RESEARCH LETTER – Physiology & Biochemistry

Differential regulation of undecylprodigiosin biosynthesis in the yeast-scavenging Streptomyces strain MBK6

Baral Bikash†, Siitonen Vilja†, Laughlin Mitchell, Yamada Keith§, Ilomäki Mikael, Metsä-Ketelä Mikko‡ and Niemi Jarmo*,†,‡

Department of Biotechnology, University of Turku, FIN-20014 Turku, Finland

*Corresponding author: Department of Biotechnology, University of Turku, FIN-20014 Turku, Finland. Tel: +358 40 078 9362; Fax: +358 29 450 5040; E-mail: jarnie@utu.fi

One sentence summary: Genome analysis of a prodigiosin producing Streptomyces isolate revealed differences in key regulatory genes, probably connected with the responsiveness of this strain to yeast.

†Bikash Baral and Vilja Siitonen should be considered joint first author
‡Mikko Metsä-Ketelä and Jarmo Niemi should be considered joint senior author

ABSTRACT

Streptomyces are efficient chemists with a capacity to generate diverse and potent chemical scaffolds. The secondary metabolism of these soil-dwelling prokaryotes is stimulated upon interaction with other microbes in their complex ecosystem. We observed such an interaction when a Streptomyces isolate was cultivated in a media supplemented with dead yeast cells. Whole-genome analysis revealed that Streptomyces sp. MBK6 harbors the red cluster that is cryptic under normal environmental conditions. An interactive culture of MBK6 with dead yeast triggered the production of the red pigments metacycloprodigiosin and undecylprodigiosin. Streptomyces sp. MBK6 scavenges dead-yeast cells and preferentially grows in aggregates of sequestered yeasts within its mycelial network. We identified that the activation depends on the cluster-situated regulator, mbkZ, which may act as a cross-regulator. Cloning of this master regulator mbkZ in S. coelicolor with a constitutive promoter and promoter-deprived conditions generated different production levels of the red pigments. These surprising results were further validated by DNA–protein binding assays. The presence of the red cluster in Streptomyces sp. MBK6 provides a vivid example of horizontal gene transfer of an entire metabolic pathway followed by differential adaptation to a new environment through mutations in the receiver domain of the key regulatory protein MbkZ.

Keywords: DNA-binding proteins; prodigiosin; antibiotic biosynthesis; Streptomyces; transcription factors

INTRODUCTION

Soil microbiota represents one of the most complex and diverse ecosystems found on our planet (Crowther et al. 2019). This nutrient scarce habitat hosts interacting communities of microorganisms, especially bacteria and fungi, competing for resources. A particularly interesting facet of these communities is the ability of the microbes to communicate with each other using secondary metabolites. Examples include initiation of an exploratory growth phase in generally stationary Streptomyces bacteria upon encountering yeast (Jones et al. 2017) and the
chemical warfare between Streptomyces and Aspergillus where the microbes respond to chemical stimuli from each partner (Nüttzmann et al. 2011; Khalil et al. 2018).

Actinobacteria are prolific producers of biologically active natural products, which have contributed approximately two thirds of antibiotics and one third of anticancer agents in clinical use (Newman and Cragg 2020). Genome sequencing projects have revealed that Streptomyces typically harbor few tens of biosynthetic gene clusters (BGC) that encode chemically diverse secondary metabolites (Bentley et al. 2002). However, many of these BGCs remain dormant under laboratory monocultures and require environmental signals from the soil microbiota community for activation of gene transcription. Numerous biotechnological applications have been developed in order to activate microbial secondary metabolic pathways for production of novel bioactive compounds (Baral, Akhgari and Metsä-Ketelä 2018).

Undecylprodigiosin is a well-studied bioactive substance (Tsao et al. 1985; Malpartida et al. 1990) produced, among others, by Streptomyces coelicolor (Cerdeño, Bibb and Challis 2001), which possesses antitumor, immunosuppressant, antifungal and antimarialar activities (Stankovic et al. 2014). Recently, the production of prodigiosins has been implicated as an agent of programmed cell death in the life cycle of S. coelicolor (Tenconi et al. 2018), but their production has also been shown to increase when in contact with Bacillus subtilis (Luti and Mavituna 2011).

We have previously noted that Streptomyces may respond to the presence of yeast by triggering production of extracellular cholesterol oxidase (Yamada et al. 2021). This study continues the characterization of Streptomyces–yeast interactions. The strain Streptomyces sp. MBK6 isolated from a soil sample was found to produce a red substance in the presence of yeast. The strain Streptomyces sp. MBK6 isolated from a soil sample was found to produce a red substance in the presence of yeast. The products were identified as undecylprodigiosin and metacycloprodigiosin when in contact with Bacillus subtilis (Luti and Mavituna 2011).

Isolation of Streptomyces sp. MBK6

Soil samples originating from south-western Finland were collected and bacteria with Streptomyces-like colony morphology were isolated by serial dilution and tested for antibiotic activity against Kocuria rhizophila ATCC 9341 (Micrococcus luteus (Tang and Gillevet 2003)) by an agar plug – zone of inhibition test as described (Barnard 1994). Streptomyces sp. MBK6 was noted due to its strong red color.

MATERIALS AND METHODS

Biological materials

The yeast interaction media were Y2 (20 g/L glucose, 2.5 g/L autoclaved bakery yeast, 2.5 g/L yeast extract, 1 g/L K2HPO4, 1 mL/L trace salts solution [FeSO4·7H2O 1 g/L, MnCl2·4H2O 1 g/L, ZnSO4·7H2O 1 g/L]) and YE (the same without yeast). The compounds were analysed by high resolution mass (MicroOTOF-Q, Bruker Daltonics, Bremen, Germany) by direct injection in positive ionization mode. For NMR analysis the purified and desiccated compounds were dissolved in MeOD or CDCl3. 1D measurements: 1H and 13C NMR and 2D measurements (COSY, heteronuclear multiple bond correlation, HMBC, heteronuclear single quantum coherence HSQCDE, heteronuclear single quantum correlation, edited) were performed with following instruments: 600 MHz Bruker AVANCE-III NMR-system with a liquid nitrogen cooled Prodigy TCI (inverted CryoProbe) and a 500 MHz Bruker AVANCE-III NMR-system with a liquid nitrogen cooled Prodigy BBO (CryoProbe). The signals were internally referenced to tetramethylsilane. Topspin (Bruker Biospin) was used for spectral analysis.

Isolation and purification of the red pigments

A 3 l fermentation was inoculated with 50 mL of Streptomyces sp. MBK6 preculture in 26 g/L whole yeast, 9 g/L glucose, 2 g/L CaCO3, 2 g/L NH4NO3 in tap water. The fermentation continued for 5 days at 30 ºC with stirring and aeration. Cells were collected by centrifugation for 20 min at 7025 × g. The red compounds were extracted from centrifuged cell pellets with 1:1 methanol: toluene: 0.1 M phosphate buffer pH 7. The toluene phase was subsequently washed with phosphate buffer. The samples were dried and dissolved in a minimal volume of 9:1 chloroform: methanol before applying to a normal-phase silica column and a gradient from 10 to 100% methanol in chloroform was run. Fractions of interest were further purified by preparative HPLC (LC-20AP, model; CBM-20A, Shimadzu, SunFire Prep C18, 5 µm 10 × 250 mm, Waters) using a gradient from 15% methanol with 0.1 formic acid to 100% methanol.

Analyses of compounds

The compounds were analysed by HPLC (SCL-10AChr HPLC with an SPD-M10AChr diode array detector, Shimadzu, Tokyo, Japan) using 15% MeOH with or without 0.1% formic acid to 100% MeOH using a KINETEX column (2.6u C18 100A; 10 × 4.6 mm, Phenomenex, Torrance, CA, USA). Purified compounds were analysed by high resolution mass (MicroOTOF-Q, Bruker Daltonics, Bremen, Germany) by direct injection in positive ionization mode. For NMR analysis the purified and desiccated compounds were dissolved in MeOD or CDCl3. 1D measurements: 1H and 13C NMR and 2D measurements (COSY, heteronuclear multiple bond correlation, HMBC, heteronuclear single quantum coherence HSQCDE, heteronuclear single quantum correlation, edited) were performed with following instruments: 600 MHz Bruker AVANCE-III NMR-system with a liquid nitrogen cooled Prodigy TCI (inverted CryoProbe) and a 500 MHz Bruker AVANCE-III NMR-system with a liquid nitrogen cooled Prodigy BBO (CryoProbe). The signals were internally referenced to tetramethylsilane. Topspin (Bruker Biospin) was used for spectral analysis.

Genome sequencing

Streptomyces sp. MBK6 was cultured in 30 mL of GYM media with 0.5% glycine at 30 ºC for 2 days shaking at 300 rpm and then pelleted. Genomic DNA was extracted (Nikodinovic, Barrow and Chuck 2003 with slight modifications). Quality control and the PCR-free shotgun library (Illumina, San Diego, CA, USA) was prepared at the Finnish Functional Genomics Centre (Turku, Finland). A single lane of an Illumina MiSeq v3 sequencer was used to produce 2 × 300 bp reads. The quality of the reads was manually checked before and after error correction using FASTQC (v0.11.2; Andrews 2010). The reads were assembled using A5-miseq (v20150522; Coin, Jospin and Darling 2015), contiguated with ABACAS (v1.3.1; Assefa et al. 2009) using Streptomyces albus NK660 (CP007574.1) as the reference, and the gaps were filled using IMAGE (v2.4.1; Tsai, Otto and Berriman 2010). The final assembly was annotated using RAST (Brettin et al. 2015) and evaluated for completeness using BUSCO (v1.2; Simão et al. 2015). All programs were used with the default parameters and ran on the CSC—IT Center for Science’s Taito super-cluster (Espoo, Finland).

The sequencing of Streptomyces sp. MBK6 resulted in 4 530 672 reads that were error corrected and trimmed down to 4 379 905 reads, which were then de novo assembled into 65
contigs. ABACAS ordered and aligned the contigs into 25 scaffolds with an N50 of 664 525 bp. The final genome assembly is 7.6 Mbp with a GC content of 72.7% and median coverage of 144x.

The BUSCO analysis searched for 40 single-copy orthologs and found 40 (100%) were complete. Out of the 40 complete BUSCOs, two were found multiple times throughout the assembly. Furthermore, no BUSCO was identified as fragmented. The genome was deposited in DDBJ/ENA/GenBank under the accession number JACERG000000000. The version described in this paper is version JACERG100000000. The biosynthetic gene cluster was deposited in MIBiG under the accession number BGC0002090.

Expression of mbkZ

The gene of size 1045 bp (Supplementary methods) was purchased from Genewiz (South Plainfield, NJ 07080, USA) and separately subcloned as a BamHI-HindIII fragment in pIJ486 and pIJ486 to create pIJ486-MbkZsyn (ermEp-mbkZp) and pIJ486-MbkZsyn (mbkZp) plasmids, respectively. To delete the native promoter (pIJ486-MbkZsynop (ermEp)) the KpnI-HindIII fragment was first subcloned in pBADHisBΔ (Kallio et al. 2006), and subsequently subcloned as a BamHI-HindIII fragment in pIJ486. These plasmids were then transformed into S. coelicolor M145 and S. lividans TK24 through protoplast transformation (Kieser et al. 2000). The plasmids were also transformed into Streptomyces sp. MBK6 by the protoplast technique, but only for the pIJ486-MbkZsyn (ermEp-mbkZp) construct was the transformation successful.

Assay of total prodigiosins in cultures

Cells from 1 mL of culture were pelleted at 20 000 × g for 5 min, and the pellet extracted for 4 h with a mixture of methanol and toluene (400 μL each) in a rotary mixer. 400 μL of 1 mol/L NaOH was added, and after mixing and centrifugation (20 000 × g, 1 min) the toluene layer was transferred to a new tube. 400 μL of 1 mol/L HCl was added, mixed and centrifuged as before, and the toluene layer was separated. After diluting to 1 mL, prodigiosin concentration was assessed as absorption at 538 nm (Multiskan). Toluene (400 μL) the toluene layer was transferred to a new tube. 400 μL of 1 mol/L HCl was added, mixed and centrifuged as before, and the toluene layer was separated. After diluting to 1 mL, prodigiosin concentration was assessed as absorption at 538 nm (Multiskan).

Protein expression and purification

Escherichia coli TOP10 cells transformed with pBADHisBΔ-MbkZ were used for protein expression. Precultures were grown overnight in 40 mL of Luria–Bertani (LB) medium with 100 μg/mL ampicillin in 250 mL Erlenmeyer flasks at 30°C and 250 rpm. 400 mL cultures of LB-medium were inoculated with 4 mL of preculture and grown at 30°C and 250 rpm to an OD600 of 0.6, whereupon protein production was induced with addition of 0.02% (v/v) 1-arabinose and the cultures were left to grow overnight at room temperature and shaking at 180 rpm. Cells were collected through centrifugation at 12 000 × g for 20 min at 4°C, resuspended in 20 mL wash buffer (5 mM imidazole, 10% glycerol, 50 mM Tris, 300 mM NaCl and pH 7.5) and disrupted by sonication (Soniprep 150, MSE). 1% Triton X-100 was added, and samples were centrifuged for 50 min at 4°C for 1 h in pre-cooled 2% TBE agarose gel and run at 4°C for 1 h in pre-cooled 1x TBE. The fluorescence of the DNA was visualized with a Li-Cor Odyssey 9120 imaging system (LI-COR).

RESULTS

Isolation and growth of Streptomyces sp. MBK6

Streptomyces sp. MBK6 was isolated from a soil sample from south-western Finland and produced a bright red antibiotically active compound on plates and in liquid culture when grown in a medium containing whole autoclaved yeast cells. The initial aim was to find new Streptomyces strains that would be stimulated by yeast, like the cholesterol oxidase-producing Streptomyces lavendulae YAKB-15 (Yamada et al. 2019). Under co-culture conditions with yeast, the strain grows as a highly dispersed culture, the mycelium adheres to the yeast cells and the yeast is sequestered in the growing mycelial clumps (Fig. 1). In the presence of yeast, Streptomyces sp. MBK6 produces red pigments early, typically on the second day of culture.

Identification of the pigment as a mixture of undecylprodigiosin and metacycloprodigiosin

Streptomyces sp. MBK6 and yeast were co-cultivated in large scale and two red compounds were purified using multiple two-phase organic extractions and chromatography techniques to produce samples suitable for analysis by NMR. Both compounds were analysed by 1D (1H and 13C) and 2D measurements by NMR and by HR-MS.

The compounds were identified as metacycloprodigiosin (1) (Wasserman, Rodgers and Keith 1969) and undecylprodigiosin (2) (Tsao et al. 1985; Fig. 2). Detailed structural analysis can be found in Supporting information (Table S1 and Figures S1–S12, Supporting Information).
Figure 1. (A) Parallel cultures of MBK6 in Y2 (with yeast) and YE medium (without yeast) after 4 days of culture, demonstrating stimulation of red prodigiosin production. (B) Sample of Y2 medium, inoculated with a spore suspension of MBK6, after 3 days of culture. Phase contrast microscopy, 100x magnification. The spherical objects are yeast cells; nearly all of them have been sequestered by the growing mycelium.

Figure 2. Compounds related to the study. (A) metacycloprodigiosin (1) and undecylprodigiosin (2). (B) HPLC chromatogram of MBK6 culture extract shown at 530 nm. (C) UV/VIS spectra of the compounds.
A draft genome sequence of Streptomyces sp. MBK6 acquired using Illumina MiSeq was analysed with antiSMASH (Blin et al. 2019). An undecylprodigiosin-type cluster was readily identified (90% of genes showing similarity) among the 26 identified BGCs. The undecylprodigiosin cluster is essentially colinear with the well-characterized red cluster of S. coelicolor (Malpartida et al. 1990; Cerdeño, Bibb and Challis 2001; Hu et al. 2016; Fig. 3A). The most significant differences are the lack of a homolog of the oxi-doreductase redD of unknown function, translational fusion of the type I polyketide synthase redL and the oxi-doreductase redK (Cerdeño, Bibb and Challis 2001) and extension of sequence similarity to SCOS899, which in some publications has been named redE (Hu et al. 2016). Importantly, we noted that the N-terminal part of redZ, the primary regulator of the red BGC (Guthrie et al. 1998), was not conserved.

A BLAST (Altschul et al. 1990) search of the Streptomyces clade of the refseq_genomes database at NCBI with the Streptomyces sp. MBK6 undecylprodigiosin cluster indicated a 98.91% nucleotide sequence identity with a Streptomyces griseoaurantiacus strain M045 (Li et al. 2011) sequence (NZ_AEXY1000041.1). The 16S rRNA sequence of Streptomyces sp. MBK6 has only one base difference from the 16S rRNA of S. griseoaurantiacus M045. Comparison of the genomes using Easyfig (Sullivan, Petty and Beatson 2011) revealed extensive colinearity and high sequence similarity, indicating the two strains are closely related (Figure S13, Supporting Information). Production of prodigiosins by S. griseoaurantiacus M045 has been observed (Li et al. 2005). S. griseoaurantiacus M045 has been isolated from marine sediment in China, whereas Streptomyces sp. MBK6 has been isolated from a soil sample in Finland.

**Cross-regulation of S. coelicolor prodigiosin production by the putative regulator mbkZ**

RedZ of S. coelicolor is a member of the ‘orphan response regulator’ family (Guthrie et al. 1998; Liu et al. 2013), in which the conserved phosphorylation site and the cognate histidine kinase gene are missing, and it is the master regulatory protein of the red cluster. Thus, the non-conservation of the nucleotide sequence with mbkZ suggested different regulation of the prodigiosin production between the strains. On the amino acid level (Fig. 3B and C), the highest similarity is within the DNA-binding domain (Liu et al. 2013), whereas the receiver domain shows weaker similarity.

To test this, we decided to transfer mbkZ with its own and/or a constitutive promoter to S. coelicolor M145. The synthetic gene with promoter was subcloned in pIJ486 (YlihONko et al. 1996; ermEp-mbkZp), in pIJ486 (Ward et al. 1986; mbkZp) and in pIJ486 without its own promoter (ermEp) in S. lividans TK24 as an intermediate host. Once the plasmids were transformed to S. coelicolor M145, the strains were grown in parallel cultures in Y2 medium (containing 2.5 g/L fresh, autoclaved yeast and 2.5 g/L yeast extract) and YE medium (containing 2.5 g/L yeast extract), together with wild-type S. coelicolor M145 and Streptomyces sp. MBK6. Total prodigiosin production was assayed by absorbance at 538 nm in toluene after alkaline extraction to remove actinorhodin and acidification (adapted from Kim et al. 2007). The constructs were also transformed into Streptomyces sp. MBK6 itself, but only for pIJ486-MbkZsym (ermEp-mbkZp) was the transformation successful, despite several attempts. Production of prodigiosins by the various strains in both media is shown in Fig. 4. Wild-type S. coelicolor produced 30 times, and Streptomyces sp. MBK6 75 times more prodigiosins in Y2 medium than in YE medium. Introduction of pIJ486-MbkZsym (mbkZp) in S. coelicolor stimulated production in both Y2 and YE. The construct pIJ486-MbkZsym (ermEp-mbkZp), containing the strong constitutional, wild-type ermEp promoter preceding the mbkZ promoter region strongly suppressed the production level both in Y2 and YE in S. coelicolor, whereas in Streptomyces sp. MBK6 it stimulated production in both media. The repression was observed in S. coelicolor also with the construct containing only the ermEp promoter [pIJ486-MbkZnop (ermEp)].

The visible effect on the production of prodigiosins of mbkZ led us to investigate the binding of MbkZ to the promoter regions of known and putative, respectively, Streptomyces antibiotic regulatory proteins (SARP) redD and mbkD by electrophoretic mobility shift assay (EMSA). Histidine tagged recombinant MbkZ produced in Escherichia coli and purified to near homogeneity by affinity chromatography binds to the promoter region of mbkD as well as to the promoter region of S. coelicolor redD (Fig. 5).

**DISCUSSION**

Streptomyces possess a great potential to produce new bioactive substances from latent BGCs, which typically outnumber 10-fold the clusters for observed products. Finding the conditions and signals, which trigger the production of these normally unseen products, is a central research problem. This study originated from experiments attempting to use yeast cells as a stimulus for production. Wild isolates from soil samples were cultured in a medium containing yeast extract as a nitrogen source with or without the supplement of whole autoclaved yeast. The strain designated Streptomyces sp. MBK6 was found to produce a red substance in yeast-containing medium. It has subsequently been observed, that at high cell density the pigment is produced also without yeast, but as can be seen in Fig. 4, the production in Y2 medium is around 75 times that observed in YE medium.

The genome sequence of MBK6 confirmed the presence of a prodigiosin BGC, and further revealed that the genome shows a high similarity to a Streptomyces strain, S. griseoaurantiacus M045 (Li et al. 2011) isolated from marine sediment in China. Therefore, Streptomyces sp. MBK6 should be classified as a member of the species S. griseoaurantiacus. The similarity emphasizes the wide geographical and ecological distribution of Streptomyces; search of new isolates has concentrated on exotic habitats, but an essentially identical strain could be isolated from a stereotypical source, ordinary soil.

The two major pigmented products of Streptomyces sp. MBK6 were identified as undecylprodigiosin and metacycloprodigiosin. In S. coelicolor the cyclized prodigiosin is butylo-cloheptylprodigidine (Streptorubin B; Taso et al. 1985). Reaction specificity difference at MbkG, the homolog of RedG, a non-haem iron dependent dioxygenase (Sydor et al. 2011), may cause the structural difference. It is notable, that MbkG is more like sydorA, the haemirondependentdioxygenase(Sydor 2011), than RedG (Figure S14, Supporting Information). Generally, the Streptomyces sp. MBK6 prodigiosin BGC is almost colinear with that of S. coelicolor, except that the gene homologous to redF is absent from both the gene cluster and the genome. This suggests that the gene is not essential, whereas SCOS899 (redE) may have a role in the biosynthesis, as it is conserved in the gene cluster. However, it appears to be an acyltransferase family protein; it has been suggested (Cerdeño, Bibb and Challis 2001) that redF is the O-methyltransferase that complements the redE mutation. Therefore, the function of

**Genome sequencing of Streptomyces sp. MBK6**

Streptomyces sp. MBK6 is a new strain from a soil sample in Finland. The synthetic gene cluster is essentially colinear with the well-characterized red cluster of S. coelicolor (Malpartida et al. 1990; Cerdeño, Bibb and Challis 2001; Hu et al. 2016; Fig. 3A). The most significant differences are the lack of a homolog of the oxi-doreductase redD of unknown function, translational fusion of the type I polyketide synthase redL and the oxi-doreductase redK (Cerdeño, Bibb and Challis 2001) and extension of sequence similarity to SCOS899, which in some publications has been named redE (Hu et al. 2016). Importantly, we noted that the N-terminal part of redZ, the primary regulator of the red BGC (Guthrie et al. 1998), was not conserved.

A BLAST (Altschul et al. 1990) search of the Streptomyces clade of the refseq_genomes database at NCBI with the Streptomyces sp. MBK6 undecylprodigiosin cluster indicated a 98.91% nucleotide sequence identity with a Streptomyces griseoaurantiacus strain M045 (Li et al. 2011) sequence (NZ_AEXY1000041.1). The 16S rRNA sequence of Streptomyces sp. MBK6 has only one base difference from the 16S rRNA of S. griseoaurantiacus M045. Comparison of the genomes using Easyfig (Sullivan, Petty and Beatson 2011) revealed extensive colinearity and high sequence similarity, indicating the two strains are closely related (Figure S13, Supporting Information). Production of prodigiosins by S. griseoaurantiacus M045 has been observed (Li et al. 2005). S. griseoaurantiacus M045 has been isolated from marine sediment in China, whereas Streptomyces sp. MBK6 has been isolated from a soil sample in Finland.

Streptomyces sp. MBK6 75 times more prodigiosins in Y2 medium than in YE medium. Introduction of pIJ486-MbkZsym (mbkZp) in S. coelicolor stimulated production in both Y2 and YE. The construct pIJ486-MbkZsym (ermEp-mbkZp), containing the strong constitutional, wild-type ermEp promoter preceding the mbkZ promoter region strongly suppressed the production level both in Y2 and YE in S. coelicolor, whereas in Streptomyces sp. MBK6 it stimulated production in both media. The repression was observed in S. coelicolor also with the construct containing only the ermEp promoter [pIJ486-MbkZnop (ermEp)].

The visible effect on the production of prodigiosins of mbkZ led us to investigate the binding of MbkZ to the promoter regions of known and putative, respectively, Streptomyces antibiotic regulatory proteins (SARP) redD and mbkD by electrophoretic mobility shift assay (EMSA). Histidine tagged recombinant MbkZ produced in Escherichia coli and purified to near homogeneity by affinity chromatography binds to the promoter region of mbkD as well as to the promoter region of S. coelicolor redD (Fig. 5).
Figure 3. (A) EASYFIG (Sullivan et al. 2011) comparison of the S. coelicolor red cluster and the MBK6 prodigiosin cluster. (B) Alignment (Clustal Omega, Sievers and Higgins 2014) of RdmZ and MbkZ annotated with Espript (Robert and Gouet 2014); the secondary structures are derived from the model in panel C. (C) Homology model of RedZ, generated by the SwissModel (Waterhouse et al. 2018) server using as template 4LDZ (Bacillus subtilis response regulator DesR), with amino acids conserved with MbkZ colored red.

Figure 4. Comparison of prodigiosin production (measured as A_{538} of extracts) of wild-type S. coelicolor and Streptomyces sp. MBK6 as well as the same transformed with indicated mbkZ containing constructs in Y2 medium (with yeast, A) and in YE medium (without yeast, B). Panel B is shown with magnified Y-axis.
SC05899 remains unclear. In Streptomyces sp. MBK6, redL and redK homologs are part of the same ORF.

The most striking difference is the lack of similarity in the N-terminal part of redZ and its counterpart mbkZ. Prodigiosin biosynthesis in S. coelicolor is regulated in many ways at a global level (Liu et al. 2013). The transcription of the cluster situated redZ is prevented during primary metabolism by two-component sensor kinases systems that control cell division (Som et al. 2017) and antibiotic biosynthesis (Sheeler, MacMillan and Hodwell 2005; Lewis et al. 2019), such as mtrA/mtrB and absA1/absA2, respectively. In addition, the presence of nutrients such as N-acetylglucosamine derived from chitin prevents the activation of redZ through the action of DasR (Rigali et al. 2008). The interesting feature of prodigiosins is that these cytotoxic metabolites are found intracellularly in S. coelicolor, which have led to the proposal that these compounds act to induce controlled cell death in Streptomyces (Rigali et al. 2008; Tenconi et al. 2018). The hypothesis is that upon nutrient depletion certain parts of the mycelium are sacrificed to ensure development of aerial hyphae and spores that guarantee the survival of the colony. Under these circumstances our observation that the presence of yeast induces production of prodigiosins also in S. coelicolor is noteworthy.

The situation seems to be different in Streptomyces sp. MBK6. This strain appeared to respond to the presence of yeast and sequester yeast cells to the mycelium, which S. coelicolor did not do. Therefore, it may be that this physical interaction leads to use of prodigiosins as antifungal agents, which is an activity that has been demonstrated (Stankovic et al. 2014).

These possible fundamental differences (Fig. 6) may also be reflected on the results from the expression studies and cross-regulation attempts that are presented here. In Streptomyces sp. MBK6/pJE486-MbkZsyn (ermEp-mbkZp) constitutive expression of mbkZ initiates production of prodigiosins already during day 1 and promotes biosynthesis even in the absence of intact yeast cells. This indicates that the cluster-situated regulatory network is likely to be similar in Streptomyces sp. MBK6 and S. coelicolor, where MkbZ binds to the promoter region of mbkD, which in turn activates the biosynthetic genes. However, the situation seems to be more complex on a global level as revealed by the expression trials of mbkZ in S. coelicolor. Early expression of mbkZ from a constitutive promoter (either S. coelicolor/pJE486-
MbKZyn(ermEp-mbkZp) or S. coelicolor/pIJ486-MbkZsyn(ermEp) seems to lead to repression of prodigiosin production. This may be due to binding of MbKZ to the redDp region, which we could observe to occur experimentally, but the inability of MbKZ to function with the S. coelicolor RNAP and other transcription factors may prevent activation of prodigiosin production. In addition, MbKz may also bind to other regions in the genome of S. coelicolor that may influence production of antibiotics in unexpected ways. In contrast, expression of mbkz from its natural promoter in S. coelicolor/pIJ486-MbkZsyn(mbkZp) enhances production of prodigiosins. We speculate that this may be due to a gene dose effect and particularly the presence of multiple mbkZp promoter sequences in the multi-copy number plasmid (Kieser et al. 1982). These trans acting elements may bind the natural repressors of the prodigiosin pathway such as MtrB, AbsA2 and DasR, which would allow production of RedZ and activation of redD transcription.

In conclusion, the work provides an intriguing account on the prevalence and diversification of soil microbes. On the one hand, we show the isolation of near identical Streptomyces strains from vastly different geographical locations and habitats, and the presence of a prodigiosin gene cluster that is highly similar to the one residing in the model organism S. coelicolor. However, changes in the receiver domain of the key regulatory protein MbKZ has led to adaptation and differential regulation of production of prodigiosins in response to varied environmental signals.

ACKNOWLEDGMENTS

Streptomyces coelicolor M145 strain was kindly provided by Dr Mark Buttnner at the John Innes Institute, Norwich, UK. The authors wish to acknowledge CSC—IT Center for Science, Finland, for computational resources.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

FUNDING

This work was supported by the Jane and Aatos Erikko Foundation (to M. M.-K.); the Turku University Foundation (to B. B. and K. Y.) and the Finnish Cultural Foundation (to K. Y.).

Conflicts of Interest. None declared.

REFERENCES

Altschul SF, Gish W, Miller W et al. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
Assefa S, Keane TM, Otto TD et al. ABACAS: algorithm-based automatic contiguation of assembled sequences. Bioinformatics 2009;25:1968–9.
Baral B, Akhgari A, Metsä-Ketelä M. Activation of microbial secondary metabolic pathways: avenues and challenges. Synth Syst Biotechnol 2018;3:163–78.
Barnard B. Isolation of antibiotic-producing organisms from soil. 1994. https://www.accessexcellence.org/AE/AEC/AEF/1994/barnard_isolation.html?
Bentley SD, Chater KF, Cerdeño-Tárraga A- et al. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature 2002;417:141–7.
Blin K, Shaw S, Steinke K et al. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res 2019;47:W81–7.
Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
Brettin T, Davis JJ, Disz T et al. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep 2015;5:8365.
Cerdeño AM, Bibb MJ, Challis GL. Analysis of the prodiginine biosynthesis gene cluster of Streptomyces coelicolor A3(2): new mechanisms for chain initiation and termination in modular multienzymes. Chem Biol 2001;8:817–29.
Coil D, Jospin G, Darling AE. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics 2015;31:587–9.
Crowther TW, van den Hooogen J, Wan J et al. The global soil community and its influence on biogeochemistry. Science 2019;365:eaav0550.
Guthrie EP, Flaxman CS, White J et al. A response-regulator-like activator of antibiotic synthesis from Streptomyces coelicolor A3(2) with an amino-terminal domain that lacks a phosphatase binding pocket. Microbiology 1998;144:727–38.
Hu DX, Withall DM, Challis GL et al. Structure, chemical synthesis, and biosynthesis of prodiginine. Nat Prod Chem Rev 2016;116:7818–53.
Jones SE, Ho L, Rees CA et al. Streptomyces exploration is triggered by fungal interactions and volatile signals. Elife 2017;6:21738.
Kallio P, Sultana A, Niemi J et al. Crystal structure of the polyketide cyclase AknH with bound substrate and product analogue: implications for catalytic mechanism and product stereoselectivity. J Mol Biol 2006;357:210–20.
Khalil ZG, Cruz-Morales P, Licona-Cassani C et al. Inter-Kingdom beach warfare: microbial chemical communication activates natural chemical defences. ISME J 2019;13:147–58.
Kieser T, Hopwood DA, Wright HM et al. The John Innes Foundation, Norwich, UK. 2000.
Kim YJ, Sa SO, Chang YK et al. Overexpression of Shi-norhizobium melloti hemoprotein in Streptomyces lividans to enhance secondary metabolite production. J Microbiol Biotechnol 2007;17:2066–70.
Lewis RA, Wahab A, Bucca G et al. Genome-wide analysis of the role of the antibiotic biosynthesis regulator AbsA2 in Streptomyces coelicolor A3(2). PLoS One 2019;14:e0200673.
Li F, Jiang P, Zheng H et al. Draft genome sequence of the marine bacterium Streptomyces griseorauranticus M045, which produces novel manumycin-type antibiotics with a pABA core component. J Bacteriol 2011;193:3417–8.
Li F, Maskey RP, Qin S et al. Chinkomycins A and B: isolation, structure elucidation, and biological activity of novel antibiotics from a marine Streptomyces sp. isolate M045. J Nat Prod 2005;68:349–53.
Liu G, Chater KF, Chandra Govind et al. Molecular regulation of antibiotic biosynthesis in Streptomyces. Microbiol Mol Biol Rev 2013;77:112–43.
Luti K, Mavituna F. *Streptomyces coelicolor* increases the production of undecylprodigiosin when interacted with *Bacillus subtilis*. Biotechnol Lett 2011;33:113–8.

Malpartida F, Niemi J, Navarrete R et al. Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. Gene 1990;93:91–9.

Newman DJ, Cragg GM. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J Nat Prod 2020;83:770–803.

Nikodinovic J, Barrow KD, Chuck J. High yield preparation of genomic DNA from *Streptomyces*. BioTechniques 2003;35:932–6.

Nützmann H, Reyes-Dominguez Y, Scherlach K et al. Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc Natl Acad Sci 2011;108:14282–7.

Rigali S, Titgemeyer F, Barends S et al. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*. EMBO Rep 2008;9:670–5.

Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res 2014;42:W320–4.

Sheeler NL, MacMillan SV, Nodwell JR. Biochemical activities of the absA two-component system of *Streptomyces coelicolor*. J Bacteriol 2005;187:687–96.

Sievers F, Higgins DG. Clustal omega. Curr Protocols Bioinformatics 2014;48. DOI: 10.1002/0471250953.bi0313s48.

Simão FA, Waterhouse RM, Ioannidis P et al. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 2015;31:3210–2.

Som NF, Heine D, Holmes N et al. The MtrAB two-component system controls antibiotic production in *Streptomyces coelicolor* A3(2). Microbiology 2017;163:1415–9.

Stankovic N, Senerovic L, Ilic-Tomic T et al. Properties and applications of undecylprodigiosin and other bacterial prodigiosins. Appl Microbiol Biotechnol 2014;98:3841–58.

Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. Bioinformatics 2011;27:1009–10.

Sydor PK, Barry SM, Oudalov OM et al. Regio- and stereodivergent antibiotic oxidative carbocyclizations catalysed by Rieske oxygenase-like enzymes. Nat Chem 2011;3:388–92.

Tang JS, Gillevet PM. Reclassification of ATCC 9341 from *Micrococcus luteus* to *Kocuria rhizophila*. Int J Syst Evol Microbiol 2003;53:995–7.

Tenconi E, Traxler MF, Hoebreck C et al. Production of prodigines is part of a programmed cell death process in *Streptomyces coelicolor*. Front Microbiol 2018;9:1742.

Tsai JJ, Otto TD, Berriman M. Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps. Genome Biol 2010;11:R41.

Tsao SW, Rudd BA, He XG et al. Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. J Antibiot (Tokyo) 1985;38:128–31.

Ward JM, Janssen GR, Kieser T et al. Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol Gen Genet MGG 1986;203:468–78.

Wasserman HH, Rodgers GC, Keith DD. Metacycloprodigiosin, a tripyrrole pigment from *Streptomyces longisporus ruber*. J Am Chem Soc 1969;91:1263–4.

Waterhouse A, Bertoni M, Bienert S et al. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res 2018;46:W296–303.

Yamada K, Koroleva A, Laughlin M et al. Characterization and overproduction of cell-associated cholesterol oxidase ChoD from *Streptomyces lavendulae* YAKB-15. Sci Rep 2019;9:11850–8.

Ylihonko K, Tuikkanen J, Jussila S et al. A gene cluster involved in nogalamycin biosynthesis from *Streptomyces nogalater*: sequence analysis and complementation of early-block mutations in the anthracycline pathway. Mol Gen Genet 1996;251:113–20.