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Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin

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Abstract:	Prodigiosin, a red linear tripyrrole pigment and a member of the prodiginine family, is normally secreted by the human pathogen Serratia marcescens as a secondary metabolite. Studies on prodigiosin have received renewed attention as a result of reported immunosuppressive, antimicrobial and anticancer properties. High-level synthesis of prodigiosin and the bioengineering of strains to synthesise useful prodiginine derivatives have also been a subject of investigation. To exploit the potential use of prodigiosin as a clinical drug targeting bacteria or as a dye for textiles, high-level synthesis of prodigiosin is a prerequisite. This review presents an overview on the biosynthesis of prodigiosin from its natural host Serratia marcescens and through recombinant approaches as well as highlighting the beneficial properties of prodigiosin. We also discuss the prospect of adopting a synthetic biology approach for safe and cost-effective production of prodigiosin in a more industrially compliant surrogate host.

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Pusat Pengajian Biosains dan Bioteknologi | ***School of Biosciences and Biotechnology***

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Prof. Dr. Alexander Steinbuchel
Editor-in-Chief
Applied Microbiology and Biotechnology

Dear Prof. Steinbuchel,

Revised Manuscript Submission to Applied Microbiology and Biotechnology

Thank you for sending us the comments and suggestions from the review process on our mini-review "Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin".

We thank the reviewers for the suggestions to improve the content of this review on prodigiosin production from its natural host and from heterologous hosts. We have made all the suggested changes which have been itemized in the Response to Reviewers' Comments document and the major changes have been highlighted in the revised manuscript.

We feel that the current version of the mini-review is greatly improved from the original version and will be of importance to those interested in prodigiosin as well as microbiologists working on large-scale production of multi-gene-encoded recombinant proteins. All authors approve the manuscript in its current form for resubmission to Applied Microbiology and Biotechnology. We hope that the Editorial Board will consider this revised manuscript suitable for publication in Applied Microbiology and Biotechnology.

Thank you

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Authors' Response to Reviewers' Comments
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Response to Reviewers' Comment

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We would like to thank the reviewers for the comments and suggestions to improve this mini-review. We have addressed all the points raised by the individual reviewers below:

Reviewer A

The mini-review paper entitled "Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin" presents an overview on prodigiosin research in last decades. The mini-review is comprehensive and well written, however it is not sufficiently novel to set it apart from few recent reviews within the field and there is not sufficient material to warrant publication in AMB in its current form. The intention of Yip and co-authors was to give overview on the prodigiosin production in its natural host *Serratia marcescens*, through recombinant and synthetic biology approaches for safe and cost-effective production of prodigiosin. However, the most of the review has been dedicated to description of the biosynthetic genes that has been done on numerous occasions in similar reviews. Thus, out of 97 references in total, 58 are older than 2014.

We thank the reviewer for the comments. We have re-aligned the focus of the mini-review to the recombinant and synthetic approaches previously undertaken to produce prodigiosin and concurrently edited the description that relates to the biosynthesis of prodigiosin in its original host, Serratia marcescens. In doing this, we have also updated the literature and the related references.

In addition, I was **not able to recognise references for the original work done by the authors within the subject.**

The Malaysian authors in collaboration with the co-authors in the United Kingdom are currently working on synthesising prodigiosin in Escherichia coli using a synthetic biology approach. In parallel with the work on cloning the 12 pig genes into E. coli, we are also developing new tools for gene cassette integration as well as codon optimization for expression in E. coli. These 2 aspects of the project have recently been published:

(1) Yip CH, Yarkoni O, Ajioka J, Wan KL, Nathan S (2018) Development of a codon optimization strategy using the eforRED reporter gene as a test case. AIP Conf Proc 1940. pp. 020080. doi: 10.1063/1.5027995;
(2) Yip CH, Yarkoni O, Mario J, Ajioka J, Wan KL, Nathan S. The Escherichia coli motA flagellar gene as a potential integration site for large synthetic DNA. Sains Malaysiana. In press

In addition the co-authors in the United Kingdom have published on the use of synthetic biology:

(1) Haseloff J & Ajioka J (2009) Synthetic biology: history, challenges and prospects. J R Soc Interface 6:S389-S391. doi: 10.1098/rsif.2009.0176.focus
(2) Juhas M & Ajioka JW (2015b) Identification and validation of novel chromosomal integration and expression loci in Escherichia coli flagellar region 1. PLoS One 10. doi:10.1371/journal.pone.0123007
(3) Juhas M & Ajioka JW (2016a) High molecular weight DNA assembly in vivo for synthetic biology applications. Crit Rev Biotechnol 37. doi: 10.3109/07388551.2016.1141394
(4) Juhas M & Ajioka JW (2016b) Integrative bacterial artificial chromosomes for DNA integration into the Bacillus subtilis chromosome. J Microbiol Methods 125:1-7. doi: 10.1016/j.mimet.2016.03.017
(5) Juhas M, Evans LDB, Frost J, Davenport PW, Yarkoni O, Gillian MF, Ajioka JW (2014) Escherichia coli flagellar genes as target sites for integration and expression of genetic circuits. PLoS One 9. doi:10.1371/journal.pone.0111451.

My recommendation is to **reduce the part on the biosynthesis of prodigiosin and remove or condense corresponding figures.**

We agree with the reviewer that the sections related to prodigiosin biosynthesis should be reduced. As noted above, we have condensed the text related to the biosynthetic pathway of prodigiosin (page 4, lines 33-37). Also, we have combined Figures 3, 4 and 5 into one single figure (new Figure 3) (page 5, line 14) that summarises the bifurcated pathway to produce prodigiosin.

Instead, **more information is needed on the downstream processing to obtain pure prodigiosin including overview of the extraction and separation techniques** from the original and recombinant hosts.

We agree with the reviewer that the downstream processes to obtain pure prodigiosin have not been extensively reviewed in the previous literature related to prodigiosin production. To enhance the content of this mini-review, we have included the section titled 'Isolation and Purification of Prodigiosin from S. marcescens' (page 11, lines 24-36 and page 12, lines 1-34).

Figure 1 should contain **characterised prodigiosin derivatives** in addition to prodigiosin.

We have improved Figure 1 by incorporating several prodigiosin derivatives: undecylprodigiosin, cycloprodigiosin, metacycloprodigiosin, prodigiosin R1 and streptorubin B) (page 2, line 10).

Reviewer B

Overall the review is nice, and very thorough. I would recommend that **figure 3, 4 and 5 is joined into one figure**. That would make it easier to follow.

We thank the reviewer for his/her suggestion. We have combined the original Figures 3, 4 and 5 into one single figure (new Figure 3) (Page 5, line 14).

Else, there are only **minor spelling mistakes**, which should be corrected.

We thank the reviewer for highlighting the spelling errors. We have proofread the manuscript and edited the spelling errors.

The revised manuscript with major changes highlighted is appended below.

Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin

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Keywords

Prodigiosin, beneficial secondary metabolite, gene cluster, bifurcated pathway, high-level synthesis

Abstract

Prodigiosin, a red linear tripyrrole pigment and a member of the prodiginine family, is normally secreted by the human pathogen *Serratia marcescens* as a secondary metabolite. Studies on prodigiosin have received renewed attention as a result of reported immunosuppressive, antimicrobial and anticancer properties. High-level synthesis of prodigiosin and the bioengineering of strains to synthesise useful prodiginine derivatives have also been a subject of investigation. To exploit the potential use of prodigiosin as a clinical drug targeting bacteria or as a dye for textiles, high-level synthesis of prodigiosin is a prerequisite. This review presents an overview on the biosynthesis of prodigiosin from its natural host *Serratia marcescens* and through recombinant approaches as well as highlighting the beneficial properties of prodigiosin. We also discuss the prospect of adopting a synthetic biology approach for safe and cost-effective production of prodigiosin in a more industrially compliant surrogate host.

Introduction

Prodigiosin is a red bacterial pigment that is secreted as a secondary metabolite. It is the most prominent member of the prodiginine family (other members include undecylprodigiosin, cycloprodigiosin, metacycloprodigiosin, prodigiosin R1 and streptorubin B) (Fig. 1) (Stankovic et al. 2014). Prodigiosin has long been a subject of research interest due to its many potential beneficial properties that include anticancer (Elahian et al. 2013), antimicrobial (Ibrahim et al. 2014) and antimalarial (Papireddy et al. 2011) properties. This metabolite is secreted by a number of microorganisms such as *Hahella chejuensis* (Kim et al. 2006), *Streptomyces coelicolor* (Do and Nguyen 2014), *Streptomyces grisiovirides* (Kawasaki et al. 2008), *Serratia nematodiphila* (Darshan and Manonmani 2016), *Serratia rubidae* (Siva et al. 2011) and *Serratia marcescens* (Casullo de Araujo et al. 2010). Driven by the potential development and application of prodigiosin as a clinical drug, many studies have been undertaken to dissect its biosynthetic pathways as a means to increase

production of this pigment. Nevertheless, attempts to develop prodigiosin as a therapeutic molecule have been hampered by reports of toxicity on eukaryotic cells (Pandey et al. 2009), its ability to intercalate and cause double stranded DNA breaks (Kimyon et al. 2016) as well as cell cycle arrest (Soto-Cerrato et al. 2007). Furthermore, purified prodigiosin is reportedly immunosuppressive on the human immune response (Liu and Nizet 2009). Taken together, prodigiosin was presumed to be cytotoxic towards eukaryotic cells and therefore, not suitable to be developed as a clinical drug.

Fig.1 Members of the prodiginine family. A) prodigiosin B) undecylprodigiosin C) cycloprodigiosin D) metacycloprodigiosin E) prodigiosin R1 and F) streptorubin B (Adapted from PubChem (Kim et al. 2016)).

Nevertheless, recent studies have shown that prodigiosin is not genotoxic (Guryanov et al. 2013). Furthermore, bacterial prodigiosins and their synthetic derivatives are effective pro-apoptotic agents against various cancer cell lines where multiple cellular targets include multi-drug resistant cells with little or no toxicity towards normal cell lines (Elahian et al. 2013; Kavitha et al. 2010). These findings pave the way for prodigiosin to be developed as a promising drug candidate and efficient production of this microbial pigment is the prerequisite to its full clinical evaluation. However, large bacterial cultivation of *S. marcescens* for high prodigiosin production is not safe and there are limited reports on the expression of prodigiosin in a safer heterologous host for high-level synthesis.

Hence, this review focuses on the organisation of the prodigiosin biosynthesising or pigment (*pig*) cluster of *Serratia marcescens* ATCC 274 (Sma 274), the representative strain of *S. marcescens*. We also discuss the potential applications of bacterial prodigiosins as a clinical drug and review current approaches to scale-up its synthesis, either in *S. marcescens* or using surrogate hosts and its purification from the expression hosts. Finally, a synthetic biology platform is proposed for a safer and more cost-effective large-scale synthesis of prodigiosin in *Escherichia coli*.

Prodigiosin

Prodigiosin is a red linear tripyrrole molecule (Fig. 1A) made up of 3 rings, A, B and C. The A and B rings are connected in a bipyrrole unit whereas the B and C rings are joined in a dipyrrin (Jolicoeur and Lubell 2008). The pyrrole moiety (C ring) is linked to the methoxy bipyrrole (A and B rings) by a methylene bridge (Garneau-Tsodikova et al. 2006). The nomenclature for prodigiosin is 2-methyl-3-amyl-6-methoxyprodigiosene (Roy et al. 2014) and prodigiosin secreted by Sma 274 has a molecular weight of 323.4 Dalton (Da) (Casullo de Araujo et al. 2010) with a chemical formula of $C_{20}H_{25}N_3O$ (Song et al. 2006).

Prodigiosin is a hydrophobic molecule with a log $P_{octanol\text{-}water}$ value of 5.16 (Suryawanshi et al. 2016) and has a 4-methoxypyrollic core with a cationic charge at physiological pH that enables selective binding to alternating DNA sequences without discriminating between the AT and CG sites. Interestingly, prodigiosin is a monoprotonated ligand that binds to important anions such as Cl^- and

HCO_3^- and transports these ions via an antiport (OH^-/Cl^-) mechanism (Seganish and Davis 2005) or an H^+/Cl^- symport mechanism which alters the transmembrane pH gradient. Furthermore, the close proximity of the three pyrroles enables prodigiosin to bind to metal ions such as Cu^{2+} (Park et al. 2003) whereby a prodigiosin-Cu^{2+} complex facilitates double-strand DNA oxidative cleavage (Kimyon et al. 2016; Melvin et al. 2000). The colour intensity of prodigiosin decreases when illuminated with light, suggesting that it is light-sensitive (Wang et al. 2012a). A subsequent report demonstrated that prodigiosin absorbs light and causes phototoxicity of the *S. marcescens* cytomembrane leading to leakage of prodigiosin (Wang et al. 2013).

Prodigiosin can be extracted using acidic or alkaline solvents. Under acidic conditions, purified prodigiosin appears red in colour whilst pure prodigiosin in an alkaline solution has a yellow appearance. It can be detected by liquid chromatography-mass spectrophotometry (LC-MS) (Williamson et al. 2006b). The red prodigiosin is usually detectable at a maximum of 535 nm (Casullo de Araujo et al. 2010) but may present as an additional peak at 500 nm which corresponds to the native prodigiosin-protein complex (Andreyeva and Ogorodnikova 2015). On the other hand, the yellow pigment which is attributed to the formation of β-carotene in *S. marcescens* (Wang et al. 2012a) is visualised as a sharp spectral peak at 470 nm. Prodigiosin has a pKa value of 7.2 (Drink et al. 2015) and an R_f value of 0.59 (Lapenda et al. 2014).

The amount of prodigiosin produced can be estimated using the formula developed by Haddix and Wenner (2000) as shown below:

$$\text{Prodigiosin unit/cell} = \frac{([\text{OD}499 - (1.381 \times \text{OD}620)]) \times 1000}{\text{OD}620}$$

Where, OD_{499} = pigment absorption

OD_{620} = bacterial culture absorption

1.381 = constant

This formula has since become the most commonly used method to quantify prodigiosin (Kamble and Hirawale 2012).

The physiological role of bacterial prodigiosin in vivo remains undefined. A number of reports have suggested potential functions that may provide an advantage in competition with other organisms (Gulani et al. 2012;) and the continuously challenging natural environment (Stankovic et al. 2014) as well as increased surface hydrophobicity to facilitate ecological dispersion of the bacteria (Song et al. 2006). This is in agreement with previous findings that environmental *S. marcescens* strains are normally pigmented whereas the non-pigmented strains are associated with nosocomial infections (Mahlen 2011). Although the role of prodigiosin still remains vague, the synthesis of this pigment is undoubtedly important to *S. marcescens* since a gene cluster of 20 kb is solely dedicated to its

production.

Biosynthesis of prodigiosin

The *pig* cluster is comprised of 14 genes and has a size of 20,960 bp. In Sma 274, these genes are arranged in the order of *pigA, pigB, pigC, pigD, pigE, pigF, pigG, pigH, pigI, pigJ, pigK, pigL, pigM* and *pigN* (Fig. 2) (Harris et al. 2004) and the function of each protein is listed in Table 1.

The *pig* genes in Sma 274 are flanked by the *cueR* and *copA* genes (Harris et al. 2004). The gene *cueR* is located 488 bp upstream of the start of the *pig* cluster whereas a gap of 183 bp separates *copA* and *pigN* (Harris et al. 2004; Venil et al. 2009). The gap of 183 bp between *copA* and *pigN* suggests that the expression of *copA* is independent of the *pig* gene cluster in Sma 274. A promoter for *copA* was predicted within this gap as well as a terminator that terminates the transcription of all the 14 *pig* genes. (Harris et al. 2004) (Fig. 2). From our analysis using the BPROM (Softberry) software (Solovyev and Salamov 2011), this promoter most likely serves as a binding site for a number of transcriptional factors such as arginine repressor 2 (argR2), fis protein, RNA polymerase sigma factor 15 (rpoD15), repressor protein lexA, C-reactive protein (crp) and integration host factor (ihf) (Yip, unpublished). In Sma 274, a bifurcated pathway is needed to synthesise two key intermediates, namely 2-methyl-3-n-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC) to produce prodigiosin (Harris et al. 2004; Williamson et al. 2006a; Williamson et al. 2006b).

Fig.2 The genetic organisation of the prodigiosin biosynthetic gene cluster of Sma 274. Black block arrows indicate genes involved in the biosynthesis of the monopyrrole moiety, 2-methyl-3-n-amylpyrrole (MAP) whereas white block arrows represent genes involved in synthesising the bipyrrole moiety, 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC). The arrow shaded with vertical lines is the gene that encodes for the terminal condensing enzyme, PigC. Transcriptional regulators of prodigiosin expression are indicated by arrows shaded with horizontal lines. (a) predicted promoter (Harris et al. 2004), (b) putative transcriptional terminator and (c) predicted *copA* promoter in Sma 274 (Harris et al. 2004). The -10 and -35 regions are in bold and underlined (Adapted from Harris et al. 2004).

Twelve out of the 14 genes in the *pig* cluster have previously been assigned and characterised (Table 1) based on cross-feeding experiments of individual gene mutants (Williamson et al. 2005). Genes *pigB, pigD* and *pigE* were assigned to the synthesis of the monopyrrole moiety, MAP whereas *pigA, pigF, pigG, pigH, pigI, pigJ, pigM* and *pigN* are involved in the production of the bipyrrole moiety, MBC. The gene *pigC* encodes for the terminal condensing enzyme that condenses both MAP and MBC to produce prodigiosin. PigC has N- and C-terminal domains that share sequence similarities to phosphoryl transferase domains and the ATP-binding domain of a pyruvate phosphate dikinase, respectively (Harris et al. 2004). A BLASTP analysis indicated that both PigC and pyruvate phosphate dikinase of *S. marcescens* have the pyruvate phosphate dikinase PEP/pyruvate binding domain with 99%

identity. This enzyme has a core catalytic region that binds MBC and MAP together to form prodigiosin (Williamson et al. 2006b). The functions of the gene products of *pigK* and *pigL* are currently not known but PigK may act as a molecular chaperone to assist folding of other Pig enzymes in the biosynthesis of MBC. In addition, PigL, which is a 4'-phosphopantetheinyl transferase, could be involved in the phosphopantetheinylation reaction in the MBC pathway (Williamson et al. 2005). An over view of prodigiosin biosynthesis is illustrated in Figure 3.

Table 1 Function of each Pig protein involved in the biosynthesis of prodigiosin (Adapted from Williamson et al. 2005)

Fig. 3 The bifurcated pathway of prodigiosin biosynthesis (Adapted from Williamson et al. 2006b).

Is prodigiosin suitable as a clinical drug?

Production of microbial secondary metabolites has always been associated with the survival of the producing organisms (Figueiredo et al. 2008). Prodigiosin was evaluated through various studies and shown to have many beneficial properties that make it a promising drug candidate.

Anticancer drug

Prodigiosin is proposed to have an anti-cancer effect where it up-regulates p73 that restores the p53 signalling pathway in SW480 cancer cells and subsequently induces apoptosis of these cells. Since cancer cells carry a variety of mutated p53 with different hotspot mutations in the DNA-binding domain, these p53 mutants can interact with p73 and inhibit the rescue of the p53 pathway. Interestingly, prodigiosin is capable of disrupting this mutant p53/p73 interaction, suggesting that it can be used to treat cancer regardless of the status of p53 (Hong et al. 2014). Additionally, Kavitha et al. (2010) found that prodigiosin induces apoptotic cell death in HeLa cells in a dose-dependent manner with a half maximal inhibitory concentration (IC_{50}) of 700 nM. Hong et al. (2014) demonstrated that prodigiosin selectively kills cancer cells and leaves normal cells unharmed at a low concentration range of 100 nM – 1 µM. This finding is in agreement with the previous study conducted by Montaner et al. (2000) that prodigiosin only induced apoptosis in haematopoietic cancer cells but leaves non-malignant cells unharmed. It has also been shown that prodigiosin acts independently on cancerous cells that express multidrug resistance transporter proteins such as multidrug resistance 1 (MDR1), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) transporters (Elahian et al. 2013).

The preferential killing of cancer cells by prodigiosin can be attributed to its nature as an anion carrier. As an anion transporter, prodigiosin binds to biologically important anions such as Cl^- and HCO_3^- to form a lipophilic prodigiosin-anion complex that can deacidify the pH of certain organelles such as lysosome, endosomes and Golgi apparatus by inhibiting V-ATPase via H^+/Cl^- symport mechanism. (Davis 2010). The altered transmembrane pH gradient causes the pH of the cellular

environment of the cancer cells to decrease. Since the intracellular pH of cancer cells is more alkaline than that of normal cells, prodigiosin-treated cancer cells become more acidic, and therefore, undergo apoptosis (Nakashima et al. 2005).

A synthetic prodigiosin derivative, obatoclax mesylate (GX15-070 or simply known as obatoclax) was discovered by Gemin X Pharmaceuticals Inc. (Canada) and has been used as an experimental drug on various types of cancers (Neidle 2013). Obatoclax was used in phase I clinical trials to treat advanced chronic lymphocytic leukaemia (O' Brien et al. 2009). Additionally, it binds to the hydrophobic pocket of BH3 in Bcl-2 proteins, antagonises MCL-1 and initiates apoptosis in cancer cells (Urtishak et al. 2013). Obatoclax also helps to overcome glucocorticoid resistance (common in many chemotherapy protocols) in acute lymphoblastic leukaemia (Heidari et al. 2010) whilst showing promise in patients with small cell lung cancer when used in the combination with carboplatin-epotoside (Chiappori et al. 2012).

Antibacterial drug

In general, cyclic molecules demonstrate distinct antibacterial activity compared to linear molecules (Lee et al. 2011). Kamble and Hiwarale (2012) proposed three mechanisms of prodigiosin as a potent antimicrobial agent: cleavage of bacterial DNA, cell cycle inhibition and modulation of pH. Prodigiosin has been demonstrated to exhibit antagonistic effects towards *Staphylococcus aureus, Bacillus cereus* (Gulani et al. 2012), *Acinetobacter anitratus, Agrobacterium tumefaciens, Bacillus licheniformis, B. subtilis, B. thuringiensis, Erwinia sp., E. coli, Micrococcus sp.,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis, S. saprophyticus* (Ibrahim et al. 2014), *Streptococcus pyogenes, Enterobacter faecalis* and oxacillin-resistant *S. aureus* (ORSA) (Lapenda et al. 2014).

Prodigiosin is inhibitory towards *Borrelia burgdorferi*, a pathogenic bacterium responsible for Lyme disease. *B. burgdorferi* are resistant to doxycycline or amoxicillin whilst prodigiosin had a low minimum inhibitory concentration of < 0.2 µg/mL and 24% activity against stationary phase and actively growing *B. burgdorferi* (Feng et al. 2015). A recent study conducted by Darshan and Manonmani (2016) showed that prodigiosin from *S. nematodiphila* induces programmed cell death (PCD) of *B. cereus, Pseudomonas aeruginosa, S. aureus* and *E. coli* as well as inhibits *B. cereus* and *E. coli* motility. Recently, it has been suggested that chaotropicity-mediated stress is the primary mode-of-action for prodigiosin's antimicrobial activity. The main target site for prodigiosin is the bacterial plasma membrane. As a chaotropic stressor, prodigiosin disrupts the bacterial plasma membrane and induces loss of essential intracellular substances such as sugars, amino acids, proteins and K^+ ions from prodigiosin-treated bacteria (Suryawanshi et al. 2016). Collectively, these findings show that prodigiosin is a potential broad spectrum antibacterial agent.

Antimalarial

Prodigiosin exhibits antagonistic effects on the causative agent of malaria, *Plasmodium falciparum* (Castro 1967). Papireddy et al. (2011) reported that prodigiosin is antimalarial towards *P. falciparum*

D6 (chloroquine-sensitive) and Dd2 (multidrug-resistant) strains, both in vitro and in vivo. An important finding by Patil et al. (2011) revealed that prodigiosin has larvicidal activity against the *P. falciparum* vectors: *Aedes aegypti* and *Anopheles stephensi*. Hence, prodigiosin is not only a potential candidate for post-infection treatment but also to eradicate the carriers of this parasite. Synthetic prodigiosins generated using alkyl or aryl substituents show remarkable reduction of *P. falciparum* in mice without compromising the health of the infected mice (Papireddy et al. 2011).

Anti-mycotic agent

Prodigiosin also inhibits the growth of many pathogenic fungi such as the filamentous fungi *Cryptococcus sp.* and *Candida parapsilosis* (Gulani et al. 2012), *C. albicans*, *Aspergillus niger*, *Penicillium glaucum* (Shaikh 2016) and *Didymella applanata* (Duzhak et al. 2012). Prodigiosin was more potent than Amphotericin B in impeding the growth of *A. niger*, *Trichoderma viridae*, *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Sumathi et al. 2014).

Prodigiosin does not play a role in bacterial pathogenesis

In light of the beneficial properties of prodigiosin, it is imperative to determine if prodigiosin contributes to the virulence of *S. marcescens* and if it is safe to be administered for human therapy. Carbonell et al. (2000) had initially reported that pigmented *S. marcescens* strains cause infections much less frequently than non-pigmented strains, thus reducing any potential risk of infection during mass production of pigment.

As RpoS sigma factor plays an important role in bacterial pathogenesis and stress response (Dong and Schellhorn 2010), its role in regulating prodigiosin production was investigated (Wilft and Salmond 2012). The *rpoS* mRNA contains an inhibitory stem-loop region that is altered by the binding of Hfq-dependent RprA which exposes the ribosome binding site for the translation of RpoS (σ^S). RpoS (σ^S) then represses transcription of the *pig* cluster. When *Caenorhabditis elegans* were challenged with *Serratia rpoS* mutant strains, prodigiosin production by the *rpoS* mutants increased but the mutants were attenuated in *C. elegans*, suggesting that prodigiosin is not vital for bacterial pathogenesis (Wilft and Salmond 2012). Recently, Seah et al. (2016) also reported that the mean time-to-death of *C. elegans* challenged with pigmented and non-pigmented *S. marcescens* does not differ significantly suggesting that prodigiosin is non-toxic towards *C. elegans*. In a parallel study, silkworm larvae were treated with purified prodigiosin. The median lethal dose (LD_{50}) for larvae injected with prodigiosin did not differ significantly from the control (untreated) larvae confirming that prodigiosin is not an essential virulence factor of entomopathogenic *S. marcescens* strains (Zhou et al. 2016). Suryawanshi and co-workers (2016) also suggested that prodigiosin is not a secreted toxin. These findings validate that prodigiosin is an innocuous metabolite that does not play a significant role in the pathogenesis of its native host.

High-level production of prodigiosin from *S. marcescens*

As reviewed above, prodigiosin has a number of potential applications as a therapeutic drug. However, a full clinical evaluation of the efficacy and safety of administering prodigiosin to patients is necessary and this would require large-scale production of prodigiosin. Therefore, parameters such as media composition and pH, temperature and incubation period have been extensively studied for high-level prodigiosin production from its natural host.

Media composition

Many types of differential and selective media have been developed for the isolation and confirmation of *Serratia*. Liquid media previously used for prodigiosin biosynthesis include nutrient broth (Haddix and Werner 2000), peptone glycerol broth (Montaner et al. 2000) and production medium (Bae et al. 2001). Peptone glycerol broth supports higher prodigiosin production compared to nutrient broth and other synthetic media such as Luria-Bertani (LB), tryptone soy, tryptone yeast extract, yeast malt extract and glycerol extract broth (Gulani et al. 2012) whereby the addition of glycerol is essential for high production of prodigiosin (Chang et al. 2011).

To lower the costs of prodigiosin production, many studies have been conducted using cheap and easily available substrates. The addition of 0.4% (w/v) ram horn peptone in control medium (yeast extract and mannitol) led to the production of 0.28 mg/mL prodigiosin by the *S. marcescens* MO-1 strain (Kurbanoglu et al. 2015). Prodigiosin production levels of 38.75 mg/mL and 16.68 mg/mL were reported when peanut seed broth and powdered sesame seed broth were used, respectively (Giri et al. 2004). The enhanced pigment production in peanut seed broth and sesame seed broth is due to the high fatty acid content in the media. Fatty acids promote cell growth and subsequently, higher pigment production (Chang et al. 2011). Saturated fatty acids are responsible for hyperpigmentation of *S. marcescens* because the saturated fatty acid content is relatively high in peanut seed broth compared to sesame seed broth. The role of unsaturated fatty acids is insignificant because growth in media containing peanut oil or sesame oil resulted in lower prodigiosin concentrations (Giri et al. 2004). The presence of fatty acids in the medium also yields 2-octenal (Spiteller et al. 2001), the precursor of the MAP biosynthesis pathway (Williamson et al. 2005), thereby, enhancing prodigiosin production. When cassava mannitol medium supplemented with 2% maltose was used as the culture medium, *S. marcescens* prodigiosin production levels of 49.5 mg/mL were recorded (Casullo de Araujo et al. 2010). Recently, Elkenawy and co-workers used crude glycerol with the addition of 1% (w/v) peptone and 10^9 cells/mL inoculum size for high production of prodigiosin (870 units/cell) from *S. marcescens* MN5 (Elkenawy et al. 2017). The presence of maltose, as an additional carbon source in nutrient broth has been shown to favour prodigiosin production compared to other carbon sources such as glucose (Giri et al. 2004), sucrose, mannitol, lactose, fructose and glucose in peptone glycerol broth (Gulani et al. 2012). Moreover, glucose was found to inhibit prodigiosin production in *S. marcescens* (Fender et al. 2012).

pH of media

Pigment synthesis is also inhibited at high pH (Fender et al. 2012). Prodigiosin production occurs when *S. marcescens* is grown in a pH range of 4 to 10 (Raj et al. 2009) but not at pH 3 and higher than pH 10

(Wang et al. 2012b). Prodigiosin biosynthesis has been reported to be optimum at pH 7.0 - 8.5 (Lapenda et al. 2014; Ramani et al. 2014).

Temperature

Maximum synthesis of prodigiosin was consistently recorded at temperatures between 22°C - 30°C (Elkenawy et al. 2017, Lapenda et al. 2014). PigC, the terminal-condensing enzyme in the bifurcated pathway, is significantly affected by temperature and reduced pigment biosynthesis is well documented at higher temperatures (Giri et al. 2004; Gulani et al. 2012). Temperatures > 30°C denature PigC, leaving MAP and MBC moieties uncondensed and no red pigment formation occurs. At temperatures < 22°C, the lack of pigment formation is due to low PigC enzyme activity. Interestingly, pigment production is restored when there is a shift from a higher temperature (37°C) to a lower temperature (30°C) (Haddix and Wenner 2000) most likely a result of the renaturation of PigC which restores its enzymatic activity.

Incubation period

Generally, higher pigment production is observed after extended incubation periods as prodigiosin is a secondary metabolite produced during the stationary phase of bacterial growth (Elkenawy et al. 2017). Nonetheless, the incubation period for pigmentation in *S. marcescens* is strain-dependent and ranges from 36 hours (Giri et al. 2004) to 96 hours (Ramani et al. 2014). High prodigiosin synthesis was also reported after 144 hours (6 days) of incubation (Elkenawy et al. 2017). This unusually long incubation period is attributed to the low temperature (22°C) which delays the accumulation of sufficient *S. marcescens* cell biomass to synthesise prodigiosin.

Scale-up production of prodigiosin from S. marcescens

Production of prodigiosin is generally undertaken on a small scale and this is not cost effective due to the low levels of in vivo expression by *S. marcescens* and costly downstream purification. More recently, production of *S. marcescens* prodigiosin in culture was scaled-up in a study by Chen et al. (2013). They reported that production of prodigiosin increased from 2.3 mg/mL to 15.6 mg/mL when starch and peptone were utilised as carbon and nitrogen sources, respectively. The six-fold increase in pigment synthesis was attributed to a 6:4 starch:peptone ratio and supplementation with 0.56 mM $FeSO_4 \cdot 4H_2O$, 3.25 mM $MnSO_4 \cdot 4H_2O$ and immobilisation of *S. marcescens* onto 3% calcium alginate beads. In addition, El-Bialy and El-Nour (2015) increased prodigiosin synthesis from an ethyl methanesulfonate (EMS) generated mutant of *S. marcescens*. Prodigiosin synthesis by the EMS-variant strain was eight-fold higher (658 ± 46.0 mg/L) compared to the parent strain (88.4 ± 4.4 mg/L) and the metabolite from the mutant strain was stable at alkaline pH and 80°C (El-Bialy and El-Nour 2015).

Large-scale synthesis of prodigiosin using a synthetic biology approach

High-level synthesis of prodigiosin directly from its original host incurs high costs whilst large-scale cultivation of *S. marcescens* is generally not regarded as safe. Although clinical strains are normally non-pigmented (Mahlen 2011), comparative genome analyses of pigmented and non-pigmented strains

indicate that the genome content of both strains is highly conserved (Li et al. 2015) and thus, the use of large-scale *S. marcescens* culture for prodigiosin synthesis is not encouraged. The high production costs are attributed to the longer incubation period required for *S. marcescens* to synthesise prodigiosin (Elkenawy et al. 2017; Kamble and Hirawale 2012). To overcome these drawbacks, prodigiosin could be synthesised using a synthetic biology approach. Synthetic biology is a recent platform technology that enables high-level production of proteins and metabolites from DNA constructs. This can be achieved by transferring the related pathway from the original host into an industrial compliant host. This would enable safe and cost-effective synthesis of the desired product in large quantities. This approach provides a convenient platform to engineer complex biological systems for the production of food, drugs, polymers, fuels and biomass (Haseloff and Ajioka 2009). Using this approach, useful products such as the precursor to the antimalarial artemisinin (Keasling 2008), terpenoids (Chang et al. 2007) and green biofuels (Atsumi et al. 2008) were successfully synthesised in high quantities.

The availability of the Registry of Standard Biological Parts (http://partsregistry.org) and BioBricks Foundation (http://bbf.openwetware.org), provides a platform for using and sharing of standardised synthetic parts such as promoters, ribosome binding sites and terminators (Haseloff and Ajioka 2009). This platform allows easy selection of regulators for the overexpression of desired products from any DNA construct. Also, engineered microbial hosts and gene assembly techniques such as Gibson Assembly (Gibson et al. 2009) and Golden Gate Assembly (Engler et al. 2008) have enabled the development of more efficient and robust systems for use in this discipline. To enable easy selection of methods to build large DNA constructs, different DNA assembly techniques for synthetic biology applications have recently been reviewed (Juhas and Ajioka 2016a).

Several research teams have integrated the synthetic biology approach for the production of prodigiosin. The prodigiosin of *H. chejuensis* was successfully expressed in *E. coli* by Kwon et al. (2010). The main focus of their study was to identify the positive regulators that upregulate the biosynthesising *hap* cluster (prodigiosin gene cluster in *H. chejuensis*) and understand the biosynthetic pathways of prodigiosin. Although this study did not report increased pigment production in *E. coli*, this research outcome will be useful for future studies to overexpress prodigiosin in the heterologous host, *E. coli*, as well as in *H. chejuensis* (Kwon et al. 2010). Domrose et al. (2015) reported the synthesis of recombinant *S. marcescens* prodigiosin in *Pseudomonas putida* KT2440. In this work, random insertion of the *pig* cluster by transposition into the chromosome of *P. putida* was performed and generated a constitutive prodigiosin-producing *P. putida* strain. The standard parameters for the growth of *P. putida* were improved using Terrific Broth (TB) under high aeration at 20°C and 48 hours incubation. Under these conditions, the heterologous host was able to synthesise up to 14 ± 1 mg/g dry cell weight (DCW) of recombinant prodigiosin (Domrose et al. 2015).

High undecylprodigiosin production was also reported in its non-pathogenic native host *S. coelicolor* using a synthetic biology approach. The production of undecylprodigiosin in *S. coelicolor* M145 increased five times compared to that of the wild-type strain using the ϕBT1 and ϕC31 integrase

strategy. This simple and straightforward strategy utilises the ϕBT1 integrase-mediated multisite recombination to delete part of the calcium-dependent antibiotic (CDA) and actinorhodin (ACT) biosynthesising gene clusters. The absence of endogenous gene clusters overexpressed undecylprodigiosin synthesis suggesting that the CDA, ACT and undecylprodigiosin biosynthesising (*red*) gene clusters compete for common precursors (Zhang et al. 2013). An enhanced undecylprodigiosin-producing *S. coelicolor* strain was recently developed by Liu et al. (2017). In their work, they inactivated the repressor gene, *ohkA*, and adopted the same approach as Zhang et al. (2013) which was to delete both the CDA and ACT gene clusters. Then, three copies of the *red* cluster were integrated into the chromosome of *S. coelicolor* and results showed that the developed strain had a 12-fold increase in undecylprodigiosin production (96.8 mg/g DCW) relative to the wild-type strain *S. coelicolor* M-145 (Liu et al. 2017).

Isolation and Purification of Prodigiosin from *S. marcescens*

Prodigiosin is normally extracted from *S. marcescens* cultures using acidic (Williamson et al. 2006b) or basic solvents (Darshan and Manonmani 2016). Recently, Khanam and Chandra (2018) reported higher yield of prodigiosin was obtained using acidic extraction compared to alkaline extraction, with Methanol being a preferred solvent (Chen et al. 2018). This is due to acid hydrolysis that breaks down the bacterial cell wall and lipid bonds, thereby, enhancing the release of prodigiosin from *S. marcescens* (Khanam and Chandra 2018). Prodigiosin is also insoluble in water but soluble in organic solvents such as acetone (Sun et al. 2015), methylene chloride, dioxane, pyridine, chloroform, hexane and methanol (Juang and Yeh 2014). During acidic extraction, *S. marcescens* is pelleted, and resuspended in methanol. The bacterial suspension is homogenised, centrifuged and the organic portion is filtered through a 0.2 µm filter paper before concentrating. The crude product is then resuspended in methanol before purification by column chromatography (Chen et al. 2018). Both Dozie-Nwachukwu et al. (2017) and Chen et al. (2018) used silica gel as the stationary phase for adsorption and hexane as the mobile phase to elute pure prodigiosin. The eluate was then dried at 45°C to obtain pure prodigiosin in powder form (Chen et al. 2018).

In an earlier report, Sun et al. (2015) extracted prodigiosin from dried *S. marcescens* jx1 cells using ultrasound-assisted extraction (with acetone as the extraction solvent) optimised by response surface methodology (RSM). Results from the RSM suggested that prodigiosin extraction is optimal at 23.4°C using a solvent-to-solute ratio of 1:27.2 over an extraction period of 17.5 minutes. From their findings, 4.3 ± 0.02 g of prodigiosin was harvested from 100 g of dried cells (Sun et al. 2015). However, extraction and purification of prodigiosin are limiting factors in large-scale synthesis of prodigiosin. These downstream processes using organic solvents are costly and energy consuming (Arivizhivendhan et al. 2016), making downstream processing of prodigiosin not feasible which prevents the production of sufficient quantities of pure prodigiosin for clinical evaluation. Furthermore, the organic solvents used for prodigiosin extraction and purification are carcinogenic, making them an occupational hazard (Campo et al. 2013).

To overcome these limitations, Arivizhivendhan et al. (2016) imparted a quaternary amine group onto iron oxide, Fe_3O_4 to become functionalised Fe_3O_4 ($[Fe_3O_4]_F$) that increased the adsorption efficiency of prodigiosin. Using this method, efficiency of prodigiosin extraction was as high as 98% whilst the use of organic solvent was reduced by 95%. Khanam and Chandran (2018) reported that ultrasonication gave the highest yield of prodigiosin (2.54 ± 0.41 mg/mL) from 50 mg of dried *S. marcescens* biomass compared to other extraction methods when the following pipeline was used: heat treatment (60°C), 0.1 N HCl, 96% ethanol, homogenisation, and freezing and thawing.

To increase the yield of extracted and purified prodigiosin, high-level prodigiosin synthesis in the producing host requires increasing the production of key products in the biosynthetic pathway. You et al. (2018) optimised the production of the key limiting enzyme in the pathway, PigC, in *E. coli* using RSM (optimal synthesis parameters were 0.73 g/L glucose, 13.17 g/L yeast extract and 5.86 g/L lactose in auto-induction medium). The extracted PigC had an optimal activity of 179.3 U/mL (You et al. 2018). We suggest that the extracted PigC (You et al. 2018) could be added to a co-culture of two *S. marcescens* mutant strains (each mutant producing either MAP or MBC ((Chen et al. 2018)) to obtain higher yield of extracted and purified prodigiosin.

Prospective Direction for the Synthesis of Prodigiosin

Future work on prodigiosin production will shift from the traditional method of optimising the standard growth conditions of its native host to high-level pigment synthesis in a surrogate as well as non-pathogenic native host using the synthetic biology approach. Previously, prodigiosin was successfully expressed in *P. putida* (Domrose et al. 2015) and *S. coelicolor* (Liu et al. 2017). Since prodigiosin possesses pharmaceutical value, large-scale synthesis of prodigiosin is required for full clinical evaluation. However, *S. coelicolor* and *P. putida* are not suitable hosts to be used in the synthesis of prodigiosin at industrial scale. The *pig* cluster of *S. marcescens* can be expressed in a safer and more industrial-compliant recombinant host such as *E. coli.*

E. coli was used as a synthetic biology chassis for the successful production of artemisinin (Keasling 2008), biofuels (Atsumi et al. 2008) and terpenoids (Chang et al. 2007). Expression of prodigiosin in *E. coli* was attempted by Dauenhauer et al. (1984) but was not successful. The unsuccessful heterologous synthesis of prodigiosin in *E. coli* could be due to the use of an incomplete *pig* cluster (Dauenhauer et al. 1984) or inefficient protein expression of the gene cluster in *E. coli.* To enable high recombinant synthesis of prodigiosin in *E. coli,* the *pig* genes should be codon optimised based on the codon bias of *E. coli.* Recently, we (Yip et al. 2018) developed a codon optimisation strategy that improved recombinant protein synthesis in *E. coli* without negatively affecting the growth rate of the expression host. This strategy is suitable to be applied to any gene of interest for heterologous production in *E. coli*. Refactoring the gene cluster by codon optimisation and grouping the genes into synthetic operons under the control of well-characterised genetic parts available in the

BioBricks Registry could promote heterologous prodigiosin synthesis in *E. coli.*

The *pig* cluster is large (~20 kb) and recombinant DNA normally causes high metabolic burden and requires constant selective pressure (Cunningham et al. 2009) in a surrogate host. Nonetheless, current methods in synthetic biology allow for the integration of large synthetic DNA fragments into bacterial chromosomes for protein expression in *E. coli* (Juhas et al. 2014; Juhas and Ajioka 2015a; Juhas and Ajioka 2015b) and *B. subtilis* (Juhas and Ajioka 2016b). Recently, we also identified the *E. coli motA* flagellar gene as a suitable chromosomal integration site for synthetic DNA (Yip et al., in press). *E. coli* has greater potential to be developed as a synthetic biology chassis for the biosynthesis of prodigiosin compared to *B. subtilis*. This is because prodigiosin is antimicrobial towards *Bacillus* species by inducing autolysins in actively growing *B. subtilis* and other *Bacillus* species (Danevčič et al. 2016b). On the other hand, studies have reported that prodigiosin is not inhibitory towards *E. coli* (Danevčič et al. 2016a; Lapenda et al. 2015). With these approaches, prodigiosin synthesis using *E. coli* as a chassis may surpass the highest production obtained using *S. marcescens* cultures. The overall synthetic biology approach for the recombinant synthesis of prodigiosin in *E. coli* is summarised in Fig. 4.

Fig. 4 Synthetic biology approach for the synthesis of prodigiosin in a safer and more industrial-compliant host, *E. coli*. A) The genes in the *pig* cluster are codon optimised based on the codon bias of *E. coli* and refactored according to the function of each gene product in prodigiosin biosynthesis. B) The gene cluster is refactored into three sub-clusters, namely MAP, MBC and *pigC* operon. In each operon, well-characterised synthetic parts such as promoter, ribosome binding site and terminator are incorporated to regulate gene expression. The genes arrangement in each operon is based on the order of the enzymes in the bifurcated pathway. In MBC operon, *pigK* and *pigL* are removed from the refactored cluster because they are not directly involved in the MBC synthesis. C) The three synthetic operons are then introduced into *E. coli* and successful expression of each operon enables production of prodigiosin in *E. coli*.

Conclusion

The beneficial properties of prodigiosin highlight the potential of this secondary metabolite as a clinical drug. This has prompted many research teams to study its biosynthesising gene cluster, elucidate its bifurcated pathways and optimise the standard growth parameters of *S. marcescens* to achieve high pigment production. Large-scale bacterial cultivation of the human pathogen *S. marcescens* is not encouraged as it is generally not considered as safe. With a thorough understanding on how prodigiosin is produced in *S. marcescens*, scientists can tap into the information for application using a synthetic biology approach. This would allow for cost-effective and safe large-scale synthesis of prodigiosin for a full clinical evaluation of its biological effects.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Table 1 Function of each Pig protein involved in the biosynthesis of prodigiosin (Adapted from Williamson et al. 2005)

Protein	Pathway	Function
PigA	MBC	$_{L}$-prolyl-PCP dehydrogenase
PigB	MAP	H_2MAP dehydrogenase
PigC	Condensing	Condensing enzyme
PigD	MAP	2-Acetyloctanal synthase
PigE	MAP	2-Acetyloctanal aminotransferase
PigF	MBC	HBC *O*-methyl transferase
PigG	MBC	Peptidyl carrier protein
PigH	MBC	HBM synthase
PigI	MBC	$_{L}$-prolyl-AMP ligase
PigJ	MBC	Pyrrolyl-β-ketoacyl ACP synthase
PigK	MBC	Specific function unknown but may act as molecular chaperone to other Pig enzymes in protein folding
PigL	MBC	4'-Phosphopantetheinyl transferase
PigM	MBC	HBM dehydrogenase
PigN	MBC	Specific function unknown but is believed to methylate HBC to MBC

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Recent advancements in high-level synthesis of the promising clinical drug, prodigiosin

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Keywords

Prodigiosin, beneficial secondary metabolite, gene cluster, bifurcated pathway, high-level synthesis

Abstract

Prodigiosin, a red linear tripyrrole pigment and a member of the prodiginine family, is normally secreted by the human pathogen *Serratia marcescens* as a secondary metabolite. Studies on prodigiosin have received renewed attention as a result of reported immunosuppressive, antimicrobial and anticancer properties. High-level synthesis of prodigiosin and the bioengineering of strains to synthesise useful prodiginine derivatives have also been a subject of investigation. To exploit the potential use of prodigiosin as a clinical drug targeting bacteria or as a dye for textiles, high-level synthesis of prodigiosin is a prerequisite. This review presents an overview on the biosynthesis of prodigiosin from its natural host *Serratia marcescens* and through recombinant approaches as well as highlighting the beneficial properties of prodigiosin. We also discuss the prospect of adopting a synthetic biology approach for safe and cost-effective production of prodigiosin in a more industrially compliant surrogate host.

Introduction

Prodigiosin is a red bacterial pigment that is secreted as a secondary metabolite. It is the most prominent member of the prodiginine family (other members include undecylprodigiosin, cycloprodigiosin, metacycloprodigiosin, prodigiosin R1 and streptorubin B) (Fig. 1) (Stankovic et al. 2014). Prodigiosin has long been a subject of research interest due to its many potential beneficial properties that include anticancer (Elahian et al. 2013), antimicrobial (Ibrahim et al. 2014) and antimalarial (Papireddy et al. 2011) properties. This metabolite is secreted by a number of microorganisms such as *Hahella chejuensis* (Kim et al. 2006), *Streptomyces coelicolor* (Do and Nguyen 2014), *Streptomyces grisiovirides* (Kawasaki et al. 2008), *Serratia nematodiphila* (Darshan and Manonmani 2016), *Serratia rubidae* (Siva et al. 2011) and

Serratia marcescens (Casullo de Araujo et al. 2010). Driven by the potential development and application of prodigiosin as a clinical drug, many studies have been undertaken to dissect its biosynthetic pathways as a means to increase production of this pigment. Nevertheless, attempts to develop prodigiosin as a therapeutic molecule have been hampered by reports of toxicity on eukaryotic cells (Pandey et al. 2009), its ability to intercalate and cause double stranded DNA breaks (Kimyon et al. 2016) as well as cell cycle arrest (Soto-Cerrato et al. 2007). Furthermore, purified prodigiosin is reportedly immunosuppressive on the human immune response (Liu and Nizet 2009). Taken together, prodigiosin was presumed to be cytotoxic towards eukaryotic cells and therefore, not suitable to be developed as a clinical drug.

Fig.1 Members of the prodiginine family. A) prodigiosin B) undecylprodigiosin C) cycloprodigiosin D) metacycloprodigiosin E) prodigiosin R1 and F) streptorubin B (Adapted from PubChem (Kim et al. 2016)).

Nevertheless, recent studies have shown that prodigiosin is not genotoxic (Guryanov et al. 2013). Furthermore, bacterial prodigiosins and their synthetic derivatives are effective pro-apoptotic agents against various cancer cell lines where multiple cellular targets include multi-drug resistant cells with little or no toxicity towards normal cell lines (Elahian et al. 2013; Kavitha et al. 2010). These findings pave the way for prodigiosin to be developed as a promising drug candidate and efficient production of this microbial pigment is the prerequisite to its full clinical evaluation. However, large bacterial cultivation of *S. marcescens* for high prodigiosin production is not safe and there are limited reports on the expression of prodigiosin in a safer heterologous host for high-level synthesis.

Hence, this review focuses on the organisation of the prodigiosin biosynthesising or pigment (*pig*) cluster of *Serratia marcescens* ATCC 274 (Sma 274), the representative strain of *S. marcescens*. We also discuss the potential applications of bacterial prodigiosins as a clinical drug and review current approaches to scale-up its synthesis, either in *S. marcescens* or using surrogate hosts and its purification from the expression hosts. Finally, a synthetic biology platform is proposed for a safer and more cost-effective large-scale synthesis of prodigiosin in *Escherichia coli*.

Prodigiosin

Prodigiosin is a red linear tripyrrole molecule (Fig. 1A) made up of 3 rings, A, B and C. The A and B rings are connected in a bipyrrole unit whereas the B and C rings are joined in a dipyrrin (Jolicoeur and Lubell 2008). The pyrrole moiety (C ring) is linked to the methoxy bipyrrole (A and B rings) by a methylene bridge (Garneau-Tsodikova et al. 2006). The nomenclature for prodigiosin is 2-methyl-3-amyl-6-methoxyprodigiosene (Roy et al. 2014) and prodigiosin secreted by Sma 274 has a molecular weight of 323.4 Dalton (Da) (Casullo de Araujo et al. 2010) with a chemical formula of $C_{20}H_{25}N_3O$ (Song et al. 2006).

Prodigiosin is a hydrophobic molecule with a log $P_{octanol\text{-}water}$ value of 5.16 (Suryawanshi et al. 2016) and has a 4-methoxypyrollic core with a cationic charge at physiological pH that enables selective binding to alternating DNA sequences without discriminating between the AT and CG sites. Interestingly, prodigiosin is a monoprotonated ligand that binds to important anions such as Cl^- and HCO_3^- and transports these ions via an antiport (OH^-/Cl^-) mechanism (Seganish and Davis 2005) or an H^+/Cl^- symport mechanism which alters the transmembrane pH gradient. Furthermore, the close proximity of the three pyrroles enables prodigiosin to bind to metal ions such as Cu^{2+} (Park et al. 2003) whereby a prodigiosin-Cu^{2+} complex facilitates double-strand DNA oxidative cleavage (Kimyon et al. 2016; Melvin et al. 2000). The colour intensity of prodigiosin decreases when illuminated with light, suggesting that it is light-sensitive (Wang et al. 2012a). A subsequent report demonstrated that prodigiosin absorbs light and causes phototoxicity of the *S. marcescens* cytomembrane leading to leakage of prodigiosin (Wang et al. 2013).

Prodigiosin can be extracted using acidic or alkaline solvents. Under acidic conditions, purified prodigiosin appears red in colour whilst pure prodigiosin in an alkaline solution has a yellow appearance. It can be detected by liquid chromatography-mass spectrophotometry (LC-MS) (Williamson et al. 2006b). The red prodigiosin is usually detectable at a maximum of 535 nm (Casullo de Araujo et al. 2010) but may present as an additional peak at 500 nm which corresponds to the native prodigiosin-protein complex (Andreyeva and Ogorodnikova 2015). On the other hand, the yellow pigment which is attributed to the formation of β-carotene in *S. marcescens* (Wang et al. 2012a) is visualised as a sharp spectral peak at 470 nm. Prodigiosin has a pKa value of 7.2 (Drink et al. 2015) and an R_f value of 0.59 (Lapenda et al. 2014).

The amount of prodigiosin produced can be estimated using the formula developed by Haddix and Wenner (2000) as shown below:

$$\text{Prodigiosin unit/cell} = \frac{([OD499 - (1.381 \times OD620)]) \times 1000}{OD620}$$

Where, OD_{499} = pigment absorption

OD_{620} = bacterial culture absorption

1.381 = constant

This formula has since become the most commonly used method to quantify prodigiosin (Kamble and Hirawale 2012).

The physiological role of bacterial prodigiosin in vivo remains undefined. A number of reports have suggested potential functions that may provide an advantage in competition with other organisms (Gulani et

al. 2012;) and the continuously challenging natural environment (Stankovic et al. 2014) as well as increased surface hydrophobicity to facilitate ecological dispersion of the bacteria (Song et al. 2006). This is in agreement with previous findings that environmental *S. marcescens* strains are normally pigmented whereas the non-pigmented strains are associated with nosocomial infections (Mahlen 2011). Although the role of prodigiosin still remains vague, the synthesis of this pigment is undoubtedly important to *S. marcescens* since a gene cluster of 20 kb is solely dedicated to its production.

Biosynthesis of prodigiosin

The *pig* cluster is comprised of 14 genes and has a size of 20,960 bp. In Sma 274, these genes are arranged in the order of *pigA, pigB, pigC, pigD, pigE, pigF, pigG, pigH, pigI, pigJ, pigK, pigL, pigM* and *pigN* (Fig. 2) (Harris et al. 2004) and the function of each protein is listed in Table 1.

The *pig* genes in Sma 274 are flanked by the *cueR* and *copA* genes (Harris et al. 2004). The gene *cueR* is located 488 bp upstream of the start of the *pig* cluster whereas a gap of 183 bp separates *copA* and *pigN* (Harris et al. 2004; Venil et al. 2009). The gap of 183 bp between *copA* and *pigN* suggests that the expression of *copA* is independent of the *pig* gene cluster in Sma 274. A promoter for *copA* was predicted within this gap as well as a terminator that terminates the transcription of all the 14 *pig* genes. (Harris et al. 2004) (Fig. 2). From our analysis using the BPROM (Softberry) software (Solovyev and Salamov 2011), this promoter most likely serves as a binding site for a number of transcriptional factors such as arginine repressor 2 (argR2), fis protein, RNA polymerase sigma factor 15 (rpoD15), repressor protein lexA, C-reactive protein (crp) and integration host factor (ihf) (Yip, unpublished). In Sma 274, a bifurcated pathway is needed to synthesise two key intermediates, namely 2-methyl-3-n-amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC) to produce prodigiosin (Harris et al. 2004; Williamson et al. 2006a; Williamson et al. 2006b).

Fig.2 The genetic organisation of the prodigiosin biosynthetic gene cluster of Sma 274. Black block arrows indicate genes involved in the biosynthesis of the monopyrrole moiety, 2-methyl-3-n-amylpyrrole (MAP) whereas white block arrows represent genes involved in synthesising the bipyrrole moiety, 4-methoxy-2,2'-bipyrrole-5-carbaldehyde (MBC). The arrow shaded with vertical lines is the gene that encodes for the terminal condensing enzyme, PigC. Transcriptional regulators of prodigiosin expression are indicated by arrows shaded with horizontal lines. (a) predicted promoter (Harris et al. 2004), (b) putative transcriptional terminator and (c) predicted *copA* promoter in Sma 274 (Harris et al. 2004). The -10 and -35 regions are in bold and underlined (Adapted from Harris et al. 2004).

Twelve out of the 14 genes in the *pig* cluster have previously been assigned and characterised (Table 1) based on cross-feeding experiments of individual gene mutants (Williamson et al. 2005). Genes *pigB, pigD*

and *pigE* were assigned to the synthesis of the monopyrrole moiety, MAP whereas *pigA, pigF, pigG, pigH, pigI, pigJ, pigM* and *pigN* are involved in the production of the bipyrrole moiety, MBC. The gene *pigC* encodes for the terminal condensing enzyme that condenses both MAP and MBC to produce prodigiosin. PigC has N- and C-terminal domains that share sequence similarities to phosphoryl transferase domains and the ATP-binding domain of a pyruvate phosphate dikinase, respectively (Harris et al. 2004). A BLASTP analysis indicated that both PigC and pyruvate phosphate dikinase of *S. marcescens* have the pyruvate phosphate dikinase PEP/pyruvate binding domain with 99% identity. This enzyme has a core catalytic region that binds MBC and MAP together to form prodigiosin (Williamson et al. 2006b). The functions of the gene products of *pigK* and *pigL* are currently not known but PigK may act as a molecular chaperone to assist folding of other Pig enzymes in the biosynthesis of MBC. In addition, PigL, which is a 4'-phosphopantetheinyl transferase, could be involved in the phosphopantetheinylation reaction in the MBC pathway (Williamson et al. 2005). An over view of prodigiosin biosynthesis is illustrated in Figure 3.

Table 1 Function of each Pig protein involved in the biosynthesis of prodigiosin (Adapted from Williamson et al. 2005)

Fig. 3 The bifurcated pathway of prodigiosin biosynthesis (Adapted from Williamson et al. 2006b).

Is prodigiosin suitable as a clinical drug?

Production of microbial secondary metabolites has always been associated with the survival of the producing organisms (Figueiredo et al. 2008). Prodigiosin was evaluated through various studies and shown to have many beneficial properties that make it a promising drug candidate.

Anticancer drug

Prodigiosin is proposed to have an anti-cancer effect where it up-regulates p73 that restores the p53 signalling pathway in SW480 cancer cells and subsequently induces apoptosis of these cells. Since cancer cells carry a variety of mutated p53 with different hotspot mutations in the DNA-binding domain, these p53 mutants can interact with p73 and inhibit the rescue of the p53 pathway. Interestingly, prodigiosin is capable of disrupting this mutant p53/p73 interaction, suggesting that it can be used to treat cancer regardless of the status of p53 (Hong et al. 2014). Additionally, Kavitha et al. (2010) found that prodigiosin induces apoptotic cell death in HeLa cells in a dose-dependent manner with a half maximal inhibitory concentration (IC_{50}) of 700 nM. Hong et al. (2014) demonstrated that prodigiosin selectively kills cancer cells and leaves normal cells unharmed at a low concentration range of 100 nM – 1 µM. This finding is in agreement with the previous study conducted by Montaner et al. (2000) that prodigiosin only induced apoptosis in haematopoietic cancer cells but leaves non-malignant cells unharmed. It has also been shown that prodigiosin acts independently on cancerous cells that express multidrug resistance transporter proteins

such as multidrug resistance 1 (MDR1), breast cancer resistance protein (BCRP) and multidrug resistance-associated protein (MRP) transporters (Elahian et al. 2013).

The preferential killing of cancer cells by prodigiosin can be attributed to its nature as an anion carrier. As an anion transporter, prodigiosin binds to biologically important anions such as Cl^- and HCO_3^- to form a lipophilic prodigiosin-anion complex that can deacidify the pH of certain organelles such as lysosome, endosomes and Golgi apparatus by inhibiting V-ATPase via H^+/Cl^- symport mechanism. (Davis 2010). The altered transmembrane pH gradient causes the pH of the cellular environment of the cancer cells to decrease. Since the intracellular pH of cancer cells is more alkaline than that of normal cells, prodigiosin-treated cancer cells become more acidic, and therefore, undergo apoptosis (Nakashima et al. 2005).

A synthetic prodigiosin derivative, obatoclax mesylate (GX15-070 or simply known as obatoclax) was discovered by Gemin X Pharmaceuticals Inc. (Canada) and has been used as an experimental drug on various types of cancers (Neidle 2013). Obatoclax was used in phase I clinical trials to treat advanced chronic lymphocytic leukaemia (O' Brien et al. 2009). Additionally, it binds to the hydrophobic pocket of BH3 in Bcl-2 proteins, antagonises MCL-1 and initiates apoptosis in cancer cells (Urtishak et al. 2013). Obatoclax also helps to overcome glucocorticoid resistance (common in many chemotherapy protocols) in acute lymphoblastic leukaemia (Heidari et al. 2010) whilst showing promise in patients with small cell lung cancer when used in the combination with carboplatin-epotoside (Chiappori et al. 2012).

Antibacterial drug

In general, cyclic molecules demonstrate distinct antibacterial activity compared to linear molecules (Lee et al. 2011). Kamble and Hiwarale (2012) proposed three mechanisms of prodigiosin as a potent antimicrobial agent: cleavage of bacterial DNA, cell cycle inhibition and modulation of pH. Prodigiosin has been demonstrated to exhibit antagonistic effects towards *Staphylococcus aureus, Bacillus cereus* (Gulani et al. 2012), *Acinetobacter anitratus, Agrobacterium tumefaciens, Bacillus licheniformis, B. subtilis, B. thuringiensis, Erwinia sp., E. coli, Micrococcus sp.,* Methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis, S. saprophyticus* (Ibrahim et al. 2014), *Streptococcus pyogenes, Enterobacter faecalis* and oxacillin-resistant *S. aureus* (ORSA) (Lapenda et al. 2014).

Prodigiosin is inhibitory towards *Borrelia burgdorferi*, a pathogenic bacterium responsible for Lyme disease. *B. burgdorferi* are resistant to doxycycline or amoxicillin whilst prodigiosin had a low minimum inhibitory concentration of < 0.2 µg/mL and 24% activity against stationary phase and actively growing *B. burgdorferi* (Feng et al. 2015). A recent study conducted by Darshan and Manonmani (2016) showed that prodigiosin from *S. nematodiphila* induces programmed cell death (PCD) of *B. cereus, Pseudomonas aeruginosa, S. aureus* and *E. coli* as well as inhibits *B. cereus* and *E. coli* motility. Recently, it has been suggested that chaotropicity-mediated stress is the primary mode-of-action for prodigiosin's antimicrobial

activity. The main target site for prodigiosin is the bacterial plasma membrane. As a chaotropic stressor, prodigiosin disrupts the bacterial plasma membrane and induces loss of essential intracellular substances such as sugars, amino acids, proteins and K^+ ions from prodigiosin-treated bacteria (Suryawanshi et al. 2016). Collectively, these findings show that prodigiosin is a potential broad spectrum antibacterial agent.

Antimalarial

Prodigiosin exhibits antagonistic effects on the causative agent of malaria, *Plasmodium falciparum* (Castro 1967). Papireddy et al. (2011) reported that prodigiosin is antimalarial towards *P. falciparum* D6 (chloroquine-sensitive) and Dd2 (multidrug-resistant) strains, both in vitro and in vivo. An important finding by Patil et al. (2011) revealed that prodigiosin has larvicidal activity against the *P. falciparum* vectors: *Aedes aegypti* and *Anopheles stephensi*. Hence, prodigiosin is not only a potential candidate for post-infection treatment but also to eradicate the carriers of this parasite. Synthetic prodigiosins generated using alkyl or aryl substituents show remarkable reduction of *P. falciparum* in mice without compromising the health of the infected mice (Papireddy et al. 2011).

Anti-mycotic agent

Prodigiosin also inhibits the growth of many pathogenic fungi such as the filamentous fungi *Cryptococcus sp.* and *Candida parapsilosis* (Gulani et al. 2012), *C. albicans*, *Aspergillus niger*, *Penicillium glaucum* (Shaikh 2016) and *Didymella applanata* (Duzhak et al. 2012). Prodigiosin was more potent than Amphotericin B in impeding the growth of *A. niger*, *Trichoderma viridae*, *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Sumathi et al. 2014).

Prodigiosin does not play a role in bacterial pathogenesis

In light of the beneficial properties of prodigiosin, it is imperative to determine if prodigiosin contributes to the virulence of *S. marcescens* and if it is safe to be administered for human therapy. Carbonell et al. (2000) had initially reported that pigmented *S. marcescens* strains cause infections much less frequently than non-pigmented strains, thus reducing any potential risk of infection during mass production of pigment.

As RpoS sigma factor plays an important role in bacterial pathogenesis and stress response (Dong and Schellhorn 2010), its role in regulating prodigiosin production was investigated (Wilft and Salmond 2012). The *rpoS* mRNA contains an inhibitory stem-loop region that is altered by the binding of Hfq-dependent RprA which exposes the ribosome binding site for the translation of RpoS (σ^S). RpoS (σ^S) then represses transcription of the *pig* cluster. When *Caenorhabditis elegans* were challenged with *Serratia rpoS* mutant strains, prodigiosin production by the *rpoS* mutants increased but the mutants were attenuated in *C. elegans*, suggesting that prodigiosin is not vital for bacterial pathogenesis (Wilft and Salmond 2012). Recently, Seah et al. (2016) also reported that the mean time-to-death of *C. elegans* challenged with pigmented and non-pigmented *S. marcescens* does not differ significantly suggesting that prodigiosin is non-toxic towards

C. elegans. In a parallel study, silkworm larvae were treated with purified prodigiosin. The median lethal dose (LD_{50}) for larvae injected with prodigiosin did not differ significantly from the control (untreated) larvae confirming that prodigiosin is not an essential virulence factor of entomopathogenic *S. marcescens* strains (Zhou et al. 2016). Suryawanshi and co-workers (2016) also suggested that prodigiosin is not a secreted toxin. These findings validate that prodigiosin is an innocuous metabolite that does not play a significant role in the pathogenesis of its native host.

High-level production of prodigiosin from *S. marcescens*

As reviewed above, prodigiosin has a number of potential applications as a therapeutic drug. However, a full clinical evaluation of the efficacy and safety of administering prodigiosin to patients is necessary and this would require large-scale production of prodigiosin. Therefore, parameters such as media composition and pH, temperature and incubation period have been extensively studied for high-level prodigiosin production from its natural host.

Media composition

Many types of differential and selective media have been developed for the isolation and confirmation of *Serratia*. Liquid media previously used for prodigiosin biosynthesis include nutrient broth (Haddix and Werner 2000), peptone glycerol broth (Montaner et al. 2000) and production medium (Bae et al. 2001). Peptone glycerol broth supports higher prodigiosin production compared to nutrient broth and other synthetic media such as Luria-Bertani (LB), tryptone soy, tryptone yeast extract, yeast malt extract and glycerol extract broth (Gulani et al. 2012) whereby the addition of glycerol is essential for high production of prodigiosin (Chang et al. 2011).

To lower the costs of prodigiosin production, many studies have been conducted using cheap and easily available substrates. The addition of 0.4% (w/v) ram horn peptone in control medium (yeast extract and mannitol) led to the production of 0.28 mg/mL prodigiosin by the *S. marcescens* MO-1 strain (Kurbanoglu et al. 2015). Prodigiosin production levels of 38.75 mg/mL and 16.68 mg/mL were reported when peanut seed broth and powdered sesame seed broth were used, respectively (Giri et al. 2004). The enhanced pigment production in peanut seed broth and sesame seed broth is due to the high fatty acid content in the media. Fatty acids promote cell growth and subsequently, higher pigment production (Chang et al. 2011). Saturated fatty acids are responsible for hyperpigmentation of *S. marcescens* because the saturated fatty acid content is relatively high in peanut seed broth compared to sesame seed broth. The role of unsaturated fatty acids is insignificant because growth in media containing peanut oil or sesame oil resulted in lower prodigiosin concentrations (Giri et al. 2004). The presence of fatty acids in the medium also yields 2-octenal (Spiteller et al. 2001), the precursor of the MAP biosynthesis pathway (Williamson et al. 2005), thereby, enhancing prodigiosin production. When cassava mannitol medium supplemented with 2% maltose was used as the culture medium, *S. marcescens* prodigiosin production levels of 49.5 mg/mL were

recorded (Casullo de Araujo et al. 2010). Recently, Elkenawy and co-workers used crude glycerol with the addition of 1% (w/v) peptone and 10^9 cells/mL inoculum size for high production of prodigiosin (870 units/cell) from *S. marcescens* MN5 (Elkenawy et al. 2017). The presence of maltose, as an additional carbon source in nutrient broth has been shown to favour prodigiosin production compared to other carbon sources such as glucose (Giri et al. 2004), sucrose, mannitol, lactose, fructose and glucose in peptone glycerol broth (Gulani et al. 2012). Moreover, glucose was found to inhibit prodigiosin production in *S. marcescens* (Fender et al. 2012).

pH of media

Pigment synthesis is also inhibited at high pH (Fender et al. 2012). Prodigiosin production occurs when *S. marcescens* is grown in a pH range of 4 to 10 (Raj et al. 2009) but not at pH 3 and higher than pH 10 (Wang et al. 2012b). Prodigiosin biosynthesis has been reported to be optimum at pH 7.0 - 8.5 (Lapenda et al. 2014; Ramani et al. 2014).

Temperature

Maximum synthesis of prodigiosin was consistently recorded at temperatures between 22°C - 30°C (Elkenawy et al. 2017, Lapenda et al. 2014). PigC, the terminal-condensing enzyme in the bifurcated pathway, is significantly affected by temperature and reduced pigment biosynthesis is well documented at higher temperatures (Giri et al. 2004; Gulani et al. 2012). Temperatures > 30°C denature PigC, leaving MAP and MBC moieties uncondensed and no red pigment formation occurs. At temperatures < 22°C, the lack of pigment formation is due to low PigC enzyme activity. Interestingly, pigment production is restored when there is a shift from a higher temperature (37°C) to a lower temperature (30°C) (Haddix and Wenner 2000) most likely a result of the renaturation of PigC which restores its enzymatic activity.

Incubation period

Generally, higher pigment production is observed after extended incubation periods as prodigiosin is a secondary metabolite produced during the stationary phase of bacterial growth (Elkenawy et al. 2017). Nonetheless, the incubation period for pigmentation in *S. marcescens* is strain-dependent and ranges from 36 hours (Giri et al. 2004) to 96 hours (Ramani et al. 2014). High prodigiosin synthesis was also reported after 144 hours (6 days) of incubation (Elkenawy et al. 2017). This unusually long incubation period is attributed to the low temperature (22°C) which delays the accumulation of sufficient *S. marcescens* cell biomass to synthesise prodigiosin.

Scale-up production of prodigiosin from S. marcescens

Production of prodigiosin is generally undertaken on a small scale and this is not cost effective due to the low levels of in vivo expression by *S. marcescens* and costly downstream purification. More recently, production of *S. marcescens* prodigiosin in culture was scaled-up in a study by Chen et al. (2013). They

reported that production of prodigiosin increased from 2.3 mg/mL to 15.6 mg/mL when starch and peptone were utilised as carbon and nitrogen sources, respectively. The six-fold increase in pigment synthesis was attributed to a 6:4 starch:peptone ratio and supplementation with 0.56 mM $FeSO_4 \cdot 4H_2O$, 3.25 mM $MnSO_4 \cdot 4H_2O$ and immobilisation of *S. marcescens* onto 3% calcium alginate beads. In addition, El-Bialy and El-Nour (2015) increased prodigiosin synthesis from an ethyl methanesulfonate (EMS) generated mutant of *S. marcescens*. Prodigiosin synthesis by the EMS-variant strain was eight-fold higher (658 ± 46.0 mg/L) compared to the parent strain (88.4 ± 4.4 mg/L) and the metabolite from the mutant strain was stable at alkaline pH and 80°C (El-Bialy and El-Nour 2015).

Large-scale synthesis of prodigiosin using a synthetic biology approach

High-level synthesis of prodigiosin directly from its original host incurs high costs whilst large-scale cultivation of *S. marcescens* is generally not regarded as safe. Although clinical strains are normally non-pigmented (Mahlen 2011), comparative genome analyses of pigmented and non-pigmented strains indicate that the genome content of both strains is highly conserved (Li et al. 2015) and thus, the use of large-scale *S. marcescens* culture for prodigiosin synthesis is not encouraged. The high production costs are attributed to the longer incubation period required for *S. marcescens* to synthesise prodigiosin (Elkenawy et al. 2017; Kamble and Hirawale 2012). To overcome these drawbacks, prodigiosin could be synthesised using a synthetic biology approach. Synthetic biology is a recent platform technology that enables high-level production of proteins and metabolites from DNA constructs. This can be achieved by transferring the related pathway from the original host into an industrial compliant host. This would enable safe and cost-effective synthesis of the desired product in large quantities. This approach provides a convenient platform to engineer complex biological systems for the production of food, drugs, polymers, fuels and biomass (Haseloff and Ajioka 2009). Using this approach, useful products such as the precursor to the antimalarial artemisinin (Keasling 2008), terpenoids (Chang et al. 2007) and green biofuels (Atsumi et al. 2008) were successfully synthesised in high quantities.

The availability of the Registry of Standard Biological Parts (http://partsregistry.org) and BioBricks Foundation (http://bbf.openwetware.org), provides a platform for using and sharing of standardised synthetic parts such as promoters, ribosome binding sites and terminators (Haseloff and Ajioka 2009). This platform allows easy selection of regulators for the overexpression of desired products from any DNA construct. Also, engineered microbial hosts and gene assembly techniques such as Gibson Assembly (Gibson et al. 2009) and Golden Gate Assembly (Engler et al. 2008) have enabled the development of more efficient and robust systems for use in this discipline. To enable easy selection of methods to build large DNA constructs, different DNA assembly techniques for synthetic biology applications have recently been reviewed (Juhas and Ajioka 2016a).

Several research teams have integrated the synthetic biology approach for the production of prodigiosin. The prodigiosin of *H. chejuensis* was successfully expressed in *E. coli* by Kwon et al. (2010). The main focus of their study was to identify the positive regulators that upregulate the biosynthesising *hap* cluster (prodigiosin gene cluster in *H. chejuensis*) and understand the biosynthetic pathways of prodigiosin. Although this study did not report increased pigment production in *E. coli*, this research outcome will be useful for future studies to overexpress prodigiosin in the heterologous host, *E. coli*, as well as in *H. chejuensis* (Kwon et al. 2010). Domrose et al. (2015) reported the synthesis of recombinant *S. marcescens* prodigiosin in *Pseudomonas putida* KT2440. In this work, random insertion of the *pig* cluster by transposition into the chromosome of *P. putida* was performed and generated a constitutive prodigiosin-producing *P. putida* strain. The standard parameters for the growth of *P. putida* were improved using Terrific Broth (TB) under high aeration at 20°C and 48 hours incubation. Under these conditions, the heterologous host was able to synthesise up to 14 ± 1 mg/g dry cell weight (DCW) of recombinant prodigiosin (Domrose et al. 2015).

High undecylprodigiosin production was also reported in its non-pathogenic native host *S. coelicolor* using a synthetic biology approach. The production of undecylprodigiosin in *S. coelicolor* M145 increased five times compared to that of the wild-type strain using the ϕBT1 and ϕC31 integrase strategy. This simple and straightforward strategy utilises the ϕBT1 integrase-mediated multisite recombination to delete part of the calcium-dependent antibiotic (CDA) and actinorhodin (ACT) biosynthesising gene clusters. The absence of endogenous gene clusters overexpressed undecylprodigiosin synthesis suggesting that the CDA, ACT and undecylprodigiosin biosynthesising (*red*) gene clusters compete for common precursors (Zhang et al. 2013). An enhanced undecylprodigiosin-producing *S. coelicolor* strain was recently developed by Liu et al. (2017). In their work, they inactivated the repressor gene, *ohkA*, and adopted the same approach as Zhang et al. (2013) which was to delete both the CDA and ACT gene clusters. Then, three copies of the *red* cluster were integrated into the chromosome of *S. coelicolor* and results showed that the developed strain had a 12-fold increase in undecylprodigiosin production (96.8 mg/g DCW) relative to the wild-type strain *S. coelicolor* M-145 (Liu et al. 2017).

Isolation and Purification of Prodigiosin from *S. marcescens*

Prodigiosin is normally extracted from *S. marcescens* cultures using acidic (Williamson et al. 2006b) or basic solvents (Darshan and Manonmani 2016). Recently, Khanam and Chandra (2018) reported higher yield of prodigiosin was obtained using acidic extraction compared to alkaline extraction, with Methanol being a preferred solvent (Chen et al. 2018). This is due to acid hydrolysis that breaks down the bacterial cell wall and lipid bonds, thereby, enhancing the release of prodigiosin from *S. marcescens* (Khanam and Chandra 2018). Prodigiosin is also insoluble in water but soluble in organic solvents such as acetone (Sun et al. 2015), methylene chloride, dioxane, pyridine, chloroform, hexane and methanol (Juang and Yeh 2014). During acidic extraction, *S. marcescens* is pelleted, and resuspended in methanol. The bacterial

suspension is homogenised, centrifuged and the organic portion is filtered through a 0.2 µm filter paper before concentrating. The crude product is then resuspended in methanol before purification by column chromatography (Chen et al. 2018). Both Dozie-Nwachukwu et al. (2017) and Chen et al. (2018) used silica gel as the stationary phase for adsorption and hexane as the mobile phase to elute pure prodigiosin. The eluate was then dried at 45°C to obtain pure prodigiosin in powder form (Chen et al. 2018).

In an earlier report, Sun et al. (2015) extracted prodigiosin from dried *S. marcescens* jx1 cells using ultrasound-assisted extraction (with acetone as the extraction solvent) optimised by response surface methodology (RSM). Results from the RSM suggested that prodigiosin extraction is optimal at 23.4°C using a solvent-to-solute ratio of 1:27.2 over an extraction period of 17.5 minutes. From their findings, 4.3 ± 0.02 g of prodigiosin was harvested from 100 g of dried cells (Sun et al. 2015). However, extraction and purification of prodigiosin are limiting factors in large-scale synthesis of prodigiosin. These downstream processes using organic solvents are costly and energy consuming (Arivizhivendhan et al. 2016), making downstream processing of prodigiosin not feasible which prevents the production of sufficient quantities of pure prodigiosin for clinical evaluation. Furthermore, the organic solvents used for prodigiosin extraction and purification are carcinogenic, making them an occupational hazard (Campo et al. 2013).

To overcome these limitations, Arivizhivendhan et al. (2016) imparted a quaternary amine group onto iron oxide, Fe_3O_4 to become functionalised Fe_3O_4 ($[Fe_3O_4]_F$) that increased the adsorption efficiency of prodigiosin. Using this method, efficiency of prodigiosin extraction was as high as 98% whilst the use of organic solvent was reduced by 95%. Khanam and Chandran (2018) reported that ultrasonication gave the highest yield of prodigiosin (2.54 ± 0.41 mg/mL) from 50 mg of dried *S. marcescens* biomass compared to other extraction methods when the following pipeline was used: heat treatment (60°C), 0.1 N HCl, 96% ethanol, homogenisation, and freezing and thawing.

To increase the yield of extracted and purified prodigiosin, high-level prodigiosin synthesis in the producing host requires increasing the production of key products in the biosynthetic pathway. You et al. (2018) optimised the production of the key limiting enzyme in the pathway, PigC, in *E. coli* using RSM (optimal synthesis parameters were 0.73 g/L glucose, 13.17 g/L yeast extract and 5.86 g/L lactose in auto-induction medium). The extracted PigC had an optimal activity of 179.3 U/mL (You et al. 2018). We suggest that the extracted PigC (You et al. 2018) could be added to a co-culture of two *S. marcescens* mutant strains (each mutant producing either MAP or MBC ((Chen et al. 2018)) to obtain higher yield of extracted and purified prodigiosin.

Prospective Direction for the Synthesis of Prodigiosin

Future work on prodigiosin production will shift from the traditional method of optimising the standard growth conditions of its native host to high-level pigment synthesis in a surrogate as well as non-pathogenic native host using the synthetic biology approach. Previously, prodigiosin was successfully expressed in *P. putida* (Domrose et al. 2015) and *S. coelicolor* (Liu et al. 2017). Since prodigiosin possesses pharmaceutical value, large-scale synthesis of prodigiosin is required for full clinical evaluation. However, *S. coelicolor* and *P. putida* are not suitable hosts to be used in the synthesis of prodigiosin at industrial scale. The *pig* cluster of *S. marcescens* can be expressed in a safer and more industrial-compliant recombinant host such as *E. coli.*

E. coli was used as a synthetic biology chassis for the successful production of artemisinin (Keasling 2008), biofuels (Atsumi et al. 2008) and terpenoids (Chang et al. 2007). Expression of prodigiosin in *E. coli* was attempted by Dauenhauer et al. (1984) but was not successful. The unsuccessful heterologous synthesis of prodigiosin in *E. coli* could be due to the use of an incomplete *pig* cluster (Dauenhauer et al. 1984) or inefficient protein expression of the gene cluster in *E. coli.* To enable high recombinant synthesis of prodigiosin in *E. coli*, the *pig* genes should be codon optimised based on the codon bias of *E. coli.* Recently, we (Yip et al. 2018) developed a codon optimisation strategy that improved recombinant protein synthesis in *E. coli* without negatively affecting the growth rate of the expression host. This strategy is suitable to be applied to any gene of interest for heterologous production in *E. coli*. Refactoring the gene cluster by codon optimisation and grouping the genes into synthetic operons under the control of well-characterised genetic parts available in the BioBricks Registry could promote heterologous prodigiosin synthesis in *E. coli.*

The *pig* cluster is large (~20 kb) and recombinant DNA normally causes high metabolic burden and requires constant selective pressure (Cunningham et al. 2009) in a surrogate host. Nonetheless, current methods in synthetic biology allow for the integration of large synthetic DNA fragments into bacterial chromosomes for protein expression in *E. coli* (Juhas et al. 2014; Juhas and Ajioka 2015a; Juhas and Ajioka 2015b) and *B. subtilis* (Juhas and Ajioka 2016b). Recently, we also identified the *E. coli motA* flagellar gene as a suitable chromosomal integration site for synthetic DNA (Yip et al., in press). *E. coli* has greater potential to be developed as a synthetic biology chassis for the biosynthesis of prodigiosin compared to *B. subtilis*. This is because prodigiosin is antimicrobial towards *Bacillus* species by inducing autolysins in actively growing *B. subtilis* and other *Bacillus* species (Danevčič et al. 2016b). On the other hand, studies have reported that prodigiosin is not inhibitory towards *E. coli* (Danevčič et al. 2016a; Lapenda et al. 2015). With these approaches, prodigiosin synthesis using *E. coli* as a chassis may surpass the highest production obtained using *S. marcescens* cultures. The overall synthetic biology approach for the recombinant synthesis of prodigiosin in *E. coli* is summarised in Fig. 4.

Fig. 4 Synthetic biology approach for the synthesis of prodigiosin in a safer and more industrial-compliant host, *E. coli*. A) The genes in the *pig* cluster are codon optimised based

on the codon bias of *E. coli* and refactored according to the function of each gene product in prodigiosin biosynthesis. B) The gene cluster is refactored into three sub-clusters, namely MAP, MBC and *pigC* operon. In each operon, well-characterised synthetic parts such as promoter, ribosome binding site and terminator are incorporated to regulate gene expression. The genes arrangement in each operon is based on the order of the enzymes in the bifurcated pathway. In MBC operon, *pigK* and *pigL* are removed from the refactored cluster because they are not directly involved in the MBC synthesis. C) The three synthetic operons are then introduced into *E. coli* and successful expression of each operon enables production of prodigiosin in *E. coli*.

Conclusion

The beneficial properties of prodigiosin highlight the potential of this secondary metabolite as a clinical drug. This has prompted many research teams to study its biosynthesising gene cluster, elucidate its bifurcated pathways and optimise the standard growth parameters of *S. marcescens* to achieve high pigment production. Large-scale bacterial cultivation of the human pathogen *S. marcescens* is not encouraged as it is generally not considered as safe. With a thorough understanding on how prodigiosin is produced in *S. marcescens*, scientists can tap into the information for application using a synthetic biology approach. This would allow for cost-effective and safe large-scale synthesis of prodigiosin for a full clinical evaluation of its biological effects.

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Table 1 Function of each Pig protein involved in the biosynthesis of prodigiosin (Adapted from Williamson et al. 2005)

Protein	Pathway	Function
PigA	MBC	$_L$-prolyl-PCP dehydrogenase
PigB	MAP	H_2MAP dehydrogenase
PigC	Condensing	Condensing enzyme
PigD	MAP	2-Acetyloctanal synthase
PigE	MAP	2-Acetyloctanal aminotransferase
PigF	MBC	HBC *O*-methyl transferase
PigG	MBC	Peptidyl carrier protein
PigH	MBC	HBM synthase
PigI	MBC	$_L$-prolyl-AMP ligase
PigJ	MBC	Pyrrolyl-β-ketoacyl ACP synthase
PigK	MBC	Specific function unknown but may act as molecular chaperone to other Pig enzymes in protein folding
PigL	MBC	4'-Phosphopantetheinyl transferase
PigM	MBC	HBM dehydrogenase
PigN	MBC	Specific function unknown but is believed to methylate HBC to MBC

Figure 1

Click here to access/download;Figure;Yip et al AMAB-D-02760 Fig1.jpg

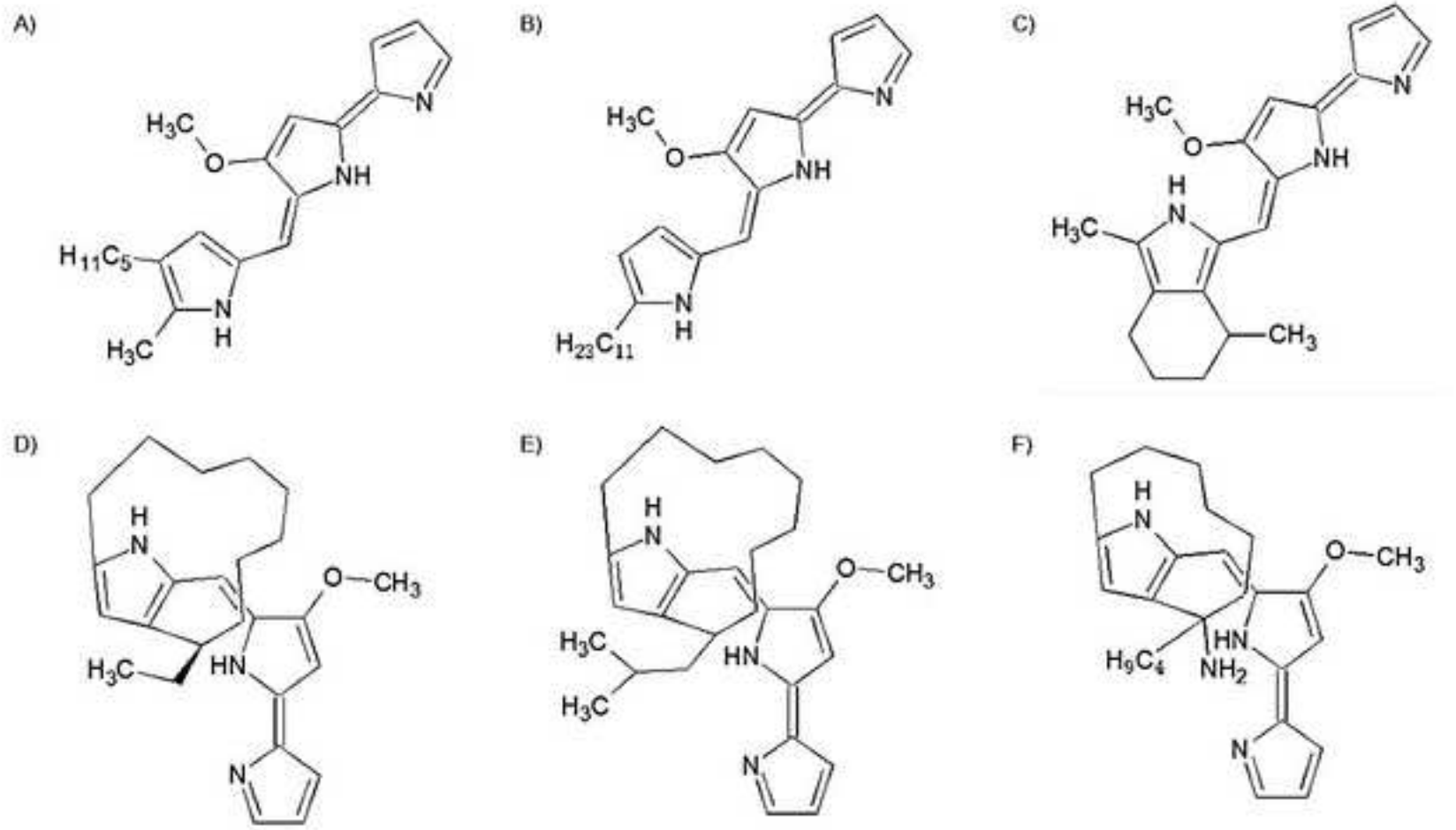

Figure 2

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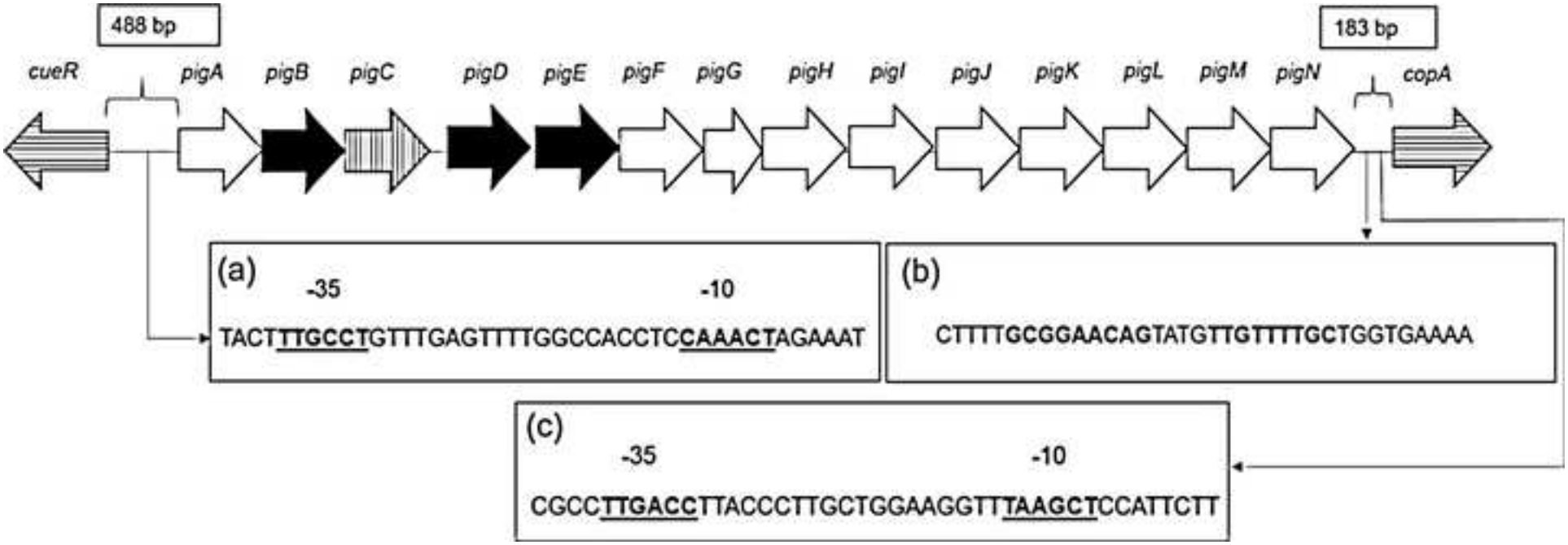

Figure 3

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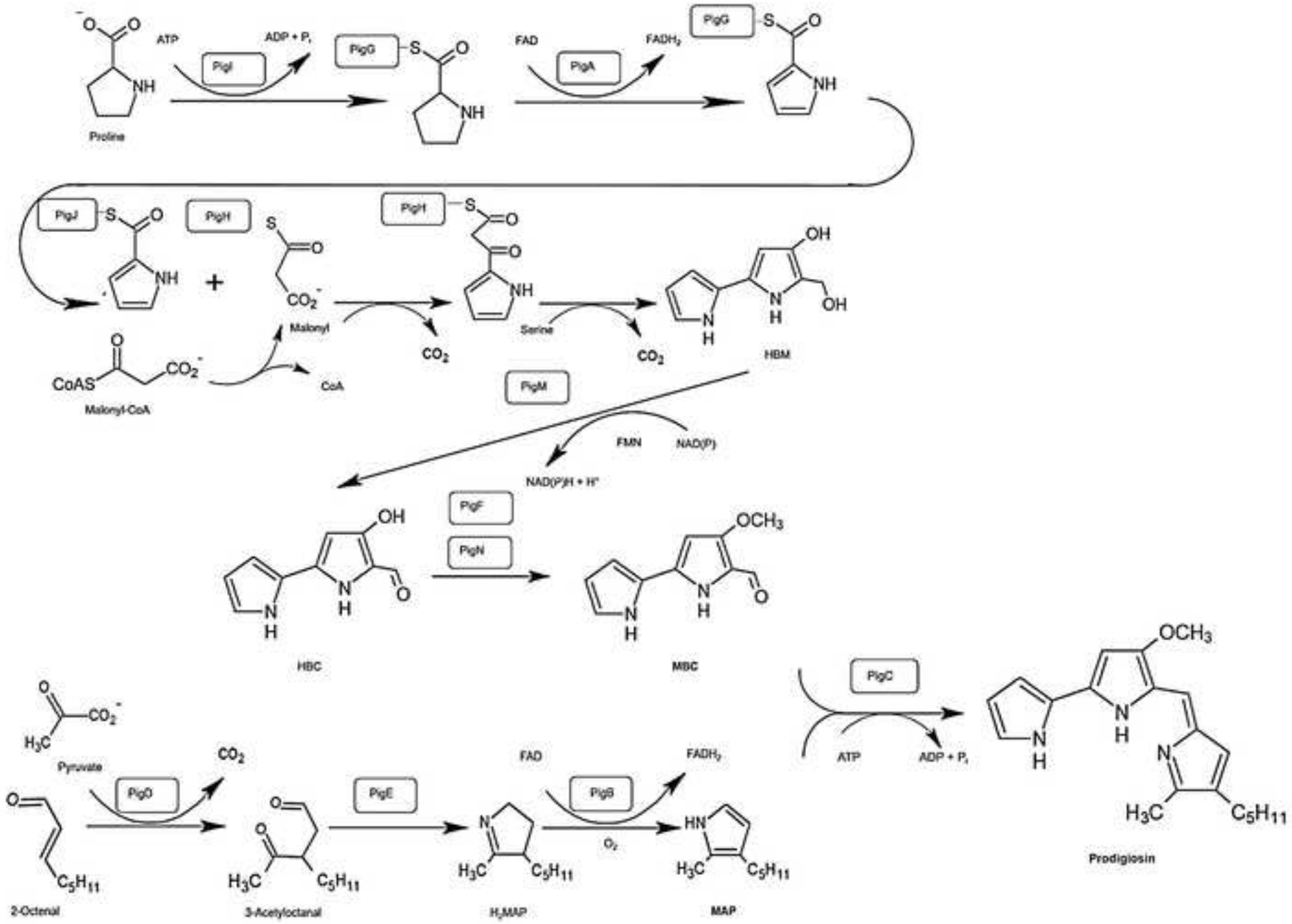

Figure 4

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