IDENTIFICATION OF ANTIBIOTICS OF ITURIN GROUP IN VARIOUS STRAINS OF *BACILLUS SUBTILIS*

FRANÇOISE BESSON, FRANÇOISE PEYPOUX, GEORGES MICHEL and LUCIEN DELCAMBE*

Laboratoire de Biochimie Microbienne, Université Claude Bernard, Lyon I, 43 Boulevard du 11 Novembre 1918, 69621 Villeurbanne, France
*Centre National de Production et d'Etude des Substances d'Origine Microbienne (C.N.P.E.M.), 32 Boulevard de la Constitution, 4020-Liège, Belgique

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Thirty eight strains of *B. subtilis* were tested for the presence of antifungal antibiotics of the iturin group. The characterization of these antibiotics was made on the basis of: antifungal activity against *P. chrysogenum*, isolation and purification of the active substance, quantitative analysis of α-aminoacids and identification of the liposoluble dipeptide obtained by partial hydrolysis. The only antibiotics of the iturin group found were: iturin A, mycosubtilin and bacillomycin L.

Iturin was isolated by DELCAMBE from a strain of *Bacillus subtilis*[1]. More recently the fractionation of crude iturin was undertaken and three components were separated and purified: iturin A, iturin B and iturin C[2]. Iturin A has a strong antifungal activity, iturin B and iturin C have no activity.

Iturin A is a cyclic peptidolipid with seven D and L α-aminoacids and a liposoluble β-aminoacid, iturinic acid[2,3,4]. Iturin C has a similar structure differing only from iturin A by the nature of one α-aminoacid residue[5]. Other antibiotics of the same group were studied and the structures of mycosubtilin[6] and bacillomycin L[7] were recently determined. All these antibiotics have the same type of structure as iturin A including a liposoluble β-aminoacid.

In a previous work, we have shown that several antifungal preparations which were formerly obtained from various strains of *Bacillus subtilis* and described under various names had, in fact, iturin A as their only antifungal component[8]. This paper describes systematic investigations on a large variety of strains of *Bacillus subtilis* from various origins in view of the detection of antibiotics of the iturin group. The nature of each antibiotic has been determined by making use of precise criteria of identification.

Material and Methods

Strains of *B. subtilis*.

Strains n°1 to 12 were obtained from Dr. J. B. BARR, Royal Victoria Hospital, Belfast, U.K., strains n°13 to 17 from Dr. F. GUILLERMET, Institut Pasteur, Lyon, France, strains n°18 to 23 from Dr. H. LECHEVALIER, Rutgers University, New Jersey, U.S.A., strains n°24 to 27 from Dr. M. C. MYNARD, Institut Merieux, France, strains n°28 to 32 from Dr. J. SZULMAJSTER, Gif-sur-Yvette, France, strain A14 from Dr. E. P. ABRAHAM, University of Oxford, U.K., strain n°34 from Dr. WIAME, Institut de Recherche C.E.R.I.A., Bruxelles, Belgium, strain n°35 from Dr. NANDI, Bose Institute, Calcutta, India, strains n°36 and n°38 from Dr. SCHAEFFER, Institut Pasteur, Paris, France, strain n°37 from Dr. CERCOS, Inta, Castelar, Rep. of Argentina.

Cultures of Microorganisms and Antibiotics

The strains of *B. subtilis* were seeded on brain-heart medium (31 g/liter) and the growth was carried out from these cultures in the medium of LANDY *et al.*[9] at 33°C. *Penicillium chrysogenum* was seeded on the following medium, for 1 liter: glucose 10 g, saccharose 10 g, peptone 2 g, malt extract 4 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, agar 10 g. The methods of isolation and purification of antibiotics are described elsewhere[8].

Chromatography

Thin-layer chromatographies were performed on silica gel 60 or on cellulose powder (Merck, Darmstadt, GFR). Solvent systems used were: chloroform - methanol - water (65: 25: 4, in vol.) = solvent A; isopropanol - pyridine - acetic acid - water (40: 40: 5: 20, in vol.) = solvent B; pyridine - *tert.*-amylalcohol - water (35: 35: 30, in vol.) = solvent C; chloroform - methanol - water (56: 24: 3, in vol.) = solvent D. The products were detected by PAULY reagent or by ninhydrin reagent according to RUSSELL[10] at 140°C.

Hydrolysis

Total hydrolysis of antibiotics was performed by 6 N HCl at 150°C for 8 hours, partial hydrolysis by 6 N HCl at 105°C for 15 hours.

Tests for an Antibiotic Activity

The antibiotic activity of the *Bacillus subtilis* strains was tested on *Penicillium chrysogenum*.

(1) Direct method: *P. chrysogenum* was seeded on an agar medium in a Petri dish. The strain of *B. subtilis* was inoculated in the center of the dish, and incubated first for three days at 37°C then for two days at 28°C. An antibiotic activity of the strain of *B. subtilis* is characterized by a circular zone of inhibition of the growth of *P. chrysogenum*.

(2) Culture Medium: The strain of *B. subtilis* was grown for five days on the medium of LANDY *et al.*[9]. One ml of the culture was filtered on a Millipore filter (0.45 μ) and an aliquot (200 μl) was tested for antibiotic activity by the cylinder method. The inhibition of growth was observed after 4 days at 28°C.

(3) Thin-layer Chromatography of Culture Medium: An aliquot of the filtrate (50 μl) was submitted to thin-layer chromatography on silica gel in solvent A. The plate was overlayered with nutrient agar seeded with *P. chrysogenum*. After incubation for 4 days at 28°C, the spots of antibiotics were detected as zones of inhibition of growth of *P. chrysogenum*.

(4) Acid Precipitate: When a negative result was obtained with the preceding methods, another test was performed with a more concentrated preparation. Bacteria were eliminated from the culture by centrifugation and the clear supernatant was acidified to pH 2.0. The precipitate was collected, lyophilized and tested by thin-layer chromatography on silica gel as above.

Identification of the Antibiotic

(1) Thin-layer chromatography, either of culture media or of acid precipitates gave spots with characteristic Rf of either iturin, mycosubtilin or bacillomycin L (see Table 1). A further identification was carried out on pure antibiotics obtained from culture media after purification by chromatography according to the method described previously[8].

(2) A total hydrolysis of the purified products gave liposoluble and hydrosoluble moieties. The liposoluble part was extracted with chloroform and identified as β-aminoacids by thin-layer chromatography on silica gel with solvent A in comparison with standard β-aminoacids. The hydrosoluble part was analyzed qualitatively by thin-layer chromatography on cellulose powder with solvents B or C and quantitatively in an autoanalyser Technicon.

(3) The structure of the unknown antibiotics was confirmed by the following procedure: Each pure antibiotic was submitted to a partial hydrolysis (6 N HCl 105°C, 15 hours) and the liposoluble moiety was extracted with chloroform. This moiety was fractionated by thin-layer chromatography on silica gel with solvent A or D and gave, in addition to β-aminoacid, a peptide with a characteristic Rf. Previous work had shown that, in usual conditions of hydrolysis, liposoluble dipeptides were obtained owing to a more resistant peptide bond between the COOH group of an α-aminoacid and the NH_2 group of a β-aminoacid. The following peptides had been characterized: Ser→β-aminoacid

in iturin A, Asp→β-aminoacid in mycosubtilin and Thr→β-aminoacid in bacillomycin L.

The peptides obtained from the unknown antibiotics were identified by thin-layer chromatography on silica gel with solvents A and D in comparison with peptides prepared from authentic antibiotics (see Table 2).

Peptides prepared from thin-layer chromatograms gave, after a more drastic hydrolysis, liposoluble β-aminoacids which were characterized by thin-layer chromatography on silica gel in solvent A, and an α-aminoacid, which was identified by thin-layer chromatography on cellulose powder with solvents B and C in comparison with standard aminoacids.

Experimental Results

The results obtained with the various methods described under tests for antibiotic activity are summarized in Table 1.

Table 1. Characterization of the antibiotic activity in strains of *Bacillus subtilis*

Reference number	Strain of *B. subtilis*	Method 1	Method 2	Method 3*	Method 4*	Antibiotic
1	NCIB 8872	++	+	+ (0.16)		Bacillomycin L
2	2a	−	−	−		
3	4a	+	+	+ (0.16)		Bacillomycin L
4	7a	−	−	−		
5	8a	++	+	+ (0.16)		Bacillomycin L
6	9a	++	+	+ (0.16)		Bacillomycin L
7	13a	++	+	+ (0.16)		Bacillomycin L
8	14a	++	+	+ (0.16)		Bacillomycin L
9	2b	−	−	−		
10	15b	−	−	−		
11	2c	−	−	−		
12	MC	++	+	+ (0.16)		Bacillomycin L
13	877	−	−	−		
14	878	−	−	−		
15	3250	−	−	−		
16	9297	−	−	−		
17	9138	−	−	−		
18	IMRU 356 var. *niger*	+	−	−	+ (0.26)	Mycosubtilin
19	LL-CW 12a	−	−	−		
20	LL-Suka 2Bs	++	+	+ (0.26)		Mycosubtilin
21	LL-G66	−	−	−	+ (0.26)	Mycosubtilin
22	CW 2a	−	−	−		
23	7	−	+	+ (0.26)		Mycosubtilin
24	1005	++	−	−	+ (0.26)	Mycosubtilin
25	1804	−	−	−		
26	546	−	−	−		
27	547	−	−	−		
28	A_4	−	−	−		
29	A_5	−	−	−		
30	A_8	−	−	−		
31	B_1	−	−	−		
32	C_{20}	−	−	−		
33	A14	++	+	+ (0.16)		Bacillomycin L
34	$C_{44}B_9$ (glu-, pur-)	−	−	−		
35	443	+	−	−	+ (0.35)	Iturin A
36	SMYW (Marburg wild type)	−	−	−		
37	168 (Marburg indol-)	−	−	−		
38	Fungocine	++	−	−	+ (0.35)	Iturin A

* The values are Rf on thin-layer chromatography with solvent A.

Table 2. Identification of the antibiotic from strains of *Bacillus subtilis*

Strains of *B. subtilis* (Ref. Number see Table 1)	Composition of the antibiotic	Structure of liposoluble peptides*	Rf of liposoluble peptides		Nature of the antibiotic
			solvent A	solvent D	
	Asp_2, Glu_1, Ser_2, Thr_1, Tyr_1	Thr-βAA	0.40	0.37	Standard bacillomycin L
	Asp_4, Glu_1, Pro_1, Ser_1, Tyr_1	Asp-βAA	0.63	0.52	Standard mycosubtilin[6]
	Asp_3, Glu_1, Pro_1, Ser_1, Tyr_1	Ser-βAA	0.33	0.31	Standard iturin A[3]
1, 3, 5, 6, 7, 8, 12, 33	Asp_2, Glu_1, Ser_2, Thr_1, Tyr_1	Thr-βAA	0.40	0.33	Bacillomycin L
18, 20, 21, 23, 24	Asp_4, Glu_1, Pro_1, Ser_1, Tyr_1	Asp-βAA	0.63	0.52	Mycosubtilin
35, 37	Asp_3, Glu_1, Pro_1, Ser_1, Tyr_1	Ser-βAA	0.29	0.31	Iturin A

* βAA is the abbreviation for the liposoluble β-aminoacid.

Those recorded by the methods described under identification of antibiotics are shown in Table 2 together with the aminoacid composition of hydrolysates of standard antibiotics.

Discussion and Conclusions

(1) The only antibiotics which were found are: iturin A, mycosubtilin or bacillomycin L, mycosubtilin being the most widely distributed. The rate of production is variable with the strains; thus, the production of mycosubtilin by strain n°18 is very low.

(2) It is very likely that many non-purified antibiotics formerly isolated from several strains of *B. subtilis* and described under various names are actually identical with one of the three antibiotics of the iturin group. Thus the "bacilipins" which were found in 1949 by ABRAHAM *et al.*[11] in *B. subtilis* A 14 and not further studied are probably bacillomycin L which we have shown to be produced by this strain.

(3) The strain NCIB 8872 (n°1) is known to produce the bacillomycin of LANDY *et al.*[9] which has been recently named bacillomycin L. Strains n°2 to n°17 are "variants" obtained from leaf scars of plants sprayed with strain n°1. They differ from the original strain in their abilities to ferment glucose, grow anaerobically in glucose broth and reduce nitrate aerobically[12]. Only six amongst these variants produce bacillomycin L; thus, in five strains, a site of the biosynthesis of bacillomycin L has been affected by the mutation.

(4) The microorganism *P. chrysogenum* which was used in our tests for antifungal activity is very sensitive to all the known antibiotics of the iturin group. A lack of activity against this fungus can exclude the presence of an antibiotic of this group in a preparation, while of course leaving open the possibility of production of one or more antibiotics from other families.

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References

1) DELCAMBE, L.: Iturine. I. Préparation, purification et poids moléculaire. Bull. Soc. Chim. Belg. 74: 315~328, 1965
2) PEYPOUX, F.; M. GUINAND, G. MICHEL, L. DELCAMBE, B. C. DAS, P. VARENNE & E. LEDERER: Isolement

de l'acide 3-amino 12-méthyl tétradécanoïque et de l'acide 3-amino 12-méthyl tridécanoïque à partir de l'iturine, antibiotique de *Bacillus subtilis*. Tetrahedron 29: 3455~3459, 1973

3) PEYPOUX, F.: Thèse Doctorat ès-Sciences. Lyon, 1974
4) DELCAMBE, L.; F. PEYPOUX, M. GUINAND & G. MICHEL: Iturin and iturinic acids. Rev. Ferment. et Ind. Alimentaires 31: 148~151, 1977
5) PEYPOUX, F.; F. BESSON, G. MICHEL, L. DELCAMBE & B. C. DAS: Structure de l'iturine C de *Bacillus subtilis*. Tetrahedron, in press.
6) PEYPOUX, F.; G. MICHEL & L. DELCAMBE: Structure de la mycosubtiline, antibiotique isolé de *Bacillus subtilis*. Eur. J. Biochem. 63: 391~398, 1976
7) BESSON, F.; F. PEYPOUX, G. MICHEL & L. DELCAMBE: Structure de la bacillomycine L, antibiotique de *Bacillus subtilis*. Eur. J. Biochem. 77: 61~67, 1977
8) BESSON, F.; F. PEYPOUX, G. MICHEL & L. DELCAMBE: Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. J. Antibiotics 29: 1043~1049, 1976
9) LANDY, M.; G. H. WARREN, S. B. ROSEMAN & L. G. COLIO: Bacillomycin: an antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proc. Soc. Exp. Biol. & Med. 67: 539~541, 1948
10) RUSSELL, D. W.: Révélation des N-méthylaminoacides. J. Chromatogr. 4: 251~252, 1960
11) NEWTON, G. G. F.: Antibiotics from a strain of *Bacillus subtilis*: Bacilipin A and B and bacilysin. Brit. J. Exper. Pathol. 30: 306~319, 1949
12) SWINBURNE, T. R.; S. G. BARR & A. E. BROWN: Production of antibiotics by *Bacillus subtilis* and their effect on fungal colonists of apple leaf scars. Trans. Br. Mycol. Soc. 65: 211~217, 1975