**Additional file 1. Overexpression/deletion of mtmL**

*Generation of plasmid pΔL*: In order to generate the mutant strain *S. argillaceus* ΔL, the plasmid pΔL was constructed as follows. A 10 Kb BamHI DNA fragment containing *mtmD* to *mtmP* genes and including *mtmL*, was isolated from cosmid cosAR7 and subcloned into the vector pBSKT previously digested with BamHI, generating the construct pDZ4. From this, a BglII-Sacl DNA fragment containing the 5’-end of *mtmL*, *mtmQ*, *mtmX* and the 5’-end of *mtmP* was subcloned into pUK21 previously digested with the same restriction enzymes, generating pDZL1. In the following step, a PstI-BglII DNA fragment from pDZ4 containing the 3’-end of *mtmD*, *mtmE*, *mtmTII*, *mtmOII*, *mtmOIII*, and the 3’-end of *mtmL* was subcloned into pUK21 previously digested with PstI-BamHI to generate the construct pDZL2. Then, an apramycin resistance cassette isolated from pEFBA was subcloned as a HindIII-Ndel DNA fragment into the plasmid pDZL1, previously digested with the same restriction enzymes, generating the construct pDZL3. The next step was subcloning the whole insert from pDZL2 as a BglII-XbaI fragment into the same sites of pDZL3 downstream of the apramycin resistance cassette, to generate pDZL4. Finally, the whole insert in pDZL4 was rescued as a SpeI DNA fragment and subcloned into the same sites of pBSKT, which is a suicide plasmid in *Streptomyces*, to generate the final plasmid pΔL.

*Generation of plasmid pDZL10*: This plasmid was constructed to overexpress *mtmL* into *S. argillaceus* wild type strain and to complement *S. argillaceus* ΔL mutant. To generate pDZL.10 the gene *mtmL* was subcloned from plasmid pDZ4 as a BclI-EcoRI fragment into the vector pIJ2925 previously digested as BamHI-EcoRI to obtain the construct pDZL6. From this plasmid, *mtmL* was subcloned as an XbaI-EcoRI fragment into the same sites of pEM4, generating the construct pDZL7. Finally, the *mtmL* gene was subcloned from pDZL7 as a BamHI-EcoRI fragment into the multicopy, conjugative, bifunctional *Streptomyces- E.coli* vector pEM4T, downstream of the erythromycin resistance gene promoter (*ermEp*) to generate the final plasmid pDZL10.
Figure S1. Comparison of MtmL with putative Acyl-CoA Ligases

CAE17553 (CmmLII); ACN64850 (PokL); ADE34493 (SsfL2)
Figure S2. Mithramycin production in R5A solid cultures by *Streptomyces argillaceus* pEM4T (control) and *S. argillaceus* pDZL10

![Bar graph showing mithramycin production](image)

Figure S3. Generation and analysis of mutant *S. argillaceus* ΔL. (A) Scheme representing the replacement event for generation of mutant ΔL. WT, wild type strain; *aac(3)IV*, apramycin resistance gene; (B) PCR analysis of mutant ΔL. PCR products from the wild type (WT) strain and from mutant ΔL, using oligonucleotides Q (ACAGCCAGCAGTACTCCGT) and LF (ATGCGCTGGTGCGGAGAGCA). λ, Pstl-digested Lambda DNA.

![PCR analysis](image)