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Sequence-based classification of type II polyketide synthase biosynthetic gene clusters for antiSMASH

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Abstract

The software antiSMASH examines microbial genome data to identify and analyze biosynthetic gene clusters for a wide range of natural products. So far, type II polyketide synthase (PKS) gene clusters could only be identified, but no detailed predictions for type II PKS gene clusters could be provided. In this study, an antiSMASH module for analyzing type II PKS gene clusters has been developed. The module detects genes/proteins in the type II PKS gene cluster involved with polyketide biosynthesis and is able to make predictions about the aromatic polyketide product. Predictions include the putative starter unit, the number of malonyl elongations during polyketide biosynthesis, the putative class and the molecular weight of the product. Furthermore, putative cyclization patterns are predicted. The accuracy of the predictions generated with the new PKSII antiSMASH module was evaluated using a leave-one-out cross validation. The prediction module is available in antiSMASH version 5 at <https://antismash.secondarymetabolites.org>.

Keywords Type II polyketide synthases · PKS · Aromatic polyketides · Secondary metabolite · Natural product · Genome mining

Introduction

Natural products are of huge interest due to their diverse bioactivities, and many are harnessed as medical drugs. New secondary metabolites are continuously discovered in bacteria, fungi and plants and still constitute the most important drug discovery resource, especially for antibiotics [23]. In the last decade, genome mining has been established as a complementary technology to “classical” approaches to

identify novel compounds [22, 37] that is routinely used by academic and industrial research teams.

The antibiotics and secondary metabolite analysis shell (antiSMASH) is one of the leading and most widely used secondary metabolite biosynthetic gene cluster (BGC) detection tools first introduced in 2011 and continuously developed and expanded since then [2, 4, 20, 32]. With antiSMASH’s rule-based approach, the software can identify gene clusters responsible for the biosynthesis of 47 different classes of secondary metabolites including thiopeptides, lanthipeptides, lassopeptides, non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS) and many more. For lanthi-, thio-, sacti- and lassopeptides, terpenes, NRPSs, and some PKSs, antiSMASH can also provide very detailed annotations and predictions on putative biosynthetic products. Of these, NRPS and PKS BGCs are the most well studied due to their products’ interesting applications as antibiotics, immunosuppressants and anticancer drugs [6, 15]. For NRPS and modular type I PKS pathways, which rely on the principle of an assembly-line biosynthesis, the domain composition of the PKS and NRPS megasynthase enzymes is used to predict putative PKS/NRPS precursors purely based on DNA/protein sequence information.

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Type II PKS gene clusters, some of which are responsible for the biosynthesis of the antibiotics tetracycline [25] and the anti-cancer drug daunorubicin [24], can be detected with antiSMASH, but no deeper analysis of these gene clusters is carried out in the current version of antiSMASH (version 4).

Several type II PKS pathways, such as those responsible for the biosynthesis of actinorhodin or tetracycline have been extensively studied [12, 34]. Key components of any PKS II pathway are a “minimal PKS”: A ketosynthase (KS/KS α) catalyzes the Claisen condensation of the extender units. It closely interacts with the chain length factor (CLF/KS β), which contributes to determining the polyketide chain length [31], and an acyl carrier protein (ACP), which provides a phosphopantetheine co-factor to bind the intermediates. The minimal PKS is responsible for assembling the polyketide backbone. Ketoreductases (KR), and cyclase/aromatase (CYC) subsequently are involved in “shaping” the molecule and leading to the characteristic polycyclic structures [13].

For some pathways, additional functions are required. Polyketides that use starter units other than acetyl-CoA often use ketosynthase III (KSIII), acyltransferases (AT) or acyl-Coenzyme A ligase (LIG) to load the starter units to the PKSII enzyme complex. Furthermore, auxiliary enzymes often are involved in tailoring steps, such as methyltransferase (MET) and glycosyltransferase (GT) that add methyl groups or sugars to the polyketide scaffold. In addition, a few other enzymes have been found in association with polyketide biosynthesis in some type II PKS gene clusters. Amidotransferases (AMID) are responsible for generating the alternative starter unit malonamyl-CoA in the oxytetracycline cluster [26]. Aminotransferases (AMIN), as found in the chelocardin and oxytetracycline clusters, attach amino groups to the polyketide [19, 26]. Halogenases (HAL) attach chlorine to the polyketide, as found in AZ154 [9], chlortetracycline [36], and lysolipin [18] BGCs. Finally, dimerases (DIMER), as encoded in the actinorhodin [10] BGC, catalyze a dimerization between two polyketide products forming a heterodimer.

This study extends antiSMASH to improve the predictive capabilities of the software concerning type II PKS gene clusters. Based on in silico analyses of the genes encoding the above-mentioned enzymes, the module can make predictions about the number of malonyl elongations during polyketide biosynthesis, the starter unit utilized when priming the PKS, the product class as one of the classes described by Zhou et al. [35], and finally provides suggestions on the putative molecular weight of the polyketide core-structure.

To assess the general prediction performance of the module a leave-one-out cross-validation has been performed. For every cluster, a predictive model was developed based on all other clusters, and it was used to make a prediction regarding the left-out cluster. This resulted in a prediction for every

cluster made from a predictive model developed independently from the cluster being predicted.

Methods

Type II PKS gene cluster dataset construction

A defined dataset of 89 type II PKS BGCs with knowledge of the encoded biosynthetic pathways has been constructed in this study. It served as the foundation on which the module was developed. The clusters were collected from several sources, such as secondary metabolite databases (MIBiG [21], DoBiscuit [14], IMG/ABC [11]), literature regarding other type II PKS prediction tools [29] and the recent literature concerning newly identified clusters [16, 33]. Sequences of enzymes involved in polyketide biosynthesis were extracted from the BGCs and assembled in FASTA files, one for each type of protein. Annotations were added to each protein individually regarding its cluster of origin, product, gene name and function (Table S1 and S2).

Detection of proteins involved with polyketide biosynthesis

A profile hidden Markov model (pHMM) was built for each protein type known to be involved with polyketide biosynthesis in type II PKS clusters. Protein sequences for each protein type were aligned using MUSCLE 3.8.31 [8], and a pHMM was built based on the alignment utilizing HMMER 3.1b2 [7]. To reduce false positive hits of the pHMMs, two trusted cut-off bitscores were computed for each pHMM, one for hits and one for domains, using the formula:

$$TC = \min(\text{bitscores}) - c \cdot \text{abs}(\min(\text{bitscores}))$$

where bitscores is a list of resulting bitscores of hits or domains from the given pHMM trusted cut-offs wish to be computed, run on all protein sequences on which it is based. For pHMMs based on more than five protein sequences c was set to 0.2, otherwise 0.5. The increased c value for pHMMs based on five or less protein sequences was set to counteract overfitting of the pHMM due to the small number of protein sequences. All pHMMs and their trusted cut-off bitscores are shown in Table S3. A validation of the pHMMs for protein detection showed good precision and recall, see Figure S1 of the supplementary material.

Identification of specific protein function

Profile HMMs were also built for identifying the specific function of CLFs, CYCs, KRs and METs. Trees were constructed based on the multiple sequence alignment of each protein type using FastTree 2.1.10 [27]. Each tree was

visualized, and clades were defined and manually assigned to groups of tentative protein function (Figures S2–5). The protein sequences of each clade group were extracted and aligned using MUSCLE 3.8.31. An individual pHMM was built for each clade group based on the alignment with HMMER 3.1b2. Two trusted cut-off bitscores were also computed for each pHMM to reduce false positive hits. Information about the pHMMs can be seen in the supplementary material (Table S4). The protein function pHMMs were validated in a similar manner as the protein detection pHMMs (Figure S6).

For the starter unit primers KSIII, AT, AMID and LIG, protein BLAST databases were created utilizing BLAST+ 2.7.1 [5], as too few protein sequences were available for pHMMs to be applicable. Validations of the protein BLAST databases were not performed here, but were indirectly assessed in the leave-one-out cross-validation.

Aromatic polyketide product predictions

The antiSMASH module utilizes the pHMMs and protein BLAST databases to make predictions about the product of a type II PKS BGC. The number of malonyl elongations during polyketide biosynthesis is predicted based on the CLF(s) found in the cluster. Starter unit(s) are predicted based on protein BLAST hits. Protein BLAST hits of proteins associated with the same starter unit priming are combined by summing their bitscores and keeping the lowest *E* value. If no BLAST hits are found, the starter unit is assumed to be acetyl-CoA with a bitscore and *E* value of zero. The resulting prediction is a list of the starter unit(s) and their associated bitscore and *E* value. The product class is predicted based on CLF(s) and CYC(s) pHMM hits. Each specific pHMM hit is associated with a set of product classes (Table S5). A list of sets of potential product classes is generated from the pHMM hits and the intersection of the sets is the predicted product class(es).

The molecular weight is estimated by taking into account the predicted starter unit(s), the number of malonyl elongations during biosynthesis, and pHMM hits of enzymes known to alter the molecular weight of the polyketide product (Tables S6 and S7). If ambiguous starter units and/or multiple possible malonyl elongations are predicted, a molecular weight is computed for every combination. The molecular weight is computed using the formula:

$$MW = mw_{\text{starter unit}} + n \cdot mw_{\text{malonyl ext.}} + \sum_p a_p,$$

where *mw* is the molecular weight of a given moiety, *n* is the number of malonyl elongations, *p* is a list of all proteins found in the cluster known to alter polyketide weight, and *a* is the polyketide weight alteration of a given protein.

Evaluation of module performance

The module's performance has been evaluated using leave-one-out cross-validation: For each cluster, a set of pHMMs and protein BLAST databases were created based on all other clusters except the one to test, i.e. the training data-set was created independently from the cluster. The antiSMASH module then is used to annotate the gene cluster based on the set of pHMMs and protein BLAST databases devoid of the entries of the cluster to be analyzed. In the end, a collection of predictions for each BGC was available based on a prediction model developed independently from the cluster. As defining and grouping clades in the trees could not be done entirely independently from the cluster, a suitable method for automation was not found. Thus, the clade groups based on the trees of all clusters were used. The results of the leave-one-out cross-validation were compared to the actual cluster information to assess the general performance of the antiSMASH module.

Results and discussion

Analyzing PKS II gene clusters with the new PKSII module of antiSMASH

The PKSII analysis module is fully integrated in version 5 of antiSMASH (released 12/2018). The module is available in both the downloaded version and online at <https://antismash.secondarymetabolites.org/>. The source code of antiSMASH 5, including the PKSII module (named t2pks) is available at <https://github.com/antismash/antismash>.

When antiSMASH identifies a PKSII BGC, the new module is triggered and used to provide further information on the identified BGC (Fig. 1). The detailed PKSII predictions include putative starter units, the number of elongation steps, the possible product classes, and the molecular weight estimations. Additionally, the predicted cyclization patterns are annotated on the identified cyclases or ketoreductases. A complete documentation of all detectable features is included within the antiSMASH documentation system at <https://docs.antismash.secondarymetabolites.org/modules/t2pks/>.

For programmatic access to the annotation, antiSMASH version 5 introduced a new JSON data structure, which contains all the information derived from this (and also all other) antiSMASH module. An excerpt of a type II PKS JSON object, obtained by analyzing the lysolipin BGC [18], is included in §13 of the Supporting Information.

Type II PKS gene cluster prediction details

Cluster section:

5496473 to 5567376

Starter unit(s):

acetyl-CoA (Score: 0.0; E-value: 0)

Malonyl elongations:

7 (Score: 743.5; E-value: 1.2e-226)

Product class(es):

benzoisochromanonequinone

Product molecular weight(s):

acetyl-CoA_7: 342.38 Da

SCO5072

KR (Score: 159.4; E-value: 3.1e-49)

SCO5079

DIMER (Score: 426.8; E-value: 8.7e-131)

SCO5080

OXY (Score: 44.7; E-value: 2.1e-14)

SCO5086

KR C9 (Score: 438.4; E-value: 1.9e-134)

SCO5087

KS (Score: 768.6; E-value: 3.5e-234)

SCO5088

CLF 7 (Score: 743.5; E-value: 1.2e-226)

SCO5089

ACP (Score: 114.2; E-value: 5e-36)

SCO5090

CYC C7-C12 (Score: 404.0; E-value: 7.8e-124)

SCO5091

CYC C5-C14 (Score: 467.3; E-value: 4.4e-143)

SCO5094

MET (Score: 32.7; E-value: 9.8e-11)

SCO5097

KR (Score: 129.8; E-value: 3.3e-40)

Fig. 1 Screenshot of the type II PKS prediction model output panel in the antiSMASH5 results view. The example shows the analysis of the actinorhodin BGC of *Streptomyces coelicolor* A3(2) (NCBI RefSeq-ID: NC_003888). The output contains information on putative starter unit(s), number of malonyl-CoA elongations, putative product class(es) and a rough prediction of the putative molecular weight. Furthermore, classifications on the individual enzymes, which the above predictions are based on, are displayed. For a description of these classifications, please see the antiSMASH online documentation at <https://docs.antismash.secondarymetabolites.org/modules/t2pks/>

Detection of PKSII features

antiSMASH uses a rule-based detection algorithm to identify clusters based on core biosynthetic enzymes. When antiSMASH identifies a hit against its KS α and KS β

profiles within 20 kbp of each other it predicts the presence of a type II PKS cluster. As a next step in type II PKS analysis, all genes in the neighborhood of the KS α and KS β hits are analyzed to provide a more detailed prediction of the cluster product. As the biosynthetic enzyme sequences cluster according to their specific activity, we have used these clusters to build activity-specific pHMMs in addition to more generic pHMMs for a general classification. Genes with translations matching the KSIII, AT, AMID, and LIG profiles are then compared to a BLAST database of similar enzymes with known starter unit specificities to predict the potential starter units of the cluster product. Depending on which activity-specific CLF profile matched best, the number of chain elongation steps is predicted. The same CLF prediction, along with the identified CYC matches, is used to predict the product class(es). Based on the predicted starter unit, the number of elongation steps, and further tailoring enzymes identified in the cluster, an estimate of the molecular weight of the product is generated.

Validation of starter unit predictions

Most type II PKS pathways that have been characterized so far use acetyl-CoA as starter units, with only a few other starter units described. A challenge in the computational prediction of such units is the very small and unbalanced dataset for some of the BGCs. Of the 12 starter units which have been described so far, four have only been identified in a single cluster making it impossible to validate them using our leave-one-out cross-validation. In fact, only propionyl-CoA and acetyl-CoA are present in more than five clusters of our 82 BGC test set with known starter units (Table S2). The 82 BGCs utilize 86 different starter units, due to a starter unit promiscuity in the frenolicin (acetyl- and butyryl-CoA) and R118 (acetyl-, propionyl-, isobutyryl-, and butyryl-CoA) biosynthetic pathways. After excluding the four starter units that can't be validated, 60 out of 82 starter units were predicted correctly in the leave-one-out validation (73%, Table 1). It is also noteworthy that the predictions for starter units utilized in only two clusters are suboptimal, again due to the limitations of cross validation on small datasets. If a starter unit is utilized by more than three clusters, then the amount of correct predictions increases. The prediction accuracy for this sub-group of better represented starter units is 77%. As acetyl-CoA is predicted as a starter unit for a BGC only if no genes coding for proteins associated with alternative starter unit priming are found, it is occasionally not predicted despite being the correct starter unit. Once more PKSII clusters have been described, the predictions can be improved without having to change the prediction method.

Table 1 Starter unit predictions of the type II PKS module from leave-one-out cross-validation

Starter unit (–CoA)	Number <i>n</i>	Predicted							
		2-meth-ylbutyryl	Acetyl	Butyryl	Glycyl	Hexadienyl	Isobutyryl	Malonamyl	Propionyl
True									
2-Methylbutyryl	2	<i>1</i>	0	0	0	1	1	1	1
Acetyl	52	0	<i>39</i>	3	0	0	1	10	4
Butyryl	4	1	0	<i>3</i>	0	0	0	1	2
Glycyl	2	0	2	0	<i>0</i>	0	0	0	0
Hexadienyl	3	1	0	0	0	2	0	2	1
Isobutyryl	2	1	0	1	0	0	<i>0</i>	1	1
Malonamyl	5	0	1	1	0	0	1	5	3
Propionyl	12	0	1	1	0	0	0	1	<i>10</i>
Total	83	4	43	9	0	3	4	21	22

The *n*-column shows the number of clusters utilizing each starter unit. Each row represents a starter unit and its predictions. Cells containing correct predictions are highlighted in italics. The rightmost column contains the total number of correct predictions. The sum of predicted starter units is greater than total *n* due to some clusters having multiple starter unit predictions

Table 2 Predictions from the type II PKS module of the number of malonyl elongations during polyketide biosynthesis

Number of malonyl elongations	Number <i>n</i>	Predicted			
		7	8 9	11 12	
True					
2	1	0	0	1	
7	11	<i>10</i>	1	0	
8	5	0	<i>5</i>	0	
9	50	0	<i>45</i>	0	
11	5	0	0	<i>3</i>	
12	11	0	0	<i>11</i>	
Total	83	10	51	15	74

The *n*-column shows the number of clusters performing the defined number of malonyl elongations. Each row represents a number of malonyl elongations during biosynthesis and its predictions. Cells containing correct predictions are highlighted in italics. The rightmost column contains the total number of correct predictions

Predictions of the number of malonyl elongations during biosynthesis

In a clustering analysis, CLF sequences group into clusters according to the number of catalytic elongation cycles the PKS carries out. Therefore, the putative chain length of an unknown PKS can be predicted by assigning it to one of these clusters.

A classification can be made for CLFs involved in PKSs with 7, 8 or 9 and 11 or 12 elongations due to the clustering observed in the cluster tree. In cross validations (Table 2), predictions for 74 of the 83 clusters are correct, corresponding to 89%. The CLF AuaD of the unique aurachin PKSII [28] which catalyzes the condensation of anthranilic acid

with 2 malonyl-CoA, is classified incorrectly. There are also two incorrect predictions of clusters performing a common 11 malonyl elongations during biosynthesis. The explanation for these lies within the clustering of the cluster tree where the two CLFs from these clusters are not located in the clade group associated with 11 or 12 malonyl elongations. The same explains the 5 incorrect predictions for clusters where 9 malonyl elongations are performed during biosynthesis.

Aromatic polyketide product class predictions

The class of the putative product is defined by the presence or absence of specific CYCs or CLFs. Lists of potential product classes for a cluster are generated based on the classification of the CYCs and CLFs identified in the cluster. The intersection of the lists obtained for the CYC and CLF prediction is used to predict the product class.

The product class for 61 of the 79 BGCs is predicted correctly (Table 3), corresponding to 77%. Excluding clusters with a unique class from the validation set increases the prediction accuracy to 95% (61/64). It is important to note that a product class prediction can have multiple product classes. The product classes tetracycline, aureolic acid and anthracyclone are often predicted together due to their similar cyclization patterns and chain lengths. When more information about the tailoring steps leading to these different product classes becomes available, it will be possible to significantly improve the detail of the product class predictions.

Aromatic polyketide molecular weight predictions

For validating the molecular weight predictions, every predicted molecular weight of the product for a cluster was

Table 3 Product class predictions from the type II PKS module

Product class	<i>n</i>	Predicted						
		Angucycline	Anthracycline	Aureolic acid	Benzoisochromanone	Pentagonal polyphenol	Tetracycline	Unknown
True								
Angucycline	22	21	4	4	0	0	4	1
Anthracycline	11	2	10	6	0	1	6	0
Aureolic acid	2	0	2	2	0	0	2	0
Benzoisochromanone	7	0	0	0	6	0	0	1
Pentagonal polyphenol	10	0	0	0	0	10	0	0
Tetracycline	5	1	1	1	0	2	1	0
Unique	15	4	3	3	3	5	3	3
Total	79 (64)	29	27	23	9	18	23	5
								61

The *n*-column shows the number of clusters having a particular product class and its predictions. Cells containing correct predictions are highlighted in italics. The rightmost column contains the total number of correct predictions. The sum of predicted product classes is greater than total *n* due to some clusters having multiple product class predictions

compared to every molecular weight of its known products, and only the closest pair of a predicted and a known product molecular weights was considered in the evaluation. The pairs showed a clear linear correlation with a Pearson correlation coefficient of 0.785. However, a trend of overestimating the molecular weight of smaller ($MW < \sim 630$) and underestimating the molecular weight of larger ($MW > \sim 630$) compounds was observed (Figure S7). This is due to the fact that the molecular weight prediction cannot take all tailoring reactions into account, such as further reductions, oxidations, ring-rearrangements caused by Baeyer–Villiger oxidations or subsequent dimerizations of the polyketide precursor molecule. Although with the currently available methods only rough predictions of the putative molecular weights can be generated, this information may be helpful for mapping analytic/mass spectrometric data of a strain to specific BGCs in the genome.

Conclusion

So far, only very few tools exist to analyze and annotate type II PKS BGCs. While PKSII gene clusters can be identified by the common genome mining pipelines, such as antiSMASH v4 [4] or PRISM [30], no further information is extracted from the sequence data. To the best of our knowledge, the only other tool dedicated to PKSII analysis is PKMiner [17], a closed-source dedicated web-based PKSII genome mining tool and PKSII BGC database.

The new type II PKS gene cluster analysis antiSMASH module is able to identify and annotate genes/proteins involved in polyketide biosynthesis. Analysis of these data allows to make predictions about the starter unit priming the PKS, the number of malonyl elongations during iterative polyketide biosynthesis, the product class and a rough estimation of the putative molecular weight range of the aromatic polyketide precursor. All this information is directly integrated in the antiSMASH framework and the annotations generated with this module will also be incorporated in the next version (v3) of the antiSMASH database [1, 3]. In addition to RiPPs, NRPS and modular type I PKS gene clusters, with this module antiSMASH now capable of providing detailed information on type II PKS BGCs, which hopefully will aid scientists in finding novel aromatic polyketides with beneficial bioactivities.

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