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Streptomyces

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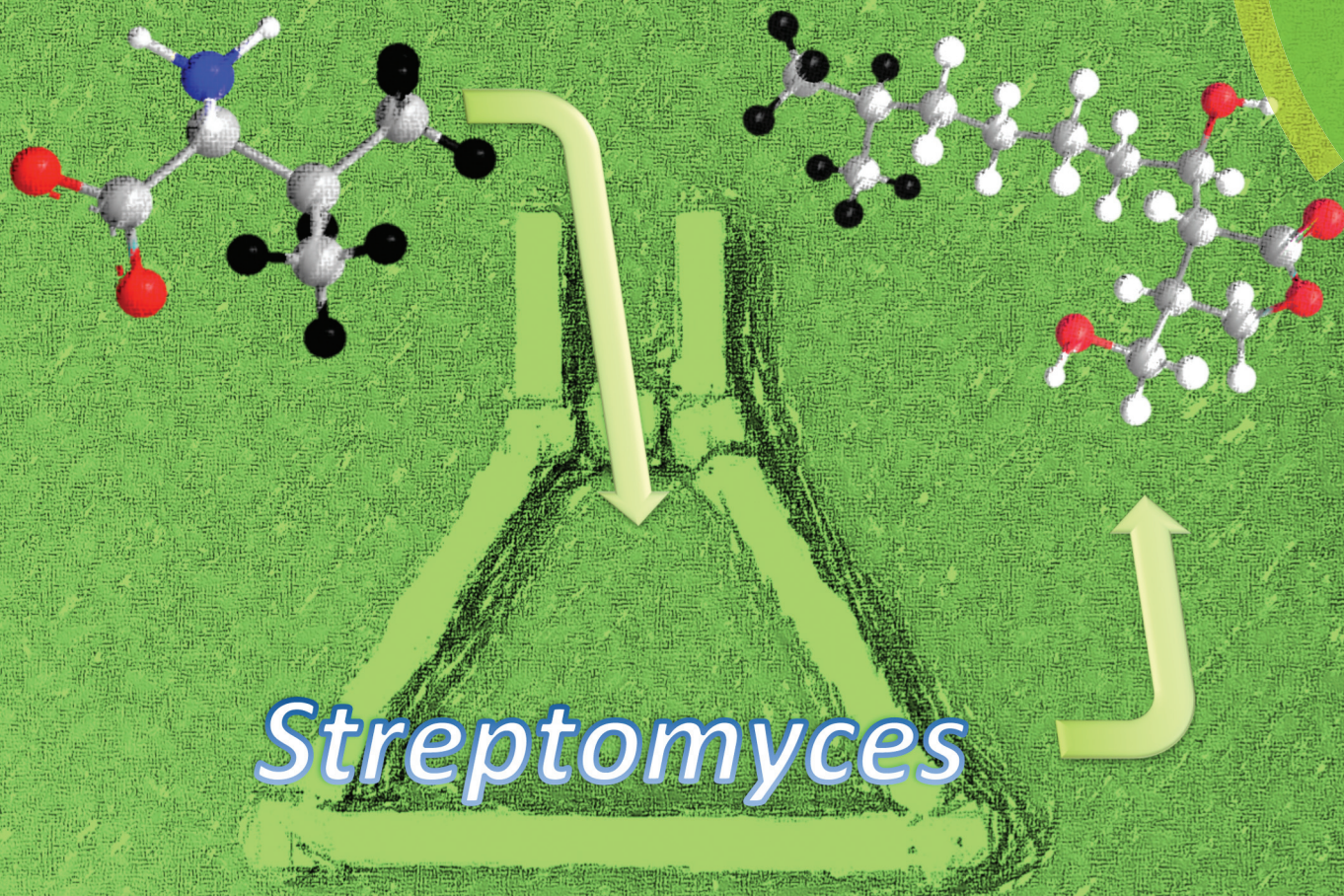
COMMUNICATION
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antibiotic production superhost *Streptomyces* M1152

175 YEARS

Overproduction and identification of butyrolactones SCB1–8 in the antibiotic production superhost *Streptomyces* M1152†

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Gamma-butyrolactones (GBLs) are signalling molecules that control antibiotic production in *Streptomyces* bacteria. The genetically engineered strain *S. coelicolor* M1152 was found to overproduce GBLs SCB1–3 as well as five novel GBLs named SCB4–8. Incorporation experiments using isotopically-labelled precursors confirmed the chemical structures of SCB1–3 and established those of SCB4–8.

Introduction

Genome mining has revealed that *Streptomyces* bacteria typically harbour 20–30 machineries or gene clusters dedicated to the production of bioactive natural products. Many of these compounds find use in human or veterinary medicine as well as in agriculture.[1] Hundreds of gene clusters proposed to direct the biosynthesis of unknown (cryptic) antibiotic-like molecules are currently available in publicly accessible databases. The characterisation of these cryptic metabolites is often challenged by the presence of transcriptional repressor proteins that prevent the expression of biosynthetic genes when bacteria are grown in laboratory culture conditions.[1]

Since the discovery of the γ-butyrolactone (GBL) A-factor in 1967 and subsequent characterisation of its intracellular target, the transcriptional repressor ArpA, distinct classes of antibiotic production inducers have been discovered.[2,3] In particular butenolides and 2-alkyl-4-hydroxymethylfuran-3-carboxylic acids (AHFCAs) have been shown to trigger the expression of antibiotic-like biosynthetic gene clusters by interacting with specific ArpA-like transcriptional repressors.[4–6]

Early investigations of signalling molecules in *S. coelicolor* A3(2) reported at least seven GBL-like signalling molecules produced by this strain.[7] The chemical structures of *S. coelicolor* GBLs SCB1–3 were elucidated in the 2000s and the *scbA* gene, an *afsA*-like butenolide synthase, was shown to direct their biosynthesis.[8–11] SCBs act as antibiotic production inducers and directly control the expression of a cluster of genes, named *cpk*, responsible for the biosynthesis of the coelimycin polyketide antibiotic in *S. coelicolor*.[12,13]

The molecular mode of action of SCBs involves direct binding to the transcriptional repressor ScbR. In the absence of SCBs, ScbR binds to specific operator sequences in particular upstream of the transcriptional activator *cpkO* and prevents coelimycin production.[14] An additional transcriptional repressor, ScbR2, is also responsible for repressing the expression of *cpk* genes.[14] Both *scbR* and *scbR2* genes are adjacent to the *cpk* gene cluster but on opposite sides (Fig. 1). Genetic inactivation of *scbR2* resulted in overproduction of coelimycin and a related yellow pigment.[15,16] In addition ScbR2 is known to bind to the *scbA–scbR* intergenic region and repress expression of *scbA*.[16] Consequently SCB1 was found to be overproduced in the *S. coelicolor* M145 *scbR2* mutant.[16]

The *S. coelicolor* superhost strain M1152, derived from *S. coelicolor* M145, has been genetically optimised for the production of antibiotic-like natural products whose biosynthesis is directed by heterologous metabolic pathways.[17] Construction of this strain involved deletion of multiple biosynthetic gene clusters, including ≈50 kb of the coelimycin gene cluster. This deletion included the gene *scbR2* but not the GBL-biosynthetic genes *scbA* and *scbB* (Fig. 1).

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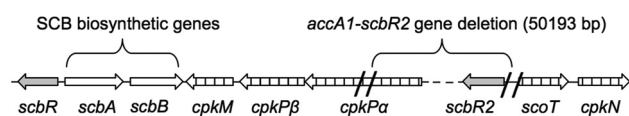

Fig. 1 Genetic organisation of the *scb/cpk* gene cluster in *Streptomyces coelicolor* highlighting the deleted DNA fragment (~50 kb) during the construction of the superhost *S. coelicolor* M1152.

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In this study we reveal that the engineered M1152 over-produces GBLs fortuitously. We report the overproduction of known *S. coelicolor* GBLs SCB1–3 as well as production of five new SCBs, SCB4–8 in M1152.[17] The chemical structures of SCB1–8 were determined by a combination of incorporation experiments using stable isotope-labelled precursors and mass spectrometry analyses.

Results and discussion

Identification of SCBs in *Streptomyces* M1152

SCBs are produced at nanomolar concentration in the parent strain *S. coelicolor* M145: ≈320 µg of SCB1 were purified from a 300 L culture in the original study.[8] In *S. coelicolor* M1152 we expected the *scbR2* deletion to result in *scbAB* overexpression and in turn overproduction of SCBs. *S. coelicolor* M1152 was therefore grown on the minimal culture medium AlaMM and ethyl acetate extracts of the culture supernatant were submitted to liquid chromatography-mass spectrometry, LC-MS, analyses (Fig. 2).

Based on the calculated m/z values for the sodium adducts of SCB1 (1) and SCB2 (2), $m/z = 267.1567$ $[C_{13}H_{24}O_4 + Na]^+$, and that of SCB3 (3) $m/z = 281.1723$ $[C_{14}H_{26}O_4 + Na]^+$, positive ion mode chromatograms were extracted for these ions. In addition, m/z values consistent with derivatives carrying shorter or longer alkyl chains ($m/z = 239.1$, 253.1 and 295.2) were also extracted and revealed the presence of metabolites 4–8. Each of the compounds 1–8 exhibited fragmentation patterns consistent with those previously reported for SCBs,[18] *i.e.* daughter ions corresponding to $[M - H_2O + H]^+$ and $[M - 2H_2O + H]^+$ in addition to $[M + H]^+$ and $[M + Na]^+$ molecular ions (Fig. 2 and S1†).

The high accuracy of the mass spectrometer used in these experiments combined with the isotopic pattern identified for each of these ions allowed masses to be generated within 5 ppm of those corresponding to the predicted molecular formulae for 1–8 (Fig. 2 and Table S1†). The molecular formulae of compounds 1 and 2 matched that of SCB1–2 ($C_{13}H_{24}O_4$) while for compound 3 it was consistent with that expected for SCB3 ($C_{14}H_{26}O_4$). For the previously uncharacterised metabolites, a common $C_{12}H_{22}O_4$ molecular formula was determined for 4–6, while 7 and 8 exhibited formulae of $C_{14}H_{26}O_4$ and $C_{11}H_{20}O_4$ respectively (Table S1†). In addition to fragmentation patterns and molecular formulae, the respective retention times on reverse phase HPLC were also consistent with 1–3 corresponding to SCB1–3. The identity of SCB1 was also confirmed by HPLC purification and NMR analysis (Fig. S11 and S12†).

The structural diversity between 1–8 was proposed to directly derive from the nature of the precursor incorporated into SCBs. The enzyme ScbA is the only butenolide synthase encoded in *S. coelicolor* M1152 genome and has been shown to be essential for SCB1–3 biosynthesis.[10] ScbA is an orthologue of AfsA that has been shown to catalyse the condensation of dihydroxyacetone phosphate (DHAP) with a β-ketothioester intermediate hijacked from fatty acid metabolism to yield a butenolide phosphate intermediate.[11] This intermediate is then reduced by the butenolide phosphate reductase BprA in *Streptomyces griseus* to generate A-factor.[11] Located downstream of *scbA* is *scbB*, which encodes for a homologue of BprA (76% identity, 84% similarity over 279 amino acids).

The SCB-like molecules 1–8 observed in this culture extract were therefore proposed to each result from the incorporation of different β-ketothioester intermediates. Specific primary metabolites, namely leucine, isoleucine, valine, propionic and butyric acids, are known to act as precursor of starter units in fatty acid biosynthesis and are converted into β-ketothioester intermediates that are subsequently incorporated into butenolides and GBLs such as SCB1 (Scheme 1).

Structural characterisation of SCB1–8 using stable-isotope labelled precursors

To unambiguously determine the nature of the alkyl side chains in each of the SCB-like compounds 1–8, *S. coelicolor* M1152 was grown in AlaMM media supplemented with 1 mM final concentration of deuterium-labelled d_8-DL-valine, d_{10}-L-leucine, d_{10}-L-isoleucine, d_5-propionic acid or d_7-butyric acid. This method was used previously to probe incorporation of specific deuterium-labelled precursor molecules into the AHFCA signalling molecules.[5,19] Metabolites were extracted from each of the supplemented cultures and analysed by LC-MS. As expected, feeding with each labelled precursor altered the mass spectra of specific SCB-like metabolites.

Feeding with d_8-DL-valine resulted in a proportion of compound 1 (SCB1) to specifically contain seven deuterium atoms, suggesting the intact incorporation of a β-ketothioester primed with isobutyryl-CoA. The molecular formula of 1 $[M + Na]^+$
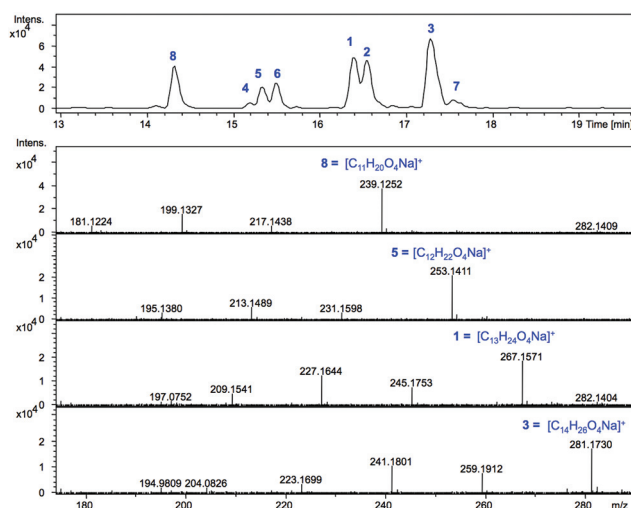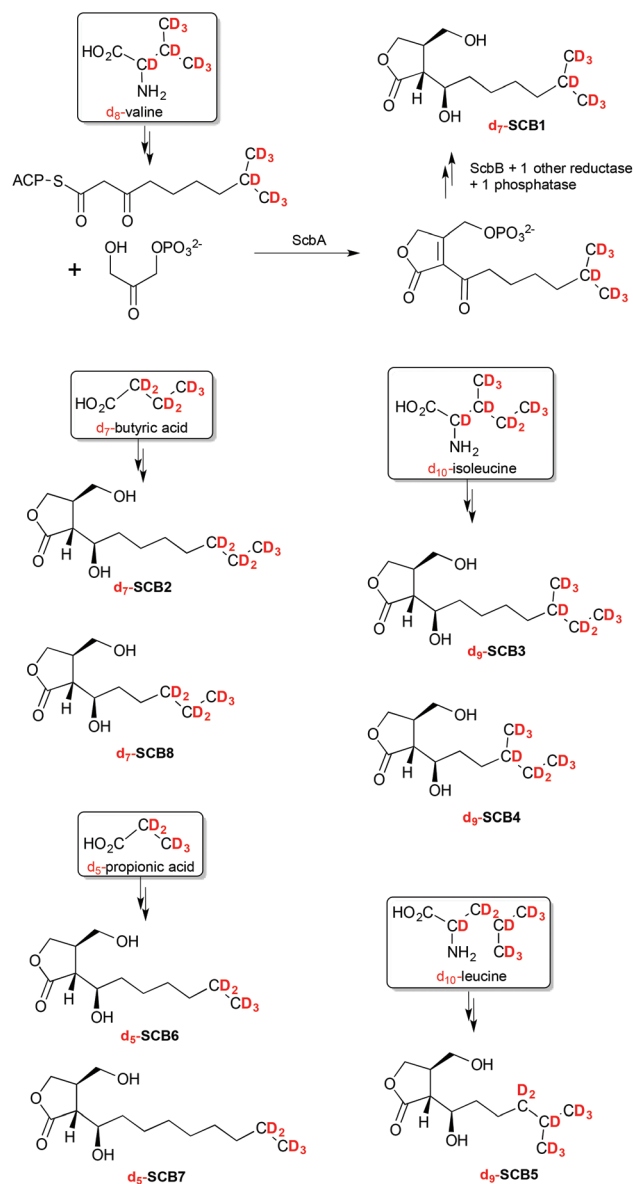

Fig. 2 LC-MS analysis representing the extracted ion chromatogram ($m/z = 239.1$, 253.1, 267.2, 281.2 in positive ion mode) corresponding to SCB sodium adducts produced by *S. coelicolor* M1152 and mass spectra (HR-MS) for compounds 8, 5, 1 and 3.

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Scheme 1 Specific incorporation of deuterated precursors into compounds **1–8** revealing the chemical structure of SCB1–8.

with m/z = 274.2 was confirmed to be $C_{13}H_{17}D_7O_4Na$ (Fig. S5†). The m/z values of $[M + H]^+$, $[M − H_2O + H]^+$ and $[M − 2H_2O + H]^+$ presented a similar pattern where seven deuterium atoms were incorporated (Fig. S5 and Table S2†). In this particular experiment, **1** was the only metabolite amongst **1–8** revealing an intact incorporation of the labelled precursor. This experiment confirmed that the alkyl chain of **1** terminates with an isopropyl group, which agrees with the published structure for SCB1 (Scheme 1). In a similar way the linear nature of the alkyl chain in **2**, SCB2, was confirmed by detecting the intact incorporation of seven deuterium atoms from d₇-butyric acid (Fig. S6†). Compound **3**, revealing the incorporation of 9 deuterium atoms from d₁₀-isoleucine, was confirmed to correspond to the previously reported SCB3 (Fig. S7†).

Feeding with d₁₀-isoleucine also resulted in specific labelling of compound **4**. The difference between SCB3 (**3**) and **4** rises from the fact that their β-ketothioester precursors have gone through a different number of chain extensions: 3 extensions in the case of SCB3 (**3**) and only 2 for compound **4**. Metabolites **5** and **6** were found to specifically incorporate nine deuterium atoms from d₁₀-leucine and five deuterium atoms from d₅-propionic acid, respectively (Fig. S3 and S4†). Compound **7** was specifically labelled upon addition of d₅-propionic acid and **8** specifically incorporated d₇-butyric acid (Fig. S8 and S2†).

The structures of the new natural products **4–8**, named SCB4–8 respectively, were therefore as indicated in Scheme 1. All the molecular formulae generated from UHR-LC-MS analyses of the labelled and unlabelled compounds **1–8** are reported in Tables S1 and S2.†

Compounds **4–8** have not been previously reported from natural sources, however **6–8** were previously synthesised and tested on the dissociation of ScbR from its DNA binding site using an *in vivo* kanamycin reporter assay.[9] Compounds **6** and **7** were shown to be 10 times less effective than SCB1 and **8** was 480 times less effective at stimulating dissociation of ScbR.[9]

Conclusion

In conclusion, we have established that the superhost strain *Streptomyces* M1152, increasingly used as a heterologous host for the expression of bacterial gene clusters, overproduces SCB1–3 (mg L^{-1} scale compare to µg L^{-1} scale in the wild-type strain) and at least five additional SCBs. The chemical nature of SCB1–8 was determined using a combination of incorporation experiments and mass spectrometry.

Overproduction of SCB antibiotic production inducers in *S. coelicolor* M1152 might provide this strain an advantage in triggering the expression of otherwise silent GBL-dependent metabolic pathways from heterologous origin. However, in most cases, the cost of GBL overproduction in terms of precursor supply and energy is more likely to impair the yield of metabolite of interest. In addition, we observed that **1–8** interfered significantly with the purification of novel metabolites co-eluting with SCB1–8. As a result of the present work, construction of an improved superhost strain has been undertaken. Inactivation of the biosynthetic gene *scbA* in the *S. coelicolor* M1152 background is expected to particularly improve the production of polyketide natural products as they share the same pool of precursors as those used in SCB biosynthesis.

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