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Functional Expression of Genes Involved in the Biosynthesis of the Novel Polyketide Chain Extension Unit, Methoxymalonyl-Acyl Carrier Protein, and Engineered Biosynthesis of 2-Desmethyl-2-Methoxy-6-Deoxyerythronolide B

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The assembly of macrolides, ansamycins, and other polyketides on type I modular polyketide synthases (PKSs) involves chain extension of an acyl starter unit by sequential addition of malonyl-CoA, methylmalonyl-CoA, or ethylmalonyl-CoA with simultaneous decarboxylation in the carbon−carbon bond-forming step to add "acetate", "propionate", or "butyrate" units, respectively.[1] In rare cases, isotopic tracer experiments have revealed the presence of another extender unit, a "glycolate" unit, consisting of two carbons in the chain which are not labeled by acetate but by glucose or glycerol, with an oxygen substituent on the α-carbon.[2−8] Such "glycolate" units are seen at C9+10 of maytansinoids[3] and C13+14 and C15+16 of FK506 and FK520 (Figure 1),[5] as well as, for example, in geldanamycin,[2] leucomycin,[4] and soraphen.[6] In most cases, the α-oxygen of these extender units is methylated,[2−6] although there are exceptions to this rule, as in the aflastatins.[7]

Analogy to the other chain extension reactions would predict that the substrate for the incorporation of these "glycolate" units should be a thioester of 2-hydroxymalonate or 2-methoxymalonate. Recently, similar sets of five genes, *asm13−17* [9] and *fkbG-K*, have been identified in the biosynthetic gene clusters for ansamitocin from *Actinosynnema pretiosum*[10,11] and FK520 from *Streptomyces hygroscopicus*,[12] respectively. Inactivation of *asm15* led to the formation of 10-desmethoxy-ansamitocin P-3 instead of ansamitocin P-3 in *A. pretiosum*,[11] implicating at least this gene in the formation of the "glycolate" extender unit. The presence of genes encoding an acyl carrier protein (ACP), *asm14* and *fkbJ*, and an *O*-methyltransferase, *asm17*and *fkbG*, suggests[11,12] that this subcluster is responsible for the synthesis of a 2-methoxymalonyl moiety on the activated[13] ACP, which then delivers the chain extension substrate to the PKS. To examine the validity of this hypothesis we decided to express *asm13−17* heterologously in *Streptomyces* and test for the formation of methoxymalonyl- or hydroxymalonyl-ACP.

An expression plasmid, pHGF 9251, was constructed from the *Escherichia coli−Streptomyces* shuttle vector pHGF7505[14] to express *asm13−17*, with *asm14* carrying a C-terminal His_6-tag, under the control of the p*actI*/p*act*III promoter and *act*II-ORF4 regulator. The plasmid was passed through the *dam*$^-$ *dcm*$^-$ *E. coli* strain, SCS-110, and introduced into *S. lividans* ZX7 or *S. coelicolor* Yu105 by PEG-mediated protoplast transformation.[15] Although the transformant strains were grown on several media (YEME, TSB, R5, and MM) for incubation periods up to 14 days, no significant band at about 10 kDa, corresponding to His-tagged methoxymalonyl- or hydroxymalonyl-ACP or uncharged ACP, was detected by SDS−PAGE of their cell-free extracts.[16] Apparently, the Asm13−17 proteins were not expressed at levels high enough for detection by the methods used.

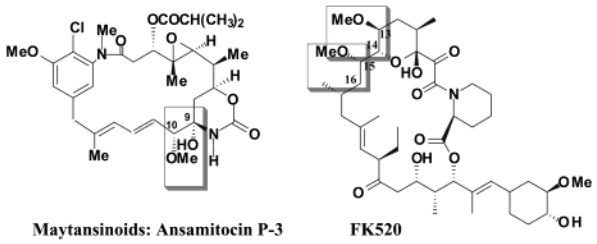

Figure 1. Structures of ansamitocin P-3 and FK 520. The locations of the unusual "glycolate" extender units are indicated by boxes.

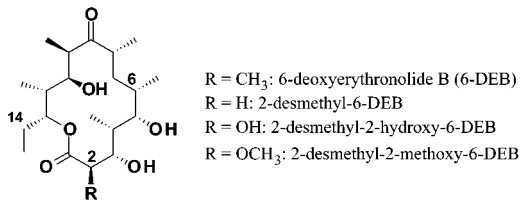

Figure 2. Structures of 6-deoxyerythronolide B (6-DEB) and analogues.

In parallel, we tried to demonstrate the function of *asm13−17* by incorporating the produced extender unit into a polyketide, using a modified PKS. The plasmid pHGF 9251 was introduced into *S. lividans* K4.114 harboring the previously constructed[17] plasmid pKOS 38-187. This plasmid carries an altered version of the *eryA* genes, encoding the 6-deoxyerythronolide B polyketide synthase (DEBS) in which the AT6 domain was replaced by the presumably hydroxymalonate-specifying *fkbA*-AT8 domain.[18] The original transformant *S. lividans* K4.114/pKOS 38-187 produced 1.5 mg/L of 2-desmethyl-6-deoxyerythronolide B (2-nor 6-DEB) and 0.5 mg/L of 6-DEB itself (resulting from the incorporation of either malonyl-CoA or methylmalonyl-CoA extender units by module 6 of DEBS[20]), whereas the co-transformant *S. lividans* K4.114/pKOS 38-187/pHGF9251 produced neither compound, but showed 3 mg/L of a new product of MW 402.[21] This novel compound was purified[22] and identified spectroscopically[23] as 2-desmethyl-2-methoxy-6-DEB (Figure 2), evidently resulting from the incorporation of 2-methoxymalonate in the last chain elongation step on the modified DEBS. The new compound has the same stereochemistry at C-2 as 6-DEB, clearly indicated by identical coupling constants between 2-H and 3-H (10.7 vs 10.5 Hz). Interestingly, no 2-desmethyl-2-hydroxy-6-DEB was detected in the fermentation.

The above results demonstrate that *asm13−17* are sufficient to allow the formation of the substrate for the hydroxy/methoxymalonate chain extension reaction, that the methoxymalonate AT8 domain of the FK520 cluster promotes the incorporation of this

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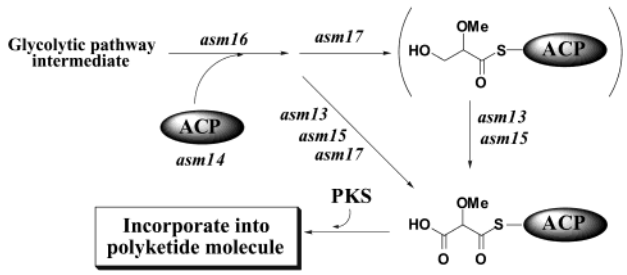

Figure 3. Possible biosynthesis of methoxymalonyl-ACP catalyzed by the *asm13−17* gene products.

substrate into a polyketide, and that the two systems can function in heterologous environments. The *asm17* gene product is identified as the methyltransferase in the *asm* gene cluster, which catalyzes the methylation of the oxygen at C-10 of ansamitocin. The result does not clearly establish whether this methylation occurs before or after polyketide assembly, although the former is more likely, since it seems unlikely that the same methyltransferase can methylate the OH-group in such different structural environments as the 6-DEB and ansamitocin backbones. The notion that the methylation catalyzed by Asm17 occurs prior to incorporation into the polyketide was confirmed by observing the effect of deleting *asm17*. The plasmid pHGF 9263 was derived by deleting from the *asm13−17* cassette a large part (314 bp) of *asm17* using suitable *Kpn*I sites. The co-transformant *S. lividans* K4.114/pKOS 38-187/pHGF 9263 produced the same metabolites as *S. lividans* K4.114/pKOS 38-187, 2-nor-6-DEB, and 6-DEB, in similar amounts; no 2-desmethyl-2-hydroxy-6-DEB (Figure 2) or 2-desmethyl-2-methoxy-6-DEB were detected. Earlier work[11] had shown no utilization of 2-hydroxy- or 2-methoxymalonyl *N*-acetylcysteamine thioester in ansamitocin biosynthesis and had demonstrated, by gene inactivation, an essential role for the ACP, Asm14, in the process. It is therefore suggested that the substrate for the "glycolate" unit should be 2-methoxymalonyl-ACP and that the 2-hydroxymalonyl-ACP (or a precursor of it) must be methylated by the product of *asm17* before its incorporation into the polyketide chain. This *O*-methylation may be a mechanistic requirement for the incorporation of the "glycolate" substrate into the polyketide.[24] However, as the normal polyketide assembly was not disturbed by the presence of *asm13−16* in *S. lividans* K4.114/pKOS 38-187, a functional interaction between the 2-hydroxymalonyl-ACP, if it is formed at all, and the modified DEBS polyketide synthase is ruled out.

The results reported here identify a set of genes involved in the biosynthesis of the novel α-methoxymalonate polyketide chain extension unit, setting the stage for unraveling the pathway and mechanism of its formation. Taking into consideration some indications that *O*-methylation may be an early step in the formation of 2-methoxymalonyl-ACP,[11] the pathway can be proposed as shown in Figure 3. The work also provides a new building block for the combinatorial biosynthesis of novel polyketides carrying a methoxy substituent at the α-carbon.

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Supporting Information Available: Construction and maps of pHGF9251 and pHGF9263, and quantitation of 6-DEB and analogues (PDF). This material is available free of charge via the Internet at http://pbs.acs.org

References

(1) (a) Khosla, C.; Gokhale, R. S.; Jacobsen, J. R.; Cane, D. E. *Annu. Rev. Biochem.* **1999**, *68*, 219−253. (b) Bentley, R.; Bennett, J. W. *Annu. Rev. Microbiol.* **1999**, *53*, 411−446. (c) Rawlings, B. J. *Nat. Prod. Rep.* **1999**, *16*, 425−484. (d) Rawlings, B. J. *Nat. Prod. Rep*. **2001**, *18*, 190−227. (e) Rawlings, B. J. *Nat. Prod. Rep*. **2001**, *18*, 231−281.
(2) Haber, A.; Johnson, R. D.; Rinehart, K. L., Jr. *J. Am. Chem. Soc*. **1977**, *99*, 3541−3542.
(3) Hatano, K.; Mizuta, E.; Akiyama, S.-I.; Higashide, E.; Nakao, N. *Agric. Biol. Chem*. **1985**, *49*, 327−335.
(4) Omura, S.; Tsuzuki, K.; Nakagawa, A.; Lukacs, G. *J. Antibiot.* **1983**, *36*, 611−613.
(5) Byrne, K. M.; Shafiee, A.; Nielsen, J. B.; Arison, B.; Monaghan, R. L.; Kaplan, L. *Dev. Ind. Microbiol*. **1993**, *32*, 29−45.
(6) Hill, A. M.; Harris, J. P.; Siskos, A. P. *J. Chem. Soc., Chem. Commun.* **1998**, 2361−2362.
(7) Ono, M.; Sakuda, S.; Ikeda, H.; Furihata, K.; Nakayama, J.; Suzuki, A.; Isogai, A. *J. Antibiot*. **1998**, *51*, 1019−1028.
(8) Bindseil, K. U.; Zeeck, A. *Liebigs Ann. Chem.* **1994**, 305−312.
(9) Genes *asm13*, *-14*, *-15*, *-16*, and *-17* show homology with 3-hydroxybutyryl-CoA dehydrogenase of *Clostridium acidobutylicum* (Gene Bank accession number P45856), D-alanyl carrier protein of *Streptococcus pyogenes* M1 GAS (T34915), a short-chain acyl-CoA dehydrogenase of *S. coelicolor* A3 (T36802), part of CO dehydrogenase of *C. thermoaceticum* (B41670), and an *O*-methyltransferase of *S. mycarofaciens* (B42719), respectively.[10]
(10) Yu, T.-W.; Bai, L.; Clade, D.; Hoffmann, D.; Toelzer, S.; Trinh, K. Q.; Xu, J.; Moss, S. J.; Leistner, E.; Floss, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002** In press.
(11) Carroll, B. J.; Moss, S. J.; Bai, L.; Kato, Y.; Toelzer, S.; Yu, T.-W.; Floss, H. G. *J. Am. Chem. Soc*. **2002**. In press.
(12) Wu, K.; Chung, L.; Revill, W. P.; Katz, L.; Reeves, C. D. *Gene* **2000**, *251*, 81−90.
(13) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber, P.; LaCelle, M.; Marahiel, M. A.; Khosla, C.; Walsh, C. T. *Chem. Biol*. **1996**, *3*, 923−936.
(14) Yu, T.-W.; Müller, R.; Müller, M.; Zhang, Z.; Draeger, G.; Kim, C.-G.; Leistner, E.; Floss, H. G. *J. Biol. Chem*. **2001**, *276*, 12546−12555.
(15) Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, U.K., 2000.
(16) Attempts to separate the His-tagged protein on Ni-NTA columns also gave no proteins in the expected molecular-weight range upon elution with various concentrations of imidazole.
(17) Reeves, C. D.; Chung, L. M.; Liu, Y.; Xue, Q.; Carney, J. R.; Revill, W. P.; Katz, L. *J. Biol. Chem*. **2002**. In press.
(18) The plasmid is derived from pCK7,[19] an *E. coli−Streptomyces* shuttle vector carrying all three *eryA* genes, by replacing the *ery*AIII AT6 domain with the AT8 domain of *fkbA* from the FK520 gene cluster.[17]
(19) Lu, L.; Thamchaipenet, A.; Fu, H.; Betlach, M.; Ashley, G. *J. Am. Chem. Soc*. **1997**, *119*, 10553−10554.
(20) Kao, C. M.; Katz, L.; Khosla, C. *Science* **1994**, *265*, 509−512.
(21) The transformants were grown in liquid R5 or R6 medium, supplemented with thiostrepton (50 mg/L) and kanamycin (200 mg/L), for 6−10 days at 28−30 °C and 200 rpm. The culture broths were extracted with EtOAc and the extracts analyzed by TLC and ES-MS. Titers were determined by LC−MS, using 13-propyl-6-DEB as an internal standard.
(22) The crude extract (135 mg) from 2 L of culture was separated on silica gel (5 mL, elution with hexane/EtOAc 2:1, then 1:1). The main product (15 mg) was further subjected to preparative TLC (silica gel, hexane/EtOAc 1:1, twice), followed by HPLC (Beckman) on an ODS-AQ column (10 × 250 mm) with an increasing linear gradient of MeOH/water (50−100% MeOH), flow rate 3 mL/min, detection at 215 nm.
(23) 2-Desmethyl-2-methoxy-6-DEB: ^{1}H NMR ($CDCl_3$, 500 MHz) δ (ppm) 5.41 (dd, 1H, J = 4.7, 10.5 Hz), 3.87 (t, 1H, J = 9.4 Hz), 3.87 (dd, 1H, J = 4.3, 10.3 Hz), 3.83 (t, 1H, J = 9.2 Hz), 3.59 (d, 1H, J = 10.7 Hz), 3.43 (s, 3H), 2.87 (m, 1H), 2.52 (m, 1H), 1.63−1.89 (m, 7H), 1.13 (d, 3H, J = 6.8 Hz), 1.09 (d, 3H, J = 6.6 Hz), 1.07 (d, 3H, J = 7.0 Hz), 1.02 (d, 3H, J = 6.7 Hz), 0.96 (t, 3H, J = 7.3 Hz), 0.93 (d, 3H, J = 6.9 Hz); HRMS (ESI): Calcd for $C_{21}H_{38}O_7Na$ 425.2515, found 425.2526.
(24) Carbon−carbon bond formation in the ketosynthase reaction involves an intermediate carbanion at C-2 of the chain extension unit. In the presence of a free OH-group at C-2 this carbanion would be prone to rearrangement to the nonproductive oxyanion.

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