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**Heterologous expression of biosynthetic gene clusters
for secondary metabolites in *Streptomyces*:**

Recent results, limitations and future challenges

Bachelor Thesis

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Abstract

The search for novel antibiotics with high yields is urgent because of antibiotic resistance by microbial pathogens. Discovery of novel secondary metabolites, activation of cryptic (silent) gene clusters and enhancement of yield is not only necessary for medical reasons but also of scientific interest and for (commercial) use in agriculture, fuel and food industry.

Traditional techniques like optimizing culture conditions and mutagenesis were used to improve secondary metabolites yields. Modern methods like biosynthetic engineering in combination with homologous and heterologous expression are used to start expression of silent gene clusters and to increase titer of valuable compounds.

Research in heterologous expression showed successful results. High yields of secondary metabolites were produced using minimal host engineered from three workhorse strains of *Streptomyces* species. Phylogenetic related species, related genes clusters and transfer of complete gene clusters are additional factors for success.

Adding traditional techniques and methods successful in homologous expression will improve heterologous expression even more.

Introduction

Streptomyces

Streptomyces are Gram-positive (Campbell and Reese, 2008) bacteria. They are the largest genus of Actinomycetes that belong to the phylum of Actinobacteria (Madigan et al., (2012). They are aerobic bacteria living in soil and on decaying vegetation. To humans they are (rarely) pathogenic. In three important aspects *Streptomyces*, like other Gram-positive bacteria, differ from most other microorganisms (Omura et al., 2001). A morphological feature is formation of filamentous mycelia, aerial hyphae and spores. A fungi-like behaviour before sporulation requires specialized and complex metabolism. More than this *Streptomyces* species are known for their ability to produce numerous secondary metabolites using a diversity of pathways. Common to *Streptomyces* is linearity of the chromosome. Depending on the species their DNA contains 8-10 MB base pairs and a GC content of 70%. Both figures are higher for *Streptomyces* than for any other microorganism specie. The three aspects make *Streptomyces* efficient and reliable producers of secondary metabolites and therefore an object of interest for scientific research and in industrial settings (Omura et al., 2001). During vegetative phase *Streptomyces* produce primary metabolites (Hwang et al. 2013) used for growth. Stress causes transition into reproductive sporulation phase during which *Streptomyces* start to produce secondary metabolites (Nieselt et al., 2010). Vegetative phase means exponential growth and sporulation phase has stationary growth.

Secondary metabolites

Secondary metabolites are a group of valuable compounds (Nguyen et al., 2012) being used in medicine, agriculture, biofuels and food. Secondary metabolites are often thought to be synonymous with antibiotics (Martin and Liras, 2010) but much more compounds belong to the group of secondary metabolites. Drugs like antitumor compounds, immunosuppressors, antiviral and antiparasitic agents and enzyme-inactivators (Olano et al., 2008) are pharmaceuticals. Insecticides and pesticides are important in agriculture. In food industries secondary metabolites are used as food additives and preservatives while biofuels are useful additions to or replacements of traditional fossil fuels.

Estimated is that 23,000 secondary metabolites are produced by microorganisms of which 16,500 are antibiotics (Nguyen et al., 2012) and of which *Streptomyces* produce 7,600. Only 150 have found their way into pharmacy, food, agriculture and other fields (Bérdy, 2005). During the golden age of antibiotics discovery (late 1940s to 1960s) *Streptomyces* were the main source of isolation (Hwang et al., 2013). Nowadays only 20-30% of newly discovered antibiotics are isolated from *Streptomyces*.

Because of emerging antibiotic resistance the need for novel antibiotics with high yields is great. Novel secondary metabolites with useful antibiotic applications are part of a large collection optimized for stability and activity by evolution (Medema et al., 2011). But native producers of secondary metabolites often have low or no yield at all and they often grow (too) slow or not at all under laboratory conditions and in industrial settings.

To start production or enhance yield of secondary metabolites *Streptomyces* can play an important role for three reasons. First, various species of *Streptomyces* (*coelicolor*, *avermitilis* and *lividans*) are producers of secondary metabolites with high yield (including antibiotics) and are expected to be sources for novel and useful compounds. Second, complete genomes are sequenced (Nett et al., 2009) in 2002 and 2003 making *Streptomyces* more suitable for further research and DNA manipulations than other microorganisms. Third, growth rates are stable of *Streptomyces* (Hwang et al. 2013) and compound extraction is easy compared to other microorganisms and less complex than from plants (Xu, 2011).

Wild-type *Streptomyces* species are low yield secondary metabolite producers while high titters are required. Using workhorses have increased titter 1000-fold up to 100,000-fold (Demain, 2006).

Genes of microorganisms coding for secondary metabolites are often organized in (biosynthetic) gene clusters located away from the (stable) central part of the chromosome (Martin, 2004) near the telomeres (Takano, 2006). Gene clusters for secondary metabolites make up 6.4% of total DNA (Omura et al., 2001) in *Streptomyces* species.

Gene clusters contain all necessary parts for secondary metabolite biosynthetic pathways (Nguyen, 2011) having genes for regulators, enzymes (biosynthesis) and transport systems. Regulators are for starting, enhancing, downsizing and stopping biosynthesis. Enzymes are responsible for biosynthesis of secondary metabolites and protein transport systems (PTS) are for transporting compounds out (secretion) of the cell.

Theoretically the switch-like and module-like constructions of gene cluster make it possible to regulate gene clusters in native organisms and to transfer complete gene clusters (Medema et al., 2011) from organisms (donor) to other organisms (host). These two methods are used to start expression of cryptic gene clusters (homologous) and to enhance yield of useful compounds (heterologous).

Heterologous expression is the main subject of this thesis. An overview of recent results using three species of *Streptomyces* (*coelicolor*, *avermitilis* and *lividans*) is given in the next chapter. Explanations for successful or failed heterologous expression are described including additional adaptations made to turn failure into success.

The final chapter shows an overall picture of the factors for success (and failure) and gives an advice how to commence with heterologous expression.

Heterologous expression of gene clusters for secondary metabolites

Native producers of secondary metabolites often have low or no yield at all and grow (too) slow or not at all under laboratory conditions and in industrial settings. Strains like *Streptomyces coelicolor*, *Streptomyces avermitilis* or *Streptomyces lividans* proved that production of (novel) these compounds at large scale is possible.

Research on heterologous expression of gene clusters for secondary metabolites in *Streptomyces* strains is described in this chapter. Success and failure are explained when possible and future challenges are mentioned.

Streptomyces coelicolor

Streptomyces coelicolor is the most studied, fully sequenced and easy manipulated strain of *Streptomyces* (Gomez-Escribano & Bibb, 2013). This strain is used often as a host because it proved to be capable of expression of various gene clusters and be able to obtain high yields.

Production of polyketides and non-ribosomal peptides is native to (most) *Streptomyces coelicolor* strains. Meaning that precursors are supplied by primary metabolism.

Heterologous expression was used to prove that gene clusters were transferred, to obtain higher yield of gene clusters from native organisms that are difficult to grow or to express (cryptic) gene clusters (Gomez-Escribano & Bibb, 2012).

Table 1 gives an overview of heterologous expression in *Streptomyces coelicolor*.

Table 1 Gene clusters expressed in the optimized *S. coelicolor* hosts strains M1146, M1152, and M1154

Metabolite	Metabolite class	Natural producing micro-organism	Aims	Yield
Cypemycin	Linaridin (RiPP)	<i>Streptomyces</i> sp. OH-4156	2, 3	Not reported
Grisemycin	Linaridin (RiPP)	<i>Streptomyces griseus</i> IFO 13350	2, 4	Not reported
Actagardine	Lantibiotic (RiPP)	<i>Actinoplanes garbadinensis</i> ATCC31049	2, 3	Not reported
Planosporicin	Lantibiotic (RiPP)	<i>Planomonospora alba</i>	2	Not reported
GE37468	Thiopeptide (RiPP)	<i>Streptomyces</i> sp. ATCC 55365	6	Not reported
Napsamycin	Uridylpeptide	<i>Streptomyces</i> sp. DSM5940	2	Not reported
Clorobiocin	Aminocoumarin	<i>Streptomyces roseochromogenes</i> var. <i>oscitans</i> DS 12.976	5	158 mg/l
Coumermycin A1	Aminocoumarin	<i>Streptomyces rishiriensis</i> DSM 40489	5	160 mg/l
Caprazamycin	Liponucleoside	<i>Streptomyces</i> sp. MK730-62F2	5	152 mg/l
FK506/FK520 (tacrolimus)	Polyketide	<i>Streptomyces tsukubaensis</i> NRRL 18488	2	2.81 mg/l
Merochlorins	Polyketide-terpenoid	<i>Streptomyces</i> sp. CNH-189	2, 3	Not reported
Gougerotin	Peptidyl nucleoside	<i>Streptomyces gramineus</i> CGMCC 4.506	2, 3	Not reported
Endophenazine	Phenazine	<i>Streptomyces anulatus</i> 9663	2, 3	20 mg/l
Roseoflavin	Riboflavin (vitamin B2)	<i>Streptomyces davawensis</i> JCM 4913	2	0.4 mg/l
Staurosporine and streptocarbazoles	Indolocarbazoles	<i>Streptomyces sanyensis</i> FMA	2	Not reported
Holomycin	Thiopeptide (NRPS)	<i>Streptomyces clavuligerus</i> ATCC27064	2, 3	Not reported
Chloramphenicol	Shikimic acid pathway	<i>Streptomyces venezuelae</i>	1	40 mg/l
Congocidine	Pyrrole-amide (oligopyrrole)	<i>Streptomyces ambofaciens</i>	1	Not reported
Tunicamycin	Fatty-acyl nucleoside	<i>Streptomyces chartreusis</i> NRL3882	2, 3	Not reported
Unsuccessful product detection				
Microbisporicin	Lantibiotic	<i>Microbispora coralina</i>	2	0
Platencin	Platencin and platensimycin	<i>Streptomyces platensis</i> MA7327	2, 3	0

1, validation of host strains; 2, cloning and characterization of biosynthetic gene cluster; 3, study of biosynthetic pathway; 4, characterization of metabolite; 5, optimization of production; 6, generation of unnatural compounds; *RiPP*, ribosomally synthesized post-translationally modified peptide

To obtain success in some cases adaptations were made to gene clusters. Only after changing regulators or promoters gene clusters were expressed and compounds were produced.

Expression of endophenazine gene cluster (Saleh et al., 2012) from *Streptomyces anulatus* gave very different results in various strains of *Streptomyces coelicolor*. Endophenazine A was produced by M512 but not by two other strains.

Placentin biosynthetic gene cluster (Smanski et al., 2012) from *Streptomyces platensis* MA7327 was well expressed in *Streptomyces lividans* but not in any other strain though production in *Streptomyces lividans* was detected after deletion of regulator *ptnR1*. No reason was given why deletion did not work in other strains.

Silencing complete gene clusters used for endogenous secondary metabolism was also an important and successful strategy.

Replacing native promoters by *hrdB* resulted (Du et al., 2013) in substantial increased levels of production in *Streptomyces coelicolor* because promoter *hrdB* is much stronger than native promoters.

Bottromycin production in *Streptomyces coelicolor* is normally low because bottromycin is toxic. Production was remarkably increased when a promoter

(*ermEp*) encoding for a transport system was introduced. Phanosporicin production was achieved after removing a (negative) regulator. Generally expression of gene clusters from phylogenetic distant organisms is difficult in *Streptomyces coelicolor* because immunity mechanisms fail or fail-safe systems prevent production.

The *spc* gene cluster from a marine-derived *Streptomyces sanyensis* FMA was first successfully expressed in *Streptomyces coelicolor* M1152. In an experiment (Li et al., 2013) to the biosynthetic machinery, rather than to the yield, heterologous expression was used. The chromosome of host *Streptomyces coelicolor* does not have any gene cluster for ICZ biosynthesis. This *spc* gene cluster is responsible for biosynthesis of indolocarbazole (ICZ) alkaloids in *Streptomyces sanyensis* FMA (Fu et al., 2008). ICZ are a group of molecules with unique structures. More than 130 different ICZ have been isolated in the last 35 years and they are capable of inhibiting protein kinases, topoisomerases and ATP-binding transporters and arresting cell cycle (Sanchez et al., 2006).

The *spc* gene cluster is a 34.6-kb DNA region. The *spc* gene cluster has 19 open reading frames (OFR) and 15 of them have biosynthetic function. Importance of the various *spc* genes was proven by inactivation.

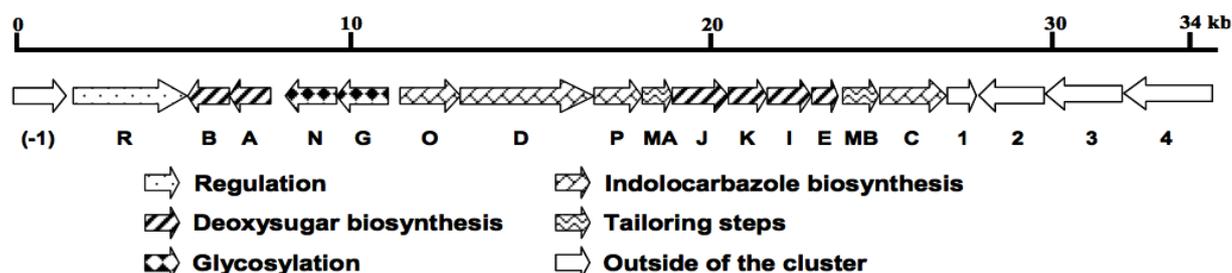


Figure 1. Genetic organization of the *spc* biosynthetic gene cluster.

The *spc* gene cluster contains genes for ICZ ring formation (*spcO*, *D*, *P*, *C*), sugar formation (*spcA*, *B*, *E*, *K*), glycosylation (*spcN*, *G*), methylation (*spcMA*, *MB*) and regulation (*spcR*).

All ICZ rings are synthesized from two molecules of tryptophan in a series of oxidation steps through halogenation, oxidation or reduction of the C-7 carbon. The glycosylation of indole nitrogen varies between different ICZ. Enzymes (FAD-dependent monooxygenases) involved in ICZ ring formation are highly conserved. This information was used to make primers a probe to isolate the *spc* gene cluster. Cosmid pWL1615 containing the *spc* gene cluster was adapted with *oriT* and *attP* for integration at the *attB* site of the chromosome of *Streptomyces coelicolor*. New cosmid pWL1617 was transferred by conjugation to the host using *Escherichia coli* ET12567/pUZ8002.

Expression of the *spc* gene cluster is influenced by culture conditions. Different culture conditions led to metabolic changes and to different ICZ profiles. Resulting yield of the host was comparable to that of the native

organism (*Streptomyces sanyensis* FMA). No explanation was giving for successful transfer and expression of the *spc* gene cluster.

A cryptic streptothricin (ST) cluster in *Streptomyces* TP-A0356 was discovered (JinE et al., 2013) comparing the DNA sequence with other *Streptomyces* strains. The ST gene cluster was heterologously expressed in *Streptomyces coelicolor* M145. Research goal was to discover and identify ST gene clusters and propose biosynthetic pathways.

Streptothricin inhibits protein synthesis. In agriculture streptothricin is used for treatment (Inamori et al., 1990) against bacterial and fungal diseases.

Streptothricin is not used in medicine because of severe cytotoxicity (Witte, 2000).

Streptothricin contains a streptolidine base, a carbamoylated glucosamine and a poly-Beta-Lys chain. The streptolidine base is made of L-Arg, carbamoylated glucosamine from glucosamine and the poly-Beta-Lys chain is produced by a non-ribosomal peptide synthetase (NPRS) mechanism from L-Lys.

The ST gene cluster is a 30.5 Kb DNA fragment. A total of 24 genes are involved in ST biosynthesis. Working of the ST gene cluster was proven by inactivation of gene *stnO* encoding for an aminomutase responsible for Lys supply for the poly-Beta-Lys chain. All (*stn*) genes showed high identity to ST genes in other *Streptomyces* strains. Though the ST gene cluster from *Streptomyces* TP-A0356 contains two extra MerR type regulators.

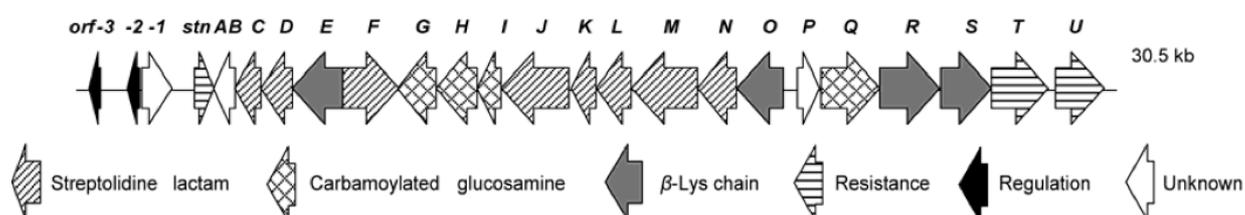


Figure 2. Organization of the streptothricin biosynthetic gene cluster from *Streptomyces* TP-A0356 (JinE et al. 2013)

Four genes (*stnE*, *stnO*, *stnR* and *stnS*) are involved in the elongation of the poly-Beta-Lys chain. Another four genes (*stnG*, *stnH*, *stnI* and *stnQ*) are responsible for the carbamoylated glucosamine and a group of genes (*stnC*, *stnD*, *stnF*, *stnJ*, *stnK*, *stnL*, *stnM* and *stnN*) are necessary for the streptolidine base. Self-protection of the ST producer is often achieved by secretion. Three transporter genes (*stnA*, *stnT* and *stnU*) are involved in this function. Regulation of the complete gene cluster is done by two genes (*orf-2* and *orf-3*) while three genes (*stnB*, *stnP* and *orf-1*) have unknown function. Cosmid (pTG6001) containing ST gene cluster was propagated in *Escherichia coli* JM109 and transferred to *Streptomyces coelicolor* M145 by conjugation. Together with the expected compounds ST-D and ST-F two new analogues were expressed and discovered using HPLC (High Performance Liquid Chromatography). Both new compounds are acetylated streptothricins named

Ac-ST-A and Ac-ST-B. Acetylation results in low antibacterial activity because this is detoxification strategy (Krugel et al., 1988).

A fragment with the complete gougerotin gene cluster was cloned from *Streptomyces gramineus* and expressed in *Streptomyces coelicolor*. Heterologous expression was used to identify the borders off the gene cluster and to discover biosynthetic pathways (Niu et al., 2012).

Gougerotin (aspiculamycin or asteromycin) is a peptidyl nucleoside (Cone et al., 2003) and is produced by various *Streptomyces* strains. Gougerotin has antitumor, antiviral and antibacterial activities making it an interesting antibiotic. Gougerotin inhibits amino acid incorporation during protein synthesis (Clark & Gunther, 1963). Peptidyl nucleoside is made from simple nucleoside and amino acid building blocks and consists of cytosine and 4-amino-4-deoxyglucoronamide forming the skeleton and a sarcosyl-D-serine dipeptide.

The fragment cloned was a 28.7 Kb DNA fragment containing 25 open reading frames (ORF) of which 15 are necessary for gougerotin production. Through inactivation and re-activation (genes *gouH* and *gouL*) the complete gene cluster was determined.

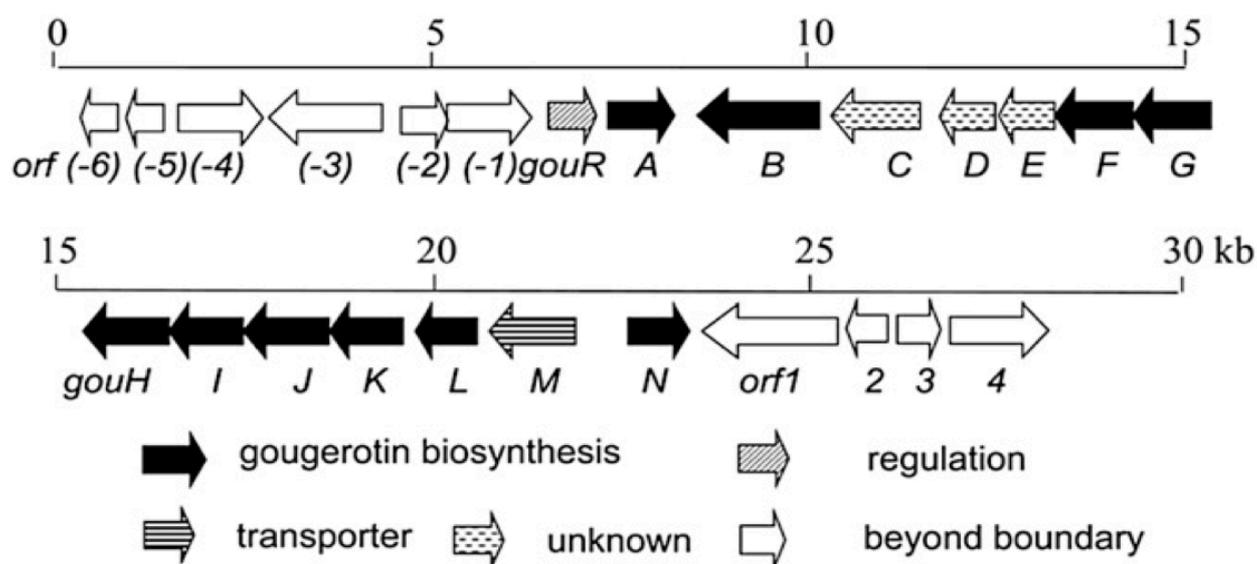


Figure 3. Organization of the Gougerotin Biosynthetic Gene Cluster in Fosmid D6-4H. Gene cluster spans 28.77 Kb fragment and contains 25 ORF (Niu et al. 2012)

Deletions in the gene cluster itself showed that 15 genes are necessary (13 production, 1 regulation, 1 secretion) to produce gougerotin.

Four genes (*gouA*, *gouB*, *gouF* and *gouH*) are involved in nucleotide skeleton biosynthesis and various genes (*gouG*, *gouI*, *gouL*, *gouN*, *gouJ* and *gouK*) are responsible for the peptidyl part using glycine and serine as precursors. Gene *gouR* is identified the regulator. Because gougerotin may be toxic to its

producer secretion is necessary. Gene *gouM* encodes for a membrane protein transporter (MFS) transporting a variety of substrates (Law et al., 2008).

Compounds gougertin and blasticidin S share a common skeleton.

Sequence of genes coding for blasticidin S are known. This knowledge was used to search the gougertin gene cluster and to create a probe for cloning from a fosmid library (no reference given). Fosmid (D6-4H) contained the gene cluster and was cloned into plasmid (pSET152). Plasmid (pSET152) was inserted in *Streptomyces coelicolor* by conjugation using donor *Escherichia coli* ET12567.

Detection of gougertin by HPLC (High-performance liquid chromatography) was first interfered by four endogenous secondary metabolites. When *Streptomyces coelicolor* M1146 (Gomez-Escribano and Bibb, 2011) was used gougertin was detected because these four gene clusters were deleted. Yield was unknown.

Streptomyces avermitilis

Streptomyces strains are known for genetic instability (Chen et al, 2002). Compared to them *Streptomyces avermitilis* is stable plus the complete genome sequence is known making it a good candidate for research and industrial production. For research reasons *Streptomyces avermitilis* was constructed as a model host (Komatsu et al., 2013) and used for heterologous expression of gene clusters for secondary metabolites.

In large-deletion hosts 1.5 Mb from the 9 Mb genome is deleted. Genes for endogenous secondary metabolites were removed leaving only essential genes. This deletion makes analyses easy and reduces competition for precursors and energy. One striking character of *Streptomyces avermitilis* is the short terminal inverted repeats (TIR) near the telomeres (Alberts, B. et al., 2008) only 49 Kb long and shortest (Ikeda et al., 2003) among *Streptomyces* strains. Most strains have TIR from 1000 KB up to 100,000 Kb. About 6-6.5 Mb DNA is highly conserved in all *Streptomyces* strains (Ikeda et al., 201). This backbone is the region of essential genes and important for the development of a genome-minimized host.

More than 20 secondary metabolites were produced by *Streptomyces avermitilis* strains SUKA17 and SUKA22 (Komatsu et al., 2010). Both minimal hosts have the right side of the deleted region replaced by gene *loxP*. In SUKA17 wt *loxP* was used and in SUKA22 a mutant *loxP* was inserted to prevent recombination. SUKA17 and SUKA22 grow better and produce more biomass than native *Streptomyces* strains.

Table 1 shows results and methods used.

Based on the type of pathway results (yield) of various compounds are mentioned. Gene clusters for amino-glycoside, polyketide-peptide hybrid and shimate-derived compounds are normally absent in *Streptomyces avermitilis* while others are native.

Sugar Pathway

donor	gene cluster	size	genes	yield	method
<i>S. griseus</i> IFO 13350	Streptomycin			176	
<i>S. ribosidificus</i> ATCC 212942	Ribostamycin	35		8	pKU465cos
<i>S. kasugaensis</i> MB 273	Kasugamycin	20		5 - 7	single kastT
<i>S. kasugaensis</i> MB 273	Kasugamycin	20		7 - 12	multi kastT
<i>S. clavuligerus</i> ATCC 27062	Pholipomycin	26	17	20	pPHM1

Polyketide Pathway

donor	gene cluster	size	genes	yield	method
<i>S. rimosus</i> NRRL 2234	Oxytetracycline	25	22	20	
<i>Streptomyces</i> NA97	Resistomycin		18	360	pKU492
<i>Sacchropolyspora eryrthea</i>	Erythromycin	60		4	pKU503
<i>Kitasatospora setea</i> KM 6054	Bafilomycin B1	72		16	pKU503
<i>S. cyaneogriseus</i> NRRL 15774	Nemadectin	80		104	pKU503
<i>Streptomyces</i> MM53	Aureothin	35		116	pKU465
<i>Streptomyces</i> EM52	Leptomycin	80		5	pKU503

Amino Acid Pathway

donor	gene cluster	size	genes	yield	method
<i>S. clavuligerus</i> ATCC 27065	Holomycin	18	13	8	pHLM1
<i>S. lactacystinaeus</i> OM 6519	Lactacystin	12		30	pLTC1 + rpsJ
<i>S. clavuligerus</i> ATCC 27065	Clavulanic acid	25		16	pCLV1 + ccaR
<i>S. clavuligerus</i> ATCC 27065	Cephamicynin C			85	pCLV1 + ccaR

Shikimate Pathway

donor	gene cluster	size	genes	yield	method
<i>Lechevalieria aerolonigenes</i>	Rebeccamycin	16	10	7	pREB1
<i>S. anulatus</i> 3533 SV4 GM95	Novobiocin	29	23	1	pKU492
<i>S. venezuelae</i> ATCC 10712	Chloramphenicol	24	19	262	pCML1

MVA or MVE Pathway

donor	gene cluster	size	genes	yield	method
<i>S. exfoliatus</i> U5319	Pentalenolactone			12	- <i>ptl</i> + <i>penM</i>
<i>S. arenae</i> TU469	Pentalenolactone			23	+ <i>pntD</i> + <i>pntE</i>

Table 1. Heterologous expression of exogenous biosynthetic gene clusters in large-deletion mutants of *Streptomyces avermitilis*.

Size (Kb) and yield (mg/L).

Column **method** shows plasmids used or genes added (+) or removed (-).

Total of 18 gene clusters were expressed without adaptations, 2 gene clusters received an alternative promoter and 3 gene clusters were mutated (codon change) to achieve expression and production (Ikeda et al., 2013). Expression of native and not-native gene clusters in *Streptomyces avermitilis* were successful and showed not difference.

Lactacystin was not produced because *Streptomyces avermitilis* lacks the regulator located outside the gene cluster. Solution came from an alternative promoter sequence upstream.

While *Streptomyces clavuligerus* produces only traces of holomycin the large deletion host produced a more than 40-fold higher yield when cephamycin C or clavulanic acid biosynthesis was blocked.

Streptomyces lividans

The *lab* gene cluster from *Actinomadura namibiensis* was transferred to five different (Krawczyk et al., 2013) strains of *Streptomyces*. In two expression of *lab* gene cluster succeeded while in three cases expression failed. Though expression was successful in two strains some adaptations were made to produce (correct) labyrinthopeptins. Because mutagenesis in *Actinomadura namibiensis* was not possible suitable hosts for (engineered) labyrinthopeptins needed to be found.

Labyrinthopeptins are class III lantibiotics produced and posttranslational modified ribosomal peptides. Precursor peptide consists of a (N-Terminal) leader peptide and a core peptide (Willey and van der Donk, 2007). Core peptide is changed posttranslational by installing a triamino triacid labionin. Labionin is a structural variation of lanthionine. Antibiotic activity of labyrinthopeptin is against retroviruses and neuropathic pain (Meindl et al., 2010).

The *lab* gene cluster is a small 6.4 Kb DNA fragment containing only 5 genes.



Figure 4. Organization of the labyrinthopeptin (*lab*) gene cluster.

Structural genes *labA1* and *labA2* are precursor production genes, gene *labKC* encodes for a modifying enzyme and genes *labT1* and *labT2* encode for two different ABC transporters needed for secretion.

The complete *lab* gene cluster was put under control of promoter *ermE** and cloned in plasmid pUWLoriTapra. Resulting plasmid pUWLab was transferred to five strains of *Streptomyces* using *Escherichia coli* as a donor. Production of labyrinthopeptin was shown by HPLC.

Only *Streptomyces lividans* and *Streptomyces albus* produced labyrinthopeptin while *Streptomyces avermitilis*, *Streptomyces coelicolor* and *Streptomyces griseus* did not produce this compound. Failure of the latter three was not explained.

Streptomyces lividans produced more than *Streptomyces albus* and was therefor further investigated. Labyrinthopeptin A1 and A2 produced were mutated with additional amino acids (combinations of Asn, Arg, Asp and Ala) attached. Reason for this mutation was an incorrect cleavage of leader peptide. Absence of a protease gene in the *lab* gene cluster is responsible (Krawczyk et al., 2013) for the problem.

The problem was solved after introducing a codon for amino acid Met located between leader and core peptide to induce a better cleavage (Kaiser and Metzka, 1999). Production of correct labyrinthopeptin A1 was restored but no labyrinthopeptin A2 was produced at all. Only after switching the sequence of genes *labA1* and *labA2* and using leader peptide of labyrinthopeptin A1 production of correct labyrinthopeptin A2 was achieved.

Yields of labyrinthopeptin A1 (86 mg/L) and labyrinthopeptin A2 (14 mg/L) achieved by *Streptomyces lividans* are less compared to yields (90 mg/L respectively 39 mg/L) by the native producer *Actinomadura namibiensis*.

The thioviridamide biosynthesis gene cluster from *Streptomyces olivoviridis* was cloned into *Streptomyces lividans* and successfully expressed. Goal of the experiment (Izawa et al., 2013) was to identify the complete gene cluster. From *Streptomyces olivoviridis* NA05001 the thioviridamide biosynthesis gene cluster was transferred to *Streptomyces lividans* TK23. The thioviridamide gene cluster is native to *Streptomyces lividans*.

Compound thioviridamide is a N-acylated undecapeptide with five thioamide bonds as its most distinguishing mark. A thioamide bond is a C-S double bond replacing regular C-O double bond in amides. By posttranslational modification of a ribosomal precursor peptide compound thioviridamide is biosynthesized.

Antibiotic function of thioviridamide is inducing apoptosis in (E1A) transformed cells (Hayakawa et al., 2006). Transformed cells are cells that have taken up exogenous DNA.

The complete thioviridamide gene cluster is a 14.5 KB DNA fragment with 14 genes involved in the biosynthesis.

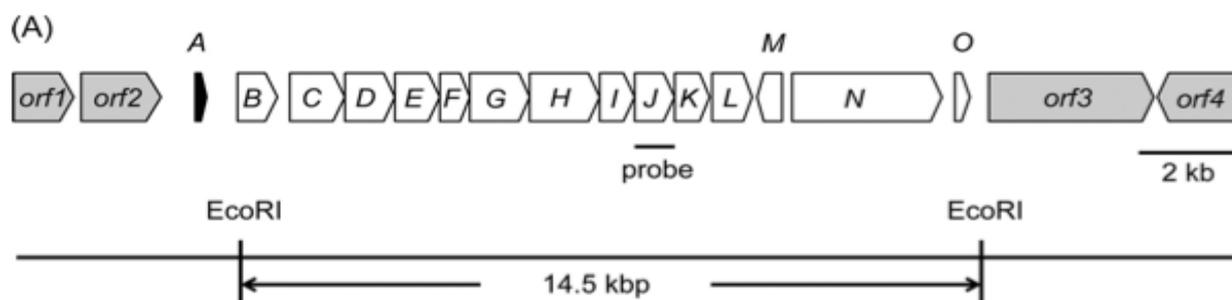


Figure 5. Thioviridamide biosynthesis gene cluster *Streptomyces olivoviridis* NA05001.

Gene (*tvaA*) is not part of the gene cluster but responsible for the production of a precursor peptide. Range *tvaC* – *tvaL* is for biosynthesis, except for gene *tvaI* that is a regulator. Three genes *tvaB*, *tvaM* and *tvaN* are also regulators while function of gene *tvaO* is unknown.

The 2.0 KB fragment *tvaA* and the 14.5 KB fragment *tvaB* – *tvaO* were cloned together into plasmid pWHM3 (Vara et al., 1989). Plasmid pWHM3-TVA was transferred from *Escherichia coli* JM110 to *Streptomyces lividans* TK23.

HPLC analysis showed thioviridamide production by *Streptomyces lividans* TK23.

Neither exact yield nor success factors for heterologous expression were mentioned.

Conclusions

Overview

The search for novel antibiotics with high yields is urgent because of antibiotic resistance by microbial pathogens. Discovery of novel secondary metabolites, activation of cryptic (silent) gene clusters and enhancement of yield is not only necessary for medical reasons but also of scientific interest and for (commercial) use in agriculture, fuel and food industry.

Increasing production of (bioactive) secondary metabolites is achieved through different ways (Olano et al., 2008)). The oldest method is by optimizing culture conditions (physico-chemical factors) like nutrition, oxygen, pH value and the temperature. A second option is traditional mutagenesis used to create strains with higher yield and improved resistance. Biosynthetic engineering is a third technique aimed at pathway improvement by redirecting precursors, mutating genes for regulators or promoters and expressing enzymes for biosynthesis. It can be used in combination with homologous and heterologous expression.

For all three methods proper function of gene clusters and pathways depend on factors influencing temporal and special control (Medema et al., 2011). Temporal control is dynamic regulation (timing) of expression of gene clusters and biosynthetic pathways. Temporal control is achieved by (a combination of) allosteric control, enzyme expression and synchronization. Allosteric control is influence of concentration of precursors or compounds on levels of expression (Holtz and Keasling, 2010). More sensitive regulators are used to switch on/off production or secretion of (toxic) compound. Producing enzymes (biosynthesis) is energy consuming for the cell. Just-in-time enzyme production saves energy. A synchronized transition from primary to secondary metabolism by all organisms in a population makes more efficient use of resources (Nieselt et al., 2010). All organisms in a population coordinate their action like in 'quorum sensing'.

Spatial control is prevention of leakage of intermediates during biosynthesis and is obtained by scaffolds, compartments and syntropy. Scaffolds fix intermediates during biosynthesis (Menzella et al., 2006). Compartments keep intermediates in captivity during processing and also prevent toxic compounds from harming the cell. Syntropy is co-operation between different species. Output from specie is input to another organism. Species specialized in partial biosynthesis have no need to possess all pathways. This makes syntropy efficient (Kim et al., 2008).

An optimized combination of temporal and special control results in higher yields.

Homologous expression of (silent) gene clusters was realized using a variety of techniques.

In *Streptomyces ansochromogenes* TH322 production of compound Nikkomycin (peptidyl nucleoside antibiotic) was increased by over expression

of structural genes (Li et al. 2005), multi-copies of regulator gene *sanG* (Liu et al. 2005) or additional copies of complete gene clusters (Liao et al. 2010).

Use of improved promoters (*hrdB* and *tcp830*) resulted in increased transcription of regulator gene *sanG* (Du et al., 2013) followed by enhanced activity of structural genes leading to nikkomycin production and yield of gougerotin was improved in *Streptomyces gramineus* after Phrb-driven (improved) promoters replaced native promoters (Jiang et al, 2013).

Duplication of gene clusters resulted in improved biosynthesis of antibiotics in *Streptomyces*. Lincomycin in *Streptomyces lincolnensis* (Peschke et al. 1995), kanamycin in *Streptomyces kanamyceticus* (Yanai et al. 2006) and nikkomycin in *Streptomyces ansochromogenes* (Liao et al. 2010) are some examples.

In *Streptomyces ahygroscopicus* production of tetramycin is in competition with Nystatin A₁ for precursors acyl-CoAs (Ren et al., 2013). Normally Nystatin A₁ yield is low but was improved when tetramycin biosynthesis was disrupted.

These methods do not conflict with heterologous expression. A combination of methods can support heterologous expression as research showed. Improved regulators and promoters (Gomez-Escribano and Bibb, 2013) and transport systems (Niu et al., 2013) increased expression of gene clusters in heterologous expression too.

Heterologous expression of (transferred) gene clusters was realized also using a diversity of techniques.

Phylogenetic related species (donor and host) improved chances for successful heterologous expression. Gene clusters (donor) related to native gene clusters (host) also improved success factors (Gomez-Escribano and Bibb, 2013). In this cases primary and secondary metabolisms are harmonized (Adrio and Demain, 2006) and presence of useful precursors and absence of toxic intermediates have increased success.

Content of transferred gene clusters is important in two aspects (Niu et al., 2013 / Izawa et al., 2013 / Komatsu et al., 2013). Incomplete gene clusters malfunction or remain silent. Over-complete gene clusters disturb biosynthesis or use too much valuable resources (Komatsu et al., 2013).

Occasionally additional genes outside gene clusters are needed to guaranty function (Izawa et al., 2013) or a combination of gene clusters (Krawczyk et al., 2013) has mutual dependence. In these situations transfer of separate gene clusters is not sufficient.

Minimal hosts are optimal acceptors of gene clusters. Non-essential genes and endogenous secondary metabolism gene clusters are removed. All resources (precursors and energy) are available for exogenous secondary metabolism of transferred gene clusters (Ikeda et al., 2013 / Komatsu et al., 2013).

Size (Kb) of transferred gene clusters matters. Transfer and expression of gene clusters up to 45 Kb is often successful. DNA fragments of 100 KB are difficult or even impossible to transfer and bring to expression (Ikeda et al., 2013).

Advice

Successful heterologous expression of (biosynthetic) gene clusters depend first and foremost on factors solely related to heterologous expression. The following three factors are essential starting points:

- Use of phylogenetic related species (donor and host) and the use of minimal hosts engineered from workhorses like *Streptomyces coelicolor*, *avermitilis* and *lividans* gave the best results.
- Transfer of exact and complete gene clusters resulted in minimal distortion and optimal use of resources.
- Maximum size of transferred gene clusters was 50 Kb.

Improved expression of gene clusters and increasing yield depend furthermore on techniques used in traditional methods to increase yield and success factors from homologous expression of (silent) gene clusters. The following two options should be added to the heterologous starting points:

- Creation of optimal culture conditions for host specie.
- Replacement or addition of improved promoters and regulators in the gene cluster for increased expression and biosynthesis.

Streptomyces species are not the sole solution to find novel (silent) secondary metabolites and express exogenous gene clusters with high yield. Also other microorganisms and chemical production of antibiotics will be necessary in the future.

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