J Ind Microbiol Biotechnol (2016) 43:277–291
DOI 10.1007/s10295-015-1685-7

NATURAL PRODUCTS

Identification and activation of novel biosynthetic gene clusters by genome mining in the kirromycin producer *Streptomyces collinus* Tü 365

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Received: 16 July 2015 / Accepted: 9 September 2015 / Published online: 3 October 2015

Abstract Streptomycetes are prolific sources of novel biologically active secondary metabolites with pharmaceutical potential. *S. collinus* Tü 365 is a *Streptomyces* strain, isolated 1972 from Kouroussa (Guinea). It is best known as producer of the antibiotic kirromycin, an inhibitor of the protein biosynthesis interacting with elongation factor EF-Tu. Genome Mining revealed 32 gene clusters encoding the biosynthesis of diverse secondary metabolites in the genome of *Streptomyces collinus* Tü 365, indicating an enormous biosynthetic potential of this strain. The structural diversity of secondary metabolisms predicted for *S. collinus* Tü 365 includes PKS, NRPS, PKS-NRPS hybrids, a lanthipeptide, terpenes and siderophores. While some of these gene clusters were found to contain genes related to known secondary metabolites, which also could be detected in HPLC–MS analyses, most of the uncharacterized gene clusters are not expressed under standard laboratory conditions. With this study we aimed to characterize the genome information of *S. collinus* Tü 365 to make use of gene clusters, which previously have not been described for this strain. We were able to connect the gene clusters of a lanthipeptide, a carotenoid, five terpenoid compounds, an ectoine, a siderophore and a spore pigment-associated gene cluster to their respective biosynthesis products.

Keywords Genome mining · *Streptomyces* · Natural products · Secondary metabolites · Isorenieratene · Deoxydehydrochorismic acid

Special Issue: Natural Product Discovery and Development in the Genomic Era. Dedicated to Professor Satoshi Ōmura for his numerous contributions to the field of natural products.

We would like to dedicate this publication to Alfred Pühler on the occasion of his 75th birthday.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-015-1685-7) contains supplementary material, which is available to authorized users.

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Introduction

Members of the genus *Streptomyces* are ubiquitous gram-positive soil bacteria of the order *Actinomycetales.* They contain DNA with a high G+C-content (over 70 %) and exhibit a complex life cycle by forming filamentous mycelia and sporulating aerial hyphae [10]. Besides their role in soil ecology, streptomycetes are of particular significance because of their capability to produce natural compounds with a diverse set of bioactivities, including antibiotic, antitumor, antiviral, antifungal, antihypertensive and immunosuppressant properties. About 75 % of all known antibiotics are produced by actinomycetes with the major contribution of the genus *Streptomyces* [14].

The availability of genome sequences of a large number of microorganisms opened a new area of research by genome mining. Genome mining is defined as the process of technically translating secondary metabolite-encoding gene sequence data into purified molecules in tubes [3].

That *Streptomyces* are an unexhausted source of natural compounds was highlighted by the identification of 25 and 38 biosynthetic secondary metabolite gene clusters after the complete genome sequences of *S. coelicolor* and *S. avermitilis* [4, 19], respectively, became available. Bioinformatics software such as antiSMASH [5, 30, 56], a platform for automated genome mining of secondary metabolite producers, allows the rapid genome-wide identification, analysis and annotation of biosynthesis gene clusters and the identification of similar characterized gene clusters in the MIBiG repository [31]. Another applicable web tool, the Natural Product Domain Seeker (NaPDoS), provides a rapid method to assess secondary metabolite biosynthetic gene diversity, based on the phylogenetic relationships of sequence tags derived from polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes [66].

Although biosynthetic genes are encoded in the producers' genomes, they are often not expressed under laboratory conditions. Yet, there are techniques available in order to successfully activate such silent gene clusters, optimize production yields and manipulate biosynthesis pathways [57]. One approach is to "awaken" pathways in the native host strain by insertion of additional promoters upstream of the biosynthesis genes, which are either independently regulated or constitutively expressed. Otherwise, expression of gene clusters in heterologous hosts can lead to expression and biosynthesis of the products and thus represents a promising alternative for activation of secondary metabolite gene clusters present in less amenable strains.

Streptomyces collinus Tü 365 has been described as the producer of the antibiotic kirromycin, a narrow-spectrum antibiotic which has activity against some bacterial pathogens and the malaria parasite *Plasmodium falciparum* [60]. Its biosynthesis has been analyzed in great detail in previous studies [35, 58].

Here, we provide an extended analysis of the potential of *S. collinus* Tü 365 to synthesize further secondary metabolites.

Genome mining of *S. collinus* Tü 365

Recently, the genome of *S. collinus* Tü 365 was sequenced using a combination of Sanger, 454 and illumina technology (Genbank ID: CP006259) [43]. *S. collinus* Tü 365 has a linear chromosome and contains two linear plasmids, SCO1 (85,041 bp, 68.3 % G+C), and SCO2 (19,314 bp, 69.9 % G+C). The total genome size is 8.37 Mb with a G+C-content of 72.6 %. The genome is predicted to encode 7193 proteins. A long terminal inverted repeat was found at both ends of the chromosome. Its size was determined to be 630 kb [43]. Genome sequencing indicated that both ends have identical sequences.

An analysis with antiSMASH using the standard cluster rule based approach resulted in the identification of a range of different natural product classes in *S. collinus* Tü 365 (Table 1). About 14 % of *S. collinus* Tü 365 genome is dedicated to secondary metabolism. The secondary metabolite gene clusters are distributed uniformly across the chromosome (Fig. 1). In total, 32 putative biosynthetic gene clusters could be identified (Table 1). Interestingly, three clusters (a lanthipeptide, an atypical NRPS cluster, and the kirromycin biosynthetic gene cluster) are situated on the long terminal inverted repeats of the *S. collinus* Tü 365 chromosome, and thus are present in two identical copies and gene organizations. Four clusters of *S. collinus* Tü 365 encode the biosynthesis of polyketide synthase (PKS) compounds (two type I, one type II PKS and one type III PKS cluster) and four clusters encode the synthesis of non-ribosomal peptides. Five clusters code for the biosynthesis of hybrid PKS/NRPS compounds. The modular PKS and NRPS are enzyme assembly lines for the synthesis of chemically diverse natural products. The knowledge on well-characterized PKS and NRPS clusters can be used to predict the encoded secondary metabolites. In *S. collinus* Tü 365, out of the 13 PKS, NRPS and hybrid clusters, 10 have no significant similarities to characterized pathways and thus either code for known molecules, whose biosynthetic pathways were not yet associated or new compounds that have not been described before. One of the duplicated NRPS/PKS hybrid clusters is known to encode the synthesis of kirromycin. Cluster 16, which codes for a type II PKS, shows strong similarity to the spore pigment cluster of *S. coelicolor* A3(2) [13].

Among the 32 identified clusters, six biosynthetic gene clusters are predicted to be involved in the biosynthesis of terpene compounds (Table 1) widely distributed among streptomycetes. The terpene biosynthetic gene clusters predicted from *S. collinus* Tü 365 seem to direct the biosynthesis of the carotenoid isorenieratene (cluster 4), 2-methylisoborneol (cluster 6), albaflavenone (cluster 20), geosmin (cluster 25), pentalenolactone (cluster 27) and hopene/squalene (cluster 28). The production of ectoine, a secondary metabolite playing an important role in protection against osmotic stress is also encoded on the chromosome of *S. collinus* Tü 365. Furthermore, the genome harbors three clusters involved in biosynthesis of siderophores. One of them, cluster 15, encodes the biosynthesis of desferrioxamine E whose synthesis is based on an NRPS-independent (NIS) mechanism. The remaining gene clusters might encode genes related to bacteriocins (clusters 22 and 24), melanin (cluster 14), and three other compounds (clusters 5, 9 and 29).

In the following sections we present detailed descriptions on the deduced biosynthetic routes of the predicted metabolites as well as results on the induction of the biosynthesis and the chemical characterization of the products.

Table 1 Biosynthetic gene clusters for secondary metabolites in *S. collinus* Tü 365 predicted by antiSMASH

No.	Cluster type	Predicted product[a]	Gene cluster location[b]	Detected ion (method/*m*/*z*)
1	*Lanthipeptide class IV*	**Streptocollin**	182318–188335	
2	*NRPS*	–	207493–266936	
3	*NRPS-T1PKS-TRANSATPKS*	**Kirromycin**	342132–504108	HPLC–ESI–MS/$[M\text{-}H]^-$ = 795.5 *m*/*z*
4	*Terpene*	**Isorenieratene**	697069–705924	HPLC-APCI-MS/$[M]^-$ = 528.5 *m*/*z*
5	*Other*	–	748533–793157	
6	*Melanin-Terpene*	2-Methylisoborneol (2-MIB)	1072633–1095928	
7	*NRPS-T1PKS*	–	1113176–1170734	
8	*Bacteriocin*[c]	–	1176894–1189129	
9	*Other*	–	1453944–1500565	
10	*T3PKS*	–	1500561–1542530	
11	*T1PKS-otherKS*	–	1599772–1653831	
12	*Ectoine*	Ectoine	2276855–2279943	
13	*T2PKS-NRPS-other KS-oligosaccharide*	–	3054126–3126687	
14	*Melanin*	–	3299247–3310578	
15	*Siderophore*	**Desferrioxamin E**	3392183–3399257	HPLC–ESI–MS/$[M + H]^+$ = 601.5 *m*/*z*
16	*T2PKS*	Spore pigment	4065884–4075248	
17	*NRPS-T1PKS*	–	4541525–4597710	
18	*Menaquinone*[d]	**Deoxydehydrochorismic acid**	4926976–4951524	HPLC–ESI–MS/$[M\text{-}H]^-$ = 206.16 *m*/*z*
19	*NRPS*	–	5425082–5484342	
20	*Terpene*	Albaflavenone	5652053–5654433	
21	*Siderophore*	–	6264326–6276435	
22	*Bacteriocin*	–	6404617–6418291	
23	*T1PKS*	–	6428451–6484132	
24	*Bacteriocin*	–	6547932–6560625	
25	*Terpene*	Geosmin	6588041–6590203	
26	*Siderophore*	–	6757853–6772221	
27	*Terpene*	**Pentalenolactone**	6863068–6874754	GC–MS/204 *m*/*z*
28	*Terpene*	**Hopene/squalene**	7128021–7140892	GC–MS/410 *m*/*z*
29	*Other*	–	7415940–7458418	
30	*NRPS*	–	7541482–7617035	
31	*NRPS-T1PKS-TRANSATPKS*	**Kirromycin**	7773632–7930793	
32	*NRPS*	–	8005988–8065431	
33	*Lanthipeptide class IV*	**Streptocollin**	8084590–8090607	

Based on sequence similarity and gene organization, the duplicated clusters (1–33, 2–32, 3–31) localized on the long terminal inverted repeats encode the same compounds

[a] Products detected are indicated in bold

[b] Based on antiSMASH prediction

[c] False-positive hit

[d] Menaquinone cluster was not predicted by antiSMASH but identified by blast of individual genes

Kirromycin

Kirromycin production in *S. collinus* Tü 365 has been described more than 40 years ago [60]. The compound, also referred to under the alternative name mocimycin [53], has been identified in a screening program for narrow-spectrum antibiotics, being the first antibiotic identified targeting the elongation factor EF-Tu. Together with several structurally related compounds, kirromycin belongs to the family of elfamycin antibiotics. Interestingly, so far no kirromycin resistance factor could be unambiguously identified in *S. collinus* Tü 365. An ABC transporter (FacT) has been reported to be involved in resistance against the related elfamycin antibiotic factumycin [51]. While there is a homologue encoded in the kirromycin biosynthetic gene cluster (KirT, 67 % amino acid identity/80 % similarity),

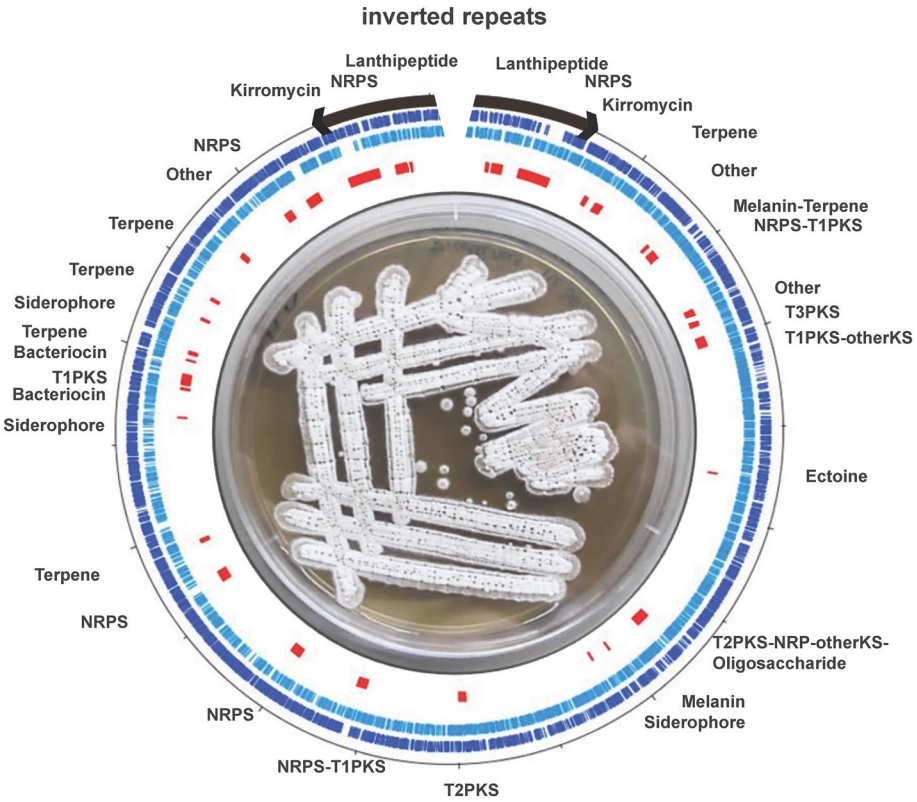

Fig. 1 Representation of the linear chromosome of *S. collinus* Tü 365 and predicted secondary metabolite gene clusters. *Dark blue* and *light blue circles* represent all genes (reverse and forward strands, respectively). The *black arrows* symbolize the terminal inverted repeats of the chromosome. Secondary metabolite biosynthetic gene clusters are indicated by *red blocks*. *T1PKS*, *T2PKS* and *T3PKS* represent type I, II, and III PKS pathways

which could have a similar role, we isolated mutations with interrupted *kirT* gene (unpublished). These strains still are able to produce kirromycin levels comparable to the wild type, indicating additional resistance mechanisms. A different kirromycin producer, *S. ramocissimus*, codes for 3 EF-Tu genes (*tuf1*, *tuf2* and *tuf3*) [52], of which the gene product of *tuf3* (EF-Tu3) displays a kirromycin-resistant phenotype [38]. The *S. collinus* Tü 365 genome codes for two elongation factors, EF-Tu1 (WP_020941664.1), and EF-Tu3 (WP_020938678.1) including a homolog to *S. ramocissimus* EF-Tu3. However, it had been experimentally demonstrated in *S. ramocisimus* that the EF-Tu3 conferred resistant phenotype is recessive, i.e., the ribosome is still blocked if there are sensitive EF-Tu species present. This is the case during kirromycin production in both, *S. ramocissiumus* [52], and *S. collinus* Tü 365 [33]. Thus, kirromycin-resistant EF-Tu seems not to be a main resistance mechanism.

The gene cluster encoding the biosynthesis of kirromycin (MIBiG ID: BGC0001070) was isolated prior to the availability of the *S. collinus* Tü 365 whole-genome sequence using a *S. collinus* Tü 365 cosmid library [58, 59]. Kirromycin biosynthesis was the first described example of an alliance between a cis-ATP-type PKS with trans-AT-type PKS in a single pathway [58]. Its biosynthesis, as well as its application for synthetic biological approaches has been reported in recent years [25, 27, 40, 61].

Genome sequencing data now revealed that the gene cluster encoding kirromycin is present in two copies in the terminal inverted repeats of *S. collinus* Tü 365 genome. This is an explanation for the great difficulty in mutant construction observed in earlier studies. Such duplicated gene clusters, located in the terminal inverted repeats of the chromosome have been identified in different *Streptomyces* strains, as for example, the type II PKS cluster encoding the biosynthesis of the antibiotic kinamycin in *S. amobofaciens* [6].

Lanthipeptide

The duplicated cluster situated in the long terminal inverted repeat of *S. collinus* Tü 365, named *stc* cluster (MIBiG ID: BGC0001226), codes for a putative lanthipeptide. Lanthipeptides are a prominent group of ribosomally synthesized and post-translationally modified peptides, containing thioether cross-links termed lanthionines and methyllanthionines.

A group of four genes localized on a 6.01-kb cluster shows high homology to biosynthesis genes of the class IV lanthipeptide venezuelin [17] encoded by *S. venezuelae*. The identified gene cluster contains a short reading frame for the precursor lanthipeptide designated *stcA*. Genes typically found in lanthipeptide biosynthetic gene clusters

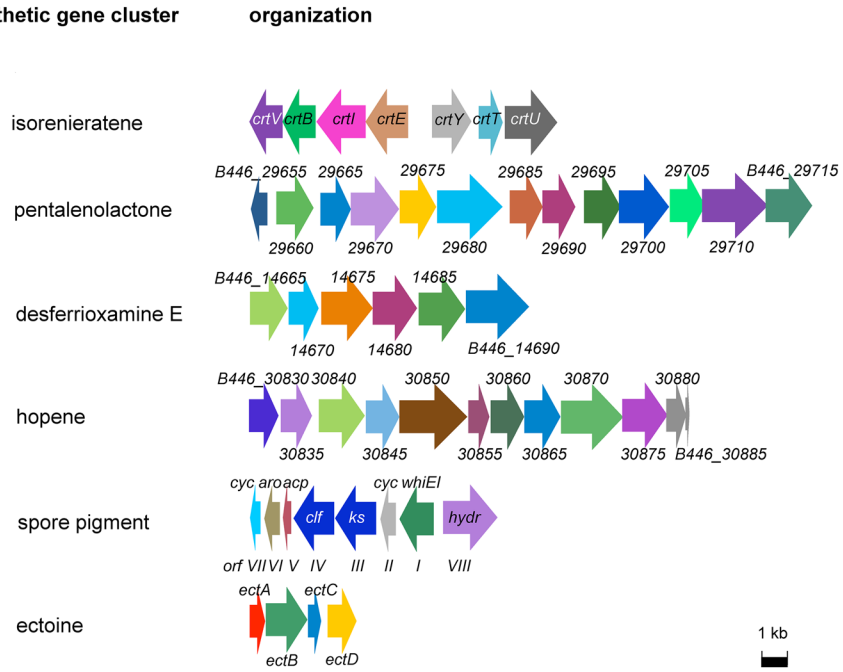

Fig. 2 Organization of the biosynthetic carotenoid isorenieratene, pentalenolactone, desferrioxamine, hopanoid, spore pigment, and ectoine gene cluster of *S. collinus* Tü 365

are present up- and downstream of *stc*A, including genes encoding a two component ABC transporter (*stcT* the ATP-binding element and *stcH* the permease subunit) and a lanthipeptide synthetase gene *stcL*. According to their organization and structure, the identified genes encode the biosynthesis of a type IV lanthipeptide (Iftime et al. manuscript submitted).

Terpenoids

Many terpenoid secondary metabolites are known to play significant roles in defense against predators, pathogens, or competitors, or are involved in conveying messages [15]. Terpenes are widespread in *Streptomyces*. Five clusters of *S. collinus* Tü 365 encoding terpenes are apparently responsible for the biosynthesis of the carotenoid isorenieratene, of the pentacyclic triterpenoid hopene, three sesquiterpenes such as pentalenolactone, albaflavenone and geosmin and the volatile monoterpene alcohol 2-methylisoborneol.

Isorenieratene

Carotenoids are a class of natural fat-soluble terpenoid pigments, characterized chemically by a long aliphatic polyene chain composed of eight isoprene units. Carotenoids are produced in a wide variety of plants, fungi, algae and bacteria. Besides their role as accessory light harvesting pigments in photosynthetic microorganisms and their potential function as antioxidant in bacteria [2, 11], carotenoids are of high interest in human health as anticancer agents, as immune response stimulants, in prevention of arteriosclerosis or macular degeneration [47]. In this context, the aromatic bicyclic carotenoid isorenieratene is important. 3,3′-Dihydroxyisorenieratene and isorenieratene seem to have multifunctional photoprotective properties and may be suitable natural compounds for the prevention of skin cancer [54]. So far the carotenoid isorenieratene has only been found in green photosynthetic bacteria and in a few actinomycetes [26].

One of five identified terpene clusters of *S. collinus* Tü 365 codes for putative isorenieratene biosynthesis genes (MIBiG ID: BGC0001227). Seven genes organized in two convergent operons *crtVBIE* and *crtYTU* (Fig. 2) could be referred to the isorenieratene cluster of *S. collinus* Tü 365, which is similar to the isorenieratene gene cluster of *S. griseus*. Five of these genes, *crtBIEYU* (Table 2), were demonstrated to be involved in biosynthesis of isorenieratene in *S. griseus* [29, 46]. The biosynthesis of isorenieratene starts with CrtE, a geranylgeranyl-pyrophosphate synthase. A second enzyme, CrtB, generates phytoene out of two molecules of geranylgeranyl-pyrophosphate. CrtI, a phytoene desaturase, converts phytoene to lycopene, which is the key intermediate for the biosynthesis of most carotenoids. Cyclization of lycopene by CrtY generates β-carotene. In a final step, CrtU, a β-carotene desaturase converts β-carotene to isorenieratene. CrtV and CrtT were proposed to function as methylesterase and methyltransferase, respectively, but they are dispensable for isorenieratene biosynthesis in *S. griseus* [26].

Compared to other microorganisms with constitutive carotenogenesis, in streptomycetes, carotenogenesis may be induced by light as found in *S. coelicolor* [49], or by

Table 2 Genes involved in isorenieratene biosynthesis and their similarity

Locus_tags in *S. collinus* Tü 365	Homologous gene in *S. griseus*	Protein function in *S. griseus*	% Identity/% coverage
B446_02725	*crtV*	Methylesterase	72/94
B446_02730	*crtB*	Phytoene synthase	73/85
B446_02735	*crtI*	Phytonene dehydrogenase	75/96
B446_02740	*crtE*	Geranylgeranyl pyrophosphate synthase	52/90
B446_02745	*crtY*	Lycopene cyclase	60/98
B446_02750	*crtT*	Methyltransferase	64/96
B446_02755	*crtU*	β-Carotene dehydrogenase	73/98

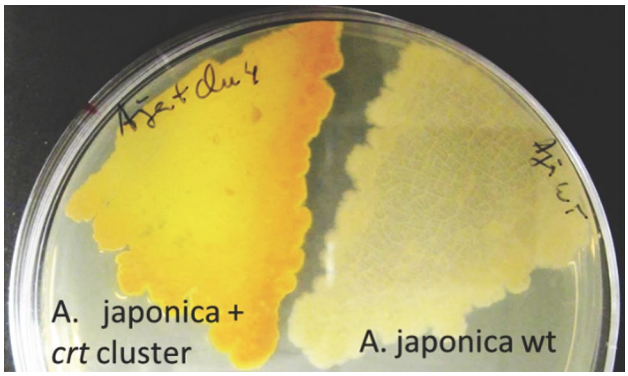

Fig. 3 Growth of *A. japonica* wild type and *A. japonica* harboring the isorenieratene cluster of *S. collinus* Tü 365 on HA agar

some oxidative stressors as has been described for *S. setonii* or *S. griseus* [28].

The *crt* genes of *S. collinus* Tü 365 are not expressed under standard laboratory conditions. In this study, we investigated whether the isorenieratene accumulation in *S. collinus* Tü 365 can be stimulated by treatment with hydrogen peroxide as response to oxidative damage. Therefore, a 3-day culture of *S. collinus* Tü 365 was supplemented with H_2O_2 to an end concentration of 100 µM. Extracts prepared from strain *S. collinus* Tü 365 after oxic shock were analyzed by atmospheric pressure chemical ionization mass spectrum (APCI-MS), revealing traces of isorenieratene in the native strain (Figure S1).

In order to produce greater amounts of isorenieratene a cosmid including the *crt* genes contained in a region of 25 kb was introduced by conjugation into *Amycolatopsis japonica,* which does not contain an intrinsic isorenieratene biosynthetic gene cluster [48]. Cultivation of a recombinant *A. japonica* strain harboring the *crt* cluster for 5 days on HA agar resulted in orange pigmentation of the mycelium (Fig. 3). The production profile was analyzed by HPLC/APCI-MS (Figure S2). The produced carotenoids were identified as lycopene, β-carotene and isorenieratene by comparing masses and UV–Vis spectra to literature data.

Pentalenolactone

Our screening of the *S. collinus* Tü 365 genome resulted in the detection of a 11.6-kb spanning region, containing 13 candidate genes that were assigned to the biosynthesis of pentalenolactone (Fig. 2). The closest homologs of these genes were found in the *ptl* cluster of *S. avermitilis* (Table 3), which was shown to produce pentalenolactone [50, 62].

Pentalenolactone belongs to the family of sesquiterpene antibiotics that are biosynthesized by a variety of *Streptomyces*. The compound was first isolated from *Streptomyces roseogriseus* 1957 and later from over 30 *Streptomyces* species. This isoprene-derived natural product exhibits various biological activities which include diverse antibacterial and antifungal activity, as well as potent inhibitory activity toward the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase [8]. It was also reported to inhibit the replication of DNA viruses such as HSV-1 and HSV-2 [37].

Based on data available for *S. avermitilis*, we postulate the following pentalenolactone synthesis pathway in *S. collinus* Tü 365 (Table 3): The first step in the biosynthesis of pentalenolactone is the cyclization of the precursor farnesyl diphosphate (FPP) to the sesquiterpene hydrocarbon pentalenene [9], catalyzed by a pentalenene synthase, in *S. collinus* Tü 365 encoded by B446_29695. The oxidative conversion of pentalenene to pentalenolactone requires the presence of four redox enzymes such as cytochrome P450 B446_29700, the non-heme iron, α-ketoglutarate-dependent hydroxylase analog, encoded by gene B446_29665, the 1-deoxy-11-beta-hydroxypentalenate dehydrogenase B446_29675, the flavin-dependent monooxygenase B446_29680 and the dioxygenase B446_29685.

Of the four remaining ORFs, *B446_29690* appears to encode a farnesyl diphosphate synthase. Gene *B446_29670* was predicted to code for a transmembrane efflux protein, while the *B446_29660* gene situated at the 5′-end of the *ptl* cluster is similar to *gap1* of *S. avermitilis,* which was described as a resistance related gene [50]. Finally, *B446_29655* resembles *ptlR*, a MarR-family transcriptional

Table 3 Genes involved in pentalenolactone biosynthesis in *S. collinus* Tü 365 and comparison with related genes of *S. avermitilis*

Locus_tags in *S. collinus* Tü 365	Homolog gene in *S. avermitilis*	Locus_tags in *S. avermitilis*	Protein function in *S. avermitilis*	% Identity/% coverage
B446_29655	*ptlR*	SAV_2989	MarR-family transcriptional regulator	73/89
B446_29660	*gap1*	SAV_2990	Glycerin-3-phosphate-dehydrogenase	88/100
B446_ 29665	*ptlH*	SAV_2991	1-Deoxypentalenic acid 11-beta-hydroxylase	78/100
B446_29670	*ptlG*	SAV_2992	MFS transport protein	75/99
B446_29675	*ptlF*	SAV_2993	1-Deoxy-11-beta-hydroxypentalenate dehydrogenase	69/97
B446_29680	*ptlE*	SAV_2994	Monooxygenase	73/96
B446_29685	*ptlD*	SAV_2995	Dioxygenase	75/100
B446_29690	*ptlB*	SAV_2997	Polyprenyldiphosphate synthase	84/100
B446_29695	*ptlA*	SAV_2998	Pentalenene synthase	76/99
B446_29700	*ptlI*	SAV_2999	Cytochrome P450 pentalene hydroxylase	80/96
B446_32515		SAV_7469	Cytochrome P450 monooxygenase	78/97

regulator that was shown to be responsible for the regulation of the pentalenolactone biosynthesis [65] in *S. avermitilis*.

A group of two genes located at the 3′-end of the *ptl* cluster, *ptlJL,* in *S. avermitilis* that were assigned to encode a lyase and a hypothetical protein are lacking in *S. collinus* Tü 365. In contrast to the pentalenolactone cluster of *S. avermitilis,* the cluster in *S. collinus* Tü 365 contains three additional genes (*B446_29705*, *B446_29710* and *B446_29715*) related to isoprene biosynthetic genes belonging to the non-mevalonate pathway. These findings indicate that the pentalenolactone building blocks may be synthesized via the alternative non-mevalonate pathway in this strain. In *S. avermitilis* the gene *sav_3006* is located near the *ptl* cluster. This gene encodes a putative polyprenyl diphosphate synthase, which may be responsible for the biosynthesis of FPP through the mevalonate pathway. Therefore, we speculate that despite the most notable similarity between the pentalenolactone gene clusters of *S. collinus* Tü 365 and *S. avermitilis*, the precursors for pentalenolactone are provided by different pathways in *S. avermitilis* and *S. collinus* Tü 365.

To prove the functionality of the pentalenolactone biosynthetic pathway in *S. collinus* Tü 365, we examined different culture extracts of *S. collinus* Tü 365 by GS-MS.

The production of pentalenene, an intermediate in biosynthesis of pentalenolactone was detected in *S. collinus* Tü 365 (Figure S3), suggesting that this pathway indeed encodes pentalenolactone biosynthesis.

Hopanoids

Our genome mining revealed a cluster of genes encoding the hopene and aminotrihydroxybacteriohopane (ATBH; also referred to as aminobacteriohopanetriol) biosynthesis in *S. collinus* Tü 365.

Hopanoids are a class of pentacyclic triterpenoid lipids. They are important components in bacterial membranes being involved in membrane fluidity, stability and permeability, similar to sterols in eukaryotic membranes [22]. They are composed of isoprene units and are present in a wide range of Gram-positive and Gram-negative bacteria. Hopanoids are synthesized by cyclization of the linear precursor squalene to pentacyclic hopene in a reaction catalyzed by the hopene-squalene cyclase enzyme. Also many *Streptomyces* strains have been described to produce hopanoids [42], including the model organism *S. coelicolor* A3(2) [41].

The hopanoid gene cluster of *S. collinus* Tü 365 contains 12 genes (Fig. 2) transcribed in one orientation. Based on the 12 identified genes and their similarity to hopanoid biosynthesis genes from *S. coelicolor* A3(2) (Table 4), a biosynthetic pathway can be proposed: The first step implies the IPP synthesis in a non-mevalonate pathway by involvement of 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (B446_30865) and a 1-deoxy-D-xylulose-5-phosphate synthase (B446_30870) gene. Apparently, genes belonging to the non-mevalonate pathway are present twice in the *S. collinus* Tü 365 genome, once for pentalenolactone production (see above) and once for hopanoid

Table 4 Proposed function of genes involved in hopane synthesis in *S. collinus* Tü 365

Locus_tags in *S. collinus* Tü 365	Locus_tags in *S. coelicolor* A3(2)	Protein function in *S. coelicolor* A3(2)	% Identity/% coverage
B446_30830	SCO6759	Phytoene/squalene synthase	84/100
B446_ 30835	SCO6760	Phytoene/squalene synthase	91/94
B446_30840	SCO6762	Phytoene/squalene dehydrogenase	85/93
B446_30845	SCO6763	Polyprenyl synthase	94/100
B446_30850	SCO6764	Squalene-hopene cyclase	87/95
B446_30855	SCO6765	Lipoprotein (putative phosphorylase)	88/100
B446_30860	SCO6766	Radical SAM	95/100
B446_30865	SCO6767	4-Hydroxy-3-methylbut-2-en-1-yl-diphosphate	91/99
B446_30870	SCO6768	1-Deoxy-D-xylulose 5-phosphate synthase	90/98
B446_30875	SCO6769	Aminotransferase	90/100
B446_30880	SCO6770	DNA-binding protein	77/100
B446_30885	SCO6771	Hypothetical protein	72/97

synthesis. Sequence alignment between these two pair of genes revealed 60 and 88 % gene sequence similarity, respectively.

In analogy to the hopanoid biosynthetic pathway *S. coelicolor* A3(2), the biosynthesis of hopene in *S. collinus* Tü 365 probable starts with the polyprenyldiphosphate synthase gene *B446_30845* required for the formation of FPP. A second step consists in a head-to-tail elongation of FPP to squalene catalyzed by two phytoene synthases B446_30830 and B446_30835. Finally, the cyclization reaction of squalene to hopene is carried out by B446_30850, a squalene-hopene cyclase in a complex reaction.

Since the genes mentioned above are sufficient for the biosynthesis of hopene, we assumed that the radical SAM enzyme B446_30860 is responsible for the addition of an adenosyl radical to the C=C of hopene, while the remaining enzymes, a putative phosphorylase B446_30855 and an aminotransferase B446_30875 should generate the aminotrihydroxy moiety of the ATBH.

We therefore hypothesized that *S. collinus* Tü 365 can produce both hopene and ATBH. While the intermediates tetrahydrosqualene, dihydrosqualene, and squalene, as well as hopene were experimentally detected in *S. collinus* Tü 365 (Figure S4), the experimental confirmation of ATBH production still remains elusive.

Albaflavenone

The *S. collinus* Tü 365 genome also includes a second sesquiterpene cluster consisting of two genes encoding albaflavenone biosynthesis. The deduced gene products of the genes *B446_24610* and *B446_24615* have 84 and 82 % identity to SCO5222 and SCO5223 from in *S. coelicolor* A3(2) encoding a terpene cyclase and a cytochrome P450. In *S. coelicolor* A3(2) (SCO5222 and SCO5223) and *S. avermitilis* (SAV_3032 and SAV_3031) these enzymes are involved in albaflavenone biosynthesis. As shown for the homologous enzymes B446_24615 catalyzes the cyclization of farnesyl diphosphate to epi-izozizaene [1], while cytochrome P450 directs the two-step oxidation of epi-izozizaene to albaflavenone via the intermediate albaflavenole [64].

Even though efforts were made to isolate albaflavenone and derivatives, none of these compounds could be identified by GS-MS in cultures of *S. collinus* Tü 365 wild type. Further studies should support the hypothesis that albaflavenone is the metabolic product of this gene cluster identified in *S. collinus* Tü 365.

Geosmin

The gene *B446_28510* located in cluster 25 revealed 79 % identity to *geoA* (SAV_2163) from *S. avermitilis* and 83 % identity to *SCO6073* from *S. coelicolor* A3(2), which was demonstrated to mediate the entire conversion of FPP to geosmin in *S coelicolor* A3(2) [20]. Thus we assume that *B446_28510* is probable responsible for the biosynthesis of geosmin in *S. collinus* Tü 365. The earthy smell of geosmin was detectable on agar plates cultivated with *S. collinus* Tü 365.

2-Methylisoborneol

2-Methylisoborneol (2-MIB) is an odorous metabolite that causes the earthy taste in drinking water [21]. Actinobacteria are the main producers of these terpenoid compounds in soil [63], biosynthesis genes for 2-MIB being first identified in these organisms [24]. A gene cluster encoding the biosynthesis of 2-MIB was identified in *S. collinus* Tü 365. In *Streptomyces* strains known for its production, 2-MIB is biosynthesized in a two-step reaction mechanism, in which a methyltransferase converts

first geranyl diphosphate to methyl-geranyl diphosphate and in a second step 2-MIB is synthesized by a 2-MIB-synthase via methyl-geranyl diphosphate cyclization [24]. B446_04425 and B446_04430 from *S. collinus* Tü 365 were associated to a 2-MIB-synthase and a methyltransferase, based on protein similarity to SAM23877_0409 (53 %) and SAM23877_0410 (60 %), respectively, from *S. amobofaciens.*

Siderophore biosynthesis: desferrioxamine E

Iron plays an essential role for metabolic processes in microorganisms. To fulfill their iron needs, many microorganisms acquire iron by biosynthesis of high affinity iron chelators called siderophores [55]. Two pathways have been predominantly described to be involved in siderophore biosynthesis [45], a NRPS dependent route [12], and an NIS-route [39]. An example of NIS-encoded siderophores are the desferrioxamines [34], tris-hydroxamate ferric-iron-chelating metabolites produced by many *Streptomyces* species [32].

We discovered a desferrioxamine E gene cluster in *S. collinus* Tü 365 (Fig. 2) similar to that of *S. coelicolor* A3(2). By mass spectrometry we were able to provide evidence for the presence of desferrioxamine E in culture extracts of *S. collinus* Tü 365 (Figure S5). Based on the high similarity of the *S. collinus* Tü 365 genes with that of *S. coelicolor* A3(2) (Table 5), we propose an analogous pathway: in the first step, L-lysine is decarboxylated by the enzyme B446_14675 to yield cadaverine. In a second step, cadaverine can be oxidized by B446_14680 to produce *N*-hydroxycadaverine. The acyltransferase enzyme, B446_14685 then adds a succinyl group resulting in formation of the *N*-hydroxy-*N*-succinylcadaverine. B446_14690 catalyzes the final NTP-dependent reaction, which converts 3 molecules of *N*-hydroxy-*N*-succinylcadaverine into desferrioxamine E.

Spore pigment

Spore pigmentation in many *Streptomyces* strains is accompanied by the biosynthesis of an aromatic polyketide during the maturation of the spores in the aerial hyphae. The first identified cluster involved in spore pigmentation is the *whiE* cluster of *S. coelicolor* A3(2). The Chater group showed in 1990, that the expression of the minimal type II PKS of *S. coelicolor* A(3)2 resulted in the production of the dodecaketide naphtophenone TW95a [13].

Like many other *Streptomyces* species, *S. collinus* Tü 365 has grey-pigmented spores. Sequence analysis of the *S. collinus* Tü 365 genome revealed the presence of a gene cluster that has notable similarity to the *whiE* gene cluster of *S. coelicolor* A3(2). The spore pigment-associated gene cluster of *S. collinus* Tü 365 also consists of seven unidirectionally transcribed genes *(ORF I–ORF VII)* and one divergently transcribed gene *ORF VIII* (Fig. 2).

Consistent with the high level of similarity (Table 6), genes *B446_17640, B446_17635 and B446_17630* are predicted to encode the three components of the minimal PKS: ketosynthase (KS), chain length factor (CLF) and acyl carrier protein (ACP). Gene *B446_17645* is proposed to encode an aromatase while *B446_17625* and *B446_17620* are likely responsible for the cyclization of the nascent chain. *B446_17655* apparently directs the introduction of a hydroxyl group into the cyclized polyketide. In *S. coelicolor* A3(2) it was proposed that *whiE* I encodes a protein needed for targeting the spore pigment within the spores [23]. Moreover, it was speculated that additional genes involved in late tailoring steps may be distributed independently in the linear chromosome [44]. Although the color of *S. collinus* Tü 365 spores changed to deep grey during the sporulation, the corresponding type II polyketide compound was not isolated so far.

Ectoine

Ectoine biosynthesis is widespread in many bacteria, conferring the ability to microorganisms to survive harsh osmotic stress conditions. In *S. collinus* Tü 365, a gene cluster resembling the *ectABCD* operon in *S. coelicolor* A3(2) [7] was identified (Fig. 2), suggesting that *S. collinus* Tü 365 is capable to induce the biosynthesis of the water-attracting organic osmolytes ectoine and hydroxyectoine under conditions of high salt concentration.

In concordance to homologous gene function in *S. coelicolor* A3(2) (Table 7), the first step in hydroxyectoine biosynthesis in *S. collinus* Tü 365 can be catalyzed by EctB (B446_09705), a diaminobutyric acid aminotransferase, which converts L-aspartate β-semialdehyde to L-diaminobutyrate. The acetyltransferase EctA (B446_09700) is assumed to add an acetyl group to L-diaminobutyrate resulting in acetyl-L-diaminobutyrate, while the ectoine synthase EctC (B446_09710) is proposed to accomplish the formation of ectoine. Finally, EctD (B446_09715) may be responsible for the conversion of ectoine to hydroxyectoine. Until now, the production of ectoine in *S. collinus* Tü 365 was not experimentally confirmed.

Table 5 Genes involved in desferrioxamine biosynthesis

Locus_tags in *S. collinus* Tü 365	Homologous gene in *S. coelicolor* A3(2)	Protein function in *S. coelicolor* A3(2)	% Identity/% coverage
B446_14665	*desE*	Secreted protein	79/100
B446_14670	*desF*	Siderophore-interacting protein	75/92
B446_14675	*desA*	Pyridoxal-dependent decarboxylase	88/100
B446_14680	*desB*	Monooxygenase	76/100
B446_14685	*desC*	Acetyltransferase	80/99
B446_14690	*desD*	Siderophore-biosynthetic-enzyme	84/100

Table 6 Feature of the spore-associated pigment gene cluster in *S. collinus* Tü 365

Locus_tags in *S. collinus* Tü 365	WhiE (ORF I-ORF VIII)	Locus_tags in *S. coelicolor* A3(2)	Protein function in *S. coelicolor* A3(2)	% Identity/% coverage
B446_17650	ORF I	SCO5320	Polyketide-based spore pigment WhiEI	71/99
B446_17645	ORF II	SCO5319	Cyclase	79/86
B446_17640	ORF III	SCO5318	Beta-ketoacylsynthase subunit alpha	84/99
B446_17635	ORF IV	SCO5317	Beta-ketoacylsynthase subunit beta/chain length factor	79/99
B446_17630	ORF V	SCO5316	Acyl carrier protein	65/98
B446_17625	ORF VI	SCO5315	Aromatase	74/100
B446_17620	ORF VII	SCO5314	Cyclase	81/100
B446_17655	ORF VIII	SCO5321	Polyketide hydroxylase	69/96

Deoxydehydrochorismic acid: an intermediate in menaquinone biosynthesis

While isolating kirromycin for biochemical studies by preparative chromatography, we observed antibacterial activity against *E. coli* and *B. subtilis* in an additional fraction not containing kirromycins. Follow-up of this active fraction yielded deoxydehydrochorismic acid (Figure S6), an intermediate of menaquinone biosynthesis. The single enzyme responsible for biosynthesis of deoxydehydrochorismic acid is MqnA, which converts chorismate to 3-((1-carboxyvinyl)oxy) benzoic acid. Bioinformatical analysis searching for MqnA, revealed the presence of a gene cluster probably encoding menaquinone biosynthesis in *S. collinus* Tü 365, since it exhibits high similarity to the menaquinone biosynthesis genes of *S. coelicolor* (Table 8). However, in contrast to *S. coelicolor*, where the *mqn* genes were found to be scattered across the genome [16], in *S. collinus* Tü 365 most genes are clustered with exception of *mqnB* and *mqnD* orthologs.

Menaquinone or vitamin K is a lipid-soluble molecule essential for commuting electrons between membrane bound redox enzymes in the electron-transport chain in prokaryotes [18]. In humans, vitamin K plays an important role as cofactor in the biosynthesis of proteins essential for blood clotting and bone health. We detected traces of vitamin K2 in *S. collinus* Tü 365 extracts, but it might be possible that this compound originates from the media components used for cultivating the strain.

Conclusions

Streptomycetes are a prolific source of secondary metabolites, being an important reservoir for novel bioactive compounds. In this work, we evaluate the capacity of *S. collinus* Tü 365 to produce secondary metabolites. By in silico analysis of the genome sequence of *S. collinus* Tü 365 using the antiSMASH genome mining software [5, 30, 56] and a manual curation, a total of 32 putative secondary metabolite biosynthetic gene clusters were identified.

Optimization of the cultivation conditions for the native strain and heterologous expression of two clusters enabled the identification of natural compounds previously not described for this strain.

In addition to the already known kirromycin gene cluster, ten secondary metabolites gene clusters encoded in the *S. collinus* Tü 365 genome share similarity to well described clusters allowing us to propose a product (Fig. 4). In addition to kirromycin, for five of them, in silico prediction successfully directed the identification of corresponding natural products: a lanthipeptide, the carotenoid isorenieratene,

Table 7 Feature of the ectoine gene cluster in *S. collinus* Tü 365 and *S. coelicolor* A3(2)

Locus_tags in *S. collinus* Tü 365	Homologous gene in *S. coelicolor* A3(2)	Locus_tags in *S. coelicolor* A3(2)	Function in *S. coelicolor* A3(2)	% Identity/% coverage
B446_09700	*ectA*	SCO1864	Acyltransferase	68/96
B446_ 09705	*ectB*	SCO1865	Diaminobutyric acid aminotransferase	85/99
B446_09710	*ectC*	SCO1866	Ectoine synthase	82/97
B446_09715	*ectD*	SCO1867	Ectoine hydroxylase	84/98

Table 8 Genes involved in menaquinone biosynthesis

Locus_tags in *S. collinus* Tü 365	Homologous gene in *S. coelicolor* A3(2)	Locus_tags in *S. coelicolor* A3(2)	Gene function in *S. coelicolor* A3(2)	% Identity/% coverage
B446_21435	*MqnA*	SCO4506	Chorismate dehydratase	93/100
B446_17345[a]	*MqnB*	SCO4327	Aminofutalosine deaminase	85/87
B446_21370	*MqnE*	SCO4494	Radical SAM	97/100
B446_17350[a]	*MqnD*	SCO4326	1,4-Dihydroxy-6-naphthoate synthase	93/100
B446_21460	*UbiE*	SCO4556	Methyltransferase	91/99
B446_21360	*UbiX*	SCO4492	Polyprenyl decarboxylase	91/97
B446_21350	*UbiD*	SCO4490	Decarboxylase	93/100
B446_21355	*UbiA*	SCO4491	Prenyltransferase	91/100
B446_21450	*MqnC*	SCO4550	Radical SAM	93/100

[a] Genes encoded outside of the gene cluster

pentalenolactone, hopene and desferrioxamine E. Five gene clusters were putatively associated to the biosynthesis of albaflavenone, geosmin, 2-methylisoborneol, ectoine and a spore pigment. Furthermore, bioactivity-guided fractionation of strain extracts yielded a bioactive small molecule, deoxydehydrochorismic acid, which is an intermediate in menaquinone biosynthesis. Nine of the remaining uncharacterized secondary metabolites clusters in *S. collinus* Tü 365 are polyketides, non-ribosomal peptides or hybrids of the two structures, confirming that *S. collinus* Tü 365 is a prolific producer of such molecules. Hence, the activation and enhancement of cryptic pathways in *S. collinus* Tü 365 is of high interest. Although there are increasing numbers of secondary metabolites being isolated from *S. collinus* Tü 365, a substantial fraction of the encoded secondary metabolites and their functional roles in this strain remains to be elucidated.

Materials and methods

Cosmid library screening

A cosmid library of *S. collinus* Tü 365 was constructed using the integrative vector pOJ436 in cooperation with Combinature Biopharm AG, Berlin, Germany. Samples were automatically processed in 384-well plates and transferred to high-density clone arrays. Two identical arrays were used for hybridization experiments to identify cosmids harboring the whole *crt* gene cluster encoding the biosynthesis of the carotenoid isorenieratene as described earlier [59].

Detection of carotenoids

Extraction of carotenoids was performed using a protocol described for *S. albus* by Myronovskyi et al. 2014 [36]. The separation and identification of carotenoids was carried out using an Agilent 1200 HPLC System (Agilent Technologies, Waldbronn, Germany) coupled to LC/MSD Ultra Trap System XCT 6330, Agilent Technologies, Waldbronn, Germany) by UV-HPLC/APCI-MS. HPLC separation was carried out at a flow rate of 400 µl/min on a nucleosil column (Nucleosil 100 C18 3 µm, 100 × 2 mm ID fitted with a precolumn 10 × 2 mm, same stationary phase, Dr. Maisch GmbH, Ammerbuch) with the mobile phase composed of formic acid (A = 0.1 %), and formic acid in acetonitrile (B = 0.06 %). A gradient from 70 to 100 % of B in 10 min with a 30-min hold at 100 % for solvent B was used. The column was maintained at 40 °C.

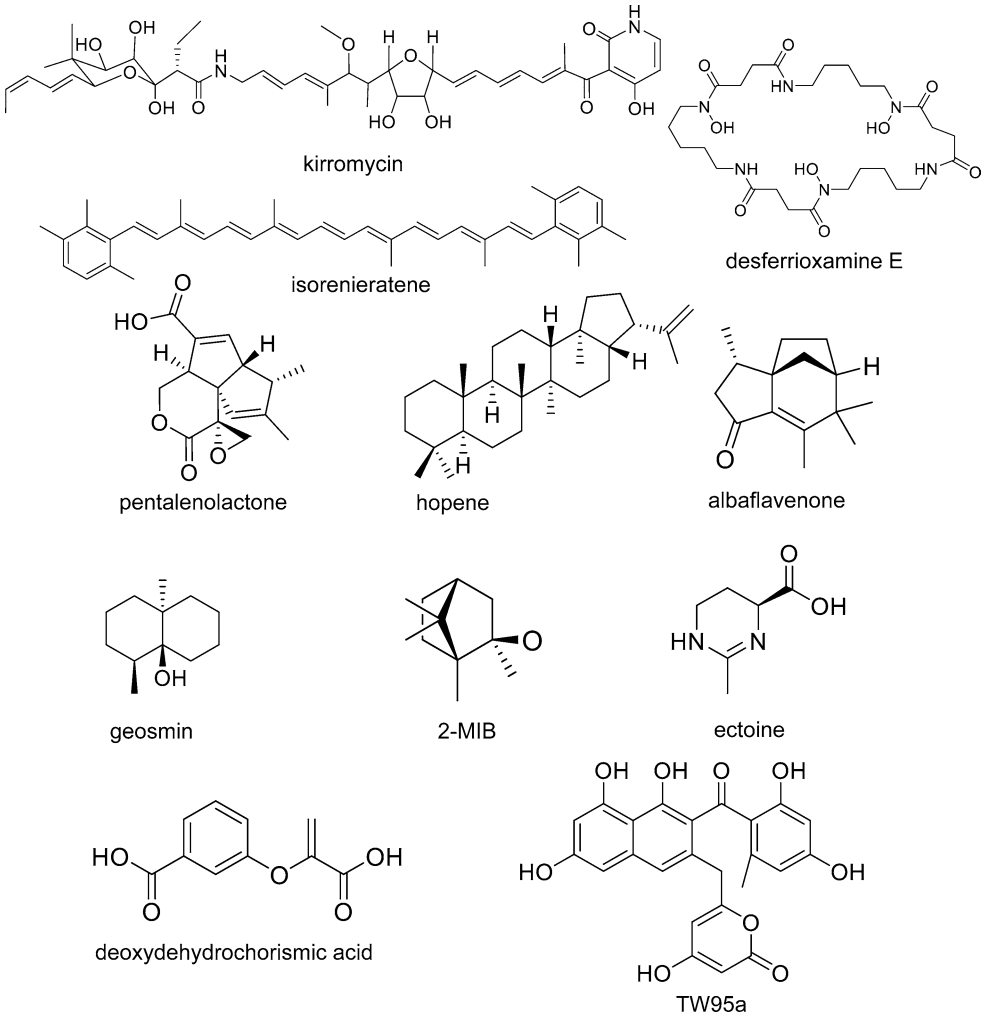

Fig. 4 Structures of identified and predicted secondary metabolites encoded in *S. collinus* Tü 365 genome

Identification of produced terpene products

We identified the production of terpene compounds by cultivation of *S. collinus* Tü 365 for 6 days on cellophane-covered mannitol-soy agar plates as well as in liquid cultures. For liquid cultures *S. collinus* Tü 365 was inoculated into 50 ml TSB medium and cultivated for 2 days on a rotary shaker at 27 °C and 180 rpm. Then 200 µl of seed culture were transferred into 200 ml KPM medium (for recipe, see Supporting Information) and incubated for 5 days at 27 °C and 180 rpm for secondary metabolites production. Culture broth was harvested by centrifugation for 20 min at 5000 rpm. Extraction of lipids from *S. collinus* Tü 365 was carried out using the method described by Bligh and Dyer (1959). The terpene fractions were separated by GS-MS (Shimadzu GC-17A) on a capillary column (Optima 5 MS, 0.25 mm × 15 m, Macherey–Nagel). The temperature program was 45 °C for 5 min isotherm with a temperature ramp of 45–200 °C at 18 °C/min and 200–340 °C at 4 °C/min.

Pentalenene was observed at *m/z* 204 Da. The observed mass spectrum was compared with those of pentalenene identified from *S. avermitilis*.

Hopanoids were identified by comparison of their retention times in GS-MS and fragmentation patterns with those of analytical standards. The mass peak at *m/z* 410, the base peak at *m/z* 189/191 and the fragmentation pattern are characteristic for hopene.

Identification of desferrioxamine E

Streptomyces collinus Tü 365 was inoculated with a TSB preculture (10 ml) in 500 ml of modified ancovenin production broth (for recipe, see Supporting Information) and incubated for 3 days at 27 °C/180 rpm. The culture broth was harvested by centrifugation for 20 min at 5000 rpm. The supernatant was extracted twice with XAD1180 resin. The XAD1180 resin was then extracted with methanol–acetone (1:1) and the extract was dried in vacuo. The extract was then resolved in 500 µl of MeOH and analyzed using an

Agilent 1200 HPLC System (Agilent Technologies, Waldbronn, Germany) coupled to an LC/MSD Ultra Trap System XCT 6330, Agilent Technologies, Waldbronn, Germany).

Chromatographic separation was performed at a flow rate of 400 µl/min using stationary phase C18 column Nucleosil 100 3 µm (100 × 2 mm ID, fitted with a precolumn 10 × 2 mm, same stationary phase, Dr. Maisch GmbH, Ammerbuch) with the mobile phase composed of formic acid (A = 0.1 %), and formic acid in acetonitrile (B = 0.06 %). A gradient from 10 to 100 % of B in 15 min with a 2-min hold at 100 % for solvent B, was used.

Detection of deoxydehydrochorismic acid

Streptomyces collinus Tü 365 was inoculated with a TSB preculture (10 %) in 100 ml of KPM production broth and incubated for 5 days at 27 °C/180 rpm in 500-ml shaking flasks. The culture was extracted twice with 1 volume ethyl acetate at pH 4 and dried in vacuo. The extract was resolved in appropriate volumes of MeOH (200 µl for 50 ml extraction volume).

The samples were analyzed using an Agilent 1200 HPLC System (Agilent Technologies, Waldbronn, Germany) coupled to LC/MSD Ultra Trap System XCT 6330, Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at a flow rate of 400 µl/min using stationary phase C18 column Nucleosil 100 3 µm (100 × 2 mm ID, fitted with a precolumn 10 × 2 mm, same stationary phase, Dr. Maisch GmbH, Ammerbuch) with the mobile phase composed of formic acid (A = 0.1 %), and formic acid in acetonitrile (B = 0.06 %). A gradient from 10 to 100 % of B in 15 min with a 2-min hold at 100 % for solvent B, was used.

Acknowledgments This work was supported by ERA-IB-GenoDrug (BMBF FKZ 0315930), The German Center for Infection Research (DZIF) (TTU 09.802) and the Graduate College 1708 (Bacterial Survival Strategies). T. Weber is supported by a grant from the Novo Nordisk Foundation. The authors acknowledge P. Schmieder (FMP Berlin-Buch) for recording the NMR spectra for deoxydehydrochorismic acid.

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