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NOTE

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Influence of Leucine and Valine on Ramoplanin Production by *Actinoplanes* sp. ATCC 33076

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Abstract Ramoplanin is a glycolipodepsipeptide antibiotic obtained by fermentation of the *Actinoplanes* sp. ATCC 33076, isolated as a complex of three closely related components A1, A2 and A3, which differ in their fatty acid moiety. We have investigated the influence of L-leucine and L-valine, the biosynthetic precursors of the fatty acids in A2 and A3 factors, on the complex composition and antibiotic productivity. Addition of 5 g/litre of L-leucine at the time of inoculation increases antibiotic production and improves the production of A2 factor, which represents the active principle component under clinical development. Addition of L-valine in the same conditions modifies the composition of the complex towards the A3 factor but does not improve total antibiotic productivity. A possible explanation for the different actions of the two amino acids is presented.

Keywords ramoplanin, antibiotic production, biosynthetic precursors, L-leucine, L-valine

Ramoplanin is a glycolipodepsipeptide antibiotic produced by fermentation of an *Actinoplanes* strain deposited in the American Type Culture Collection as ATCC 33076. Its structure consists in a cyclic peptide formed from sixteen amino acids and one side branch amino acid, which is acylated by a doubly unsaturated fatty acid. A disaccharide residue (D-mannosyl-D-mannose) is attached to the cyclic peptide [1] (Fig. 1). The antibiotic was first isolated as a complex of three closely related components A1, A2 and A3 of which A2 is the most abundant. These factors differ in the length and branching of the fatty acid residue [2] (Fig. 1). Ramoplanin component A2 is highly active against numerous Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) [3, 4], vancomycin-resistant *Enterococcus faecium* (VREF) and those Gram-positive bacteria resistant to ampicillin and erythromycin [1, 4]. Ramoplanin is presently in Phase III clinical trials for eradication of VRE and MRSA, and in Phase II for the treatment of *Clostridium difficile*-associated diarrhea. (http://biotechnologyhealthcare.com, source: Genome Therapeutics, June 2004).

Previous observations reported in an US patent by Lancini *et al.* [5] showed that the addition of different biosynthetic precursors (*i.e.* isobutyric acid, valine, leucine, isovaleric acid and ethyl butyrate) to the fermentation medium changes the composition of the complex. This paper deals with the influence of leucine and valine on the relative and absolute amounts of the complex components, and on their role as the biosynthetic precursors of fatty acid chains in components A2 and A3, respectively.

Materials and Methods

Strains and Cultural Conditions

Actinoplanes sp. ATCC 33076 was maintained lyophilised in vials stored at 4°C. The content of lyophilised vials was suspended in physiological solution and used to seed

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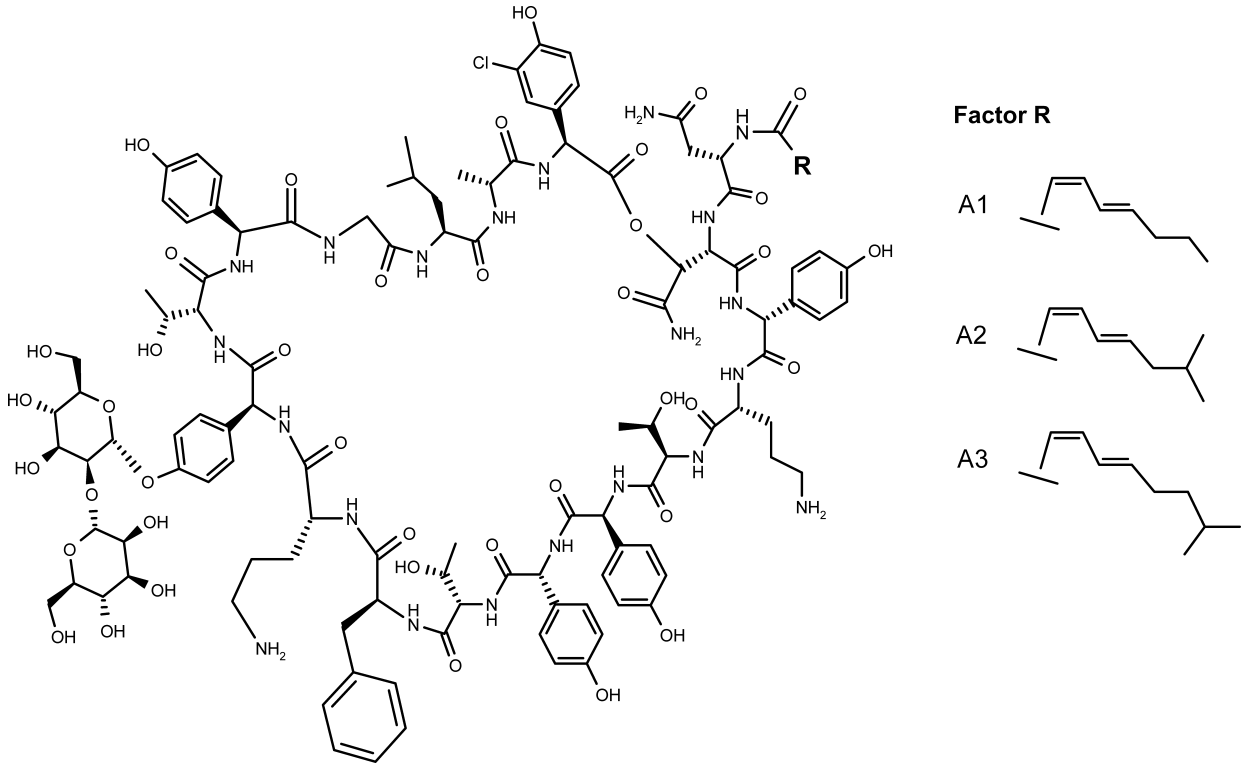

Fig. 1. Structure of the different components in the ramoplanin complex, varying in the fatty acid moiety.

oatmeal agar slants (grams per litre: oatmeal, 60; agar filii, 20; tap water up to 1 litre). The slants were incubated at 28°C for 8 days and then used to inoculate 100 ml of vegetative medium VB (grams per litre: glucose, 12; soluble starch, 13; soybean meal, 13; $CaCO_3$, 4; demineralised water up to 1 litre) in 500-ml baffled flasks. Flasks were incubated at 28°C for 96 hours on a rotary shaker set at 200 rpm. 10% of pre-culture was used to inoculate 30 ml production medium PB (grams per litre: glucose, 4; maltose, 20; starch, 4; glycerol, 20; sucrose, 20; soybean meal, 30; $CaCO_3$, 6; tap water up to 1 litre) incubated in 300-ml baffled flasks at 28°C at 200 rpm. Every 24 hours two flasks were harvested and extracted to measure PMV, pH and antibiotic production. Data are reported as the mean values of at least two replicates for each experiment.

PMV, pH and Ramoplanin Extraction

10 ml of culture were collected from the fermentation flask, centrifuged at 3250×*g* for the determination of Packed Mycelium Volume (PMV (%)) and pH. The whole flask culture (30 ml) was mixed with 3 volumes of acetone : water 2 : 1 brought to pH 2.2 with 1 N HCl, shaken for 20 minutes at room temperature and then filtered through paper filter. 4 ml of the filtered solution were extracted by an equal volume of ethyl acetate and centrifuged at 3250×*g* for 5 minutes. The organic phase was eliminated and the residual water phase was analyzed by HPLC.

Analytical HPLC

HPLC analyses were performed on a 5 μm particle size Hypersil ODS (Supelco Inc.) column (4.6×250 mm) with a guard column Symmetry C_{18} 5 μm, 3.9×20 mm (Waters) and eluted at 1 ml/minute flow rate with a 35-minute linear gradient from 7% to 20% of Phase B. Phase A was 25 mM $NH_4H_2PO_4$ pH 3.5 : CH_3CN 70 : 30 (v/v) and Phase B was 25 mM $NH_4H_2PO_4$ pH 3.5: CH_3CN 20 : 80 mixture. The chromatography was performed with a Hewlett Packard mod 1082 HPLC system and detection was at 268 nm. An authentic sample of ramoplanin antibiotic was used as internal standard.

Determination of the Potency

The ramoplanin factors potency was determinate by the following mathematical formula:

$$\text{Potency } (\mu\text{g/ml}) = \frac{\text{Cstd}\times\text{Pstd}\times\text{PAx}\times\text{AAx}\times\text{Vacq}\times 4^*}{\text{Astd}\times 4}$$

Cstd = standard solution concentration (μg/ml)
Pstd = standard potency (%)
PAx = Ax (x=1 or 2 or 3) component potency (%)

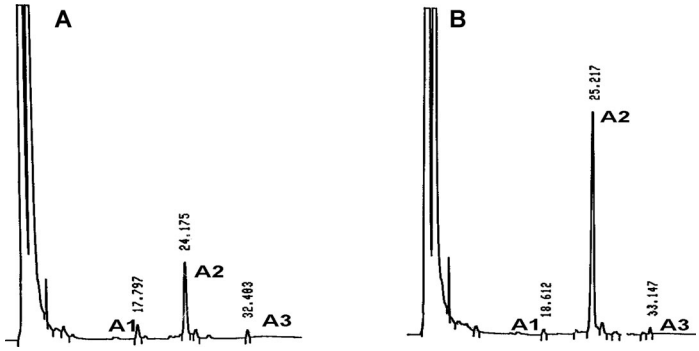

Fig. 2. HPLC profiles of the ramoplanin complexes produced without (A) and with (B) L-leucine addition to the fermentation broth. A1, A2 and A3 represent the ramoplanin components.

AAx = sample (A2, A1 or A3 peak area)
Astd = standard (A2, A1 or A3 peak area)
Vacq = Aqueous volume
4 = extract (ml)
4* = dilution

Areas for factors A1, A2 and A3 were considered separately and added to give the total complex estimation.

Results

Actinoplanes sp. ATCC 33076 cultivated in PB medium produced more than 100 mg/litre of ramoplanin after 192 hours of growth in which 15%, 75% and 10% of the complex was represented by A1, A2 and A3 components respectively, as reported in the HPLC profile shown in Figure 2A. The time course for the fermentation reported in Figure 3A shows that almost all ramoplanin was produced between 72 and 96 hours after the inoculum.

As previously reported for other antibiotics produced as a complex of molecules differing in the length and branching of the fatty acid residue [6~9], the addition of specific acids or of the corresponding amino acids may direct the biosynthesis towards the desired factors. In the case of ramoplanin, acetate or butyrate, isovalerate and isobutyrate are the putative starting molecules of the polymerisation process leading to the acids of A1, A2 and A3 components, respectively. Since butyric and valeric acids or salts are generally toxic to microorganisms, they could be supplied as esters in the case of *iso*-acids or efficiently replaced by the amino acids valine and leucine from which they are derived by common metabolic pathways [5,10]. It may be noted that Wallace *et al.* demonstrated that in actinomycetes deuterated labelled leucine is incorporated into odd carbons *iso*-fatty acids [11]. L-leucine was added to the *Actinoplanes* sp. ATCC 33076 cultures at various times after the inoculum and at concentrations ranging from 0.5 to 5 g/litre with the aim of increasing the production of the A2 component which bears the *iso*-nonadienoic acid (7-methylocta-2,4-dienoic acid) and represents the active principle under clinical development. The best results were obtained after the addition of 5 g/litre of L-leucine at the time of inoculation. Under these conditions a total complex productivity of more than 400 mg/litre was achieved, with A2 component contributing more than 96% (HPLC reported in Fig. 2B). The time course of the fermentation reported in Fig. 3B shows that addition of L-leucine stimulated production of ramoplanin, which started 48 hours after inoculation and then continued until 192 hours of fermentation, when a maximum of 433 mg/litre was achieved.

Thus, a three-fold improvement was induced by the addition of L-leucine in comparison to the control culture. L-leucine also stimulated an increase in biomass production, with a PMV of 26 at the end of fermentation *versus* a value of 20~23 obtained without L-leucine (data not shown). Considering however, the extent of production improvement, this increase in yield is mainly due to the increase of specific productivity. This suggests that fatty acid biosynthesis may represent a limiting factor in the ramoplanin production, which can be overcome by the addition of the appropriate precursor. To better investigate this hypothesis, L-valine, which is the precursor of the *iso*-decadienoic acid (8-methylnona-2,4-dienoic acid), in the A3 component, was supplied to the system under the same fermentation conditions, *i.e.* 5 g/litre at the time of inoculation. In this case, the addition of the precursor changed the complex composition but it did not increase the antibiotic productivity (Fig. 3C). Maximum total antibiotic productivity was slight less than 120 mg/litre as in the control cultures without L-valine addition. After 192 hours of fermentation, the complex contained 10% of A1,

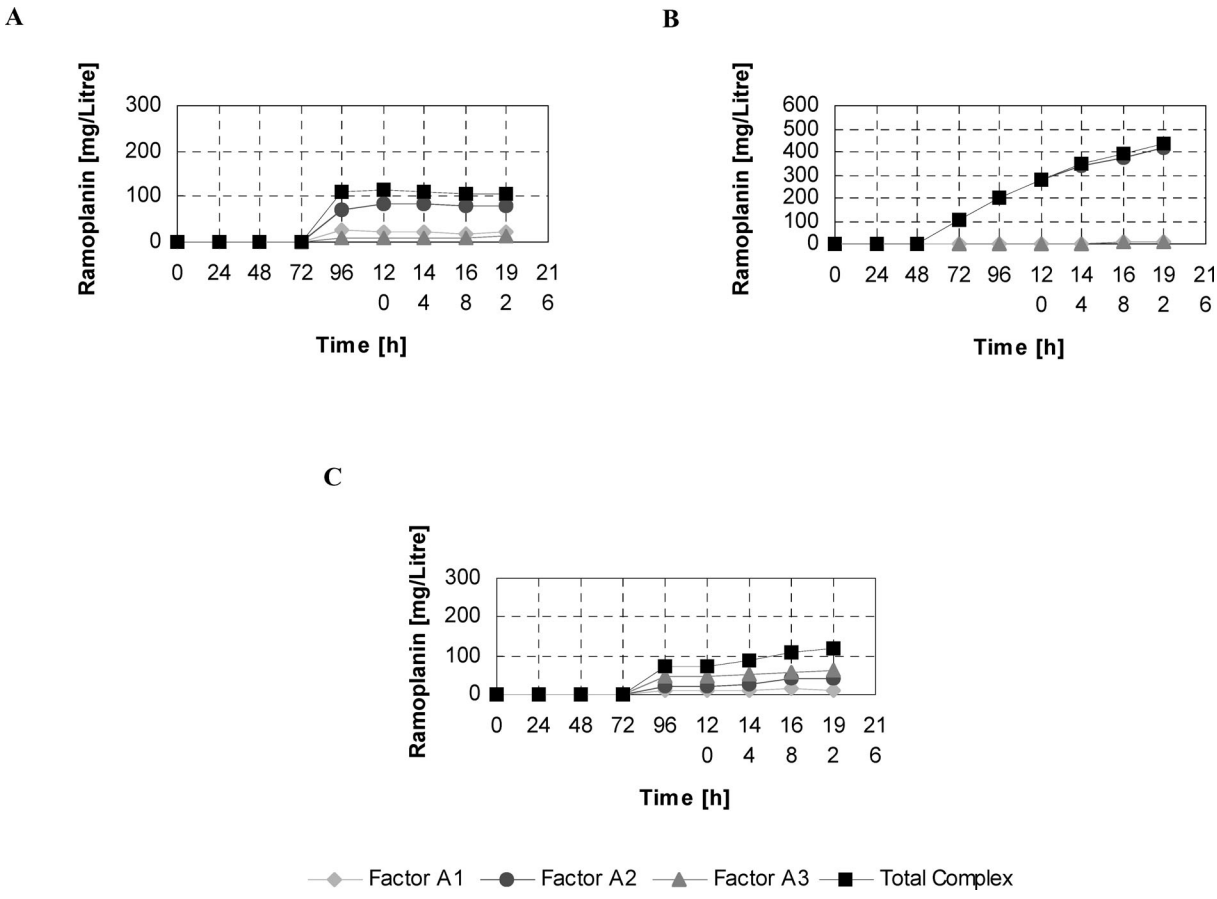

Fig. 3. Time course of the fermentation of *Actinoplanes* sp. in PB medium without (A) and with (B) L-leucine or (C) L-valine (5 g/litre) addition to the fermentation medium.

36% of A2 and 54% of A3. L-valine also exerted an inhibitory effect on the growth of the microorganism, a maximum PMV of 16 being achieved.

Discussion

Beltrametti *et al.* [6] recently described the effect of L-valine addition to the glycopeptide A40926 production by *Nonomuraea* sp. ATCC 39727. A40926 and the structurally similar teicoplanin, from *Actinoplanes teichomyceticus* ATCC 31121, are also produced as a complex of factors structurally differing in the length and branching of their fatty acid moieties [10]. The addition of L-valine in concentrations ranging from 1 to 3 g/litre changed the A40926 complex composition, increasing the production of the B0 component, which bears the even carbon *iso*-acid, and decreasing the production of the B1 factor, characterized by an *n*-acid residue. The L-valine effect in increasing the yield of the major component in the A40926 complex is due to its conversion into the *iso*-butyric acid, the precursor of the branched even carbon fatty acids. In similar conditions, the addition of L-valine to the fermentations of *Actinoplanes teichomyceticus* ATCC 31121 (concentrations from 1 to 2 g/litre) substantially increased both the relative and absolute yields of the major component T-A2-2 in the teicoplanin complex, characterised by the *iso*-C10:0 moiety [12]. In this case, it has been demonstrated that the acyl chains of the teicoplanin components are not directly synthesised from the amino acid precursors, but derive from the shortening of membrane fatty acids or of exogenous C_{18} acids fed in the fermentation medium [13]. Addition of L-leucine to the teicoplanin fermentation changed the complex composition but did not improve total complex productivity. On the basis of these reports [6,12,13], it is reasonable to suggest

that L-leucine and L-valine also influence the composition of the pool of membrane fatty acids in the *Actinoplanes* sp. producer of ramoplanin, and through this action change the ramoplanin complex composition. This contention is supported by the sequence analysis of the gene cluster governing ramoplanin production, where the PKS genes needed for the production of antibiotic fatty acids are absent. The di-unsaturated fatty acids of ramoplanin likely derive from the membrane pool of saturated fatty acids by the β-oxidative degradation mechanism and of by the action of two dehydrogenases whose genes are indeed present in the gene cluster [14]. In addition, the analysis of the membrane fatty acid composition revealed that the percentage of *iso*-even fatty acids (iC15:0 and iC17:0)–from which *iso*-nonadecadienoic acid of A2 derives–was increased from *ca.* 36% to 63% of the total fatty acids upon the addition of L-leucine to the *Actinoplanes* sp. cultures (A. Borghi, personal communication). The fact that L-leucine enhances both ramoplanin production and cell growth, whereas L-valine does not improve total antibiotic productivity and exerts an inhibitory effect on cell growth, is possibly due to the extent of the membrane perturbation caused by the massive addition of the two amino acids to the different producer organisms. This constitutes a difference from what happens for teicoplanin. In fact, in the case of A40926 and teicoplanin, the major component in the membrane fatty acid, in the absence of precursors, is the one originating from L-valine. According to our hypothesis, its increase upon the L-valine addition causes a less perturbing effect on composition of *Nonomuraea* sp. and *Actinoplanes teichomyceticus* membranes. In contrast, addition of L-leucine substantially increases a minor fatty acid component significantly altering the membrane's properties. In the case of *Actinoplanes* sp. producing ramoplanin, the major components of the cell fatty acids derive from L-leucine and thus addition of this amino acid does not significantly affect membrane composition as much as L-valine, which indeed exerts an inhibitory action on cell growth.

Conclusions

Addition of L-leucine and L-valine to *Actinoplanes* sp. ATCC 33076 fermentations represent a method for selectively enhancing the production of factor A2 or A3 in the antibiotic complex. Addition of 5 g/litre of L-leucine at the time of inoculation directs and improves the production of A2 factor, which represents the active principle under clinical development. Beside the positive impact in reducing cost and time of fermentation and purification, single component antibiotics or at least complexes with a standardized and reproducible composition better meet the quality guidelines for pharmaceutical product development.

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