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Influence of dimethylsulfoxide on tylosin production in *Streptomyces fradiae*

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The polyketide aglycone, tylactone (protylonolide), does not normally accumulate during tylosin production in *Streptomyces fradiae*, suggesting that the capacity of the organism to glycosylate tylactone exceeds the capacity for polyketide synthesis. Consistent with this model, tylosin yields were significantly increased (due to bioconversion of the added material) when exogenous tylactone was added to fermentations. However, tylosin yield improvements were also observed (albeit at lower levels) in solvent controls to which dimethylsulfoxide (DMSO) was added. At least in part, the latter effect resulted from stimulation of polyketide metabolism by DMSO. This was revealed when the solvent was added to fermentations containing the *tylA* mutant, *S. fradiae* GS14, which normally accumulates copious quantities of tylactone. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 46–51.

Keywords: dimethylsulfoxide; polyketide metabolism; *Streptomyces fradiae*; tylosin production

Introduction

Macrolide antibiotics, such as tylosin, are composed of polyketide lactones substituted with one or more 6-deoxyhexose moieties. A wide range of such secondary metabolites has been characterized. Several have important applications in human and/or animal health care, others have been widely used as growth promoters in livestock feeds [16,17], and the novel macrolide, spinosyn ("spinosad"; previously known as A83543), has emerged as an insecticide of unrivalled potential in agriculture [18,25]. Gene clusters encoding the synthesis of antibiotics that contain 6-deoxyhexose substituents have been analyzed in various actinomycetes, including macrolide producers [3,4,14,15,20,23,24,28]. Such studies have revealed interesting variations in the patterns of assembly of deoxyhexose biosynthetic genes and, not least, in the number and nature of regulatory elements associated with respective clusters. In contrast, the polyketide-biosynthetic megagenes in macrolide producers conform to a common pattern. Linear arrays of partially repetitive sequences encode giant multifunctional "type I" polyketide synthase enzymes, within which discrete catalytic domains sequentially extend and reductively manipulate nascent polyketides [11].

As yet, relatively little is known about the regulation of macrolide biosynthesis nor is it yet obvious whether a common pattern of control might emerge. For example, the intensively studied erythromycin-biosynthetic (*ery*) gene cluster of *Saccharopolyspora erythraea* contains no regulatory genes and none that control erythromycin production has yet been identified elsewhere in the *Sacc. erythraea* genome. In contrast, the tylosin-biosynthetic (*tyl*) gene cluster of *Streptomyces fradiae* contains at least five candidate regulatory genes [2]. Although the precise functions of the latter remain to be established, a current model [7] proposes that tylosin production is controlled in pathway-specific fashion by regulatory protein(s) of the SARP family [26] and/or by the product of *tylR* [2] and also by one or more gamma-butyrolactone signalling factors [12]. The TylR protein appears to mediate global control of tylosin production, since both polyketide and deoxysugar metabolism were abolished in a *tylR*-disrupted strain of *S. fradiae* [2]. As an added complication, glycosylated precursors of tylosin are required to stimulate production of the aglycone, tylactone [8]. In this respect, glycosylated macrolides act catalytically (in an uncharacterized manner) to turn a trickle of polyketide metabolism into a flood [9]. However, none of this implies that the polyketide aglycone and the three tylosin sugars are necessarily produced in stoichiometrically equivalent amounts. The primary purpose of the present work was to address this point and to determine whether tylosin production in *S. fradiae* is limited by availability of the aglycone or of the sugars. It was also of interest to ascertain whether such limitation has changed significantly during the empirical selection of enhanced production strains.

The starting point for the present work was the observation that the polyketide aglycone, tylactone (synonym, protylonolide), does not accumulate during tylosin-production fermentations with wild-type *S. fradiae* (strain C373.1) or with empirically selected strains (such as C4) that produce elevated levels of tylosin. Accordingly, tylosin-production fermentations were supplemented exogenously with the aglycone to determine the extent to which *S. fradiae* might possess "excess" capacity to glycosylate tylactone. Since the latter was added to fermentations as a dimethylsulfoxide (DMSO) solution, the solvent was also added to controls — with unexpected consequences.

Materials and methods

Bacterial strains, growth conditions, and genetic manipulation

S. fradiae T59235 (also known as C373.1, and referred to here as wild type), the enhanced production strain C4 derived from C373.1 by sequential mutagenesis [21] and the nonproducing *tylA* mutant, GS14, derived from strain C4 *via* mutagenesis [1], were

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maintained and propagated at 37°C on AS-1 agar [27] or at 30°C in tryptic soy broth (Difco).

Fermentation analysis

Fermentation of *S. fradiae* for 7 days at 28°C, extraction of products, and HPLC analysis with internal standards, were carried out as described elsewhere [5]. Tylactone, dissolved in DMSO, was added to selected fermentation flasks and the solvent alone was also added to controls.

Results

Studies with the ancestral strain C373.1

To ascertain whether *S. fradiae* could glycosylate extra tylactone, in addition to that being produced endogenously, varying amounts of the aglycone were added to cultures of *S. fradiae* after 2 days of fermentation in tylosin production medium and the solvent (DMSO, 1% v/v final concentration) was added to controls. Fermentation was then continued for a further 5 days. The results (Figure 1) were dramatic on two counts. First, the yield of tylosin was more than doubled in solvent controls to which no exogenous tylactone was added (Figure 1a,b). (In additional controls, it was established that these effects were not due to enhanced recovery of tylosin in the presence of organic solvent.) Second, the yield of tylosin could be further increased by exogenous addition and complete bioconversion of tylactone (Figure 1c,d). This level of tylosin production appeared to represent a plateau value because unmodified aglycone was recovered from fermentations when greater amounts of tylactone were added (Figure 1e). Collectively, these data revealed that *S. fradiae* wild type could support glycosylation of tylactone to at least four times the levels normally seen in shake flask fermentations under these conditions, although it was not clear whether the presence of DMSO had any effect on the glycosylation capacity. In contrast, DMSO clearly had an effect on the total amount of macrolide produced. The observation that

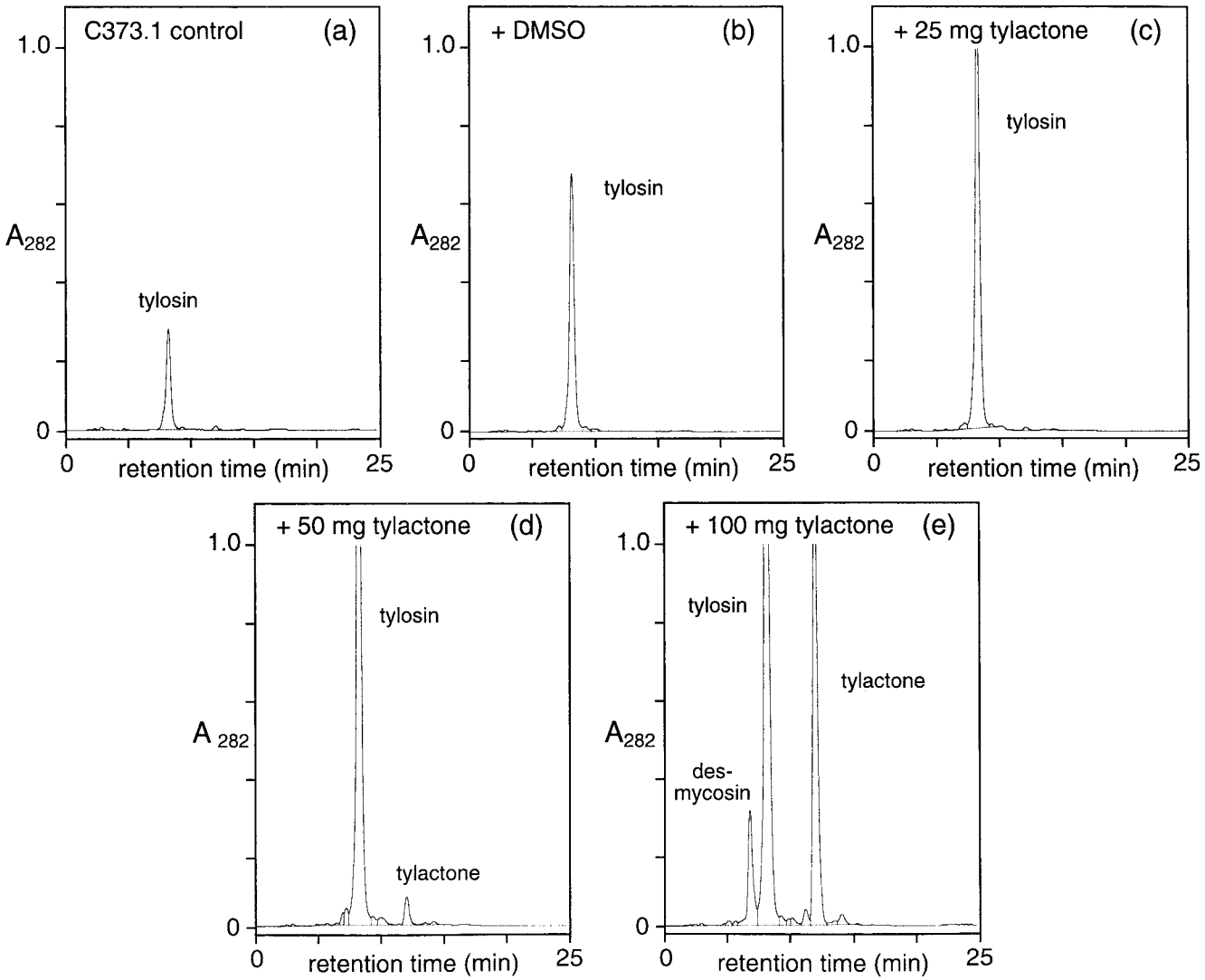

Figure 1 Effects of DMSO and exogenous tylactone on tylosin production by *S. fradiae* wild type (strain C373.1). DMSO (1% v/v) or tylactone dissolved in DMSO was added to fermentations at day 2 and incubation was continued for a further 5 days. HPLC analysis of material produced in media containing: (a) no additions — control, (b) 1% v/v DMSO, (c) 25 mg tylactone, (d) 50 mg tylactone, (e) 100 mg tylactone. Each scan represents products from approximately 0.2 ml of culture.

polyketide metabolism limits tylosin yields in *S. fradiae* has obvious implications for engineered strain improvement and will be addressed elsewhere. The unexpected effect(s) of DMSO on tylosin production were further addressed here.

Thus far, DMSO had been added to fermentations exclusively at 1% v/v (final concentration); however, this proved to be suboptimal. The increase in tylosin yield was significantly greater when DMSO was added to fermentations at 3% and appeared to plateau at 3–5% input (Figure 2). Such concentrations of DMSO did not obviously affect the total biomass generated during fermentation and had no obvious effect on growth or sporulation of strain C373.1 when added to solid AS-1 agar medium (data not shown). Other experiments (data not shown) examined the influence of lower inputs of DMSO and revealed that the solvent did not affect tylosin yields when added to fermentations at 0.1% v/v or less. Although organic solvents in general were not screened, dimethylformamide (DMF) at 1% v/v elicited an effect closely similar to that seen with 1% DMSO (Figure 2e). At this level of input, DMF did not appear to be growth inhibitory, but was obviously so at 3% v/v, under which conditions the yield of tylosin was greater than that in the unsupplemented control, but much less than that seen with 1% DMF. At 5% v/v input, DMF severely inhibited growth and very little tylosin was produced (data not shown).

Studies with the developed strain C4

This strain normally produces about five times more tylosin than does strain C373.1 during shake flask fermentations under the present conditions (Figure 3a). Addition of DMSO at 1% v/v final concentration (Figure 3b) increased the tylosin yield by approximately 40%, but the total amount of macrolide produced was approximately doubled because desmycosin (demycarosyl-tylosin) also accumulated in the cultures (confirmed by spiking samples with authentic material). At 3% input (Figure 3c), DMSO more than doubled the tylosin yield and also caused accumulation of a complex mixture of tylosin precursors, including desmycosin

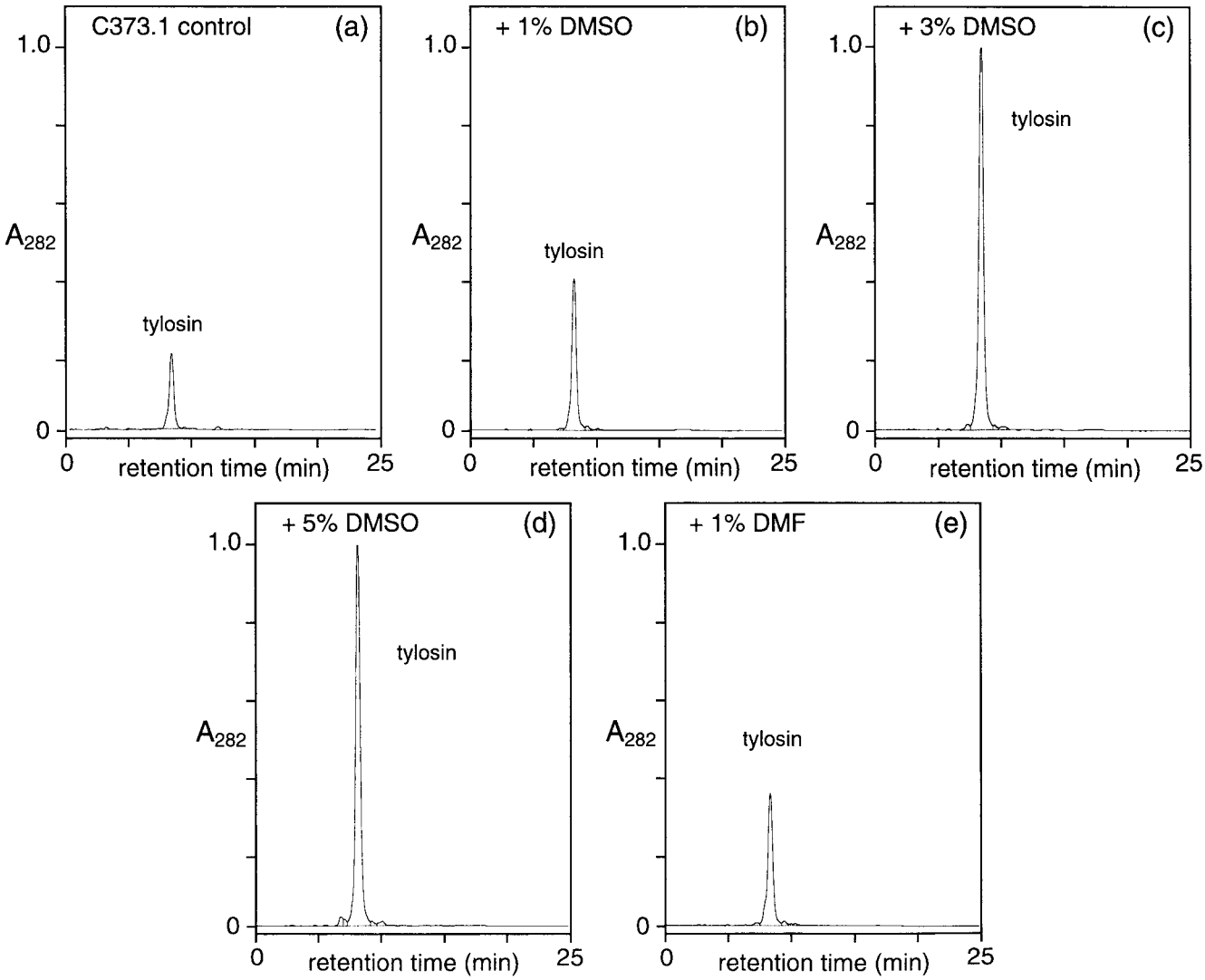

Figure 2 Effects of DMSO and DMF on fermentation of *S. fradiae* wild type. HPLC analysis of material produced in media containing: (a) no organic solvent, (b) 1% v/v DMSO, (c) 3% v/v DMSO, (d) 5% v/v DMSO, (e) 1% v/v DMF. Each scan represents products from approximately 0.1 ml of culture.

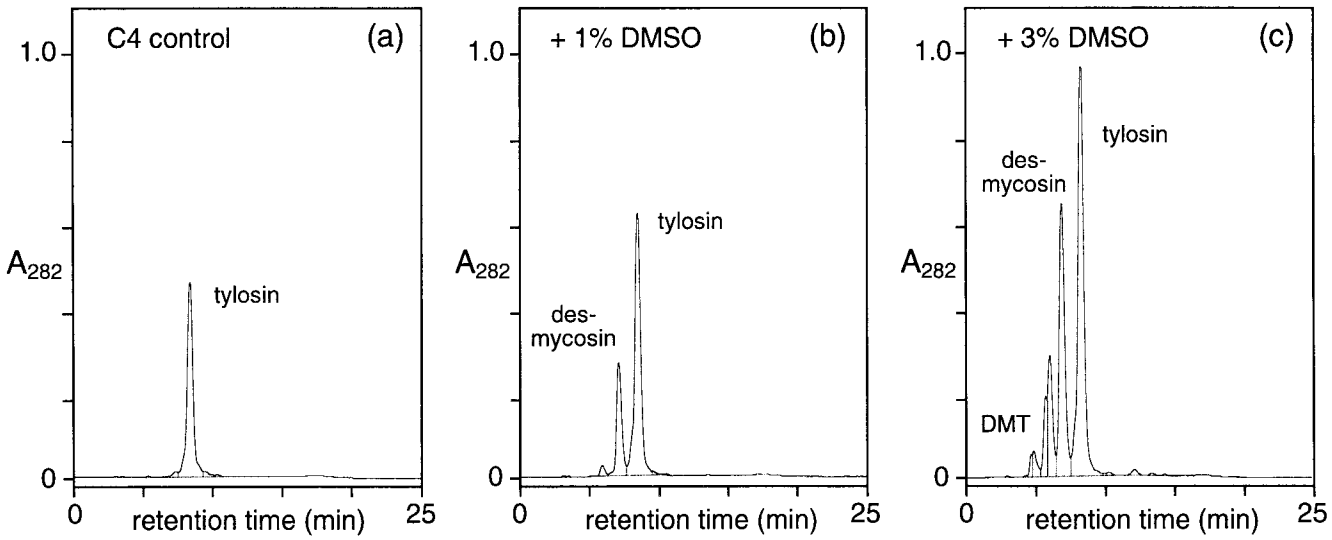

Figure 3 Effect of DMSO on fermentation of the developed strain, *S. fradiae* C4. HPLC analysis of material produced in media containing: (a) no organic solvent, (b) 1% v/v DMSO, (c) 3% v/v DMSO. Each scan represents products from approximately 0.1 ml of culture. Abbreviation: DMT, demycinosyl-tylosin.

and demycinosyl-tylosin. These were present in substantial amounts, so that the total yield of macrolide material was increased four- to five-fold. Under these conditions, deoxyhexose biosynthesis appeared to be limiting, although the continued absence of tylactone suggested that the supply of mycaminose still (at least) matched that of the aglycone. Increasing the DMSO concentration to 5% (data not shown) did not significantly change the profile, or the yields, of compounds produced.

Studies with the tylA mutant, GS14

Data obtained with strains C373.1 and C4 pointed directly to stimulation of polyketide metabolism by DMSO during tylosin production. However, given the unresolved interplay between polyketide metabolism and deoxyhexose availability, it was not clear to what extent these results might have stemmed from unsuspected effects of DMSO on biosynthesis of the tylosin sugars. It was therefore decided to investigate the effect of DMSO on polyketide biosynthesis in the virtual absence of deoxyhexose metabolism. This was done using the *tylA* mutant, *S. fradiae* GS14, which was originally derived from strain C4 by mutagenesis [1]. This strain harbours a missense mutation within *tylAII* (which normally encodes NDP-glucose dehydratase [19], an enzyme common to the biosynthesis of all three tylosin sugars), and can be successfully complemented by DNA fragments containing wild type *tylAII* (AR Butler, unpublished). (In Ref. [19], *tylAII* was inappropriately designated "*tylA2*.") Strain GS14 displays a leaky phenotype and can still produce minute levels of bioactive material, barely distinguishable by HPLC, but sufficient to trigger the production of copious quantities of tylactone during fermentation (Figure 4a). The amount of tylactone produced by strain GS14 was significantly enhanced by the addition of DMSO at 1–3% v/v final

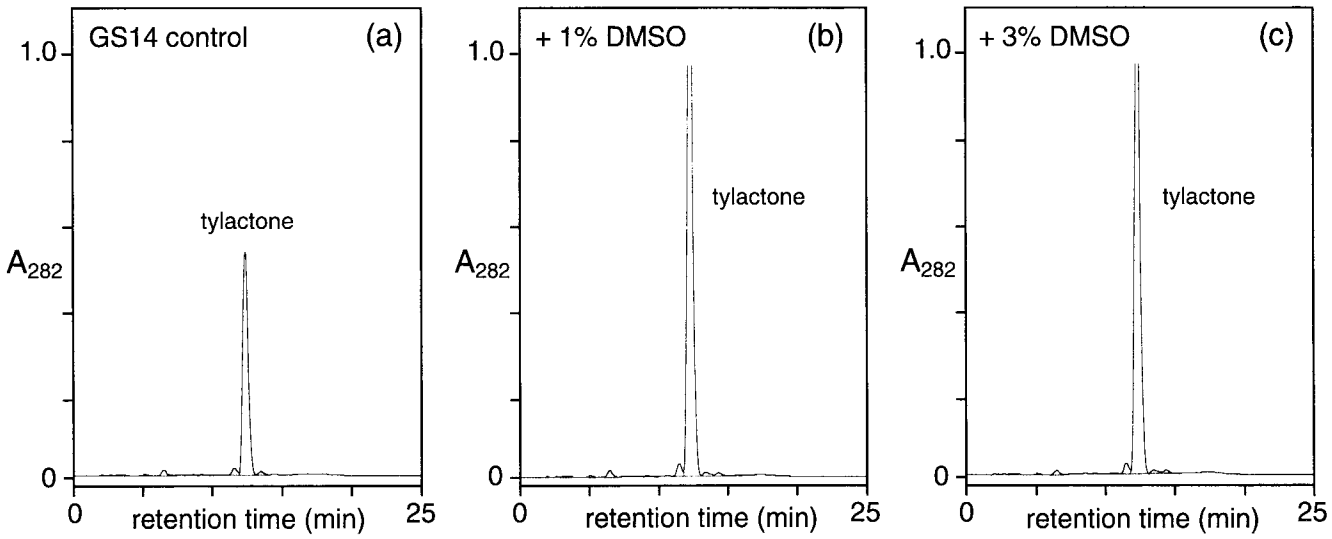

Figure 4 Effect of DMSO on fermentation of the *tylA* mutant of *S. fradiae* GS14. HPLC analysis of material produced in media containing: (a) no organic solvent, (b) 1% v/v DMSO, (c) 3% v/v DMSO. Each scan represents products from approximately 0.1 ml of culture.

concentration (Figure 4b,c); again, this appeared to represent a plateau level.

Discussion

It is not clear from the present work whether addition of DMSO to tylosin-production fermentations had any significant effect on deoxyhexose metabolism in *S. fradiae*. However, it is clear that *S. fradiae* possesses a greater capacity to glycosylate tylactone than is normally utilized in shake flask fermentations under the present conditions. It is also clear that DMSO stimulates polyketide metabolism in *S. fradiae* and that, even without changing the fermentation medium, strains C373.1 and C4 are inherently capable of producing significantly elevated yields of tylosin. It would be interesting to ascertain whether similar yield improvements could be achieved with current industrial production strains and whether the "DMSO effect" (which is presently obscure) could be mimicked in some other way that might be more amenable to commercial exploitation.

During the final stages of this work, we learned that others [6] had observed stimulation of antibiotic production by DMSO in a much broader context, involving several chemically different antibiotics produced by natural and recombinant strains of actinomycetes and by bacilli. The present work reinforces their observations. Those workers used DMSO at concentrations similar to those employed here and, like us, they concluded that stimulation of secondary metabolism by DMSO was not due to changes in biomass accumulation during fermentations. There were, however, some minor differences between their data and ours. In the present work, there was no obvious effect of 1% DMSO on growth or sporulation of *S. fradiae* on solid medium, whereas Chen et al [6] reported morphological changes and effects on the timing and density of sporulation with several organisms exposed to DMSO. Perhaps more interestingly, 1% DMF mimicked the effect of 1% DMSO on tylosin production in the present work, but apparently did not do so in other contexts [6].

The nature of the DMSO effect is obscure. In an expansive discussion of their results, Chen et al [6] consider various scenarios ranging from possibly specific effects relating to DMSO *per se* (they point out, for example, that DMSO is a natural product) to a more general model invoking the possibility of a causal link between perturbation of translational accuracy and secondary metabolism. Our observation that DMF can mimic DMSO in enhancing tylosin production might weigh against the former. The revelation that DMSO affects the accuracy of protein synthesis *in vitro* (unpublished data of some antiquity, reported in Ref. [6]) might link the present phenomena to recent data from Hesketh and Ochi [10], Hosoya et al [13], and Shima et al [22], who have observed stimulatory effects on secondary metabolism due to ribosomal changes (involving streptomycin-resistance mutations that affect protein S12) that, likewise, perturb translational fidelity.

Acknowledgements

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