Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-coenzyme A from S-adenosyl-L-methionine

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Polyketides are among the major classes of bioactive natural products used to treat microbial infections, cancer, and other diseases. Here we describe a pathway to chloroethylmalonyl-CoA as a polyketide synthase building block in the biosynthesis of salinosporamide A, a marine microbial metabolite whose chlorine atom is crucial for potent proteasome inhibition and anticancer activity. S-adenosyl-L-methionine (SAM) is converted to 5'-chloro-5'-deoxyadenosine (5'-CIDA) in a reaction catalyzed by a SAMdependent chlorinase as previously reported. By using a combination of gene deletions, biochemical analyses, and chemical complementation experiments with putative intermediates, we now provide evidence that 5'-CIDA is converted to chloroethylmalonyl-CoA in a 7-step route via the penultimate intermediate 4-chlorocrotonyl-CoA. Because halogenation often increases the bioactivity of drugs, the availability of a halogenated polyketide building block may be useful in molecular engineering approaches toward polyketide scaffolds.

actinomycete | biological halogenation | marine natural product | proteasome inhibitor | *Salinispora tropica*

Polyketides are abundant microbial metabolites that possess a remarkable diversity in chemical structure and biological function. The enzymes that catalyze the assembly of these natural products, namely polyketide synthases (PKSs), belong to 3 protein families that similarly use small carboxylic acid building blocks as substrates. Polyketide biosynthetic pathways have evolved a myriad of ways to accommodate changes in the number and composition of their substrates, the manner in which they are assembled, and the further biochemical modification of the PKS product by tailoring enzymes to synthesize these often very complex organic molecules (1–4). The assembly line organization of modular type I PKSs in particular has facilitated their rational reengineering through combinatorial biosynthesis and mutasynthesis to yield new compound scaffolds that further extends their natural biosynthetic prowess (5).

Although PKSs exploit a wide assortment of priming carboxylic acid substrates to initiate the polyketide biosynthetic process (6), they are relegated to a small number of extending dicarboxylic acid units needed to elongate the growing polyketide chain via successive Claisen condensation reactions (7). The most common PKS extender units are malonyl-CoA, methylmalonyl-CoA, and, to a much lesser extent, ethylmalonyl-CoA that are selected by and attached to the PKS domain acyl carrier protein (ACP) by dedicated acyltransferases (ATs). These CoAtethered PKS building blocks impart unreactive, aliphatic substituents (proton, methyl, and ethyl, respectively) to the polyketide backbone and contrast the second class of dedicated ACP-linked PKS extender units that instead harbor functionalized side chains. Methoxymalonyl-ACP, hydroxymalonyl-ACP, and aminomalonyl-ACP are relatively rare extender units that

supply methoxy, hydroxyl, and amino groups, respectively, to the polyketide molecule (8). The programmed introduction of these ACP-bound extender units results in the strategic placement of functional groups that confers important structural and biological properties to the polyketide.

We recently proposed that the PKS extender unit chloroethylmalonyl-CoA was involved in the biosynthesis of the anticancer agent salinosporamide A in the marine bacterium Salinispora tropica (9). This CoA-linked halogenated metabolite provides the reactive chloroethyl side chain germane to salinosporamide A's irreversible binding mechanism against the 20S proteasome (10). The biosynthesis of salinosporamide's chlorinated building block is initiated by the S-adenosyl-L-methionine (SAM)dependent chlorinase (11), which catalyzes the conversion of SAM to 5'-chloro-5'deoxyadenosine (5'-ClDA). Herein, we detail an 8-step biosynthetic pathway to chloroethylmalonyl-CoA and firmly establish this chlorinated metabolite as a PKS extender unit. Because halogen atoms not only favorably influence the bioactivity of drugs (12) but also offer chemically reactive handles for lead optimization by semisynthetic chemistry, this pathway to a halogenated PKS building block may facilitate the bioengineering of polyketide molecules for drug development.

Results

Analysis of the sal Gene Cluster. Complete genome sequence analysis of *S. tropica* CNB-440 revealed 19 secondary metabolic gene clusters (13), including a 41-kb hybrid PKS-nonribosomal peptide synthetase (NRPS) gene set consistent with salinosporamide A biosynthesis (Fig. 1 and *SI Text*, Table S1). Before genome sequencing of CNB-440 was completed, we attempted to clone and sequence the *sal* cluster from *S. tropica* strain CNB-476 by using PCR-amplified PKS, NRPS, and crotonyl-CoA carboxylase/reductase (CCR) gene fragments as probes. Library screening led to the identification of a pOJ446 cosmid clone containing a 33-kb genomic insert 99% identical in DNA sequence to strain CNB-440 (Fig. 1).

Central to the sal cluster is the salA gene that codes for a

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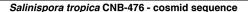
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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. CP000667 (*S. tropica* CNB-440 complete genome sequence) and EF397502 (partial *sal* cluster from *S. tropica* CNB-476)].

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Salinispora tropica CNB-440 - genome sequence ORF4 ORF3 **ORF1 ORF2** Z YXWV UTS RIII Q RIIONML K FG

Fig. 1. Organization of the sal biosynthetic gene cluster from Salinispora tropica. The sal DNA sequence in strains CNB-476 and CNB-440 is 99% identical. Genes putatively involved in the chloroethylmalonyl-CoA pathway (red), construction of the core γ -lactam- β -lactone ring system (gray), assembly of the nonproteinogenic amino acid L-3-cyclohex-2'-enylalanine (blue), regulation and resistance (yellow), unknown (white), and 2 partial transposases (black) are color-coded.

bimodular PKS of unusual domain organization harboring contiguous acyltransferase loading (AT_L) and extender (AT₁) (Fig. 1) domains rather than the standard AT_L-ACP_L-KS₁-AT₁-ACP₁ assembly as observed in prototypical PKSs such as 6-deoxyerythronolide B synthase 1 (14). This noncanonical domain architecture is, however, found in several myxobacterial megasynthases such as those involved in stigmatellin, soraphen, and aurafuranone biosynthesis (15–17). Stable isotope feeding studies showed that salinosporamide A is biosynthesized from the building blocks acetate, the nonproteinogenic amino acid β-hydroxy-L-3-cyclohex-2'-enylalanine (CHA) and a sugar-derived chlorinated molecule that we hypothesized was a previously unknown PKS extender unit, namely chloroethylmalonyl-CoA (9).

We propose that the hexa-domained SalA is involved in the selection, attachment and condensation of acetyl-CoA and chloroethylmalonyl-CoA, to generate a β -ketothioester intermediate. The loading module is of the ACP_L/AT_L-type that recognizes monocarboxylic acid starter units such as acetyl-CoA rather than the KSQ-type that accepts dicarboxylic acids like malonyl-CoA (6). The possibility of chloroethylmalonyl-CoA as an extender unit prompted us to examine the phylogeny of AT₁ in more detail (SI Text and Fig. S1), because AT domain divergence is a critical factor in the evolution of polyketide structural diversity (18). Malonyl-CoA- and methylmalonyl-CoA-specific AT domains share a common ancestor, diverging at some point of evolution to form 2 distinct groups, whereas the relatively rare ethylmalonyl-CoA and methoxymalonyl-ACP ATs appear to have evolved more than once because the known sequences reside in either clade (18). Our analysis with representative AT domains reconstructs that scenario and places SalA_AT₁ in the methylmalonyl-CoA group, more closely related to some myxobacterial ATs although forming its own subclade (SI Text and Fig. S1). In addition, the distinct signature motifs (14) apparent from sequence alignments are also in agreement with AT₁ accepting an unreported extender unit (SI *Text* and Fig. S1B).

Moreover, the detection of salinosporamide analogs with different side chains at C2 (ethyl and methyl, Fig. 2), points to the promiscuity of AT₁ in accepting not only chloroethylmalonyl-CoA (salinosporamide A), but also other substituted malonyl extender units such as ethylmalonyl-CoA (salinosporamide B) and methylmalonyl-CoA (salinosporamide D). To probe the central role of salA, we disrupted it via a single-cross-over homologous recombination event. Inactivation of salA abolished the biosynthesis of all salinosporamides, thereby confirming that this family of β -lactones is indeed derived from a PKS pathway (Fig. 2).

salinosporamide A

The Chloroethylmalonyl-CoA Pathway. Based on the gene organization of the sal cluster, we propose a route to chloroethylmalonyl-CoA as illustrated in Fig. 3A. Biosynthesis of ethylmalonyl-CoA as a precursor of salinosporamide B is shown for comparison (Fig. 3B) and is not encoded in the sal locus but constitutes rather a primary metabolic pathway for acetate assimilation and a source of building blocks for secondary metabolite production (19, 20).

We recently reported that chlorine incorporation into salinosporamide A is catalyzed by the SAM-dependent chlorinase SalL in an orthogonal manner to other known enzymatic chlorination reactions, but analogous to fluorinase FIA of Streptomyces cattleya, a fluoroacetate and 4-fluorothreonine producer (11, 21). Earlier studies using ¹³C-labeled glucose showed an incorporation pattern in the chlorobutyrate moiety of salinosporamide A consistent with the ribose unit of SAM being the ultimate precursor (9). Interestingly, the sal cluster also harbors the FIB homolog SalT. FIB is the second enzyme in the pathway to fluorometabolites in S. cattleya, catalyzing the phosphorolytic cleavage of 5'-FDA to produce

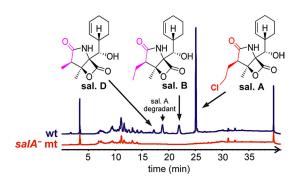


Fig. 2. Inactivation of the PKS gene salA completely abolishes salinosporamide (sal.) production. HPLC chromatograms of culture extracts with detection at 210 nm. Mt, mutant; wt, wild-type.

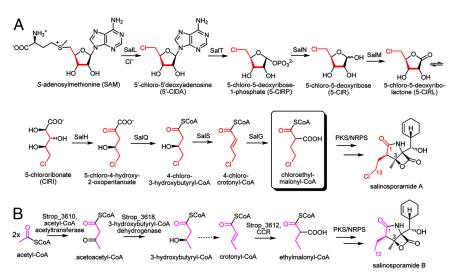


Fig. 3. Comparison of chloroethylmalonyl-CoA and ethylmalonyl-CoA biosynthetic pathways. (A) Proposed pathway to chloroethylmalonyl-CoA as a PKS extender unit in salinosporamide A biosynthesis. (B) The corresponding ethylmalonyl-CoA moiety in salinosporamide B is derived from acetate (9). Crotonyl-CoA carboxylase/reductase (CCR) is a key enzyme in the ethylmalonyl-CoA pathway (19).

5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FRP) (22). Although SalT likely catalyzes the analogous conversion of 5'-ClDA to 5-ClRP (Fig. 3A), the 2 pathways appear to diverge at this point as no other structural homologous genes are shared between the *sal* and *fl* clusters (22). For fluorometabolites it has been speculated that an isomerase catalyzes the ring opening of 5-FRP to 5-fluoro-5-deoxy-D-ribulose-1-phosphate, in analogy to the known metabolism of 5'-methylthioadenosine (23). Indeed, the in vitro reconstitution of 4-fluorothreonine biosynthesis has been recently achieved by using besides FlA and FlB, 3 enzymes not coded in the *fl* cluster, that is, an isomerase, a surrogate aldolase and a pyridoxal (PLP)-dependent transaldolase (24).

For chloroethylmalonyl-CoA, we propose instead that the phosphatase homolog SalN and the dehydrogenase/reductase SalM catalyze the dephosphorylation of 5-ClRP to 5-chloro-5-deoxy-Dribose (5-ClR) followed by oxidization to 5-chlororibonate (5-ClRI) possibly via the intermediate 5-chloro-5-deoxy-D-ribono-1,4-lactone (5-ClRL) (Fig. 3A). SalH, a dihydroxyacid dehydratase homolog, then putatively converts 5-ClRI to 5-chloro-4-hydroxy-2-oxopentanoate, which is then subjected to SalQ-catalyzed oxidative decarboxylation to 4-chloro-3-hydroxybutyryl-CoA. This product importantly results in a 4-carbon chlorometabolite consistent with the C1/C2/C12/C13 salinosporamide A fragment (9). SalQ shares >50% sequence identity with α-oxoacid ferredoxin oxi-

doreductases known to catalyze the oxidative decarboxylation of α -ketoacids with reduction of ferredoxin (Fd) to the corresponding CoA derivative, the prototype of which is pyruvate ferredoxin oxoreductase (25).

Domain analysis of the putative biosynthetic enzyme SalS suggests that it belongs to the hotdog fold superfamily that includes FabZ, a β -hydroxyacyl-ACP dehydratase involved in bacterial fatty acid biosynthesis (26). SalS's closest characterized homolog (47% amino acid identity) is Rv0130 from *Mycobacterium tuberculosis*, a 16-kDa protein shown to catalyze the reversible hydration of crotonyl-CoA to hydroxybutyryl-CoA in vitro (27). However, it has been shown that these enzymes function as dehydratases in vivo when coupled to a reductase (28). Hence, we propose that SalS catalyzes the reversible dehydration of 4-chloro-3-hydroxybutyryl-CoA to 4-chlorocrotonyl-CoA.

The final reaction toward chloroethylmalonyl-CoA is putatively catalyzed by SalG, which shows sequence identity (>60%) to crotonyl-CoA carboxylase/reductases (CCR). The biological function of CCR was recently revised to catalyze the last step of ethylmalonyl-CoA biosynthesis (19) (Fig. 3B), and similarly we hypothesize that SalG catalyzes the reductive carboxylation of 4-chlorocrotonyl-CoA to chloroethylmalonyl-CoA.

Gene Inactivation and Chemical Complementation. To functionally identify the chloroethylmalonyl-CoA pathway enzymes, we inacti-

Table 1. Salinosporamide production by S. tropica mutants compared to the wild-type

Strain	Protein function	Sal. A, % [†]	Sal. B, % [†]	Strop homolog, sequence identity
wild-type	_	100 ± 10	100 ± 13	_
salA-	PKS	n.d.	n.d.	_
salL-	Chlorinase (11)	n.d.	90 ± 20	Strop_1405, 35%
salT ⁻	Purine nucleoside phosphorylase	50 ± 8	91 ± 10	Strop_0986, 69%
salN-	Phosphatase	16 ± 3	92 ± 9	· <u> </u>
salM ⁻	Dehydrogenase/reductase	2.2 ± 0.2	120 ± 20	Strop_2799, 35%
salH ⁻	Dihydroxyacid dehydratase	3.8 ± 0.7	70 ± 15	Strop_1231, 36%
salQ-	α -ketoacid decarboxylase	25 ± 6	98 ± 16	Strop_1050 [‡]
salS-	Acyl dehydratase	39 ± 14	95 ± 30	· <u>-</u>
salG ⁻	CI-CCR	n.d.	94 ± 30	Strop_3612, 53%
Strop_3612 ⁻	CCR	112 ± 8	52 ± 6	salG, 53%

Sal., salinosporamide; n.d., not detected (<0.2%).

 $^{^{\}dagger}$ Values are average of at least two independent experiments \pm deviation.

[‡]Strop_1050 represents a partial duplication of *salQ* (894 of 3,450 nt).

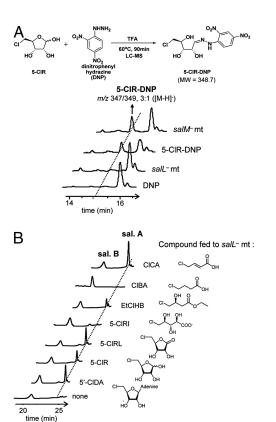


Fig. 4. Chromatographic analysis of intermediates in the chloroethylmalonyl-CoA pathway. (A) 5-CIR as a key intermediate in the chloroethylmalonyl-CoA pathway. Scheme of the method used to derivatize 5-CIR for UV detection (352 nm) (Upper). LC/MS analysis of derivatized 5-CIR and water extracts of the salL (negative control) and salM mutants (Lower). (B) Feeding experiments with postulated intermediates (or their counterparts) in the chloroethylmalonyl-CoA pathway restore salinosporamide A production in the salL mutant. See Results for details.

vated each postulated gene by replacement with an apramycin resistance (aac (3)IV) marker using λ -Red recombination (11, 29). Inactivation led to the selective loss of salinosporamide A production in relation to salinosporamide B in case of the first and last committed pathway enzymes encoded by the chlorinase salL (11) and the 4-chlorocrotonyl-CoA carboxylase/reductase (Cl-CCR) salG, respectively. The remaining 6 mutants, however, all resulted in significant reductions in salinosporamide A yields, suggesting partial complementation by housekeeping enzymes (Table 1).

salM deletion in particular led to a substantial loss in salinosporamide A production to approximately 2% wild-type levels. This low level of residual biosynthesis may result from biochemical cross-talk with homologous dehydrogenases such as Strop 2799 (35% sequence identity). We hypothesized that the absence of SalM might furthermore result in the accumulation of its putative substrate 5-CIR. To probe whether 5-CIR is indeed accumulated in the blocked mutant, we derivatized an organic extract of the supernatant with 2,4-dinitrophenylhydrazine and analyzed by LC/MS. The corresponding hydrazone of 5-CIR was detected in the salM but not in the salL mutant as expected (Fig. 4A). This result suggests that 5-CIR is indeed a key metabolite in the chloroethylmalonyl-CoA pathway and the substrate of SalM.

Putative intermediates were next chemically synthesized and administered to the salL- and salG-defective mutants and salinosporamide A production analyzed. In accordance with the pathway proposed in Fig. 3A, 5'-ClDA (11), 5-ClR, 5-ClRL, ethyl 4-chloro-3-hydroxybutyrate (EtClHB) and 4-chlorocrotonic acid (ClCA) selectively restored salinosporamide A production in the salL mutant (Fig. 4B) but not in the salG mutant, providing further support that these compounds are intermediates either directly or indirectly in the biosynthesis of the presumed chloroethylmalonyl-CoA extender unit. The anticipated intermediate 5-ClRI, however, did not restore salinosporamide A biosynthesis, which may be a consequence of poor cellular uptake because of its polarity. Furthermore, saturated 4-chlorobutyric acid (CIBA) did not chemically complement either mutant and restore salinosporamide A production, which is consistent with the proposed in vivo role of SalG as a reductive carboxylase and not simply as a reductase.

Semiquantitative RT-PCR analysis was conducted to test the possibility of polar effects on downstream genes caused by each relevant gene replacement (SI Text and Fig. S2). Transcription of salS, salO, and salL in the salT, salN, and salM disruption mutants, respectively, was equivalent to the wild type. However, salG gene replacement by the apramycin resistance marker did have a negative effect on downstream salH transcript levels. The construction of a salG mutant in which salH was put directly under control of the putative salA promoter (see SI Text and Figs. S3 and S4) was able to revert the originally observed polar effect and restore salH transcription to wild-type levels. Nonetheless, salinosporamide A production was still not detected in this mutant, thereby confirming the dedicated role of SalG in 4-chloroethylmalonyl-CoA biosynthesis.

Characterization of SalG, a Chlorocrotonyl-CoA Carboxylase/Reductase. To demonstrate the function of SalG as a 4-chlorocrotonyl-CoA carboxylase/reductase and establish the authenticity of chloroethylmalonyl-CoA as an intermediate, we biochemically characterized recombinant SalG, which was expressed as an octahistidyl-tagged protein in Escherichia coli. Chlorocrotonyl-CoA was chemically synthesized and converted by recombinant SalG to chloroethylmalonyl-CoA in a NADPH-dependent reductive carboxylation reaction as detected by LC/MS analysis. The apparent kinetic constants (Table 2) support a preference for the chlorinated substrate, whereas the enzyme also accepts crotonyl-CoA with 7-fold decrease in catalytic efficiency (k_{cat} / $K_{\rm M}$). Moreover, the turnover ($k_{\rm cat}$) for the reductive carboxylation of chlorocrotonyl-CoA is ≈10-fold faster than for the reductive reaction in the absence of bicarbonate, which is consistent with the kinetic properties of the known CCR from Rhodobacter sphaeroides (19).

Table 2. Apparent kinetic constants of SalG

	Reductive carboxylation†		Reduction only [‡]		
Substrate	Crotonyl-CoA	Chlorocrotonyl-CoA	Crotonyl-CoA	Chlorocrotonyl-CoA	
k_{cat} , min ⁻¹	15.4 ± 0.9	23.1 ± 2.6	4.7 ± 0.2	2.2 ± 0.2	
K_{M} , μM	20.7 ± 4.2	4.4 ± 1.8	9.8 ± 1.4	1.9 ± 0.7	
$k_{cat}/K_{\rm M}$, min ⁻¹ $\mu \rm M^{-1}$	0.75	5.3	0.48	1.1	
Relative catalytic efficiency, %	14	100	44	100	

 $^{^{\}dagger}$ (chloro)crotonyl-CoA + CO₂ + NADPH → (chloro)ethylmalonyl-CoA + NADP⁺.

[‡](chloro)crotonyl-CoA + NADPH → (chloro)butyryl-CoA + NADP⁺.

In addition to *salG*, the *S. tropica* genome harbors only one other CCR gene (*Strop_3612*), which likely encodes the housekeeping CCR responsible for ethylmalonyl-CoA biosynthesis. To test this hypothesis we carried out a separate deletion of *Strop_3612*. Although gene inactivation of *Strop_3612* had no significant effect on salinosporamide A yields, salinosporamide B production fell 50% in this mutant (Table 1), which is consistent with its biosynthesis from a CCR-derived ethylmalonyl-CoA unit (Fig. 3*B*).

Discussion

We present in vivo and in vitro evidence for an exquisite pathway to a halogenated PKS building block composed of 8 enzymes in total, some of which are unique and others whose function can be replaced by primary metabolic counterparts. SalG, for instance, appears to have singularly evolved from a common CCR ancestor to accept a halogenated substrate. Its relaxed substrate discrimination, evident from the kinetic data presented from the fact that fluorinated (30) and brominated (31) salinosporamide analogs can be generated in vivo, is useful when attempting to engineer structural analogs. The residual biosynthesis of salinosporamide B from ethylmalonyl-CoA in the housekeeping CCR (Strop 3612) deletion mutant may derive from SalG-catalyzed reductive carboxylation of crotonyl-CoA. Alternatively, an orthogonal pathway for acetate assimilation may function in S. tropica (7, 20) as suspected in Streptomyces cinnamonensis in which genetic inactivation of its CCR likewise resulted in the reduced production of the ethylmalonyl-CoA-derived polyketide monensin A rather than abolishing it completely (32).

Inactivation of the chlorinase gene *salL* also abolished salinosporamide A biosynthesis (11) despite the presence of the *salL* homolog *Strop_1405*, which contains a domain of unknown function (DUF62, Table 1). We recently showed that a DUF62 ortholog from *Salinispora arenicola* (86% identity to Strop_1405) has SAM hydrolase but no halogenase activity in vitro (33), which was independently reported with an archaeal DUF62 (34). Together with *in silico* analysis, these data suggest divergent evolution of SAM-dependent halogenases from SAM hydrolases (33) and clarify the *salL* mutant phenotype.

Although the function of SalL and SalG appear unparalleled, the activities of the 6 other pathway-committed enzymes can be replaced to varying levels by primary metabolic counterparts (Table 1). For instance, another purine nucleoside phosphorylase involved in nucleotide metabolism (35), 5'-methylthioadenosine phosphorylase (Strop_0986, 69% identity to SalT), is encoded by the *S. tropica* genome and is likely the enzyme complementing the *salT* mutation. Similarly, *S. tropica*'s chromosome codes for several short-chain dehydrogenases/reductases and dihydroxyacid dehydratases with sequence similarity to SalM and SalH, respectively (Table 1).

However, SalN and SalS have no clear homologs (<23% sequence identity to other *Strop* genes). In the case of SalS, primary metabolic enoyl thioester dehydratases such as the FabZ-like Strop_2797 and the unknown 3-hydroxybutyryl-CoA dehydratase (Fig. 3B) may include possible candidates to replace its function. It is noteworthy that FabZ enzymes, despite having a preference for ACP-bound substrates, also accept CoA analogs at lower rates, with crotonyl-CoA often used as a substrate to evaluate FabZ activity (28, 36, 37). Likewise, other phosphatases present in the *S. tropica* chromosome such as the putative alkaline phosphatase Strop_2394 may account for the *salN* mutant phenotype, because such enzymes have broad substrate specificity (38). Indeed a commercial alkaline phosphatase (EC 3.1.3.1; Sigma Chemical Co. A2356) catalyzes the phosphorylic cleavage of chemo-enzymatically prepared 5-CIRP to 5-CIR.

Although α -keto acid ferredoxin oxidoreductase SalQ is partially duplicated in the *S. tropica* genome as Strop_1050 (corresponding to 298 of 1,149 amino acids), its expression cannot explain the salinosporamide A levels observed in this mutant, because the

C-terminal catalytic domain is not complete and the thiamine pyrophosphate binding site is missing in Stop_1050 (39). Alternatively, α -keto acid oxidative decarboxylation to form acyl-CoA can be mediated by 2-oxo acid dehydrogenases (OADH), multienzyme complexes consisting of 3 components, decarboxylase (E1), transacetylase (E2) and dehydrogenase (E3) (40). The *S. tropica* genome codes for a few OADHs, i.e., Strop_2097 (E1) to Strop_2099 (E3), Strop_0109 to Strop_0107 and Strop_3690 (E1-E3), putatively encoding pyruvate or branched-chain oxoacid and 2-oxoglutarate dehydrogenase complexes, respectively. The relaxed substrate specificity of OADHs (40) is a possible explanation for *salQ*'s mutant phenotype.

To the best of our knowledge, the salinosporamide system is at present the only PKS machinery known to use chloroethylmalonyl-CoA as an extender unit. The discovery of chloroethylmalonyl-CoA expands our current knowledge of known PKS extender units and more significantly represents a functionalized CoA-tethered malonyl extender (7). In concert with the availability of an additional PKS building block, the divergence of AT domains is an important factor to the evolution of structural diversity in polyketides (18). Therefore, chloroethylmalonyl-CoA and SalA AT₁ extend the repertoire of tools to be used in polyketide assembly, significantly impacting our ability to generate polyketide scaffolds and possibly improve biological activity. Although the number of known extender units has expanded in recent years, a true increase in available building blocks may come from those biosynthesized by a CCR-like route (7), which could possibly lead to previously unknown extender units through conversion of a variety of α, β , unsaturated acyl-CoA thioesters. The chloroethylmalonyl-CoA pathway presented here and SalG in particular is clearly representative of this scenario and points the way toward the genetic engineering of new polyketide scaffolds with strategically placed halogens that may serve key mechanistic roles or provide functional handles in the semisynthesis of derivatives.

Materials and Methods

Chemicals. 5'-CIDA, butyric acid and 4-chlorobutyric acid were obtained from Sigma. 5-CIR, 5-CIRL, 5-CIRI, ethyl 4-chloro-3-hydroxybutyrate and CICA were synthesized according to modified literature preparations (see *SI Text*). Compounds were administered to the *S. tropica* mutant strains to a final concentration of 0.3 mM and products analyzed by HPLC-MS as described in ref. 11. The 2,4-DNP derivatization of synthetic 5-CIR and water extracts of 100-mL cultures of *S. tropica salL* and *salM* deficient mutants and monitoring by HPLC-MS followed literature precedence (41).

Inactivation of sal Genes. *salA* was inactivated by homologous recombination via a single cross-over (42) after introducing pAEM3 into *S. tropica* CNB-440 by conjugation from *E. coli* ET12567/pUZ8002. Other genes were inactivated by using the PCR targeting system (29) with some modifications as described in ref. 11. See *SI Text* for details.

Characterization of SalG. His₈-tagged SalG protein was purified from *E. coli* BL21(DE3) by using Ni-affinity chromatography and gel-filtration (see *SI Text* for details) and stored in 50 mM phosphate buffer pH 7.2, 100 mM NaCl and 10% glycerol at -80 °C. Determination of apparent k_{cat} and $K_{\rm M}$ for each substrate and reaction was performed by continuous assay measuring NADPH consumption at 340 nm using a UV-1700 spectrophotometer (Shimadzu). Assay conditions involved SalG (47 nM) incubated with various concentrations of crotonyl-CoA and 4-chlorocrotonyl-CoA in reaction buffer (100 mM Tris-HCl, pH 7.9) under saturating levels of NADPH. Assays were carried out in the absence and presence of NaHCO₃ (80 mM, final concentration) to determine the kinetics values for the reductive and the reductive-carboxylation reaction, respectively. Data were collected in triplicate and averaged. Confirmation of products was carried out by LC/(+)ESI-MS analysis using a water to methanol gradient containing 5 mM ammonium acetate. See *SI Text* for further methods.

For additional details see a list of gene sequences used in phylogenetic analysis (Table S2) and a summary of synthesized chlorinated substrates (Fig. S5).

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