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Research review paper

Biotechnological advances on Penicillin G acylase: Pharmaceutical implications, unique expression mechanism and production strategies

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ABSTRACT

In light of unrestricted use of first-generation penicillins, these antibiotics are now superseded by their semisynthetic counterparts for augmented antibiosis. Traditional penicillin chemistry involves the use of hazardous chemicals and harsh reaction conditions for the production of semisynthetic derivatives and, therefore, is being displaced by the biosynthetic platform using enzymatic transformations. Penicillin G acylase (PGA) is one of the most relevant and widely used biocatalysts for the industrial production of β-lactam semisynthetic antibiotics. Accordingly, considerable genetic and biochemical engineering strategies have been devoted towards PGA applications. This article provides a state-of-the-art review in recent biotechnological advances associated with PGA, particularly in the production technologies with an emphasis on using the *Escherichia coli* expression platform.

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Abbreviations: 6-APA, 6-aminopenicillianic acid; 7-ACA, 7-aminocephalosporanic acid; 7-ADCA, 7-amino-desacetoxycephalonsporic acid; AA, amino acid; AfPGA, PGA from *A. faecalis*; βLA, β-lactam antibiotics; BRP, bacteroicin release protein; CRP, cAMP receptor protein; D-(−)-PGA, D-(−)-phenylglycine amide; D-(−)-PGM, D-(−)-phenylglycine methyl ester; D-(−)-HPGA, D-(−)-4-hydroxyphenylglycine amide; D-(−)-HPGM, D-(−)-4-hydroxyphenylglycine methyl ester; DNA, deoxyribonucleic acid; DO, dissolved oxygen; DAO, D-amino acid oxidase; EcPGA, PGA from *E. coli*; GA, glutaryl acylase; HSP, heat-shock protein; IM, inner membrane; IPTG, isopropyl-β-D-thio-galactoside; KcPGA, PGA from *K. cryocrescens*; LL, leaderless; mRNA, messenger-RNA; Ntn, N-terminal nucleophilic; nt, nucleotides; OM, outer membrane; PAA, phenylacetic acid; PEG, polyethylene glycol; PenG, penicillin G; PG, peptidoglycan; PGA, penicillin G acylase; PrPGA, PGA from *P. rettgeri*; PVA, penicillin V acylase; RBS, ribosome binding site; RNA, ribonucleic acid; SSC, semi-synthetic cephalosporins; SSP, semi-synthetic penicillin; (S/H), synthesis/hydrolysis; Tat, Twin-Arg translocation; TCA, tricarboxylic acid; TF, trigger factor; TFF-AMEC, tangential flow filtration anion-exchange membrane chromatography; tRNA, transfer-RNA; TtPGA, PGA from *T. thermophiles*.

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1. Introduction

Penicillin is the first β-lactam antibiotic (βLA) to be discovered and, approximately 80 years later, is still one of the most common antibiotics in terms of annual bulk production (~3×10^7 kg/year), annual sales (~$15 billion), and market share (~65% of the total antibiotic market) (Chandel et al., 2008; Parmar et al., 2000; Peñalva et al., 1998). However, the unfettered use of first-generation penicillins (i.e. penicillin G and penicillin V) in the second half of the 20th century led to the development of many penicillin-resistant pathogens. Consequently, only a small fraction of penicillins produced today are used for therapeutic purposes, whereas the majority are used as raw materials for the production of semisynthetic penicillins (SSPs) (e.g. amoxicillin and ampicillin) to further augment the potency of penicillins and broaden their antimicrobial range (Bush, 2007; Parmar et al., 2000). In comparison to their first-generation counterparts, SSPs are engineered to confer novel properties, such as an improved side effect profile, lower toxicity, and superior pharmacokinetics (Chandel et al., 2008; Parmar et al., 2000; Peñalva et al., 1998). SSPs are commonly produced in a two-step fashion (Fig. 1). First, bulk penicillins are transformed into 6-aminopenicillianic acid (6-APA) either chemically or enzymatically. Next, 6-APA is further processed into SSPs enzymatically by condensation with the amide or ester of D-(−)-4-hydroxyphenylglycine and D-(−)-phenylglycine derivatives, respectively (Bruggink et al., 1998). On the other hand, upon expanding the 5-membered thiazolidine ring (which is fused to the β-lactam ring) to a 6-membered thiazine ring, penicillins can be converted to cephalosporin G and then another intermediate of 7-amino-desacetoxycephalonsporic acid (7-ADCA) for subsequent enzymatic production of semisynthetic cephalosporins (SSCs). Alternatively, SSCs can be produced using the raw material of cephalosporin C via another intermediate of 7-aminocephalosporanic acid (7-ACA) (Bruggink et al., 1998). A schematic overview of chemical and enzymatic reactions for the production of a selection of semisynthetic β-lactam antibiotics is presented in Fig. 1.

Given that 6-APA is the key intermediate for the production of SSPs, its production technology and availability directly impact the stability of the world's antibiotic markets. Accordingly, to meet the demand of bulk SSPs, continual effort has been made to improve the scalability, economics, and efficacy of the 6-APA production platform. Traditionally, 6-APA was produced through a laborious yet effective chemical process, in which penicillins were hydrolyzed through the use of hazardous chemicals and solvents, such as trimethylchlorosilane, phosphorous pentachloride, and dichloromethane, at unusually low temperatures (Bruggink et al., 1998). Presently, nearly all bulk penicillins are enzymatically transformed into 6-APA using penicillin acylase (E.C. 3.5.1.11, also known as either penicillin amidase or penicillin amidohydrolase). While the feasibility of this enzymatic approach for the production of 6-APA has been known since the 1950s, it was not economically favorable, primarily due to low conversion yields and high costs of biocatalysts, until its full-scale implementation in the late 1980s. Apart from being an environmentally amicable production process, enzymatic conversions are regio- and stereo-specific, energetically benign, and devoid of undesirable byproducts (Bruggink et al., 1998; Rajendhran and Gunasekaran, 2004). As aforementioned, penicillin acylases may also be used to synthesize a number of SSPs and SSCs by catalyzing the fusion of novel acyl groups with a proper intermediate (i.e. 6-APA, 7-ACA, or 7-ADCA) (Table 1 and Fig. 1).

Broadly, penicillin acylases can be grouped into two classes according to substrate specificity. Type I penicillin acylases hydrolyze penicillin V [thus referred as penicillin V acylase (PVA)], while Type II penicillin acylases hydrolyze penicillin G [thus referred as penicillin G acylase (PGA)]. Moreover, PGA can be further sub-classified into Type IIa specific to an aromatic phenylacetyl moiety and Type IIb specific to an aliphatic moiety (Schmidt, 2010; Sudhakaran et al., 1992). Type IIa PGAs (specifically referred as PGA herein) are the most industrially relevant enzymes and it is estimated that ~85% of enzymatically produced 6-APA (~7650 tons) originates from penicillin G, with the rest from penicillin V (Rajendran et al., 2011; Sudhakaran et al., 1992). While PGA activity has been detected in approximately 40 different microorganisms (including yeast, filamentous fungi, and bacteria), cell factories employed for large-scale production of PGA are limited to a few bacterial platforms with *Escherichia coli* as the major one (Rajendran et al., 2011; Sudhakaran et al., 1992). Being a relatively mature industrial biotechnology, microbial production of PGA, either in native or recombinant hosts, remains riddled with numerous technological issues and limitations. Earlier studies concentrated on bioprocessing and commercial aspects, whereas recent efforts in genetic and protein engineering have been aimed at constructing novel recombinant host/vector systems for PGA overproduction. Herein, we review various novel cellular, molecular, and bioprocessing approaches undertaken to enhance microbial production of PGA.

2. Molecular aspects of PGA

2.1. Gene expression and regulation

Bacterial genes encoding PGAs and their gene expression and regulation mechanisms have been extensively examined, particularly for PGA from *E. coli*. In the native PGA-producing *E. coli* strain of ATCC 11105, phenylacetic acid (PAA) induces the expression of its PGA-encoding gene (*pac*) whereas glucose represses it and the associated regulatory elements have been identified (Radoja et al., 1999). The physiological and metabolic functions of these gene regulations might be associated with the catabolism of carbonaceous aromatic compounds during the organism's ‘free-living mode’ (Duggleby et al., 1995; Rajendhran and Gunasekaran, 2004). However, regulation of the *pac* gene varies from one organism to another. To exemplify, in *Alcaligenes faecalis*, the expression of *pac* is induced by PAA but not repressed by any carbons (Spence and Ramsden, 2007). On the other hand, the expression of *pac* is also induced by PAA, but repressed by tricarboxylic acid (TCA) cycle intermediates (i.e. succinate, fumarate and malate) in *Providencia rettgeri* (Spence and Ramsden, 2007) and by glucose in *Bacillus megaterium* cultivated with complex media (Pinotti et al., 2000). Apart from PAA induction and catabolite repression, *in vivo* PGA synthesis can be temperature-dependent as well (Deshpande et al., 1994). Based on organisms examined so far, this thermo-regulation appears to be universal in all fungi,

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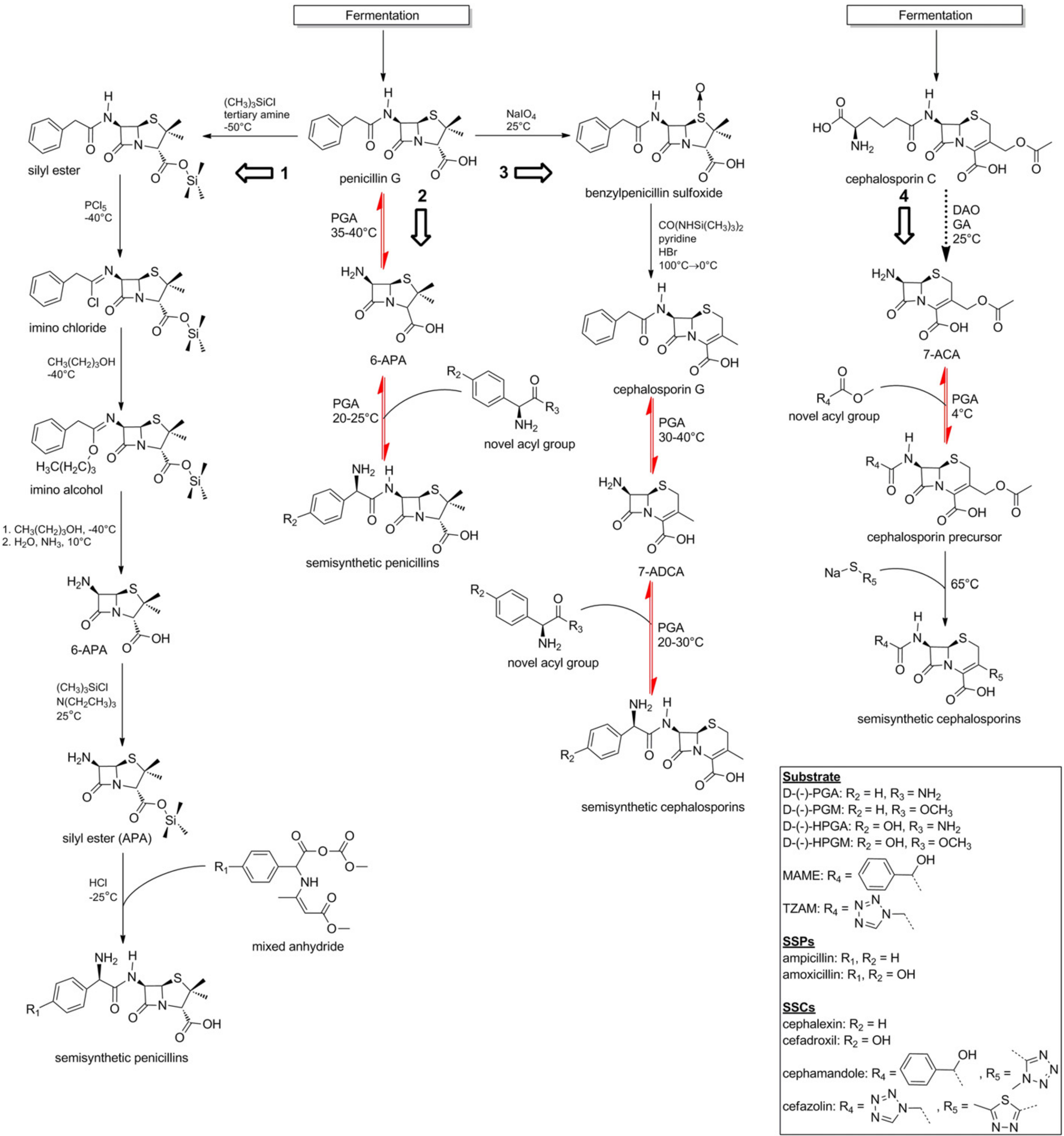

Fig. 1. Chemical and biosynthetic pathways for the production of semisynthetic penicillins (SSPs) and cephalosporins (SSCs). Red/bold arrows: enzymatic reactions catalyzed by PGA; bold/dashed arrow: enzymatic reaction catalyzed by D-amino acid oxidase (DAO) and glutaryl acylase (GA). Both methods for SSP production begin with the formation of penicillin nucleus, 6-aminopenicillanic acid (6-APA), which can be acylated to produce a variety of SSPs (Bruggink et al., 1998). The traditional chemical approach proceeds under unusually low temperatures to prevent splitting of the highly labile β-lactam ring. Chlorosilanes are required to protect the penicillin C(3)-carboxyl, and PCl_5 facilitates the formation of imino bond at the cleavage site for deacylation (Verweij and de Vroom, 1993; Weissenburger and van der Hoeven, 1970). 6-APA is then silated and converted to the desired SSP with the appropriate reagent (e.g. mixed anhydride) (path **1**) (Verweij and de Vroom, 1993). Alternatively, enzymatic synthesis of 6-APA, and SSPs from 6-APA, via PGA does not require harsh reagents or extreme operating conditions (path **2**) (Ospina et al., 1996; Parmar et al., 2000; Wu et al., 2010). Chemical synthesis of the cephalosporin G nucleus, 7-amino-desacetoxycephalonsporic acid (7-ADCA), proceeds with oxidative ring expansion of penicillin G (path **3**) followed by similar removal of the phenylacetic acid (PAA) side chain (not shown) (de Koning et al., 1975; Verweij and de Vroom, 1993). 7-ADCA can be converted to SSCs using silyl protection followed by acylation with a mixed anhydride in a process similar to that shown for SSP production (Verweij and de Vroom, 1993). PGA can also readily hydrolyze cephalosporin G, derived chemically from penicillin G, yielding 7-ADCA (Erarslan, 1993; Li and Cao, 2011). PGA catalyzed synthesis of 7-ADCA, and SSCs from 7-ADCA, occurs under mild conditions (path **3**) (Li and Cao, 2011; Schroen et al., 2001). 7-aminocephalosporanic acid (7-ACA), the cephalosporin C nucleus, can be produced by enzymatic hydrolysis of cephalosporin C using DAO and GA (Justiz et al., 1997). PGA can convert 7-ACA to SSC precursors, which are readily converted to SSCs upon the addition of appropriate reagent (path **4**) (Justiz et al., 1997; Terreni et al., 2001). The chemical route to 7-ACA is analogous to 6-APA and 7-ADCA, whereby choroacetyl chloride is used for carboxyl protection in place of a silating agent (not shown) (Henderson et al., 2008).

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Table 1
Selected β-lactam antibiotics fabricated by PGA biocatalysis.

Antibiotic	Structure	PGA substrates	Advantages	Disadvantages	References
Ampicillin		Phenylglycine amide & 6-APA	• Broad host range • Active against Gram-negative organisms	• Low activity against Gram-positive organisms • Easily degraded by *Stapholococci* Penicillinases • High resistance rates	(Bruggink et al., 1998; Hamad, 2010; Moellering, 1995; Schnarr and Smaill, 2008; Youshko et al., 2004)
Amoxicillin		Hydroxyphenyl-glycine amide & 6-APA	• Highly stable, minimal degradation • Production is feasible with a high substrate concentration	• Limited activity against Gram-negative organisms	(Alemzadeh et al., 2010; Bruggink et al., 1998)
Cefaclor		Phenylglycine methyl ester & 7-ACA	• Broad spectrum efficacy • Safe and well tolerated	• Expensive enzymatic synthesis • pH sensitivity (unstable above pH 6.5)	(Aguirrea et al., 2010; Bruggink et al., 1998; Spencer, 2008)
Cephalexin		Phenylglycine methyl ester & 7-ADCA	• Safe for use during pregnancy	• Difficult synthesis due to pH restrictions and undesirable side products • Not active against *Enterococcus* spp.	(Bruggink et al., 1998; Dashe and Gilstrap, 1997; Einarson et al., 2001; Schnarr and Smaill, 2008; Yang and Wei, 2003)
Cefadroxil		Hydroxyphenyl-glycine methyl ester & 7-ADCA	• Broad spectrum efficacy (both Gram-positive and negative organisms) • Oral administration	• High solubility • Subject to chemical degradation and enzymatic hydrolysis	(Bruggink et al., 1998; Wegman et al., 2001)

yeast, and bacteria. Therefore, PGA-producing strains are often cultivated at temperatures lower than 30 °C. Using *E. coli pac* as the model gene, it was shown that high temperatures tend to affect translation and potentially posttranslational processing steps, but not transcription (Spence and Ramsden, 2007). While the mature enzyme of PGA has an optimal temperature of 40 °C (Table 2), its precursor polypeptide can misfold without being properly processed at elevated temperatures (Spence and Ramsden, 2007).

2.2. Protein structure and maturation

PGAs often have a heterodimeric structure (Table 2) derived from a rather peculiar protein formation mechanism. In general, the *pac* gene is first transcribed and translated into an inactive polypeptidyl precursor, which then undergoes an extensive posttranslational processing to become active. This type of protein maturation is commonly found in eukaryotes (cf. human insulin synthesis), but seldom in prokaryotes. All PGA producers examined thus far are believed to share this type of posttranslational processing, which has been thoroughly investigated only for PGA from *E. coli* (McVey et al., 2001; Thöny-Meyer et al., 1992; Wallace, 1993). Comparative analysis of several bacterial PGAs (Fig. 2) shows considerable sequence homology, especially the α and β subunits (Tishkno, 2010), implying the potential similarity in protein maturation.

In *E. coli*, *pac* is first transcribed and translated in the cytoplasm into a polypeptidyl precursor (i.e. preproPGA) consisting of four domains from N-terminus to C-terminus: (1) 26-AA (amino acid) signal peptide; (2) 208-AA α-subunit; (3) 54-AA endopeptide spacer; and (4) 557-AA β subunit (Sudhakaran et al., 1992; Thöny-Meyer et al., 1992). The signal peptide is responsible for shuttling the precursor into the periplasmic space and mutations in this region can cause the accumulation of nascent preproPGA in the cytoplasm (Schumacher et al., 1986). After translocation into the periplasm, the signal peptide is removed to form another precursor of proPGA. Subsequently, proPGA starts to fold on itself while autoproteolytically removing the spacer region between α and β subunits in the periplasm (Kasche et al., 1999). It has been demonstrated that such autoproteolysis can also occur in the cytoplasm of *E. coli* (Xu et al., 2005a) or *in vitro* (Schumacher et al., 1986). Molecular insights into the multifaceted intra- and intermolecular autoproteolytic reactions and folding to yield the active enzyme are still incomplete (Deshpande et al., 1994; Ignatova et al., 2003; Spence and Ramsden, 2007). Nevertheless, it is postulated that the α subunit starts to fold first, whereas the junction between the spacer region and the β subunit gets dissected, effectively exposing the catalytically active Ser residue (see Section 2.3). The two segments of α + spacer and β subunits assemble to form a premature heterodimeric structure with a partial enzyme activity. Subsequently, the spacer region is removed from the C-terminus by proteolysis, resulting in the active heterodimer (Spence and Ramsden, 2007; Wallace, 1993). This complex maturation is critical as much of the enzyme activity can be lost due to: (1) improper translocation of preproPGA; (2) non-specific proteolysis by cytoplasmic and/or periplasmic peptidases; and (3) improper folding in the periplasm (Chou, 2007; Ignatova et al., 2000, 2003).

2.3. Catalytic mechanism

X-ray crystallographic structures enable the modeling of the molecular aspects of PGA. High-resolution crystallographic structures of PGA from *E. coli* (Chilov et al., 2008; Duggleby et al., 1995), *P. rettgeri* (McVey et al., 2001), and, more recently, *A. faecalis* (Varshney et al., 2012) have been reported to reveal intimate enzyme details, such as overall protein structure, substrate binding site, active center, and catalytic mechanism. Although PGA is a serine hydrolase, it does not possess the catalytic triad characteristic of many other serine proteases

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Table 2
Biochemical properties of industrially relevant class II penicillin G acylases.

Origin: strain	Location	Structure	Protein sequence homology (%)		Optimal hydrolysis		Additional information	References
			Gram −	Gram +	Temp (°C)	pH		
Gram negative bacteria								
Escherichia coli ATCC 11105	Periplasm	Heterodimer, 23 & 62 kDa	100	29	40	8.0	Model industrial PGA	(Erarslan et al., 1990)
Kluyvera cryocrescens ATCC 21285	Periplasm	Heterodimer, 23 & 62 kDa	85	29	40	7.5	Highest homology to *E. coli* PGA	(Alvaro et al., 1992; Bodhe and Sivaraman, 1987)
Providencia rettgeri ATCC 31052	Periplasm	Heterodimer, 24 & 62 kDa	62	32	55	7.5		(Ljubijankic et al., 2002)
Achromobacter xylosoxidans & sp. CCM 4824	Intracellular	Heterodimer, 27 & 62 kDa	51	28	60	7.5	High thermal stability	(Skorb et al., 2003)
Alcaligenes faecalis ATCC 19018 & CICC AS1.767	Periplasm	Heterodimer, 23 & 62 kDa	40	29	60	8.0	High thermal stability	(Kasche et al., 2003; Verhaert et al., 1997; Zhou et al., 2003)
Gram positive bacteria								
Bacillus megaterium ATCC 14945	Extracellular	Heterodimer 27 & 59 kDa	29	100	37	8.0		(Chiang and Bennett, 1967; Kang et al., 1991)
Arthrobacter viscosus ATCC 15294	Extracellular	Heterodimer, 24 & 60 kDa	28	97	45–50	6.0–7.5		(Ohashi et al., 1988)
Bacillus badius PGS10	n/a	Heterodimer 25 & 62 kDa	29	72	50	7.0	Broad substrate specificity	(Rajendran et al., 2011)

(e.g. lipases and proteases) (Alkema et al., 2002; McVey et al., 2001). Thus, it is a member of the structural superfamily of N-terminal nucleophilic (Ntn) hydrolases which contain a catalytic nucleophile (generally a Ser, Cys, or Thr) at the N-terminus as the active site for cleaving an amide bond (Dodson and Wlodawer, 1998). Most bacterial PGAs have a consensus Ser residue (β1), with its hydroxyl group acting as a nucleophile, in the enzyme active center (Duggleby et al., 1995; McVey et al., 2001). PGA mediates the hydrolysis of penicillin G to yield 6-APA and PAA under slightly alkaline pH, resulting in the transfer of the phenylacetyl moiety from 6-APA to water. Mechanistically, this deacylation process is similar to that of serine proteases (Fig. 3). Namely, a nucleophilic attack is brought upon the carbonyl carbon of the amide bond by the Oγ hydroxyl group of Ser (β1), resulting in the formation of a covalent intermediate of an acyl-enzyme complex via a tetrahedral transition state. When this transition state collapses through the involvement of another two AAs of Asn (β241) and Ala (β69), the first product, 6-APA, is released from the active site. Next, the acyl-enzyme complex is deacylated via a nucleophilic attack by water (or another nucleophile), yielding the second product, PAA, and the free enzyme (Arroyo et al., 2003; Spence and Ramsden, 2007). Given that all steps are reversible, the condensation of acyl groups with a β-lactam nuclei becomes possible under low water activities and acidic pH, yielding SSPs (Spence and Ramsden, 2007). Such catalytic reversibility solidifies the importance of PGA as a generic biocatalyst for producing a variety of β-lactam semisynthetic antibiotics.

3. Microbial platforms for PGA production

3.1. Production of heterologous PGA

The biosynthetic capacities associated with PGA entail prosperous development of technologies for large-scale production of PGA over the past few decades. While several microbial hosts have been explored for the production of heterologous PGA, *E. coli* is unarguably the most facile host system due to robust growth characteristics, high fecundity on inexpensive feedstock, well known physiology and metabolism, and genetic tractability. Although certain *E. coli* strains (e.g. ATCC 11105) possess an endogenous *pac* gene whose expression is induced by PAA, the native *pac* promoter is rather weak and unsuitable for large-scale production. Recombinant DNA technology has offered a powerful tool for enhancing PGA production. Among several microbial PGA sources, PGA from *E. coli* (EcPGA) is the most extensive one for both academic study and industrial applications (Rajendran et al., 2011; Sudhakaran et al., 1992). Theoretically, the *pac* gene dosage has to be maximized and all gene expression steps leading to mature PGA (i.e. transcription, translation, translocation, periplasmic processing, and folding) have to be simultaneously effective in order to overproduce PGA. However, such optimal scenario hardly occurs and different host/vector systems might be subject to different expressional limitations. Hence, it is critical that the step(s) limiting overall PGA production can be identified for development of effective expression strategies.

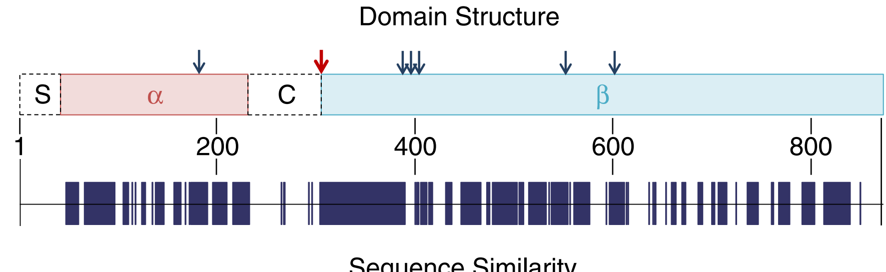

Fig. 2. Domain structure of the preproPGA from *E. coli* ATCC 1105 and its corresponding sequence similarity to other industrially significant PGAs displayed in Table 2. Active site is indicated with a red arrow and calcium binding sites are indicated with blue arrows. The multiple sequence alignment of PGAs utilizing sequences from *E. coli* (P06875.2), *K. cryocrescens* (P07941.1), *P. rettgeri* (AAP86197.1), *A. xylosoxidans* (AAP20806.1), *A. sp. CCM 4824* (AAY25991.1), *A. faecalis* (ABZ91986.1), *B. megaterium* (Q60136.1), *B. badius* (AAZ20308.1), *and A. viscosus* (P31956.1) is constructed using AlignX with a threshold of 0.5 (Vector NTI Advance, Invitrogen, Carlsbad, CA). A full multiple sequence alignment is available in Supplementary Fig. 1.

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Fig. 3. Proposed mechanism of hydrolysis of penicillin G mediated by PGA to yield PAA and 6-APA. Refer Section 2.3 for more detailed description. Refer the section of Abbreviations for the full names of various abbreviations in the figure legend.

For the production of recombinant PGA, the *pac* gene from *E. coli* or other microbial species is often overexpressed in high-copy episomal plasmids to augment gene dosage under the regulation of a strong promoter-operator system to enhance its transcription. In addition to limitations at the transcriptional level, functional overexpression of *pac* is also hampered by several other factors associated with translation, posttranslational modifications as well as physiological impacts on the host. These can result in unexpected misfolding, aggregation or proteolysis of various PGA species in the cytoplasm and/or periplasm. It is estimated that up to 90% of the expressed PGA species, including precursors, subunits, and heterodimers, could eventually become degraded or inactive in any stages of protein maturation due to the above limitations (Ignatova et al., 2000, 2003). Fig. 4 provides a schematic overview of various genetic strategies associated with the manipulation of the *E. coli* expression system to alleviate or even eliminate the above limitations for the overproduction of recombinant PGA. While several of them were developed based on general strategies for enhancing recombinant protein production in *E. coli* (Baneyx, 1999; Jana and Deb, 2005; Sørensen and Mortensen, 2005), the unique PGA formation mechanism significantly contributes to the increased level of technical challenges and complications.

3.1.1. Limitations for pac expression in E. coli

Heterologous gene expression for recombinant protein production in *E. coli* tends to be limited by transcription. Hence, the well-regulated and strong *lac* (and its derivatives such as *tac*, *trc*, and *lacUV5*) and T7 promoter-operator systems have been extensively utilized to enhance gene expression at a transcriptional level (Jana and Deb, 2005). To prevent the undesired glucose catabolite repression upon PGA production, the putative cAMP receptor protein (CRP)-binding site should be excluded from the structural *pac* gene cloned into an expression plasmid (Chou et al., 1999d). Being a natural inducer of all *lac*-derived promoter systems, lactose might be unsuitable for *E. coli* host strains with several *Δlac* mutations, which potentially interfere with lactose transport and/or metabolism. Hence, the non-metabolizable lactose analog, isopropyl-β-D-thiogalactoside (IPTG), is used as an inducer instead. Unfortunately, the use of IPTG can be economically unfavorable for large-scale cultivation. Moreover, PGA overproduction in *E. coli* using various *lac*-derived or T7 promoter systems with IPTG induction generally leads to the formation of insoluble PGA as inclusion bodies in the cytoplasm and/or periplasm due to an imbalance in the flux of PGA formation pathway, particularly at the stages of translocation and periplasmic processing (Scherrer et al., 1994; Sriubolmas et al., 1997). In addition, the accumulation of inclusion bodies, which appear to be physiologically toxic, significantly compromises the integrity of outer membrane, resulting in high levels of cell lysis and growth inhibition (Pan et al., 2003).

Since IPTG-induction for PGA overproduction is fraught with technical hurdles, the use of other sugars, in particular arabinose and galactose, as an alternative inducer is explored. While the binding affinity of galactose to LacI repressor is significantly less than that of IPTG, it

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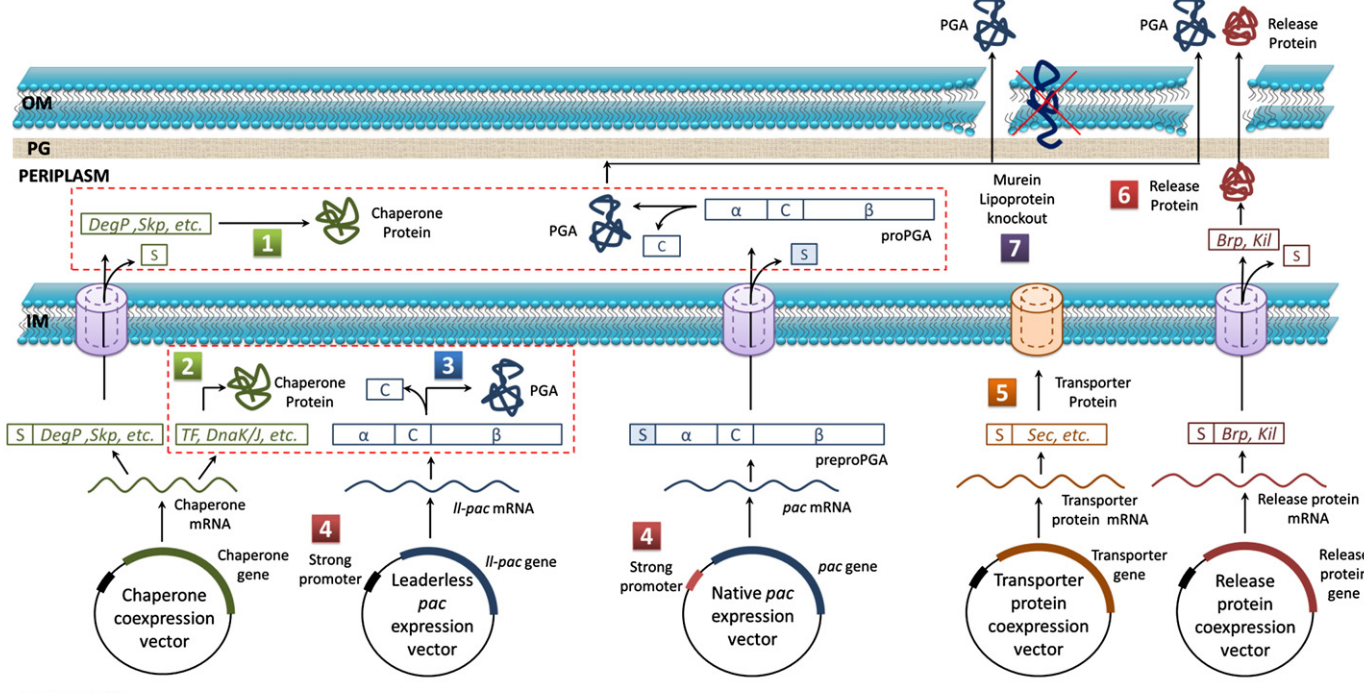

Fig. 4. Genetic strategies for enhancing the production of PGA in *E. coli*. Refer Section 2.1 for detailed description of PGA maturation. Genetic strategies displayed are discussed in detail in the following sections: (1) Coexpression of periplasmic chaperones, Section 3.1.2. (2) Coexpression of cytoplasmic chaperones, Section 3.1.2. (3) Cytoplasmic PGA expression, Section 3.1.2. (4) Transcriptional regulation, Section 3.1.1. (5) Coexpression of translocational machinery, Section 3.1.3. (6) Coexpression of release proteins for extracellular PGA production, Section 3.1.4. (7) Knockout of outer membrane protein or murien lipoprotein for extracellular PGA production, Section 3.1.4.

can act both as an inducer and carbon source for the production of PGA (De Leon et al., 2003a). In *E. coli* cultures supplemented with 0.5% w/v galactose, the volumetric and specific activities of PGA were ~3.3- and 6.8-fold, respectively, those of the conventional cultures induced with IPTG (De Leon et al., 2003a). Moreover, galactose can be even used as inducer in cultures where glucose is the primary carbon source to achieve volumetric and specific activities comparable to cultures induced by IPTG without any impact on cell growth (De Leon et al., 2003a). While galactose-induction certainly resolves technical issues associated with IPTG-induction, little is known as to the particular expression step(s) or mechanism to be improved.

On the other hand, arabinose has not only been demonstrated as an effective inducer for the *trc* and T7 promoters upon PGA production in both the periplasm (by expressing the native *pac* gene) and the cytoplasm [by expressing the leaderless *pac* gene (LL *pac*)], but the induction yields high levels of active PGA with minimal formation of insoluble bodies and hardly any physiological impact (Narayanan et al., 2006a; Xu et al., 2006). Based on superior cell growth and similar or even higher specific *pac* expression levels, culture performance for PGA production for arabinose-induced cultures is significantly better than that for IPTG-induced cultures. Moreover, in an extracytoplasmic stress monitoring study in *E. coli* (Narayanan et al., 2008), it was observed that upon IPTG-induction, there is an upregulation of several extracytoplasmic stress response genes, such as *degP*, *cpxP* and *rpoH*, as the formation of insoluble inclusion bodies within the periplasm triggers a local stress response. These stress-responsive reporter systems were generally less activated with no growth arrest being observed upon arabinose-induction, implying reduced levels of physiological stress experienced by cells (Narayanan et al., 2008). No studies at molecular levels have been conducted to mechanistically illustrate the inducibility and physiological improvement of arabinose for PGA production. Nevertheless, it is generally believed that the flux imbalance upon PGA overproduction *in vivo* is primarily caused by fast transcription of the *pac* gene upon IPTG-induction. Arabinose, like galactose, might have a lower binding affinity to LacI repressor; but the binding is still effective enough to drive slow *pac* transcription without causing subsequent posttranslational processing issues to overwhelm cells.

Given the positive features associated with the use of arabinose for inducing the expression of the *pac* gene regulated by the *trc* and T7 promoters, the use of another common strong promoter system of *araB*, for which arabinose is a native inducer, to regulate *pac* expression is worth investigation. When arabinose is used to induce the expression of *pac* regulated by the *araB* promoter, technical issues similar to IPTG-induction for the *trc* and T7 promoters are observed, implying fast transcription can still be a potential culprit leading to poor culture performance (Narayanan et al., 2006b). The results also support the previous hypothesis that the enhanced PGA production with minimum formation of inclusion bodies upon arabinose-induction for the *trc* and T7 promoter systems is likely associated with the slow *pac* transcription rather than any physiological or metabolic consequences of arabinose supplementation.

Selection of proper *E. coli* host strains for PGA production can be critical since the promoter inducibility and arabinose effect appears to be largely host-dependent. Out of common *E. coli* host strains for recombinant protein production, HB101 and JM109 perform well for the production of PGA, whereas MC4100 and BL21 perform poorly even though the same *pac* expression vectors are used (Narayanan et al., 2006b). Genotypes affecting *pac* expression performance have not been specifically characterized. However, certain mutations, such as *araD* in MC4100, could potentially affect arabinose assimilation because of potential accumulation of L-ribulose-5-phosphate, a known toxic metabolite, and strains with such mutations should not be used as the expression host.

Heterologous gene expression for recombinant protein production in *E. coli* is seldom limited by translation due to the abundance of

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ribosomes/tRNAs and effective coupling between transcription and translation. However, translation can still become limiting by several genetic factors, such as the efficiency of translation initiation and the stability and secondary structure of the nascent mRNA. Since the initiation of translation is often regarded as the limiting step and the most highly regulated phase in translation, researchers have focused on augmenting translation by modifying the region of ribosome binding site (RBS). The spacer region between the RBS and the start codon of *pac* mRNA is only four nucleotides and this may potentially limit translation given that the optimal spacer region is between 5 to 13 nucleotides (Stormo, 2000). PGA production was greatly enhanced, presumably through the improved efficiency of translation initiation by increasing the number of nucleotides of the spacer region (Akkaya et al., 2012; Chou et al., 1999d). The translation efficiency can be also enhanced by increasing the stability of *pac* mRNA, leading to higher levels of PGA activity (Viegas et al., 2005).

In addition to the efficiency of transcription and translation, another important parameter limiting recombinant protein production is gene dosage, which is often associated with plasmid copy number and stability (Valesova et al., 2004). The overproduction of recombinant PGA in *E. coli* can be achieved using certain segregationally stable host/vector systems. It is discerned that the *pac* expression vector pKA18 can be stably maintained in an *E. coli* strain RE3 without plasmid loss for prolonged PGA production for up to 8 successive batch cultivations (approximately equivalent to 108 generations) (Valesova et al., 2004). Additionally, the *pac* gene cloned in an *asd*$^{+}$-expression vector can be stably overexpressed without plasmid loss even in the absence of antibiotic selection (Vohra et al., 2001).

3.1.2. "Engineering" E. coli cell physiology

As mentioned above, *E. coli* cells overproducing recombinant PGA are often under immense physiological and metabolic burdens, particularly upon the accumulation of misfolded PGA aggregates as inclusion bodies. Ultimately, they are subject to growth arrest, cell lysis and even cell death, encroaching on the cells' capacity for recombinant protein production. Nevertheless, cells are equipped with several stress-responsive mechanisms interplaying at various levels in order to survive and proliferate under ever-changing physiological and metabolic stresses (Chou, 2007). These mechanisms and the involved molecules can be manipulated to reduce intracellular stresses, improve cell physiology, and, most importantly, enhance culture performance in PGA production.

The majority of insoluble PGA aggregates reside in the periplasm in the form of proPGA, implying periplasmic processing (for removing the spacer region) is a critical step limiting the overall production of PGA (Scherrer et al., 1994; Sriubolmas et al., 1997). This periplasmic processing is an autoproteolytic step (Ignatova et al., 2003) with proper folding of proPGA as a prerequisite for successful maturation. Cells overwhelmed by the accumulation of periplasmic inclusion bodies experience high levels of extracytoplasmic (which includes the inner membrane, periplasm, and outer membrane) stress and are subject to lysis and growth arrest. A natural mechanism for cells to overcome physiological stress is to selectively express heat-shock proteins (HSPs), in either cytoplasm or periplasm depending on the stress location, with protease (to degrade misfolded proteins) and/or chaperone (to assist or recover close-to-misfolded proteins) activities (Chou, 2007). To date, several genetic stratagems have been implemented to suppress the physiological stress and assuage multifaceted complexities arising from recombinant protein overproduction based on improving protein solubility, stability, secretion efficacy, and even disulfide bond formation. Generally, these involve the concomitant expression of recombinant protein with various HSPs.

Among various periplasmic HSPs, DegP (which possesses both protease and chaperone activities) has been demonstrated extremely effective in assisting PGA maturation and reducing the amount of inclusion bodies (Lin et al., 2001a). Physiological stress and growth arrest associated with PGA overproduction were significantly reduced upon DegP coexpression, though it was discerned that DegP was not required for PGA maturation based on the observation that the Δ*degP* mutant strain was still capable of producing active PGA (Lin et al., 2001a). These findings are in agreement with the current understanding that the periplasmic processing from proPGA to heterodimeric PGA is an autoproteolytic cleavage on the Thr_{263}–Ser_{264} bond. Interestingly, the physiological improvement and enhanced PGA production were associated with the protease activity of DegP since the coexpression of $DegP_{Ser210Ala}$, a DegP mutant lacking the protease activity whilst retaining the chaperone activity, was incapable of suppressing the physiological stress caused by *pac* overexpression (Pan et al., 2003). It is then speculated that DegP plays a role in aiding the autoproteolysis of proPGA, thus effectively reducing the inundation of precursor proteins and streamlining the PGA maturation process in the periplasm. Nevertheless, molecular details of the interaction between DegP with the nascent proPAC polypeptides remain largely unclear. For instance, co-expression of DegP-homologous periplasmic proteases, DegQ or DegS, could not rescue the curtailed culture performance upon *pac* overexpression (Pan et al., 2003). The effect of another periplasmic chaperone of FkpA on *pac* overexpression was also investigated (Wu et al., 2007). Compared to the wild-type strain, the Δ*fkpA* mutant strain overexpressing *pac* had deteriorated cell physiology, but the *pac* expression level was slightly affected. While exogenous coexpression of *fkpA* did not significantly enhance *pac* expression in the wild-type strain, coexpression of either *degP* or *fkpA* was able to complement the deteriorated cell physiology and *pac* expression in the Δ*degP*/Δ*fkpA* double mutant strain (Wu et al., 2007), implying the two periplasmic chaperones share certain overlapping functions.

Though the protease activity of DegP has been identified to be associated with the enhancement of *pac* overexpression, the contribution from the chaperone function cannot be completely excluded. To address this issue, the event of PGA maturation was relocated from the periplasm to cytoplasm, in which several known chaperones can be readily tested. The relocation of PGA maturation was achieved through the expression of the leader-less *pac* gene (LL *pac*) and the primary expression product of proPAC was overexpressed for maturation in the cytoplasm (Kang et al., 2005; Xu et al., 2005a, 2005b). While maturation can occur to form active PGA in the cytoplasm of *E. coli*, most of the expressed proPGA polypeptides aggregate into insoluble inclusion bodies, similar to the technical issue associated with periplasmic processing. Coexpression of cytoplasmic chaperones of DnaK/J-GrpE and GroEL/ES and/or trigger factors potentially enhanced *pac* expression performance and relieved physiological stress, implying proper folding of proPAC can be critical for successful maturation (Xu et al., 2005a, 2005b).

3.1.3. Effect of translocation efficiency in E. coli

Scherrer et al. (1994) and Sriubolmas et al. (1997), observed that misfolded PGA precursors accumulated in both the cytoplasm and periplasm as inclusion bodies under *pac* overexpression conditions. It was suggested that, in addition to periplasmic processing, translocation machineries may also be overwhelmed. While most protein translocations from the cytoplasm to the periplasm occur via the Sec system, which recognizes N-terminal signal peptides of distinct sizes and compositions (Berks et al., 2000), the Tat (Twin-Arg translocation) transport system can specifically export proteins with signal peptides containing the Twin-Arg signature motif [i.e. (Ser/Thr)-Arg-Arg-x-Phe] in the N-domain (Berks, 1996). The signal peptide of preproPGA has two N-domain Arg residues which are non-consecutive, and, therefore, does not conform to the typical Tat recognition motif (Schumacher et al., 1986). Despite this fact, preproPGA translocation may be Tat-dependent in *E. coli* since translocation of preproPGA was completely blocked in a mutant strain (JARV15) bearing deletions of Tat machinery proteins TatA and TatE (Ignatova et al., 2002). However, multiple pathways for translocation of preproPAC were proposed since no translocation incompetence was observed for the expression of a PGA derivative, with a single

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mutation (i.e. R6S) in the signal peptide of preproPGA, in JARV15 (Ignatova et al., 2002). Interestingly, translocation inhibition can also be suppressed by replacing the native PGA signal peptide with the Sec-targeting signal peptide of OmpT so the resulting fusion of OmpT-proPGA can still be exported to the periplasm in JARV15, presumably via the Sec pathway (Ignatova et al., 2002). With the use of the OmpT-proPGA fusion, coexpression of Sec components (i.e. SecA, SecB, and SecF) enhanced the production of PGA (Ignatova et al., 2003). Though Sec-dependent translocation typically occurs more effectively than Tat-dependent translocation (Berks et al., 2000), depending on the host strain, translocation of PGA precursors via the Sec-pathway does not necessarily lead to an increased PGA activity (Ignatova et al., 2003). However, the fate of preproPGA with regard to translocation is presently unclear as new evidence suggests that the Sec-pathway is the dominant translocation route (Akkaya et al., 2012). PGA activity in total cell preparations of mutant strain DADE, bearing deletions in TatA, B, C, D and E, was ~70% of that observed in the parent strain (MC4100). Western blotting confirmed the presence of similar levels of the PGA β-subunit, indicating successful translocation and post-processing of preproPGA (Akkaya et al., 2012). As aforementioned, PGA precursors can be translocated across the inner membrane via multiple secretion systems, including the Sec-pathway. Among several Sec-components, SecB is a cytoplasmic chaperone known to stabilize precursors of proteins destined for translocation (Topping et al., 2001). SecB appears to be critical for functional *pac* expression, presumably at the stage of translocation, since minimal PGA activity was detected when *pac* was expressed in a *secB* mutant and functional expression of *pac* can be restored upon basal level of *secB* coexpression in a *secB* mutant (Chou et al., 1999b). However, overexpression of *secB* resulted in increased levels of PGA precursors, either soluble or insoluble, located primarily in the periplasm with minimal improvement in PGA activity (Chou et al., 1999b). The results suggest that SecB can potentially assist translocation of preproPGA by mediating their stability even though the translocation is primarily Tat-dependent. Nevertheless, the improvement in translocation does not seem to improve the overall PGA production, suggesting that translocation might not be a limiting step. In fact, periplasmic processing tends to be a key step limiting the overall PGA production since the majority of misfolded PGA precursors often accumulated in the periplasm upon *pac* overexpression (Scherrer et al., 1994; Sriubolmas et al., 1997). Finally, the potential importance of cofactor Ca^{2+} on the translocation and periplasmic processing of PGA precursors was noted base on the observations that (1) Ca^{2+} can potentially facilitate periplasmic processing (Ignatova et al., 2005) and (2) preproPGA accumulated in the cytoplasm during fedbatch cultivation with modified M9 media lacking Ca^{2+} and PGA production was greatly enhanced with minimal accumulation of PGA precursors when Ca^{2+} was supplemented (Kasche et al., 2005). This obviously represents a simple and cost-effective biochemical approach to improve PGA maturation and culture performance.

3.1.4. Extracellular secretion in E. coli

Extracellular secretion has been proposed as an alternative strategy for the production of PGA. In addition to typical advantages associated with recombinant protein secretion (Choi and Lee, 2004), the strategy should be theoretically effective because (1) PGA is a periplasmic protein, which only requires another export step across the outer membrane to be extracellularly secreted, and (2) extracellular release of PGA is expected to reduce the overwhelming accumulation of misfolded PGA species in the periplasm and thus the associated extracytoplasmic stress. Two main genetic approaches, i.e. coexpression of outer-membrane permeation proteins and genetic manipulation of host outer membrane components (Fig. 4), have been employed for extracellular secretion of PGA with varying success.

Several outer-membrane permeation proteins, such as bacteriocin release protein (encoded by *brp*) and colicin E1 lysis protein (encoded by *kil*), can mediate the permeation of the outer membrane of *E. coli* through the interaction with outer-membrane phospholipids or protein components (van der Wal et al., 1995). Periplasmic proteins are extracellularly secreted as a result. PGA can be extracellularly secreted upon coexpressing the *kil* gene, but the host cell physiology was severely deteriorated, resulting in growth inhibition and reduced cell viability (Ignatova et al., 2003). Similarly, BRP-mediated extracellular secretion of PGA was also investigated (Lin et al., 2001b). However, secretion levels above 40% of the total PGA activity were accompanied with serious physiological deterioration and growth inhibition of the PGA-producing cells, implying the presence of extracytoplasmic stress. This strategy is considered ineffective since it neither increases the overall PGA activity as compared to the control of intracellular PGA production nor reduces inclusion body formation.

Some success has been reported for the extracellular production of recombinant PGA by *E. coli* mutant strains defective in the outer membrane. A promising application of this strategy is the use of L-form *E. coli* strains which completely lack the outer membrane (Gumpert et al., 1996). PGA maturation can still occur in the absence of a defined periplasmic space possibly because the processing is an autoproteolytic step. Interestingly, active PGA can be produced even at elevated temperatures (e.g. 37 °C). While extracellular secretion of PGA appears to be efficient, these L-form *E. coli* strains are extremely sensitive to environmental conditions, making them unsuitable for industrial applications. Several 'leaky' *E. coli* mutants bear mutations in one or more genes encoding outer-membrane protein components. An *E. coli* mutant strain (JE5505) deficient in the *lpp* gene encoding murein lipoprotein was employed for the extracellular production of PGA (Orr et al., 2012a). With proper modulation of medium composition, PGA was overproduced with an extremely high secretion efficiency, i.e. 90% of the total PGA activity was detected in the extracellular medium. However, the extracytoplasmic stress and inclusion body formation appear to be persistent even though most of the overproduced PGA gets secreted extracellularly. While this host strain of JE5505 can still suffer physiological deterioration to some extents under PGA-overproducing and secretion conditions, its sensitivity to environmental conditions is relatively low and, therefore, the application for high-cell-density cultivation for PGA production is believed to be feasible. Also importantly, simultaneous overproduction and extracellular secretion of PGA significantly facilitates downstream recovery and purification primarily based on the application of ion-exchange chromatography (Orr et al., 2012a).

3.1.5. Heterologous expression of various bacterial pac genes in E. coli

To date, in addition to *pac* from *E. coli* (encoding EcPGA), a selection of other bacterial *pac* genes have been heterologously expressed in *E. coli*, including *pac* from *B. megaterium* (Kang et al., 1991), *P. rettgeri* (Cheng et al., 2006; Chou et al., 2000; Huang et al., 2002), *K. cryocrescens* (Cheng et al., 2006; Garcia and Buesa, 1986; Jiang et al., 2007; Wen et al., 2005), *A. faecalis* (Cheng et al., 2006, 2007; Deak et al., 2003; Wang et al., 2006), *Arthrobacter viscosus* (Ohashi et al., 1989), *Achromobacter xylosoxidans* (Cai et al., 2004) and *Thermus thermophilus* (Torres et al., 2012). These bacterial PGAs can outperform EcPGA in terms of certain enzymatic properties, such as high molecular stability, wide operation range, broad substrate specificity, and high environmental tolerance, etc. For example, PGA from *A. faecalis* (AfPGA) is an attractive enzyme due to its broad pH optimum, greatly enhanced enantioselectivity (van Langen et al., 2000), and excellent thermostability presumably resulting from a unique disulfide bridge in the β-subunit (Verhaert et al., 1997). High-level expression of AfPGA has been achieved in a high-cell-density *E. coli* batch culture with dextrin as the sole carbon source (Cheng et al., 2007). Similar to the approach based on chaperone coexpression to enhance the production of EcPGA, coexpression of isoaspartate methyltransferase (PIMT), an enzyme recognizing L-isoaspartyl residues resulting primarily from the spontaneous rearrangement of aspartyl and asparaginyl residues (Kern et al., 2005), can suppress inclusion body formation under physiological stress to significantly enhance the production of AfPGA (Wang et al., 2006). PGA

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from *A. xylosoxidans* (AxPGA) is another attractive PGA with a thermostability [$t_{1/2,55\ °C}$ (enzyme half-life at 55 °C) of 55 min] significantly outperforming EcPGA ($t_{1/2,55\ °C}$ of 5 min) and AfPGA ($t_{1/2,55\ °C}$ of 15 min) and its heterologous expression in *E coli* has been demonstrated (Cai et al., 2004).

Heterologous expression of a novel PGA from *T. thermophilus* (TtPGA), an inherently thermostable enzyme with a half-life of 9.2 h at an optimal temperature of 75 °C, in *E. coli* has been recently reported (Torres et al., 2012). However, the function expression suffered a major limitation of the lack of periplasmic processing when the signal peptide from EcPGA was used to translocate TtPGA precursors into the periplasm of *E. coli*. Instead, the protein was targeted in the cytoplasm of *E. coli* by expressing the leaderless *pac* gene from *T. thermophilus* (Torres et al., 2012). Various cytoplasmic chaperones, i.e. DnaK/J and GrpE, trigger factor (TF) and GroEL/ES, were coexpressed to facilitate TtPGA maturation in *E. coli*. Although the production of TtPGA precursors increased upon chaperone coexpression, particularly TF and GroEL/ES in combination provided the greatest enhancement, PGA activity did not increase proportionally. Similar to the observation for the production of EcPGA, Ca^{2+} supplementation markedly improved the heterologous expression of TtPGA in *E. coli* (Torres et al., 2012). Continued effort for functional expression of TtPGA in *E. coli* is warranted given its exceptional stability at elevated temperatures.

Heterologous expression of PGA from *P. rettgeri* (PrPGA) in *E. coli* was feasible at elevated temperatures (e.g. 37 °C) more favorable for cell growth (Chou et al., 2000; Huang et al., 2002). Unlike the case of EcPGA production, functional expression of PrPGA in *E. coli* was not limited by posttranslational processing as insoluble PrPGA precursors were hardly detected, even at elevated temperatures which can significantly impact posttranslational processing and folding. Given the advantage of high-temperature cultivation for heterologous expression of PrPGA in *E. coli*, PrPGA may exhibit a lower enzyme activity towards synthesis of SSCs as compared to PGAs from other species (Cheng et al., 2006). PGA from *Kluyvera cryocrescens* (KcPGA) may provide certain technical advantages over EcPGA, such as easier immobilization, enhanced stability upon exposure to elevated temperatures, pH fluctuations, and organic solvents (Wen et al., 2005). Heterologous expression of KcPGA in *E. coli* has been reported (Cheng et al., 2006). In a comparative study using four heterologously expressed PGAs (i.e. EcPGA, PrPGA, AfPGA, and KcPGA), KcPGA showed the highest synthesis/hydrolysis (S/H) ratio towards cephalexin synthesis, suggesting its high industrial applicability (Cheng et al., 2006).

3.2. Production of recombinant PGA in platforms alternative to E. coli

Primarily due to the lack of recombinant DNA tools, most natural microbial PGA producers are not genetically amenable and, therefore, are seldom employed as an expression host for large-scale PGA production. While *E. coli* represents the most common host for the production of recombinant PGA, other expression systems, primarily Gram-negative *Bacillus* and eukaryotic yeast, have been explored but with limited success and are briefly reviewed herein. Other hosts, such as *Pseudomonas aeruginosa* which was used for expressing *pac* from *E. coli* (Krzeslak et al., 2009), appear to be uncommon and therefore are not reviewed.

3.2.1. Gram-positive Bacillus expression systems

The potential of using *Bacillus* strains for recombinant protein production has recently gained much attention (Westers et al., 2004). A major technical advantage for this microbial expression system is the capacity for extracellular secretion of recombinant proteins due to the lack of an outer membrane. This biological feature can potentially resolve the major limitation associated with *pac* overexpression in *E. coli*, i.e. the periplasmic accumulation of PGA precursors and associated extracytoplasmic stress. Two PGA-producing *Bacilli*, *B. megaterium* and *B. subtilis* have been explored (Rajendhran et al., 2003; Yang et al., 2001, 2006). Due to the existence of natural PGA-producing *Bacillus* species (Table 2), these *Bacillus* strains potentially possess the necessary posttranslational processing mechanisms for PGA maturation. In addition, unlike *E. coli* and other microbes, PGA production in *Bacillus* is not subject to glucose catabolite repression (Yang et al., 2006) and this can facilitate large-scale cultivation. Similarly to *Bacillus*, the Gram-positive *A. viscosus* extracellularly secretes PGA into the culture medium and, therefore, it is believed that the two microbes share a compatible mechanism for PGA maturation. Cloning and expression of recombinant PGA from *A. viscosus* in *B. subtilis* as a host has been demonstrated (Ohashi et al., 1988). In fact, compared to PGA production in native *A. viscosus* which normally requires PAA-induction, recombinant *Bacillus* did not require PAA-induction but had a much higher PGA productivity.

Although PGA production in *Bacillus* in early works relied on PAA induction at low temperatures (25-30 °C), recently developed *Bacillus* strains were capable of constitutive fermentation for PGA production at an optimal growth temperature of 37 °C, (Yang et al., 2001). This was accomplished through cloning the native *pac* gene from *B. megaterium* into a plasmid for subsequent transformation into the more genetically docile *B. subtilis*. Though plasmid instability is a common issue for large-scale cultivation of *B. subtilis*, this recombinant *B. subtilis* demonstrated a high plasmid stability during PGA production. In addition, deficient in six protease genes, the *B. subtilis pac* expression system had low extracellular protease activities, contributing its high PGA productivity. The effect of medium composition for *Bacillus* species has been studied in detail as a means to improve PGA production. Various carbon and nitrogen sources were analyzed for their effect on both bacterial growth and PGA production, which tends to be repressed by high glucose levels and is closely related to nitrogen sources under low glucose concentrations (Pinotti et al., 2000; Silva et al., 2006). Inorganic nitrogen salts have no major effect on PGA production while urea has negative effects (Rajendhran et al., 2003). It is therefore ideal to supplement the medium with more complex nitrogen sources, such as free amino acids or alcalase digested casein. Casein hydrolysis makes key amino acids available for assimilation (in particular, tryptone is preferentially consumed) and represents a cost-effective means of increasing nitrogen resources in the medium, leading to enhanced PGA production (Pinotti et al., 2007). Also, several carbon sources suitable for PGA production in *Bacillus* expression systems have been identified, including partially degraded starch (Zhang et al., 2006), sucrose, fructose, mannitol, xylose (Rajendhran et al., 2003), and cheese whey (Pinotti et al., 2007; Silva et al., 2006).

3.2.2. Eukaryotic expression systems

The complex posttranslational processing for PGA maturation warrants exploration of functional expression of PGA in yeast hosts, such as *Saccharomyces cerevisiae* and *Pichia pastoris* which typically possess diversified capacities for posttranslational processing/modification of proteins. Yeast platforms also offer technical advantages of high-cell-density cultivation and well-characterized expression vectors (Cregg et al., 2000; Maresova et al., 2010; Mattanovich et al., 2012). *S. cerevisiae* CBL1-30 has been explored as a host for expression of the *pac* genes from *E. coli* and *P. rettgeri* with both gene products being extracellularly secreted into the medium (Ljubijankic et al., 2002; Ljubijankić et al., 1999). While the production of *P. rettgeri* PGA in *S. cerevisiae* significantly outperformed bacterial expression systems (possibly due to partial glycosylation of the α-subunit of *P. rettgeri* PGA), this is not the case for the production of *E. coli* PGA in *S. cerevisiae* (Ljubijankić et al., 1999).The production of *P. rettgeri* PGA in *S. cerevisiae* was further enhanced by manipulating culture conditions for high-cell-density cultivation and the extracellular secretion of *P. rettgeri* PGA also facilitated the subsequent purification (Ljubijankic et al., 2002). Heterologous production of *P. rettgeri* PGA in *P. pastoris* was far more effective than the production in *S. cerevisiae* and the PGA product had a high thermostability possibly due to extensive glycosylation (Senerovic et al., 2009;

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Ševo et al., 2002). Stable integration of multiple copies of the *pac* gene into the *P. pastoris* genome resolved the issue of episomal plasmid instability in *S. cerevisiae*, resulting in enhanced PGA production (Cregg et al., 2000; Ševo et al., 2002). Heterologous production of *E. coli* PGA in the cytoplasm of *P. pastoris* was ineffective and the PGA product was less active possibly because of partial glycosylation and non-specific proteolysis at the N-terminus of the α-subunit (Maresova et al., 2010).

4. Bioprocess development for PGA production in *E. coli*

Given the availability of various expression platforms, to date, large-scale production of PGA is almost exclusively performed using recombinant *E. coli* containing a bacterial *pac* gene, of which *E. coli pac* is the most popular one. High-level *pac* expression and high-cell-density cultivation have to be simultaneously conducted to optimize the culture performance for subsequent harvest and purification of PGA. Extensive purification of PGA is unnecessary for most of industrial applications. The unique gene expression and protein maturation mechanisms make large-scale production of PGA challenging and major biochemical engineering approaches focusing on strain manipulation, cultivation method, and downstream processing are summarized herein.

4.1. Strain manipulation

Like almost all bioprocesses, development of superior and robust biological strains is critical for large-scale production of PGA. Recombinant DNA technology has been extensively applied to construct novel *E. coli* host/vector systems to enhance *pac* expression by eliminating various potential expression limitations described in Section 3. Basically, all intracellular steps leading to PGA synthesis and maturation, i.e. transcription, translation, translocation, periplasmic processing, folding, and extracellular secretion (if applied), have to be effective. In addition, the physiological stress associated with *pac* overexpression has to be minimized so that these PGA-overproducing cells can be cultivated to a high cell density. Since native *pac* expression in *E. coli* ATCC 11105 and *B. megaterium* ATCC 14945 is subject to the induction by PAA, an unfavorable carbon potentially inhibiting cell growth, and the repression by glucose, a common and favorable carbon source for microbial cultivations, these undesired regulations can be modified at the stage of the construction of expression vectors by using a strong promoter or by truncating the native *pac* regulatory region (Chou et al., 1999c; Merino et al., 1992; Spence and Ramsden, 2007). Novel *E. coli* mutant strains with an enhanced PGA-producing capacity and more robust physiological state can be screened for being used as the expression host (Arshad et al., 2010a,b; Chou et al., 1999a; Quratulain et al., 2006). Genetically engineered *E. coli* host/vector systems with superior adaptability to harsh culture environment associated with PGA overproduction can be strategically derived to optimize culture performance (Arroyo et al., 2003; Rajendhran and Gunasekaran, 2004).

4.2. Cultivation method

Medium formulation often represents a major task for high-cell-density microbial cultivation and it should be tailored to sustain both cell growth and *pac* overexpression. For cultivations with extracellular production of PGA, medium development is of utmost importance as it can potentially affect the stability, recovery, and purification of secreted PGA. Glucose can be used as the primary carbon source for production strains which are not subject to catabolite repression.

Other carbons, such as glycerol, sucrose, lactose, etc. had varying effects (Pinotti et al., 2007; Rajendhran et al., 2003). PGA production is also affected by nitrogen source as well as the carbon-to-nitrogen ratio (Bhattacharya et al., 1993; Chou et al., 1999c). High PGA activities have been reported with the use of peptide nitrogen sources, such as tryptone and peptone, as well as complex sources, such as yeast extract and beef extract (Gumpert et al., 1996; Orr et al., 2012a; Rajendhran et al., 2003). Replacing peptides with free amino acids as the nitrogen source significantly increases the production of PGA in *B. megaterium* ATCC 14945 (Pinotti et al., 2007). Proper supplementation of Ca^{2+}, an identified cofactor involved in translocation and periplasmic processing, in the feed medium for fedbatch cultivation of *E. coli* BL21(DE3) resulted in a significant increase in PGA activity (Kasche et al., 2005).

To produce PGA (either native or heterologous) in microbial expression systems, cultivation is often conducted at mild temperatures (e.g. 25–30 °C) as translation and posttranslational processing are negatively impacted by high temperatures (e.g. 37 °C) (Bhattacharya et al., 1993; Chou et al., 2000; Keilmann et al., 1993; Spence and Ramsden, 2007). An exception is heterologous expression of *pac* from *P. rettgeri* in *E. coli* HB101, which is feasible at 37 °C as long as pH remains neutral (Chou et al., 2000; Huang et al., 2002). While culture pH might not have a major effect and is generally maintained near neutral to promote optimal cell growth, increased PGA production in *E. coli* at slightly basic pH was reported (Gale and Epps, 1942; Spence and Ramsden, 2007). Approximately eight-fold increase in volumetric PGA activity was observed upon increasing pH from 7 to 8.5 in batch cultivation of *E. coli* ATCC 11105, whereas hardly any PGA activity was detected at pH 6 (Bhattacharya et al., 1993).There are conflicting reports on the effect of dissolved oxygen (DO) on PGA production. Specific PGA activity reached a maximum when DO was maintained at 20% (air saturation) in fedbatch cultures of *E. coli* ATCC 9637, but declined with decreasing DO (Liu et al., 1999). Hence, common industrial practice is to maintain DO above 15% to enhance PGA production (Spence and Ramsden, 2007). In contrast, a maximum PGA activity (both specific and volumetric) was observed at 1% DO of *E. coli* JM101 cultivation and the activity declined with increasing DO until plateauing at ~10% DO (De Leon et al., 2003b). Similar DO effect was reported in another study, where PGA activity (both specific and volumetric) was maximized at a low DO (<10%) in *E. coli* ATCC 11105 cultivation. However, cell growth is often arrested under low DO conditions, impacting the industrial feasibility of such cultures (Gebauer et al., 1987).

The popular use of various *lac*-based and T7 promoters for the production of recombinant PGA drives the exploration of novel induction strategies, particularly the inducer type in light of technical limitations discussed in Section 3. For the conventional inducer of IPTG, the culture is often induced to initiate PGA production in the exponential phase though late induction in the early stationary phase sometimes can lead a better culture performance (Jiang et al., 2007). In fact, the production of recombinant PGA in *E. coli* appears to be mixed-growth-associated (De Leon et al., 2003b; Ramirez et al., 1994), implying the feasibility of late induction during a slow growth phase, even for fedbatch cultivations. Alternatively, sugars, such as arabinose and galactose, may prove to be a suitable inducer to replace IPTG for certain *E. coli* host strains since high PGA activities can be obtained without causing severe physiological stress, growth arrest, and inclusion body formation (De Leon et al., 2003a; Narayanan et al., 2006a). Application of these alternative inducers for large-scale production of PGA certainly warrants more explorations.

4.3. Purification

With the exception of Gram-positive bacteria or fungi, PGA is often produced intracellularly in Gram-negative bacteria. As a result, purification of PGA entails a conventional multistep cascade (which is not reviewed here). Downstream processing commences with the preparation of crude cell lysate by cell harvest, washing, lysis, and clarification. Subsequently, PGA is partially purified using ammonium sulphate precipitation followed by desalting. If necessary, this partially purified PGA can be administered, such as PGA immobilization, for industrial applications without further chromatographic purification. Virtually all types of chromatography have been successfully applied for recovery and

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purification of PGA (Aguilar et al., 2006; Fonseca and Cabral, 2002; Liu et al., 2003; Sudhakaran and Shewale, 1987). Direct immobilization without purification has also been investigated using immobilized metal affinity membranes to capture and retain PGA from crude *E. coli* lysate (Chen et al., 2011). Conventional purification of PGA has also targeted the reduction of process complexity by selectively harvesting the periplasmic fraction instead of the whole cell lysate through the preparation of spheroplasts (De León et al., 2003; Rodriguez et al., 1992). However, this approach is effective for analytical purposes, but not large-scale process. Recently, a single-step downstream setup based on tangential flow filtration anion-exchange membrane chromatography (TFF-AMEC) was applied to process unaltered *E. coli* culture for direct harvest and purification of extracellularly produced PGA at a high yield and high purity (Orr et al., 2012b). This represents a major bioprocess improvement for effective production, harvest, and purification of PGA by systematically considering key aspects of all bioprocess stages, i.e. strain development, cultivation, and downstream processing.

5. Prospects

High demand for β-lactam semisynthetic antibiotics will continue to drive biotechnological advances in identification, molecular engineering, and large-scale production of novel bacterial PGAs. Recombinant DNA technology has been extensively applied for biochemical characterization and large-scale production of a selection of bacterial PGAs, with *E. coli* as the dominating host system for their heterologous expression. The unique maturation process of PGAs in *E. coli* has driven the development of novel genetic, biochemical, and bioprocessing strategies for PGA production. Recent insights into the molecular structure and catalytic mechanism of PGAs suggest ample potential to further engineer these enzymes towards the generation of novel antibiotics and effective catalytic conversion. Physiological stress associated with the formation of non-recoverable inclusion bodies upon PGA overproduction remains a major technological hurdle. Strategies based on genetic manipulation of stress response proteins; such as chaperone co-expression, appear to be feasible. The poorly understood interplay between biochemical cultivation conditions, gene expression profiles, and physiological stress responses will require major investigation. Advances in engineering cell physiology lead to manipulative strategies for stress alleviation and high-cell-density cultivation to enhance PGA production. Attempts in extracellular production of PGAs by exploring various secretion mechanisms in *E. coli* have been somewhat successful. Recently, this approach was extensively developed in combination with downstream processing. The integrated bioprocess appears to be promising since it enables simultaneous high-level production, effective harvest, and one-step purification of PGA. Finally, the atypical expression mechanisms of PGAs suggests that optimal control of metabolic flux can be critical for effective PGA synthesis and thus this production system will both benefit from and contribute to the development of modern systems biology and metabolic engineering.

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