Relationship between threonine dehydratase and biosynthesis of tylosin in *Streptomyces fradiae*

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To elucidate the repression mechanism of ammonium ions on the biosynthesis of tylosin in *Streptomyces fradiae* NRRL 2702, enzyme activities involved in the metabolism of the aspartate family of amino acids were evaluated in relation to the ammonium ion concentration and tylosin production. It was found that aspartate aminotransferase was essential for both cell growth and tylosin production. However, both threonine dehydratase and valine dehydrogenase were repressed by supplemented ammonium ions at concentrations higher than 50 mM. Threonine dehydratase was purified from cell-free extracts by acetone precipitation, ion-exchange chromatography and gel filtration, and its molecular mass was estimated to be 67 200 Da. The optimum pH and temperature for threonine dehydratase activity were 7.5 and 25 °C, respectively, and the K_m value for threonine under these optimum conditions was 21 mM. The inhibition pattern of ammonium ions on the activity of threonine dehydratase appeared to be a mixed type.

Introduction

Tylosin is a macrolide antibiotic produced by *Streptomyces fradiae*. It has been reported that the major precursor of an aglycone moiety, tylactone, was built up with propionate, acetate and butyrate as unit molecules. The formation of the unit molecules represented limiting steps in the control of the overall biosynthetic rate of tylosin (Corcoran, 1974; Morin & Gorman, 1964; Omura *et al.*, 1977). In addition it was found that tylosin production was apparently repressed by ammonium ions and the repression could be eliminated by the addition of acetate, isopropionate and butyrate (Omura *et al.*, 1984). These observations indicated that the formation of the essential unit molecules resulted from the conversion of amino acids to the corresponding carboxylic acids (viz. acetate, propionate and butyrate) and ammonium ions, where ammonium ions might play an important role in repressing the expression of enzyme(s) involved in the conversion. Valine dehydrogenase, which converted valine to 2-oxoisovalerate (subsequently converted to butyrate and propionate) was identified as the repressible site (Omura *et al.*, 1983). It was also thought that threonine dehydratase, which converts threonine to propionate, might be another repressible enzyme (Bode & Birnbaum, 1986; Vancura *et al.*, 1988). However, no

enzymic evidence has been reported to support this idea and we therefore proposed to elucidate the effect of ammonium ions on the formation of threonine dehydratase and to determine their effect on the activity of the purified enzyme.

Methods

Micro-organisms and preservation. *Streptomyces fradiae* NRRL 2702 was used as the parent, wild-type strain. An aspartate auxotroph strain (SMF 305) was obtained from the parent strain by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), and a spontaneous revertant strain (SMF 306) from the auxotroph was also selected. The strains were grown aerobically in a seed culture medium which consisted of (w/v): 2.0% glucose, 1.0% corn steep liquor, 0.5% yeast extract, 0.3% CaCO₃ (the pH was adjusted to 7.2 before sterilization) (Seno *et al.* 1971). Cells in the exponential growth phase in a batch culture were harvested aseptically, suspended in sterile 10% (v/v) glycerol and rapidly frozen to –70 °C.

Culture conditions. The frozen stock cells were used as inocula for the seed cultures that were used for inocula (5%, v/v) of the main cultures; the seed cultures were incubated at 30 °C for 3 d. A 3.0 litre, *in situ* steam sterilizable fermenter (Chemap CF) and a synthetic medium consisting of (w/v): 2.0% soluble starch, 0.5% glucose, 0.27% sodium lactate, 0.05% MgSO₄·7H₂O, 0.47% (NH₄)₂SO₄, 0.3% CaCO₃, 0.0003% trace elements (CuSO₄·5H₂O, ZnSO₄·7H₂O, FeSO₄·7H₂O, CoCl₂·6H₂O and MnCl₂·4H₂O) were used for the main cultures (Vancura *et al.* 1988). Solutions of L-aspartate and (NH₄)₂SO₄ were always sterilized separately and added before inoculation. The temperature was maintained at 30 °C and the pH was adjusted to 7.0 before inoculation, while aeration was fixed to 1 vol. vol.⁻¹ min.⁻¹ and

Abbreviations: ASAT, aspartate aminotransferase; TDT, threonine dehydratase; VDH, valine dehydrogenase.

agitation was controlled to give a dissolved oxygen tension above 40% (air saturation).

Cell growth. Cell concentration was estimated as cell dry weight, which was determined as follows: cells were collected by vacuum filtration (Azumi filter paper no. 2), washed with 2 vols distilled water, and then dried at 80 °C for 24 h.

Tylosin assay. Tylosin concentration was determined by HPLC (Bio-Rad) using a μ Bondapak C₁₈ column and a UV detector (280 nm). The mobile phase was 40% (v/v) acetonitrile and 2% (v/v) monoethanolamine (Kennedy, 1978).

Measurement of ammonium ions. Concentration of ammonium ions in the culture broths was determined by the phenate method (Taras *et al.*, 1981).

Enzyme assays. The activities of various enzymes were determined in cell-free extracts. Cells were disrupted by sonication (100 W, 5 min) at 2–5 °C, then centrifuged at 21 000 g for 30 min; the supernatant was used as crude enzyme solution. Activity of aspartate aminotransferase (ASAT; EC 2.6.1.1) was determined by coupling the ASAT with a reaction catalysed by malate dehydrogenase, and measuring the oxidation of NADH (Bergmeyer *et al.*, 1983). Threonine dehydratase (TDT; EC 4.2.1.16) activity was assayed by determining the 2-oxobutyric acid produced by its colour reaction with 2,4-dinitrophenylhydrazine (Vancura *et al.*, 1988), while valine dehydrogenase (VDH; EC 1.4.1.8) activity was measured as the increase of NADH absorbance at 340 nm (Vancurova *et al.*, 1988). One unit (U) was defined as the quantity of each enzyme producing 1 μ mol of product in 1 min under specified assay conditions, and protein concentrations were determined by the method of Bradford (1976).

Purification of TDT. Acetone (–20 °C) was added slowly to the crude enzyme solution with gentle stirring to give a concentration of 35% (v/v). After gentle stirring for 30 min at 4 °C followed by centrifugation at 21 000 g for 30 min, the precipitate was discarded and acetone (–20 °C) was further added to the supernatant to give a final concentration of 55% (v/v). Again after gentle stirring for 30 min at 4 °C, the precipitate collected by centrifugation at 21 000 g for 30 min was dissolved in 0.01 M-potassium phosphate buffer (pH 7.5) and dialysed against the same buffer. The samples after dialysis were applied to a column (2.5 $\times$ 110 cm) of Sephadex G-100-120 equilibrated with 0.01 M-phosphate buffer (pH 7.5), collecting 5 ml fractions. The active fractions were pooled and applied to a DEAE-Sephadex A-50 column (5.7 $\times$ 17.5 cm), equilibrated with the same buffer system as used for the gel filtration, and after eluting the column with the same buffer, 5 ml fractions were collected. The active fractions following ion-exchange were concentrated using Centriprep-10 and the concentrated enzyme solutions were applied to a Sephadex G-75-120 column (2.0 $\times$ 60 cm) equilibrated with 0.01 M-phosphate buffer (pH 7.5) for further purification.

Molecular mass determination. SDS-PAGE was performed using the method of Laemmli (1970); the concentration of the running gel was 12% (w/v) and that of the stacking gel was 5% (w/v). After electrophoresis, the gel was silver-stained (Morrissey, 1981). The molecular mass markers were rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

Determination of optimum pH and temperature. One unit of the purified TDT was added to 1.0 ml of reaction mixture in the following buffers: 0.5 M-sodium acetate (pH 4.0–6.0), 0.5 M-potassium phosphate (pH 6.0–8.0), 0.5 M-Tris/HCl (pH 8.0–9.0). Enzyme activities at different pH values were compared after incubation at 25 °C for 30 min; enzyme activities at different temperatures (4–60 °C) were determined at pH 7.5.

Effect of ammonium ions. Ammonium chloride was added to the reaction mixtures to obtain final concentrations ranging from 10 to 50 mM and the enzyme activities of each of these mixtures were compared with that of the control where no ammonium chloride was added. The K_m value and inhibition pattern were determined from a Lineweaver–Burk plot.

Chemicals, reagents and reproducibility. NAD⁺, NADH, all amino acids and specialized reagents were purchased from Sigma. All other chemicals were of reagent grade. Each experiment was repeated three times and mean values are shown.

Results

Repression of tylosin biosynthesis by ammonium ions

As described previously, cell growth was associated with higher activities of aspartate aminotransferase (ASAT) and citrate synthase (CS), while tylosin production was accompanied not by increased activities of ASAT and CS but by higher activity of methylmalonyl-CoA carboxytransferase (MMCT) (Kang & Lee, 1987a, b; Lee & Lee, 1987). These results were interpreted as arising from the greater conversion of oxaloacetate, the common substrate of these enzymes, to methylmalonyl-CoA by the higher activity of MMCT. Since methylmalonyl-CoA may be the essential starting precursor of ty lactone, it was thought that mutants in which the activities of CS and ASAT were reduced but that of MMCT was increased might produce higher levels of tylosin. We therefore tried to isolate such mutants by NTG mutagenesis of *S. fradiae* NRRL 2702.

As a result, an auxotroph (SMF 305) requiring aspartate for growth was isolated and a revertant (SMF 306) was obtained, which had spontaneously reverted to prototrophy from the auxotroph. The cell growth and tylosin production of these strains were then compared in batch cultures (Table 1). ASAT activity could not be detected in the cell-free extracts of the auxotroph. Furthermore the growth of the auxotroph was apparently retarded and tylosin production was completely repressed. However, the revertant SMF 306 showed ASAT activity comparable to the parent strain, and its cell growth and tylosin production were apparently increased. It was not clear why the revertant was superior to the parent strain in both cell growth and tylosin production and why the growth of the aspartate auxotroph was so much lower than that of the parent even in aspartate-supplemented medium.

Since tylosin production had been shown to be greatly increased by the addition of threonine and valine to the fermentation medium (Lee & Lee, 1987; Vancura *et al.*, 1987), the activities of enzymes involved in threonine and valine catabolism were determined in the three strains (wild-type, revertant and auxotroph). As shown in Table 2, the revertant showed the largest tylosin

Table 1. Comparison of tylosin production and ASAT activity in various strains of *S. fradiae*

Cultures were grown in synthetic medium with aspartate (25 mM) for 3 d. Biomass was estimated as cell dry weight and tylosin concentration was determined by HPLC (Bio-Rad) using a μ Bondapack C₁₈ column and UV detector (280 nm). The enzyme activity was determined by coupling with the reaction of malate dehydrogenase (25 °C; pH 7.4), and measuring the oxidation of NADH (340 nm).

Strain	Biomass (g l ⁻¹)	Tylosin (mg l ⁻¹)	Tylosin production [mg (g biomass) ⁻¹]	ASAT [U (mg protein) ⁻¹]
Wild-type	7.2	18.5	2.5	1.6
Revertant	10.9	74.0	6.8	1.8
Aspartate auxotroph	3.1	ND	ND	ND

ND, Not detected.

Table 2. Comparison of VDH, TDT and tylosin production in various strains of *S. fradiae*

Cultures were grown in synthetic medium with aspartate (25 mM) and ammonium sulphate (25 mM) for 3 d. Residual ammonium ion concentration in the culture broth was analysed by the phenate method and tylosin concentration was determined as in Table 1. The activity of TDT was measured by determining 2-oxobutyric acid produced with 2,4-dinitrophenylhydrazine at 540 nm (pH 8.0, 25 °C). VDH activity was determined in the direction of oxidative deamination by measuring the appearance of NADH at 340 nm (pH 10.6, 25 °C).

Strain	Residual NH ₄ ⁺ ions (μ M)	Biomass (g l ⁻¹)	Tylosin production [mg (g biomass) ⁻¹]	TDT*	VDH*
Wild-type	2.4	10.2	1.3	0.096	15.3
Revertant	3.9	12.9	5.6	0.152	21.2
Aspartate auxotroph	1.3	9.6	ND	0.062	9.6

ND, Not detected.

* Specific activity [U (mg protein)⁻¹].

production and activities of VDH and TDT, while the aspartate auxotroph showed the smallest values for all three parameters. The increased enzyme activities in the revertant might give rise to the increase in the production of biomass and tylosin. These results indicated that TDT and VDH were important enzymes in the tylosin biosynthetic pathway, by which threonine and valine were hydrolysed to produce ammonium ions and the corresponding fatty acids. As indicated in Table 2, the growth of the auxotroph was similar to that of the wild-type strain when the synthetic medium was supplemented with aspartate (25 mM) and ammonium sulphate (25 mM). Therefore we suggest that there has been a second mutational event affecting the ammonium utilization system in the aspartate auxotroph. Since TDT activity and tylosin production of the revertant strain were superior to both the wild-type strain and the auxotrophic strain, the revertant was used in the following experiments.

The effects of the initial concentration of ammonium ions on biomass formation and tylosin production in batch cultures were evaluated with the revertant, and the activities of VDH and TDT were compared as well. As shown in Fig. 1(a-e), cell growth was enhanced with increasing initial concentrations of ammonium ions and it continued until the second day of the batch cultures (Fig. 1a). In the media where the initial concentrations of added ammonium ions were 25 mM and 50 mM, nearly all of the ammonium ions were consumed by the second day. However, in the medium where the initial added ammonium ion concentration was 100 mM, ammonium ions remained throughout the fermentation (Fig. 1b). Tylosin production began on the second day and increased steadily to the seventh day (Fig. 1c). Maximum production of tylosin was obtained when the initial concentration of ammonium ions added was 50 mM, whereas 100 mM-ammonium ions significantly repressed production. This indicated that tylosin biosynthesis was

Fig. 1. Effects of ammonium ion concentration on tylosin production and on VDH and TDT formation by the revertant strain (SMF 306) of *S. fradiae*. Strain SMF 306 was grown in synthetic medium containing 25 (●), 50 (■) or 100 (▲) mM-ammonium ion. An aliquot was taken from each fermenter and assayed for (a) cell growth (—) and pH (---); (b) residual ammonium ion concentration; (c) tylosin production ($Y_{p,x}$); (d) intracellular VDH activity; and (e) intracellular TDT activity.

greatly repressed by ammonium ions, which might exert nitrogen catabolite repression on an essential enzyme(s) involved in tylosin biosynthesis. The revertant strain differed from the wild-type strain in that its tylosin production was highest not at the lowest concentration of initially added ammonium ions tested (25 mM) but rather at the higher concentration of 50 mM. Also, the revertant strain was superior to the wild-type strain in the formation of enzymes involved in ammonium assimilation, especially glutamine synthetase (EC 6.3.1.2),

although the formation of these enzymes was not related to tylosin production (data not shown). Since the supplemented ammonium ions (50 mM) were completely assimilated, we assumed that ammonium ions up to a concentration of 50 mM might have all been used for primary metabolism, thus leaving none to repress secondary metabolism, especially tylosin production. The activities of TDT and VDH, followed similar patterns to that of tylosin production in the early growth phase, although no clear pattern was apparent at later

Fig. 2. Silver-stained SDS-PAGE electrophoregram of fractions from the purification of TDT (see Table 3). Lanes: 1, Sephadex G-75 fraction (10 ng); 2, crude extract (48 ng); 3, molecular mass standards (see Methods). The acrylamide concentration was 12% (w/v). TDT is indicated by the arrow.

times (Fig. 1*d, e*). We concluded that VDH and TDT were the sites of repression by ammonium ions when supplemented at a concentration greater than 50 mM.

Purification and properties of TDT

The specific activity of TDT in cell-free extracts from the revertant strain was 0.16 unit (mg protein)⁻¹. Precipitates obtained from 35% to 55% (v/v) acetone specifically showed TDT activity, and so these active fractions were subjected to gel filtration on Sephadex G-100 and then to ion-exchange chromatography on DEAE-Sephadex A-50. The active fractions obtained from the previous step were then passed through Sephadex G-75;

Fig. 3. Effect of temperature (a) and pH (b) on the activity of TDT from *S. fradiae* strain SMF 306. (a) A constant amount of purified TDT (1 unit) was assayed at the temperatures indicated (4–60 °C). (b) A constant amount of purified TDT (1 unit) was assayed at the indicated pH: 0.5 M-sodium acetate buffer (pH 4.0–6.0; ●), 0.5 M-potassium phosphate buffer (pH 6.0–8.0; ■), 0.5 M-Tris/HCl buffer (pH 8.0–9.0; ▲).

the purified enzyme obtained showed 12450 times greater activity than the cell-free extract and the recovery yield was 10% (Table 3).

The purified enzyme showed a single band on SDS-PAGE (Fig. 2), and the molecular mass of denatured TDT was estimated to be 67200 Da. The optimum temperature and pH of threonine dehydratase were found to be 25 °C and 7.5, respectively (Fig. 3), and its

Table 3. Purification of TDT from the revertant strain of *S. fradiae*

Fraction	Activity (U)	Protein (mg)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude extract	272	1660	0.16	1	(100)
Acetone precipitation	136	194	0.70	4.4	50
Sephadex G-100	67.8	0.066	1027	6419	25
DEAE-Sephadex A-50	34.6	0.031	1116	6975	13
Sephadex G-75	25.9	0.013	1992	12450	10

K_m value for threonine was 21 mM. Finally, the enzyme activity was activated by AMP but significantly inhibited by ammonium ions, and this inhibitory effect of ammonium ions had characteristics similar to a mixed-type inhibition (data not shown).

Discussion

The aspartate auxotroph (SMF 305) was inferior to the wild-type strain in both cell growth and tylosin production, whereas the revertant (SMF 306) was superior to the wild-type strain. These results indicated that the formation of aspartate from oxaloacetate by ASAT was essential for both cell growth and tylosin biosynthesis. Since aspartate is the starting compound for the synthesis of aspartate family amino acids, aspartate should be adequately supplied for cell growth. It has been thought that, under certain conditions, amino acids could be catabolized and then the metabolites could be used for the production of tylosin. This suggestion was proposed elsewhere (Omura *et al.*, 1984; Vancura *et al.*, 1988), for valine and threonine.

In the present study we found that tylosin biosynthesis and the activities of TDT and VDH were stimulated when the initial concentration of ammonium ions was low (50 mM) and that those activities were greatly repressed at a high concentration of ammonium ions (100 mM). From these results, we can conclude that threonine was converted to 2-oxobutyrate under conditions where the initial ammonium ion concentration was low, and that the 2-ketobutyrate produced under this condition was used in the production of tylosin. TDT may be a site of nitrogen catabolite repression in the tylosin biosynthetic pathway as VDH was shown to be (Navarrete *et al.*, 1990).

Purified TDT showed a single band on SDS-PAGE. Its molecular mass was 67200 Da and its K_m value for threonine was 21 mM, which was lower than that of the TDT from *Clostridium tetanomorphum* (37 mM) (Nakazawa, 1971) and higher than those of the TDTs from *Bacillus subtilis* (4.5 mM) (Hatfield & Umbarger, 1971), *Rhodopseudomonas sphaeroides* (10 mM) (Datta, 1971) and *Escherichia coli* (11 mM) (Shizuta & Tokushige, 1971). No report has been made on the TDT from other species of *Streptomyces*. The activity of purified TDT was inhibited by 10–20 mM-ammonium ions. The inhibition pattern of ammonium ions on the activity of TDT was a mixed-type inhibition.

The intracellular activity of TDT was high when the concentration of ammonium ions was controlled to 25–50 mM (see Fig. 1). The results indicated that the ammonium ions added to the culture broth at a concentration of 50 mM were completely assimilated by

the second day; hence TDT expression and tylosin production were not repressed. However, when the initial concentration of ammonium ions was greater than 50 mM, the excess ammonium ions could play an important role in regulating TDT gene expression, although we have not verified this at the gene level.

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References

- BERGMEYER, H. U., BERGMEYER, J. & GRA, M., I. (1983). Phosphoenolpyruvate carboxylase. In *Methods of Enzymatic Analysis*, vol. 2, pp. 275–276. Edited by H. U. Bergmeyer & M. Gra I. Weinham: Verlag Chemie.
- BODE, R. & BIRNBAUM, D. (1986). Threonine dehydratase activity from several yeast species is activated and affected by phosphate. *FEMS Microbiology Letters* **37**, 189–192.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CORCORAN, J. W. (1974). Lipid and macrolide lactone biosynthesis in *Streptomyces erythreus*. *Developments in Industrial Microbiology* **15**, 83–100.
- DATTA, P. (1971). Threonine deaminase – biosynthetic (*Rhodopseudomonas sphaeroides*). *Methods in Enzymology* **17B**, 566–571.
- HATFIELD, G. W. & UMBARGER, H. E. (1971). Threonine deaminase – degradative (*Bacillus subtilis*). *Methods in Enzymology* **17B**, 571–575.
- KANG, H. A. & LEE, K. J. (1987a). Regulation of cell growth and tylosin biosynthesis through flux control of metabolite intermediate in *Streptomyces fradiae*. *Korean Journal of Microbiology* **25**, 189–197.
- KANG, H. A. & LEE, K. J. (1987b). Regulation of tylosin biosynthesis by cell growth rate in *Streptomyces fradiae*. *Korean Journal of Microbiology* **25**, 353–359.
- KENNEDY, J. H. (1978). Liquid chromatographic analysis of fermentation broths: cephalosporin C and tylosin. *Journal of Chromatographic Science* **16**, 492–495.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LEE, J. H. & LEE, K. J. (1987). Effect of growth rate on the tylosin biosynthesis in *Streptomyces fradiae*. *Korean Journal of Applied Microbiology and Bioengineering* **15**, 34–38.
- MORIN, R. B. & GORMAN, M. (1964). The partial structure of tylosin, a macrolide antibiotic. *Tetrahedron Letters* **14**, 2339–2345.
- MORRISSEY, J. H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* **117**, 307–310.
- NAKAZAWA, A. (1971). Threonine deaminase – degradative (*Clostridium tetanomorphum*). *Methods in Enzymology* **17B**, 571–575.
- NAVARRETE, R. M., VARA, J. A. & HUTCHINSON, C. R. (1990). Purification of an inducible L-valine dehydrogenase of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* **136**, 273–281.
- OMURA, S., TAKESHIMA, A., NAKAGAWA, A., PRIIOU, F. & LUKACS, G. (1977). Studies on the biosynthesis of 16-membered macrolide antibiotics using carbon-13 nuclear magnetic resonance spectroscopy. *Biochemistry* **16**, 2860–2866.
- OMURA, S., TSUZUI, K., TANAKA, Y., SAKAKIBARA, H., MAMADA, H. & MASUMA, R. (1983). Ammonium ion suppresses the biosynthesis of tylosin aglycone by interference with valine catabolism in *Streptomyces fradiae*. *Journal of Antibiotics* **36**, 1792–1794.
- OMURA, S., TAKI, A., MATSUDA, K. & TANAKA, A. (1984). Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of tylosin aglycone. *Journal of Antibiotics* **37**, 1362–1369.

- SENO, E. T., PIEPER, R. L. & HUBER, F. M. (1977). Terminal stages in the biosynthesis of tylosin. *Actinomicrobial Agents and Chemotherapy* **11**, 455–461.
- SHIZUTA, Y. & TOKUSHIGE, M. (1971). Threonine deaminase (degradative) (*Escherichia coli*). *Methods in Enzymology* **17B**, 575–580.
- TARAS, M. J., ARNOLD, E. F., HOAK, R. D. & RAND, M. C. (1981). Phenate method. In *Standard Methods for the Examination of Water and Wastewater*, pp. 350–352. Edited by M. J. Taras, E. C. Arnold, R. D. Hoak & M. C. Rand. Washington, DC: American Public Health Association.
- VANCURA, A., REZANKA, T., MARSALEK, K., VANCULAR, I., KRISTAN, V. & BASAROVA, G. (1987). Effect of ammonium ions on the composition of fatty acids in *Streptomyces fradiae*, producer of tylosin. *FEMS Microbiology Letters* **48**, 357–358.
- VANCURA, A., REZANKA, T., MARSALEK, J., MELZUCH, K., BASAROVA, G. & KRISTAN, V. (1988). Metabolism of L-threonine and fatty acids and tylosin biosynthesis in *Streptomyces fradiae*. *FEMS Microbiology Letters* **49**, 411–415.
- VANCUROVA, A. I., VOLC, J., FUSSEY, S. P. M., FLIEGER, M., NEUZIL, J., MARSALEK, J. & BEHAL, V. (1988). Valine dehydrogenase from *Streptomyces fradiae*: purification and properties. *Journal of General Microbiology* **134**, 3213–3219.