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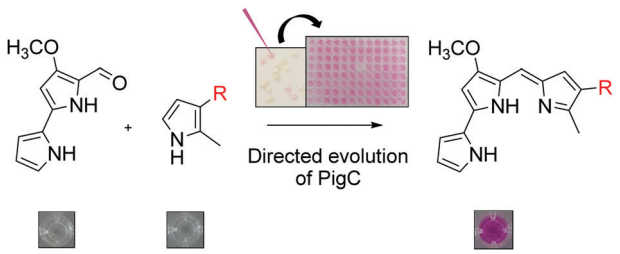

A colourimetric high-throughput screening system for directed evolution of prodigiosin ligase PigC

Stefanie Brands, Hannah U. C. Brass, Andreas S. Klein, Jörg Pietruszka, Anna Joëlle Ruff and Ulrich Schwaneberg*

A colourimetric high-throughput screening system was developed for the first directed evolution campaign on PigC towards production of artificial prodiginines.

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A colourimetric high-throughput screening system for directed evolution of prodigiosin ligase PigC†

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A colourimetric high-throughput screening system was established for directed evolution of prodigiosin ligase PigC. The two-step system consists of a colony prescreening test and a subsequent photometric 96-well plate assay. Screening PigC epPCR libraries in *Pseudomonas putida* revealed a PigC variant that achieved a 2.9× increased yield of prodiginine derivatives.

The prodigiosin synthetase PigC is a bacterial membrane-bound ligase that catalyses the final condensation step in the bifurcated biosynthesis pathway of prodigiosin (**1a**) in *Serratia marcescens*.[1] During the adenosine triphosphate (ATP)-dependent PigC reaction, two pyrrolic precursor molecules, 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC, **2**) and 2-methyl-3-amylpyrrole (MAP, **3a**), are sequentially combined (Fig. 1). The resulting tripyrrolic red pigment prodigiosin (**1a**) is a secondary metabolite produced in several bacterial species.[1–3] Homology modelling of PigC revealed a three domain structure:[4] the ATP-binding domain (amino acid residues 1–298) and phosphohistidine swivel domain (780–888) are homologous to respective domains of phosphoenolpyruvate synthases and pyruvate phosphate dikinases from different species.[4–6] The substrate-binding domain (299–779) on the other hand is unique to condensing enzymes. Apart from PigC, several homologous condensing enzymes are also able to catalyse the condensation of MBC and MAP, like HapC from *Hahella chejuensis*,[7] TreaP from *Pseudoalteromonas citrea* or TamQ from *Pseudoalteromonas tunicata*.[8,9]

Prodigiosin and its derivatives, in general the class of prodiginines, show promising bioactivities in various fields ranging from bacteriostatic[10] and immunosuppressive[2] effects to antifungal[11] and nematocidal[12] activity in agriculturally relevant applications. As prominent example for the pharmaceutical use of prodiginines, the benzyl derivative Obatoclax mesylate (GX15-070) has already been tested in phase II clinical trials in the treatment of various cancer types (leukaemia, lymphoma, myelofibrosis and mastocytosis).[13,14] Prodiginines are therefore of significant biotechnological interest and show promising potential as bioactive compounds. Their bioactivity greatly depends on the nature of side chains that decorate the tripyrrolic scaffold.[15,16] Recently, the available sequence space of prodiginine structures has been greatly expanded in combinatorial approaches of synthetic biology and chemistry.[7,9,17,18] In mutasynthesis approaches with artificial monopyrrolic substrates, a major bottleneck is the limited substrate flexibility of PigC. Especially with respect to monopyrroles that differ

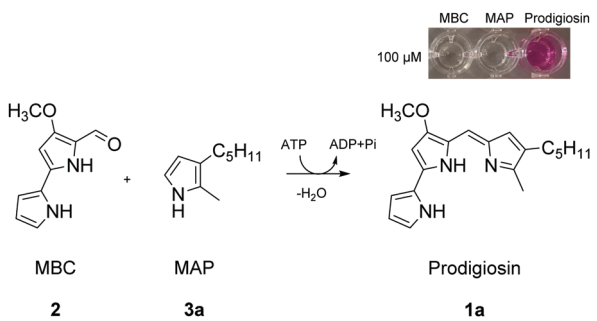

Fig. 1 Final condensation reaction of the bifurcated prodigiosin biosynthesis pathway in *S. marcescens*, catalysed by prodigiosin ligase PigC. Picture: 100 µM MBC (4-methoxy-2,2′-bipyrrole-5-carbaldehyde, **2**), 100 µM MAP (2-methyl-3-amylpyrrole, **3a**) and 100 µM prodigiosin (**1a**) in acidified ethanol.

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† Electronic supplementary information (ESI) available: Material and method section, chemical synthesis of pyrrolic compounds, assay standard deviations, prodiginine standard curves, PigC purification, catalytic efficiencies and half-life determination. See DOI: 10.1039/d0cc02181d

significantly from the natural substrate MAP (**3a**) in side chain lengths or functional moieties, PigC catalytic activity is greatly reduced.[9,17]

In the last decades, directed evolution has become a standard methodology in protein engineering to tailor enzymes toward industrial applications. Directed evolution enables experimentalists to generate tailor-made proteins by repetitive cycles of random mutagenesis, screening of protein libraries and characterization of beneficial variants.[19]

A common method for generating random mutagenesis libraries for directed evolution campaigns is error-prone PCR (epPCR).[20] In epPCR, DNA polymerases that lack proofreading capacities are employed under suboptimal conditions (*e.g.* addition of manganese ions that hamper polymerase precision) to produce randomly mutagenised PCR products. From the vast number of possible enzyme variants in mutagenesis libraries with random amino acid exchanges, beneficial variants with improved properties need to be identified. Therefore, directed evolution campaigns always strongly rely on efficient and specific screening systems, preferably with high spatiotemporal throughput. Prerequisites for suitable screening systems in directed evolution campaigns are (i) a specific detection of the wanted enzyme property to distinguish improved variants in a library, and (ii) reliability and reproducibility of quantitative results. Due to their easy and quick photometric readout, colourimetric screening methods are a useful approach for directed evolution. Numerous examples of colourimetric assays with direct and indirect product detection as well as surrogate substrate usage are published for many enzyme classes, *e.g.* laccases,[21] transaminases,[22] glycosynthases[23] and P450 monooxygenases.[24]

In case of prodigiosin ligase PigC, no high-throughput screening system that enables directed evolution is available so far, and no evolution of PigC has been published yet. Since prodiginines are easily detected and quantified by their prominent red colour, it is possible to screen PigC libraries from directed evolution campaigns for beneficial PigC variants by colourimetric endpoint measurement of red prodiginine extracts at 536 nm. The production of prodigiosin and other prodiginines has so far been established in S1 expression hosts like *Escherichia coli*[25,26] and the Gram-negative soil bacterium *Pseudomonas putida* KT2440.[10] Beside its acknowledged apathogenity, especially *P. putida* KT2440 is a suitable production chassis for bioactive prodiginines because of its high natural resistance towards antibiotic prodiginine compounds.[10]

In this work, we established and validated a colourimetric high-throughput screening system for directed evolution of PigC towards acceptance of artificial precursor molecules and enhanced stability in *P. putida* KT2440. A two-step screening system (ProdEvolve assay, Fig. 2) was established, consisting of (i) an agar-plate based colony prescreening approach to preselect active PigC clones from a library, and (ii) a colourimetric 96-well plate screening assay, which includes individual expression of active PigC clones, biotransformation of the (artificial) substrates, and simple ethanolic extraction of prodiginines from cell pellets for measurement of absorbance in a plate reader. As proof of principle, the ProdEvolve assay was applied

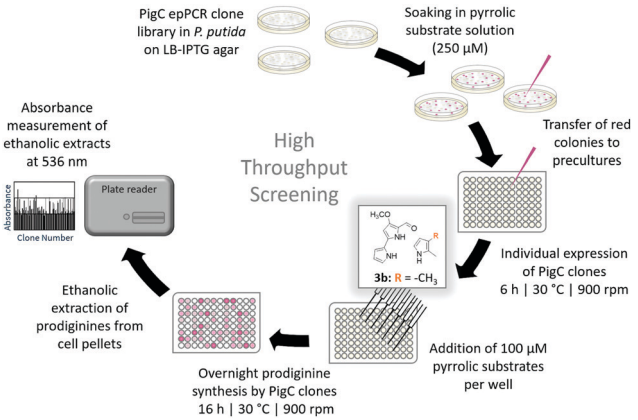

Fig. 2 Overview of the ProdEvolve screening system procedure.

to identify PigC variants with improved acceptance of the short-chain MAP derivative 2,3-dimethyl-1*H*-pyrrole (diMe-pyrrole, **3b**) at 30 °C. Efficient synthesis of derivatives such as prodiginine **1b** is of high interest, as especially analogs with reduced chain-length responded in autophagy tests with enhanced anticancer bioactivity.[17]

As first step of the ProdEvolve screening approach, a prescreening method for identification of active PigC clones on expression agar plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) was established. *P. putida* KT2440 colonies were grown on selective IPTG agar covered with nutrient-permeable nitrocellulose membranes. For identification of active PigC clones, nitrocellulose membranes with PigC expressing colonies were then transferred onto a filter paper that was soaked with substrate solution in selective LB media [250 µM MBC (**2**)/diMe-pyrrole (**3b**), 5% (v/v) dimethyl sulfoxide (DMSO) as cosolvent, 5 mM ascorbic acid as antioxidant]. After short incubation, active PigC clones turned red due to production of prodiginines, while colonies containing the empty vector or inactive PigC variants stayed colourless (Fig. 3a). This method allowed clear distinction between red active PigC clones and colourless inactive PigC clones. Only red and active clones were selected for photometric screening in 96-well plates in the next step of the ProdEvolve assay, which significantly enhanced the screening efficiency: the major background of empty vector clones as well as inactive PigC variants was left behind after this first screening step. Validation of the prescreening system was performed in a blind trial based on recognition of positive and active PigC clones. Plasmids containing *pigC* were mixed with empty pVLT33 vector in a 1 : 1 ratio prior to electroporation of *P. putida* KT2440.[27] The resulting mock clone library with 50% wild type PigC colonies and 50% empty vector clones was then incubated with substrate solution. Red colonies were transferred into wells of a 96-well preculture plate with selective LB media and screened following the ProdEvolve procedure. In the positive blind trial, 87 of 88 colonies (98.9%) were recognised as positive clones containing active wild type PigC. In the corresponding negative blind trial, 67 of 88 colonies recognised as empty vector clones (76.1%). Thus, the recognition of active PigC clones in the agar prescreening system was demonstrated to be highly reliable. Expression of select active

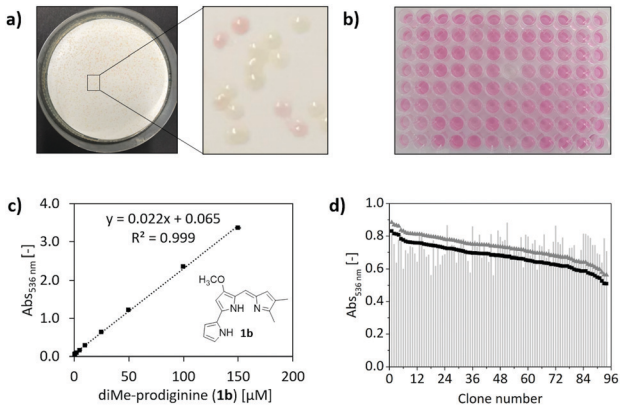

Fig. 3 (a) Prescreening colonies of an epPCR library after incubation with 250 µM substrates diMe-pyrrole (**3b**)/MBC (**2**). (b) Ethanolic prodiginine extracts from *P. putida* KT2440 *pigC* microcultures with 100 µM substrates diMe-pyrrole (**3b**)/MBC (**2**). (c) Linear detection range of the ProdEvolve assay in 96-well plate format and 100 µL acidified ethanol (4% 1 N HCl) at 536 nm. Linearity was achieved between 0.5 and 250 µM diMe-prodiginine (**1b**). (d) Standard deviation of the assay with diMe-pyrrole (**3b**)/MBC (**2**). Apparent [(▲), 9.7%] and true standard deviation [(■); 10.5% after subtraction of cell background]. The grey columns show absorbance values sorted by position in the 96-well plate.

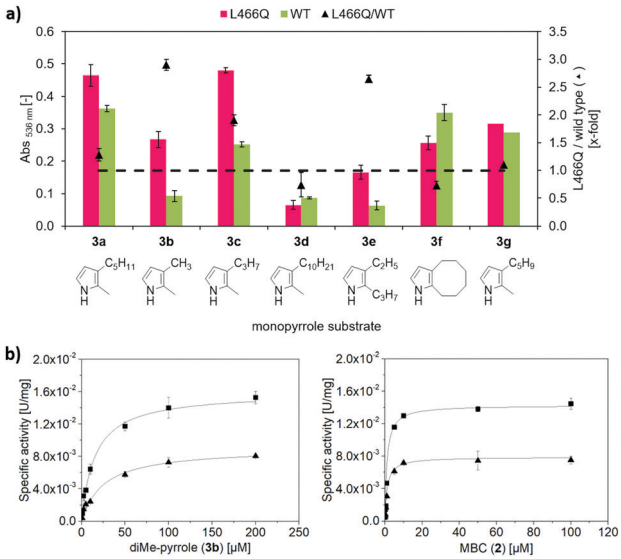

Fig. 4 (a) Substrate acceptance profiles of variant L466Q (■) and PigC wild type (■) with monopyrrolic substrates (50 µM **3a–g**, end point measurement at 536 nm). Improvement of prodiginine yield of L466Q over the PigC wild type (dashed line) is depicted in black triangles (▲). Error bars indicate standard deviation of biological triplicates. (b) Michaelis–Menten plot of PigC wild type (▲) and variant L466Q (■): specific activity [U mg_{PigC}^{-1}] with 0–200 µM diMe-pyrrole (**3b**, left)/0–100 µM MBC (**2**, right).

pigC clones for photometric screening was subsequently performed in 96-well plate format. Substrates diMe-pyrrole (**3b**) and MBC (**2**) were added in 100 µM final concentration resulting in the production of red prodiginine **1b**. After overnight incubation, prodiginines were extracted from *P. putida* KT2440 pellets by resuspension in acidified ethanol [4% (v/v) 1 N HCl, Fig. 3b] and quantified in a plate reader at 536 nm.

Photometric prodiginine **1b** quantification in acidified ethanol responded linear within 0.5–150 µM (Fig. 3c). The coefficient of variance (CV% = [standard deviation ÷ (mean) × 100%]) of the ProdEvolve assay has been determined with diMe-pyrrole (**3b**) in one 96-well plate with 94 clones of wild type PigC (n = 94; apparent CV% = 9.7%; true CV% = 10.5% after subtraction of the PigC-free cell background, Fig. 3d). On extension, the CV% was determined with four further monopyrroles (**3d–g**, Fig. S4 and Table S1, ESI†). The ProdEvolve assay thus met requirements for directed evolution campaigns with different substrates, since screening assays with CV%s under 15% are routinely and successfully applied in directed evolution.[28]

After establishment of the ProdEvolve procedure, epPCR libraries (50, 75 and 100 µM $MnCl_2$; ratio of active variants ~40–60%; mutational loads 0.37, 0.56 and 1.19 kb^{-1}, respectively) of PigC were cloned in *E. coli* DH5α. 1784 DH5α clones were pooled, the extracted plasmid library was transferred to *P. putida* KT2440, and 3168 KT2440 clones have been screened with the ProdEvolve assay towards enhanced acceptance of the short-chain monopyrrolic substrate diMe-pyrrole (**3b**). By means of the ProdEvolve assay, variant L466Q with one amino acid substitution (Leu to Gln) in the substrate binding domain has been identified, which showed 2.9× improved prodiginine yield under screening assay conditions after subtraction of cell background. For determination of substrate acceptance profiles, both PigC wild type and L466Q have been tested with monopyrrolic substrates with increasing side chain lengths at the 3-position of the pyrrole [3-methyl (**3b**), 3-propyl (**3c**), 3-amyl (**3a**), and 3-decyl (**3d**)], as well as pyrrole **3e** with an ethyl substituent in 2-position and a *n*-propyl group in 3-position, a cyclic pyrrole (8-ring pyrrole, **3f**), and 3-pentenyl pyrrole (**3g**) with a terminal double bond (Fig. 4a), applying the 96 well plate screening protocol of the ProdEvolve assay. While variant L466Q showed 2–3× improved prodiginine yield with short-chain substrates (**3b**, **3c**, **3e**) compared to PigC wild type, it showed similar acceptance of the natural substrate MAP (**3a**) and it's derivative **3g** bearing a terminal double-bond in 3-position,[29] and lower acceptance of the long-chained and bulky substrates (**3d**, **3f**). These results demonstrate that the substrate preference of L466Q was shifted towards short chain pyrrolic substrates. Variant L466Q and PigC wild type have subsequently been overexpressed in *E. coli* BL21(DE3) to obtain sufficient PigC amounts for kinetic characterization. The whole cell membrane fraction containing PigC has been isolated *via* ultracentrifugation of cell-free lysate as published before,[9] and subjected to kinetic evaluation in a buffered PigC *in vitro* assay at 30 °C and pH 7.5 [100 mM Tris–HCl, 0–200 µM diMe-pyrrole (**3b**), 0–200 µM MBC (**2**), 200 µM $ATPxNa_2$]. Variant L466Q showed 1.8-times enhanced specific activity in BL21 membrane fraction (Fig. 4b), and 2.9-times increased catalytic efficiency (k_{cat}/K_M) with diMe-pyrrole (**3b**) compared to wild type PigC (Table 1). The substrate affinity of L466Q was enhanced for diMe-pyrrole (**3b**, K_M 1.5× decreased over wild type), while the

Table 1 Kinetic parameters of PigC wild type (WT) and variant L466Q in *E. coli* BL21(DE3) membrane fraction

PigC	K_M (diMe-pyrrole) [μM]	K_M (MBC) [μM]	k_{cat} [min^{-1}]	k_{cat}/K_M (diMe-pyrrole) [L ($mmol^{-1}$ s^{-1})$^{-1}$]	Half-life time $t_{1/2}^{30\,°C}$ [min]
WT	25.2 ± 3.2	1.2 ± 0.2	0.84 ± 0.07	0.56 ± 0.05	6.5 ± 0.1
L466Q	15.4 ± 1.8	1.1 ± 0.1	1.50 ± 0.18	1.63 ± 0.20	11.2 ± 0.2

K_M for MBC (**2**) did not significantly change between L466Q (1.1 μM) and PigC wild type (1.2 μM). Also with substrate **3e**, the catalytic efficiency of L466Q exceeded the wild type efficiency (Fig. S7 and Table S2, ESI†). Beside its improved catalytic efficiency, also the stability of L466Q was enhanced over PigC wild type. As protein stability usually increases by introduction of a more rigid protein backbone, it often coincides with a loss in flexibility, and consequently a loss in catalytic activity. Notably, substitution L466Q enhanced both properties: the half-life time (Table 1; $t_{1/2}^{30\,°C}$) of PigC in *E. coli* BL21(DE3) membrane fraction at 30 °C was prolonged from 6.5 min (wild type) to 11.2 min (L466Q). From k_{cat} and half-life time, the total turnover numbers (TTN) of PigC wild type and variant L466Q in substrate excess were calculated following Rogers and Bommarius.[30] L466Q achieved a TTN (k_{cat}/k_d) of 54, which was 2.6 times increased over wild type (k_{cat}/k_d = 21). In summary, substitution L466Q located in the substrate binding domain lead to a shift in the substrate acceptance towards short-chain monopyrroles. This observation might be explainable by the substitution of a hydrophobic residue (leucine) to a polar residue (glutamine), which enables the membrane protein PigC to attract the more polar pyrrolic substrates with shorter side chains. Next to the catalytic efficiency, also the stability of L466Q was enhanced under ProdEvolve assay conditions compared to PigC wild type. Notably, L466Q showed improved catalysis independent from the expression host, in both *P. putida* KT2440 and *E. coli* BL21(DE3).

In conclusion, the herein published ProdEvolve screening assay has successfully been applied for directed evolution of prodigiosin ligase PigC in *P. putida* KT2440. A first protein engineering campaign has been conducted on PigC as proof of concept for the ProdEvolve assay, enhancing artificial substrate acceptance of PigC. By means of the ProdEvolve screening assay, a PigC variant with higher stability and shifted substrate acceptance has been identified. The overall screening efficiency was significantly increased by preselection of active PigC clones from agar plates prior to screening in 96-well plates. The rapid and simple procedure is extendable to alternative target substrates, which emphasises the flexibility of the system for efficient prodiginine production. In future, the ProdEvolve assay will enable directed evolution campaigns on condensing enzymes towards enhanced production of novel prodiginine compounds that are not yet accessible biosynthetically.

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Conflicts of interest

There are no conflicts to declare.

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