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The biosynthetic genes encoding for the production of the dynemicin enediyne core in *Micromonospora chersina* ATCC53710

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Abstract

Dynemicin is a novel anthraquinone-fused member of the 10-membered enediyne antitumor antibiotic family. The development of a genetic system for the dynemicin producer *Micromonospora chersina* confirmed, for the first time, the requirement of the putative enediyne core biosynthetic genes (*dynE8*, *U14* and *U15*) and a tailoring oxidase gene (*orf23*) for dynemicin production. Cloning and sequence analysis of a 76 kb of genomic sequence region containing *dynE8* revealed a variety of genes conserved among known enediyne loci. Surprisingly, this fragment and flanking chromosomal DNA lacked any obvious genes encoding for the biosynthesis of the anthraquinone, suggesting that the location of genes encoding for the biosynthesis of the dynemicin enediyne core and the dynemicin anthraquinone are chromosomally distinct. The demonstrated trace production of a shunt product from mutant strain QGD23 (Δ*orf23*) also sets the stage for subsequent studies to delineate the key steps in enediyne core biosynthesis and tailoring.

Keywords

dynemicin; enediyne; biosynthesis; polyketide synthase; gene disruption; cancer

Introduction

The enediyne antibiotics are appreciated for their unique molecular architectures, fascinating mechanisms of action and remarkably potent biological activities (Thorson *et al.*, 2000;

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Supplementary material

The following supplementary material is available for this article:

Table S1. Bacterial strains, plasmids and primers used in this study.

Fig. S1. Overview of the key cosmids and probes highlighted in this study.

Fig. S2. Inactivation of *dynE8*, *dynU14*, *dynU15*, *orf8*, and *orf23*.

Fig. S3. HPLC analysis of fermentation extracts from wild-type, Δ*dynU14* (QGDU14), and Δ*dynU15* (QGDU15) *M. chersina* prodigy.

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Shen, 2003; Galm *et al.*, 2005). Thirteen naturally occurring enediynes have been structurally characterized, all of which encompass the signature di-acetylenic core conjugated by (or to) a double bond. Despite their structural distinctions, all enediyne antibiotics damage DNA via a rapid enediyne cycloaromatization to form highly reactive diradical species capable of inducing oxidative DNA strand scission. Conjugation of enediynes to tumor-specific monoclonal antibodies and the application of polymer-assisted delivery devices have also led to the clinical success of enediynes (Abe & Otsuki, 2002; Damle, 2004; Jedema *et al.*, 2004; Wu & Senter, 2005).

There exist two enediyne structural subfamilies –chromoprotein (or nine-membered) enediynes and 10-membered enediynes. The chromoprotein enediynes C-1027 (**1**), neocarzinostatin (NCS **2**), maduropeptin (MAD **3**), kedarcidin (**4**) and N1999A2 (**5**) contain a bicyclo[7.3.0]enediyne nine-membered core and, with one exception (**5**), require a specific protein for enediyne stabilization and transport (for a recent review, see Galm *et al.*, 2005). The recently isolated sporolides (**6**) and cyanosporasides (**7**) (Fig. 1a) have also been proposed to derive from nine-membered enediyne precursors (Buchanan *et al.*, 2005; Oh *et al.*, 2006). The 10-membered enediynes share a common bicyclo[7.3.1]enediyne core and include calicheamicin (**9**), esperamicin (**10**), namenamicin (**11**) shishijimicin (**12**) dynemicin (**8**) and uncialamycin (**13**) (Fig. 1b) (for a recent review, see Galm *et al.*, 2005). While the 10-membered enediynes lack protein stabilizers, some producing organisms of this family rely upon a novel ‘self-sacrifice’ resistance protein (as exemplified by the calicheamicin protein, CalC) as a self-resistance mechanism (Biggins *et al.*, 2003; Singh *et al.*, 2006). This latter subgroup can be further divided into the glycosylated analogs (**9–12**) and anthraquinone-fused members (**8** and **13**).

Early metabolic labeling studies suggested the nine- and 10-membered enediynes to derive from distinct biosynthetic pathways (Hensens *et al.*, 1989; Tokiwa *et al.*, 1992; Lam *et al.*, 1993). In contrast, the recent cloning and characterization of gene clusters encoding both nine-membered and 10-membered enediynes revealed a common enediyne polyketide synthase (PKSE) gene and thereby established the first unified, divergent polyketide paradigm for enediyne core biosynthesis (Ahlert *et al.*, 2002; Liu *et al.*, 2002). Subsequent genomic scanning with nine-membered PKSE-specific or 10-membered PKSE-specific primers were consistent with a predicted biosynthetic divergence (Liu *et al.*, 2003), and PKSE genes were found to be remarkably prevalent in bacteria (Zazopoulos *et al.*, 2003). Yet, while gene loci encoding for chromoprotein (**1** and **2**) and saccharide-appended 10-membered (**9**) enediynes have been characterized (Ahlert *et al.*, 2002; Liu *et al.*, 2002, 2005), loci for the anthraquinone-fused enediynes **8** or **13** remain surprisingly elusive. Moreover, although a putative **8** PKSE-encoding gene (*dynE8*) has been identified by degenerate PCR (Liu *et al.*, 2003), this gene has not been confirmed to play a role in **8** biosynthesis.

Herein we report cloning, sequencing and characterization of a 76-kb contiguous genomic DNA region containing *dynE8* from *Micromonospora chersina* ATCC53710. The subsequent successful implementation of gene replacements for the **8**-producing *M. chersina* confirmed, for the first time, *dynE8* and other localized genes (*dynU14*, *U15* and *orf23*) as essential for the biosynthesis of the **8**. While analysis of the isolated genomic region

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surprisingly lacked additional PKS-encoding genes anticipated for the biosynthesis of the **8** anthraquinone moiety, the demonstrated trace production of a shunt product from mutant strain QGD23 (Δ*orf23*) clearly sets the stage for subsequent studies to delineate the key steps in enediyne core biosynthesis and tailoring.

Materials and methods

For details regarding bacterial strains, culture conditions, plasmids, reagents, cloning, genomic library construction and screening, DNA sequencing and analysis, gene inactivation, and product isolation and characterization, see supplemental online material. The DNA sequence reported in this paper has been deposited in GenBank under the accession number EF552206.

Results and discussion

Cloning of the *dynE8* -containing genomic region from *M. chersina* ATCC 53710

For the current study, three distinct probes based upon the putative *dynE8* sequence (Liu *et al.*, 2003, Zazopoulos *et al.*, 2003), designated A, B and D (Fig. 3a), were used to screen *c.* 4800 colonies from a *M. chersina* genomic cosmid library constructed in pOJ446 (Kieser *et al.*, 2000). All positive clones were confirmed by secondary hybridization and as templates for the amplification of the dyn A, B and D amplicons. From this analysis, seven *dynE* positive clones spanning *c.* 90 kb (based upon restriction mapping) were identified.

Sequencing and organization of the *M. chersina* dynemicin core biosynthetic genes

Shotgun sequencing of cosmids pJST1009 and pJST1059 (Supplementary Fig. S1) at approximately fivefold coverage provided 76 kb of genomic sequence (deposited in Genbank under accession number EF552206). Analysis of the sequence (72.5% GC content) by FramePlot (Ishikawa & Hotta, 1999) revealed 60 ORFs (Fig. 3a), the preliminary annotation of which derived from BLAST and NCBI Conserved Domain Database (Altschul *et al.*, 1997; Marchler-Bauer *et al.*, 2007) analysis. The putative functional assignments for each ORF, and their closest homologs, are listed in Table 1. Of the putative ORFs identified, 24 genes were notably homologous to counterparts within **9** biosynthetic gene cluster (Fig. 3b). For readers' convenience, gene denominations correlate to activity groups, E for enediyne biosynthesis, A for maturation or fusion, T for uptake and transport, R for regulation and U for unknown.

Disruption of *dynE8*, *orf8*, *dynU15*, *dynU14* and *orf23* in *M. chersina*

Before this work, a genetic system for the **8**-producing *M. chersina* was unavailable. Using PCR-targeting-mediated gene replacement (Gust *et al.*, 2003), the entire *dynE8* in cosmid pQG9B01 (a subclone carrying a 32 kb insert of pJST1009, see Supplementary Table S1) was replaced by an apramycin resistance gene to give pQGD9001 (Supplementary Fig. S2). Conjugation using pQGD9001 led to 8 exconjugants – two of which were double crossovers. Although the conjugation efficiency was low (8×10^{-8} exconjugants per recipient spores), the desired *M. chersina dynE8::AmR* mutant strain QGD01 was confirmed by both PCR and Southern hybridization (Supplementary Fig. S2) thereby establishing, for the first time, a

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gene replacement for *M. chersina*. Similar apramycin replacement experiments for genes *orf8*, *dynU15*, *dynU14* (in cosmid pQG9B01) and *orf23* (in cosmid pQG59B01) provided constructs pQGD9008, pQGD9U15, pQGD9U14 and pQGD5923, respectively (Supplementary Fig. S2). Upon conjugation, this set of mutant cosmids ultimately presented *M. cherisina* mutant strains QGD08 (*orf8::Am*^{R}), QGDU15 (*dynU15::Am*^{R}), QGDU14 (*dynU14:: Am*^{R}) and QGD23 (*orf23:: Am*^{R}) (Supplementary Table S1).

Dynemicin production in wild-type and mutant strains of *M. chersina*

Using a slight modification of previous **8** fermentation protocols (Miyoshisaitoh *et al.*, 1991), optimal **8** production (4.2 mg L^{-1}) by *M. chersina* strain ATCC53710 was provided on the third day in production media containing 1% Diaion HP-20 (Lam *et al.*, 1995). The identity of **8** was further confirmed by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis (*m/z* calculated for $C_{30}H_{19}NO_9$ $[M+H]^+$ 538.1133, observed 538.1155) and by coelution with a standard for **8**. MALDI-MS analysis ultilized an IonSpec HiResMALDI FT-MS equipped with a 7 T superconducting magnet and a nitrogen laser. For this analysis, 0.4 μL of **8** (1 mg mL^{-1}) in 50: 50 H_2O: MeOH was spotted onto a plate containing 0.4 μL of a saturated solution of a 2,5-dihydroxybenzoic acid (DHB) matrix in 50: 50 H_2O: MeOH and allowed to air dry. Seven laser shots of the sample spot were taken, and accumulated in the analyzer cell. The Fourier transformed spectrum was peak matched with a dimer of DHB to a mass accuracy of 4.24 p.p.m.

As highlighted in Fig. 2, disruption of *dynE8* completely abolished **8** production (Fig. 2, panel iii), consistent with *dynE8* as encoding the **8** PKSE. It is important to note that while the disruption of *dynU14* and *U15* presented identical phenotypes (Supplementary Fig. S3), due to the gene organization of *dynU15*, *dynU14*, *dynT3*, *dynE8*, *dynE7* and *dynS6*, the Δ*dynU15*, Δ*dynU14* and/or Δ*dynE8* disruptions could potentially induce polar effects upon other downstream genes within this small operon. In contrast, mutant strain QGD08 (*orf8*) retained antibiotic production (Fig. 2, panel iv) at a level similar to the wild type strain (Fig. 2, panel ii) (4.3 mg L^{-1}) while mutant strain QGD23 (Δ*orf23)* accumulated a new compound with a similar color, solubility and UV spectrum to **8** (Fig. 2, panel v). LC-MS analysis of this new compound was consistent with the loss of an oxygen from **8**. Notably, these results validate the genetic system and highlight the essential nature of *dynE8* (and nonessential nature of *orf8*) for **8** biosynthesis. Given the lack of validated plasmids for complementation experiments in enediyne producing *Micromonospora* (Takada *et al.*, 1994; Ahlert *et al.*, 2002; Li *et al.*, 2004; Hosted *et al.*, 2005), the *orf8* and *orf23* replacement mutants are particularly noteworthy examples as they illustrate *M. chersina* genotype alterations without abolishing metabolite production.

Biosynthesis of the dynemicin enediyne precursor

Sequence analysis of the *M. chersina* genes clustered with *dynE8* revealed six gene products, DynU15-DynS6 with homologs in **9** biosynthesis (% identity/% similarity ranging from 39/49 to 53/66; Table 1), believed critical for enediyne core formation. The genes for five of these (U15, U14, T3, E8, E7, Fig. 3b) reside within what has now become known as the ‘minimal enediyne cassette’ (Ahlert *et al.*, 2002; Liu *et al.*, 2002, 2003, 2005; Zazopoulos *et al.*, 2003). Consistent with other known enediyne loci, three conserved

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proteins of unknown function were also found to be encoded within the **8** minimal enediyne gene cassette (DynT3, DynU14 and DynU15). Unique in comparison with other enediyne loci, the characterized sequenced region contained a distinct gene (designated *orf17*) encoding a protein homologous to the PKSE KR domain (see Table 1). Beyond the genes related to minimal enediyne cassette, there exist only four additional genes (*dyn*/*calS6*, *dyn*/*calU16*, dyn/*calU20* and *dyn*/*calU21*) uniquely conserved among the enediyne loci characterized to date. Based upon homology, DynS6/CalS6 has been annotated as a putative F420 – dependent NADP oxidoreductase (see Table 1) while the remaining gene products (U16 and U20 and U21) have no homologs outside putative enediyne-associated pathways. Interestingly, three U16-like proteins (U16, Orf14 and Orf20) are encoded by the **8** locus. While these three vary in size and share modest homology, they all contain two common domains – a COG3832 (uncharacterized) and ASHA1 (activator of Hsp90 ATPase homolog 1-like protein) domain.

Dynemicin fusion/tailoring/maturation

Consistent with the postulated separation of the biosynthesis of the **8** bicyclo[7.3.1.]diyne unit and the anthraquinone unit (Tokiwa *et al.*, 1992), preliminary degenerate PCR and Southern hybridization failed to identify candidate anthraquinone biosynthetic genes near (within 20 kb) either end of 76-kb sequenced region (data not shown). It should also be noted that precedent does exist for the chromosal separation of biosynthetic loci in bacteria (Yu *et al.*, 2002; Ostash *et al.*, 2007). Based upon metabolic labeling, three putative oxygenation steps can be predicted (C18, C20 and possibly C22) as required for anthraquinone maturation/tailoring. In addition, the fact that deoxydynemicin (**14**) (Fig. 1b) and **8** were coproduced by *M. globosa* MG-331-FG (Shiomi *et al.*, 1990), and the recent elucidation of uncialamycin (**13**) (Fig. 1b), implicate the potential for C5 oxidation. Consistent with this, a number of candidate genes encoding oxidative enzymes were found to be colocalized with *dynE8*. As the most likely candidates for C18/C22 oxidation, DynA1 and DynA2 (the genes for which are transcriptionally coupled) are homologs of the small ‘cofactorless’ hydroxylases AclR (see Table 1) and SnoL (see Table 1), respectively. AclR and SnoL (also designated as SnoaL2) catalyze the C1 aromatic hydroxylation of cinerubin (*Streptomyces galilaeus*) and nogalamycin (*Streptomyces nogalater*) (Torkkell *et al.*, 2001), respectively, and also share distant sequence and architectural similarity to members of polyketide cyclases (Beinker *et al.*, 2006). Consistent with a lack of anthraquinone core PKS genes, a gene encoding a signature quinone-forming cofactor-less anthrone monoxygenase (as exemplified by TcmH) (Shen & Hutchinson, 1993; Fetzner, 2002) was not found within the sequenced region highlighted in Fig. 3.

From metabolic labeling, minimally a single oxygenation step (C5) is required during maturation of the enediyne core. The basis for this postulation derives from the identification of minor fermentation products dynemicins M, O, P and Q (**15**, **16**, **17**, and **18**, respectively) (Fig. 1b), all of which bear a C5 carbonyl oxygen (Konishi *et al.*, 1991; Miyoshisaitoh *et al.*, 1991). Isotopic labeling also revealed the C5 carboxylate to derive from a separate acetate condensation with the cyclodiynene polyketide followed by decarboxylation/oxidation and a likely enzymatic route to this convergent condensation parallels the many recently elucidated HMG-CoA-like β-alkylation pathways (Simunovic *et al.*, 2003; Chang *et al.*,

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2004; Edwards *et al.*, 2004; Pulsawat *et al.*, 2007; Wu *et al.*, 2007). While a signature HMG-CoA gene cassette was not found within the existing sequenced region, various candidates were identified that may play a role in subsequent decarboxylation/oxidation. For example, the encoded DynA5 shares similarities to RdmB, a *S*-adenosyl-L-methionine (AdoMet)-dependent aclacinomycin 10-hydroxylase responsible for decarboxylation and subsequent oxidation en route to aclacinomycin biosynthesis (Jansson *et al.*, 2005). Genes *orf21-orf23*, encode for a set of protein homologs to those known to comprise various prokaryotic molybdenum containing hydroxylases (Lehmann *et al.*, 1994; Okamoto *et al.*, 2004), the putative maturation factor for which is encoded by *dynE12* (see Table 1) (Ivanov *et al.*, 2003). Interestingly the trace production of a new product with similar UV signature to **8** was detected in mutant strain QGD23 (Δ*orf23*), the structural elucidation of which was restricted by limited abundance (see Fig. 2, panel v). The encoded DynE11, DynE14 and DynE15 also all contain conserved domains found in glyoxalase/bleomycin resistance protein/dioxygenase superfamily (Moran, 2005) while DynA3, contains a short chain dehydrogenase domain common to members of the dehydrogenase/isomerase family but also displays weak homology with proteins involved in controlling nitrogen metabolism (NmrA) and azoreductases. Finally, DynE13, a homolog of FAD dependent monooxygenases such as salicylate hydroxylase (39% identity, 49% similarity) and zeaxanthin epoxidase (see Table 1), serves as a likely candidate for the C3/C8 epoxidase (Fig. 3c), while DynO6, a homolog of CalO6 (a phenolic *O*-methyltransferase involved in calicheamicin biosynthesis; see Table 1) (Ahlert *et al.*, 2002), is the plausible dynemicin C6 *O*-methyltransferase.

While prior metabolic labeling (Tokiwa *et al.*, 1992) or the current gene sequence annotation fails to shed any additional light upon the amino donor for the key anthraquinone–enediyne bridging nitrogen, we postulate two putative cytochrome P450s may be involved in forming the C8/C9 C–C bond, and possibly, the C2–N1–C22 bridge. Specifically, DynE10 and Orf19, show strong similarity to PdmJ (Table 1), NocL (DynE10- 43% identity, 57% similarity; Orf19- 49% identity, 62% similarity; AAT09797) and CalE10 (DynE10- 41% identity, 53% similarity; Orf19-37% identity, 50% similarity). While the role of PdmJ (also refered to as PrmJ) in pradimicin biosynthesis remains undetermined (Kim *et al.*, 2007), NocL catalyzes a rare conversion of a primary amine to an oxime in nocardicin A biosynthesis (Kelly & Townsend, 2002). while CalE10 catalyzes a novel sugar amine oxidation in calicheamicin biosynthesis (H.D. Johnson and J.S. Thorson, unpublished data). Similar P450s are critical to C–C (e.g. RebP) (Onaka *et al.*, 2002; Sanchez *et al.*, 2002; Hyun *et al.*, 2003; Howard-Jones & Walsh, 2006) and C–N (e.g. StaN) (Onaka *et al.*, 2005) bond formation within indolocarbazole biosyntheses.

Conclusions

Cumulatively, this study highlights the first genetic system for *M. chersina*, direct confirmation of the participation of *dynU14*, *dynU15*, *dynE8* and *orf23* in **8** biosynthesis. Disruption of the putative tailoring oxidase gene *orf23* or the nonstructural gene *orf8* importantly illustrated the ability for *M. chersina* genetic manipulation while retaining production of **8** or corresponding shunt metabolites and thereby sets the stage for the application of genetics in delineating key biosynthetic steps. In addition, consistent with the

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postulation that **8** bicyclo[7.3.1.]diyne unit and the anthraquinone unit are biosynthesized separately and later assembled (Tokiwa *et al.*, 1992), this preliminary study suggests that the genes encoding for these two distinct dynemicin precursors may also be physically separated in *M. chersina* genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Abe S, Otsuki M. Styrene maleic acid neocarzinostatin treatment for hepatocellular carcinoma. Curr Med Chem Anticancer Agents. 2002; 2:715–726. [PubMed: 12678722]

Ahlert J, Shepard E, Lomovskaya N, et al. The calicheamicin gene cluster and its iterative type I enediyne PKS. Science. 2002; 297:1173–1176. [PubMed: 12183629]

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25:3389–3402. [PubMed: 9254694]

Beinker P, Lohkamp B, Peltonen T, Niemi J, Mantsala P, Schneider G. Crystal structures of SnoaL2 and AclR: two putative hydroxylases in the biosynthesis of aromatic polyketide antibiotics. J Mol Biol. 2006; 359:728–740. [PubMed: 16650858]

Biggins JB, Onwueme KC, Thorson JS. Resistance to enediyne antitumor antibiotics by CalC self-sacrifice. Science. 2003; 301:1537–1541. [PubMed: 12970566]

Buchanan GO, Williams PG, Feling RH, Kauffman CA, Jensen PR, Fenical W. Sporolides A and B: structurally unprecedented halogenated macrolides from the marine actinomycete *Salinispora tropica*. Org Lett. 2005; 7:2731–2734. [PubMed: 15957933]

Chang Z, Sitachitta N, Rossi JV, Roberts MA, Flatt PM, Jia J, Sherman DH, Gerwick WH. Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*. J Nat Prod. 2004; 67:1356–1367. [PubMed: 15332855]

Damle NK. Tumour-targeted chemotherapy with immunoconjugates of calicheamicin. Expert Opin Biol Thera. 2004; 4:1445–1452.

Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, Gerwick WH. Structure and biosynthesis of the jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. Chem Biol. 2004; 11:817–833. [PubMed: 15217615]

Fetzner S. Oxygenases without requirement for cofactors or metal ions. Appl Microbiol Biotechnol. 2002; 60:243–257. [PubMed: 12436305]

Galm U, Hager MH, Van Lanen SG, Ju J, Thorson JS, Shen B. Antitumor antibiotics: bleomycin, enediynes, and mitomycin. Chem Rev. 2005; 105:739–758. [PubMed: 15700963]

Gust B, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci USA. 2003; 100:1541–1546. [PubMed: 12563033]

Hensens OD, Giner JL, Goldberg IH. Biosynthesis of NCS Chrom-A, the chromophore of the antitumor antibiotic neocarzinostatin. J Am Chem Soc. 1989; 111:3295–3299.

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Hosted TJ Jr, Wang T, Horan AC. Characterization of the *Micromonospora rosaria* pMR2 plasmid and development of a high G+C codon optimized integrase for site-specific integration. Plasmid. 2005; 54:249–258. [PubMed: 16024079]

Howard-Jones AR, Walsh CT. Staurosporine and rebeccamycin aglycones are assembled by the oxidative action of StaP, StaC, and RebC on chromopyrrolic acid. J Am Chem Soc. 2006; 128:12289–12298. [PubMed: 16967980]

Hyun CG, Bililign T, Liao JC, Thorson JS. The biosynthesis of indolocarbazoles in a heterologous *E. coli* host. Chembiochem. 2003; 4:114–117. [PubMed: 12512086]

Ishikawa J, Hotta K. FramePlot: a new implementation of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. FEMS Microbiol Lett. 1999; 174:251–253. [PubMed: 10339816]

Ivanov NV, Hubalek F, Trani M, Edmondson DE. Factors involved in the assembly of a functional molybdopyranopterin center in recombinant *Comamonas acidovorans* xanthine dehydrogenase. Eur J Biochem. 2003; 270:4744–4754. [PubMed: 14622263]

Jansson A, Koskiniemi H, Erola A, Wang J, Mantsala P, Schneider G, Niemi J. Aclacinomycin 10-hydroxylase is a novel substrate-assisted hydroxylase requiring *S*-adenosyl-L-methionine as cofactor. J Biol Chem. 2005; 280:3636–3644. [PubMed: 15548527]

Jedema I, Barge RMY, van der Velden VHJ, Nijmeijer BA, van Dongen JJM, Willemze R, Falkenburg JHF. Internalization and cell cycle-dependent killing of leukemic cells by gemtuzumab ozogamicin: rationale for efficacy in CD33-negative malignancies with endocytic capacity. Leukemia. 2004; 18:316–325. [PubMed: 14614514]

Kelly WL, Townsend CA. Role of the cytochrome P450 NocL in nocardicin A biosynthesis. J Am Chem Soc. 2002; 124:8186–8187. [PubMed: 12105888]

Kieser, T., Bibb, MJ., Buttner, M., Chater, K., Hopwood, DA. Practical Streptomyces Genetics. John Innes Foundation; Norwich: 2000.

Kim BC, Lee JM, Ahn JS, Kim BS. Cloning, sequencing, and characterization of the pradimicin biosynthetic gene cluster of *Actinomadura hibisca* p157-2. J Microbiol Biotechnol. 2007; 17:830–839. [PubMed: 18051306]

Konishi M, Ohkuma H, Matsumoto K, Saitoh K, Miyaki T, Oki T, Kawaguchi H. Dynemicins, new antibiotics with the 1,5-diyn-3-ene and anthraquinone subunit. 1. Production, isolation and physicochemical properties. J Antibiot. 1991; 44:1300–1305. [PubMed: 1778782]

Lam KS, Veitch JA, Golik J, Krishnan B, Klohr SE, Volk KJ, Forenza S, Doyle TW. Biosynthesis of esperamicin-A_1 an enediyne antitumor antibiotic. J Am Chem Soc. 1993; 115:12340–12345.

Lam KS, Veitch JA, Lowe SE, Forenza S. Effect of neutral resins on the production of dynemicins by *Micromonospora chersina*. J Ind Microbiol. 1995; 15:453–456.

Lehmann M, Tshisuaka B, Fetzner S, Roger P, Lingens F. Purification and Characterization of isoquinoline 1-oxidoreductase from *Pseudomonas diminuta*, a novel molybdenum-containing hydroxylase. J Biol Chem. 1994; 269:11254–11260. [PubMed: 8157655]

Li X, Zhou X, Deng Z. Isolation and characterization of *Micromonospora* phage PhiHAU8 and development into a phasmid. Appl Environ Microbiol. 2004; 70:3893–3897. [PubMed: 15240260]

Liu W, Christenson SD, Standage S, Shen B. Biosynthesis of the enediyne antitumor antibiotic C-1027. Science. 2002; 297:1170–1173. [PubMed: 12183628]

Liu W, Ahlert J, Gao Q, Wendt-Pienkowski E, Shen B, Thorson JS. Rapid PCR amplification of minimal enediyne polyketide synthase cassettes leads to a predictive familial classification model. Proc Natl Acad Sci USA. 2003; 100:11959–11963. [PubMed: 14528002]

Liu W, Nonaka K, Nie L, et al. The neocarzinostatin biosynthetic gene cluster from *Streptomyces carzinostaticus* ATCC 15944 involving two iterative type I polyketide synthases. Chem Biol. 2005; 12:293–302. [PubMed: 15797213]

Marchler-Bauer A, Anderson JB, Derbyshire MK, et al. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res. 2007; 35:D237–D240. [PubMed: 17135202]

Miyoshisaitoh M, Morisaki N, Tokiwa Y, Iwasaki S, Konishi M, Saitoh K, Oki T. Dynemicin-*O*, dynemicin-*P* and dynemicin-Q – novel antibiotics related to dynemicin-A – isolation, characterization and biological-activity. J Antibiot. 1991; 44:1037–1044. [PubMed: 1955384]

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Moran GR. 4-Hydroxyphenylpyruvate dioxygenase. Arch Biochem Biophys. 2005; 433:117–128. [PubMed: 15581571]

Oh DC, Williams PG, Kauffman CA, Jensen PR, Fenical W. Cyanosporasides A and B, chloro- and cyanocyclopenta[a]indene glycosides from the marine actinomycete "*Salinispora pacifica*". Org Lett. 2006; 8:1021–1024. [PubMed: 16524258]

Okamoto K, Matsumoto K, Hille R, Eger BT, Pai EF, Nishino T. The crystal structure of xanthine oxidoreductase during catalysis: implications for reaction mechanism and enzyme inhibition. Proc Natl Acad Sci USA. 2004; 101:7931–7936. [PubMed: 15148401]

Onaka H, Taniguchi S, Igarashi Y, Furumai T. Cloning of the staurosporine biosynthetic gene cluster from *Streptomyces* sp. TP-A0274 and its heterologous expression in *Streptomyces lividans*. J Antibiot. 2002; 55:1063–1071. [PubMed: 12617516]

Onaka H, Asamizu S, Igarashi Y, Yoshida R, Furumai T. Cytochrome P450 homolog is responsible for C–N bond formation between aglycone and deoxysugar in the staurosporine biosynthesis of *Streptomyces* sp. TP-A0274. Biosci Biotechnol Biochem. 2005; 69:1753–1759. [PubMed: 16195595]

Ostash B, Saghatelian A, Walker S. A streamlined metabolic pathway for the biosynthesis of moenomycin A. Chem Biol. 2007; 14:257–267. [PubMed: 17379141]

Pulsawat N, Kitani S, Nihira T. Characterization of biosynthetic gene cluster for the production of virginiamycin M, a streptogramin type A antibiotic, in *Streptomyces virginiae*. Gene. 2007; 393:31–42. [PubMed: 17350183]

Sanchez C, Butovich IA, Brana AF, Rohr J, Mendez C, Salas JA. The biosynthetic gene cluster for the antitumor rebeccamycin: characterization and generation of indolocarbazole derivatives. Chem Biol. 2002; 9:519–531. [PubMed: 11983340]

Shen B. Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol. 2003; 7:285–295. [PubMed: 12714063]

Shen B, Hutchinson CR. Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A. Science. 1993; 262:1535–1540. [PubMed: 8248801]

Shiomi K, Iinuma H, Naganawa H, Hamada M, Hattori S, Nakamura H, Takeuchi T, Iitaka Y. New Antibiotic Produced by *Micromonospora globosa*. J Antibiot. 1990; 43:1000–1005. [PubMed: 2211346]

Simunovic V, Gherardini FC, Shimkets LJ. Membrane localization of motility, signaling, and polyketide synthetase proteins in *Myxococcus xanthus*. J Bacteriol. 2003; 185:5066–5075. [PubMed: 12923079]

Singh S, Hager MH, Zhang CS, Griffith BR, Lee MS, Hallenga K, Markley JL, Thorson JS. Structural insight into the self-sacrifice mechanism of enediyne resistance. ACS Chem Biol. 2006; 1:451–460. [PubMed: 17168523]

Takada Y, Inouye M, Morohoshi T, Muto N, Kato F, Kizuka M, Tanaka M, Koyama Y. Establishment of the host-vector system for *Micromonospora griseorubida*. J Antibiot (Tokyo). 1994; 47:1167–1170. [PubMed: 7961169]

Thorson JS, Sievers EL, Ahlert J, Shepard E, Whitwam RE, Onwueme KC, Ruppen M. Understanding and exploiting nature's chemical arsenal: the past, present and future of calicheamicin research. Curr Pharm Des. 2000; 6:1841–1879. [PubMed: 11102565]

Tokiwa Y, Miyoshisaitoh M, Kobayashi H, Sunaga R, Konishi M, Oki T, Iwasaki S. Biosynthesis of dynemicin-a, a 3-ene-1,5-diyne antitumor antibiotic. J Am Chem Soc. 1992; 114:4107–4110.

Torkkell S, Kunnari T, Palmu K, Mantsala P, Hakala J, Ylihonko K. The entire nogalamycin biosynthetic gene cluster of *Streptomyces nogalater*: characterization of a 20-kb DNA region and generation of hybrid structures. Mol Genet and Genomics. 2001; 266:276–288. [PubMed: 11683270]

Wu AM, Senter PD. Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol. 2005; 23:1137–1146. [PubMed: 16151407]

Wu J, Cooper SM, Cox RJ, Crosby J, Crump MP, Hothersall J, Simpson TJ, Thomas CM, Willis CL. Mupirocin H, a novel metabolite resulting from mutation of the HMG-CoA synthase analogue, mupH in *Pseudomonas fluorescens*. Chem Commun. 2007:2040–2042.

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Yu TW, Bai L, Clade D, Hoffmann D, Toelzer S, Trinh KQ, Xu J, Moss SJ, Leistner E, Floss HG. The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. Proc Natl Acad Sci USA. 2002; 99:7968–7973. [PubMed: 12060743]

Zazopoulos E, Huang K, Staffa A, Liu W, Bachmann BO, Nonaka K, Ahlert J, Thorson JS, Shen B, Farnet CM. A genomics-guided approach for discovering and expressing cryptic metabolic pathways. Nat Biotechnol. 2003; 21:187–190. [PubMed: 12536216]

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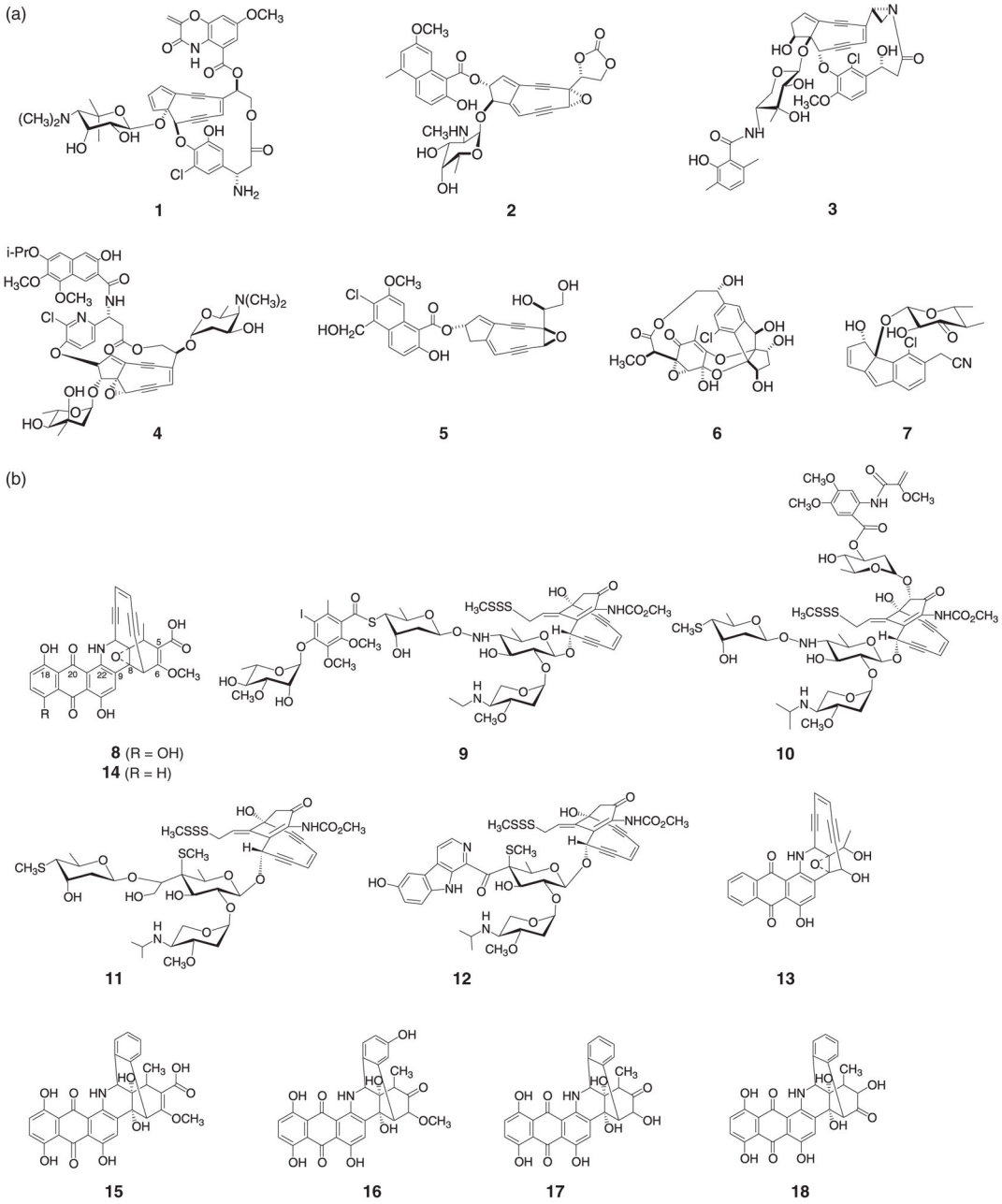

Fig. 1.

Representative naturally occurring enediynes. (a) The nine-membered enediynes: C-1027 (**1**), neocarzinostatin (**2**), maduropeptin (**3**), kedarcidin (**4**), N1999A2 (**5**) and putative members sporolide A (**6**) and cyanosporaside A (**7**). (B) The 10-membered enediynes: dynemicin A (**8**), calicheamicin γ_1^I (**9**), esperamicin A_1 (**10**), namenamicin (**11**), shishijimicin A (**12**), uncialamycin (**13**), deoxydynemicin A (**14**), dynemicin N (**15**), dynemicin O (**16**), dynemicin P (**17**), dynemicin Q (**18**).

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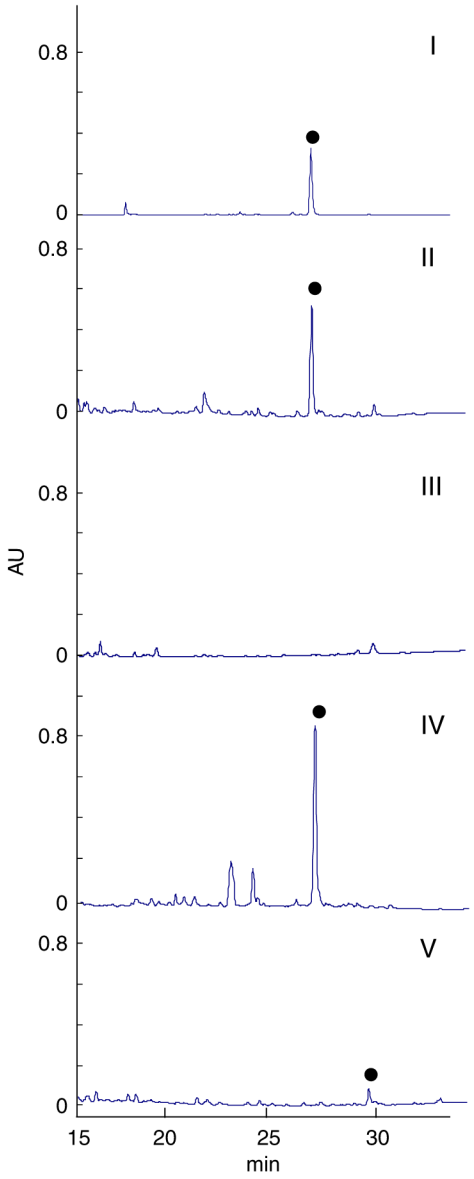

Fig. 2.

HPLC analysis of fermentation extracts from wild-type, Δ*dynE8* (QGD01), Δ*orf8* (QGD08) and Δ*orf23* (QGD23) *Micromonospora chersina* strains: (i) **8** authentic standard, (ii) wild-type, (iii) QGD01, (iv) QGD08, and (v) QGD23. The violet color, solubility and UV-vis signature of the QGD23 putative **8** shunt metabolite [CH_3CN: 233.4, 279 (sh), 543 nm] are similar to that of **8** [CH_3CN: 237, 280 (sh), 543 nm]. Parameters for analytical HPLC and product characterization are described in Materials and methods.

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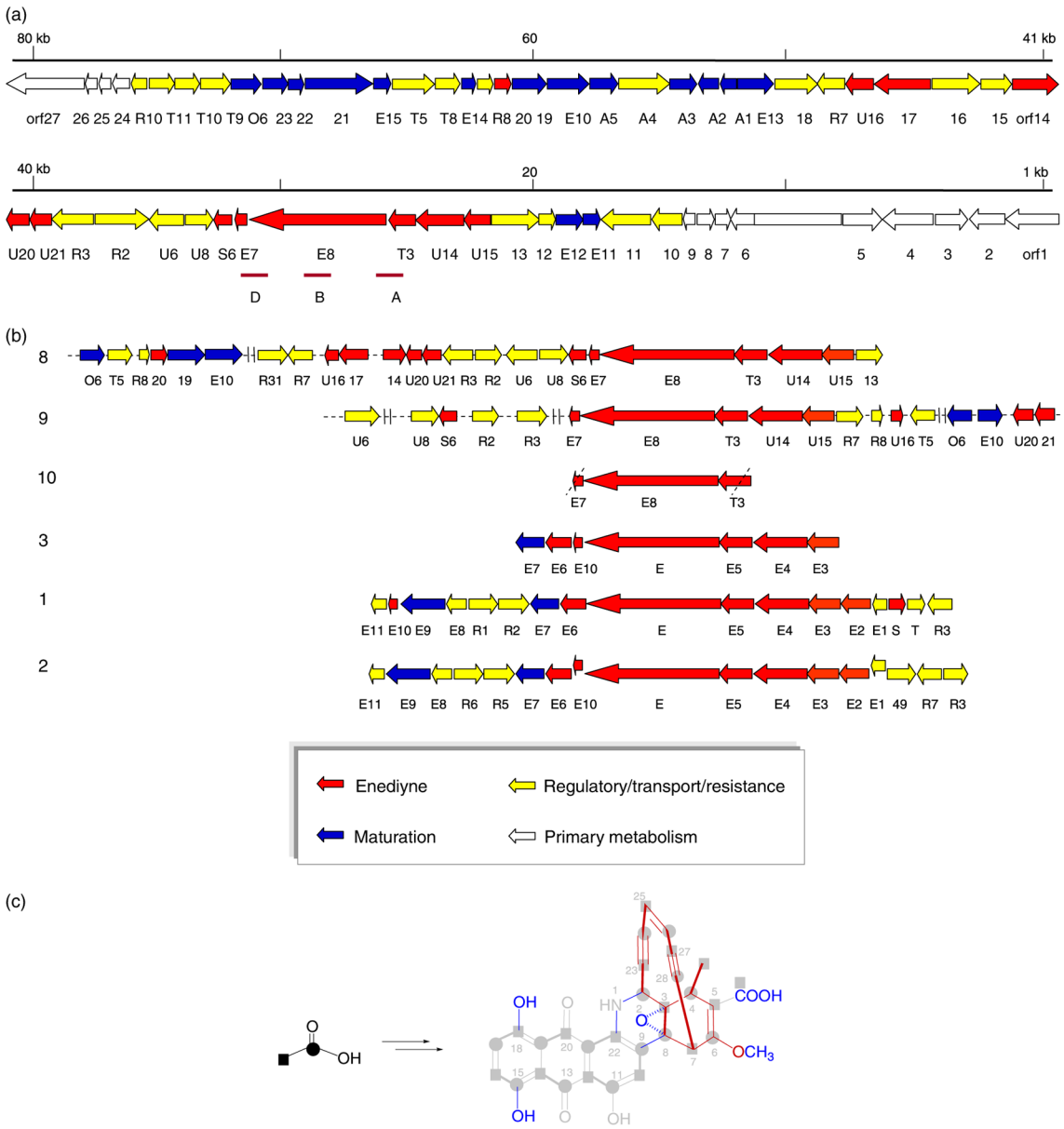

Fig. 3.

Enediyne biosynthetic genes. (a) The organization of *dynE8*-containing sequenced region. (b) Comparison of nine and 10-membered enediyne biosynthetic genes. Numbers on the left correspond to enediyne numbering in Fig. 1. Arrows represent the relative ORF size and direction of transcription. Colors designate putative functionality based upon homology to gene products of known or putative functions (see Table 1). (c) Summary of metabolic labeling in the context of genes found clustered with *dynE8*. The anthraquinone and enediyne core derive from distinct intermediates, the latter of which is constructed by the minimal enediyne machinery encoded within the *dynE8*-containing sequence region (highlighted in red). While the core genes responsible for the anthraquinone biosynthesis are not clustered with *dynE8*, a variety of putative maturation genes (highlighted in blue)

located adjacent to *dynE8* are likely involved in oxidation and possibly the key enediyne–anthraquinone fusion event.

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Table 1

Deduced functions of the dynemicin ORFs illustrated in Fig. 3

Gene	Amino acid	Putative function	Closest homology (Identity, similarity %)	Accession number
orf1	698 (partial)	Unknown protein	Strop_0190 (50/65)	ABP52675
orf2	409	Unknown protein	Strop_0188 (54/63)	ABP52673
orf3	306	Putative NUDIX hydrolase	Strop_0187 (66/57)	ABP52672
orf4	605	Putative resistance protein	Conserved hypothetical protein (71/83)	CAG38681
orf5	215	NADP oxidoreductase, F420-dependent	ORF MecP21.23c (75/88)	CAG38682
rRNA	7244-12 447	rRNA operon with 5S, 16S and 23S rRNA genes	*M. echinospora* strain DSM 43036	AJ628149
orf6	159	Hypothetical protein	GntT (52/58)	AAR98538
orf7	116	Putative alkylated DNA repair protein	TobX protein (76/88)	YP_035491
orf8	155	Unknown protein	–	–
orf9	151	Unknown protein	SAV740 (60/72)	BAC68450
orf10	352	Tryptophanyl tRNA synthetase	GntU (83/90)	AAR98539
orf11	572	Arginyl-tRNA synthetase	SareDRAFT_0146 (73/83)	EAX25545
dynE11	144	Putative glyoxalase/bleomycin resistance protein/ dioxygenase	YraH (34/46)	CAB46394
dynE12	386	Putative dehydrogenases maturation factor; XdhC/ CoxF family	MSMEG_0743 (61/72)	ABK69650
orf12	195	Hypothetical protein	SareDRAFT_0187 (60/66)	EAX25586
orf13	623	Transcriptional regulatory	CalR2 (45/59)	AAM94777
dynU15	328	Unknown conserved protein	CalU15 (39/49)	AAM70330
dynU14	632	Unknown conserved protein	CalU14 (47/57)	AAM70329
dynT3	329	Unknown conserved protein	CalT3 (53/66)	AAM94795
dynE8	1900	Enediyne core forming iterative polyketide synthase	CalE8 (44/54)	AAM94794
	4–453	KS domain	CalE8-KS (59/70)	
	476–895	AT domain	CalE8-AT (49/55)	
	933–1003	ACP domain	CalE8-ACP (45/59)	
	1148–1402	KR domain	CalE8-KR (49/59)	
	1409–1549	DH domain	CalE8-DH (50/59)	
	1685–1891	Putative PPTase domain	CalE8-Sfp	
dynE7	144	Putative thioesterase	CalE7 (48/64)	AAM94793
dynS6	219	NADP oxidoreductase, F420-dependent	CalS6 (44/54)	AAM94776
dynU8	224	Putative transcriptional regulator	CalU8 (66/79)	AAM94775
dynU6	307	Putative transporter	CalU6 (61/75)	AAM94767
dynR2	627	Putative transcriptional regulator	CalR2 (43/58)	AAM94777
dynR3	454	Putative regulator	CalR3 (49/61)	AAM94780
dynU21	188	Hypothetical protein	CalU21 (65/78)	AAM70363
dynU20	191	Unknown protein	CalU20 (31/46)	AAM70362
orf14	405	Unknown protein	CalU16 (29/41)	AAM70339
orf15	210	Putative aminopeptidase	CAB720 (22/41)	CAH64167
orf16	441	Hypothetical protein	Strop_0601 (53/71)	ABP53081

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Gene	Amino acid	Putative function	Closest homology (Identity, similarity %)	Accession number
orf17	516	Ketone reductase	CalE8-KR (42/51)	AAM94794
dynU16	283	Unknown protein	CalU16 (29/43)	AAM70339
dynR7	283	Putative transcriptional regulator	CalR7 (37/47)	AAM70331
orf18	418	Putative regulator	CalR3 (46/60)	AAM94780
dynE13	378	FAD-dependent oxidoreductases	MXAN_3398 (32/47)	ABF93079
dynA1	142	Putative hydroxylase	AclR protein (41/64)	CAJ87106
dynA2	167	Putative hydroxylase	SnoL (32/45)	AAF01808
dynA3	298	NmrA family protein	Aave_3935 (38/50)	ABM34478
dynA4	509	Putative peptidases and hydroxylases	ORF9 (36/48)	AAP85358
dynA5	370	Putative hydroxylase	RdmB	AAA83421
orf19	403	Cytochrome P450 hydroxylase	PdmJ (45/61)	ABM21756
dynE10	400	Cytochrome P450 hydroxylase	LnmA (49/65)	AAN85514
orf20	146	Unknown protein	CalU16 (29/39)	AAM70339
dynR8	114	Putative transcriptional regulator	CalR8 (39/55)	AAM70335
dynE14	123	Putative glyoxalase/dioxygenase	NB231_07547 (42/57)	EAR21006
dynT8	269	Putative ABC transporter	ABC-2 (44/59)	ABD10850
dynT5	333	Transporter ATP-ATPase subunit	CalT5 (46/64)	AAM7034
dynE15	123	Putative glyoxalase/dioxygenase	RHA1_ro02544 (45/58)	ABG94349
orf21	786	Molybdenum-containing oxidoreductase-large subuit, molybdopterin binding	F.DRAFT_4713 (47/62)	EAN16489
orf22	164	Molybdenum-containing oxidoreductase-small subunit, (2Fe–2S)-binding subunit	Mjls_4986 (67/76)	ABO00752
orf23	292	Molybdenum-containing oxidoreductase-medium subunit, FAD-binding subunit	kdhM (42/56)	CAD47945
dynO6	349	*O*-methyltransferase	CalO6 (37/51)	AAM70356
dynT9	339	Transmembrane transport protein	ORF31 (55/72)	ABD65951
dynT10	305	ABC transporter ATP-binding protein	CcmA (70/83)	ABD65952
dynT11	331	Transmembrane transport protein	ORF31 (53/68)	ABD65951
dynR10	189	Two-component system sensor kinase	ORF43(48/65)	ABD65963
orf24-orf26	218 141 141	Unknown protein	–	–
orf27	1138	Putative restriction enzyme	Mflv_0803 (60/75)	ABP43287