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MINI-REVIEW

Efflux systems in bacteria and their metabolic engineering applications

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Abstract The production of valuable chemicals from metabolically engineered microbes can be limited by excretion from the cell. Efflux is often overlooked as a bottleneck in metabolic pathways, despite its impact on alleviating feedback inhibition and product toxicity. In the past, it has been assumed that endogenous efflux pumps and membrane porins can accommodate product efflux rates; however, there are an increasing number of examples wherein overexpressing efflux systems is required to improve metabolite production. In this review, we highlight specific examples from the literature where metabolite export has been studied to identify unknown transporters, increase tolerance to metabolites, and improve the production capabilities of engineered bacteria. The review focuses on the export of a broad spectrum of valuable chemicals including amino acids, sugars, flavins, biofuels, and solvents. The combined set of examples supports the hypothesis that efflux systems can be identified and engineered to confer export capabilities on industrially relevant microbes.

Keywords Efflux · Toxicity · Metabolic engineering · Synthetic biology · Export · Transporters

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Introduction

Efflux pumps are essential components of the bacterial cell envelope which enable an adaptive response to counteract toxic compounds encountered in the environment. The role of efflux systems in multidrug resistance has received broad attention (Nikaido and Pages 2012; Du et al. 2015); however, efflux systems are also responsible for maintaining homeostatic levels of essential metabolites in bacteria (Eggeling and Sahm 2003). This broad substrate specificity has given rise to the notion that tolerance toward exotic molecules can be endowed to microorganisms if a specific efflux system can be identified and heterologously expressed (Dunlop et al. 2011). Therefore, given the growing interest in producing non-native chemicals in industrial microbes, efforts to better understand and engineer metabolite export are increasingly being undertaken to optimize metabolic pathway function. This review will highlight some of the lessons learned from studying natural efflux systems, particularly for amino acids, as well as recent attempts to develop methods of identifying and engineering efflux pumps for enhanced secretion capabilities of non-native metabolites.

Metabolite efflux

Amino acids

Export plays an essential role in metabolic engineering strategies for producing amino acids. Active efflux, catalyzed by microbial efflux pumps, reduces intracellular amino acid concentrations and thereby alleviates feedback inhibition and circumvents toxicity associated with high amino acid concentrations (Eggeling and Sahm 2003). Many efflux pumps have been identified by exploiting the toxicity caused by high

concentrations of amino acids and/or associated molecules. In this section, we highlight examples of known amino acid efflux systems and how they have been used to increase product titers.

Cysteine Global demand for L-cysteine, in excess of 4000 t per year, is mostly met by the acid hydrolysis of animal-supplied keratin or by chemical synthesis (Wada and Takagi 2006). Microbial synthesis would bypass the problems associated with current routes, i.e., racemic mixtures from chemical synthesis (Martens et al. 1981) and risk of contamination by prions or pathogens (Wenda et al. 2011). In *E. coli*, L-cysteine biosynthesis is tightly controlled by L-cysteine levels (*O*-acetyl serine synthase has a K_i of 1 μM cysteine) (Kredich and Tomkins 1966). Introduction of a feedback-insensitive *O*-acetyl serine synthase into *E. coli* resulted in the extracellular accumulation of approximately 2 g/L L-cysteine (Takagi et al. 1999). However, high intracellular L-cysteine levels are toxic toward *E. coli*, suggesting that titers could be further increased by facilitating the export of L-cysteine (Harris 1981; Park and Imlay 2003). Several lines of evidence indicate that *E. coli* mitigates L-cysteine toxicity by employing efflux pumps. First, overexpression of the *ydeD* gene in *E. coli*, encoding for a membrane protein belonging to the DMT (PecM) family, results in the extracellular accumulation of L-cysteine whereas no extracellular L-cysteine is observed from wild-type (wt) cells (Dassler et al. 2000) (Table 1). *YdeD* is upregulated in response to H_2O_2 stress, and a Δ*ydeD* mutant is more sensitive to peroxide (Ohtsu et al. 2010). Together, it has been suggested that *ydeD* is part of redox balancing mechanism wherein *E. coli* exports cysteine to counteract oxidation stress in the periplasm. The oxidized product, L-cystine, is then reimported into the cytoplasm by a process dependent on the L-cystine periplasmic binding protein FliY (Ohtsu et al. 2010). This hypothesis is corroborated by the fact that the glutathione (GSH) export system *cydDC*, which typically maintains the redox balance of the periplasm in *E. coli*, also exports L-cysteine, albeit with reduced kinetics compared to GSH (Pittman et al. 2005). Second, the *E. coli* gene, *yfiK*, encoding a transporter belonging to the RhtB family, was identified in a screen for L-cysteine overproduction. Overexpression of *yfiK* in a cysteine-producing strain resulted in elevated levels of extracellular L-cysteine as well the L-cysteine precursor *O*-acetylserine (Franke et al. 2003) (Fig. 1). Externally applied *O*-acetylserine is toxic to wt *E. coli*, and overexpression of either *yfiK or ydeD* alleviates this toxicity, suggesting that L-cysteine exporters might also function as metabolic release valves (Franke et al. 2003). Using an *E. coli* mutant deficient in L-cysteine degradation, Yamada et al. examined the effect of overexpressing 33 known drug transporter genes in *E. coli*. Of a small subset that decreased the intracellular accumulation of L-cysteine, *bcr*, encoding a membrane protein of the MFS family, displayed the highest L-cysteine export rates. Indeed, overexpressing *bcr* in an L-cysteine production strain resulted in a 5-fold increase in L-cysteine titers, from 100 to 500 mg/L over a 48-h period (Yamada et al. 2006). Lastly, with regard to L-cysteine export across the outer membrane in *E. coli*, *tolC* mutants are highly susceptible to externally applied L-cysteine, whereas single-deletion mutants of genes encoding the outer membrane proteins *ompA*, *ompC*, *ompF*, *ompT*, and *ompX* are not (Wiriyathanawudhiwong et al. 2009). These data suggest that L-cysteine efflux depends on the active extrusion across the outer membrane carried out by TolC-dependent systems (Fig. 1).

Threonine Over 100,000 metric tons of L-threonine was produced in 2012 as a supplement for animal feed (Ajinomoto 2013). *E. coli* has been engineered to excrete L-threonine at high titers (Debabov et al. 1997). In the first-generation L-threonine production strains, the intracellular concentration of L-threonine was higher than in wt cells, but the amount of extracellular L-threonine was lower than expected, indicating that L-threonine export was limiting (Kruse et al. 2002). *E. coli* exports L-threonine via efflux pumps encoded by the genes *rhtA*, *rhtB*, and *rhtC* with varying degrees of specificity (Dong et al. 2011). Overexpression of *rhtC* increases resistance to externally supplied L-threonine, decreases accumulation of threonine-derived metabolites, and leads to a 3-fold increase in exported L-threonine in an engineered production strain (Kruse et al. 2002) (Fig. 1). Overexpression of *rhtA* had no effect on the resistance to externally supplied L-threonine; however, a mutant, *rhtA23*, with a A-G mutation −1 to the start codon, displayed a 3-fold increase in resistance to L-threonine, making it a likely candidate for L-threonine export (Livshits et al. 2003). Indeed, in a threonine-producing *E. coli* strain with a reduced genome, overexpression of *rhtA23* resulted in an approximate 3-fold increase in titer, from 80 to 230 mg/L (Lee et al. 2009). Overexpression of *rhtB* in *E. coli* leads to a slight increase in resistance to externally supplied L-threonine (1.67-fold) (Zakataeva et al. 1999) and a 2.4-fold increase in L-threonine titer (Kruse et al. 2002). However, resistance to the L-threonine biosynthetic precursor, homoserine, increases 80-fold when *rhtB* was overexpressed, implying a strong preference for homoserine over threonine (Zakataeva et al. 1999). Additionally, overexpression of *rhtB* resulted in a 1.6-fold increase in exported homoserine, suggesting that *rhtA*, *rhtB*, and *rhtC* might function as metabolic release valves, mitigating the transient accumulation of the threonine precursor, homoserine (Zakataeva et al. 1999). This hypothesis is supported by the pleotropic substrate specificity of these transporters. For example, overexpression of *rhtA23* increases the resistance of *E. coli* to toxic concentrations of L-cysteine, L-serine, L-glutamate, and toxic amino acid analogues, S(2-aminoethyl)-L-cysteine, L-glutamic acid-γ-hydrazide, and DL-hydroxynorvaline, strongly suggesting that *rhtA* transports molecules other than L-threonine. Given this,

Table 1 Efflux systems highlighted in this review

Efflux system	Relevant substrate[a]	Organism	Family	Mechanism of export[b]	Applied to metabolic engineering?[c]	Titer improvement[d]	Reference
YdeD	Cysteine (o-acetyl serine)	*E. coli*	DMT	n/a	No	n/a	Dassler et al. (2000)
YfiK	Cysteine (o-acetyl serine)	*E. coli*	LysE	H^+ antiport	Yes	10-fold	Franke et al. (2003)
Bcr	Cysteine	*E. coli*	MFS	H^+ antiport	Yes	5-fold	Yamada et al. (2006)
RhtC	Threonine	*E. coli*	LysE	H^+ antiport	Yes	3-fold	Kruse et al. (2002)
RhtA	Threonine (5-aminolevulinic acid)	*E. coli*	DMT	H^+ antiport	Yes	3-fold (1.4-fold)	Lee et al. (2009; Kang et al. (2011)
RhtB	Homoserine	*E. coli*	LysE	H^+ antiport	No	n/a	Zakataeva et al. (1999)
YgaW	Alanine	*E. coli*	n/a	n/a	Yes	1.5-fold	Hori et al. (2011b)
MscCG	Glutamate	*C.glutamicum*	MscS	diffusion	Yes	1.05–1.24-fold	Nakamura et al. (2007)
BrnFE	Valine, isoleucine, leucine, methionine	*C.glutamicum*	LIV-E	H^+ antiport	Yes	1.6-fold Ile, 1.6-fold Val, 1.3-fold Met	Kennerknecht et al. (2002)
YgaZH	Valine, isoleucine	*E. coli*	n/a	n/a	Yes	1.75-fold Val, 3.4-fold Ile	Park and Lee (2010)
YeaS	Leucine	*E. coli*	LysE	n/a	Yes	2.5-fold	Kutukova et al. (2005)
LysE	Lysine (arginine)	*C.glutamicum*	LysE	H^+ antiport	Yes	1.4-fold	Vrljic et al. (1996)
Cg2893	1,5-diaminopentane	*C.glutamicum*	MFS	n/a	Yes	1.2-fold	Kind et al. (2011)
LysO (YbjE)	Lysine	*E. coli*	n/a	H^+ antiport	No	n/a	Pathania and Sardesai (2015)
ArgO (YggA)	Arginine	*E. coli*	LysE	ATP hydrolysis	Yes	3-fold	Ginesy et al. (2015)
YddG	Tryptophan (Phe, Tyr)	*E. coli*	DMT	n/a	Yes	1.1-fold Trp	Liu et al. (2012)
AaeAB	p-Hydroxybenzoate	*E. coli*	FUSC	n/a	No	n/a	Van Dyk et al. (2004)
SetA (YabM)	Hexoses, disaccharides	*E. coli*	MFS	H^+ antiport	No	n/a	Liu et al. (1999b)
YdeA	Arabinose	*E. coli*	MFS	H^+ antiport	No	n/a	Bost et al. (1999)
CscB	Sucrose	*E. coli*	MFS	H^+ symport[e]	Yes	34 mg/L/h[f]	Ducat et al. (2012)
YeeO	Flavins	*E. coli*	MATE	Likely H^+ antiport[g]	Yes	36-fold	McAnulty and Wood (2014)
TtgABC	Toluene	*P. putida*	RND	H^+ antiport	No	n/a	Dunlop et al. (2011)
AcrAB-TolC	Isoprenoids	*E. coli*	RND	H^+ antiport	Yes	2-fold	Wang et al. (2013a)
MsbA	Isoprenoids[h]	*E. coli*	ABC	ATP hydrolysis	Yes	2–4-fold	Doshi et al. (2013)

DMT drug/metabolite superfamily, *MFS* major facilitator superfamily, *LysE* lysine export superfamily, *MscS* mechanosensitve channel protein, small conductance family, LIV-E family, *FUSC* resistance to fusaric acid family, *MATE* multi-antimicrobial extrusion family, *RND* resistance-nodulation-cell division family, *ABC* ATP-binding cassette family

[a] Molecule that was either identified as a substrate of the indicated efflux pump or molecule that was engineered to be overproduced wherein overexpression of indicated efflux pump leads to an increase in titers

[b] Mechanism of export reported from the Transport Classification Database (Saier et al. 2014)

[c] Overexpressing the indicated efflux pump to demonstrate an increase in export of the indicated substrate or used in a metabolic engineering application to increase titers of the indicated substrate

[d] Improvement in titers from overexpressing the indicated efflux pump relative to a control

[e] CscB is a sucrose importer (H+ symport) in *E. coli*, expressing *cscB* in *Synechococcus elongatus* results in sucrose export due to a reverse proton motive force (Ducat et al. 2012)

[f] Titer was not reported; therefore, the volumetric productivity is given

[g] Eighty-one percent identical EmmdR (He et al. 2011)

[h] MsbA typically exports LPS (King and Sharom 2012)

it might be possible to exploit that the Rht family of transporter to facilitate the export of molecules displays close structural similarities to amino acids. This hypothesis is supported by the fact that expression of *rhtA* in a strain engineering to produce 5-aminolevulinic acid resulted in 1.4-fold increase in titers compared to a strain that did not express *rhtA* (Kang et al. 2011) (Fig. 1).

Alanine Alanine efflux pumps have been discovered by exploiting toxicity to dipeptide L-alanine-L-alanine (Ala-Ala). When high concentrations (1–10 mg/ml) of the Ala-Ala are applied to *E. coli*, a brief increase in the extracellular concentrations of L-alanine is observed, indicating that Ala-Ala is taken up by *E. coli* and degraded and L-alanine is reexported (Hori et al. 2011a). Mutants deficient in L-alanine export are susceptible to Ala-Ala with minimum inhibitory concentrations (MICs) of 40 μg/ml whereas the wt MIC is >10 mg/ml (Hori et al. 2011b). The gene *ygaW*, encoding a putative membrane protein, was identified in a screen that confers resistance to *E. coli* mutants sensitive to Ala-Ala (Hori et al. 2011b). Genomic deletion of *ygaW* rendered strains sensitive to Ala-Ala, causing elevated intracellular L-alanine levels with decreased extracellular L-alanine when supplied a sublethal concentration of Ala-Ala. Importantly, overexpressing *ygaW* in a wt genetic background resulted in extracellular L-alanine accumulation whereas no L-alanine was observed from wt cultures. Lastly, overexpression of *ygaW* in a strain that overproduces L-alanine resulted in a 1.5-fold increase in titers (Hori et al. 2011b) (Fig. 1).

Glutamate Approximately 2.5 million tons of L-glutamate are produced each year by the industrial fermentation of glucose by *Corynebacterium glutamicum* (Hirasawa et al. 2012). Glutamate production in *C. glutamicum* is dependent on the mechanosensitive channel MscCG (Nakamura et al. 2007) which opens in response to membrane stress (Hoischen and Kramer 1990) either caused by biotin limitation (Gutmann et al. 1992), the addition of surfactants (Eggeling et al. 2001), sublethal concentrations of penicillin (Demain and Birnbaum 1968), or the anti-TB drug ethambutol (Radmacher et al. 2005). Genomic deletion of *mscCG* (*NCgl1221*) in *C. glutamicum* ATCC 13869 abolishes nearly all glutamate production, indicating that MscCG is the primary glutamate export protein in *C. glutamicum*; however, glutamate export is not completely eliminated, indicating that additional efflux systems are present (Nakamura et al. 2007). Overexpression of *mscCG* in *C. glutamicum* ATCC 13869 leads to a 1.05–1.24-fold increase in L-glutamate titers (Nakamura et al. 2007) (Fig. 2). MscCG belongs to the small conductance mechanosensitive ion channel family, a group of osmoregulated ion channels represented by MscS in *E. coli* (Bass et al. 2002). To prevent membrane rupture under hypoosmotic conditions, MscS channels open to allow the efflux of ions. It has been suggested that the excretion of glutamate in *C. glutamicum* functions in a similar manner (Borngen et al. 2010). MscCG also exports L-aspartate, albeit to a lesser extent than L-glutamate. For example, in a 9-h fermentation run, *C. glutamicum* ATCC 13868 produced 7 mM L-aspartate and 180 mM L-glutamate in a MscCG-dependent manner (Nakamura et al. 2007). This finding was corroborated by electrophysiological studies demonstrating that conductance is observed in MscCG-loaded patch clamps when L-aspartate or L-glutamate is present in the buffer solution whereas, relatively, no current is observed in the absence of either amino acid (Hashimoto et al. 2012). These experiments suggest the possibility of using MscCG to export amino acids other than L-glutamate. Indeed, expression of the constitutively active MscCG point mutant, MscCG A111V, in an *E. coli* strain engineered to overproduce L-phenylalanine resulted in approximately a 2-fold increase in the amount of L-phenylalanine secreted into the media compared to control strains lacking MscCG A111V (Hashimoto et al. 2012). Similarly, *C. glutamicum* engineered to produce gamma-amino butyrate (GABA) by the heterologous expression of glutamate decarboxylase produced approximately 8 g/L GABA after 96 h of fermentation. However, export of GABA occurred under high-biotin conditions (500 μg/ml) and was insensitive to the addition of Tween 40, conditions

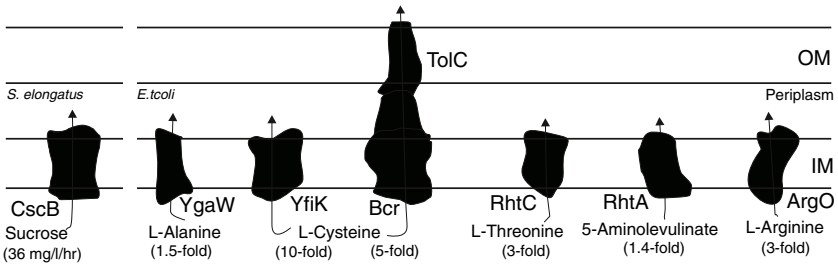

Fig. 1 Examples of efflux pumps from *E. coli* used to increase titers of valuable chemicals. Each efflux pump is indicated along with its cognate substrate generated by a metabolic engineering strategy mentioned in the text. Pump shapes were arbitrarily created and do not reflect actual structures. The fold increase in product titer when the efflux system is overexpressed relative to control strains is indicated in *parenthesis*. *S. elongatus* does not export sucrose; however, introduction of the sucrose permease, *cscB*, facilitated sucrose efflux. L-Cysteine is exported across the outer membrane via TolC. However, the membrane fusion protein involved in the process is not known. Furthermore, it is not known how any of the other products listed here are exported across the outer membrane (see text for details)

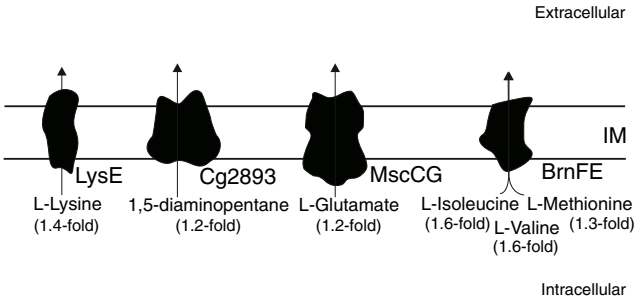

Fig. 2 Examples of efflux systems from *Corynebacterium glutamicum* used to increase the production of valuable chemicals. Each efflux pump is indicated along with its cognate substrate generated by a metabolic engineering strategy mentioned in the text. Pump shapes were arbitrarily created and do not reflect actual structures. The fold increase in product titer when the efflux system is overexpressed relative to control strains is indicated in *parenthesis*. The cell envelope of *C. glutamicum* was omitted for clarity (see text for details)

that typically promote L-glutamate export via MscCG (Takahashi et al. 2012). These results indicate that GABA is exported by a process independent of MscCG. Therefore, more research is necessary to determine the substrate specificity of MscCG and, thus, the utility of MscCG as a general exporter of amino acids.

Isoleucine, valine, leucine, and methionine The dipeptide, Ile-Ile, when supplied at high concentrations, is toxic to *C. glutamicum*. This toxicity is counteracted by expressing two membrane proteins, encoded by the genes *brnE* and *brnF* (Kennerknecht et al. 2002). Deletion of *brnFE* results in decreased export rates as well as elevated intracellular levels of L-isoleucine, L-valine, and L-leucine when supplied high concentrations of the respective dipeptides (i.e., Ile-Ile, Ala-Val, Leu-Leu) (Kennerknecht et al. 2002). BrnFE displays homology to AzlCD in *Bacillus subtilis*, the founding members of the LIV-E family of transporters, a group of two-component membrane proteins hypothesized to actively export branched chain amino acids in bacteria (Belitsky et al. 1997). Taken together, these data suggest that *brnFE* mediates the extrusion of all branched chain amino acids in *C. glutamicum* and would thus make excellent candidates for metabolic engineering applications. Indeed, overexpression of *brnFE* along with its transcriptional activator *lrp* (Lange et al. 2012) in an L-isoleucine production strain of *C. glutamicum* resulted in a 1.6-fold increase in titer after 72 h of shake-flask fermentation (Yin et al. 2013). Furthermore, overexpressing *brnFE* in *C. glutamicum* 13869 gave rise to an approximate 1.6-fold increase in L-valine titer after 96 h compared to control strains (Chen et al. 2015). *BrnFE* was also found to export L-methionine in *C. glutamicum* (Trotschel et al. 2005), and overexpressing *brnFE* in a methionine-producing strain of *C. glutamicum* causes an approximate 1.3-fold increase in L-methionine titers (Qin et al. 2014) (Fig. 2). The homolog of *brnFE* in *E. coli* is *ygaZH*. Deletion of *ygaZH* renders *E. coli* sensitive to the non-metabolizable valine analogue norvaline, whereas overexpression of *ygaZH* renders *E. coli* more resistant to norvaline, strongly suggesting that *ygaZH* mediates valine export in *E. coli* (Park et al. 2007). Supporting this hypothesis, a valine-overproducing *E. coli* overexpressing *ygaZH* increased titer by 1.75-fold relative to control strains (Park et al. 2007) (Fig. 3). YgaZH also exports L-isoleucine. Overexpressing *ygaZH* leads to a 3.4-fold increase in the amount of extracellular L-isoleucine from an L-isoleucine production strain of *E. coli* (Park et al. 2012) (Fig. 3). Lastly, *E. coli* exports L-leucine via the *yeaS* gene product belonging to the RhtB family of exporters (Kutukova et al. 2005). Deletion of *yeaS* in *E. coli* causes sensitivity to leucine analogues, L-α-amino-*n*-butyric acid and 4-aza-DL-leucine, whereas overexpression of *yeaS* renders *E. coli* more resistant to these compounds, suggesting that *yeaS* would make for good candidate to improve titers from a metabolic engineering standpoint. Indeed, overexpression of *yeaS* results in an approximate 2.5-fold increase in L-leucine titers, with minor increases in histidine and methionine also observed (Kutukova et al. 2005) (Fig. 3).

Lysine and arginine Over 600,000 t of L-lysine is produced each year from the industrial fermentation of glucose by a metabolically engineered strain of *C. glutamicum* (Pfefferle et al. 2003). L-Lysine is exported in this organism by the *lysE* gene product, representing the canonical transporter of this family (Burkovski and Kramer 2002). *C. glutamicum* mutants deficient in *lysE* accumulate intracellular L-lysine 3.5-fold above wt levels, and overexpression of *lysE* results in an approximate 1.4-fold increase in the export rate of L-lysine compared to wt (Vrljic et al. 1996) (Fig. 2). LysE also transports L-arginine in *C. glutamicum* (Bellmann et al. 2001). The dual-substrate specificity of LysE suggests that this transporter could be used to export molecules that display structural similarities toward these basic amino acids. However, the production of 1,5-diaminopentane by *C. glutamicum* heterologously expressing the lysine decarboxylase, *ldcC*, was unaffected by deletion of *lysE*, refuting the hypothesis. The diamine was instead exported via the uncharacterized MFS transporter Cg2893 (Kind et al. 2011) (Fig. 2).

E. coli exports lysine via the transporter, YbjE (LysO) (Pathania and Sardesai 2015), but the impact of overexpressing *lysO* in a L-lysine-producing strain has yet to be reported. L-Arginine is exported in *E. coli* by the product of *yggA* (*argO*), a homolog of LysE from *C. glutamicum* (Nandineni and Gowrishankar 2004). *ArgO* mutants are hypersensitive to the arginine anti-metabolite canavanine, and overexpression of *argO* rescues an arginine auxotrophic strain in cross feeding studies indicating that *argO* is an excellent candidate to improve titers from engineered *E. coli* strains (Nandineni and Gowrishankar 2004). Indeed, introduction of *argA214*, a feedback-insensitive enzyme catalyzing the first step in

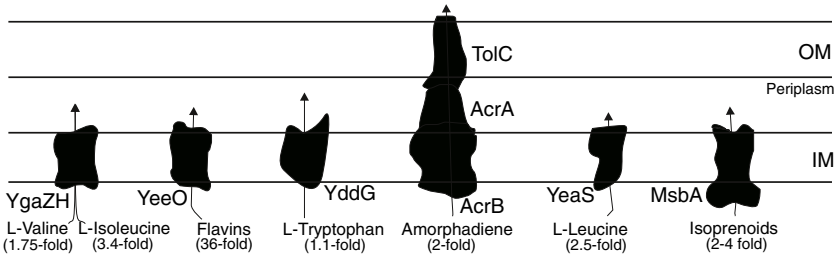

Fig. 3 Examples of efflux pumps from *E. coli* used to increase titers of valuable chemicals. Each efflux pump is indicated along with its cognate substrate generated by a metabolic engineering strategy mentioned in the text. Pump shapes were arbitrarily created and do not reflect actual structures. The fold increase in product titer when the efflux system is overexpressed relative to control strains is indicated in *parenthesis*. Amorphadiene export was increased by expressing *acAB-tolC*; however, how the other products are being exported across the outer membrane remains to be determined (see text for details)

arginine biosynthesis, into a bicistronic operon with *argO* resulted in a 3-fold increase in titers compared to *argA214* expression alone (Ginesy et al. 2015) (Fig. 1), demonstrating that in *E. coli* L-arginine production is limited by L-arginine export.

Phenylalanine, tyrosine, and tryptophan Overexpression of the *rhtA* paralog *yddG* in *E. coli* leads to a slight increase in the resistance to high concentrations of L-phenylalanine and 5-flouro-tryptophan, suggesting that this membrane protein might be involved in the export of aromatic amino acids (Doroshenko et al. 2007). In further support of this hypothesis, overexpression of *yddG* in *E. coli* strains engineered to produce L-phenylalanine, L-tyrosine, or L-tryptophan leads anywhere from 2- to 4-fold increases in the respective aromatic amino acid. However, deletion of *yddG* in *E. coli* did not render the cells sensitive to high concentrations of these amino acids nor did it fully diminish the export of these amino acids from their respective production strains (Doroshenko et al. 2007). These data suggest that either there are redundant exporters for the aromatic amino acids, or due to their hydrophobic nature, passive diffusion through the membranes contributes to their extrusion from *E. coli*. Additional studies demonstrate that only a 1.1-fold increase in titers is observed from L-tryptophan-producing strains of *E. coli* when *yddG* is overexpressed (Liu et al. 2012; Wang et al. 2013b), suggesting that *yddG* makes a minor contribution to the export of L-tryptophan (Fig. 3). Along similar lines, addition of the aromatic acid para-hydroxybenzoate (PHB) to *E. coli* causes upregulation of *yhcP* gene product (renamed *aaeB*) encoding a membrane protein belonging to the fusaric acid resistance family (Van Dyk et al. 2004). Deletion of *aaeB* increases the sensitivity to externally applied PHB, and overexpression of *aaeB* renders the cells more resistant to PHB, providing strong evidence that *aaeB* exports aromatic acids in *E. coli* (Van Dyk et al. 2004). In further support of this hypothesis, addition of para-hydroxycinnaminic acid also results in a strong upregulation of *aaeB* (Sariaslani et al. 2005).

Sugars

Much like amino acid efflux, export of a rich energy source like monosaccharides and disaccharides seems paradoxical; however, sugar efflux may serve as an additional level of control to balance metabolites. For example, addition of lactose to a mid-logarithmically growing culture of *E. coli* in the presence of glycerol will quickly result in the production of the lactose-derived metabolites, glucose, galactose, and allolactose in the culture filtrate (Huber et al. 1980). The activities emanate from whole cells and not from inadvertent lysis, indicating that lactose is taken up and hydrolyzed and products are secreted (Huber et al. 1980). The efflux system responsible for this activity was identified in a search for genes that suppress the toxicity of overexpressing the inner membrane protein TetA with an Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (Liu et al. 1999a). Overexpression of the gene *yabM* via an orthogonal induction system led to an increase in the efflux of IPTG, thus alleviating the toxicity of LacI-regulated *tetA* (Liu et al. 1999a). *YabM* and its ortholog, *yeiO*, were renamed *setA* and *setB* and are responsible for the efflux of lactose and IPTG based on the desensitization of LacI-regulated promoters when either *setA* or *setB* is overexpressed in *E. coli* (i.e., higher concentrations of inducer are necessary to achieve the same transcriptional activity as wt) (Liu et al. 1999b). Additionally, it was shown, by the transport of radiolabeled substrates within energized inside-out membrane vesicles, that SetA is also capable of exporting the disaccharides maltose and cellobiose in addition to lactose, but sucrose was not transported (Liu et al. 1999b). Moreover, SetA also facilitated the transport of radioactive phenyl-glucosides and alky-galactosides in inside-out membrane transport studies (Liu et al. 1999b). Lastly, many hexoses like glucose, galactose, mannose, and fructose were transported in similar experiments by SetA, but none of the 3-, 4-, 5-, or 7-carbon sugars like glyceraldehyde, erythrose, arabinose, or glucoheptose was transported (Liu et al. 1999b). Arabinose is exported by the membrane protein YdeA, belonging to the MFS superfamily (Carole et al. 1999). Deletion of *ydeA* diminishes the rate of radiolabeled arabinose

efflux (Carole et al. 1999), and overexpression of *ydeA* prevents the accumulation of arabinose which results in the desensitization of pBAD-based promoters (Bost et al. 1999). Interestingly, the multidrug efflux pump Cmr (mdfA) is implicated in arabinose export, as deletion of *cmr* sensitizes pBAD-based promoters, presumably due to intracellular arabinose accumulation from lack of efflux (Koita and Rao 2012).

Sugar export from a photosynthetic organism could be used to produce sustainable feedstocks for biofuels and other chemicals. A step toward achieving this goal was made by expressing the sucrose permease (CscB) from *E. coli* in the cyanobacteria *Synechococcus elongatus* PCC 7942 (Ducat et al. 2012). Normally, this pump imports sucrose by using protons from the proton motive force (PMF), but *S. elongatus* has an inverted PMF at the inner membrane and thus can drive the reverse process. Sucrose production occurs naturally in cyanobacteria to balance salt stress; therefore, simultaneous expression of *cscB* under high salt stress should result in sucrose export (Ducat et al. 2012). Indeed, sucrose production was greatest in these experiments when *cscB* was expressed in the presence of high salt (150 mM NaCl) whereas no sucrose was detected from wt cultures under identical conditions. In addition, the pH of the media influences the ability to export sucrose (below pH 7.8, the exported sucrose decreases dramatically) consistent with the hypothesis that CscB is using an inverted PMF to drive the process. With this approach and the deletion *invA*, a gene involved in the breakdown of sucrose as well as deletion of the glycogen synthase *glgC*, sucrose productivities reached as high as 36.1 mg/L/h in engineered strains (Ducat et al. 2012) (Fig. 1).

Vitamins

Riboflavin (vitamin B2) is an important dietary supplement produced in industrial fermentation processes by either the fungus *Ashbya gossypii*, the yeast *Candida famata*, or an engineered *B. subtilis* strain (Stahmann et al. 2000). *A. gossypii* is a natural riboflavin overproducer wherein the secretion of riboflavin is thought to protect hyphae against UV damage (Stahmann et al. 2001). *C. famata* secretes riboflavin under low-iron conditions possible to reduce insoluble Fe^{3+} to the more accessible Fe^{2+} (Dmytruk and Sibirny 2012). However, the export of riboflavin in these organisms is not well understood. The expression of the permease *ribM*, involved in riboflavin uptake from *Streptomyces davawensis*, from a riboflavin production strain of *B. subtilis* resulted in a 1.18-fold increase in titers relative to control strains (Hemberger et al. 2011). In *E. coli*, overexpression of the membrane protein *yeeO*, belonging to the MATE family, leads to an increase in extracellular flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) by 36- and 17-fold, respectively (McAnulty and Wood 2014) (Fig. 3).

Hydrocarbons

In this section, we highlight bacterial tolerance to linear and aromatic hydrocarbons as an example of the benefits that efflux pumps can impart upon microorganisms used in biotechnological applications.

Microbial production of industrial hydrocarbons has long been pursued, but frequently, these products are toxic to producing cells. Aliphatic molecules displaying log P_{ow} between 1.5 and 4 partition within biological membranes (Vermue et al. 1993; Ramos et al. 2002) leading to loss of ions, dissipation of the PMF, and the disruption of membrane proteins (Sikkema et al. 1995). Yet, many bacteria occupy environmental niches in close proximity to hydrocarbons, indicating that they have evolved mechanisms to counteract these toxic effects. For example, *Alcanivorax borkumensis* cannot use sugars as carbon sources but instead thrives at the oil-liquid interface in the world's oceans (Yakimov et al. 1998). Analogously, a strain of *Pseudomonas aeruginosa* (ATCC 33988) recently isolated from a jet fuel storage tank (Brown et al. 2014) grows better than *P. aeruginosa* PAO1 in the presence of Jet A-1 fuel (Gunasekera et al. 2013). Pan-inhibition of RND efflux pumps by treatment with the RND pump inhibitor, PAβN, prevents the growth of *P. aeruginosa* ATCC 33988 in the presence of Jet A-1 fuel but has no effect when grown with glycerol as the carbon source, indicating that efflux pumps are necessary for jet fuel tolerance in this organism (Gunasekera et al. 2013). Interestingly, the pseudomonads seem to be particularly suited for hydrocarbon tolerance, as some strains of *Pseudomonas putida* grow in the presence of toluene at concentrations as high as 50–90 % (*v*/*v*) (Inoue and Horikoshi 1989; Faizal et al. 2005).

The potential of transferring hydrocarbon tolerance to industrial microbes has motivated the search for underlying molecular mechanisms. Much has been written about the tolerance mechanisms of *P. putida* to organic solvents (reviewed in (Ramos et al. 2015)). Many authors emphasize the role of membrane reorganization, chaperones, and oxidative stress mitigation in addition to the role of efflux pumps in these organisms, presumably due to their upregulation from proteomic or transcriptomic studies; however, phenotypic analysis of individual mutants tells a different story. In laboratory settings, sudden toluene shocks of 0.3 % (*v*/*v*) to *P. putida* DOT-T1E result in an approximate 4-log reduction in viability; however, culturing the cells in toluene supplied via the gas phase before the toluene shock only reduces viability by 0.3 log, indicating that a strong and robust adaptation to the solvent occurs (Ramos et al. 1998). Deletion of cis-trans isomerase, *cti*, responsible for decreasing membrane fluidity in *P. putida* DOT-1E, rendered the cells just as sensitive as wt strains to sudden toluene shock and subsequent toluene exposure (Bernal et al. 2007). Similarly, genomic deletion of cardiolipin synthase in *P. putida* DOT-1E renders cells more

sensitive to growth when toluene is supplied in the gas phase, but ultimately, the cells recover and are as viable as wt upon subsequent toluene exposure (Bernal et al. 2007). Likewise, there was no significant difference in the viability of a mutant deficient in cyclopropane synthase in *P. putida* DOT-T1E when exposed to a sudden toluene shock compared to its parent strain; however, exposure to m-xylene resulted in moderate reduction in viability (Pini et al. 2009). Lastly, deletion of the gene encoding for the cold shock protein CspA2 did not change the sensitivity of cells to toluene shocks relative to wt (Segura et al. 2005).

We emphasize these phenotypes because they are in stark contrast to genomic deletion of any one of the three toluene efflux systems in *P. putida* DOT-T1E encoded by the *acrAB-tolC* homologs, *ttgABC*, *ttgDEF*, and *ttgGHI* (Rojas et al. 2001). For example, disrupting the function of *ttgABC* results in an 8-log reduction in cell viability when toluene is supplied via the gas phase. Further, the viability of the *ttgABC* mutant was reduced by 3–4 orders of magnitude in a subculture containing 0.3 % (*v*/*v*) toluene (Ramos et al. 1998). Cells with a disruption in *ttgDEF* responded to toluene in the gas phase the same as wt, but cell viability was reduced by 2–3 orders of magnitude after a 0.3 % toluene shock (Mosqueda and Ramos 2000). Disrupting *ttgGHI* resulted in an 8-log reduction in viability during culture of toluene in the gas phase and 7-log reduction in viability upon a 0.3 % toluene shock (Rojas et al. 2001). Disrupting all three efflux systems resulted in non-viable cells after exposure of toluene in the gas phase, highlighting their absolute essentiality in the tolerance to organic solvents (Rojas et al. 2001). However, to our knowledge, no findings have been reported on whether or not these efflux systems are sufficient to confer toluene tolerance toward other bacteria. *TtgABC* was expressed in a Δ*acrAB* strain of *E. coli*, and *ttgABC* expression functionally complimented the *acrAB* null phenotype in the presence of *n*-butanol, geranyl acetate, α-pinene, and farnesyl hexanoate, but tolerance to toluene was not reported (Dunlop et al. 2011).

Despite extensive work to uncover the regulation of these key efflux systems (Ramos et al. 2009; Fillet et al. 2012), little is known about their roles in the physiology of *P. putida* DOT-T1E beyond their necessity in conferring toluene tolerance. In response to a sublethal challenge of phenol ($log\ P_{ow}$=1.5), the solvent-sensitive *P. putida* KT2440 decreases the abundance of the porins OprB, OprF, OprG, and OprQ while increasing expression of efflux systems TtgA (35-fold), *ttgC*, ttg2A, and ttg2C and the uncharacterized RND transporters NP_1516 and NP_1517 (Roma-Rodrigues et al. 2010). These data suggest that *P. putida* can reorganize its cell envelope to accommodate a preponderance of mostly Ttg efflux systems. Similarly, *P. aeruginosa* PseA, in response to cyclohexane exposure ($log\ P_{ow}$=3.44), decreases the abundance of the general porins OprD and OprF while increasing the abundance of Opr86, an outer membrane protein implicated in maintaining the integrity of the outer membrane (Tashiro et al. 2008), and LptD, a protein involved in LPS biosynthesis (Hemamalini and Khare 2014). Taken together, global reorganization of the cell envelope to decrease its permeability, upregulation of efflux systems, general stress response chaperones, and redox balancing systems (Blank et al. 2008) is likely required to provide tolerance to saturating concentrations of aromatic hydrocarbons.

Given these abilities, it is no surprise that the solvent-tolerant strains of the pseudomonads are often used for the bioremediation of toxic organic solvents like 1,2,3 trichlorpropane (Samin et al. 2014) or biocatalytic transformations of aromatic hydrocarbons like the epoxidation of styrene (Volmer et al. 2014) (reviewed in (Poblete-Castro et al. 2012)). Strains of the pseudomonads are also used for the de novo production of aromatic hydrocarbons like p-hydroxybenzoic acid (Verhoef et al. 2007) and p-coumarate (Nijkamp et al. 2007). It is assumed that efflux systems are facilitating the export of the aliphatic products in these situations, yet few reports have directly examined this hypothesis. In one example, production of p-hydroxybenzoic acid (PHB) from glucose in *P. putida* S12 results in the upregulation of the tripartite efflux system PP_1271-73, which is homologous to *emrAB-tolC* in *E. coli* (Verhoef et al. 2010). Disrupting PP_1271-73 in a PHB production strain decreases the final titer of PHB almost 2-fold, along with a concomitant decrease in cell growth; however, overexpressing the tripartite system did not increase titers (Verhoef et al. 2010).

The organic solvent tolerance of certain pseudomonads also allows the use of biphasic fermentations for the production of aliphatic molecules wherein the organic phase acts as a product sink, decreasing both feedback inhibition and internal toxicity. For example, the final titers of p-hydroxystyrene from an engineered *P. putida* S12 strain increased from 4.5 to 21 mM in fed-batch fermenters when 1-decanol was added as the second phase (Verhoef et al. 2009). Additionally, in a phenol-producing strain of *P. putida* S12, addition of octanol resulted in an increase in titer from 5 to 58 mM in the organic phase (Wierckx et al. 2005). However, it should be noted that if the second phase is non-toxic, a solvent-tolerant strain does not necessarily need to be used. In an *E. coli* strain engineered to produce phenol, Kim et al. used tributyrin as a second phase to pull toxic levels of phenol out of *E. coli* such that final titers increased from 1.6 to 18 g/L in the organic phase (Kim et al. 2014). This study calls into question the use of solvent-tolerant pseudomonads for metabolic engineering of hydrophobic molecules, as long as a suitable second phase is available for use in *E. coli*. After all, *E. coli* possesses its own hydrocarbon tolerance mechanisms dependent on the *soxS*, *marA*, and *robA* stress-responsive genes. For example, constitutive activation of *marA* along with deletion of *acrR*, the repressor for *acrAB*

multidrug efflux system, is known to confer resistance to lethal concentrations of cyclohexane (log P_{ow}=3.44) (Oh et al. 2012; Watanabe and Doukyu 2012).

Another class of hydrophobic compounds that have been subject of interest in the oleochemical industry is the free fatty acids (FFA) (Lennen and Pfleger 2013). Although it is common to find excreted FFA in the media of fatty acid-producing strains, little is known about the mechanism underlying the process of export. In an effort to elucidate the FFA export mechanism and engineer the overproduction of these molecules, a series of *E. coli* multidrug efflux transporter knockouts were constructed in a strain engineered to produce dodecanoic acid at titers of 0.5 g/L (Youngquist et al. 2012; Lennen et al. 2013). Among the pumps tested were several members of the RND family (such as *acrAB*, *acrD*, *mdtEF*, and *acrEF*), MFS family (*mdtG*, *cmr*), and porins such as *tolC and ompF*. Knockouts of the RND pump *acrAB* and the porin *tolC* conferred a growth defect in the dodecanoic acid-producing strain compared to a control strain, suggesting that these proteins are involved in the export of dodecanoic acid (Lennen et al. 2013). However, overexpression of the pumps did not render the cells more tolerant to exogenously added octanoate or decanoate. In addition, overexpression of the efflux pumps did not increase titers despite the fact that expression of *acrAB* in this system fully complimented the Δ*acrAB* phenotype, indicating that the proteins from the overexpressed genes were indeed functional (Lennen et al. 2013).

Terpenes are a diverse group of molecules that are useful in many applications such as medicine, cosmetics, and biofuels (Peralta-Yahya et al. 2012). Terpenes have been produced in *E. coli*; however, little is known about how these molecules are exiting the cells (Alonso-Gutierrez et al. 2013). Overexpression of *marA*, a stress response activator in *E. coli*, confers resistance to geraniol, a monoterpene alcohol (Shah et al. 2013), and activation of *marA* leads to upregulation of the AcrAB-TolC efflux system, suggesting that AcrAB-TolC mediates the extrusion of terpenes in *E. coli* (Watanabe and Doukyu 2012). Given this and using an *E. coli* strain that produces the sesquiterpene amorphadiene or the diterpene kaurene, Wang et al. overexpressed various combinations of the RND family efflux pump components, AcrA, AcrB, MdtE, MdtF, and TolC, as well as MexA, MexB, and OprM from *P. aeruginosa*, using a T7-based system and examined the effect on product titers (Wang et al. 2013a). Overexpression of *acrA* or *acrB* alone had no effect on the titers of amorphadiene or kaurene, but overexpression of *tolC* resulted in a 1.3-fold increase in amorphadiene levels and an approximate 1.2-fold increase in kaurene levels compared to an empty plasmid control (Wang et al. 2013a). Not surprisingly, simultaneous overexpression of all three pump components, *acrA*, *acrB*, and *tolC*, resulted in the highest titers of amorphadiene and kaurene (approximately 350 mg/L amorphadiene and 30 mg/L kaurene corresponding to 1.5-fold and 2-fold increases, respectively, relative to empty plasmid controls). Additionally, according to the authors, the levels of amorphadiene were increased a further 1.16-fold merely by overexpressing two copies of *tolC* and one of *acrB* compared to simultaneous expression of all three components (Wang et al. 2013a) (Fig. 3). These data highlight the role of *tolC* in the extrusion of amorphadiene from *E. coli* and might serve as an interesting focal point for further engineering studies. In a similar investigation of using efflux pumps to increase the export of terpenes, homologs of the ABC transporter MsbA, a flippase involved in LPS export to the outer membrane in *E. coli* (King and Sharom 2012), were overexpressed in a strain that produces the isoprenoids zeaxanthin, canthaxanthin, and β-carotene (Doshi et al. 2013). Overexpressing the MsbA homolog, StMsbA*, from *Salmonella enterica* ser. *typhimurium*, leads to a 2.4-fold increase in the titer of zeaxanthin after 72 h relative to empty plasmid controls in *E. coli* Bl21(DE3) with a decane overlay. Overexpression of *E. coli* MsbA led to a 4.4-fold increase in canthaxanthin as well as β-carotene under identical conditions (Doshi et al. 2013) (Fig. 3). In the final analysis, these studies demonstrate that overexpressing efflux systems offer the possibility to increase the production of terpenes from bacteria.

Engineering efflux

Given the pleiotropic specificity of many efflux systems, multiple groups have investigated adaptive evolution techniques to identify novel pumps and select for mutations within efflux pumps that confer a higher export capacity relative to wt pumps. In this section, we highlight a few examples whereby improvements in export capacity were facilitated by selection for heterologous efflux systems or mutating known efflux pumps in the presence of valuable chemicals.

In an attempt to examine RND efflux systems that could confer biofuel tolerance to *E. coli*, Dunlop et al. cloned 43 RND efflux systems displaying homology to the toluene tolerance pump *ttgABC* from *P. putida* and expressed each in an *E. coli* Δ*acrAB* genetic background (Dunlop et al. 2011). The pooled Δ*acrAB* transformants were screened for increased tolerance based on a competitive growth assay in the presence of biofuels (Dunlop et al. 2011). Pumps that survived the competition assay complimented the growth defect of the Δ*acrAB. AcrAB* itself was included in the competition, thus affording the opportunity to determine if heterologous expression of some pumps could outperform the wt system. When pooled transformants were exposed to α-pinene, cells expressing *ttgABC* dominated the assay, whereas when cells were exposed to farnesyl hexanoate, *mexEF-oprN* from *P. putida* showed increased prevalence (Dunlop et al. 2011). As a comparison, *acrAB* dominated in the presence of limonene and

geraniol. While each of these "top performing pumps" increased tolerance relative to a Δ*acrAB* strain, it was not shown whether each would have conferred superior tolerance if overexpressed in the original AcrAB+ *E. coli* cells.

Once identified, efflux pumps can be engineered to improve their function. For example, tolerance toward short-chain alcohols was increased in *E. coli* by creating a mutant library of *acrB* on a plasmid that also contained wt *tolC* and *acrA* components (Fisher et al. 2014). This library of ~50,000 mutants was grown under a competitive assay in the presence of *n*-butanol, and mutants were identified that conferred 25 % higher growth rates in the presence of externally applied *n*-butanol compared to cells expressing wt *acrB* (Fisher et al. 2014). However, the impact of the *acrB* variants on titer from a butanol-producing strain was not reported. In a similar screen, variants of *acrB* were identified that conferred a 1.45-fold increase in the efflux rate of *n*-octane and 4–5-fold increase in the efflux rate of α-pinene (Foo and Leong 2013). However, the tolerance of these mutants toward *n*-octane or α-pinene in liquid culture was not reported. In addition, whether or not the mutated *acrB* variants improved the titers of octane- or α-pinene-producing strain of *E. coli* was also not reported. Lastly, in another study aimed at identifying *acrB* mutants that confer resistance to biofuels, a library of PCR-mutagenized *acrB* was screened in the presence of 1-hexene in a Δ*acrB* strain (Mingardon et al. 2015). Several clones were identified that conferred increased resistance relative to wt *acrB*; however, subsequent tolerance exposure tests in liquid cultures displayed virtually no significant differences in the presence of 1-hexene, styrene, or bile salts (Mingardon et al. 2015).

Concluding remarks

This review highlights the many areas where microbial efflux pumps have played a role in tolerance and improved metabolite production. These examples illustrate the importance of metabolite export in natural microbes and motivate further efforts aimed at identifying and engineering transporters for metabolites of commercial value. However, it remains to be determined to what extent productivities and cellular fitness can be increased by overexpressing efflux systems relative to wt levels. Several examples highlighted the ability to complement deletion mutants with heterologous pumps, but few studies, e.g., terpene production (Doshi et al. 2013; Wang et al. 2013a), have successfully improved wt strains by expressing pumps that act on non-native metabolites. It may be necessary to mimic pseudomonads by remodeling the abundance of other proteins in the cell membrane to increase the capacity for desired efflux pumps. Furthermore, the capacity for proper expression and insertion of efflux pumps may need to be increased, i.e., by co-expressing the SecYEG translocation machinery in addition to the efflux pump (Mulder et al. 2013). Alternatively, the use of biphasic fermentations could bypass the challenges of optimizing efflux in these strains, as was seen with phenol production in *E. coli* (Kim et al. 2014). It is clear that novel efflux pumps can be identified through screens based upon toxicity to specific compounds and heterologously expressed to confer export abilities on engineered microbes. The next generation of research in this area will determine the optimal strategies for maximizing metabolite efflux and improving product tolerance.

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