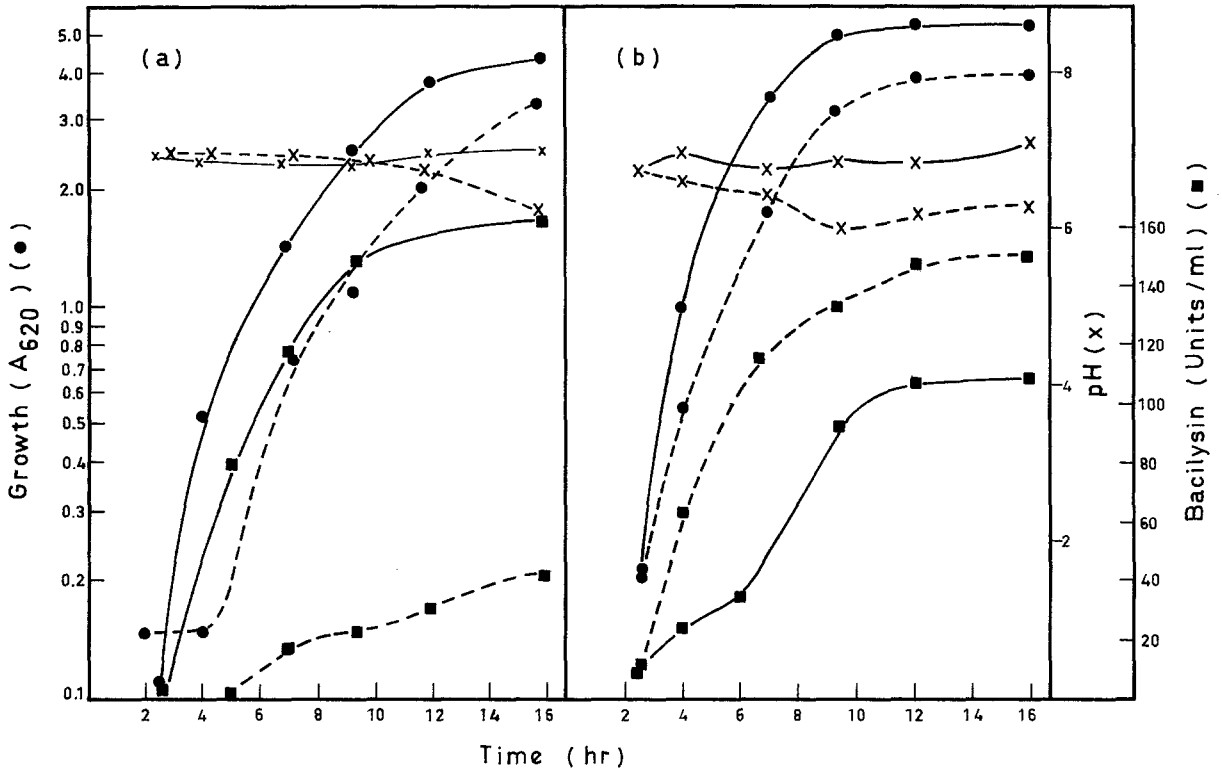

Fig. 4. Ammonium effect on bacilysin biosynthesis. (a) 10 mM ammonium sulfate (----) substituted for glutamate (———); (b) 10 mM ammonium sulfate was added to the glutamate-containing control medium at the time of inoculation (———), and to 6 h cultures (----), respectively. MOPS was included in the medium.

bacilysin production (Table 3). Histidine, valine and methionine were poor nitrogen sources for growth, and serine and threonine could not be utilized at all when supplied as sole nitrogen sources. Despite its supports of growth, alanine exerted a negative effect on production.

Casamino acids, when added to the glutamate-containing PA medium at 0.3% concentration, increased growth but completely inhibited bacilysin formation. However, a drop in pH to 5.0 was observed (Fig. 5). In the presence of MOPS, the pH drop was only minor but bacilysin production was only 20% of the control value. To study this effect further, thirteen different amino acids were tested individually as supplements to glutamate at 0.1% concentration. MOPS was included in the medium. The amino acids tested were alanine, arginine, serine, threonine, glycine, proline, leucine, valine, methionine, aspartic acid, tyrosine, phenylalanine, and tryptophan. Growth and bacilysin formation were followed throughout a 10 h fermentation. None of the amino acids significantly stimulated bacilysin production (data not shown). Serine and threonine were the only amino acids which inhibited growth; as expected, they also inhibited volumetric bacilysin production. Alanine and phenylalanine stimulated growth slightly but had a negative effect on volumetric bacilysin production by about 30–40%. Addition of tyrosine plus tryptophan reversed the negative effect of phenylalanine on antibiotic production, presumably by competing for uptake.

Control by inorganic phosphate

Growth and bacilysin formation were studied as functions of inorganic phosphate concentration from 1 to 250 mM in media buffered with 100 mM MOPS. Growth increased up to an inorganic phosphate concentration of 7.5 mM above which it decreased almost linearly (data not shown). Volumetric bacilysin production was also best at 7.5 mM phosphate and then decreased at higher levels. Thus, phosphate did not appear to have a negative regulatory influence on specific bacilysin synthesis.

TABLE 2

Growth and bacilysin formation with various organic nitrogen sources when used at different concentrations[a]

Organic nitrogenous compound	Concentration: 0.1%			0.2%			0.4%			0.8%		
	Maximum DCW (mg/ml)	Bacilysin		Maximum DCW (mg/ml)	Bacilysin		Maximum DCW (mg/ml)	Bacilysin		Maximum DCW (mg/ml)	Bacilysin	
		U/ml	U/mg		U/ml	U/mg		U/ml	U/mg		U/ml	U/mg
Glutamate	2.1	97.7	47	2.5	136	54	3.1	156	50	3.1	156	50
Soybean flour	1.1	23.7	22	1.2	52.1	43	2.5	102	41	2.7	122	45
Peptone	1.7	44.6	26	2.8	44.6	16	3.0	46.7	16	3.2	13.8	4.3
Urea	3.3	19.6	5.9	3.7	32.0	8.6	3.2	44.6	14	2.5	25.4	10

[a] MOPS was present in the medium.

TABLE 3

Growth and bacilysin production with various amino acids as sole nitrogen sources[a]

Nitrogen source	Maximum DCW (mg/ml)	Maximum volumetric bacilysin production (units/ml)	Specific bacilysin production (units/mg DCW)
Aspartic acid	2.6	152	58
Glutamic acid	3.0	152	51
Arginine	2.6	121	47
Valine	1.3	60.0	46
Proline	2.5	106	42
Histidine	1.1	36.9	34
Methionine	0.89	25.4	29
Alanine	2.9	79.2	27

[a] MOPS was present in the medium. Serine and threonine supported no growth.

Influence of precursors

We next investigated the effects of primary metabolite precursors of bacilysin: L-alanine, phosphoenolpyruvate (PEP), pyruvate and shikimic acid. The influence of anticapsin was not evaluated because of its scarcity. Shikimic acid, PEP and pyruvate did not significantly affect the rate or extent of bacilysin formation (data not shown). A marked effect was seen with alanine which delayed the onset and reduced the extent of bacilysin production. This was not surprising in view of the above results on nitrogen source regulation by alanine.

Alanine addition to the cultures was made at different times during the course of bacilysin biosynthesis (Fig. 6). It was observed that addition of alanine at 0 or 6 h markedly interfered with bacilysin production.

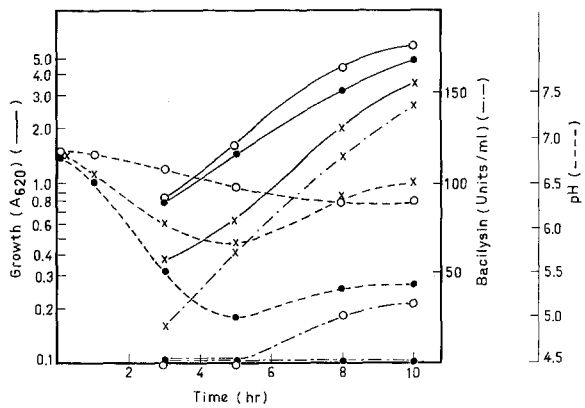

Fig. 5. The effect of casamino acids on bacilysin synthesis: No addition (x); casamino acids (●); casamino acids and MOPS (○).

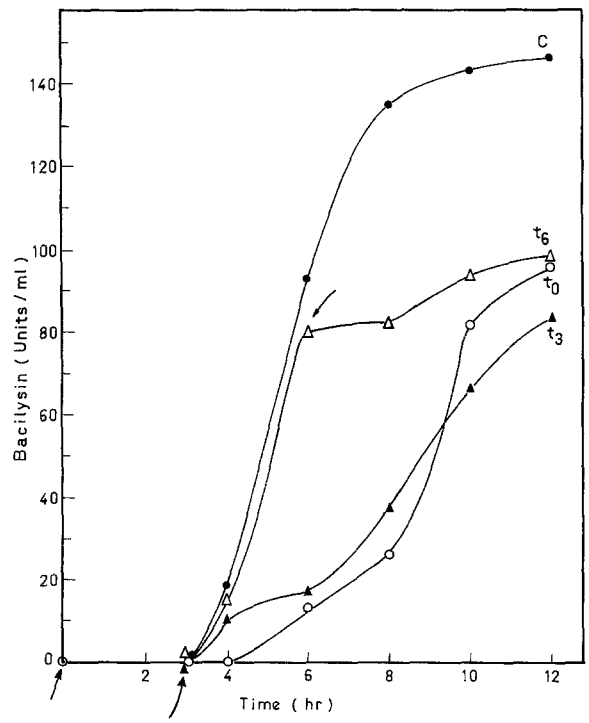

Fig. 6. The effect of time of alanine addition (→) at 0.1% final concentration on bacilysin synthesis. Control (●) contained no alanine. MOPS was included.

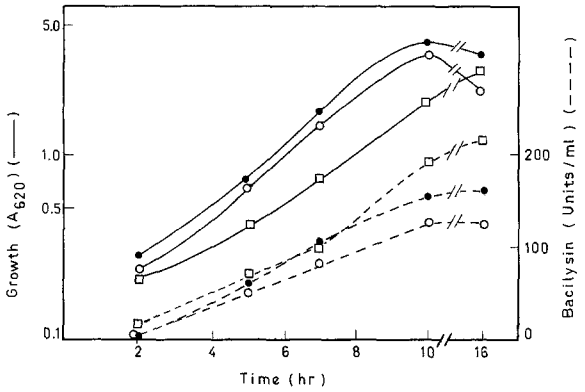

Fig. 7. Growth (———) and bacilysin synthesis (----) upon the omission of ferric citrate (□); of $ZnSO_4$, $CaCl_2$, ammonium molybdate, $MnCl_2$ and $CuSO_4$ (○) from the PA control medium (●).

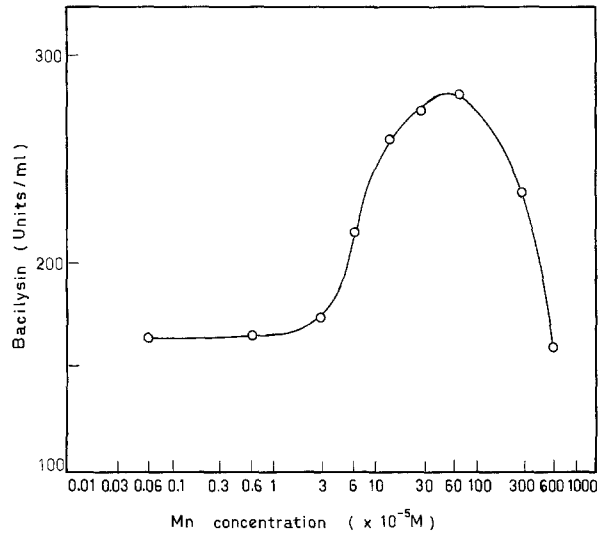

Fig. 8. The effect of manganese concentration on bacilysin biosynthesis.

Trace metal effects

The rate of growth dropped considerably when ferric citrate was omitted from PA medium, but it was not influenced by the omission of a mixture of $ZnSO_4$, $CaCl_2$, ammonium molybdate, $MnCl_2$ and $CuSO_4$ (Fig. 7). While needed for maximum growth rate, ferric citrate interfered with bacilysin biosynthesis, bacilysin titer increasing about 26% in its absence. A linear inverse relationship between ferric citrate concentration (0 to 0.15 mg/ml) and bacilysin titer ($\approx$215 to $\approx$160 μ/ml) was observed (data not shown). The antibiotic synthesized in the absence of ferric citrate was found to be bacilysin by thin layer chromatography followed by bioautography. To determine whether the effect is due to iron or to the chelating activity of citrate, sodium citrate and ferric chloride were incorporated into the MOPS-containing production medium as replacement for ferric citrate. Surprisingly, MOPS containing either sodium citrate or ferric chloride stimulated growth almost as much as ferric citrate and inhibited bacilysin synthesis to an even greater extent than ferric citrate (data not shown).

To further examine metal effects on bacilysin biosynthesis, maximum values of biomass and bacilysin were determined in media differing in metal content (Table 4). Mn, Zn, Co and Fe were chosen since these metals are usually the most important in secondary metabolism. Mn is the most important metal for bacilysin biosynthesis since specific production was decreased by 20% with its omission. Omission of Zn and Co had no effect. The negative role of ferric citrate was seen as expected.

TABLE 4

Growth and bacilysin production as a function of metal content of medium

Omitted from medium	Maximum DCW (mg/ml)	Maximum volumetric bacilysin production (units/ml)	Specific bacilysin production (units/mg DCW)
None	3.0	160	53
Mn	3.0	133	44
Zn	3.0	152	51
Co	3.0	156	52
Fe	2.3	216	94
Mn, Zn, Co	2.7	122	45
Mn, Zn, Co, Fe	2.3	122	53

Fig. 8 shows the relationship between Mn concentration and the production of bacilysin. The optimum Mn concentration was found to be 6.6×10^{-4} M, about 100 times the amount in the original medium; higher concentrations were inhibitory.

DISCUSSION

The production of bacilysin was observed to parallel active growth of *B. subtilis* when sucrose or glucose was carbon source but not when other carbon sources less effective for bacilysin synthesis (e.g., maltose, ribose, xylose) were used. These data support the concept of Martin and Demain [8] that the idiophase of secondary metabolism can occur after or during the trophophase depending on the nutritional environment presented to the culture. Repression or inhibition of enzymes by catabolism of glucose and other rapidly utilized carbon sources has been reported for many secondary metabolite systems, including actinomycin, puromycin, novobiocin, kanamycin, cephalosporin C and penicillin [4,8,14]. Disaccharides and other oligosaccharides and polysaccharides often prove to be the best carbon sources for the biosynthesis of these metabolites in batch culture. It was demonstrated in this study that glucose, even though it is a good carbon source for growth, does not negatively affect bacilysin biosynthesis even when it was added at 1.0% concentration to PA medium containing sucrose. Thus bacilysin resembles novobiocin and streptonigrin as a secondary metabolic process in which glucose is a useful carbon source. On the other hand, maltose, ribose, and xylose (and probably also glucosamine, glycerol, starch, inulin, dextrin and arabinose) are repressive or inhibitory.

Experiments involving nitrogen source regulation indicated that glutamate as the sole nitrogen source played an important role in bacilysin production. When glutamate was replaced by ammonium sulfate, specific bacilysin production was lowered by about 75% even when the medium was buffered with MOPS to retard pH changes. The suppressive effect of ammonium ions was also observed in experiments involving ammonium sulfate addition to glutamate-containing medium. The effect was much greater when addition was made at the time of inoculation than during bacilysin synthesis (at 6 h). Thus we hypothesize that repression is the mechanism. Addition of ammonium to *Streptomyces clavuligerus* cultures growing on glutamate or asparagine has been reported to suppress cephalosporin production [3]. Decreased antibiotic synthesis with ammonium or other nitrogen sources, has been well documented [2].

Among the bacilysin precursors investigated, L-alanine was the only one displaying a marked negative effect on bacilysin production. This is a puzzling observation reminiscent, however, of other antibiotic precursors interfering with production of the antibiotic [19]. It is also peculiar that the only other amino acid interfering with bacilysin synthesis is phenylalanine which resembles the anticapsin moiety in structure.

Manganese was required for maximum bacilysin biosynthesis at a concentration around 6×10^{-4} mM. This requirement is not surprising since this metal is often important in secondary metabolism and morphogenesis in the genus *Bacillus* [17,18].

In conclusion, the major regulatory controls on bacilysin synthesis appear to be nitrogen source regulation, probably repression, exerted by ammonium and probably also by nitrate and urea. The best nitrogen sources are aspartate and glutamate. Surprisingly, alanine, a precursor, and phenylalanine, structurally related to the anticapsin moiety, exerted negative effects on production. Many carbon sources appeared to interfere with bacilysin synthesis but not glucose or sucrose. Ferric citrate was inhibitory and Mn was stimulatory to production of the antibiotic.

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