STUDIES ON THE BIOSYNTHESIS OF β-AMINO ACIDS, THE LIPID MOIETY OF ITURINS A, IN *BACILLUS SUBTILIS*

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The biosynthesis of the β-amino acid components of iturins A was studied in comparison to the biosynthesis of fatty acids. Palmitic acid was incorporated into the lipid moiety of iturins A when it was added to the culture medium of the iturin producer *Bacillus subtilis*. Addition of unlabeled palmitic acid enhanced the formation of straight-chain β-amino acids and addition of valine or leucine increased the production of branched β-amino acids. These mod fications correlated with modifications in the corresponding biosynthesized fatty acids.

Iturins A, antifungal antibiotics, are produced by *Bacillus subtilis*[1]. They are cyclolipopeptides containing 7 residues of α-amino acids and one β-amino acid residue with a *n*-C_{14}, *iso*-C_{15}, *anteiso*-C_{15}, *iso*-C_{16} or *n*-C_{16} carbon chain[2,3]. The influence of the culture medium on iturins A production has been determined[4]: The best carbon sources for iturin production were mannitol, fructose and sucrose. Among the amino acids which are components of iturins A, L-asparagine was the best substrate for antibiotic biosynthesis.

In a previous study, the biosynthesis of the β-amino acid constituents of bacillomycins F were studied[5]. Bacillomycins F differ from iturins A by the presence of a threonyl instead of a seryl residue in the peptide moiety and by the nature of the carbon chain of β-amino acids; in bacillomycins F, β-amino acids are *iso*-C_{15}, *anteiso*-C_{15}, *iso*-C_{16}, *n*-C_{16}, *iso*-C_{17} and *anteiso*-C_{17}. Branched α-amino acids and threonine have been found to increase the synthesis of specific β-amino acids of bacillomycins F and specific cellular fatty acids of *B. subtilis*.

In this paper, we describe the effect of palmitic acid and α-amino acids on iturins A production and on the nature of the carbon chain of the β-amino acid components of iturins A.

Materials and Methods

Radioactive Chemicals

[1-^{14}C]Palmitic acid (2 GBq/mM) (1 GBq=27 mCi) and [^{14}C]isoleucine (9 GBq/mM) were obtained from the Commissariat à l'Energie Atomique, Saclay, France.

Organism and Cultivation

A strain of *B. subtilis* producing iturins was kindly supplied by Dr. L. DELCAMBE, C.N.P.E.M., Liège, Belgium. It was grown at 35°C for 100 hours on LANDY medium[6]. In some experiments, D,L-α-amino acids or palmitic acid were added to the medium and the pH was adjusted to 7.5 before autoclaving.

Purification and Titration of Iturins A[1,4]

Crude antibiotic preparation was obtained by acidification, pH 2, of the culture medium. The precipitate was purified by column chromatography on silicic acid. Iturins A production was determined by colorimetric measurement of β-amino acids as 2,4-dinitrophenyl derivatives.

Gas Chromatography (GC)

Fatty acid methyl esters were analyzed by GC on SP 2100 capillary column with an IGC 120 FL Intersmat apparatus. Temperature conditions were from 140 to 240°C at a rate of 2°C per minute. β-Amino acids were analyzed by GC as described previously[7].

Results

Effect of α-Amino Acids on the Biosynthesis of the Carbon Chain of β-Amino Acids

B. subtilis was grown for 100 hours in LANDY medium containing D,L-leucine, D,L-valine or D,L-isoleucine at various concentrations. Iturins A production was enhanced (Table 1) by addition of leucine and valine (1 to 8 g/liter) and by addition of isoleucine only at the lowest concentration; at the highest concentrations of isoleucine, a decrease of antibiotic production was observed.

Iturins A were isolated from the culture media, purified and hydrolyzed; β-amino acids were obtained from the hydrolysate by solvent extraction, derivatized and analyzed by GC. The results are shown in Table 2. The addition of valine or leucine increased (about 2-fold) *iso*-C_{16} or *iso*-C_{15} β-amino acids, respectively. An unexpected result was the increase of *n*-C_{14} β-amino acid caused by addition of valine and isoleucine; moreover isoleucine did not enhance the synthesis of *anteiso*-C_{15} β-amino acid.

In order to confirm the incorporation of isoleucine into the carbon chain of β-amino acids, [^{14}C]isoleucine (1.85 MBq/100 ml of LANDY medium) was added to a 2.5-hour old culture of *B. subtilis* and the radioactivities of iturins A and β-amino acids were measured after 100 hours. The results — 24×10^3 dpm/μmol for iturins A and 19×10^3 dpm/μmol for β-amino acids — show that

Table 1. Effect of α-amino acids on iturin production.

Amino acid added (g/liter)		Growth (OD at 600 nm)	Antibiotic (μM)	Antibiotic yield (μmol/OD_{600} unit)
Control	0	6.2	60	10
Valine	1	5.8	90	15
	2	6.2	80	13
	4	6.8	76	11
	8	8.0	73	9
Leucine	1	5.4	58	11
	2	3.8	70	18
	4	4.2	75	18
	8	5.3	72	14
Isoleucine	1	3.8	60	16
	2	4.4	45	10
	4	4.4	30	7
	8	3.7	15	4

Table 2. Effect of amino acids on β-amino acids of iturins A.
The results are expressed in percent of total β-amino acids.

Amino acid added (g/liter)		Nature of the carbon chain				
		n-C_{14}	*iso*-C_{15}	*anteiso*-C_{15}	*iso*-C_{16}	*n*-C_{16}
Control	0	24	40	21	9.0	6.0
Valine	1	52	20	15	7	7
	2	56	20	13	6	5
	4	45	20	11	19	5
	8	51	20	10	16	3
Leucine	1	33	52	12		3
	2	29	59	10		
	4	28	72			
	8	30	70			
Isoleucine	1	65	5	24		6
	2	64	5	24		8
	4	72		25		3
	8	68		24		7

Table 3. Effect of amino acids on cellular fatty acids.
The results are expressed in percent of total fatty acids.

Amino acid added (g/liter)		Nature of the carbon chain							
		iso-C_{14}	*n*-C_{14}	*iso*-C_{15}	*anteiso*-C_{15}	*iso*-C_{16}	*n*-C_{16}	*iso*-C_{17}	*anteiso*-C_{17}
Control	0	3	2	9	34	6	32	5	9
Valine	1			8	25	5	51		5
	2	4		10	30	8	39	4	6
	4	11		11	36	17	14	5	7
	8	14		10	33	19	14	4	6
Leucine	1	3	3	35	33		14	8	4
	2		3	43	32		11	8	3
	4		2	52	25		10	9	2
	8		3	51	25		8	10	3
Isoleucine	1		5		17		69		8
	2		5		15		60		20
	4		4		16		56		24
	8		4		23		62		11

isoleucine was mainly incorporated into β-amino acids and only to a small extent into the peptide moiety.

Threonine was also added at 8 g/liter concentration to the culture medium. Iturins A β-amino acids were analyzed by GC. Only three compounds were identified: *n*-C_{14}, *iso*-C_{15} and *anteiso*-C_{15} β-amino acids (50%, 28% and 21% of total β-amino acids respectively). Thus, threonine did not increase the *anteiso*-C_{15} β-amino acid but enhanced the *n*-C_{14} β-amino acid component by a factor two.

Effect of Branched α-Amino Acids on the Biosynthesis of Cellular Fatty Acids

The role of branched α-amino acids in the synthesis of β-amino acids was compared with their role as precursors of fatty acids. Fatty acids of *B. subtilis* grown in various media were prepared according to ITO *et al.*[8] and analyzed as methyl esters by GC. The results are shown in Table 3. The modifications of the fatty acid composition were roughly similar to those of β-amino acids: Valine or leucine increased even *iso* or odd *iso* fatty acids, respectively, and isoleucine increased unbranched C_{14} and C_{16} fatty acids. In addition, isoleucine gave an important decrease of *anteiso*-C_{15} fatty acid.

Incorporation of Palmitic Acid into β-Amino Acids

We then tested the possibility of a biosynthetic pathway from fatty acids to β-amino acids by measuring the incorporation of [1-^{14}C]palmitic acid in iturins A. [1-^{14}C]Palmitic acid (3.7 MBq/100 ml of culture medium) was added at the middle logarithmic phase of growth. Iturin production was determined after 100 hours of culture: The antibiotic concentration was 60 μM. Iturins A were purified and hydrolyzed as described previously; the radioactivities of antibiotics and lipid moieties were determined: 73% of the radioactivity incorporated in iturins A (139×10^3 dpm/μmol) was found in the β-amino acids (101×10^3 dpm/μmol).

When unlabeled palmitic acid was added to the culture medium at 0.5 g/liter concentration, the analysis of iturins A β-amino acids by GC showed an increase of straight-chain β-amino acids: 33% instead of 24% in the control for *n*-C_{14} and 11% instead of 6% in the control for *n*-C_{16}.

Discussion

In this work, we tested some potential precursors of iturins A β-amino acids.

Leucine was found to increase the production of *iso*-C_{15} β-amino acid and *iso*-C_{15} fatty acid. This result is in agreement with the effect previously reported for cellular fatty acids of *B. subtilis*[9] and β-amino acids of bacillomycins F[5]; in the latter case, leucine had been demonstrated to be the precursor of *iso*-C_{15} and *iso*-C_{17} β-amino acids.

Valine was found to increase *iso*-C_{16} and *n*-C_{14} β-amino acids. The increase of *iso*-C_{16} β-amino acid of iturins A, as that of bacillomycins F[5], corresponds to an increase of the *iso*-C_{16} fatty acid. Valine is known to be a precursor of even-chain *iso*-fatty acids through isobutyrate[9] but valine catabolism can also give *n*-butyrate[10,11] which is a precursor of straight-chain fatty acids. Thus, the effect of valine on the biosynthesis of *iso*-C_{16} and *n*-C_{14} β-amino acids could be explained by a process related to the biosynthesis of fatty acids.

A puzzling result was obtained when isoleucine or threonine was added to the culture medium of the iturin producer *B. subtilis*: The amount of *anteiso*-C_{15} β-amino acid was not significantly modified and the amount of *n*-C_{14} β-amino acid strongly increased. Similar effects were observed on the biosynthesis of fatty acids. As isoleucine and threonine are usually the precursors of odd-chain *anteiso*-fatty acids through 2-methyl butyrate[9], these results cannot be easily explained.

In the absence of isoleucine or threonine, the composition of fatty acids in the strain of *B. subtilis* producing iturins shows an unusual distribution of *n*-C_{16} and *anteiso*-C_{15} fatty acids (32% and 4% of total fatty acids, respectively) while in other studied strains of *B. subtilis*, the distribution is about 6% for *n*-C_{16} and 30~40% for *anteiso*-C_{15}[5,9]. Thus, it appears that the strain producing iturins has a poor ability to synthetize *anteiso*-C_{15} fatty acids and this fact cannot be changed by addition of isoleucine or threonine.

In any case, branched α-amino acids and threonine are precursors of the lipid moiety of iturins A as they are the precursors of fatty acids. The β-amino acids of iturins A probably arise from homologous fatty acids as suggested by the incorporation of palmitic acid into β-amino acids. The mechanism of synthesis of β-amino acids from fatty acids is currently being studied.

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