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Influence of Culture Conditions on Lipopeptide Production by *Bacillus subtilis*

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Abstract

Bacillus subtilis produces various families of lipopeptides with different homologous compounds. To produce "new molecules" with improved activities and to select strains that produced a reduced number of homologs or isomers, we studied the effects of different media on the nature of the synthesis of fatty acid chains for each lipopeptide family. This study focused on two *B. subtilis* strains cultivated in flasks. Optimized medium for lipopeptide production and Landy medium modified by replacing glutamic acid with other α-amino acids were used. We found that the intensity of production of homologous compounds depends on the strain and the culture medium. Analysis of these lipopeptides by high-performance liquid chromatography showed that the strain *B. subtilis* NT02 yielded various homologous compounds when cultivated in Landy medium (L-Glu), but primarily one homologous product in high relative amounts when cultivated in the optimized medium. Mass spectrometric analysis and determination of the amino acid composition of this molecule enabled us to identify it as Bacillomycine L c15.

Index Entries: *Bacillus subtilis*; lipopeptide; biosurfactant; bacillomycin; high-performance liquid chromatography; culture medium.

Introduction

The *Bacillus subtilis* lipopeptides are members of a particular antibiotic class formed by the iturin, surfactin, and fengycin families. The general structure of these lipopeptides is a peptide cycle of 7 (iturin and surfactin)

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or 10 amino acids (fengycin) linked to a fatty acid chain. The length of the fatty acid chains can vary from C-13 to C-16 for surfactins, from C-14 to C-17 for iturins, and from C-14 to C-18 for fengycins, giving different homologous compounds and isomers (*n*, *iso*, *anteiso*) for each lipopeptide *(1–5)*. Such molecules are of great interest because of their biologic and physicochemical properties, which can be exploited in food, oil, and pharmaceutical industries *(6–9)*. Iturin A and fengycin have a wide antifungal activity *(5,10,11)*. In addition, iturin A possesses an antibacterial spectrum, though it is restricted to a few bacteria such as *Micrococcus luteus (12,13)*. Surfactin exhibits a larger antibacterial spectrum *(14)*, but it is primarily one of the most powerful biosurfactants known *(2,15)*. At their critical micellar concentration, surfactin S1 and iturin A diminish surface tension of water from 72 to 31 and 54.5 mN/m, respectively *(16)*. Furthermore, surfactin exhibits antitumoral, antiviral, antimycoplasma, and fibrin clot-inhibiting activities *(2,17,18)*. No interfacial studies have been available until recently for fengycin.

Several studies have shown the existence of a relationship between the structure of these molecules and their properties. Hbid *(19)* and Bland et al. *(20)* demonstrated that the antifungal and hemolytic activities of these agents are enhanced with increasing number of carbon atoms of their fatty acid side chains, presumably owing to stronger interactions with biomembranes. Concerning the effect of peptide molecular attributes, the substitution of L-asparagine 1 in iturin A by acid L-aspartic in iturin C and the esterification of tyrosine 2 residue in iturin A diminish drastically their biologic activities *(10,21)*. Thimon et al. *(22)* demonstrated that the Glu-γ-methylester of surfactin has a much lower critical micellar concentration of 30 μ*M* than natural isomer (240 μ*M*) and showed 100% hemolysis at an appreciable lower concentration (12 μ*M*) compared with the nonesterified compound (200 μ*M*).

Unfortunately, applications of these biomolecules are limited by the cost of their production and purification. In addition, some *B. subtilis* strains coproduced various families and homologs *(23)*, causing additional purification problems.

To produce "new molecules" with improved activities and to select strains that produce a reduced number of homologous compound or isomers, we studied the effect of α-amino acids on the nature of the fatty acid chains for each lipopeptide family. This provided information about specificity of the biosynthetic enzymatic system for each lipopeptide family. Two *B. subtilis* strains were chosen among a collection of *Bacilli* conserved in our laboratory. *B. subtilis* S499 was chosen for its ability to produce the three lipopeptide families in order to determine the effect of α-amino acid pool on the production of each lipopeptide family of homologous compounds. Also chosen was *B. subtilis* NT02, which produces only iturins and is sensitive to the culture medium. The results obtained are presented herein.

Table 1
Optimized Medium Composition
per Liter of Distilled Water

Medium components	Quantity
Sucrose	20 g
Peptone	30 g
Yeast extract	7 g
KH_2PO_4	1.9 g
$MgSO_4$	0.450 g
Trace elements solution	9 mL[a]

[a]Composition of trace elements per liter of distilled water is 0.001 g of $CuSO_4$, 0.005 g of $FeCl_3$, 0.004 g of $NaMnO_4$, 0.002 g of KI, 0.014 g of $ZnSO_4$, 0.01 g of H_3BO_3, 0.0036 g of $MnSO_4$, 10 g of citric acid.

Materials and Methods

Strains

B. subtilis S499 was a gift from L. Delcambe (CNPEM, Liège, Belgium). This strain was collected in Ituri, Congo (formally Zaïre) *(1)*, and *B. subtilis* NT02 was isolated in CWBI (Wallon Center of Industrial Biology, Belgium) from *netetu*, a Senegalese fermented food.

Culture Media

Strains were cultivated on Jacques et al. *(23)* medium called optimized medium (Table 1), Landy medium *(24)*, and Landy media modified by replacing the L-glutamic acid with various L-α-amino acids at the same concentration (5 g/L). Landy medium contained the following per liter of distilled water: 20 g of glucose, 5 g of L-glutamic acid, 0.5 g of $MgSO_4$, 0.5 g of KCl, 1 g of KH_2PO_4, 0.0012 g of Fe_2SO_3, 0.0014 g of $MnSO_4$, 0.0016 g of $CuSO_4$. The solutions were brought to pH 7.0 with 5 *N* KOH or 5 *N* H_3PO_4, according to whether the amino acid used was acid or basic, before sterilizing.

Bacterial Culture and Extraction of Lipopeptide

The bacteria were grown in 100 mL of medium contained in 500-mL Erlenmeyer flasks stirred at 130 rpm at 30°C. For culture seeding, 10 mL of a preculture grown under the same conditions for 16 h was used. The culture was stopped after 96 h and the cell material was removed by centrifugation at 15,300*g*. Lipopeptides were extracted from the culture supernatant by solid-phase extraction on bond elut C18 (5 g) (Varian, CA) as described by Razafindralambo et al. *(25)*. The extract was brought to dryness, the residue was redissolved in $CHCl_3/CH_3OH$ (2:1 [v/v]) and separated by chromatography on silicagel 60 with the solvent $CHCl_3/CH_3OH/H_2O$ (65:25:4 [v/v/v]). Subsequently, the fengycins were eluted with $CHCl_3$/

$C_2H_5OH/CH_3OH/H_2O$ (7:3.5:3:1.5 [v/v/v/v/v]). The flow rate was about 2 mL/min, and the glass column was 30 cm long and had a 2-cm id.

Purification and Measurement of Lipopeptide

The lipopeptide extract was dissolved in methanol and analyzed by high-performance liquid chromatography (HPLC) on a C18 column (5 µm, 1 × 25 cm) (Chrompack, Middelburg, The Netherlands). Each family of lipopeptides was separately analyzed. Iturins were analyzed with the solvent acetonitrile/water/trifluoroacetic acid (TFA) (40:60:0.05 [v/v/v]), surfactins with the solvent acetonitrile/water/TFA (80:20:0.05 [v/v/v]), and fengycins with a gradient of 0.05% TFA in water and acetonitrile (60:40 for 25 min, 65:35 for 15 min). Solvents were HPLC grade. TFA was from Sigma (St. Louis, MO). The flow rate was 1 mL/min, detection wavelength was 214 nm (HP 1100; Waldbronn, Germany), and sample injection volume was 20 µL. Mass spectra analysis was performed on a VG platform (Fison, Manchester, UK) with an electrospray source. Samples were dissolved in 1 mL of a mixture of $CH_3OH/H_2O/CH_3COOH$ (50:50:0.1 [v/v/v]). Amino acid determinations were carried out according to the Stein and Moore method (Pharmacia Alpha Plus, Uppsala, Sweden).

Results

Production of Lipopeptide by B. subtilis *S499*

The production of different homologous compounds from the three families of lipopeptides produced by *B. subtilis* S499 in the optimized medium, Landy medium, and Landy media modified with L-leucine, L-valine, L-isoleucine, and L-threonine was analyzed. To avoid complex purification procedures, a proportion of the different homologous compounds was evaluated by ESI-MS. The behavioral similarity of these homologous congeners during the ionization process of mass spectrometry (MS) was confirmed by MS analysis of known samples of iturin and surfactin. This test related to *B. subtilis* S499 cultivated in the optimized medium. Figure 1A,B shows the proportions of iturin and surfactin of various homologous compounds analyzed by HPLC and electrospray ionization (ESI)-MS. The general tendency (height of the homologous peaks) for the two types of analysis is similar for surfactin (c15 > c14 > c13) and iturin homologous compounds (c14 > c15 > c16). This comparison could not be made for fengycins because their homologous compounds are not formally identified yet by hplc analysis.

Surfactin Homologous Compounds

Figure 2A–C shows the proportions of lipopeptide homologous compounds analyzed by ESI-MS. The use of Val, Leu, Ile, Thr, and optimized medium modified the percentages of the homologous compounds of surfactin (Fig. 2A). The rich medium allowed the synthesis of homologous

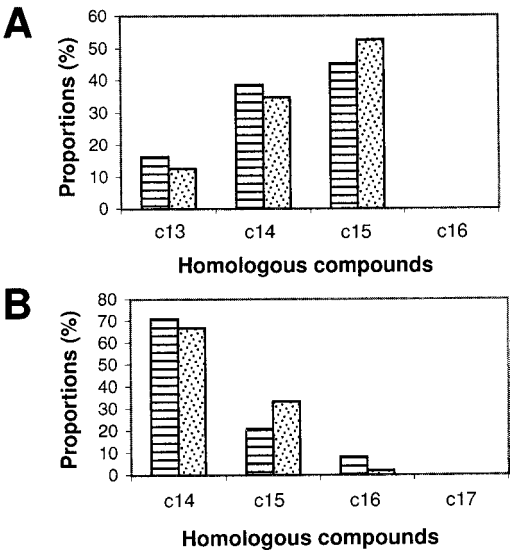

Fig. 1. Proportions of iturin and surfactin determined by HPLC (▨) and MS (▤) analysis. (**A**, surfactin homologs; **B**, iturin homologs.)

compounds c13, c14, and c15 with a higher proportion of c15 β-hydroxy fatty acid followed by the c14 chain. We did not observe production of surfactin homologous compound with the 16 carbon atom chain. The addition of Val gave equal proportions of c13 and c15 β-hydroxy fatty acid, which were less than for the c14 carbon chain. Unlike the optimized medium, we observed production of c16 homologous compound. These results agree with the literature, since Val is known to be a precursor of even fatty acid carbon chains *(26)*. The homologous S2 c13 produced with this medium is an isoform of the standard surfactin S1 c13. In this homologous S2, one of the Leu residues of surfactin S1 is replaced by a Val residue *(27)*. The addition of Leu and Ile, respectively, increases the proportion of c13 and c15 β-hydroxy fatty acid chain. This can be explained by the fact that these two α-amino acids are known to be precursors of odd β-hydroxy fatty acid chains *(28)*. Nevertheless, we observed the production of homologous congener with 16 carbon atoms. The more prominent increase was observed with c15 β-hydroxy fatty acid when we used Thr. We observed also the best proportion of c16 and the production of a small quantity of c17. Thus, this is the first time that the presence of such a long carbonaceous chain has been described according to surfactin. This positive effect of Thr on the long chains of surfactin cannot be easily explained.

Iturin Homologous Compounds

On the other hand, with the use of the optimized medium, Ile and Thr gave a strong percentage of the c14 β-amino fatty acid (>60%) of the iturin family (Fig. 2B). The proportion of c15 fatty acid chain was slightly higher

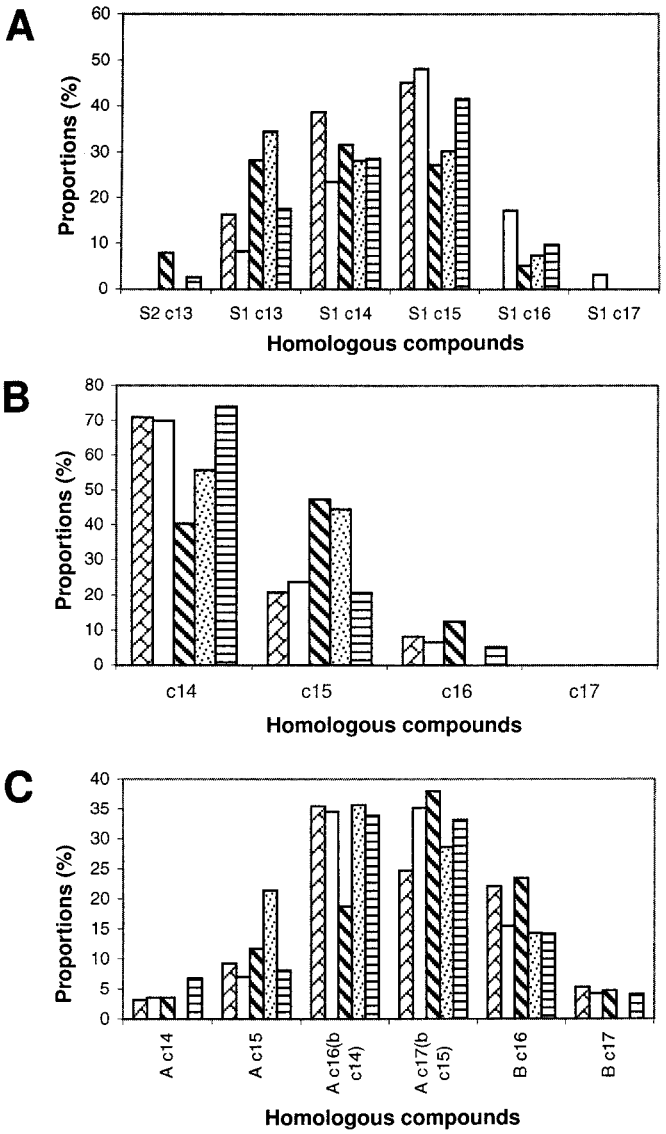

Fig. 2. MS analysis of homologous compounds produced by *B. subtilis* s499 grown in various culture media. **(A)** Surfactin homologous compounds; **(B)** iturin homologous compounds; **(C)** fengycin homologous compounds. ⊠, optimized medium; □, Landy L-threonine; ▨, Landy L-valine; ⊡, Landy L-leucine; ▤, Landy L-isoleucine.

than 20% and the c16 was <10%. The use of Leu increased the rate of c15 β-amino fatty acid (44%), but this rate remained lower than its c14 congener (56%). This result is in agreement with the effect previously reported for cellular fatty acid biosynthesis of *B. subtilis (26)*. In addition, leucine has been demonstrated to be the precursor of *iso*-c15 β-amino fatty acids *(29)*. The addition of Val gave the best production of iturin c16. This is under-

standable because Val is known to be a precursor of even fatty acid, but we had an unexpected result with a decrease in the c14 rate (40%) and an increase in the c15 rate (47%).

Fengycin Homologous Compounds

Concerning the fengycin homologous compounds (Fig. 2C), the analysis showed a tendency toward a "bell shape" according to the intensity of production of the various homologous. Fengycin A c16 (B c14) and A c17 (B c15) had the most intense peaks. They were flanked on both sides by the homologous A c14, A c15, B c16, and B c17. The most intense part of the peaks can be explained by the simultaneous presence of the homologous compounds fengycin A c16 and B c14, on the one hand, and fengycin A c17 and B c16, on the other hand. These two different types of fengycin are owing to the presence of an alanine residue (molecular weight of 71 kDa) in fengycin A and a valine residue (molecular weight of 99 kDa) in fengycin B, giving a difference of 28 kDa. The use of optimized medium and Leu medium resulted in fengycin A c16 (B c14) having the most intense production (35%), followed by the homologous A c17 (B c16), with 24–28%. In addition, the Leu medium allowed production in a strong proportion of fengycin A c15. The addition of Val gave an unexpected result, because fengycin A c17 (B c15) production was the most intense (38%). The proportions of fengycin homologous A c16 (B c14) and A c17 (B c15) were simultaneously most intense with Thr (34%) and Ile (34%). Then, the proportion decreased gradually on both sides. Note that results in the case of fengycin homologous compounds are difficult to explain because there is an overlapping of molecular mass of the homologous congeners of fengycin A and B.

Characterization of Iturin Homologous Compounds a, b, and c Produced by B. subtilis *NT02*

B. subtilis NT02 produces only antibiotic molecules belonging to the iturin family, not the surfactin or fengycin families. It was grown for 96 h in Landy medium. HPLC of the homologous compounds produced is shown in Fig. 3. To identify these homologous iturins whose retention times do not correspond to those of iturin A, they were purified with semipreparative HPLC. These purified peaks were analyzed by ESI-MS and amino acid composition was determined. The molecular ions $(M+K)^+$ of the iturin homologous compounds a, b, and c produced were, respectively, at *m/z* 1059.3, 1073.3, and 1073.3. Table 2 gives the amino acid composition of these various peaks. Six different amino acids were given with a constant ratio. Regarding these results, we concluded that these homologous compounds are, respectively, Bacillomycin L c14 and Bacillomycin L c15. The two *m/z* 1073.3 were owing to the presence of two isomers (*iso*, *anteiso*, or normal) of the c15 homolog.

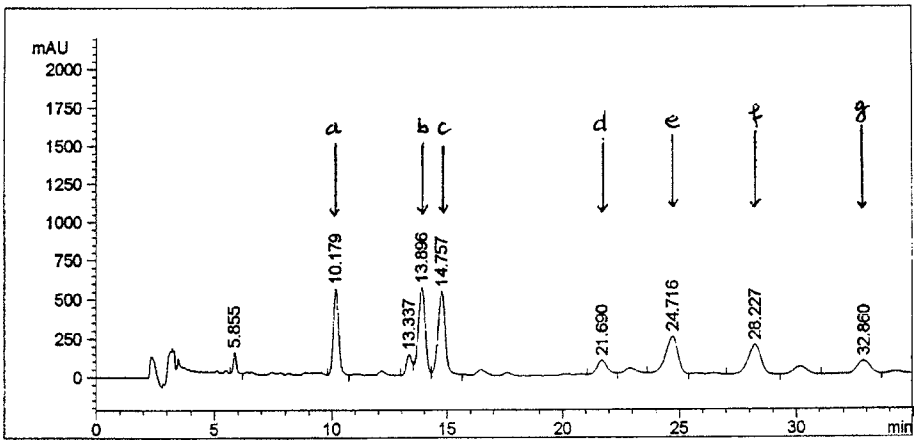

Fig. 3. HPLC analysis of iturin family lipopeptide produced by *B. subtilis* NT02 cultivated in Landy medium.

Table 2
Amino Acid Composition, Molecular Weight, and Identification of Main Different Molecules of Bacillomycin Purified by HPLC

Peak	Amino acid composition	Molecular weight $(M+K)^+$	Identification
a	2Asx, 1Tyr, 2Ser, 1Glx, 1Thr	1059	Bacillomycin L c14
b	2Asx, 1Tyr, 2Ser, 1Glx, 1Thr	1073	Bacillomycin L c15
c	2Asx, 1Tyr, 2Ser, 1Glx, 1Thr	1073	Bacillomycin L c15

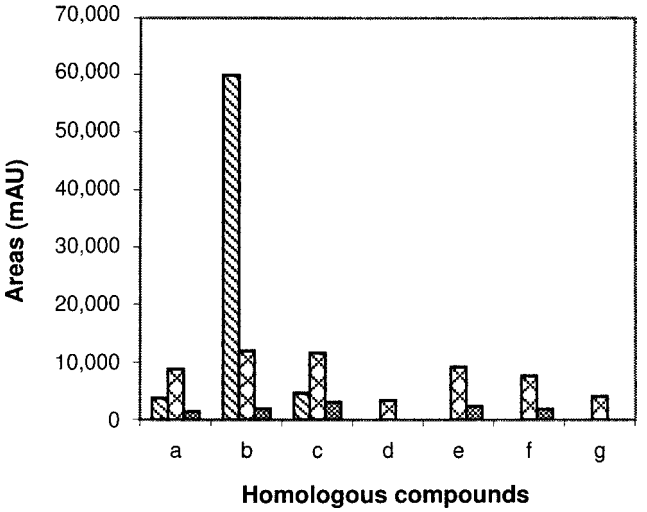

Fig. 4. Iturin family homologous compounds produced by *B. subtilis* NT02 cultivated in various media. ▨, optimized medium; ▩, Landy L-glutamic acid; ⁘, Landy L-serine.

Production of Iturin Homologous Compounds by Strain NT02 According to Culture Medium

To study the influence of the culture medium on the production of iturin homologous compounds, strain NT02 was grown for 96 h in the optimized medium, Landy L-serine, and Landy L-proline media. The intensities of the principal peaks analyzed by HPLC are represented in Fig. 4. Several iturin homologs were produced by NT02 when cultivated in Landy medium and in Landy media modified with L-serine and L-proline (data not shown). However, NT02 produced mostly one homologous compound when it was cultivated in the optimized medium.

Discussion

This work consisted of studying the influence of the culture medium on lipopeptide production by *B. subtilis*. The aim was to produce "new molecules" with improved activities and to select strains that produce a reduced number of homologous compounds or isomers according to the culture medium. It was shown that the modification of the culture media influences the proportion of surfactin homologous compounds produced by *B. subtilis* S499. This influence, as opposed to what Hourdou *(30)* revealed, can relate to the length of the carbonaceous chain, as observed with the medium of Landy L-threonine. Nevertheless, this variation in proportion does not mean the total suppression of one of the principal surfactin homologous compounds (c13, c14, c15). Nor does it produce an unusual number of carbon, except for the homologous c17 observed in a very small quantity in the production resulting from the medium Landy L-Thr, which constitutes a first.

Concerning the iturins, *B. subtilis* S499 invariably produced more homologous compound with 14 carbon atoms than 15 or 16 carbons, excluding production in Landy L-valine medium. However, this handicap was quickly overcome by the intensity of production.

Overall, the influence of the culture medium in the production of iturin homologous molecules was less significant than that of the surfactins. This was owing to the fact that the enzymatic complex responsible for surfactin biosynthesis would be less sensitive to the intracellular fatty acid chain pool than that of iturin A. Indeed, it has been shown that the enzymatic system of the biosynthesis of the surfactins is not very specific *(27)* but little is known about biosynthesis of the iturin family. Some amino acids such as threonine probably would be favorable to the lengthening of the fatty acid chain and others (ramified α-amino acid) rather than the formation of isomers *i*, *ai*, and *n (31)*. However, these α-amino acids would not have a great influence in producing high carbonaceous chains. It has been suggested that the responsible enzyme system for β-amino acid synthesis selects among the cellular fatty acids those that correspond to the carbon chain of β-amino acid *(29)*.

Of the two studied strains, *B. subtilis* NT02 was shown to be the most efficient for obtaining less homologous compounds. This strain yielded various compounds when cultivated in Landy media but primarily one homologous product in very strong intensity when cultivated in optimized medium. MS analysis and determination of amino acid composition of this compound showed that it is Bacillomycin L c15. This molecule belongs to the iturin family and possesses biologic properties similar to those of iturin A *(11)*. The length of carbon chain of this principal molecule is a great advantage. This will enable us to reduce purification problems and improve biologic properties, as suggested by Hbid *(19)*.

Conclusion

Modification of the culture medium influences the proportion of surfactin homologous compounds produced by *B. subtilis* S499. Threonine would be favorable to the lengthening of the fatty acid chain. The influence of the culture medium in the production of iturin homologous molecules was less significant than that of the surfactins. This would be owing to the fact that the enzymatic complex responsible for surfactin biosynthesis would be less sensitive to intracellular fatty acid chain pool than that of iturin A. *B. subtilis* NT02 yielded various compounds when cultivated in Landy media but primarly one homologous product (Bacillomycin L c15) in very strong intensity when cultivated in optimized medium. This will enable us to reduce purification problems. The length of carbon chain would be a great advantage for biologic properties.

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