Engineering of natural product biosynthesis in *Pseudomonas putida*

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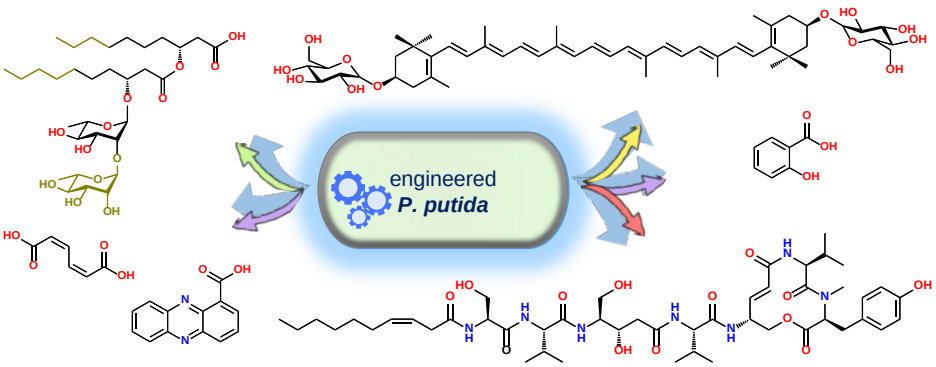
Abstract

Organisms from all kingdoms of life represent a rich source for valuable natural products with various applications. Microbial systems are useful to implement the expression of respective biosynthetic genes and establish the production and effective access to natural products, or to elucidate the biochemistry of the underlying pathways. *Pseudomonas putida*, a Gram-negative soil bacterium, appears to be particularly suitable for natural product biosynthesis because of an advantageous intrinsic metabolism and a remarkable tolerance towards various xenobiotics. This article presents the current state of engineering *P. putida* for the biosynthesis of rhamnolipids, terpenoids, polyketides and non-ribosomal peptides, and products from amino acid metabolism. Technological advances facilitating chromosomal integration and expression of biosynthetic genes, and smart metabolic engineering are key to success.

Graphical abstract



Introduction

During the past few decades, the Gram-negative soil bacterium *Pseudomonas putida* has been developed into a versatile microbial cell factory, in particular for the recombinant biosynthesis of high-value natural products and aromatic building blocks. The list of the bacterium's advantageous features includes a fast and robust growth, a low intrinsic natural product background, and a remarkable tolerance toward xenobiotics [1, 2, 3, 4]. A versatile metabolism provides various redox cofactors and enzymatic capacities. Notably, the central carbon metabolism of *P. putida* shows a unique circular architecture termed EDEMP cycle which is built from enzymes of the Entner-Doudoroff (ED), the Embden-Meyerhof-Parnas (EMP) and the pentose phosphate pathway [5,6]. Recent findings on *P. putida* KT2440 hosting parts of xylose metabolization illustrate that novel metabolic capacities are still to discover [7]. While the biological safety of environmental *P. putida* strains is controversially discussed, the prominent strain *P. putida* KT2440 is classified by regulatory institutions like the FDA as HV1 certified, that is, safe to work within appropriate facilities, similarly to *Escherichia coli* K12 [8•].

A number of recent reviews shed light on the intriguing progression of this bacterium from a soil isolate to a number of engineered platform strains, for which the development of sophisticated engineering tools has been key [2,3,9•,10]. Applications of *P. putida* for natural product biosynthesis have been comprehensively reviewed in 2015 [1].

Here, we describe the current state of engineering natural product biosynthesis in *P. putida* and summarize relevant recent developments for the production of rhamnolipids, terpenoids, polyketides, and non-ribosomal peptides, as well as other compounds derived from the amino acid metabolism. Moreover, the state-of-the-art methods for genetic engineering and heterologous gene expression are highlighted.

Novel tools for engineering recombinant biosynthesis in *P. putida*

A considerable set of technologies has become available for genetic strain engineering and the heterologous expression of biosynthetic genes in *P. putida* (Figure 1a). Single biosynthetic genes and smaller operons are currently predominantly introduced on plasmids (Figure 1b), despite the potential burden of plasmid maintenance [11,12]. This enables the rapid creation of production strains and the flexible use of one construct in multiple strains [13, 14, 15]. Several plasmid series, including a large plasmid collection with standardized architecture called SEVA (Standard European Vector Architecture), have found wide application in the field [2,15,16,17,18].

Especially for larger biosynthetic gene clusters (BGCs), a trend to chromosomal integration is evident, which resulted in the development of multiple tools with different features and chromosomal integration sites (Table 1). Random integration, which has been achieved by the use of transposon Tn5 and mariner transposons (Figure 1c), creates a library of clones wherefrom the most suitable can be selected via a transcription reporter [19•,20,21,22]. Such a randomized approach served to identify the rDNA of *P. putida* KT2440 as especially advantageous chromosomal locus for BGC expression [23•]. Site-specific integration of biosynthetic genes (Figure 1d) can target several loci in the chromosome by use of the IntB13 site-specific recombinase allowing insertion in the *attB* sites within tRNA-encoding genes [24••]. Alternatively, the unique native *att*Tn7 site is frequently exploited for gene integration in one defined locus [25], a strategy particularly advantageous in terms of inter-experiment comparability. Homologous recombination-based methods have allowed the integration of biosynthetic genes into a specific locus of choice, which can be effectively aided by recombinases like RecET [26•]. Intriguingly, this approach enables the deletion of undesired elements like pyoverdine biosynthetic genes at the same time [26•].

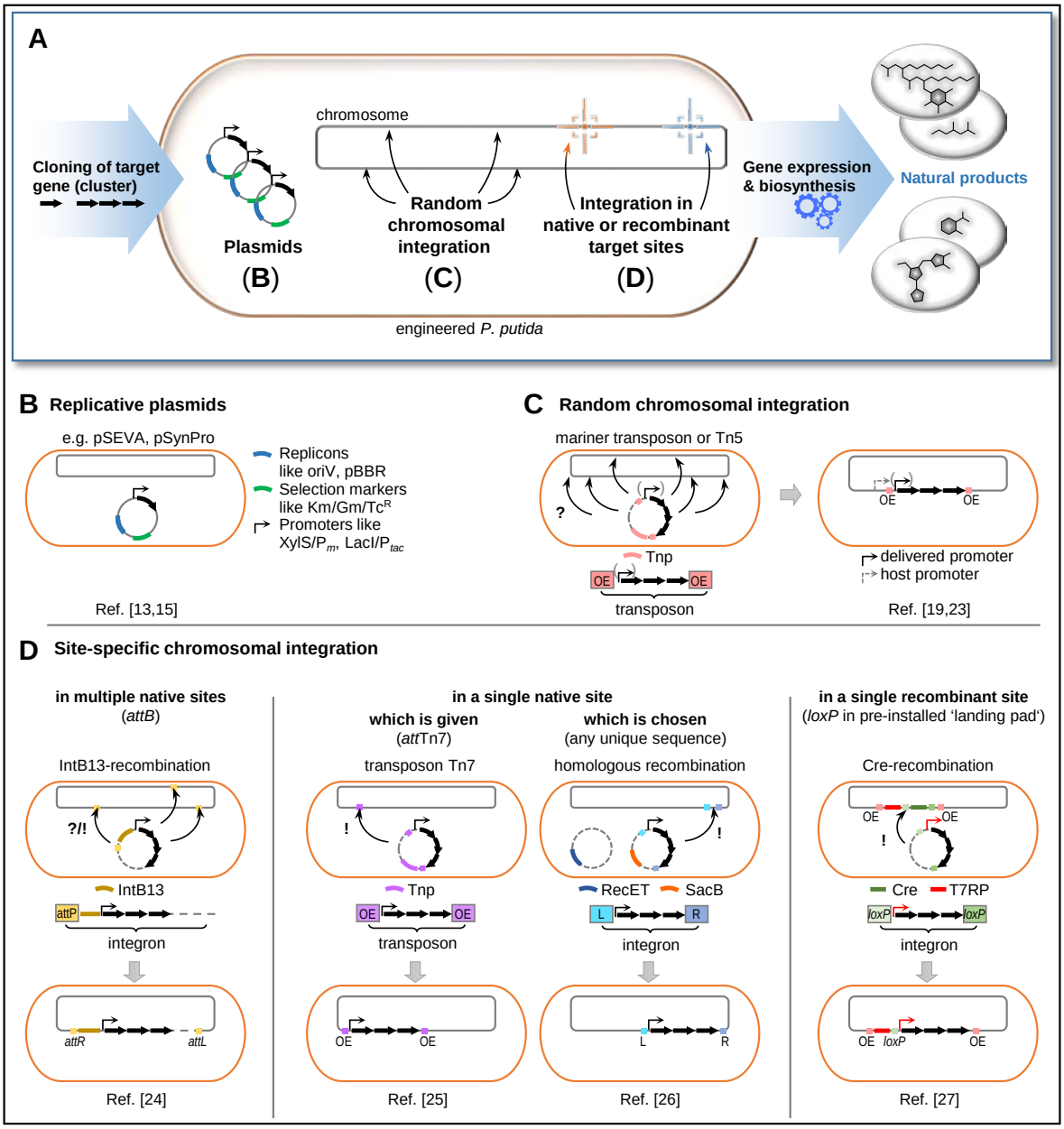


Figure 1. Prominent methods for installation and expression of heterologous biosynthetic genes in *P. putida*. (a) Overview scheme: various methods have become available to realize a 'gene to product' pipeline in *P. putida*. These rely on plasmid use or chromosomal gene integration; specific strategies applied in noteworthy studies are schematically depicted in (b–d). Genetic elements relevant for the procedures are specified. **(b)** Replicative plasmids are typically used for the expression of one gene or operons <10 kb [13,15]. Modular plasmid series with exchangeable replicons, resistance markers (e.g. Km/Gm/Tc^R against kanamycin, gentamicin, tetracycline, respectively) or suitable promoter elements are especially useful. **(c)** Gene integration at a random chromosomal position ('?') relies on transposition. The method can lead to exploitation of chromosomal promoters for BGC expression [19•,23•]. **(d)** Site-specific integration strategies are shown ordered by different modes, that is, targeting non-unique ('?/!') [24••] or unique sites ('!') [25,26•], which are native to *P. putida* or recombinantly introduced [27••]. Approaches depending on specific target sequences (IntB13/*attB*; Tn7/*att*Tn7; Cre/*loxP*) can be distinguished from those allowing to target a freely chosen site (homologous recombination). 'Aiding tools' (like RecET, SacB) grant high effectivity of the latter process [26•]. Tnp, transposase-encoding gene; OE, outside end of transposon; *att*, attachment site; RecET, exonuclease-recombinase-pair; SacB, levansucrase; L, R, here short for left and right homologous sequences to a specific genomic region; T7RP, T7 RNA polymerase; *loxP*, locus of crossover.

CRAGE (chassis-independent recombinase-assisted genome engineering) is a recent example for an elegant two-stage chromosomal integration of different BGCs for expression in various bacteria including *P. putida*. This system is based on the transposon-mediated genomic integration of a 'landing pad', which delivers the T7 RNA polymerase gene and Cre/*loxP* elements, and the subsequent Cre recombinase-mediated integration of a gene cluster under control of promoter P_{T7} [27••]. This way, a remarkable set of standardized platform strains and BGC transfer vectors was established.

Notably, the toolkit for marker-less and scar-less engineering has been expanded, facilitating and securing double crossing over in homologous recombination, for example, via the levansucrase SacB [26•], CRISPR/Cas9 [28] or homing endonuclease I-*Sce*I [29], as well as marker excision after manipulations, for example, via Cre/*loxP* recombination [26•]. After strain construction, transcription of biosynthetic genes can be driven from of a plethora of promoters (e.g. P_{tac}, P_m, P_{BAD}, or synthetic promoters [2]). Moreover, the expression of genes can be further modulated with new effective technologies, for example, for significant enhancement via specific design of untranslated regions [30•,31], or effective protein depletion via CRISPR-interference as titratable gene repression tool [32,33].

Table 1. Applicable sites for implementing heterologous pathways in the *P. putida* chromosome.

Approach	Chromosomal loci	Utilized strategies[1]	Ref
Random chromosomal integration of biosynthetic genes	e.g. rDNA (seven operons)	Transposon Tn5	[21–23,34]
	PP_0619, *fnrA*, PP_5136, PP_3698	Mariner transposon	[19,35]
Targeted chromosomal integration of biosynthetic genes	*attB* sites (in four $tRNA^{Gly}$ genes)	IntB13-mediated integration	[24,36]
	ΦC31 *attB* site at ΦCTX *attB* site in $tRNA^{Ser}$	ΦC31-mediated integration in ΦC31 *attB* (pre-installed via ΦCTX integrase)	[37,38]
	*att*Tn7 site near *glmS*	Transposon Tn7	[7,25,39]
	Chosen loci: *trpE*, *pvdD*, PP_3158, intergenic region of *fpvA* / PP_4218, 16S rRNA gene (specifically chosen for strong gene expression), *pcaHG*, *catBC* (both chosen for deletion of undesired metabolism)	Homologous recombination (options for enhanced effectivity: RecET-mediated recombination; ensuring of second crossing over with SacB or I-*Sce*I)	[26,30,40–47]
	Recombinant 'landing pad' in PP_4633	Cre/*loxP*-mediated integration in 'landing pad' (pre-installed via Tn5 transposition)	[27]
Genomic editing of (intrinsic) metabolic pathways	Multiple loci, individually chosen as appropriate	Gene integration, excision or editing via Cre/*loxP*-, λ Red-, Ssr-, CRISPR/Cas9-, SacB- or I-*Sce*I-based/aided recombineering	[9,28,29,43,44,48–51]

[1] as outlined in Figure 1

The described toolset has been utilized over the last decades for the expression of biosynthetic genes of diverse origins in *P. putida* and has enabled the biosynthesis of different classes of compounds (Figure 2). In the following, recent developments are discussed for each of these classes.

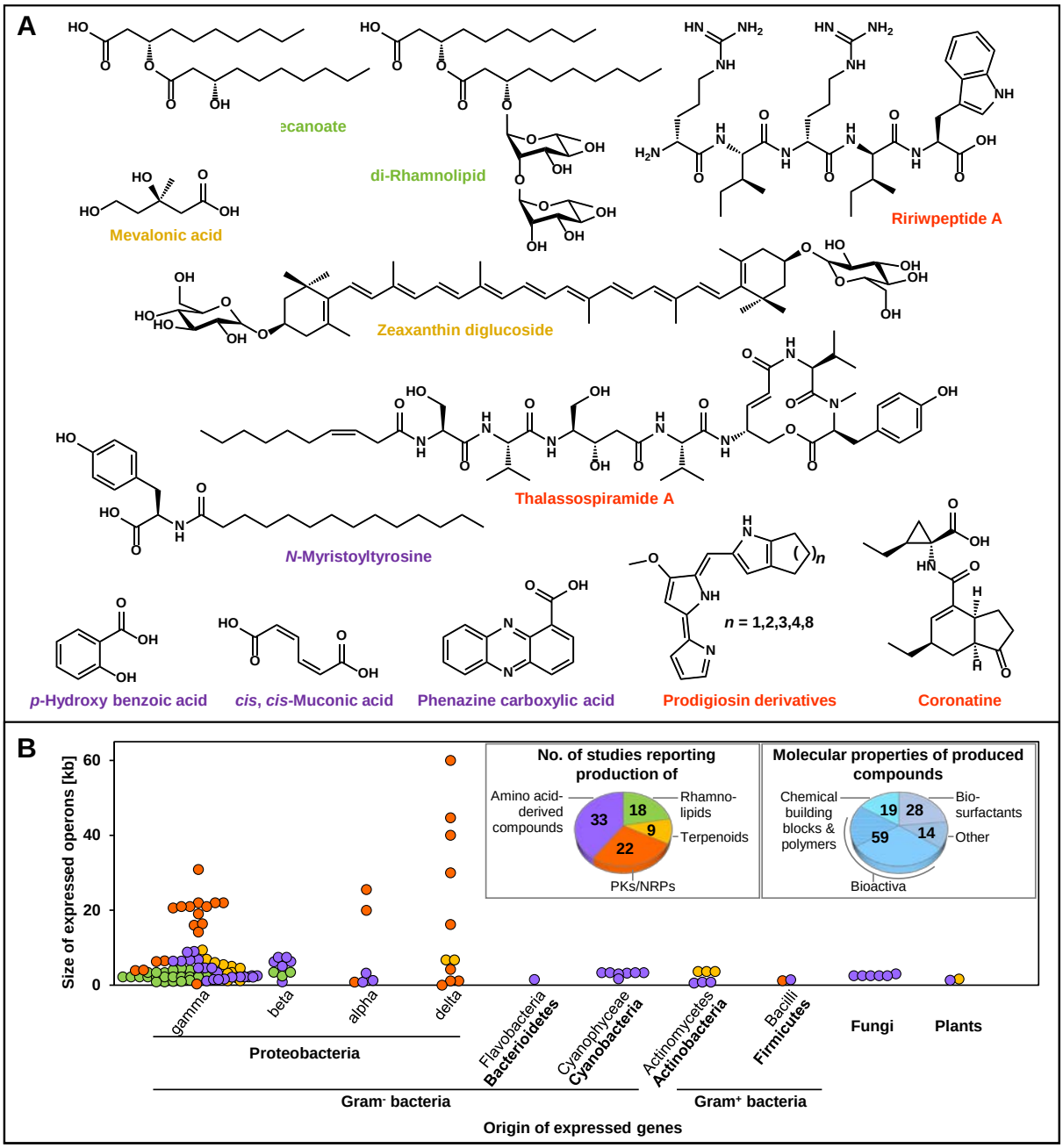


Figure 2. Reported applications of the *P. putida* natural compound production platform. (a) Examples of produced rhamnolipids (green), terpenoids (yellow), PKs/NRPs (red), and other compounds derived from amino acid metabolism (purple). **(b)** Distribution of origin, size and biosynthetic function of genes expressed in *P. putida* for natural product synthesis. The plot summarizes all reports on natural product biosynthesis as reviewed in this article and previously [1] (up to 02/2020, a total of 80 studies). Genes from different phylogenetic sources with various sizes have been employed to achieve the biosynthesis of natural products. Left inset: The distribution of the respective types of compounds is indicated in the above used color scheme. Right inset: Relevant properties of the produced compounds for potential applications. Note that categories are not sharply separable; they were defined according to the focus set by the individual studies. The fraction of new-to-nature derivatives of natural compounds (see selected structures of prodigiosin derivatives as an example) is indicated (hatched area in 'Bioactiva').

Rhamnolipid biosurfactants

Rhamnolipids are secondary metabolites produced by different bacteria [52,53], which can serve as biosurfactants providing an alternative to common oil-based surfactants [54]. Biosynthesis starts from two β-hydroxy fatty acids, building the dimer 3-(3-hydroxyalkanoyloxy)alkanoate (HAA), which is subsequently linked to one or two rhamnose units to form mono-rhamnolipids (mRL) and di-rhamnolipids (dRL) (Figure 2a). These are naturally produced as mixtures that vary in the ratio of mRL to dRL and in the length of the hydroxyl fatty acid chains [52,55]. *P. putida* KT2440 has been developed since the 1990s toward a rhamnolipid production platform because of its close relation to the natural producer *Pseudomonas aeruginosa* and the remarkable resistance toward these substances. Rhamnolipid production is usually established by expression of biosynthetic genes from *P. aeruginosa*, a human pathogen which is thus problematic itself as a production strain. Diverse patents have been filed describing the technology [52] and dRL production has recently reached the level of industrial application (see Evonik Press Release URL: https://corporate.evonik.com/en/pages/article.aspx?articleId=121470). Recent scientific studies have focused on tailoring of the product, mainly regarding the number of rhamnose units and length of fatty acids. For expression of small biosynthetic operons, these studies relied on plasmids with different promoters, including synthetic variants (Table 2). Product tailoring has been achieved, for example, by differential gene selection and expression to specifically produce HAA, mRL or dRL [56••], or by use of biosynthetic genes from *Burkholderia glumae* to introduce longer fatty acid chains [57]. The yields of rhamnolipids with long chain fatty acids, however, were so far much lower than those reported for the *P. aeruginosa*-type rhamnolipids. The *P. putida*-based rhamnolipid production is a useful model system to develop bioprocess strategies, like the utilization of alternative carbon sources [7,13,58] or novel bioreactor designs for example, with immobilized cells [59]. Furthermore, enzymatic and genetic mechanisms of rhamnolipid biosynthesis could be further explored [57,60,61].

Apart from mRL and dRL, *P. putida* may be a promising host for the production of biosurfactants in general [62]. Recently, the production of lipopeptide serrawettin W1 was successfully established in *P. putida*, with solid phase *in situ* recovery of the product yielding the highest titers reported so far [63•]. The strain has furthermore been used to uncover and characterize novel genes related to the synthesis of surface active lipoamino acids like ornithine lipids or *N*-acylated tyrosine (Figure 2a) and phenylalanine from metagenomic libraries [37•,64]. These studies showed not only that metagenomic library screenings in different hosts can yield different positive hits [65], but also that the expression of the same biosynthetic genes may even lead to different products, again indicating that host strains other than *E. coli* may prove advantageous for the expression of metagenomics libraries.

Terpenoids and precursors

Terpenoids occur in all kingdoms of life and comprise one of the largest classes of secondary metabolites with a wide range of applications reaching from the cosmetics to the pharma sector. Terpenoid biosynthesis starts from universal C_5 isoprene precursors isopentenyl diphosphate and dimethylallyl diphosphate, which are elongated step-wise before conversion by terpene synthases, whose reaction products are typically further modified by, for example, hydroxylation and glycosylation [66]. The production of terpenes using *P. putida* started with biotransformation approaches [67], and first *de novo* biosyntheses yielded the C_{10}monoterpenoid geranic acid [68], as well as C_{40} tetraterpenes β-carotene and zeaxanthin [69,70]. Recent studies have further focused on tetraterpene production in *P. putida* KT2440, mostly relying on plasmids for expression of approximately 3−10 kb biosynthetic operons (Table 2). Besides the production of the linear carbohydrate lycopene [25] and cyclic β-carotene [71•], the

recombinant production of zeaxanthin diglucoside (Figure 2a) was reported [72]. These studies did not aim at achieving industrially attractive product levels or gaining access to yet unexplored terpenoid chemistry, but rather used carotenoid formation as a readout for classical metabolic engineering and re-wiring of the chassis' core metabolism [25,71•] or for confirmation of enzyme function [72].

These recent developments corroborate the general suitability of *P. putida* as host for (tetra)terpenoid biosynthesis and highlight that product levels can be effectively enhanced by engineering precursor biosynthesis via the intrinsic 2-*C*-methylerythritol-4-phosphate (MEP) or additional installation of the mevalonate (MVA) (Figure 2a) isoprenoid pathway. Further studies addressed the upstream core metabolism providing on the one hand MEP pathway precursors glyceraldehyde 3-phosphate and pyruvate by implementation of linear Embden-Meyerhof-Parnas (EMP) glycolysis instead of the Entner-Doudoroff (ED) pathway [71•] or on the other hand MVA pathway precursor acetyl-CoA by the introduction of recombinant enzymatic steps [73]. These efforts may pave the way for *P. putida* to become a terpenoid production host complementary to established systems as *E. coli* and yeast [74].

Polyketides, non-ribosomal peptides and related compounds

Polyketides (PKs) and non-ribosomal peptides (NRPs) represent a large and diverse group of complex natural products, exhibiting diverse properties including antibiotic and cytotoxic activities. The respective biosynthetic machineries, that is, polyketide synthases (PKSs) and non-ribosomal peptide synthases (NRPSs) synthesize oligomers from acyl and aminoacyl building blocks, respectively, which can undergo numerous modifications such as cyclization. PKSs and NRPSs are modular multidomain enzymes and enzyme complexes, which also occur in the form of hybrids of the two classes of assembly systems [75]. The recombinant biosynthesis of a number of PKs, NRPs, hybrids thereof (e.g. myxochromide S [40]) and related compounds in *P. putida* has been established over the last few decades [1]. Recent studies demonstrated powerful approaches to tackle key challenges in this field, which include the cloning and effective expression of often large BGCs and the elucidation of complex biosynthetic machineries (Table 2). Expression of multiple gene clusters from a *Photorhabdus luminescens* strain via CRAGE has enabled the functional expression of six BGCs in various hosts including *P. putida* KT2440, which was identified as suitable for the biosynthesis of three complex natural products including ririwpeptides (Figure 2a) [27••]. This strategy is highly effective in indicating which pathways can be activated in certain hosts. However, a negative CRAGE result may not necessarily exclude a host from the expression of a particular BGC, as successful glidobactin A biosynthesis was previously achieved in *P. putida* via an alternative approach but not via CRAGE [76]. Different strategies have been described to achieve attractive product titers of PK-type and NRP-type compounds. Production of coronafacic acid, the precursor of coronatine (Figure 2a), could be increased remarkably by a change of the promoter and by transposon-based chromosomal BGC integration instead of plasmid use [19•]. Specific chromosomal positions which are especially suitable for gene cluster expression indeed seem to be a major determinant for obtaining high yields, as demonstrated recently in a transposon Tn5-based genome screening with a prodigiosin gene cluster [23•]. Effective pathway activation can be supported via co-expression of a suitable phosphopantetheinyl transferase (PPTase). Although the intrinsic PPTase encoded in *P. putida* KT2440 is already capable of activating a wide range of carrier proteins [77,78] and constitutes one of the advantageous features of the host, co-expression of a BGC-associated PPTase can serve to enhance product levels, as recently shown for thalassospiramides (Figure 2a) [24••,41].

These studies demonstrate that *P. putida* undoubtedly represents a valuable host for PKS/NRPS activation and elucidation of related biosynthetic pathways. This may complement *Streptomyces* host systems,

which are dominant because a multitude of PKS/NRPS pathways come from these species [79]. *Pseudomonas* robustness, fast growth, low biosynthetic background, relatively high GC-content (61.5%) and excellent genetic accessibility render the platform attractive. In particular for myxobacterial pathways, *P. putida* appears especially valuable as one of the few promising hosts thus far established [80]. In addition, high-level production strains or technology platforms can be developed as shown for recombinant prodigiosin production. Here, *P. putida* was used as a platform to access numerous new-to-nature prodiginines via mutasynthesis. This approach was based on *P. putida* harboring a truncated biosynthetic pathway, and enabled incorporation of externally added precursor analogues instead of natural precursors into new tripyrrolic compounds (Figure 2a) [81•,82].

Compounds derived from amino acid metabolism

Besides NRPs, the amino acid metabolism enables the production of numerous additional compounds including aromatics originating from the shikimate pathway that are valuable building blocks for bioactive small molecules, resins, and polymers [83,84]. *P. putida,* especially solvent tolerant strains, has been effectively engineered to produce phenolic compounds. In contrast to the production of the above discussed natural products, this requires the expression of only few enzymes from other organisms, usually introduced on plasmids. In addition, intrinsic metabolic networks have to be interrupted to diminish competing pathways and also product degradation [1,85]. Here, the metabolic versatility of the host would otherwise counteract attractive yields. Recent studies have further refined these engineering approaches (Table 2). For example, introduction of a feedback-inhibition-resistant mutant version of 3-desoxyarabinoheptulosanate-7-phosphate (DAHP) synthase AroG, which catalyzes the first step in the shikimate pathway, significantly improved the production of anthranilic acid and *p*-hydroxy benzoic acid (Figure 2a) [15,51].

The production of *cis, cis*-muconic acid (Figure 2a), an intermediate in aromatics catabolism, and a potential building block for polymers like nylon, polyurethane and polyester, represents another recent focus either by *de novo* production or bioconversion of aromatic monomers from lignin [43••,44,46]. A complete value chain starting from the widely abundant substrate to a bio-based polymer was imaged by conversion of aromatics from hydrothermally treated lignin to muconic acid using a strain equipped with a heterologous phenol hydroxylase cluster and a duplication of native product forming catechol 1,2-dioxygenases CatA and CatA2. Notably, the engineered strain was also deficient in product degradation. The product was subsequently chemically hydrogenated to adipic acid and condensed to a polyamide with the respective diamine [43••].

Violaceines and phenazines are bioactive secondary metabolites derived from the shikimate pathway [86,87]. Here, recent studies achieved product tailoring and raised product titers in *P. putida*, presumably by choice of genes and expression modes, using both, plasmids and chromosomal integration (Table 2) [20,88]. Remarkably, the production of redoxactive compounds like phenazine carboxylic acid (PCA) (Figure 2a) as electron acceptors may enable utilization of this obligate aerobic bacterium for oxygen-limited biocatalysis [88,89]. These studies document the continued interest in the *P. putida*-based production of compounds derived from amino acid metabolism and point toward bio-economic applications in the future. However, the recent reassignment of solvent-tolerant *P. putida* platform strains as potential pathogens in biosafety risk group 2, at least in Germany, has restricted their industrial applicability. Notably, *Pseudomonas taiwanensis* VLB120 is developed as an alternative chassis by transfer of the knowledge gained from studies with *P. putida* S12 [90, 91, 92, 93,94•].

Table 2. Recent natural product synthesis in engineered *P. putida*.

Product	Product titer[1]	Donor strain	Applied *P. putida* strain and expressed genes[2] Strain engineering (where applicable)[3]	Ref.
Rhamnolipids				
mRL	600 mg/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), pl	[97]
mRL	14.6 g/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), pl	[98]
HAA; mRL; mRL/dRL	1.5 g/L; 2.4 g/L; 3.3 g/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlA* (0.9 kb) or -*rhlAB* (2.2 kb) or -*rhlAB* (2.2 kb) and -*rhlC* (1 kb), pl	[56]
mRL	2.4 g/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), pl	[99]
HAA; mRL; mRL/dRL	15 µM; 10 µM; 3 µM/3.5 µM[4]	*P. aeruginosa*	KT2440, P_{tac}-*rhlA* (0.9 kb) or -*rhlAB* (2.2 kb) or -*rhlABC* (3.4 kb syn), pl	[60]
mRL; dRL	75 mg/L; 59 mg/L[y]	*B. glumae*	KT2440, P_{tac}-*rhlAB* (2.4 kb) or P_{tac}-*rhlABC* (3.4 kb syn), pl	[57]
mRL	95 mg/L	*P. aeruginosa*	KT2440, P_{tac}-*rhlAB* (2.2 kb), pl	[57]
mRL	0.08 g/L	*P. aeruginosa*	KT2440, P_{syn}(P_{rRNA} based)-*rhlAB* (2.2 kb), pl	[59]
mRL	1.2 g/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), pl	[61]
dRL	1.2 g/L	*P. aeruginosa*	KT2440, *rha*P_{BAD}-*rhlABC* (approx. 3.4 kb syn), pl	[58]
mRL	827 or 212 mg/L from glucose[y] or propionic acid[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), pl	[13]
mRL	0.72 g/L[y]	*P. aeruginosa*	KT2440, P_{syn}-*rhlAB* (2.2 kb), chr + xylose metabolism (*P. taiwanensis*)	[7]
Terpenoids and precursors				
Lycopene	n.d., µg range	*Pantoea ananatis*	KT2440, P_{crtE}-*crtEIB* (3.4 kb syn), chr + MVA pathway (*Myxococcus xanthus*) or + MEP genes (*P. putida*)	[25]
β-Carotene	372 µg/gDCW	*P. ananatis*	KT2440, XylS/P_m-*crtEBIY* (4.5 kb syn), pl - ED pathway, + EMP glycolysis module (*E. coli*)	[71]
Zeaxanthin diglucoside	121 mg/L	*Pseudomonas* sp.	KT2440, Multi-operon cluster *crtE-idi-crtXIB-orf2-crtZ-orf1* with native promoters (9.4 kb), pl	[72]
Mevalonate	4.07 g/L	*Enterococcus faecalis*, *E. coli*	KT2440, LacI/P_{trc}-*mvaE-mvaS-atoB* (4.8 kb syn), pl - Endonucleases, - ethanol dehydrogenase, + acetyl-CoA synthetase (*E. coli*)	[100]
Mevalonate	237 mg/L	*E. faecalis*	KT2440, $LacI_q$/P_{trc}-*mvaE-mvaS* (3.6 kb), pl - repression of glycerol utilization	[95]
Mevalonate	2.21 g/L	*E. faecalis*	KT2440, LacI/P_{trc}-*mvaE-mvaS-atoB* (4.8 kb syn), pl (*atoB* from *P. putida*)	[73]
Polyketides, non-ribosomal peptides and related compounds				
Flaviolin	n.d.,	*Streptomyces griseus*	KT2440, P_{tac}-*rppA* (1.2 kb), chr	[26]
Serrawettin W1	> 300 mg/L	*Serratia marcescens*	KT2440, P_{tac}-*swrW* (3.9 kb), pl	[63]
Thalassospiramides	n.d.	*Thalassospira* sp. or *Tistrella mobilis*	EM383, P_{native}-*ttcABC* (25.5 kb) or P_{native}-*ttmAB* (20 kb), chr + PPTase (*Thalassospira*)	[24]
Vioprolides	n.d.	*Cystobacter violaceus*	KT2440, P_{tet}- or P_{tn5}-*vioLABCDEFGHIJK* (44.7 kb), chr	[101]

Ririwpeptide A/B; Mevalagmapeptide B; putative pisci-bactin complex	n.d.	*P. luminescens*	KT2440, P_{T7}- *plu3123* (16.4 kb) or -*plu0897-0899* (14.2 kb) or -*plu2316-2325* (30.9 kb), chr	[27]
			+ T7 RNA polymerase (bacteriophage T7)	
Coronatine; derivatives	2.6 mg/L; < 100 µg/L	*P. syringae* pv. tomato	KT2440, XylS/P_m**-*cfl-cfa1-9* (22 kb) and XylS/P_m**-*cmaDEABCT-PSPTO_4713-cmaU* (6.3 kb), pl	[19]
			+ L-*allo*-Isoleucine biosynthesis (*P. syringae*) or + supplementation of unnatural amino acid precursors	
Coronafacic acid	92.8 mg/L	*P. syringae* pv. tomato	KT2440, P_{PrpsH}-*cfa1-9* (20.6 kb), chr	[19]
			+ flaviolin synthase as reporter (*Sorangium cellulosum*)	
Docosahexaenoic acid; *n*-6 docosa-pentaenoic acid	3.0 mg/L; 0.4 mg/L	*Aetherobacter fasciculatus*	KT2440, XylS/P_m-*pfa123* (16.2 kb), chr and XylS/P_m**-*pfa123* (16.2 kb syn), pl	[41]
			- 2,4-dienoyl-CoA reductase, + PPTase (*A. fasciculatus*), + acetyl-CoA carboxylase	
Prodigiosin	150 mg/L	*S. marcescens*	KT2440, P_{chr}-*pigABCDEFGHIJKLMN* (21 kb), chr	[20]
Prodigiosin	94 mg/L	*S. marcescens*	KT2440, P_{16S}-*pigABCDEFGHIJKLMN* (21 kb), chr	[23,34, 102]
Prodigiosin derivatives	0.6-19.8 mg/L	*S. marcescens*	KT2440, P_{16S}-*pigABCDEFGHIJKLMN* (21 kb), chr	[81,82]
			+ Supplementation of unnatural monopyrrole precursors	
Amino acid metabolism-derived compounds				
N-Acyl amino acids	n.d.	soil metagenome	KCTC 2403, P_{native}-*nasYPL* (0.9 kb), pl	[64]
Ornithin lipids	n.d.	lake sediment metagenome	MBD1, P_{native}-*olsB* (0.8 kb on a 29 kb metagenomic DNA fragment), chr	[37]
N-Methylglutamate	17.9 g/L	*Methylobacterium extorquens*	KT2440Δ*upp*, *rha*P_{BAD}-*mgsABC-gmaS* (3.2 kb), pl	[103]
			- Repression of glycerol metabolism, + glutamate dehydrogenase	
Valerolactam	92 mg/L	*Streptomyces aizunensis*	KT2440, AraC/P_{BAD}-*davBA-orf26* (3.9 kb syn), pl (*davBA* from *P. putida*)	[104]
			- Lysine catabolism, - lysine isomerase, - lactamase	
p-Coumarate	200 mg/L	*Flavobacterium johnsoniae*, *E. coli*	KT2440, XylS/P_m-*tal* (1.5 kb) and RhaS-RhaR/P_{rhaB}-*tyrA**-*aroG** (2.2 kb), pl	[15]
			- Feruloyl-CoA synthetase, - *p*-hydroxyphenylpyruvate dioxygenase	
Anthranilic acid	250 g/L	*E. coli*	KT2440, $LacI_q$/P_{trc}-*aroG**-*trpE** (2.1 kb syn), pl	[105]
			- Anthranilate phosphoribosyltransferase, - indole-3-glycerol phosphate synthase, - chorismate mutase	
p-Hydroxy benzoic acid	1.73 g/L[y]	*E. coli*	KT2440, $LacI_q$/P_{trc}-*ubiC-aroG** (1.6 kb syn), pl	[51]
			- *p*-hydroxy benzoate hydroxylase, - chorismate mutase, - anthranilate synthase, + glucose metabolism	
Phenyl ethanol; Trans-cinnamic acid	180 mg/L; 200 mg/L	*Nostoc punctiforme*, *Rhodospirillum rubrum*, *P. putida*	DOT-T1E, XylS/P_m-*pal* (1.7 kb) or XylS/P_m-*PP_0968-kdc-T1E_5478* (artificial Ehrlich pathway 4.5 kb syn), pl	[106]
			+ upregulation of phenylalanine biosynthesis, -	

			phenylalanine catabolism	
Muconic acid (→ nylon)	64.2 or 13 g/L from catechol or lignin	*Pseudomonas* sp.	KT2440, P_{syn}-*dmpKLMNOP* (4.6 kb), chr - Muconate metabolization, - endonucleases, + catechol 1,2-dioxygenase	[43]
Muconic acid (→ adipic acid)	13.5 g/L from lignin	*Pseudomonas* sp., *Enterobacter cloacae*	KT2440, P_{tac}-*aroY* (1.5 kb) and P_{tac}-*catA-dmpKLMNOP* (4.6 kb syn), chr + Catechol 1,2-dioxygenase, - protocatechuate 3,4-dioxygenase, - catechol degradation	[46]
Muconic acid	92 or 15.6 g/L from glucose[y] or coumarate[y]	*E. cloacae*, *Bacillus cereus*	KT2440, P_{tac}-*aroY-ecdB*(-*ecdD*)-*asbF* (3.3 kb syn), chr - Protocatechuate 3,4-dioxygenase, - catechol degradation, + catechol 1,2-dioxygenase	[44]
Muconic acid	2.7 g/L from coumarate[y]	*Pseudomonas* sp., *E. cloacae*	KT2440, P_{tac}-*aroY* (1.5 kb) and P_{tac}-*catA-dmpKLMNOP* (4.6 kb syn), chr + Catechol 1,2-dioxygenase, - protocatechuate 3,4-dioxygenase, - catechol degradation, - catabolite repression	[107]
Muconic acid	22 g/L[y]	*E. cloacae*, *B. cereus*	KT2440, P_{tac}-*aroY-ecdB-asbF* (2.9 kb), chr - Protocatechuate 3,4-dioxygenase, + catechol 1,2-dioxygenase, + rerouting glucose metabolism	[108]
PCA; Pyocyanin	80 mg/L; 11 mg/L	*P. aeruginosa*	KT2440, only NagR/P_{nagAa}-*phzABCDEFG* (6.4 kb) or with NagR/P_{nagAa}-*phzM-phzS* (2.2 kb), pl	[88]
PCA	424 mg/L	*P. aeruginosa*	KT2440, P_{chr}-*phzABCDEFG* (6.4 kb), chr + β-galactosidase as transcription reporter (*E. coli*)	[20,109]
Pyocyanin	30 mg/mL	*P. aeruginosa*	NagR/P_{nagAa}-*phzABCDEFGSM* (8.8 kb syn), pl + xylose metabolism (*E. coli*)	[7]
Violacein/Deoxyviolacein (dVio); dVio; Pro-dVio	105 mg/L; 21 mg/L; mg/L range	*Chromobacterium violaceum*	KT2440, P_{chr}-*vioABCDE* (7.4 kb) or -*vioABCE* (6.4 kb syn) or -*vioABE* (5 kb syn), chr	[20]
Violacein	n.d.	*C. violaceum*	KT2440, P_{tac}-*vioABCDE* (7.4 kb), chr	[26]
Violacein	10 mg/L	*Pseudoaltermonas luteoviolacea*	KT2440, P_{native}-*vioABCDE* (9 kb genomic fragment), pl	[14]

[1] Product levels are listed as highest titers stated in the respective studies; ([y]) indicates that product yields are additionally specified in the original publications. n.d., not determined; DCW, dry cell weight.

[2] Transferred biosynthetic modules are detailed. syn, synthetic operon; pl, use of expression plasmids; chr, integrated in the chromosome; */**, variant of gene or promoter.

[3] Deletion (-) or additional implementation or enhancement of features (+) is indicated separately.

[4] Products were quantified based on congeners with two C10 hydroxy fatty acids.

Conclusions

P. putida has clearly emerged as a valuable and robust host for the recombinant biosynthesis of a wide range of natural products. The bacterium remained a relevant platform for high-level compound production and significantly gained importance regarding elucidation of biosynthetic mechanisms and pathways. It is highly interesting to note that efforts are dedicated not only to exploiting native biosynthetic pathways to deliver natural compounds but also to exploring the non-natural chemical space beyond by tailoring BGCs, enzyme engineering, and mutasynthesis. The current state of research puts the community in an ideal position to tackle the next challenges ahead. Future research may further enhance and extend the available toolkits for gene and gene cluster expression. Filling of current knowledge gaps, for example, on product tolerance and export mechanisms will further strengthen *P. putida* performance.

Combined engineering of biosynthetic pathways and the bacterial strains will then lead to a next generation of *P. putida* chassis for even more effective natural product biosynthesis. Here, recently developed gene expression technologies like CRISPR-interference together with accurate metabolic models [96] will enable effective rerouting of metabolic carbon fluxes and speed up this development.

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Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Anita Loeschcke: Conceptualization, Validation, Visualization, Writing - original draft, Writing - review & editing. Stephan Thies: Conceptualization, Validation, Visualization, Writing - original draft, Writing - review & editing.

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