Influence of the Culture Medium on the Production of Iturin A by *Bacillus subtilis*

By FRANÇOISE BESSON,* CATHERINE CHEVANET AND
GEORGES MICHEL

Laboratoire de Biochimie Microbienne, Université Claude Bernard, Lyon I,
34 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France

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The production of iturin A by *Bacillus subtilis* was studied with respect to the composition of the culture medium. Increasing phosphate concentrations did not modify the antibiotic yield. Fructose, sucrose and mannitol were better carbon sources than glucose for antibiotic production. The nature of the nitrogen source was an important factor in the production of antibiotic. Among the amino acids which are components of iturin A, L-asparagine was the best substrate for the biosynthesis of iturin A; L-glutamine and L-serine were rather poor substrates while L-proline and D-tyrosine gave no antibiotic. Ammonium salts permitted good synthesis of antibiotic but the addition of calcium ions to the culture medium inhibited the excretion of antibiotic from the cells.

INTRODUCTION

Iturin A is an antifungal antibiotic produced by several strains of *Bacillus subtilis* (Besson *et al.*, 1976). Its chemical structure (Fig. 1) has been determined (Peypoux *et al.*, 1978): it is a cyclolipopeptide containing two residues of D-asparagine, one residue each of L-asparagine, L-glutamine, L-proline, L-serine and D-tyrosine and one residue of a liposoluble β -amino acid with an *n*-C₁₄, *iso*-C₁₅, *anteiso*-C₁₅, *iso*-C₁₆ or *n*-C₁₆ chain (Isogai *et al.*, 1982).

It is well known that the production of most antibiotics is dependent on the composition of the culture medium in which the producer micro-organism is grown. Carbon and nitrogen catabolite regulation, phosphate regulation and induction appear to be general regulatory mechanisms that control the onset of antibiotic synthesis (Martin & Demain, 1980). The regulatory effects of amino acids on peptide antibiotic synthesis have been reported for colistin (Ito *et al.*, 1969), actinomycin (Katz, 1960; Katz *et al.*, 1961), tyrocidin (Mach & Tatum, 1964), gramicidin (Matteo *et al.*, 1975; Vandamme & Demain, 1976), echinomycin (Formica & Waring, 1983), bacitracin (Haavik, 1979, 1981) and antibiotic K-582 (Ito-Kawaga *et al.*, 1983).

Iturin A has been isolated from a complex medium (Besson *et al.*, 1976). In order to compare the factors which might affect the production of iturin A, we screened a number of carbon and nitrogen sources.

METHODS

Culture conditions. A strain of *B. subtilis* producing iturin was kindly supplied by Dr L. Delcambe, CNPEM, Liège, Belgium. It was grown at 35 °C for 50 h on a basal medium containing (g l⁻¹): glucose, 10; KH₂PO₄, 1; MgSO₄·7H₂O, 0.5; KCl, 1; Fe₂(SO₄)₃·6H₂O, 5 × 10⁻³; CuSO₄·5H₂O, 0.16 × 10⁻³ and glutamic acid, 5 (34 mM). In some experiments, glucose was replaced by other carbon sources at the same concentration (10 g l⁻¹), and glutamic acid by other nitrogen sources at the same molarity (34 mM). In other experiments, the phosphate concentration was increased from 0.1 to 100 mM. The pH of the media was adjusted to 7.7 before autoclaving. Growth was estimated as OD₆₀₀ (10⁵ cells ml⁻¹ corresponds to an optical density of about 1).

Quantitative determination of iturin. Crude preparations of antibiotic were obtained by acidification of culture medium, and the presence of iturin was detected by the characterization of β -amino acids after total hydrolysis with 6 M-HCl at 150 °C for 8 h as described previously (Besson *et al.*, 1976). For quantitative analysis, samples of the chloroformic extract (corresponding to 100 nmol of β -amino acids) were dinitrophenylated with 1-fluoro-2,4-

Fig. 1. Structure of iturin A.

dinitro[^{14}C]benzene, specific activity 7.4 MBq mmol $^{-1}$ (Amersham) (Chevanet *et al.*, 1986); the dinitrophenyl β -amino acids were purified by chromatography on silica gel 60 (Merck) in chloroform/methanol/acetic acid (95:5:1, by vol.) and their radioactivities were measured in a liquid scintillation spectrometer (Packard Tricarb 2001) with Ready Solv EP scintillation fluid (Beckman). Standards of radioactive dinitrophenyl β -amino acids were obtained by dinitrophenylation of 25–200 nmol of pure β -amino acids.

Determination of antibiotic activity by bioautography. Antibiotic activity was located on thin-layer chromatograms by the detection of antifungal activity on Sabouraud agar cultures of *Saccharomyces cerevisiae* (Besson *et al.*, 1978).

RESULTS

Growth and iturin production. Fig. 2 shows the course of iturin production by *B. subtilis* grown in basal medium, growth and the changes in pH and glucose concentration of the culture medium. During the first 15 h of cultivation, growth was exponential. Iturin production began at the end of the exponential phase, when the glucose was almost exhausted, and increased markedly until 45 h. The relation between growth and iturin production showed a typical trophophase–idiophase pattern. The variations of pH were slight, between 5.6 and 6.8.

Effect of phosphate on iturin production. Increasing phosphate concentrations in the basal medium increased both growth and antibiotic production but did not modify the relative antibiotic yield (Table 1). The effect of increased buffer strength on iturin production was also studied by adding 50 mM-HEPES to 0.1 and 1 mM-phosphate media; the pH was maintained at 7.5 and no variation in the antibiotic production was observed.

Effect of carbon sources on iturin production. Glucose in the basal medium was replaced by the same concentration (10 g l $^{-1}$) of fructose, sucrose, glycerol, mannitol, citric acid or lactic acid (Table 2). Fructose, sucrose and mannitol were better substrates than glucose for antibiotic synthesis. With glycerol, the growth was good but the yield of antibiotic was lowered; citric acid and lactic acid were poor substrates for growth, although citric acid gave a normal yield of antibiotic.

Effect of amino acids on iturin production. The abilities of several amino acids, as sole nitrogen sources, to support growth of the organism and the production of antibiotic were tested (Table 3). All the amino acids which are components of iturin A or which could be precursors of iturin A gave normal growth except tyrosine. L-Glutamic acid, L-glutamine, D- and L-aspartic acid, L-asparagine and L-serine supported antibiotic production whereas L-proline did not. The highest yields were obtained with L-glutamic acid, L- and D-aspartic acid and L-asparagine. Replacement of L-asparagine by D-asparagine decreased iturin production.

Some structural analogues of the peptide precursors were also tested. Among them, only DL-threonine supported antibiotic production, but at a low level.

Another antibiotic related to iturin, bacillomycin F, isolated from another *B. subtilis* strain (Mhammedi *et al.*, 1982) contains a threonyl residue instead of the seryl residue in iturin A (Peypoux *et al.*, 1985). Thus, the precise nature of the antibiotic produced in the presence of threonine was determined. Crude antibiotic preparation obtained with addition of threonine in the culture medium was purified by chromatography on silicic acid columns as described previously (Peypoux *et al.*, 1973). The fractions were examined by thin-layer chromatography on silica gel 60 with the solvent chloroform/methanol/water (65:25:4, by vol.), detected by Pauly reagent (Jutisz, 1960) or by their antifungal activities on *Saccharomyces cerevisiae*. Bioautograms and Pauly reactions revealed the presence of iturin A only; no bacillomycin F was detected.

The effects of DL-alanine, DL-leucine, DL-isoleucine and DL-valine were also studied. DL-Leucine and DL-valine did not support antibiotic synthesis; with DL-isoleucine, antibiotic was

Fig. 2. Iturin A production in the basal medium. Antibiotic concentration (■) was determined in relation to growth (▲), and the pH (◆) and glucose concentration (●) of the culture medium. The data points are means of three assays.

Table 1. *Effect of phosphate on iturin production after 50 h growth*

Phosphate concn (mM)	Growth* (OD ₆₀₀)	pH*	Antibiotic in medium* (µM)	Antibiotic yield (µmol per OD ₆₀₀ unit)
0.1	3.3 ± 0.1	8.7 ± 0.1	37 ± 1	12
1	3.2 ± 0.1	8.5 ± 0.1	41 ± 3	13
10	4.6 ± 0.4	6.2 ± 0.3	59 ± 1	13
100	5.1 ± 0.1	6.5 ± 0.1	68 ± 1	13

* The values are the mean of at least two assays, ± the range of results.

Table 2. *Effect of different carbon sources on iturin production after 50 h growth*

Carbon sources	Growth* (OD ₆₀₀)	Antibiotic* in medium (µM)	Antibiotic yield (µmol per OD ₆₀₀ unit)
Glutamic acid alone†	2.4 ± 0.1	4 ± 1	2
Glutamic acid + glucose‡	4.4 ± 0.1	53 ± 2	12
+ fructose	5.0 ± 0.2	116 ± 3	23
+ sucrose	5.6 ± 0.1	118 ± 6	21
+ glycerol	6.0 ± 0.2	50 ± 7	8
+ mannitol	4.6 ± 0.5	149 ± 9	32
+ citric acid	1.5 ± 0.1	20 ± 5	13
+ lactic acid	2.4 ± 0.3	8 ± 1	3

* The values are the mean of at least two assays, ± the range of results.

† *B. subtilis* was grown for 5 d (instead of 50 h) in this medium.

‡ This medium is the basal medium.

Table 3. *Effect of amino acids on iturin production after 50 h growth*

Amino acid* (34 mM)	Growth† (OD ₆₀₀)	Antibiotic† in medium (μM)	Antibiotic yield (μmol per OD ₆₀₀ unit)
<i>L-Glutamine</i>	4.4 ± 0.2	12 ± 2	
<i>L-Glutamic acid</i> ‡	4.4 ± 0.1	53 ± 2	12
<i>D-Asparagine</i>	4.8 ± 0.3	13 ± 1	3
<i>L-Asparagine</i>	4.6 ± 0.2	65 ± 4	14
<i>D-Aspartic acid</i>	3.8 ± 0.3	40 ± 2	11
<i>L-Aspartic acid</i>	2.1 ± 0.1	23 ± 1	11
<i>L-Serine</i>	3.8 ± 0.3	16 ± 2	4
<i>DL-Threonine</i>	2.7 ± 0.4	7 ± 1	2
<i>DL-Homoserine</i>	4.4 ± 0.1	0	—
<i>D-Tyrosine</i>	0	—	—
<i>L-Tyrosine</i>	0	—	—
<i>DL-Phenylalanine</i>	2.0 ± 0.1	0	—
<i>L-Proline</i>	10.0 ± 0.4	0	—
<i>DL-Alanine</i>	5.2 ± 0.2	30 ± 3	6
<i>DL-Isoleucine</i>	1.8 ± 0.1	2 ± 1	1
<i>DL-Leucine</i>	1.8 ± 0.3	0	—
<i>DL-Valine</i>	1.4 ± 0.1	0	—

* Amino acids in italics are constituent amino acids of iturin.

† The values are the mean of at least two assays, ± the range of results.

‡ This medium is the basal medium.

Table 4. *Effect of ammonium ions on iturin production after 50 h growth*

Nitrogen source (34 mM)	Growth* (OD ₆₀₀)	pH*	Antibiotic*		β-Amino acids in cells*	
			In medium	In cells	Free	After hydrolysis
<i>L-Glutamic acid</i> †	4.4 ± 0.2	7.0 ± 0.1	53 ± 2	0	0	0
NH ₄ Cl	3.2 ± 0.1	4.2 ± 0.1	0	0	0	0
NH ₄ Cl + CaCO ₃ (5 g l ⁻¹)	3.7 ± 0.4	6.2 ± 0.1	0	48 ± 3	0	48 ± 3
(NH ₄) ₂ SO ₄ + CaCO ₃ (5 g l ⁻¹)	3.9 ± 0.4	6.2 ± 0.1	0	55 ± 4	0	55 ± 4
NH ₄ NO ₃ + CaCO ₃ (5 g l ⁻¹)	5.5 ± 0.2	6.1 ± 0.1	0	42 ± 2	0	42 ± 2
<i>L-Glutamic acid</i> + CaCl ₂ (5 g l ⁻¹)	4.8 ± 0.1	7.0 ± 0.1	0	50 ± 3	0	50 ± 3
NH ₄ Cl + phosphate (100 mM)	2.5 ± 0.3	6.7 ± 0.1	45 ± 3	0	0	0

* The values, expressed as μmol per litre of culture, are the mean of at least two assays, ± the range of results.

† This medium is the basal medium.

present as traces, and *DL*-alanine gave good growth and moderate antibiotic production (Table 3).

Effect of ammonium ions on iturin production. When glutamic acid of the basal medium was replaced by ammonium ions at the same molarity, no iturin A was detected in the culture medium (Table 4). As the pH was very low, CaCO₃ (5 g l⁻¹) was added; in these conditions, the pH was kept at 6.2 but no antibiotic was detected in the culture medium.

Ammonium chloride could inhibit the production of antibiotic in several ways: e.g. inhibition of the synthesis of β-amino acids, inhibition of the formation of cyclic lipopeptide or prevention of the excretion of the antibiotic from the cells into the culture medium. This last possibility was tested on cells grown on media containing ammonium chloride and neutralized or not with CaCO₃. Bacteria were extracted with chloroform/methanol (2:1, v/v) and the extracts were tested for the presence of iturin A in comparison with the extract obtained from cells grown in the basal medium. After thin-layer chromatography on silica gel 60 in the solvent chloroform/methanol/water (65:25:4, by vol.) and bioautography, iturin A was detected only in

the cells grown in the medium containing ammonium chloride neutralized with CaCO_3 . Iturin A was absent in the cells grown in the medium containing ammonium chloride without CaCO_3 and in the cells grown in the basal medium (Table 4). Thus, at acidic pH (4.2), there was no synthesis of iturin A; at higher pH (6.2), synthesis occurred in the presence of ammonium chloride but the addition of CaCO_3 prevented the excretion of antibiotic into the medium.

The inhibition of iturin A synthesis by ammonium chloride at low pH could occur before or beyond β -amino acid synthesis. If the latter is true, β -amino acids might be found in the cells either in the free state or as derivatives. The cell extracts obtained with chloroform/methanol (2:1, v/v) were tested for the presence of β -amino acids (Table 4). β -Amino acids were not detected in the cells grown in medium containing ammonium chloride, either in the free form, or after hydrolysis of the extract with 6 M-HCl for 8 h at 150 °C. Thus, when the pH decreased to 4.2, β -amino acid synthesis was inhibited, while at higher pH (6.2) β -amino acids were synthesized and incorporated into iturin molecules.

The influence of the nature of the anion of various ammonium salts was studied by replacing ammonium chloride by the same molarities of ammonium sulphate or ammonium nitrate in the culture medium. In all cases, when the pH was maintained at 6.2 by CaCO_3 , iturin A was not excreted into the medium but did accumulate in the cells (Table 4).

Effect of calcium ions on iturin production. The influence of calcium ions was tested more precisely. In the basal medium (final pH 6.9), the solubility of CaCO_3 was very low and it was replaced by calcium chloride (5 g l⁻¹). In these conditions, iturin A was not found in the culture medium but it accumulated in the cells (Table 4). Moreover, in the medium containing ammonium chloride, when the pH was brought to 6.7 by phosphate (100 mM) instead of CaCO_3 addition, iturin A was found in the culture medium and not in the cells (Table 4). Thus, these results show that calcium ions prevent excretion of iturin A.

DISCUSSION

Iturin A production was studied in a synthetic medium containing phosphate, glucose and glutamic acid as the main components. Mannitol, fructose and sucrose were better carbon sources than glucose for iturin A production. Limitation of growth by phosphate led to a decrease in growth and in iturin A production but did not modify the relative cell yield of antibiotic. The increased production observed at higher concentrations of phosphate is not in agreement with previous results on synthesis of other antibiotics (Martin & Demain, 1980).

The effect of amino acids that are structural components or possible precursors of iturin A, used as sole nitrogen source in the basal medium, was studied. Based on specific yield (μmol iturin per litre of culture and per unit of growth measured as OD_{600}), L-glutamic acid, D- or L-aspartic acid and L-asparagine produced equivalent yields of antibiotic. The yields obtained with L-serine, L-glutamine and D-asparagine were about threefold lower. A similar decrease in antibiotic synthesis has been reported with the D-phenylalanine and D-ornithine constituents of bacitracin (Haavik, 1979), D-valine, D-isoleucine and allo-D-isoleucine of actinomycin D (Katz, 1960), and D-phenylalanine of polymyxin B (Srinivasa & Ramachandran, 1979). This inhibitory effect was explained if the L-amino acid, instead of the D-isomer, is the substrate of the multi-enzyme system responsible for the antibiotic synthesis (Katz & Weissbach, 1963; Leung & Baxter, 1972).

The addition of ammonium chloride to the culture medium lowered the pH to 4.2 and thus inhibited the synthesis of iturin A. When the pH was maintained at 6.2 by phosphate buffer, the antibiotic was synthesized normally and excreted into the culture medium. However, when the pH was brought to 6.2 with CaCO_3 , iturin A synthesis was normal but excretion into the medium was totally inhibited. A similar result was observed when calcium ions were added to the basal medium. Thus, it seems that calcium ions modify the permeability of the cell envelope to iturin A. Although no explanation can be proposed at the present time, this observation provides an interesting model to explore the mechanism of excretion of antibiotics into the culture medium. Further work is currently in progress.

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