Factors affecting the normal and branched-chain acyl moieties of teicoplanin components produced by *Actinoplanes teichomyceticus*

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Teicoplanin, a glycopeptide antibiotic produced by *Actinoplanes teichomyceticus*, comprises five main components, denoted T-A2-1 to T-A2-5, differing in the structure of their acyl side chain, which is linear in T-A2-1 and T-A2-3 and branched in the other components. Production of T-A2-1, characterized by a linear C10:1 acyl moiety, is entirely dependent on the presence of linoleate in the fermentation medium. Addition to the medium of oleic acid esters at 2 g l⁻¹ increases the yields of T-A2-3, characterized by a linear C10:0 acyl chain, about threefold. The antibiotic linear side chains thus appear to originate from C18 unsaturated acid by β -oxidation degradation. The percentage of T-A2-2, T-A2-4 and T-A2-5, bearing the iso-C10:0, anteiso-C11:0 and iso-C11:0 acyl moieties, respectively, is strongly influenced by the presence in the medium of the amino acids known to be precursors of branched-chain fatty acids. Thus, valine increases the production of T-A2-2 whereas isoleucine or leucine increase the relative yields of T-A2-4 or T-A2-5, respectively. Analysis of the total cell lipids upon addition of the same amino acid shows corresponding increases in the proportion of the iso-C16:0, iso-C15:0 or anteiso-C17:0. A mutant *A. teichomyceticus* strain, which produces a novel teicoplanin with a linear C9:0 chain, differs from the wild strain in the presence of the linear C17:1 acid in its lipids. The relative incorporation of [¹⁴C]acetate into teicoplanin acyl moieties is substantially lower when this precursor is added to grown *A. teichomyceticus* mycelium rather than at the time of inoculation. The results suggest that teicoplanin branched acyl moieties also originate from β -oxidation degradation of cellular long-chain fatty acids.

Introduction

Teicoplanin, a glycopeptide antibiotic recently introduced in clinical practice, is produced by *Actinoplanes teichomyceticus* as a complex of several active substances (Coronelli *et al.*, 1987). The most interesting ones, originally named teichomycin A-2, were shown (Borghi *et al.*, 1984) to be a complex of at least five closely related factors, designated T-A2-1 to T-A2-5. A sixth active component, teichomycin A-3, not found in fermentation beers, is however always present in crude or purified extracts. It was shown to be a hydrolysis product of the teichomycin A-2 complex (Malabarba *et al.*, 1984).

Teichomycin A-2 components differ in the structure of a fatty acid moiety linked by an amide bond to a glucosamine residue (Barna *et al.*, 1984). As shown in

Fig. 1, the fatty acids are 4-n-decenoic acid (n-C10:1), 8-methylnonanoic acid (iso-C10:0), n-decanoic acid (n-C10:0), 8-methyldecanoic acid (anteiso-C11:0) and 9-methyldecanoic acid (iso-C11:0). In addition, four minor components referred to as Related Substances 1 to 4 (RS 1–4) have been recently isolated (Cometti *et al.*, 1988; Borghi *et al.*, 1989). Again they differ in their fatty acid moieties which are 10-methylundecanoic acid (iso-C12:0), n-dodecanoic acid (n-C12:0), 6-methyloctanoic acid (anteiso-C9:0) and n-nonanoic acid (n-C9:0), respectively (see Fig. 1).

Although the antibacterial activities of the single components of teicoplanin, determined on a number of clinical isolates, show only minor differences, it was considered desirable to have methods for controlling the composition of batches derived from different fermentations. Therefore, the factors influencing the quantitative composition of the complex in fermentation broths were studied. We report here the results of such studies which suggest a biogenetic relationship between teicoplanin acyl moieties and the fatty acids constituting the lipids of the producing organism.

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Abbreviation: FAME, fatty acid methyl ester.

plate (Merck 15682) and developed with a Na_2SO_4 (5% w/v, aqueous solution)/acetonitrile (7:3 v/v). The spots corresponding to T-A3-1 (254 nm detection) and to the acylglucosamines (iodine vapours) were separately scraped off, eluted from the adsorbent with water/methanol (1:1, v/v) and their radioactivity measured.

Analytical methods. For high-performance liquid chromatography (HPLC), a Waters instrument with a Zorbax ODS (Du Pont) column (4.6 × 150 mm) was used. A gradient of 8% to 40% phase B (0.02 M- NaH_2PO_4 /acetonitrile, 95:5, v/v) in phase A (0.02 M- NaH_2PO_4 /acetonitrile, 25:75, v/v) required 40 min at a flow rate of 1.5 ml/min⁻¹. The detector was set at 254 nm.

Radioactivity was measured with a Packard Tri-Carb 460 C automatic liquid scintillation system using Insta-Gel (Packard) counting cocktail.

Results

Preliminary experiments indicated that the relative amounts of teicoplanin components were influenced by the composition of the fermentation medium. This was especially evident for the components bearing linear fatty acids, which were enhanced by addition of fats or oils. As shown in Fig. 2, in which the HPLC profiles of teicoplanin produced under two different conditions are shown, the presence of component T-A2-1 depended on the medium composition; T-A2-1 was absent when the only nutrients were glucose and yeast extract (medium C). The determining factor for production of T-A2-1 was the presence of esters of linoleic acid in the cotton seed meal used as a nitrogen source in medium TE/20.

Typical results of systematic series of experiments (Table 1) clearly indicate that T-A2-1 is preferentially produced in the presence of trilinolein and T-A2-3 in the presence of triolein. Similar results were obtained using methyl or ethyl linoleate or oleate respectively. It is noteworthy (results not shown) that no change in total yields or in the relative amounts of components was observed when esters of saturated fatty acids such as

Fig. 2. HPLC profiles of the teicoplanin complexes produced in: (a) medium TE/20; and (b) medium C. A, B, C, D and E represent T-A2-1, T-A2-2, T-A2-3, T-A2-4 and T-A2-5, respectively.

stearate or palmitate were added to the fermentation medium.

Altogether, these results suggest that biosynthesis of the acyl moieties is a limiting step in the production of individual teicoplanin components and that the two linear ten-carbon acids derive, probably through the usual β -oxidation mechanism, from the corresponding eighteen-carbon unsaturated acids. The position of the double bond in the 4-decenoic acid corresponds to that of the double bond in position 12 of linoleic acid, assuming the loss of four acetate units by β -oxidation degradation.

A selective increase of the relative amounts of components T-A2-2, T-A2-4 and T-A2-5 by specifically stimulating the biosynthesis of their characteristic acyl moieties was then attempted.

Table 1. Effect of biosynthetic precursors on teicoplanin complex composition

Cultures were grown in medium C. Precursors were added 24 h after the inoculum. The results are the means of three flasks.

Additions	Total yield (mg l ⁻¹)	Percentage composition				
		T-A2-1 (n-C10:1)	T-A2-2 (i-C10:0)	T-A2-3 (n-C10:0)	T-A2-4 (a-C11:0)	T-A2-5 (i-C11:0)
None	380	—	32	20	26	22
Trilinolein						
2 g l ⁻¹	340	29	20	14	20	17
4 g l ⁻¹	350	31	23	17	17	12
None	440	—	40	16	22	22
Triolein						
2 g l ⁻¹	510	—	30	41	15	14
4 g l ⁻¹	460	—	25	43	17	15

Stimulation of branched fatty acid synthesis can generally be obtained by supplying a specific precursor, since it is known (Kaneda, 1977) that isobutyrate, isovalerate and 2-methylbutyrate are the starting molecules of the polymerization process leading to even-carbon iso acids, odd-carbon iso acids and odd-carbon anteiso acids, respectively. Since butyric or valeric acids themselves are often toxic to micro-organisms they can be supplied as esters, or indirectly by supplying valine, leucine or isoleucine from which they are generated by common metabolic pathways.

Addition of valine substantially increased both the relative and absolute yields of component T-A2-2, characterized by the iso-C10:0 moiety (Table 2). Similar increases were observed in the amounts of T-A2-4 (bearing anteiso-C11:0) with isoleucine, and of T-A2-5 (bearing iso-C11:0) with leucine. These results could be interpreted as evidence for a direct synthesis of these acids by an antibiotic-specific synthase. However, it is unlikely that there are two different pathways (direct synthesis and degradation of longer chains) for the branched and linear fatty acid moieties of the same antibiotic. Thus we looked for the presence of possible long chain precursors of the branched fatty acids.

Most cellular fatty acids in Gram-positive bacteria are constituents of membrane lipids. Branched fatty acids are typical membrane constituents of actinomycetes (Kroppensted, 1985), and certain other micro-organisms. GC-MS of the total fatty acids of *A. teichomyceticus* gave the pattern shown in Fig. 3. Since superimposable results were obtained when the same analysis was carried out with extracted cellular phospholipids we assume that this pattern reflects the composition of the cell membrane fatty acids. The major peaks corresponded to 14-methylpentadecanoic acid (iso-C16:0), 13-methyltetradecanoic acid (iso-C15:0) and 14-methylhexadecanoic

Fig. 3. FAME GC-MS profiles for *A. teichomyceticus* strain 44 whole-cell hydrolysates from fermentations: (a) unsupplemented (see also Fig. 4); (b) with added valine; (c) with added isoleucine; (d) with added leucine. The amino acids were added to medium C at a final concentration of 1 g l^{-1} 24 h after inoculation. Chromatographic method 1 was used.

acid (anteiso-C17:0), which are logical precursors of the iso-C10:0, iso-C11:0 and anteiso-C11:0 moieties of T-A2-2, T-A2-5 and T-A2-4, respectively. In addition, oleic acid (n-C18:1), the precursor of the n-C10:0 moiety, was present whereas linoleic acid (n-C18:2) was absent, in agreement with the observation that the decenoic moiety of T-A2-1 is produced only when linoleic acid is supplied to the fermentation medium.

Addition to the cultures of the amino acid precursors of the branched fatty acids markedly modified the lipid composition. As shown in Fig. 3, when valine (1 g l^{-1}) was added, iso-C16:0 was almost the only component of the cellular fatty acids. Superimposable results were obtained when the analysis was repeated on extracted lipids. Upon addition of leucine or isoleucine, iso-C15:0 or the anteiso-C17:0, respectively, became the major constituents. This exactly paralleled the increase in teicoplanin components T-A2-2, T-A2-5 and T-A2-4 described above and suggests, although it does not prove,

Table 2. Effect of biosynthetic precursors on teicoplanin complex composition

Cultures were grown in medium C. Precursors were added 24 h after the inoculum. The results are the means of three flasks.

Additions	Total yield (mg l^{-1})	Percentage composition			
		T-A2-2 (i-C10:0)	T-A2-3 (n-C10:0)	T-A2-4 (a-C11:0)	T-A2-5 (i-C11:0)
None	340	34	20	24	21
L-Valine					
1 g l^{-1}	650	70	12	10	7
2 g l^{-1}	790	83	9	4	3
L-Isoleucine					
1 g l^{-1}	130	24	13	40	20
L-Leucine					
1 g l^{-1}	280	35	10	8	45

a biosynthetic relationship between the antibiotic fatty acid moieties and the cellular lipids.

Further support for this hypothesis came from the isolation of *A. teichomyceticus* mutant A-184, morphologically indistinguishable from the parent strain, but producing substantial amounts of two teicoplanin analogues, RS-3 and RS-4, having an iso-C9:0 and a n-C9:0 chain, respectively (Borghi *et al.*, 1989). The presence of the iso derivative could easily be explained by a further loss of acetate from the iso-C11:0 deriving from iso-C15:0. The linear nonanoic acid should derive from a linear odd-carbon acid, probably unsaturated because saturated linear acids were shown not to be utilized as precursors. The major difference between the mutant and parent strain fatty acids was indeed, as shown in Fig. 4, the presence of a substantial amount of linear heptadecenoic acid, as well as the presence of n-pentadecanoic and n-heptadecanoic acids.

The final demonstration that cell fatty acids are precursors of antibiotic fatty acid moieties was provided by experiments with labelled acetate. *A. teichomyceticus* was grown in medium C for 30 h. At this time, when antibiotic production had just started, the mycelium was filtered, washed and resuspended in buffer. After a further 24 h, teicoplanin was isolated and hydrolysed as shown in Fig. 5.

When [2-¹⁴C]acetate was added to the growth medium, 36.4 mg of [¹⁴C]teicoplanin with a specific activity of 38850 d.p.m. mg⁻¹ was obtained. After hydrolysis,

Fig. 4. FAME GC-MS profiles for *A. teichomyceticus* whole-cell hydrolysates from: (a) strain 44 (as in Fig. 3); (b) mutant strain A-184. Chromatographic method 2 was used.

78% of the antibiotic radioactivity was found in the pseudoaglycone and 22% in the acylglucosamine moiety. This result is consistent with an approximately uniform distribution of radioactivity in the carbon atoms deriving from carbon 2 of acetate. On the basis of what is known about the biosynthesis of glycopeptide antibiotics (Hammond *et al.*, 1983; Chung *et al.*, 1986), nine carbon atoms in the pseudoaglycone and three in each acid chain are derived from carbon 2 of acetate. When [2-¹⁴C]acetate was added to the resuspended mycelium, 48.9 mg of teicoplanin with a specific activity of 159000 d.p.m. mg⁻¹ was obtained. After hydrolysis, only 4.8% of the radioactivity was associated with the acylglucosamine,

Fig. 5. Hydrolysis of T-A2-2 to T-A3-1 and *N*-(8-methylnonanoyl)glucosamine. R = 8-methylnonanoyl; R₁ = *N*-acetyl- β -glucosaminyl; R₂ = α -D-mannosyl.

demonstrating that the fatty acid moieties must have come from molecules formed during the growth phase.

Discussion

The results show that teicoplanin acyl moieties originate by degradation of fatty acids from the cell membrane or, in the case of linoleic acid, present in the fermentation medium.

This is supported by: (a) the absolute requirement for linoleic acid in the fermentation medium to obtain T-A2-1; (b) the strong increase in the production of T-A2-3 upon addition of oleic acid to the medium; (c) the consistent relationship between the amount of a given cellular acid and the yield of the corresponding component of the teicoplanin complex, both in the presence and in the absence of specific precursors; (d) the presence in a mutant strain producing a component with a linear odd carbon acyl moiety of an unsaturated linear acid with seventeen carbon atoms; (e) the low incorporation of labelled acetate in the acyl moieties when this precursor was added to cultures after completion of mycelial growth. This last result is in agreement with the report (Chung *et al.*, 1986) that [^{14}C]oleate, but not [^{14}C]acetate labelled the linear fatty acid moiety of ardacins (aridicins) when added to a fermentation after growth was complete.

The change in cellular fatty acid composition caused by precursor amino acids is of interest. Since similar results were obtained by analysing cell phospholipids, the membrane composition was altered in a parallel manner. This result is in agreement with previously reported observations on the effect of amino acids on the lipid composition of several *Bacillus* species (Kaneda, 1977) and of *Streptomyces hygroscopicus* and *Streptomyces griseus* (Graefe *et al.*, 1982a). However, the extent of the alteration caused by a moderate amount of valine is surprising.

Alteration in fatty acid content due to genetic changes in *Streptomyces* have been reported previously: Arima *et al.* (1973) described a mutant *S. fradiae* strain with an altered ratio of anteiso-C15:0 to iso-C16:0. Similarly (Graefe *et al.*, 1982a, b) reported an altered ratio of anteiso-C15:0 to iso-C16:0 in *Streptomyces* mutants defective in the production of aerial mycelium. Mutant A-184 of *A. teichomyceticus*, although almost indistinguishable from the parent strain in morphology and growth characteristics, is significantly different in lipid composition, which includes substantial amounts of odd-carbon linear fatty acids virtually undetectable in the parent strain.

The pattern of fatty acids in cell lipids has been

proposed and used as an element for taxonomic classification of bacteria (Kroppensted, 1985). Our results suggest that this criterion should be used with care since variations are possible, even within the same species.

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