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# Unusual Chemistry in the Biosynthesis of the Antibiotic Chondrochlorens

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## SUMMARY

**The antibiotic chondrochlorens A and B from the myxobacterium *Chondromyces crocatus* Cm c5 incorporate several unusual structural features, notable among them a shared chloro-hydroxy-styryl functionality and the ethoxy group of chondrochloren B. Our analysis of the chondrochloren gene cluster by targeted gene inactivation coupled with assays in vitro has shed significant light on the biosynthesis of these metabolites. Chlorination of tyrosine occurs early in the pathway, likely on a peptidyl carrier protein-bound intermediate, whereas decarboxylation to the styryl moiety appears to be accomplished by an unprecedented oxidative decarboxylase. We also show that the chondrochloren B ethoxy group arises from initial incorporation by the polyketide synthase of hydroxy malonate as an extender unit, methylation in *cis* by an *O*-methyltransferase, followed by a second methylation. This report therefore constitutes a direct demonstration of the involvement of a radical *S*-adenosylmethionine methylase in bacterial secondary metabolism.**

## INTRODUCTION

Myxobacteria are a large and growing class of Gram-negative microbes which exhibit several characteristic features, including movement by gliding in swarms, multicellular ''social'' behavior, and formation of complex fruiting bodies under nutrient-limiting conditions (Bode and Müller, 2006; Reichenbach, 2001). Recently, the secondary metabolic potential of these bacteria has emerged to rival that of more established sources for medicinally valuable natural products, such as the actinomycetes and the pseudomonads (Gerth et al., 2003). Compounds derived from myxobacteria exhibit unusual modes of activity, targeting cellular structures which are rarely hit by other metabolites (Bode and Müller, 2006). Prominent among myxobacterial secondary metabolites are several compounds which interact with the eukaryotic cytoskeleton, such as epothilone (Mulzer, 2009). Among myxobacterial strains, the genus *Chondromyces* is an especially prolific producer of natural products. For example, *Chondromyces crocatus* strain Cm c5 biosynthesizes six structurally unique groups of metabolites under laboratory conditions, including the chondramides (Kunze et al., 1995), crocacins (Jansen et al., 1999), ajudazols (Jansen et al., 2002), crocapeptins (Jansen et al., 2003), thuggacins (Steinmetz et al., 2007), and chondrochlorens (Jansen et al., 2003).

Chondrochlorens A (**1**) and B (**2**) (Figure 1) show weak antibacterial activity against *Micrococcus luteus*, *Schizosaccharomyces pombe*, *Bacillus subtilis*, and *Staphylococcus aureus* (Jansen et al., 2003). Recent structure elucidation (Jansen et al., 2003) revealed a highly functionalized $C_{14}$ carboxylic acid which is joined in amide linkage to a chloro-hydroxy-styryl functionality. Such halogenated derivatives are rare among myxobacterial metabolites (Jansen et al., 2003) and, more unusually, *C. crocatus* produces two of them (the chondramides contain chlorotryptophan). Chondrochloren B, the minor metabolite, incorporates an additional methylene group relative to chondrochloren A. Surprisingly, NMR analysis showed that the methylene is part of an ethoxy substituent which replaces the methoxy group at C-2 of chondrochloren A. Although ethoxy groups are not uncommon in plant metabolites (Shyr et al., 2006; Mallavadhani et al., 2006), to our knowledge, the presence of this functionality in bacterial compounds is highly unusual. The origin of the shared styryl moiety is also intriguing, as it appears to derive from decarboxylation of a functionalized chlorotyrosine.

In order to decipher the unusual features of chondrochloren biosynthesis and to pave the way to genetic engineering of novel derivatives, we aimed to sequence the corresponding biosynthetic gene cluster in *C. crocatus* Cm c5. ''Retrobiosynthetic analysis'' of the compounds strongly suggested their origin from a hybrid polyketide synthase (PKS)-nonribosomal polypeptide synthetase (NRPS) system. PKS and NRPS are gigantic multienzyme ''assembly lines'' which catalyze the sequential condensation of simple building blocks, acyl-CoA thioesters and amino acids, respectively (Walsh, 2007; Weissman and Leadlay, 2005). Chain extension is accomplished by successive modules of enzymes, and thus the genetic organization is colinear with the sequence of biosynthetic transformations. PKS modules minimally incorporate acyl transferase (AT), ketosynthase (KS), and acyl carrier protein (ACP) domains, and may also contain reductive activities (ketoreductase [KR], dehydratase [DH], and enoyl reductase [ER]). The analogous core functions of NRPSs include condensation (C) (or heterocyclization [HC]) and peptidyl carrier protein (PCP) domains, whereas optional modifying enzymes may comprise epimerase (E), methyltransferase (MT), and oxidase (Ox) functions. Chain termination in both systems is typically performed by an integral thioesterase (TE) activity (Kopp and Marahiel, 2007).

 

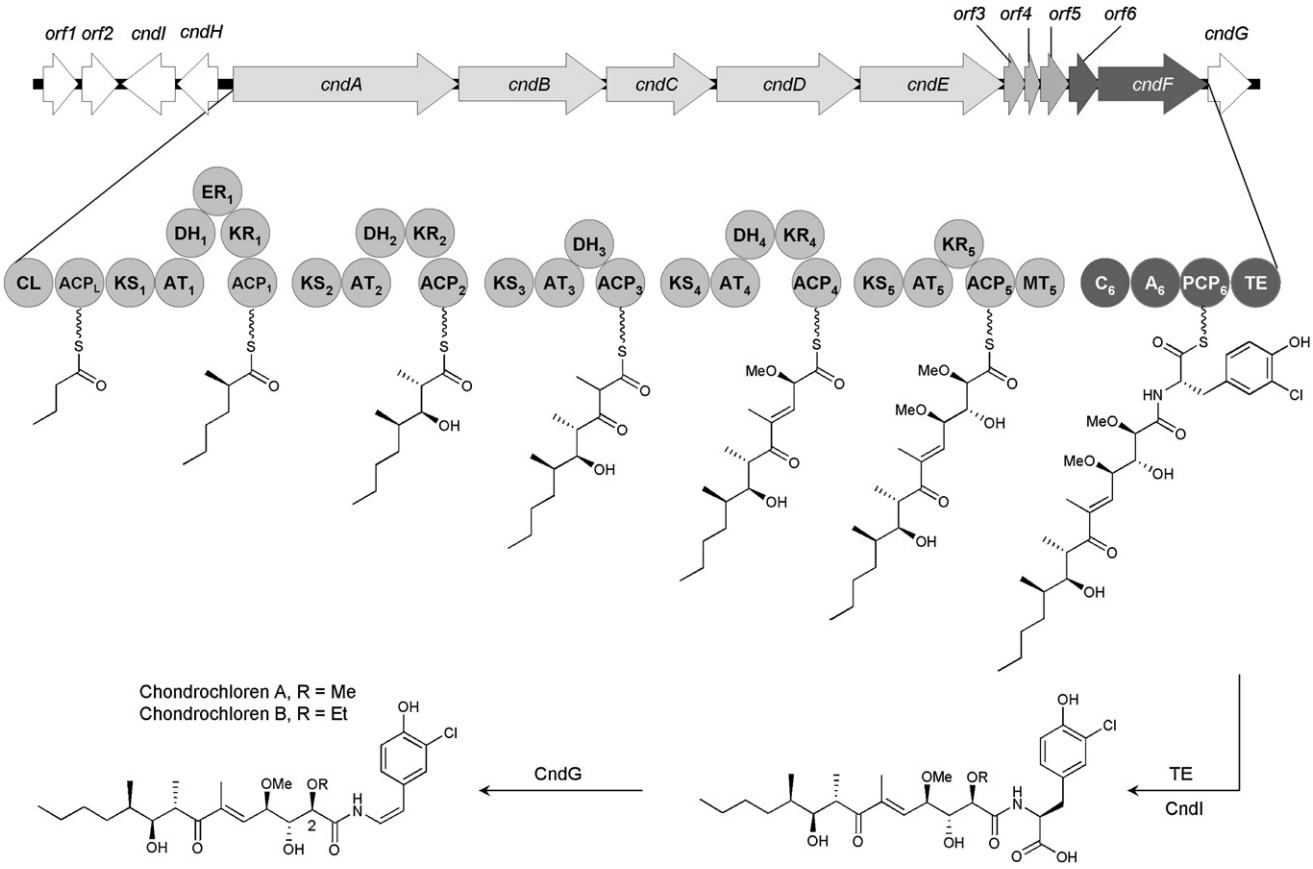


**Figure 1. Organization of the Chondrochloren Gene Locus and Proposed Biosynthetic Pathway to Chondrochlorens A and B**
PKS domains (light gray): CL, CoA-ligase; ACP, acyl carrier protein; KS, ketosynthase; AT, acyltransferase; DH, dehydratase; ER, enoylreductase; MT, methyltransferase. NRPS domains (dark gray): C, condensation; A, adenylation; PCP, peptidyl carrier protein; TE, thioesterase.

We report here the cloning and characterization of the gene cluster for chondrochloren biosynthesis in *C. crocatus* Cm c5. This analysis has provided insights into several features of the biosynthesis, including a new member of the radical *S*-adenosylmethionine (SAM) enzyme superfamily (Marsh et al., 2004) which methylates a methyl group to yield the ethoxy of chondrochloren B, an early-stage chlorination of tyrosine, and a candidate for a catalyst which accomplishes the oxidative decarboxylation of 3-chlorotyrosine. Support for our analysis has been provided by a recently developed gene inactivation methodology for *C. crocatus* (Rachid et al., 2006), coupled with assays of several key enzymes in vitro.

## RESULTS

### Identification and Analysis of the Chondrochloren Biosynthetic Gene Cluster

Based on the chemical structure of the chondrochlorens, we anticipated that the pathway would include a mixed PKS-NRPS assembly line, as well as a tyrosine chlorinase. Therefore, to identify the gene cluster in *C. crocatus* Cm c5, we probed the genome for halogenase genes (Fujimori and Walsh, 2007), with secondary screening for the colocalization of PKS and NRPS sequences. A halogenase fragment was amplified using degenerate primers designed against conserved sequences of halogenase genes from several bacterial species, and used together with specific PKS and NRPS probes to screen a 2304-clone chromosomal library of *C. crocatus*. Based on the genome size of myxobacteria (Schneiker et al., 2007), this cosmid library should represent a 10-fold coverage of the genome. This analysis highlighted seven cosmids (A:A8, B:E4, C:A22, E:I15, D:J12, B:M18, and C:L23), which were then crossanalyzed with a PKS probe. All plasmids were positive both for halogenase and PKS genes. We selected B:E4 for further study, as end sequencing revealed PKS genes on one end and genes with no predicted function in chondrochloren assembly on the other, suggesting that it contained one end of a gene cluster. To identify the remainder of the cluster, we designed oligonucleotides against the PKS end sequence of B:E4, and used the resulting PCR product to screen for overlapping cosmids within the chromosomal library. The resulting cosmid E:H19 hybridized to the PKS probe, but in addition to a degenerate probe designed against NRPS sequences. End sequencing of cosmid E:H19 again revealed the presence at one end of PKS genes and at the other of a gene with putative regulatory function with no apparent role in chondrochloren biosynthesis. Thus, this analysis suggested that the two plasmids together contained the full extent of the chondrochloren cluster.

Cosmids B:E4 and H:19 were sequenced on both strands (the nucleotide sequence has been deposited with the EMBO under

accession number AM988861). The chondrochloren gene cluster spans a contiguous stretch of approximately 68 kbp on the *C. crocatus* chromosome (Figure 1). The overall GC content of the sequenced region is 68.6%, which is characteristic of myxobacteria (Shimkets, 1993). The sequence was analyzed for the presence of putative open reading frames (ORFs) with FramePlot 2.3.2 (Ishikawa and Hotta, 1999), and preliminary functional assignments of individual ORFs were made by comparison of the deduced gene products with proteins of known functions in the database. Twelve complete ORFs were identified, including six likely structural genes for chondrochloren biosynthesis designated *cndA–cndF* (Table 1). Genes *cndA–cndE* encode for PKSs, whereas *cndF* encodes for an NRPS. Apart from genes *cndH* and *cndI*, all identified genes are transcribed in the same direction (Figure 1).

In order to identify the upstream boundary of the cluster, *orf1*, encoding a putative two-component sensor histidine kinase, was inactivated by insertional mutagenesis (see Figure S1A available online). The resulting mutant strain Cmc-orf1$^-$ produced a 3-fold lower yield of the metabolites (as judged by high-pressure liquid chromatography–mass spectrometry [HPLC-MS] analysis of extracts in triplicate), consistent with a regulatory role for Orf1 in the pathway (Figure S1F). However, inactivation of the adjacent gene *orf2*, which encodes a putative cytochrome P450 oxidase (yielding mutant Cmc-orf2$^-$), did not reduce production of the chondrochlorens to any significant extent (Figure S1G). Taken together, these results suggest that *orf1* lies at the upstream end of the cluster, although it is clearly not essential for chondrochloren biosynthesis. The downstream margin of the cluster appears to be defined by *cndG*, a gene with homology to FAD-dependent oxidases. This analysis is supported by the observation that the three genes downstream of *cndG* are transcribed in the opposite direction, and the translated products are similar, respectively, to dioxygenase, thiolase, and epimerase enzymes, with no obvious functions in chondrochloren biosynthesis.

To demonstrate unambiguously the identity of the cluster, we aimed to inactivate an essential biosynthetic gene by insertional mutagenesis. For this, plasmid pSR11 containing a fragment of the A domain from NRPS module 6 ($A_6$) was introduced into strain Cm c5 via conjugation with *Escherichia coli*. Analysis independently by PCR and southern blot (Figures S2B and S2C) confirmed the correct integration of plasmid pSR11 into the chromosome by single crossover. HPLC-MS analysis clearly showed the absence of both chondrochlorens A and B in extracts of the resulting mutant Cmc-cndF$^-$, relative to the wild-type strain (Figure S2E).

**Table 1. Predicted Functions of Proteins Present in the Chondrochloren Biosynthetic Gene Cluster of *C. crocatus* Cm c5**

| Protein | Length (aa) | Putative Function | Identity/Similarity (%) to Protein Homolog |
|---|---|---|---|
| Orf1 | 459 | Unknown | 38/57, two-component sensor histidine kinase *Sorangium cellulosum* So ce 56 |
| Orf2 | 476 | Unknown | 38/57, cytochrome P450 *Plesiocystis pacifica* SIR-1 |
| CndI | 700 | Methyltransferase | 29/44, radical SAM protein *Desulfatibacillum alkenivorans* AK-01 |
| CndH | 2198 | Halogenase | 57/69, halogenase *Amycolatopsis balhimycina* |
| CndA | 2923 | PKS: CL, $ACP_L$, $KS_1$, $AT_1$, $DH_1$, $ER_1$, $KR_1$, $ACP_1$ | 45/58, EpoD *Polyangium cellulosum* |
| CndB | 1922 | PKS: $KS_2$, $AT_2$, $DH_2$, $KR_2$, $ACP_2$ | 45/59, MxaE *Stigmatella aurantiaca* |
| CndC | 1411 | PKS: $KS_3$, $AT_3$, $DH_3$, $ACP_3$ | 48/63, MxaD *Stigmatella aurantiaca* |
| CndD | 1873 | PKS: $KS_4$, $AT_4$, $DH_4$, $KR_4$, $ACP_4$ | 46/62, MxaD *Stigmatella aurantiaca* |
| CndE | 1868 | PKS: $KS_5$, $AT_5$, $KR_5$, $ACP_5$, $MT_5$ | 40/58, JamP *Lyngbya majuscula* |
| Orf3 | 271 | Dehydrogenase | 40/55, FkbK *Streptomyces hygroscopicus* var. *ascomyceticus* |
| Orf4 | 90 | Acyl carrier protein | 38/63, FkbJ *Streptomyces hygroscopicus* var. *ascomyceticus* |
| Orf5 | 337 | Dehydrogenase | 35/50, FkbI *Streptomyces hygroscopicus* var. *ascomyceticus* |
| Orf6 | 386 | Phosphatase | 49/65, FkbH *Streptomyces hygroscopicus* var. *ascomyceticus* |
| CndF | 1400 | NRPS: $C_6$, $A_6$, $PCP_6$, TE | 43/58, nonribosomal peptide synthetase *Myxococcus xanthus* |
| CndG | 541 | Decarboxylase | 42/59, FAD-linked oxidase domain protein *Solibacter usitatus* |

### *cndI* Encodes an Unusual Radical SAM Methylase

We next addressed several unusual aspects of chondrochloren biosynthesis, including the origin of the ethoxy moiety at position C-2 in chondrochloren B (Figure 1). Although our proposed mechanism required reaction at an unactivated carbon, we hypothesized that the ethoxy group could arise by methylation of an initial methoxy function using a methyl group derived from SAM. To test this hypothesis, we incubated purified chondrochloren A with cell-free extracts of the chondrochloren nonproducer Cmc-cndF$^-$ in the presence of added SAM, and screened for production of chondrochloren B. High-resolution analysis on a Thermo LTQ Orbitrap Hybrid FT mass spectrometer revealed compounds with the expected mass for chondrochloren B (Figure 2A; HRMS [*m/z*]: $[M+Na]^+$ calculated for $C_{28}H_{42}ClNO_7$, 562.2548; observed, 562.2537; $\Delta = -0.8$ ppm). The compounds were not detected in control incubations lacking exogenous SAM (Figure 2B).

The only candidate gene in the cluster encoding for a SAM-dependent methyltransferase was *cndI*, which lies directly adjacent to the tyrosine halogenase gene *cndH*. Sequence analysis shows that CndI exhibits convincing homology to SAM radical enzymes, a large superfamily which catalyzes a diversity of reactions, notably including methylation of

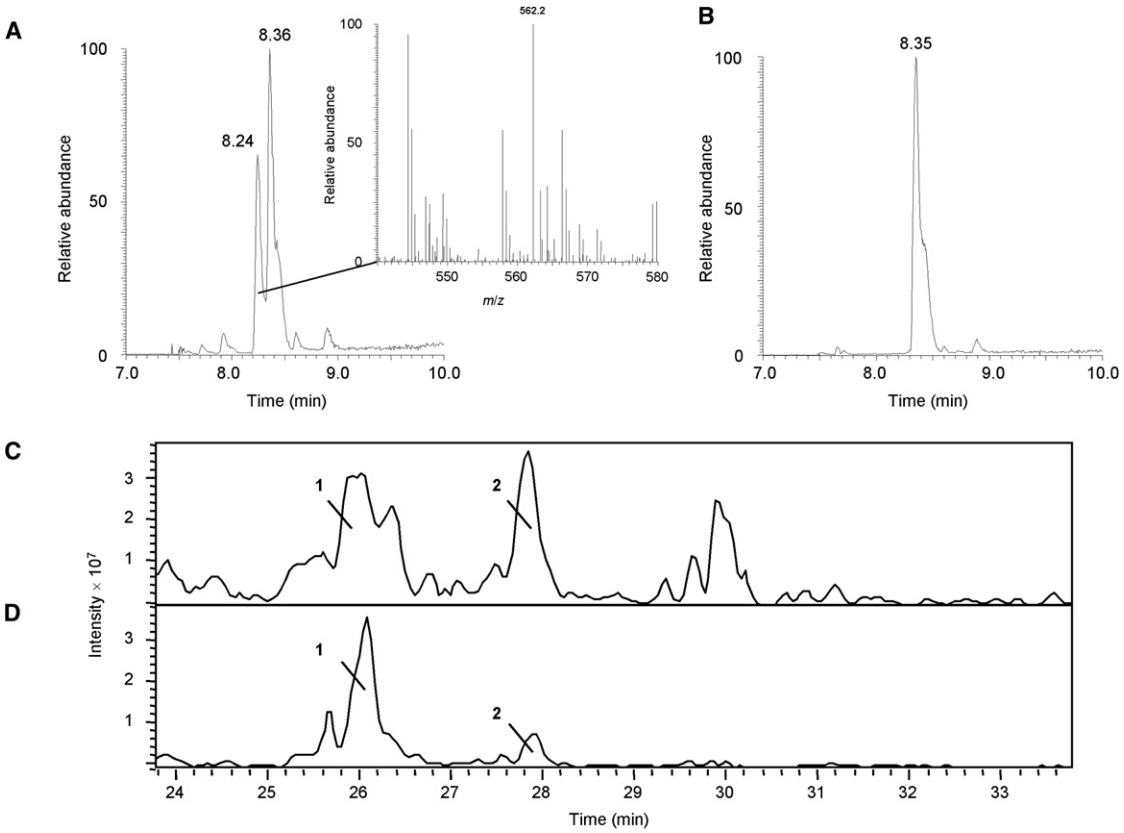


**Figure 2. *cndI* Encodes a Methylmethylase**

(A) High-resolution analysis using a Thermo LTQ Orbitrap Hybrid FT mass spectrometer revealed compounds with the expected mass for chondrochloren B in cell-free extracts of the chondrochloren nonproducer Cmc-cndF$^{-}$, following incubation with purified chondrochloren A and SAM. The administered chondrochloren A was a mixture of isomers, presumably generated by spontaneous chemistry. A peak with the expected mass of chondrochloren B was detected at 8.24 min (calculated *m/z* $[M+Na]^+$ for chondrochloren B, 562.2; this peak represented the major isomer). The peak at 8.36 min corresponds to chondrochloren A.

(B) Chondrochloren B was not detected in an incubation lacking exogenous SAM.

(C) HPLC-MS analysis of wild-type *C. crocatus* Cm c5. The extracted ion chromatogram (*m/z* = 548.2 and 562.2) is shown. Peaks corresponding to chondrochloren A (**1**) and chondrochloren B (**2**) are indicated.

(D) HPLC-MS analysis of mutant Cmc-cndI$^{-}$. The extracted ion chromatogram (*m/z* = 548.2 and 562.2) is shown. Peaks corresponding to **1** and **2** are indicated.

unreactive carbon centers (Marsh et al., 2004). The catalytic center of these enzymes incorporates an unconventional [4Fe-4S] cluster, which is coordinated by three closely spaced cysteines within a conserved CxxxCxxC motif (Marsh et al., 2004), a sequence present in CndI. We therefore inactivated *cndI* by insertional mutagenesis using plasmid pSR14, and confirmed integration into the chromosome by PCR (Figure S1C). Extracts of the resulting strain Cmc-cndI$^{-}$ contained substantially lower amounts of chondrochloren B relative to chondrochloren A, than the wild-type strain (chondrochloren A:B in wild-type 1:1.35 ± 0.09; mutant 1:0.25 ± 0.04 [5.6-fold decrease; Figures 2C and 2D]).

## CndH Is a Tyrosine 3-Chlorinase

On the basis of sequence analysis, we predicted that gene *cndH* would encode a halogenase, catalyzing chlorination of the 3-position of tyrosine in the pathway to the final chlorohydroxy-styryl moiety. To investigate this proposal, we inactivated the *cndH* gene by insertional mutagenesis using plasmid pSR12. Integration of the plasmid into the genome by single crossover was confirmed by PCR (Figure S1D). Analysis of extracts of the *cndH* mutant, Cmc-cndH$^{-}$, revealed the complete absence of chondrochlorens A and B relative to the wild-type strain (Figures 3A and 3B). This result suggested that chlorination of tyrosine occurs early in the pathway (e.g., on the free amino acid [Keller et al., 2000; Yeh et al., 2005], or on a PCP-bound species [Lin et al., 2007; Dorrestein et al., 2005]), as an alternative timing of the reaction, for example, following release of the intermediate from NRPS CndF, should have resulted in nonchlorinated analogs of the chondrochlorens. We therefore evaluated whether biosynthesis of the chondrochlorens could be restored by administration of 3-chloro-L-tyrosine to growing cultures of *C. crocatus*. Indeed, supplementation with 3-chloro-L-tyrosine resulted in essentially wild-type levels of chondrochloren A, and low but detectable levels of chondrochloren B (Figure 3C).

## The Substrate Specificity of the NRPS $A_6$ Domain

We aimed to evaluate the substrate specificity of the CndF $A_6$ domain toward a panel of potential substrates by expressing it in

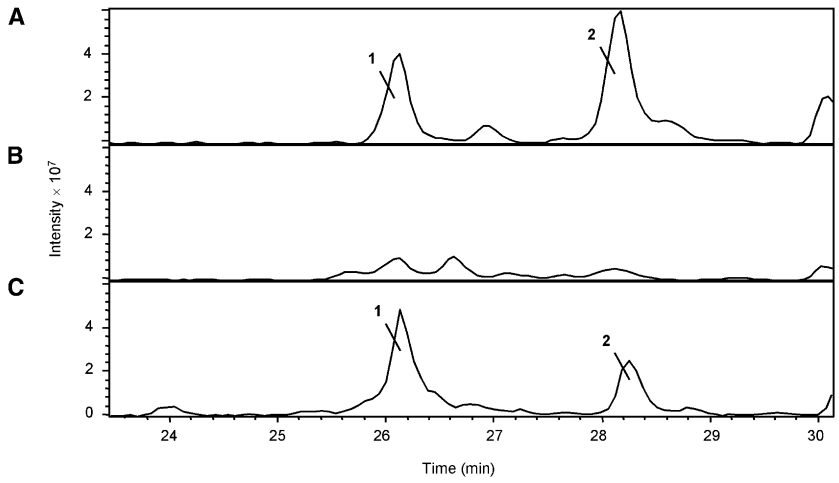


**Figure 3. CndH Acts as a Tyrosine Chlorinase**
(A) HPLC-MS analysis of wild-type *C. crocatus* Cm c5. The total ion chromatogram is shown. Peaks corresponding to chondrochloren A (**1**) and chondrochloren B (**2**) are indicated.
(B) **1** and **2** were not detected in extracts of the halogenase mutant Cmc-cndH$^{-}$.
(C) Production of **1** and **2** by mutant Cmc-cndH$^{-}$, following supplementation with 3-chloro-L-tyrosine.

recombinant form in *E. coli*. The $A_6$ domain was successfully obtained as a C-terminal translational fusion with glutathione *S*-transferase (GST) from plasmid pGEX-Cnd-Ad (Figure S3). Purification by glutathione affinity chromatography followed by digestion with PreScission Protease yielded the purified, untagged $A_6$ domain (molecular mass determined by MALDI mass spectrometry: calculated, 64,064 Da; observed, 64,100 Da; Table S1). We then investigated the substrate specificity of the $A_6$ domain using the established ATP-PPi exchange reaction (Stachelhaus and Marahiel, 1995). The $A_6$ domain was incubated in the presence of [$^{32}$P]pyrophosphate and a panel of amino acids including L-tyrosine, 3-chloro-L-tyrosine, β-tyrosine, L-tryptophan, L-phenylalanine, and L-methionine. The highest activity was observed with 3-chloro-L-tyrosine (normalized to 100%; negative controls without enzyme gave 0.1%–1% of this activity), but L-tyrosine was also activated to a very similar extent (86%) (Table 2). β-tyrosine also showed significant activity (49%), but activation of the remaining amino acids was negligible (5%, 7%, and 4%, respectively).

### CndH Shows No Activity toward Free Tyrosine

To investigate the substrate specificity of CndH directly, the enzyme was expressed as a GST fusion protein in *E. coli*. Purification by glutathione affinity chromatography followed by digestion with PreScission Protease yielded the purified, untagged CndH (Figure S4). As the native flavin reductase partner of CndH in *C. crocatus* is unknown, we used a surrogate enzyme (Fre) obtained from *E. coli* K12 to ensure that a sufficient amount of $FADH_2$ was available in the assay (Zehner et al., 2005). Fre was also obtained by expression as a GST fusion, followed by cleavage and purification to homogeneity (Figure S4). As measured spectrophotometrically by the decrease in absorbance at 340 nm, Fre catalyzed the oxidation of NADH to NAD (Figure S4); the presence of GST lowered the reaction rate, and therefore only the GST-free form was used in further assays. We next attempted to reconstitute the halogenation reaction using CndH, Fre, and free tyrosine, following the literature precedent (Zehner et al., 2005). The reaction products were derivatized with *O*-phthaldialdehyde and *N*-acetylcysteine (OPA-NAC) (Buck and Krummen, 1987), and then analyzed by HPLC-MS by comparison to OPA-NAC-derivatized commercially available 3-chlorotyrosine. This method did not reveal any evidence for chlorination of the derivatized tyrosine (data not shown).

## DISCUSSION

Obtaining insights into the detailed enzymology and structural biology of the modular multienzyme PKS, NRPS, and their hybrids will be key to improving our ability to engineer modified synthases with high efficiency (Weissman and Leadlay, 2005; Walsh, 2007). In this regard, sequencing of clusters encoding the biosynthesis of hybrid PK-NRP metabolites in myxobacteria has been particularly illuminating. Almost without exception, each new cluster challenges accepted rules of PKS and NRPS architecture while simultaneously revealing unusual chemistry accomplished by PKS and NRPS domains, or their associated post-assembly line processing activities (Wenzel and Müller, 2007). We aimed, therefore, to clone and analyze the genetic locus for chondrochloren biosynthesis in the myxobacterium *C. crocatus* Cm c5. Using techniques established during investigation of the chondramide gene cluster within the same strain (Rachid et al., 2006), we identified an approximately 68 kbp gene cluster on the chromosome whose deduced functions

**Table 2. ATP-PPi Exchange Assay of the $A_6$ Domain**

| Substrate | L-Tyr | 3-chloro-L-Tyr | (*RS*)-β-Tyr | L-Trp | L-Phe | L-Met |
|---|---|---|---|---|---|---|
| | 4613 | 5382 | 2817 | 261 | 316 | 201 |
| | 4207 | 5111 | 2422 | 234 | 401 | 200 |
| | 4433 | 4814 | 2323 | 271 | 355 | 287 |
| Average | 4418 ± 203 | 5102 ± 284 | 2521 ± 261 | 255 ± 19 | 357 ± 42 | 226 ± 50 |
| Percentage | 86 | 100 | 49 | 5 | 7 | 4 |

Counts per minute obtained by liquid scintillation counting, following incubation of purified $A_6$ domain with substrate, for 15 min at 25°C. The error is given as standard deviation.

**Table 3. Active Site and Additional Conserved Residues which Correlate with Specificity in AT Domains from the Chondrochloren Cluster**

| Domain | 11 | 63 | 90 | 91 | 92 | 93 | 94 | 117 | 200 | 201 | 231 | 250 | 255 | 15 | 58 | 59 | 60 | 61 | 62 | 70 | 72 | 197 | 198 | 199 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT1 | Q | Q | G | H | S | M | G | R | S | H | S | N | V | W | R | I | D | V | L | Q | A | D | V | A |
| AT2 | Q | Q | G | H | S | M | G | R | S | H | T | N | V | W | R | S | D | R | L | Q | A | D | V | A |
| AT3 | Q | Q | G | H | S | M | G | R | S | H | S | N | V | W | R | I | D | V | L | Q | A | D | V | A |
| AT4 | H | H | G | H | S | V | G | R | S | H | S | H | V | W | R | I | E | T | L | Q | A | D | V | A |
| AT5 | L | Q | G | Y | S | I | G | R | F | H | V | H | V | Y | R | T | F | L | A | E | A | T | H | A |

Consensus active site residues for methylmalonate- and malonate-specific AT domains are QQGHS[QMI]GRSHT[NS]V and QQGHS[LVIFAM]GR [FP]H[ANTGEDS][NHQ]V, respectively (Yadav et al., 2003). The equivalent residues in the hydroxymalonate-specific AT from the zwittermicin pathway are QQGHSIGRFHV (Chan et al., 2006; Emmert et al., 2004), and those from the niddamycin AT are QQGHSQGRGHTNV. Underlining indicates the active site.

are entirely consistent with their involvement in chondrochloren biosynthesis. Furthermore, we could confirm the identity of the cluster by insertional disruption of a gene encoding for a NRPS module.

The predicted complement of PKS and NRPS domains required to assemble the chondrochlorens matches that present in the cluster with only a few exceptions, and the genes appear to be located on the genome in the order in which they operate. Thus, the chondrochloren gene cluster, like that responsible for chondramide biosynthesis in the same strain, is one of the few from myxobacteria (Wenzel and Müller, 2007) in which there is a high degree of colinearity between the genetic architecture and the set of biosynthetic transformations. Nonetheless, there are several features of the pathway which are not evident from sequence analysis.

Biosynthesis of the chondrochlorens is likely initiated by the loading module of subunit CndA, by activation of a butyrate starter unit by the N-terminal coenzyme A ligase (CL), followed by transfer to the adjacent ACP domain. The CL domain shows strong homology to JamA (51% identity; 69% similarity), a standalone acyl-ACP synthetase domain thought to activate either 5-hexenoic acid or 5-hexynoic acid as starter unit in the pathway to the jamaicamides (Edwards et al., 2004). The presence of a CL domain is unusual, as initiation modules of modular PKS typically exhibit two alternative organizations: either a didomain AT-ACP for direct selection of acetate or propionate, or a tridomain $KS^Q$-AT-ACP, in which a carboxylated precursor (either malonate or methylmalonate) is selected by the ACP, followed by $KS^Q$-catalyzed decarboxylation to yield a typical starter unit (Bisang et al., 1999). However, the participation of a CL domain in activation and attachment of unusual starter moieties has precedent in the biosynthetic pathway to the polyketide immunosuppressant rapamycin (Schwecke et al., 1995).

Extension of the chain to the triketide stage is accomplished sequentially by module 1 of CndA, followed by the standalone modules CndB (module 2) and CndC (module 3). Extensive sequence analysis of AT domains has revealed sequence motifs which predict the specificity of the domains for the alternative extender units malonate, methylmalonate, and methoxymalonate (Haydock et al., 1995, 2005; Yadav et al., 2003). Inspection of these amino acid motifs in the $AT_1$, $AT_2$, and $AT_3$ domains (Table 3) predicts selection in each case of methylmalonyl-CoA, consistent with the observed structures of the chondrochlorens. Module 1 contains a full reductive loop (DH-ER-KR), appropriate to the level of reduction observed in the final product. In contrast, modules 2 (DH-KR) and 3 (DH) incorporate apparently superfluous reductive activities, as chain extension by these modules is expected to result in a β-hydroxy and a β-keto group, respectively. The conserved active site motif (LxxHxxxGxxxxP) characteristic of DH domains is present in $DH_2$ (Donadio and Katz, 1992), but the domain shows overall low homology to the other DH activities in the cluster, which may explain its lack of activity (Figure S5A). Alternatively, dehydration to the double bond does occur, but the functionality is subsequently rehydrated by an as yet unidentified domain. Sequence analysis of $DH_3$ also suggests that it should also be active, but the KR domain required to furnish it with β-hydroxyl substrate is absent from the module.

Construction of the remaining polyketide portion of the molecule is accomplished by CndD (module 4) and CndE (module 5). One interesting feature of the catalysis in each case is the building block used for chain extension. Both chondrochlorens A and B incorporate a methoxy functionality at the α-position established by module 4 (C-4), whereas a further methoxy is installed by module 5 in chondrochloren A (C-2). However, in chondrochloren B, an ethoxy moiety replaces the module 5 methoxy. As we anticipated that the ethoxy moiety would arise from methylation of an initial methoxy group, the extender unit used in chain extension by modules 4 and 5 was expected to be methoxymalonate. However, analysis of the gene cluster revealed only four of the five genes whose products have been shown to cooperate in the synthesis of methoxymalonyl-ACP (using FK520 nomenclature) (Wu et al., 2000): an FkbH homolog (Orf6) which binds to a glycolytic intermediate (likely 1,3-biphosphoglycerate; Wenzel et al., 2006; Dorrestein et al., 2006) and dephosphorylates it while tethering it to a standalone ACP domain (FkbJ; Orf4), forming glyceryl-ACP; an FkbK homolog (Orf3) which catalyzes the oxidation of glyceryl-ACP to 2-hydroxy-3-oxopropinyl-ACP; and an FkbI homolog (Orf5) responsible for converting 2-hydroxy-3-oxopropinyl-ACP to hydroxymalonyl-ACP. The chondrochloren gene cluster lacks a discrete homolog of FkbG (Wu et al., 2000), the expected *O*-methyltransferase, and instead an *O*-MT is present as an integral domain at the end of subunit CndE; this genetic architecture may have arisen from fusion to CndE of an ancestral discrete *O*-MT domain. Taken together, these findings support the use of hydroxymalonate instead of methoxymalonate in both modules 4 and 5, suggesting that

both ATs 4 and 5 should recruit extender units from hydroxylmalonyl-ACP (Chan et al., 2006).

We therefore anticipated that the specificity residues of ATs 4 and 5 might resemble those in the known hydroxymalonyl-ACP-specific AT domain from the niddamycin (Yadav et al., 2003) or zwittermicin PKSs (Chan et al., 2006; Emmert et al., 2004). Comparison of these residues shows that $AT_5$ exhibits convincing similarity to the zwittermicin AT, whereas $AT_4$ more closely resembles the remaining chondrochloren ATs (Table 3). Indeed, when the overall sequences are aligned, homology is greatest between $AT_5$ and the zwittermicin AT, whereas $AT_4$ clusters with the chondrochloren ATs and other domains with specificity for methylmalonate (Figure S5B). At present, we cannot reconcile the discrepancy between our biosynthetic model and the sequence analysis, but will aim in the future to directly interrogate the substrate specificity of ATs 4 and 5 through studies of the domains in recombinant form in vitro. If hydroxymalonate is used as an extender unit by module 4, however, the mechanism by which the final methoxy moiety is established becomes an issue, as module 4 lacks an *O*-MT domain. One possibility is that the *O*-MT domain in module 5 acts iteratively, carrying out *O*-methylation during chain extension by module 5 and during catalysis by the previous module. Such repetitive action of polyketide processing domains has been postulated previously to explain the absence of reductive activities in some PKS modules which appear to be required (Tang et al., 2004). Alternatively, the *O*-MT simultaneously methylates both hydroxyl groups while the intermediate is bound to the ACP of module 5.

To explain the ethoxy group of chondrochloren B, we investigated a mechanism in which an initially formed methoxy group was further methylated to yield the ethoxy moiety of chondrochloren B. Such a proposal has precedent in the biosynthesis of bacteriochlorophyll by the green sulfur bacterium *Chlorobaculum tepidum* (Gomez Maqueo Chew et al., 2007). In this pathway, the methyl group at C-2 of the chlorophyll backbone is monomethylated, whereas the ethyl group at C-8 can be modified with one or two methyl groups, ultimately giving rise to the unique side chains which characterize these compounds. The enzymes responsible for this transformation in *C. tepidum*, BchQ and BchR, have been identified as radical SAM enzymes which use SAM as a methyl donor. Members of this 600-strong superfamily are found in all three kingdoms of life, and participate in numerous biosynthetic pathways, including synthesis of thiamine, heme, molybdopterin, and nitrogenase cofactor (Marsh et al., 2004). In the case of *C. tepidum*, the need for radical chemistry is clear: the bacterium is a strict anaerobe, and therefore cannot use molecular oxygen to activate unreactive carbon centers toward the addition of methyl groups.

We reconstituted biosynthesis of chondrochloren B from chondrochloren A using cell-free extracts of a chondrochloren nonproducer, demonstrating conclusively that the ethoxy group arises from methylation of the methoxy group, and that *C. crocatus* Cmc5 contains a methylmethylase activity. Inactivation of *cndI*, the candidate methylase gene within the cluster, produced a significant decrease in the amount of chondrochloren B relative to chondrochloren A (Figure 2D), but did not abolish its biosynthesis. Furthermore, the same phenotype was observed following complementation with 3-chloro-L-tyrosine of the halogenase mutant Cmc-cndH$^-$, in which polar effects were expected to abolish transcription of *cndI* (Figure 3C). Taken together, these data strongly implicate CndI as the chondrochloren A methylase, but also suggest that *C. crocatus* contains more than one such activity. Although this result accounts for production of chondrochloren B by the strain, it remains unclear how the extent of methylation of the carboxylated precursor is controlled. Nonetheless, to our knowledge, these data constitute the first direct evidence for the involvement of a radical SAM methylase in bacterial secondary metabolism.

C-alkyl groups in several other natural products, including the hydroxyethyl functionalities of pactamycin (Kudo et al., 2007) and thienamycin (Williamson et al., 1985), have been shown by feeding studies to arise from methionine, and the corresponding clusters contain putative radical SAM enzymes (Kudo et al., 2007). It is also likely that members of this family participate in formation of additional functionalities in known natural products, including the *t*-butyl groups of the apratoxins (Luesch et al., 2001; Gutierrez et al., 2008; Matthew et al., 2008) and the bottromycins (Kaneda, 1992). Thus, methylation by radical SAM enzymes appears to be a general strategy for modification of unreactive carbon centers in natural product biosynthesis.

The terminal chloro-hydroxy-styryl moiety of the chondrochlorens was assumed to derive from tyrosine. Consistent with this expectation, the PKS genes are followed by a single NRPS gene *cndF* encoding C, A, and PCP domains, and a thioesterase to carry out chain release. Although the presence of an NRPS module accounts for incorporation of the tyrosine scaffold into the metabolites, several mechanisms can be proposed to explain the origin of the chlorine in the final products: (1) direct recruitment of chlorotyrosine from the cellular medium; (2) chlorination of a PCP-bound tyrosine, either before or after amide bond formation; and (3) chlorination of an intermediate (either carboxylated or decarboxylated) following its release by the TE.

A chlorotyrosine (or one of several derivatives) is found in a number of other metabolites of PKS and/or NRPS origin, including the cryptophycins, balhimycin, and the anabaenopeptilides. Studies on the timing of chlorination during balhimycin assembly have ruled out the participation of a free β-hydroxy chlorotyrosine, suggesting that chlorination occurs instead during the nonribosomal biosynthesis of the heptapeptide core (Puk et al., 2004). Indeed, the A domain in the associated module exhibits clear specificity for the unchlorinated β-amino acid (Recktenwald et al., 2002). Similarly, chlorination of tyrosine in the biosynthesis of the anabaenopeptilides is proposed to occur on a PCP-bound substrate (Rouhiainen et al., 2000). The requirement for substrate tethering has been shown directly in the biosynthesis of pyoluteorin (Dorrestein et al., 2005) and the enediyne C-1027 (Lin et al., 2007), where chlorination occurs, respectively, on pyrrolyl-*S*-PCP and β-tyrosyl-*S*-PCP.

Gene *cndH* exhibits convincing homology to previously described nonheme, flavin-dependent halogenases within NRPS and PKS clusters, including tryptophan and tyrosine halogenases from several glycopeptide biosynthetic pathways (Pelzer et al., 1999; van Wageningen et al., 1998; Sosio et al., 2004), as well as SgcC3, the C-1027 chlorinase (Lin et al., 2007). This major class of enzymes catalyzes formation of carbon-halogen bonds at electron-rich positions, using $FADH_2$, a halide ion (usually $Cl^-$), and molecular oxygen (Yeh

et al., 2007). Although the reduced flavin is provided by a partner flavin reductase, in common with many pathways (Yeh et al., 2005), a gene encoding for this activity could not be located within the sequenced *cnd* region. A reductase-encoding gene is also absent from the chondramide cluster (Rachid et al., 2006), suggesting that the partner for both the chondrochloren and the chondramide halogenases is located elsewhere in the genome.

To confirm involvement of CndH in the biosynthesis, we inactivated *cndH* by insertional mutagenesis. By comparison to the wild-type strain, the resulting mutants did not produce either chondrochloren A or B. This result implied that chlorination occurs before release of the intermediate from the assembly line, an expectation supported by the successful rescue of the *cndH* mutant by 3-chloro-L-tyrosine (Figure 3). To attempt to pin down further the timing of the chlorination reaction, we expressed the $A_6$ domain of CndF in recombinant form, and evaluated its substrate specificity toward a panel of amino acids, including L-tyrosine and 3-chloro-L-tyrosine, using the standard ATP/PPi exchange assay (Stachelhaus and Marahiel, 1995). Although the CndF $A_6$ domain exhibited a preference for the chloro derivative over the unchlorinated amino acid, it was not significant (100% versus 83%).

We have recently solved the crystal structure of CndH (Büdenbender et al., 2009). The structure revealed architectural features relative to the structurally characterized FAD-dependent halogenases PrnA (Dong et al., 2005) and RebH (Yeh et al., 2007), which correlate with recognition of a carrier-protein-bound tyrosine instead of the free amino acid. Indeed, we found no evidence in vitro under standard conditions that CndH can catalyze the chlorination of free tyrosine. This result, taken together with the convincing homology of CndH to SgcC3 (53% identity), makes it likely that chlorination of tyrosine in chondrochloren biosynthesis also occurs following activation of the residue as a PCP-bound species. Presumably, the tolerance of the $A_6$ domain toward 3-chloro-L-tyrosine reflects the lack of evolutionary pressure to select against this species, which is not normally available in the cell.

We hypothesized that the final step in the biosynthesis would be oxidative decarboxylation of functionalized tyrosine, to yield both chondrochlorens A and B. Inspection of the proteins encoded in the gene cluster revealed that CndG shows significant homology (31%–44% identity) to a family of putative FAD-linked oxidases identified from bacterial genomes, notably including FeeG from the fatty acid enol cluster (Brady et al., 2002). FeeG has been proposed to catalyze the decarboxylation of several long-chain *N*-acyl tyrosines, through a quinine methide intermediate. Therefore, CndG is an attractive candidate for an enzyme capable of catalyzing the decarboxylation of a highly elaborated tyrosine, suggesting that the many as yet uncharacterized members of its family may perform similar functions. Efforts to directly probe the activity of CndG by gene inactivation are currently under way in the laboratory.

## SIGNIFICANCE

**Myxobacteria are soil-dwelling microorganisms, notable for several unique behavioral features such as cellular movement by gliding and the formation of multicellular fruiting bodies. More recently, they have gained recognition as multiproducers of polyketide and nonribosomal polypeptide metabolites of potential therapeutic value. The biosynthetic pathways to these compounds are also of significant interest, as they frequently challenge established paradigms for chain assembly on modular multienzymes, as well as reveal uncommon functional group transformations. As anticipated, the deduced pathway for assembly of chondrochlorens A and B in the strain *Chondromyces crocatus* Cm c5 incorporates several unusual features, including a candidate for a novel tyrosine decarboxylase and, to our knowledge, the first example in bacterial secondary metabolism of the methylation of an unactivated carbon by a radical *S*-adenosylmethionine enzyme. The availability of the chondrochloren gene cluster sets the stage for detailed mechanistic studies in vitro of these intriguing catalysts, as well as attempts to manipulate the biological activity of the metabolites by genetic engineering.**

## EXPERIMENTAL PROCEDURES

### Materials

dNTPs, restriction enzymes, T4 DNA ligase, isopropyl-β-D-thiogalactopyranoside (IPTG), and X-gal were purchased from Fermentas, Germany. Amino acids were sourced from Merck (L-Tyr, L-Trp), Sigma (L-Met, L-Phe, 3-chloro-L-tyrosine), or Johnson Pump, UK (β-tyrosine). Ammonium persulfate, [$^{32}$P]pyrophosphate, tetrasodium pyrophosphate, perchloric acid, and charcoal were obtained from Sigma. Gel electrophoresis materials were obtained from Roth, Germany. Oligonucleotides were purchased from MWG-Biotech-AG, Germany.

### General Molecular Biological Methods

Standard methods for DNA isolation and manipulation were used (Sambrook et al., 1989; Kieser et al., 2000). DNA fragments were isolated from agarose gels using the NucleoSpin Extract gel-extraction kit (Machery-Nagel, Germany). Southern analysis of genomic DNA was performed with the DIG DNA labeling and detection kit (Roche, Germany). Hybridization was carried out with buffer containing 50% formamide at 42°C for homologous probes with stringent washing at 68°C. For heterologous probes, hybridization was performed at 37°C with stringent washing at 60°C. PCR reactions were performed with Taq DNA polymerase (Fermentas) or Pfu polymerase (Stratagene). Conditions for amplification using an Eppendorf Mastercycler were as follows: denaturation, 30 s at 95°C; annealing, 30 s at 48°C–60°C; extension, 45 s at 72°C; 30 cycles and a final extension for 10 min at 72°C. PCR products were purified using the High Pure PCR Product Purification kit (Boehringer Mannheim).

### Screening of the *C. crocatus* Cm c5 Cosmid Library for the Chondrochloren Gene Cluster

The following gene sequences were used to design degenerate primers with CODEHOP (Rose et al., 2003) for amplification of the halogenase gene (Hal-Chlst-up: 5′-GGG GCT GCA GST GGD WSA TYC CGY T-3′ and Hal-Chlst-dn: 5′-CCS STG GAT CCS CGG GTC SAB GAA GC-3′: *rebH* (DNA Data Bank of Japan, accession number AB090952; rebeccamycin biosynthesis in *Lechevalieria aerocolonigenes*); *prnC* (GenBank accession number U74493; pyrrolnitrin biosynthesis in *Pseudomonas fluorescens*); *prnA* (GenBank accession number AF161184; pyrrolnitrin biosynthesis in *Pseudomonas fluorescens*); *clohal* (GenBank accession number AF329398; clorobiocin biosynthesis in *Streptomyces roseochromogenes* subsp. *oscitans*); *bhaA* (EMBL accession number Y16952; balhimycin biosynthesis in *Amycolatopsis balhimycina*); and *tcp21* (EMBL accession number AJ605139; teicoplanin biosynthesis in *Actinoplanes teichomyceticus*). Screening was performed on a cosmid library of *C. crocatus* Cm c5, whose construction was reported previously (Rachid et al., 2006). A DIG-labeled halogenase probe approximately 270 bp in length was generated using the

oligonucleotides Hal-Trpst-up (5′-TAT CGG ATC CGG STG GAC CTG GGR ASA TYC C-3′) and Hal-Chlst-dn (5′-CCS STG GAT CCS CGG GTC SAB GAA GC-3′). Screening of the library identified seven cosmids (A:A8, B:E4, C:A22, E:I15, D:J12, B:M18, and C:L23), which were then crosshybridized with probes designed to bind to conserved regions within KS domains of PKS systems (generated using oligonucleotides KS1UP and KSD1; Beyer et al., 1999). As all seven plasmids were positive for both KS and halogenase sequences, we end-sequenced the inserts in order to identify a cosmid which contained one end of its encoded cluster. This analysis yielded cosmid B:E4, whose insert contained a PKS gene at the T3 terminus and genes with no obvious involvement in chondrochloren biosynthesis at the T7 terminus. A portion of the PKS region was then amplified from cosmid B:E4, and used to rescreen the original cosmid library for overlapping sequences. This experiment identified cosmids E:H19, A:F5, A:G6, A:M11, B:J7, C:L23, and D:I19. These cosmids were then screened with homologous NRPS probes designed to recognize the regions between the conserved A3 and A10 motifs within the NRPS adenylation (A) domains (generated using degenerate primers RevA3 (5′-CCT CCG G[GC]C C[GC]A CCG G[GC][AC] CGC C[GC]A AGG-3′) and PSLGG (5′-GCC GCC [GC]AG [GC]C[CT] GAA GAA-3′). End sequencing of the inserts in the NRPS-positive cosmids E:H19, A:G6, D:I19, and A:M11 showed that E:H19 contained one end of its encoded gene cluster. Thus, cosmids B:E4 and E:H19 together were expected to encompass the entire chondrochloren cluster. Sequencing of the cosmids was carried out on both strands, using a shotgun library containing DNA fragments approximately 1.5–2.0 kbp in length, as described previously (Silakowski et al., 1999). Briefly, sheared fragments of 3 kbp in length (GeneMachines) were subcloned separately into vector pUC19. At least 384 clones were selected from each clone library. Plasmid DNA was prepared following a standard protocol (Millipore). Cycle sequencing was performed routinely using the ABI PRISM BigDye Terminator v 3.1. Ready Reaction Cycle Sequencing kit (Applied Biosystems) and M13f/M13r primers. All reactions were analyzed on an ABI 3730XL capillary sequencer. Data were assembled and edited using the GAP4 program (Staden, 1996). All sequence similarity searches were carried out on the amino acid level in the GenBank database with the BLAST program (release 2.0). Amino acid and nucleotide sequences were aligned with the Lasergene (DNASTAR) and Vector NTI (Invitrogen) software packages.

### Inactivation of the Chondrochloren Gene Cluster

The NRPS gene within the chondrochloren cluster was targeted for inactivation by insertional disruption, using an internal fragment of its own A domain. Primers RevA3 and PSLGG were used to amplify a 1215 bp internal fragment of gene *cndF*. The PCR product was cloned into pTOPO resulting in plasmid pTOPO-E:H19, and sequenced. The cloned fragment was then excised with HindIII and EcoRV, and cloned into plasmid pSUPHyg (Rachid et al., 2006) to yield the conjugation plasmid pSR11. The plasmid was electroporated into *E. coli* strain ET12567 containing pUB307, and the resulting clones were used for conjugation of pSR11 into *C. crocatus* cm c5, as described previously (Rachid et al., 2006). Mutant strains were then grown in 50 ml MD1 medium at 30°C for isolation of chromosomal DNA. Insertion of the plasmid was verified by PCR on the mutant and wild-type strains using oligonucleotides pSUP-EV (5′-GCA TAT AGC GCT AGC AGC-3′) and RevA3. These results were further confirmed by southern blot analysis of BamHI-digested chromosomal DNA of wild-type *C. crocatus* Cm c5 and two NRPS mutants, using a DIG-labeled internal fragment (1215 bp) of the $A_6$ domain as a hybridization probe. To analyze for chondrochloren production, single exconjugants were grown in Pol03 medium containing hygromycin (100 mg/ml) at 30°C for 7 days, in the presence of 1% Adsorber-Harz (XAD 16) resin (Rohm and Haas, Germany). The XAD resin was extracted with methanol, and the extracts were analyzed using an Agilent 1100 series HPLC equipped with a photodiode array detector and coupled to a Bruker HCT plus mass spectrometer operating in positive ionization mode (scan range $m/z$ = 100–1000). A Nucleodur C18/3 μm RP column (125 × 2 mm; Machery-Nagel) was used for separation with a solvent system consisting of $H_2O$ (A) and acetonitrile (B), each containing 0.1% formic acid. The following gradient was applied: 0–2 min 5% B; 2–32 min linear from 5% to 95% B; 32–35 min isocratic at 95% B. Chondrochlorens A and B were identified by comparison to the retention times and MS data of authentic standards (chondrochloren A: retention time = 26.2 min, $m/z$ $[M+Na]^+$ = 548.2; chondrochloren B: rt = 27.9 min, $m/z$ $[M+Na]^+$ = 562.2).

### Inactivation of *orf1*, *orf2*, *cndI*, and *cndH*

Internal fragments of *orf1*, *orf2*, *cndI*, and *cndH* encoding, respectively, a putative histidine kinase, a P450, a radical SAM methyltransferase, and a halogenase were generated by PCR from chromosomal DNA of *C. crocatus* Cm c5. The following primers were used: *orf2* (His-cnd-frame-up: 5′-CTC TTG *A*TC ACC TCT GCC-3′; His-cnd-dn: 5′-GAG GGA ACT TCG AGG AG-3′); *orf3* (P450-cnd-frame-up: 5′-GAA GAC CT*G* GAC CTC GTT C-3′; P450-cnd-dn: 5′-GGA GTT CCG TAG AAA GAG C-3′); *orf4* (MT-cnd-frame-up: 5′-CTA CTA CT*G* ATG CGG TGA ACG-3′; MT-cnd-dn: 5′-GAA GAA ATA CTC CTC CCA G-3′); and the halogenase *cndH* (Cnd-Halo-frame-up: 5′-ATG AGT ACG *T*AG GCC TGA GGT G-3′; Halogenase-dn: 5′-GAA TAC GGA TCT CAC CGT AAT C-3′) (the base pair added to introduce a frame shift in each gene is indicated in italics). The resulting 951, 855, 1040, and 848 bp fragments were cloned into pCR2.1TOPO, sequenced, and then subcloned into pSUPHyg, resulting in plasmids pSR14, PSR15, PSR16, and pSR12, respectively. The plasmids were then transferred by conjugation into *C. crocatus* Cm c5, leading to gene inactivation after homologous recombination, as verified by PCR analysis. The resulting mutants Cmc-orf1$^-$, Cmc-orf2$^-$, Cmc-cndI$^-$, and Cmc-cndH$^-$ were grown in Pol03 medium supplemented with 100 μg/ml hygromycin at 30°C for 7 days, in the presence of 1% XAD 16 resin. Methanolic extracts of the resin were then analyzed by HPLC-MS for the production of chondrochlorens A and B. Each analysis was performed in triplicate or quadruplicate. The amount of chondrochlorens A and B was quantified using the characteristic fragment ion ($m/z$ $[M+H]^+$ = 480.1).

### Complementation of the Halogenase Gene *cndH*

3-chloro-L-tyrosine (Aldrich) was added to a 100 ml culture of the *cndH* mutant and in equal portions (final concentration 1 mM) at 24, 48, and 72 hr after inoculation. A culture of unsupplemented Cmc-cndH$^-$ was grown in parallel. XAD 16 resin (1% [v/v]) was added, and then the cultures were grown for a further 7 days at 30°C. The methanolic extracts of the resin were then analyzed by HPLC-MS, as described above.

### Expression, Purification, and Enzymatic Analysis of the NRPS $A_6$ Domain

The $A_6$ domain was amplified from chromosomal DNA of *C. crocatus* Cm c5 using primers Chondro-Adom-up (5′-*GGA TCC* ATG CTC TTC GAC TCC GCG-3′) and Chondro-Adom-dn (5′-*GAA TTC* AGA GGA GAT CTT CCC AG-3′) (the introduced BamHI and EcoRI sites are indicated in italics). The resulting 1786 bp fragment was cloned into pCR2.1TOPO (Invitrogen) for sequencing, and then subcloned into expression vector pGEX-6P-1 (GE Biosciences). Plasmid pGEX-Cnd-Ad was then transformed into *E. coli* BL21 cells. The cells were grown in LB medium containing 100 μg/ml ampicillin at 30°C until an $OD_{600}$ of 0.6–0.8 was reached, and then protein expression was induced with 0.1 mM IPTG. Growth was then continued at 20°C for 8 hr. Cells were disrupted by French press into 1× phosphate buffered saline (PBS) (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ [pH 7.3], 140 mM NaCl, 2.7 mM KCl), and cell debris was removed by centrifugation at 25,000 × g. The supernatant was bound to a MicroSpin GST purification module (GE Biosciences), and then the pure $A_6$ domain was released by cleavage with PreScission Protease (GE Biosciences) at 4°C in cleavage buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT), according to the manufacturer's instructions. The molecular weight of the protein was confirmed by MALDI mass spectrometry. ATP-PPi exchange assays were performed in a 100 μl total volume, containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM dATP, 150 nM $A_6$ domain, 1 mM [$^{32}$P]pyrophosphate (0.5 μCi), and 2 mM of 3-chloro-L-tyrosine, L-tyrosine, β-tyrosine, L-tryptophan, L-phenylalanine, or L-methionine. In these assays, dATP was used instead of ATP, as this substrate produced a lower background signal (Eppelmann et al., 2001). The reactions were carried out for 15 min at 25°C after addition of the purified $A_6$ domain, and then quenched by addition of a charcoal-tetrasodium pyrophosphate-perchloric acid mixture (1.6% [w/v] activated charcoal, 4.46% [w/v] tetrasodium pyrophosphate, 3.5% perchloric acid in water). The charcoal was pelleted by centrifugation, washed twice with the quenching mixture (without charcoal), and then resuspended in 0.5 ml water and analyzed by liquid scintillation counting on a liquid scintillation analyzer (Packard Biosciences). The reactions were typically performed in triplicate.

 

### Expression of the Chondrochloren Halogenase CndH

Gene *cndH* was amplified by PCR using chromosomal DNA of *C. crocatus* Cm c5 and primers Cnd-Hal-up (5′-CGG *GAT CC*A TGA GTA CGA GGC CTG AGG TG-3′) and Cnd-Hal-dn (5′-*GAA TTC* AGC GCC GGG CGC TCG GCG CGC TA-3′) (the introduced BamHI and EcoRI sites are indicated in italics). The resulting 1573 bp fragment was cloned into pCR2.1 TOPO for sequencing, and then subcloned into expression vector pGEX-6P-1. Plasmid pGEX-CndH was transformed into *E. coli* BL21 cells. Recombinant *E. coli* BL21 cells containing pGEX-CndH were grown in LB medium supplemented with 100 μg/ml ampicillin at 30°C until an $OD_{600}$ of 0.6–0.8 was reached. Protein expression was then induced with 0.5 mM IPTG, and the culture was further incubated at 30°C for 4 hr. Cells were disrupted by French press into 1× PBS (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ [pH 7.3], 140 mM NaCl, 2.7 mM KCl), and cell debris was removed by centrifugation at 25,000 × g. The supernatant was bound to a MicroSpin GST purification module and then the purified protein was released by cleavage with PreScission Protease at 4°C in cleavage buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT), according to the manufacturer's instructions. Ten percent glycerol was added, and the sample was stored at −80°C.

### Expression and Analysis of the *E. coli* Flavin Oxidoreductase Fre

Gene *fre* from *E. coli* K12 was amplified by PCR using the oligonucleotides Fre-BamHI-up (5′-CGG *GAT CC*A TGA CAA CCT TAA GCT G-3′) and Fre-EcoRI-dn (5′-G*GA ATT* CAG ATA AAT GCA AAC GC-3′) (the introduced BamHI and EcoRI sites are indicated in italics). The resulting 715 bp fragment was cloned into pCR2.1 TOPO (Invitrogen) for sequence analysis, and then into the expression vector pGEX-6P-1 (GE Biosciences). pGEX-fre was then transformed into *E. coli* BL21 cells, and cells containing plasmid were grown in LB medium supplemented with 100 μg/ml ampicillin at 30°C until an $OD_{600}$ of 0.6–0.8 was reached. Protein expression was induced with 0.5 mM IPTG, and the culture was incubated for a further 4 hr at 30°C. Protein purification was performed using a MicroSpin GST purification module, and the pure Fre protein was released by cleavage with PreScission Protease at 4°C in cleavage buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT), according to the manufacturer's instructions. Alternatively, the GST fusion protein was released from the resin using reduced glutathione (15 mM).

Fre-catalyzed oxidation of NADH was monitored spectrophotometrically by measuring the decrease in absorbance at 340 nm ($\Delta\varepsilon_{340} = -6220\ M^{-1}, cm^{-1}$), in 10 mM potassium phosphate buffer (pH 7.0) containing 35 pmol Fre, 20 μM FAD, and 300 μM NADH, at room temperature. The assay was also carried out using an equivalent amount of GST-Fre, and a control was performed in the absence of enzyme.

### Enzymatic Analysis of CndH

Analysis of the activity of CndH toward free tyrosine was performed according to the literature precedent (Zehner et al., 2005). Briefly, 100 μl of purified CndH (0.25 nmol) was added to a mixture containing 70 pmol Fre, 10 μM FAD, 2.4 mM NADH, 25 mM NaCl, and 0.6 mM L-tyrosine in 10 mM potassium phosphate buffer (pH 7.2) in a total volume of 200 μl. The mixture was incubated at 30°C for 6 hr, and then the reaction was stopped by boiling in a water bath for 5 min. Precipitated protein was removed by centrifugation, and then a 50 μl aliquot of the assay was derivatized with 200 μl OPA-NAC reagent (Fluka) (Buck and Krummen, 1987) and incubated for 10 min at room temperature prior to analysis by HPLC-MS. The mobile phase consisted of 50 mM sodium phosphate (pH 6.5) (buffer A) and a 35:65 mixture of phosphate buffer and methanol containing 5% tetrahydrofuran (buffer B). The analysis was performed using a linear gradient from 35% to 40% B over 24 min on a Nucleodur C18 column (5 μm particle size, 125 × 2 mm) (Berner et al., 2006). Separation of the OPA-NAC derivatives of commercially available 3-chloro-tyrosine (Sigma) was used as control.

## SUPPLEMENTAL DATA

Supplemental Data include five figures and one table and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00451-1.

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