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ORIGINAL PAPER

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Evidence from proteomics that some of the enzymes of actinorhodin biosynthesis have more than one form and may occupy distinctive cellular locations

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Abstract An important attribute of proteome analysis carried out with the aid of two-dimensional gel electrophoresis is that post-translational modifications of proteins can often be revealed. Large-scale proteomic analysis of *Streptomyces coelicolor* A3(2) has been made possible with the availability of its genome sequence. Here, we bring together observations on the proteins specifically associated with biosynthesis of the isochromanequinone polyketide antibiotic actinorhodin. The predicted products of 14 of the genes annotated as belonging to the *act* gene cluster were detected. They were generally present only in stationary phase cultures. Plausible explanations are presented for the absence of the other nine. For six of the gene products detected, there was evidence of either specific processing or covalent modification; in the case of the pyran ring closure enzyme ActVI-ORF3, the cleavage of the N-terminal 31 or 34 amino acids was previously shown to be associated with an extracytoplasmic location for the mature gene product [Hesketh A, et al. (2002) Mol Microbiol 46:917–932]. These observations may have implications for the regulation of actinorhodin biosynthesis, and for biochemical studies of artificially expressed Act proteins.

Keywords Polyketides · Secondary metabolism · 2D gel electrophoresis · Proteolytic processing

Introduction

A genetics-led approach to the biosynthesis of the important polyketide class of natural products began about 25 years ago with the isolation and genetic mapping of mutations in the *act* cluster responsible for biosynthesis of the blue isochromanequinone antibiotic actinorhodin that gives *Streptomyces coelicolor* its name [13]. The availability of the *S. coelicolor* genome sequence [1] has made it possible to initiate characterisation of *S. coelicolor* at the level of its global protein content, using mass spectrometry to analyse tryptic digests of protein spots separated by two-dimensional (2D) gel electrophoresis. Thus, some 920 spots have been shown to correspond to the products of 770 genes, the result of approximately 20% of gene products existing in more than one form [8]. Depending on the pattern of shifts between such related spots, it is possible to make reasonably confident discrimination between covalent modification, and forms of the protein differing only in length either because of proteolytic cleavage or because of their translation from alternative start codons.

Hesketh et al. [8] identified many of the *act* gene products among the spots on 2D gels, and noted that some of them occurred in more than one form. This raised the possibility of new insights into actinorhodin biosynthesis; individual steps might be regulated by post-transcriptional processes, the existence of which might affect the ways in which, for example, individual enzymes should be studied via their over-expression in heterologous hosts (of course, this is already understood in certain specific cases, such as acyl carrier proteins, to which phosphopantetheinyl groups need to be added to generate the active form). Here, we use proteomics to examine many of the proteins involved in actinorhodin synthesis, to clarify for each of them the extent to which post-translational processing takes place, and to find out whether it changes during the course of laboratory-scale batch culture.

Materials and methods

S. coelicolor M600 [12] was cultivated with vigorous agitation at 30°C in minimal medium supplemented with 0.2% casamino acids (SMM) as described previously [12]. Protein extracts were prepared from harvested mycelium and separated by 2D gel electrophoresis as previously described [8]. The isoelectric point range analysed was pH 5.5–6.7, which was expected to reveal many, but not all, of the

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Act proteins. The experiments were performed twice from separate cultures, with similar results. Gels were stained with Sypro Ruby (Bio-Rad, Paisley, UK) according to the manufacturer's instructions, and scanned using the Perkin-Elmer ProXPRESS proteomic imaging system (Perkin-Elmer, Foster City, Calif.) using excitation and emission wavelengths of 480 nm and 630 nm, respectively. Image analysis was performed using PHORETIX 2D version 5.1 (NonLinear Dynamics, Newcastle upon Tyne, UK): spot detection was optimised automatically using the 'Spot Detection Wizard' and then manually edited; background subtraction was performed automatically using the 'Mode of Non-Spot' setting; and images were then normalised to the total spot volume for quantification. Spot filtering was not used. Protein spots of interest were excised from gels using the Investigator ProPic robot from Genomic Solutions (Ann Arbor, Mich.), and identified by tryptic digestion and MALDI-TOF mass spectrometry as previously described [8].

Results

Gene products corresponding to *act* genes

We previously reported an initial identification of protein spots from 2D gel separations of extracts from *S. coelicolor* (strain M145) grown in liquid SMM medium [8]. This analysis revealed the products of many of the genes in the *act* cluster, as expected from the observed production of blue pigment in the cultures. In an extension of this earlier work, we examined the time-course of appearance of Act protein spots during replicate typical shake-flask batch cultures of strain M600, a prototrophic plasmid-free derivative of *S. coelicolor* A3(2) distinct from the sequenced strain, M145 [12]. Our choice of M600 was dictated by plans for further comparisons, not shown here, with mutants available only in the M600 background. Two separate sets of cultures were established from the same spore suspension and each was harvested at five time points representing mid-log phase, late log phase, transition stage, and early and late stationary phase [8]. All the samples were extracted for proteome analysis by 2D gel electrophoresis. For ease of quantitation we stained gels with Sypro Ruby, a fluorescent protein stain with a high dynamic range that also shows good sensitivity and is fully compatible with downstream protein identification by tryptic digestion and MALDI-TOF mass spectrometry. The pI range studied was expected to encompass 12 of the 23 *act* gene products. Using Phoretix software for spot matching and spot intensity measurements, we were able to recognise and quantify many of the Act proteins identified in our previous study.

Table 1 lists all gene products of the Act biosynthetic cluster together with some of their predicted character-

Table 1 Summary of proteins in the actinorhodin biosynthetic cluster. *TM* Transmembrane domains

	ORF no[a]	Gene	Annotated gene product[b]	Predicted properties[c]			Observations from two-dimensional (2D) gel analysis[d]		
				TM	pI	kDa	pI	kDa	Modification?[e]
↑	SCO5070	*act*VI-ORFB	Hydroxylacyl-CoA dehydrogenase	1	4.95	41			
↑	SCO5071	*act*VI-ORFA	Hydroxylacyl-CoA dehydrogenase	0	6.39	16	6.3, 6.5	17, 17	C
↓	SCO5072	*act*VI-ORF1	Hydroxylacyl-CoA dehydrogenase	1	5.83	33	5.9	39	
↓	SCO5073	*act*VI-ORF2	Oxidoreductase	2	6.01	34	6.1, 6.3	40, 18	L
↓	SCO5074	*act*VI-ORF3	Dehydratase	1	8.76	23	8.3, 8.3	20, 19	L
↓	SCO5075	*act*VI-ORF4	Oxidoreductase	1	5.86	31	5.9, 5.8	37, 26	L
↓	SCO5076	*act*VA-ORF1	Integral membrane protein	13	9.79	54			
↓	SCO5077	*act*VA-ORF2	Hypothetical protein	0	6.92	14	7.2	15	
↓	SCO5078	*act*VA-ORF3	Hypothetical protein	0	6.08	30	6.2, 6.2, 5.9	33, 32, 32	L and C or L
↓	SCO5079	*act*VA-ORF4	Conserved hypothetical protein	0	5.69	32	5.8	37	
↓	SCO5080	*act*VA-ORF5	Hydrolase	3	5.50	39	5.8	39	L or C
↓	SCO5081	*act*VA-ORF6	Hypothetical protein	0	5.00	12			
↑	SCO5082	*act*II-ORF1	Transcriptional regulator	0	5.16	28			
↓	SCO5083	*act*II-ORF2	Integral membrane transport protein	13	6.48	59			
↓	SCO5084	*act*II-ORF3	Integral membrane protein	12	6.17	74			
↓	SCO5085	*act*II-ORF4	Pathway-specific activator protein	0	6.01	28			
↑	SCO5086	*act*III	Ketoacyl reductase	1	5.27	27	5.3	26	
↓	SCO5087	*act*I-ORF1	Polyketide β-ketoacyl synthase α	2	5.02	49			
↓	SCO5088	*act*I-ORF2	Polyketide β-ketoacyl synthase β	1	5.35	42	5.4, 5.5	49, 57	A
↓	SCO5089	*act*I-ORF3	Polyketide synthase acyl carrier protein	0	3.98	9			
↓	SCO5090	*act*VII	Polyketide synthase cyclase/dehydratase	0	5.96	34	6.0	45	
↓	SCO5091	*act*IV	Cyclase	3	5.36	32	5.3	33	
↓	SCO5092	*act*VB	Dimerase	1	6.14	18	6.4	21	

[a]Arrows represent the gene orientation. The genes are listed in their order in the *act* cluster
[b]From SCODB at http://jiio16.jic.bbsrc.ac.uk/S.coelicolor/ (accessible from www.sanger.ac.uk/Projects/S_coelicolor/)
[c]pI and molecular weight values were calculated from ComputepI/mw at www.ca.expasy.org, and TM predicted using TMPred at www.ch.embnet.org
[d]Blank rows indicate that the protein was not detected. Observed pI and molecular weight values were estimated from the location of identified spots on 2D gels: because of variation in pI, not all proteins could be visualised on a single gel separation, and the accuracy of the estimations varies. The observations combine data from this paper with those of Hesketh et al. [8]
[e]*L* Modified in length (possible proteolytic processing), *C* modified only in charge (possible covalent modification), *A* possible aggregation/incomplete solubilisation

istics and a summary of observations made from analysis of the proteome. It combines the M600 data obtained here with those previously reported for M145, which extended into the alkaline pH range and also further into the acidic range, but which did not incorporate time-courses. Figure 1 shows an area of a representative gel on which many of the proteins discussed in this paper can be seen; Fig. 2 is a compilation of histograms revealing relative intensities of the spots illustrated in Fig. 1 during the time-course analysis. It was noticeable that the majority of the Act spots were not detectable at either of the two earliest time points. For two apparent exceptions, ActVI-ORF4 (SCO5075) and ActVA-ORF5 (SCO5080), faint spots were present in the correct 2D gel location at early time points, but not in sufficient quantities to be identified, and may correspond to unrelated proteins. The late onset of transcription previously reported for the *act* operons [6, 9] was therefore mirrored, as expected, at the protein level. Moreover, it appears that each of the proteins increased in abundance during the first 10 h of stationary phase (i.e. between time points 4 and 5 in Fig. 2). In an earlier study of *S. coelicolor* M600 grown under the same conditions, transcription of *act*III and the pathway-specific regulatory gene *act*II-ORF4 increased dramatically during transition phase but, in contrast to the protein accumulation observed here, did not show any further increase on entry into stationary phase [7].

Certain Act proteins were not detected at any time point in these or our previous proteomic studies. Notably, none of the four ActII proteins involved in regulation and transport was detected. This may reflect the prediction that ActII-ORF2 and -ORF3 are integral membrane proteins, and the likely low abundance of the ActII-ORF1 and ActII-ORF4 regulators (although we were able to detect some other regulatory proteins, including CdaR, which regulates production of the calcium-dependent antibiotic CDA and, like *act*II-ORF4, is a member of the SARP family of pathway specific regulators [16]). The case of the missing biosynthetic enzymes is discussed below.

Several of the enzymes of actinorhodin biosynthesis appear to be post-translationally processed

We previously reported that ActVI-ORF3 was detected as two spots with molecular weight and pI coordinates different from the predicted values. Peptide mass fingerprint analysis of two lower molecular weight forms revealed a major spot truncated by proteolytic cleavage between amino acids 31 and 32, and a minor one resulting from cleavage between residues 34 and 35 [8]. Interestingly, analysis of the sequence predicted a possible signal peptide cleavage site removing the first 31 amino acids, in agreement with the observed results. Analysis of a preparation obtained by externally extracting proteins from intact washed mycelium showed significant enrichment of processed ActVI-ORF3 protein, suggesting that it is exported to the cell wall.

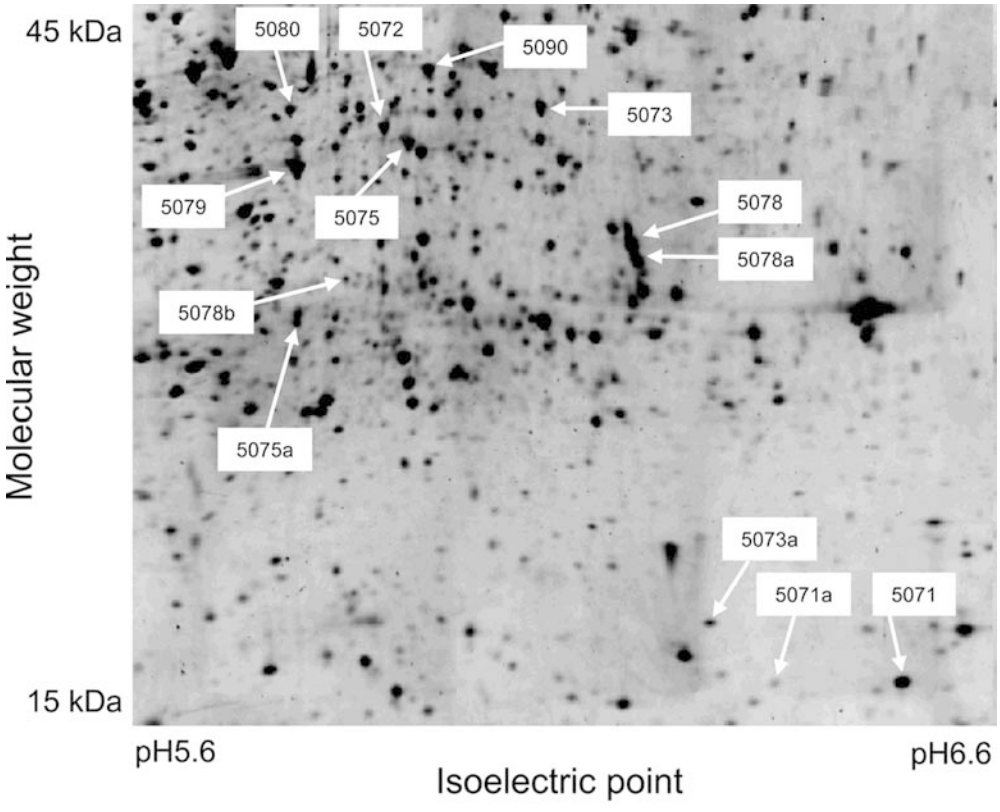

Fig. 1 Two-dimensional (2D) gel separation of proteins showing the relative abundance and possible post-translational modification of gene products from the actinorhodin biosynthetic cluster in a stationary phase culture of *Streptomyces coelicolor* M600. The gel shown corresponds to culture *B*, time point *5* in Fig. 2. Note that the gel separated only those proteins in the pI range 5.5 to 6.7

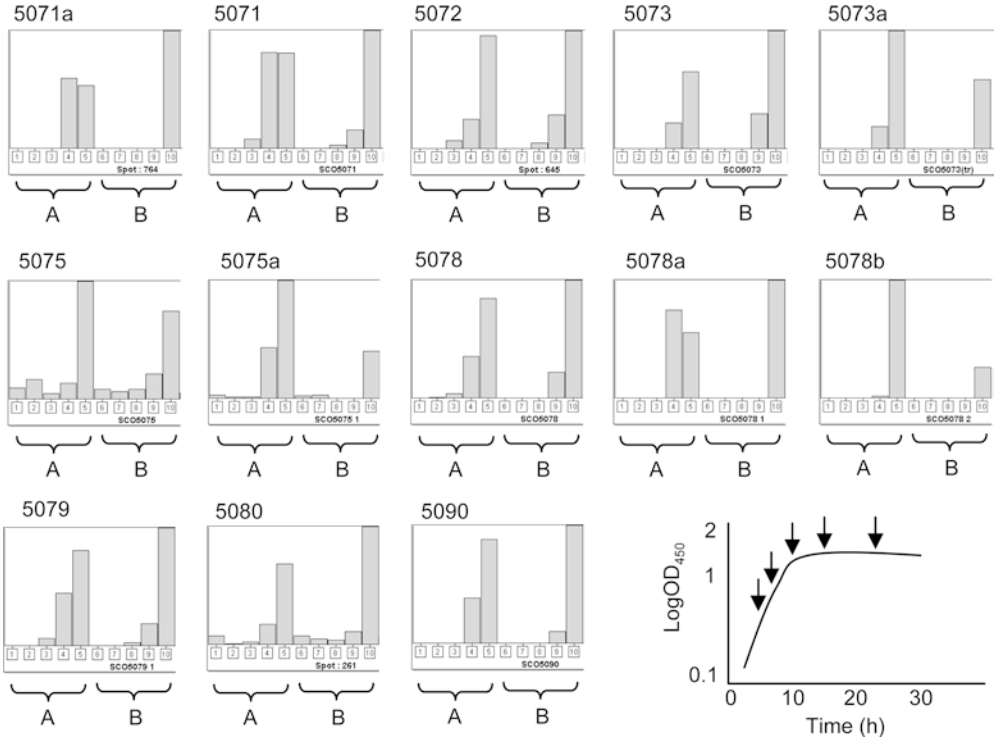

Fig. 2 Time-course abundance profiles of Act proteins during growth of *S. coelicolor* M600 in liquid minimal medium supplemented with 0.2% casamino acids (SMM). *A*, *B* Duplicate experiments in which cultures were sampled at five time points, as illustrated in the stylised growth curve shown at the bottom right. *Histogram bars* Normalised spot intensities following staining with Sypro Ruby, arranged from left to right in the same order as the *arrows* in the growth curve indicating when the five samples were taken

ActVI-ORF3 is one of only two predicted positively charged Act proteins, and was therefore not detected in Fig. 1, which displays the acidic range of proteins, including many of the other Act proteins in the cluster. Examination of Fig. 1 reveals multiple spots for several gene products, the abundance of which is plotted in Fig. 2. This indicates possible post-translational modification of proteins as summarised in Table 1, and is particularly evident for the various ActVI and ActVA proteins, which have been assigned functions late in actinorhodin biosynthesis [2, 4, 11, 15]. Analysis of shunt metabolites produced in disruptant strains suggested that the actVI region of the cluster is responsible for the stereo-specific pyran ring formation that occurs following assembly of the carbon skeleton by the multicomponent Type II polyketide synthase (PKS) complex [11], while *act*VA-ORF2 to -ORF6 have been implicated in the control of post-cyclisation ring hydroxylation reactions [2, 11].

ActVI-ORF2 and -ORF4

In addition to ActVI-ORF3, ActVI-ORF2 and -ORF4 also appear to undergo modification by proteolytic processing. The major ActVI-ORF2 spot had pI and molecular weight values consistent with predictions for the full-length protein (5073 on Fig. 1), but a less abundant spot was also present at a lower molecular weight (5073a in Fig. 1). Both spots exhibited similar accumulation profiles (Fig. 2). The absence of three tryptic peptides in the MALDI-TOF fingerprint of spot 5073a (which were present in the presumably full-length 5073) indicates that this form of the protein lacks at least the first 74 N-terminal amino acid residues, and possibly as many as 165. A similar comparison showed that the low molecular weight form of the ActVI-ORF4 protein (spot 5075a in Fig. 1) was also N-terminally truncated, with a possible range of 38 to 55 amino acids missing. The two protein species again exhibited similar abundance profiles to each other.

ActVI-ORFA

The ActVI-ORFA protein, which is believed to be involved in stabilising the PKS complex [14], was identified as two spots differing only in isoelectric point (5071 and 5071a in Fig. 1), suggesting modification by covalent addition of a low molecular weight adduct.

ActVA-ORF3 and -ORF5

These proteins have been implicated in the control of ring hydroxylation reactions, which are additional examples of post-PKS tailoring steps required for Act production [2, 11]. It was striking that ActVA-ORF3 appeared as two adjacent spots of approximately equal abundance differing slightly in both pI and molecular weight (5078 and 5078a on Fig. 1). Comparison of the MALDI-TOF peptide mass fingerprint data showed that the lower of the two spots (5078a) has a single C-terminal peptide missing, suggesting truncation by removal of between 3 and 24 amino acids from the C-terminus. A third form of the protein was also identified (5078b) with

a similar apparent molecular weight to the truncated 5078a but a more acidic pI. The MALDI-TOF data again indicated that this had been truncated at the C-terminus, and it had presumably also been further modified by covalent addition of a low molecular weight adduct. Alternatively, it is possible that a slightly different C-terminal processing of 5078b from that of 5078a might have caused the charge change. Abundance profiles show that the higher molecular weight form (spot 5078) was reproducibly detected in the cultures before either of the two C-terminally truncated proteins (Fig. 2). ActVA-ORF5 was present as an abundant spot at a significantly more basic pI than would be predicted from the protein sequence, suggesting the possibility that it had been covalently modified. It is also possible that proteolytic processing resulting in a small truncation would result in the same apparent shift in spot position.

Some spots for biosynthetic steps are absent

ActI-ORF1 protein (ActKSα) contains the conserved features of type II ketosynthases, and is presumed to be responsible for the seven rounds of addition of acetate extender units that generate the actinorhodin carbon skeleton. The *act*I-ORF1 gene overlaps with, and may well be translationally coupled to, *act*I-ORF2, which encodes a protein having extensive homology with ActI-ORF1 but lacking the catalytic site (ActKSβ). Biochemical studies have shown that the two proteins form a complex [3]. A previous study of the equivalent KSα protein, TcmM, encoded in the tetracenomycin biosynthetic gene cluster, showed that it co-fractionated with the cell membrane, and could be co-localised with the membrane by immunocytochemistry [5]. Consistent with this, ActI-ORF1 is predicted to contain two membrane-spanning domains at its N-terminus. In general, we have found that proteins with three or fewer predicted transmembrane domains are present on the 2D gels used for proteomics, and this is reflected in the successful identification of the ActIV, ActVA-ORF5 and ActVI-ORF2 proteins (Fig. 1). The absence of ActI-ORF1 in this context is difficult to explain, although it is possible that it is more strongly membrane-associated than would be predicted, due to interaction with one or more of the integral membrane proteins ActVA-ORF1, ActII-ORF2 or ActII-ORF3. These each have more than ten predicted transmembrane domains, and cannot be extracted from the membrane for visualisation on 2D gels.

Interestingly, the ActI-ORF2 KSβ, which has only one predicted transmembrane domain, was found in our initial proteomic analysis [8], although its low pI meant that it was not detected on the pH 5.5–6.7 analysis on which this paper is focussed. [In ongoing work with the pH range 4.5–5.5, ActI-ORF2 has been detected, and initial evidence of a second spot indicative of a likely covalent modification will be followed up in future work (A.H. and K.F.C., unpublished)]. The interaction between the KSα and KSβ proteins, or any interaction of ActI-ORF2 with the membrane is evidently insufficiently strong to prevent ready solubilisation under the conditions of our analysis.

ActI-ORF3, the acyl carrier protein to which the growing polyketide chain is attached, was also undetected in our analysis. This can readily be explained by its low molecular weight, which is below the range displayed in the SDS-PAGE conditions that we employed. ActVA-ORF6, the second lowest molecular weight protein in the cluster after ActI-ORF3, was also not detected, presumably for the same reason. ActVI-ORFB, however, has a predicted molecular weight of 41 kDa and its absence from the gels is consistent with the proposal, based on the observation that it is not required for normal Act production [15], that the *act*VI-ORFB gene is not part of the cluster.

Discussion

In this study, 2D gel analysis of proteins from the actinorhodin biosynthesis cluster has identified six gene products with pI and molecular weight coordinates at odds with their predicted gel positions, suggesting the possibility of a significant level of post-translational regulation in the production of this antibiotic. Modified proteins were detected for enzymes from the *act*VI and *act*VA regions of the cluster that are responsible for post-PKS tailoring reactions occurring towards the end of the pathway. It is of course well known that activation of acyl carrier proteins by covalent attachment of phosphopantetheinyl groups is necessary for the production of the polyketide skeleton, and these results suggest that post-transcriptional processes are also involved in the conversion of the polyketide into the finished antibiotic. The protein modifications identified involve differences in length in five of the cases, including three apparent N-terminal truncations (ActVI-ORF2, ActVI-ORF3, ActVI-ORF4), and one example in which the C-terminus is missing (ActVA-ORF3). Two protein spots were also identified that may be the result of covalent modification by addition of a low molecular weight adduct (5071a and 5078b in Fig. 1). In both cases the modified form was always present at relatively low abundance.

While we must be cautious in interpreting these data because of the difficulty in confidently distinguishing between biologically relevant protein modifications and artefacts resulting from sample preparation, experience gained from extensive mapping of the *S. coelicolor* M145 and M600 proteomes, where about 80% of gene products in either strain were detected as single spots, encourages us to believe that non-specific modifications are not common under the conditions used. In contrast, 6 of the 14 *act* cluster proteins detected in this study appear to be modified, a figure of around 40%. The possibility that the observed modifications are part of a mechanism for activating or deactivating enzymes involved in actinorhodin production adds a new dimen-

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ActVI-ORF3   MTSSLHHAIRLTTASAIALGGLVTLGTSAHA-ASVAV
Med-ORF5     MKKKSAAGIAAALSISAATAMLSVASPANA-TGPGTS
Gra-ORF18    MPALRSRTRTAAIAVPLALAGLMATTSGAQA-APVKT
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Fig. 3 Comparison of the predicted N-terminal signal sequences of ActVI-ORF3 with homologues from the medermycin and granaticin biosynthetic clusters. Predicted transmembrane helices are *underlined*, and the proposed cleavage site is represented by a –. ActVI-ORF3 and Gra-ORF18 sequences are from NCBI Accession Numbers NC003888 and AJ011500, respectively. The Med-ORF5 sequence was kindly supplied by Koji Ichinose (University of Tokyo)

sion to the investigation of the biosynthesis of this antibiotic. N-Terminal processing of the putative dehydratase ActVI-ORF3 appears to be 100% under the conditions analysed and, furthermore, the truncated form is enriched in a cell wall extract, suggesting that it is exported. The prediction of a signal peptide sequence for ActVI-ORF3 using the SignalP server at www.cbs.dtu.dk gave a mean *S* value of 0.81 (above 0.55 is predictive), but only three of the four scoring categories were positive. The sequence does not contain the Arg or Lys residues usually expected near the N-terminus (Fig. 3), and it is formally possible that the observed enrichment of the processed protein in the cell wall is the result of external binding following lysis of a sub-population of the culture rather than secretion. However, the observed processing site exactly coincides with the SignalP prediction, and the two homologues of ActVI-ORF3 found in the granaticin [10] and medermycin antibiotic biosynthetic clusters (*gra*-ORF18 and *med*-ORF5, respectively) both have strongly predicted signal sequences with mean *S* values >0.9 and all four scoring categories positive (Fig. 3). Granaticin and medermycin belong to the same family of benzoisochromanequinone antibiotics as actinorhodin. The suggestion that these enzymes are exported has interesting implications for the assigned functions of these proteins, and for the mechanism of production of this family of antibiotics.

Since we are proposing that the multiple protein products from some of the *act* genes are specific and are not artefacts of extraction, they may have functional significance. It follows that the modifications should be controlled. This raises questions of what are the agents of modification? Are they specific to the relevant Act proteins or do the Act proteins interact with more widely acting protein-modifying enzymes? Do different forms of particular Act proteins differ in their biochemical activity? And what is the significance of these observations for the biochemical analysis of artificially expressed Act proteins?

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