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A novel reporter system for bacterial and mammalian cells based on the non-ribosomal peptide indigoidine

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ABSTRACT

The biosynthesis of non-ribosomal peptides, many of which have pharmaceutical activities, is an evolutionary privilege of microorganisms. Capitalizing on the universal set of the *Streptomyces lavendulae* non-ribosomal peptide synthase BpsA and the *Streptomyces verticillus* 4′-phosphopantetheinyl transferase Svp, we have engineered *Escherichia coli* as well as mammalian cells, including human stem cells, to produce the blue 3,3'-bipyridyl pigment keto-indigoidine and the reduced colorless but fluorescent leuco-isoform. Detailed characterization of a tailored substrate-free chromogenic assay and FACS analysis showed that indigoidine's blue color and fluorescence could be reliably profiled in bacteria and mammalian cells using standard multiwell-compatible detection equipment. Besides serving as an inexpensive, reliable, versatile and easy-to-assay cross-kingdom reporter system, the potential of having mammalian cells produce non-ribosomal peptides, preferably ones with biopharmaceutical activities, may provide novel treatment opportunities in future gene- and cell-based therapies.

1. Introduction

Non-ribosomal peptides are exclusively produced by microorganisms and belong to a class of peptide secondary metabolites that includes some of the most valuable medical products such as immunosuppressants (e.g., cyclosporine A), antibiotics (e.g., vancomycin), antiviral compounds (e.g., luzopeptin A) and anticancer drugs (e.g., echinomycin) (Watanabe et al., 2006). Unlike RNA-encoded proteins that are assembled by ribosomes from a set of proteinogenic amino acids, each non-ribosomal peptide is produced by a specific non-ribosomal peptide synthase (NRPS). NRPS are very large proteins containing sets of modules, each consisting of various catalytic domains that form a chemical assembly line that synthesizes peptides in a sequential multi-step enzymatic process (Marahiel and Essen, 2009). NRPS is produced as an inactive apoform that requires activation by a superfamily of 4′-phosphopantetheinyl transferases (PPTases), which transfer the phosphopantetheinyl group of coenzyme A (CoA) to a conserved serine residue of their thiolation (T-) domain (Lambalot et al., 1996; Sanchez et al., 2001). The resulting holoform of NRPS activates individual amino acids as aminoacyl-adenylates that are fixed at the thiol group of the module's T-domain before it is condensed at the C-domain with the amino acid residues of adjacent modules. The growing peptide chain moves then from one module to the next until the final peptide is released by the catalysis of the thioesterase domain (Strieker et al., 2010). Since many non-ribosomal peptides are clinically relevant, several metabolic engineering-based approaches have been devised to increase production titer and simplify the synthesis in non-mammalian host systems (Kosec et al., 2012; Olano et al., 2008; Qiao et al., 2011) or to enable simplified production in heterologous hosts (Siewers et al., 2009; Watanabe et al., 2006).

The blue 3,3'-bipyridyl pigment indigoidine is a powerful radical scavenger which enables phytopathogens such as *Streptomyces lavendulae* to tolerate H_2O_2, organic peroxides and superoxides produced as part of the plant defense program (Reverchon et al., 2002). Indigoidine is synthesized by condensation of two L-glutamines by a PPTase-activated NRPS (Reverchon et al., 2002; Takahashi et al., 2007). Although NRPS and fatty acid synthases share common enzymatic principles and components of equivalent activities, mammalian cells have so far not been reported to produce any type of non-ribosomal peptides. Here we show that mammalian cells engineered for concomitant expression of the *S. lavendulae* blue pigment synthase A (BpsA) and the *Streptomyces verticillus* PPTase (Svp) are able to produce indigoidine. This blue pigment can be used

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Table 1
Plasmids used and designed in this study.

Plasmid	Description and cloning strategy	Reference or source
pcDNA3.1	Mammalian expression vector (P_{hCMV}-MCS-pA)	Life Technologies, Carlsbad, CA
pCMV-GLuc	Constitutive GLuc expression vector (P_{hCMV}-GLuc-pA)	New England Biolabs, Ipswich, MA
pColaDuet-1	ColA replicon-based bacterial vector for P_{T7lac}-driven transgene expression	Merck, Darmstadt, Germany
pETDuet-1	ColE1 replicon-based bacterial vector for P_{T7lac}-driven transgene expression	Merck, Darmstadt, Germany
pMF111	Tetracycline-responsive SEAP expression vector ($P_{hCMV^{*}-1}$-SEAP-pA)	Fussenegger et al., 1997
pSS158	Constitutive SAMY expression vector (P_{hCMV}-SAMY-pA)	Schlatter et al., 2002
pTet-ON	Constitutive rtTA expression vector (P_{hCMV}-rtTA-pA)	Clontech, Palo Alto, CA
pUC57	Bacterial expression vector	GenScript, Piscataway, NJ
pDA43	Tetracycline-responsive GLuc expression vector ($P_{hCMV^{*}-1}$-GLuc-pA). GLuc was excised from pCMV-GLuc using *BamH*I/*Not*I and cloned into the corresponding sites (*BamH*I/*Not*I) of pMF111	This work
pMM45	pUC57-derived vector containing C-terminally HA-tagged BpsA (BpsA-HA) Insert synthesized by GenScript (Piscataway, NJ)	This work
pMM46	pUC57-derived vector containing C-terminally c-Myc-tagged Svp (Svp-c-Myc) Insert synthesized by GenScript (Piscataway, NJ)	This work
pMM47	Mammalian BpsA expression vector (P_{hCMV}-BpsA-HA-pA). BpsA-HA was excised from pMM45 using *Eco*RI/*Xba*I and cloned into the corresponding sites (*Eco*RI/*Xba*I) of pcDNA3.1	This work
pMM48	Mammalian Svp expression vector (P_{hCMV}-Svp-c-Myc-pA). Svp-c-Myc was excised from pMM46 using *Eco*RI/*Xba*I and cloned into the corresponding sites (*Eco*RI/*Xba*I) of pcDNA3.1	This work
pMM64	Bacterial BpsA expression vector (P_{T7lac}-BpsA-HA-pA). BpsA-HA was excised from pMM45 using *Eco*RI/*Hin*dIII and cloned into the corresponding sites (*Eco*RI/*Hin*dIII) of pETDuet-1	This work
pMM65	Bacterial Svp expression vector (P_{T7lac}-Svp-c-Myc-pA). Svp-c-Myc was excised from pMM46 using (*Eco*RI/*Hin*dIII) and cloned into the corresponding sites (*Eco*RI/*Hin*dIII) of pColaDuet-1	This work
pMM80	Tetracycline-responsive mammalian BpsA expression vector ($P_{hCMV^{*}-1}$-BpsA-HA-pA). BpsA-HA was excised from pMM45 using *Eco*RI/*Hin*dIII and cloned into the corresponding sites (*Eco*RI/*Hin*dIII) of pMF111	This work
pMM81	Tetracycline-responsive mammalian Svp expression vector ($P_{hCMV^{*}-1}$-Svp-c-Myc-pA). Svp-c-Myc was excised from pMM45 using *Eco*RI/*Hin*dIII and cloned into the corresponding sites (*Eco*RI/*Hin*dIII) of pMF111	This work
pMM95	Tetracycline-responsive SAMY expression vector ($P_{hCMV^{*}-1}$-SAMY-pA). SAMY was excised from pSS158 using *Spe*I/*Hin*dIII and cloned into the complementary sites (*Xba*I/*Hin*dIII) of pMF111	This work

Abbreviations: **BpsA**, blue pigment synthetase A of *Streptomyces lavendulae* (ATCC 11924) codon-optimized for *homo sapiens*; **c-Myc tag**, protein tag derived from the *c-myc* gene; **GLuc**, *Gaussia* Luciferase codon-optimized for *homo sapiens*; **HA tag**, protein tag derived from the human influenza hemagglutinin; **MCS,** multiple cloning site; **P_{hCMV}**, human cytomegalovirus immediate early promoter; **$P_{hCMV^{*}-1}$**, tetracycline-responsive promoter; **P_{T7lac}**, lac-inducible variant of the Phage T7 RNA Polymerase promoter; **rtTA,** reverse tetacycline-dependent transactivator; **SAMY**, *Bacillus stearothermophilus*-derived heat-stable secreted α-amylase; **SEAP**, human placental secreted alkaline phosphatase; **Svp**, *Streptomyces verticillus* (ATCC 15003) 4′-phosphopantetheinyl transferase (PPTase) codon-optimized for *homo sapiens*.

as a visual indicator peptide, a FACS-compatible fluorescent marker and a reporter compound to precisely measure gene expression using a custom-designed simple, robust, cheap, scalable and high-throughput-compatible assay. Functioning in bacteria as well as in mammalian cells including human stem cells, the indigoidine-based reporter assay could be used as a universal cross-kingdom reporter system. Also, the pioneering example that engineered mammalian cells can in principle be engineered to produce the new compound class of non-ribosomal peptides, many of which have therapeutic activities, may provide novel treatment opportunities for gene- and cell-based therapies.

2. Materials and methods

2.1. Vector design

Comprehensive design and construction details for all expression vectors are provided in Table 1.

2.2. Bacterial strains and indigoidine production

Escherichia coli strain XL10-Gold® (XL10-Gold® ultracompetent cells, Agilent Technologies, Basel, Switzerland; cat. no. 200314) was used for cloning and plasmid propagation. *E. coli* strain BL21 (DE3) Gold (Agilent Technologies; cat. no. 230132) was used for indigoidine production. All *E. coli* strains were grown at 37 °C on LB agar plates or in liquid LB medium (Beckton Dickinson, NJ, USA; cat. no. 244610) supplemented with appropriate antibiotics (Ampicillin, 100 mg/mL, cat. no. A9518; Kanamycin, 30 mg/mL, cat. no. K1377, both from Sigma-Aldrich, Munich, Germany). In transgenic *E. coli* liquid cultures (10 mL, cultivated for 4 h) expression of the indigoidine-producing enzymes BpsA and Svp was induced for 6 h using 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma-Aldrich, Munich, Germany; cat. no. I6758).

2.3. Cell culture and transfection

Human embryonic kidney cells (HEK293-T, ATCC: CRL-11268 (Mitta et al., 2002)) and human bone marrow stromal cells transgenic for the catalytic subunit of human telomerase (hMSC-TERT; (Simonsen et al., 2002)) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Basel, Switzerland; cat. no. 52100-039) supplemented with 10% (v/v) fetal calf serum (FCS; Bioconcept, Allschwil, Switzerland; cat. no. 2-01F10-I; lot no. PE01026P) and 1% (v/v) penicillin/streptomycin solution (Biowest, Nuaillé, France; cat. no. L0022-100). Human embryonic kidney cells adapted for growth in suspension (FreeStyle 293-F, Invitrogen, Carlsbad CA, USA) were cultivated in GIBCO® FreeStyle 293-F Expression Medium (Invitrogen, Carlsbad CA, USA; cat. no. 12338-018). All cell lines were cultivated at 37 °C in a 5% CO_2-containing humidified atmosphere. Cells grown in suspension were cultivated on an orbital shaker (IKA KS 260 basic, IKA®-Werke GmbH & CO. KG, Staufen, Germany) at 100 rpm. Whenever indicated, the culture medium was adjusted to specific pH and buffered using 25 mM HEPES (AppliChem GmbH, Darmstadt, Germany; cat. no. A3724). The pH was monitored in real time using sensor dish-plates and the corresponding sensor dish reader (SDR SensorDish Reader, PreSens Precision Sensing GmbH, Regensburg, Germany; cat. no. 200000431).

All cell lines were transfected using an optimized polyethyleneimine (PEI)-based protocol. In brief, a transfection solution containing 1 μg of plasmid DNA mixtures, 2 μL PEI (PEI "max", 1 mg/mL in water; Polysciences, Eppelheim, Germany; cat. no.