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Rapid generation of recombinant *Pseudomonas putida* secondary metabolite producers using yTREX

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ABSTRACT

Microbial secondary metabolites represent a rich source of valuable compounds with a variety of applications in medicine or agriculture. Effective exploitation of this wealth of chemicals requires the functional expression of the respective biosynthetic genes in amenable heterologous hosts. We have previously established the TREX system which facilitates the transfer, integration and expression of biosynthetic gene clusters in various bacterial hosts. Here, we describe the yTREX system, a new tool adapted for one-step yeast recombinational cloning of gene clusters. We show that with yTREX, *Pseudomonas putida* secondary metabolite production strains can rapidly be constructed by random targeting of chromosomal promoters by Tn5 transposition. Feasibility of this approach was corroborated by prodigiosin production after yTREX cloning, transfer and expression of the respective biosynthesis genes from *Serratia marcescens*. Furthermore, the applicability of the system for effective pathway rerouting by gene cluster adaptation was demonstrated using the violacein biosynthesis gene cluster from *Chromobacterium violaceum*, producing pathway metabolites violacein, deoxyviolacein, prodeoxyviolacein, and deoxychromoviridans. Clones producing both prodigiosin and violaceins could be readily identified among clones obtained after random chromosomal integration by their strong color-phenotype. Finally, the addition of a promoter-less reporter gene enabled facile detection also of phenazine-producing clones after transfer of the respective phenazine-1-carboxylic acid biosynthesis genes from *Pseudomonas aeruginosa*. All compounds accumulated to substantial titers in the mg range. We thus corroborate here the suitability of *P. putida* for the biosynthesis of diverse natural products, and demonstrate that the yTREX system effectively enables the rapid generation of secondary metabolite producing bacteria by activation of heterologous gene clusters, applicable for natural compound discovery and combinatorial biosynthesis.

1. Introduction

Microorganisms exhibit an immense biosynthetic capability for the production of valuable compounds offering versatile bioactivities, applicable in sectors like human medicine or agriculture [1]. A vast multitude of gene sequences has become available, in which more and more gene clusters are identified that encode secondary metabolite biosynthetic pathways [2]. One key technology enabling effective exploration of the encoded chemical wealth is the functional expression in amenable heterologous hosts [3]. Therefore, increasing efforts are put in the development of diverse genetic systems for accessing natural compounds by heterologous expression of biosynthetic genes and gene clusters [4]. Here, the critical determinants for successful heterologous compound production currently represent (i) the efficient gene cluster cloning and (ii) the functional expression of all pathway genes

Abbreviations: yTREX, yeast recombinational cloning-enabled pathway transfer and expression tool; CIS, cluster integration site; *pig*, prodigiosin biosynthesis genes; *vio*, violacein biosynthesis genes; *phz*, phenazine biosynthesis genes; PCA, phenazine-1-carboxylic acid.

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requiring an appropriate host strain which offers a genetic codon usage compatible with the genes to be expressed, can provide metabolic precursors and is tolerant against putative toxicity of heterologous biosynthetic products [5].

Regarding cloning, restriction-independent methods have proven to be a key enabling technology in natural product research [6]. Phage enzyme-dependent recombination in *E. coli* and *in vitro* homology-based methods have been developed and successfully applied for gene cluster cloning and engineering [7–10], and recently, increasing use of yeast-based recombination cloning highlights the value of such approaches [6,11].

Regarding heterologous expression, the number of sophisticated tools refined for the use in different hosts increases likewise. Here, especially *P. putida* KT2440 represents one promising host for heterologous secondary metabolite biosynthesis [12,13]. Valuable tools include diverse vector and promoter systems enabling calibrated gene expression [14,15]. Furthermore, we have previously established the pathway transfer and expression (TREX) system which allows the straight-forward generation of stable expression strains in different species, employing random chromosomal integration of the heterologous gene cluster in the host by transposition and bidirectional expression of all biosynthetic genes by T7 RNA polymerase [16]. Moreover, we recently applied the tool for random integration of a unidirectional gene cluster into the chromosome of *P. putida* which resulted in strains exhibiting effective heterologous expression *via* a chromosomal promoter [17]. Nonetheless, the lack of suitable advanced cloning and expression systems for gene clusters was identified as one drawback hampering the broad utilization of this bacterium [5]. Thus, novel easy to apply tools for the fast activation of heterologous pathways in the host are needed.

Here, we describe the yTREX system, a new tool which like TREX enables the transfer, chromosomal integration and expression of gene clusters, but is enhanced by the key feature of fast one-step yeast recombinational cloning. As an application example, we moreover present the rapid generation of *P. putida* secondary metabolite production strains based on yTREX-mediated random chromosomal integration of biosynthetic genes. Employing the biosynthetic gene clusters of prodigiosin from *Serratia marcescens*, of violacein from *Chromobacterium violaceum*, and of phenazines from *Pseudomonas aeruginosa*, we demonstrate the system's applicability not only for i) the rapid transfer of metabolic pathways to the host, but also for ii) straightforward pathway engineering *via* targeted gene cluster re-design, and iii) the implementation of reporter systems for indication of biosynthetic gene expression.

2. Materials and methods

2.1. Bacterial and yeast strains

Escherichia coli strains DH5α [18] and S17-1 [19], applied for cloning and conjugation, were cultivated in shake flasks under constant agitation (120 rpm) at 37 °C in LB liquid medium (Carl Roth®, Karlsruhe, Germany: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) or on LB agar plates (15 g/L Agar-Agar, Kobe I, Carl Roth®, Karlsruhe, Germany). *Pseudomonas putida* KT2440 [20] was cultivated in shake flasks under constant agitation (120 rpm) at 30 °C in either LB or TB liquid medium (Terrific broth, modified, Carl-Roth Karlsruhe, Germany: 12 g/L Casein, enzymatically digested, 24 g/L yeast extract, 9.4 g/L dipotassium phosphate, 2.2 g/L monopotassium phosphate, 4 mL/L glycerol) or on LB agar plates. Antibiotics were added to the culture medium to the following final concentrations [μg/mL]: *E. coli*: kanamycin 50, tetracycline 10; *P. putida*: tetracycline 50, irgasan 25.

Saccharomyces cerevisiae strain VL6-48 [21,22] (ATCC® MYA-3666, LGC Standards GmbH, Wesel, Germany) was cultivated in shake flasks under constant shaking (120 rpm) at 30 °C in YPD (yeast peptone dextrose) liquid medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) [23] or on YPD agar plates (20 g/L Agar-Agar). Plasmid-bearing VL6-48 were cultivated in SD (synthetic dextrose) minimal medium [23] without uracil (SD_{-Ura}), composed of 6.7 g/L Yeast-Nitrogen-Base (without Amino Acids) (Carl Roth®, Karlsruhe, Germany), 1.926 g/L Kaiser Uracil drop-out mixture [24] (Formedia™, Norfolk, United Kingdom), 20 g/L glucose, and for SD_{-Ura} agar plates 20 g/L agar.

2.2. Standard molecular genetic methods

Standard recombinant DNA techniques were performed essentially as previously described [25]. Plasmid DNA was amplified using *E. coli* DH5α and isolated with innuPREP Plasmid Mini Kit (Analytik Jena AG, Jena, Germany). Genomic DNA was isolated with DNeasy Blood & Tissue Kit (Quiagen® GmbH, Hilden, Germany). Restriction endonuclease enzymes and phosphatase FastAP (ThermoFisher Scientific GmbH, Walkham, USA) were applied according to the manufacturer's instructions. DNA fragments were purified using innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany). Commercial services were engaged for DNA synthesis of primer oligonucleotides and yTREX cassettes, and for DNA sequencing (Eurofins Genomics GmbH, Ebersberg, Germany).

2.3. Yeast recombinational cloning

DNA fragment generation: DNA fragments with homology arms ranging from 28 bp to 52 bp were generated by PCR or restriction hydrolysis and, if necessary, purified by agarose gel electrophoresis and spin column purification. Vector fragments were furthermore treated with FastAP. All DNA fragments were combined to a total volume of 20 μL (5 μL yTREX vector, 15 μL insert solution; amounts of all DNA parts were adjusted to 0.1–1 μg) and co-transformed into *S. cerevisiae* for assembly by yeast recombinational cloning.

Yeast transformation: *S. cerevisiae* cells were transformed with DNA fragments using a protocol based on the high-efficiency yeast transformation LiAc/SS carrier DNA/PEG method developed by Gietz and Schiestl in the most recent version [26], with the following adaptions: Prior to transformation, cells were cultivated in YPD liquid medium (Gietz step 1/3). Instead of water, 1 mL 100 mM LiAc was used to wash yeast cells and for aliquotation (Gietz step 7). Heatshock was performed for 30 min at 42 °C followed by a regeneration phase of 30 min at 30 °C under constant shaking (120 rpm) (Gietz step 9). Subsequently, transformation mixtures were plated on SD_{-Ura} medium for selection and incubated for 2 days at 30 °C (Gietz step 12) [26].

Plasmid isolation: For plasmid isolation from yeast cells, colonies were transferred from plates to 1 mL SD_{-Ura} medium and incubated in FlowerPlates® (m2p-labs GmbH, Baesweiler, Germany) at 30 °C under constant shaking (1400 rpm) over night. Cells were harvested by centrifugation and solved in resuspension buffer from innuPREP Plasmid Mini Kit, supplemented with 12.5 U of Zymolyase (Zymo Research Europe GmbH, Freiburg, Germany), incubated for 2 h at 37 °C, pelleted again and subjected to plasmid isolation using innuPREP Plasmid Mini Kit. Plasmid DNA was finally introduced into *E. coli* DH5α for amplification, isolation and analysis for correct assembly.

2.4. Generation of the yTREX vector

The vector yCP50-poly [27] (ATCC® 87555™, LGC Standards GmbH, Wesel, Germany) was employed as a starting point to create the shuttle vector backbone, which was modified by deletion of the β-lactamase gene (AmpR) together with the MCS, and integration