ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Efflux Transporter Engineering Markedly Improves Amorphadiene Production in *Escherichia coli*

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ABSTRACT: Metabolic engineering aims at altering cellular metabolism to produce valuable products at high yields and titers. Achieving high titers and productivity can be challenging if final products are largely accumulated intracellularly. A potential solution to this problem is to facilitate the export of these substances from cells by membrane transporters. Amorphadiene, the precursor of antimalarial drug artemisinin, is known to be secreted from *Escherichia coli* overexpressing the biosynthetic pathway. In order to assess the involvement of various endogenous efflux pumps in amorphadiene transport, the effects of single gene deletion of 16 known multidrug-resistant membrane efflux transporters were examined. The outer membrane protein TolC was found to be intimately involved in amorphadiene efflux. The overexpression of *tolC* together with ABC family transporters (*macAB*) or MFS family transporters (*emrAB* or *emrKY*) enhanced amorphadiene titer by more than threefold. In addition, the overexpression of transporters in the lipopolysaccharide transport system (*msbA*, *lptD*, *lptCABFG*) was found to improve amorphadiene production. As efflux transporters often have a wide range of substrate specificity, the multiple families of transporters were co-expressed and synergistic benefits were observed in amorphadiene production. This strategy of screening and then rationally engineering transporters can be used to improve the production of other valuable compounds in *E. coli*.

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KEYWORDS: efflux pump; transporter engineering; amorphadiene; TolC; lipopolysaccharide transport system; artemisinin

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Introduction

A central goal of metabolic engineering is to alter cellular metabolism by re-directing the distributions of metabolic fluxes in biochemical pathways to produce a variety of products at high yields and titers (Woolston et al., 2013). Many target compounds typically accumulate intracellularly. As such, concentration of some products can rise to saturated levels which may limit production levels (Dunlop, 2011; Wang et al., 2013). A potential solution to this challenge is to remove these compounds by facilitating their efflux through the engineering of cellular transporters. Transporters provide a direct route to separate the products from microbial cells, thus, not only reduce product inhibition and function as a pull on the biosynthetic pathway (Boyarskiy and Tullman-Ercek, 2015; Dunlop, 2011; Mukhopadhyay, 2015).

About 7% of the *Escherichia coli* (*E. coli*) inner membrane proteins are involved in the efflux of a variety of compounds (Daley et al., 2005). These efflux transporters are classified into five families which include the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) superfamily, the resistance-nodulation cell division (RND) superfamily, and the multidrug and toxic compound extrusion (MATE) superfamily (Li and Nikaido, 2004; Li et al., 2015; Venter et al., 2015). Most of these efflux transporters are composed of three subunits. A subunit (e.g., MacB) is located in the inner membrane where substrates are bound and exported through the outer membrane subunit (e.g., TolC). The membrane-fusion protein located in the periplasmic space (e.g., MacA) connects and stabilizes the inner and outer membrane units (Fig. 1; Piddock, 2006). Unlike importers which are known to transport only a small class or specific substances (Saier, 2000), many efflux transporters have a wider range of substrate profiles. Such promiscuous efflux mechanisms not only allow the removal of a broad range of toxic substances but also enable cost-effective recovery, and drive biochemical reactions

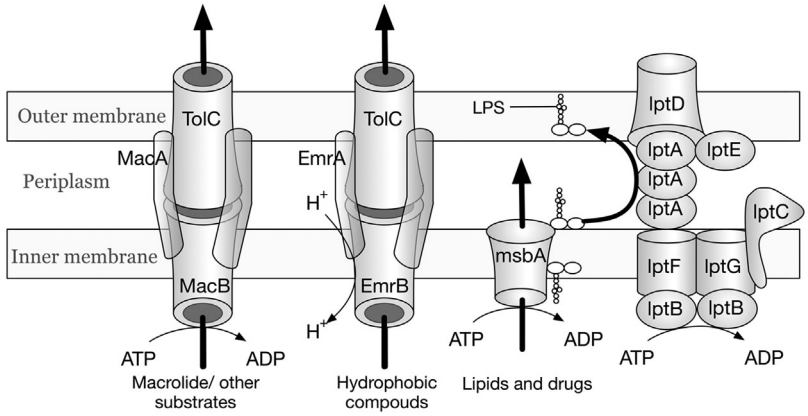

Figure 1. Illustration of MacAB-TolC (ABC family), EmrAB-TolC (MFS family), and lipopolysaccharide (LPS) transport system (ABC family). MFS transporters are capable of transporting small solutes in response to chemiosmotic ion gradients, exchanging one H^+ ion for one drug molecule. In contrast to the RND, MSF, and MATE families of transporters driven by proton motive force, efflux of ABC transporters is driven by ATP hydrolysis. LPS transporters are ABC exporters that are known to export extremely hydrophobic compounds, such as lipids, drugs, and steroids.

forward as products are separated from the rest of the cells (Allen et al., 2010; Boyarskiy and Tullman-Ercek, 2015; Zhang et al., 2011).

There is an increasing number of reports of engineering transporters to improve tolerance of hosts to different adverse conditions and some of these transporters can be further applied to increase titers of various products in *E. coli* (Foo et al., 2014; Mukhopadhyay, 2015). In general, all these studies can be broadly classified into two types. The first approach is to generate variants of potentially useful efflux pumps, for example, the directed evolution of RND family efflux pump (*acrB*) was found to improve tolerance to target compounds (Fisher et al., 2014; Foo and Leong, 2013; Mingardon et al., 2015). The second approach is to screen individual transporters through heterologous expression. A large number of RND transporters from different hosts have been overexpressed and some have been identified to improve biofuel tolerance in *E. coli* (Dunlop et al., 2011). By screening a series of ABC family transporters, the overexpression of *msbA* was found to significantly increase in the secretion of canthaxanthin and β-carotene in *E. coli* (Doshi et al., 2013). Similarly, the production of cyanidin 3-O-glucoside, an anthocyanin, was increased by 15% with the efflux pump *yadH* overexpression (Lim et al., 2015). And the production of isoprenoids, including amorphadiene (AD), was enhanced by the overexpression of RND family transporters of native AcrAB-TolC and the heterologous MexAB-OprM pump in *E. coli* (Wang et al., 2013). However, none of these studies systematically explored the contribution of different endogenous families and the combinations of endogenous transporters in *E. coli* in AD production. Interestingly, the overexpression of many heterologous efflux pumps were less effective in the efflux of many compounds as compared to the native transporters (Doshi et al., 2013; Dunlop et al., 2011). One possibility for this contrasting difference may simply be due to the poor expression/functional activities of these heterologous efflux pumps and, hence, limiting the use of these heterologous systems.

In this study, a number of endogenous multidrug-resistant efflux transporters and components of the lipopolysaccharide (LPS) transport system involved in AD transport were systematically analyzed and the putative homologous transporters were then expressed individually or co-expressed in combinations to enhance AD production.

Materials and Methods

Strain and Plasmid Construction

BW25113 (Δ[araD-araB] 567 ΔlacZ4787[::rrnB-3] λ- rph-1 Δ [rhaD-rhaB] 568 hsdR514) and its single multidrug-resistant-gene knockout mutants were used in the screening and *tolC* reconstitution experiments. All of them were from Keio collection of gene knockout mutants (Baba et al., 2006). *K12 MG1655 ΔrecAΔendA DE3* strain (Ajikumar et al., 2010) was used for AD production with overexpression of transporters.

Selected genes and operons were amplified by PCR from *MG1655 ΔrecAΔendA DE3* genomic DNA with their putative upstream ribosome binding sites. All the genes and operons were inserted into modified RK2 (amp) plasmid with the Pm promoter replaced with TM1 promoter (T7 promoter variant; Zhang et al., 2015a). *TolC* was inserted into RK2 vector firstly to generate RK2-*tolC* plasmid. Selected operons/genes (*macAB*, *acrAB*, *emrAB*, *arcEF*, *emrKY*, *entS*, *acrD*, *mdtEF*, *lptD*, and *msbA*) were also inserted into RK2-*tolC* to generate artificial operons together with *tolC*. The genes (*msbA* and *lptD*) were inserted directly into RK2 plasmid. The operons *lptCAB* and *lptFG* were co-inserted into RK2 plasmid, resulting in RK2-*lptCABFG* plasmid. Furthermore, *lptE* and *lptD* were co-inserted into RK2-*lptCABFG* plasmid to form RK2-*lptCABFGED* plasmid. The gene ADS was cloned into modified pAC plasmid with the original constitutive promoter replaced with araBAD promoter to obtain pACM-ADS. Plasmid pBAD-*dxs-idi-ispDF* (Zhang et al., 2013) and pACM-*ADS* (araBAD promoter) were co-transformed into *BW25113* strain. RK2 series of plasmids were con-transformed with pACM-SAAI (T7 promoter; Zhang et al., 2015a) into *MG1655* strain. All the

constructions used CLIVA methods (Zou et al., 2013) and the phosphorothioated primers used are shown in Supplementary Table SI. The information about strains and plasmids is summarized in Supplementary Table SII.

Media and Culture Conditions

All the cells were grown in 2 xpy media (20 g/L Peptone, 10 g/L Yeast extract, and 10 g/L NaCl, with pH adjusted to 7), supplemented with 10 g/L glycerol, 0.5% (v/v) Tween 80, and 50 mM HEPES. Fresh cells (10 μL) were inoculated into 1 mL fresh media in 14 mL BD Falcon™ tube and covered with 200 μL dodecane (with 100 mg/L β-caryophyllene as internal control). Cells were grown at 28 °C with the shaking speed of 300 rpm and were induced by 0.01–0.03 mM IPTG (T7 promoter) or 3 mM L-arabinose (araBAD promoter) when OD_{600} reached 0.5–1.0. The media were supplemented with appropriate antibiotics (100 mg/L ampicillin, 34 mg/L chloramphenicol, or 100 mg/L kanamycin) to maintain corresponding plasmids.

Quantification of Intracellular and Extracellular AD

Extracellular AD was captured into the organic layer (dodecane) during cell incubation and intracellular AD was extracted using organic solvent ethyl acetate. Briefly, at 8th and 24th h after induction, 5 μL dodecane was transferred into 495 μL ethyl acetate (extracellular AD). And 400 μL cell culture (OD_{600} was 3.0–8.0) was transferred into 1.5 mL Eppendorf® microcentrifuge tube and was centrifuged to separate cell pellets from the media. The cell pellets were washed once by 1 mL PBS and were re-suspended into 100 μL double distilled water. Sixty microliter ethyl acetate and 10 mg of 0.1 mm glass beads (BioSpec, Bartlesville, OK) were added into the cell suspension. The extraction of intracellular AD was performed with a VWR high-duty vortex platform for 10 min at 4 °C. After centrifugation, 30-μL ethyl acetate (top layer, intracellular AD) was collected for GC/MS measurement. AD was measured using Agilent 7980A gas chromatograph equipped with an Agilent 5975C mass spectrometer (GC/MS) as previously reported (Zhang et al., 2013).

Quantification of Plasmid Copy Number

The plasmid copy number was calculated based on real-time quantitative PCR data. It was calculated by normalizing the copy number of plasmid-resistant genes (ampicillin-resistant gene for pBAD-*dxs-idi-ispDF* and chloramphenicol-resistant gene for pACM-*ADS*) to the copy number of chromosomal gene *cysG* (Zhang et al., 2013).

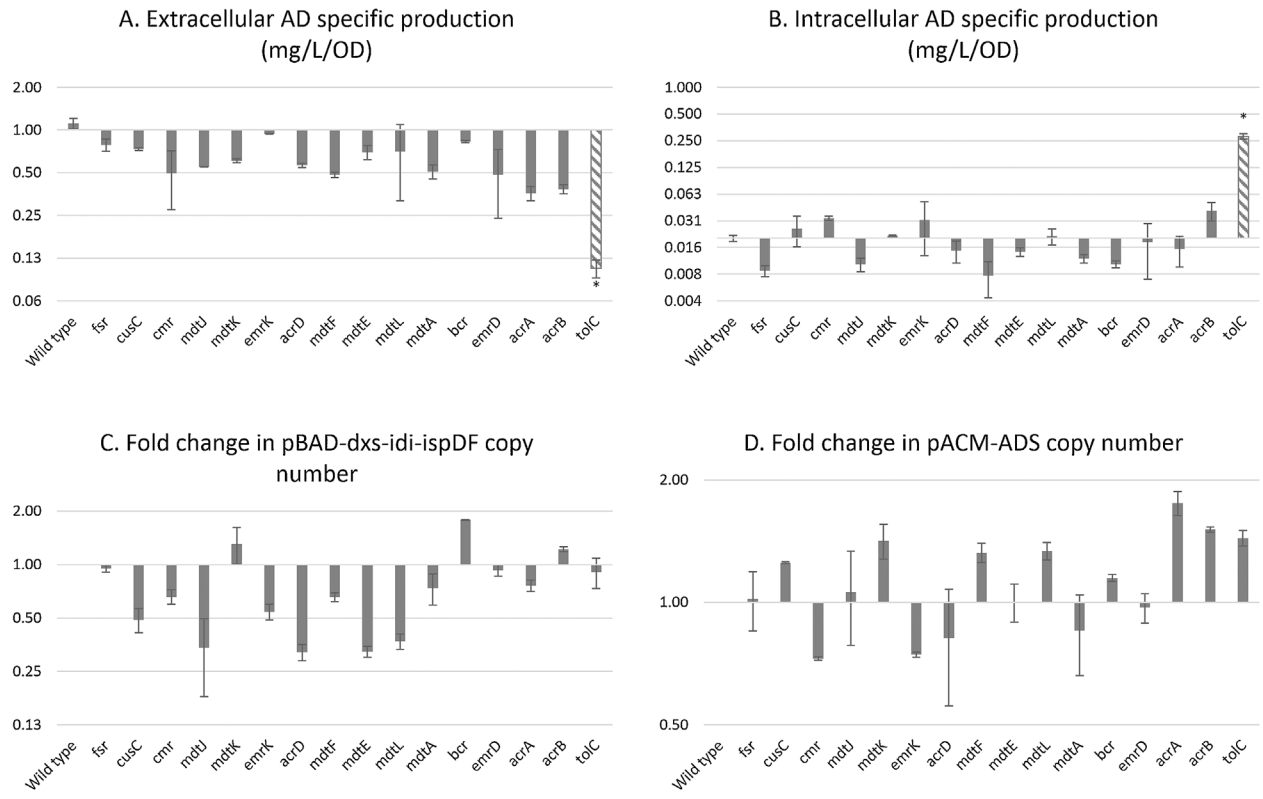

Figure 2. The effect of single-transporter-gene deletion on intracellular and extracellular AD-specific production and plasmid copy number. (**A**) The effect of gene deletion on extracellular AD-specific production (in dodecane phase); (**B**) the effect of gene deletion on intracellular AD-specific production (* *P*-value < 0.01 versus wild-type, as shown in Supplementary Table SIV); (**C**) the effect of gene deletion on the copy number of plasmid pBAD-dxs-idi-ispDF; (**D**) the effect of gene deletion on the copy number of plasmid pACM-ADS.

Results

Identifying Transporter Candidates Using Single-Gene Knockout Mutants

The *BW25113* strain (WT) and its 16 single multidrug-resistant-gene knockout mutants (Baba et al., 2006) were transformed with two plasmids, pBAD-dxs-idi-ispDF and pACM-ADS (Supplementary Table SII). The 16 pump-encoding genes included four families of efflux pumps in *E. coli* (Supplementary Table SIII), MFS family (*bcr*, *cmr*, *emrD*, *emrK*, *fsr*, *mdtL*), RND family (*mdtA*, *cusC*, *acrD*, *mdtF*, *mdtE*, *acrA*, and *acrB*), SMR family (*mdtJ*), and MATE family (*mdtK*). In addition, the outer membrane protein (OMP) *tolC* mutant was also included in the study. The immiscible organic solvent, dodecane, was used as an overlay during cell culture so as to extract AD from the media. As shown in Figure 2 and Supplementary Table SIV, the specific extracellular AD production (mg/L/OD) was significantly decreased (P-value < 0.01, P-values were shown in Supplementary Table SIV) only when *tolC* was deleted. Similarly, only *tolC* mutant showed a significant change in specific intracellular AD titer (mg/L/OD, Fig. 2). Quantitatively, the knockout of *tolC* resulted in an 8.2-fold increase in intracellular AD titer and a concomitant decrease of 90% of extracellular AD titer (Fig. 2A and B). Although for different mutant strains, the copy numbers of the plasmid pBAD-dxs-idi-ispDF and pACM-ADS were maintained as compared to wild-type strain (Fig. 2C and D), indicating the change of AD efflux was not due to the variation in the expression of *dxs-idi-ispDF* and *ADS*. These data suggested that *tolC* plays a significant role in the efflux of AD.

Reconstitution of *TolC* Increased AD Efflux in *tolC* Mutant

If *tolC* was involved in the AD efflux, the reconstitution of *tolC* in the *tolC* mutant (*tolC*− strain) should predictably increase the AD efflux. The *tolC* gene was subcloned into the low copy-number (4–7 copies per cell) RK2 plasmid (Supplementary Table SII) and the resulting plasmid was then transformed into the *tolC*− strain. As expected, the reconstitution of *tolC* in the mutant (*tolC*+ strain) restored the level of AD efflux. The knockout of *tolC* in *BW25113* strain resulted in the decrease in extracellular AD-specific production from 0.3 mg/L/OD (wild-type strain) to 0.15 mg/L/OD (*tolC*− strain) with an increase of intracellular AD-specific production from 0.01 mg/L/OD (wild-type strain) to 0.48 mg/L/OD (*tolC*− strain; Fig. 3A). The reconstitution of *tolC* resulted in the increase in extracellular AD, from the specific yield of 0.15 mg/L/OD (*tolC*− strain) to 0.28 mg/L/OD (*tolC*+ strain), a level similar to that found in the wild-type strain. Likewise, a concomitant decrease in the intracellular-specific yield of AD, from 0.48 mg/L/OD (*tolC*− strain) to 0.03 mg/L/OD (*tolC*+ strain), was observed. The reconstitution of *tolC* in the *tolC*− strain also increased cell growth, as evident in the final cell density (Fig. 3B). Similar to

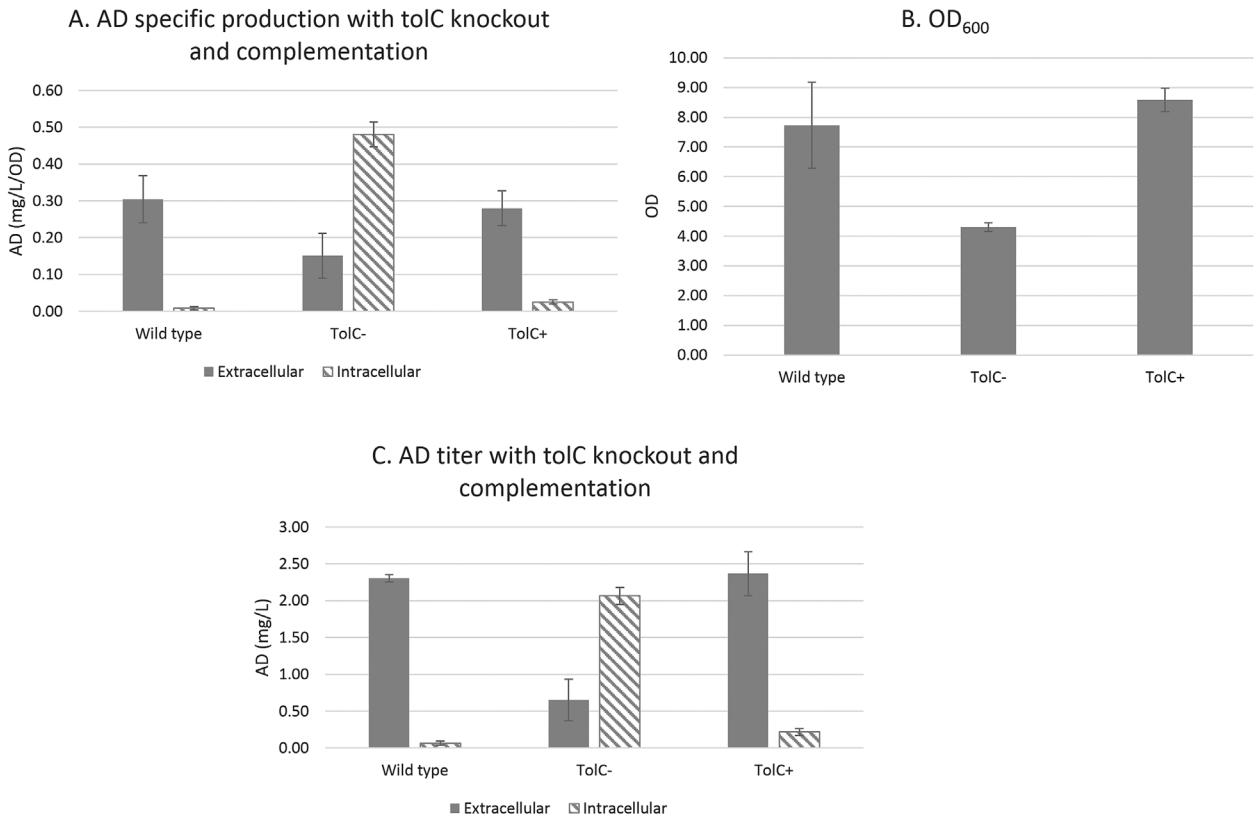

Figure 3. Complementation of *tolC* in *tolC* mutant strain. (**A**) Specific AD production (mg/L/OD); (**B**) cell density at OD600; (**C**) AD titer (mg/L).

specific yield, the AD titers in the *tolC* reconstitution experiment had the same trends with *tolC* deletion and complementation (Fig. 3C). These results strongly supported the proposal that AD did not passively diffuse out of cells but was transported with the aid of *tolC*.

Overexpression of *tolC*-Dependent Transporters Enhanced AD Production

As the deletion of *tolC* resulted in the remarkable decrease in the efflux of AD, it was worth investigating whether the overexpression of *tolC* could increase the efflux and, thus, the production. Therefore, we first overexpressed *tolC* in *MG1655 ΔrecAΔendA DE3* strain carrying pACM-*SAAI* and pET-*lacI* plasmids (Supplementary Table SII). As expected, the overexpression of *tolC* resulted in greater than twofold increase in AD-specific yields and titers, both at late exponential phase (8 h) and stationary phase (24 h) of culture (Fig. 4). As *tolC* lacks substrate specificity, associated inner membrane protein/proteins may serve to recognize and regulate the export of AD across the inner membrane. TolC protein is known to form complexes with three types of transport systems in *E. coli*: ABC family (MacAB), RND family (AcrAB, AcrEF, MdtABC, AcrAD, and MdtEF), and MFS family (EmrAB, EmrKY, and EntS; Supplementary Table SV). The deletion of individual associated inner membrane proteins did not affect the overall titer of AD (Fig. 2), raising the possibility that these proteins may play redundant roles in transporting AD. Interestingly, AD titer was increased significantly both in late exponential phase (8 h) and stationary phase (24 h) with the overexpression of all of the *tolC* combinations, except *entS-tolC*. The overexpression of *emrAB-tolC*, *emrKY-tolC*, or *macAB-tolC* combinations resulted in the increase in AD yield in early stage (8 h) from 5.1 to ∼20.8 mg/L/OD and the increase in the final stage (24 h) from 5.8 to ∼22.4 mg/L/OD (Fig. 4). These results suggested that AD efflux could be regulated by multiple transporters.

Effect of Overexpressing LPS Transporters on AD Production

Of the TolC-related transporters, AcrAB, AcrEF, AcrAD, and EmrAB are known to transport many hydrophobic compounds, and their overexpression significantly improved AD production. Thus, we hypothesized that other native transporters of hydrophobic compounds may also be involved in AD efflux and their overexpression might enhance AD production. LPS transporters (Fig. 1) are ABC exporters that are known to export extremely hydrophobic compounds, such as lipids, drugs, and steroids (Pohl et al., 2005). Furthermore, the overexpression of MsbA, a LPS flippase, has been reported to enhance the secretion of hydrophobic carotenoids (Doshi et al., 2013). Therefore, we hypothesized that AD

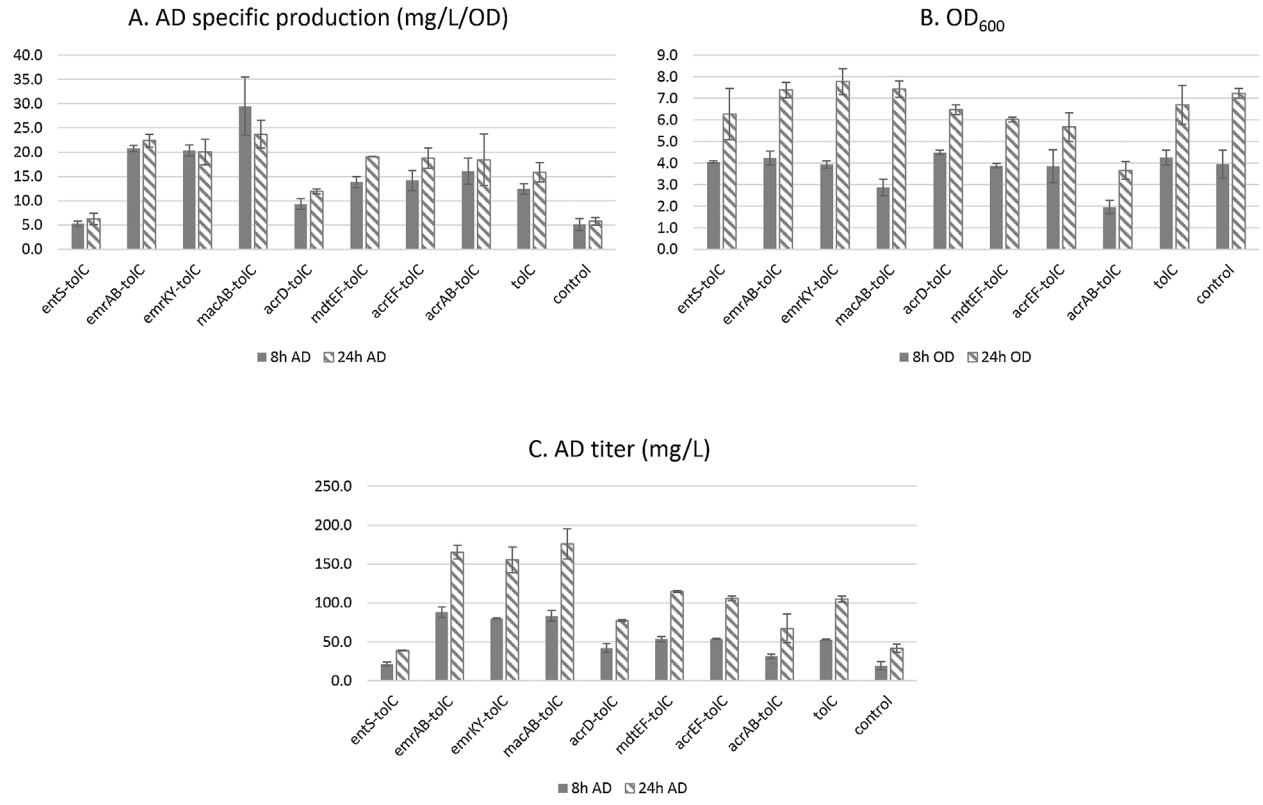

Figure 4. AD-specific production and titers in strains with overexpression of *tolC*-dependent efflux pumps. Among the nine efflux pump systems that are related to the OMP TolC, MdtABC-TolC system could be not constructed successfully in our system due to unknown reasons. (**A**) Specific AD production (mg/L/OD); (**B**) cell density at OD600; (**C**) AD titer (mg/L).

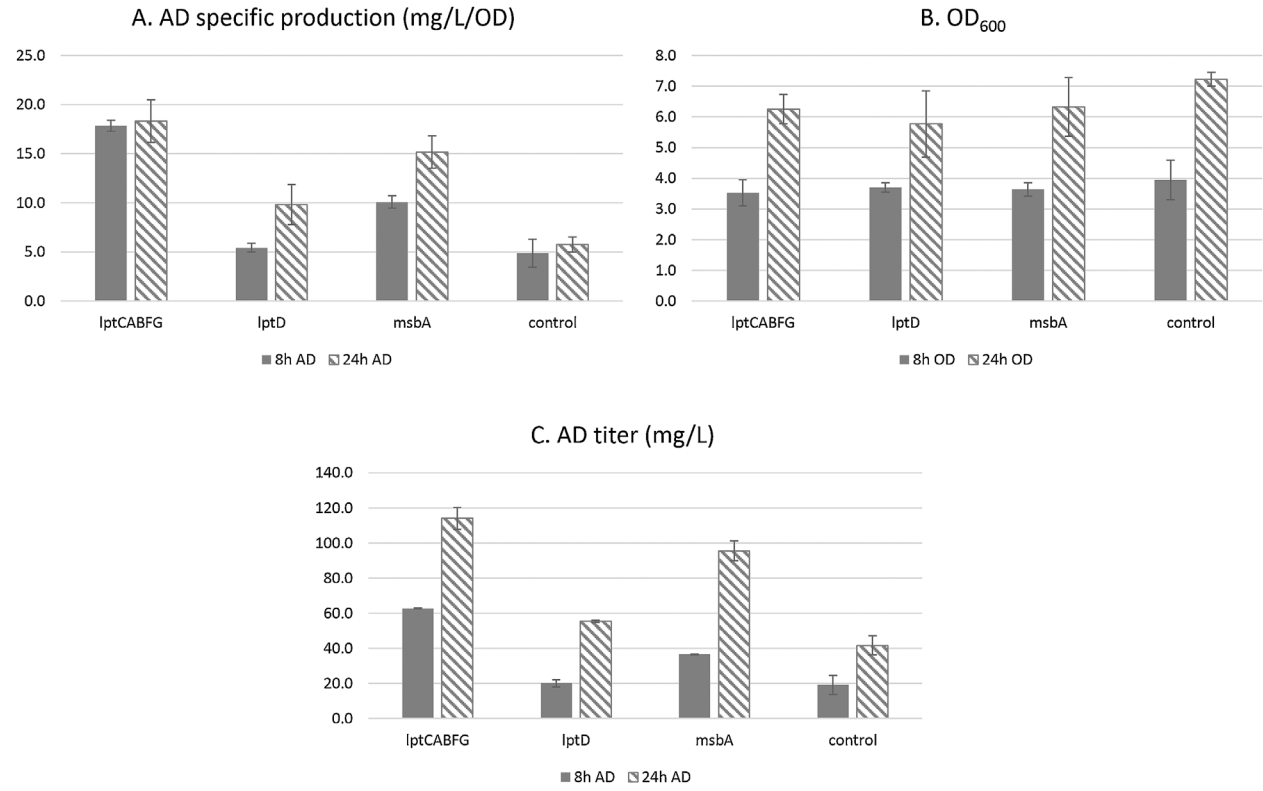

Figure 5. AD-specific production and titers in strains with overexpression of LPS efflux pumps. (**A**) Specific AD production (mg/L/OD); (**B**) cell density at OD600; (**C**) AD titer (mg/L).

might be exported through the LPS transporters. To test this hypothesis, we overexpressed the transporters in LPS transport system (LptD, LptCABFG, and MsbA). Indeed, as shown in Figure 5, AD production in both early stage (8 h) and final stage (24 h) were increased by more than twofold in the strains that overexpressed *lptCABFG* or *msbA*. AD production was also increased slightly when the OMP *lptD* was overexpressed only at 24 h. Collectively, these results indicated that the LPS transport system is also involved with AD efflux and their overexpression can be used to increase AD production.

Combination of Multiple Types of Transporters on AD Production

Our results suggested that multiple transporters were involved with the AD efflux and their overexpression could be used to enhance AD production. We next asked whether a combination of multiple types of transporters had synergistic effect on AD production. Thus, we co-expressed MsbA (inner membrane transporter) with TolC (OMP), LptCABFG (periplasmic space and inner membrane protein) with LptD (OMP), and two OMPs LptD with TolC. As shown in Figure 6, AD-specific production was significantly higher with the co-overexpression of *msbA* and *tolC* (20.3 mg/L/OD) than either *tolC* (16.1 mg/L/OD) or *msbA* (16.2 mg/L/OD) alone. These observations demonstrated the synergetic effects of the co-overexpression of multiple types of transporters. Noteworthy, the inner membrane LPS transport protein MsbA has not been reported to interact with the OMP TolC, yet the co-overexpression of TolC and MsbA has synergistic benefit in AD production. However, co-overexpression of LptCABFG and LptD or LptD and TolC did not enhance AD-specific production synergistically, despite the fact that the AD titer was increased mainly due to the increased cell density.

Discussion

Transporter engineering has become an important tool to improve both strain tolerance and products (Boyarskiy and Tullman-Ercek, 2015; Mukhopadhyay, 2015). In addition, it also contributes to reduce recovery costs as products are removed from cells (Doshi et al., 2013). Many studies focused on screening heterologous transporters or directed evolution under toxic substances. Here, we directly explored native transporters in *E. coli* especially multidrug efflux pumps. Microbial efflux pumps were able to export a wide range of compounds both native and heterologous, such as antibiotics, solvents, cationic, and lipophilic products (Brown and Skurray, 2001; Nishino and Yamaguchi, 2001; Ramos et al., 1997, 2002). With this, we hypothesized that *E. coli* has the capability to export AD or other heterologous hydrophobic compounds via native efflux pumps or other transport mechanisms.

It was once assumed that the volatile heterologous AD passively diffuses out of cells through membranes in *E. coli*. However, this is incorrect as our results indicated that *tolC* is closely involved in AD

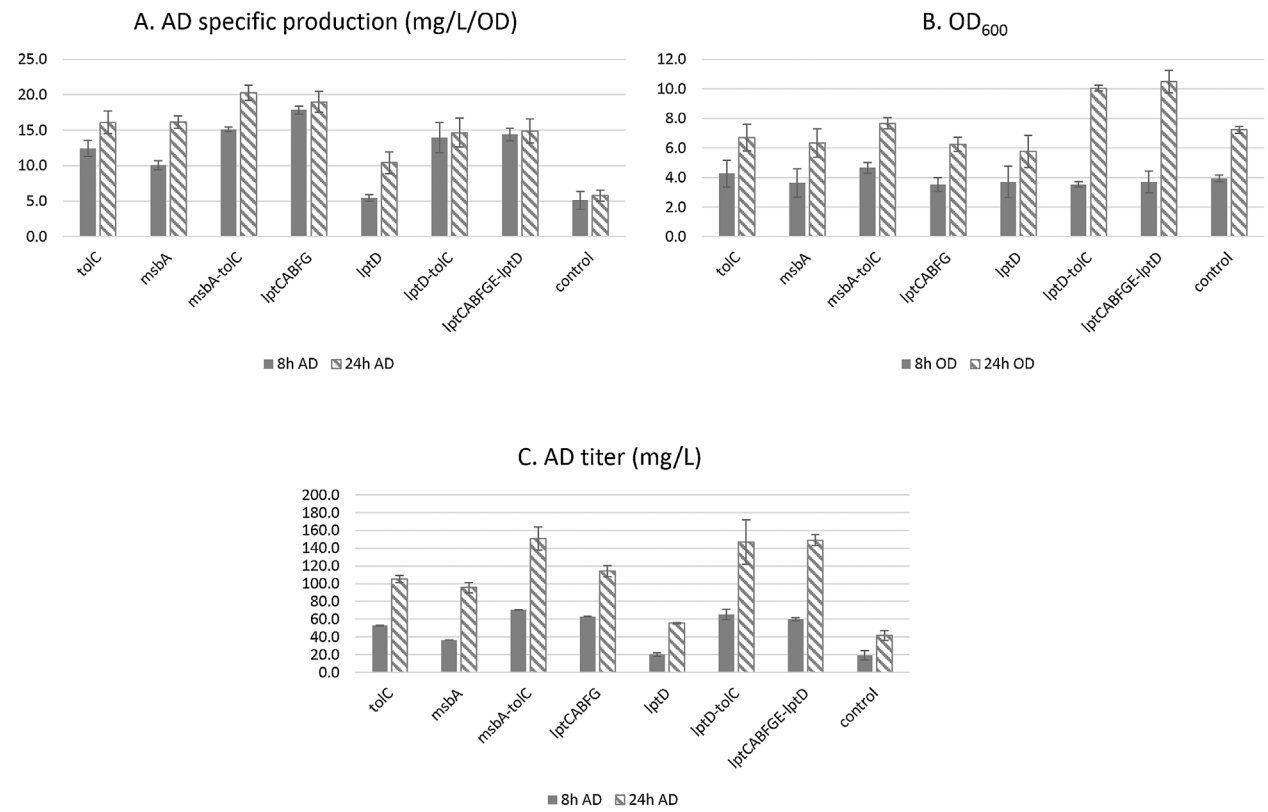

Figure 6. The synergistic benefit of combinations of multiple types of transporters on AD production. (**A**) Specific AD production (mg/L/OD); (**B**) cell density at OD600; (**C**) AD titer (mg/L).

efflux. Without *tolC*, even adding an organic layer (dodecane) onto the cell culture to extract AD, the majority of AD (more than 80%) was found to be entrapped intracellularly (Fig. 2). The OMP TolC is known to be involved in the extrusion of various harmful substances such as antibiotics (Nikaido, 1996), dyes (Tegos and Hamblin, 2006), organic solvents (Aono et al., 1998), and detergents (Sulavik et al., 2001). Furthermore, it was found that the export of some metabolites (porphyrin; Tatsumi and Wachi, 2008), cysteine (Wiriyathanawudhiwong et al., 2009), and siderophores (Bleuel et al., 2005)) also involved *tolC*. TolC is also responsible for the extrusion of intracellular signaling molecules such as cyclic AMP (Hantke et al., 2011). It was reported that *tolC* deletion almost abolished free fatty acid production in *E. coli* (Lennen et al., 2013). Thus, *tolC* mutant has various effects in increasing the susceptibility to antibiotics, cell division defects, changes in the expression of outer membrane porins, sensitivity to acid, and decreasing virulence (Zgurskaya et al., 2011).

TolC deletion inhibited AD efflux and *tolC* overexpression increased AD titer. This led us to further examine the effects of the overexpression of *tolC* together with its related transporter systems. As a result, we found multiple transporters resulted in significant increases in AD titer, especially with the overexpression of *emrAB-tolC*, *emrKY-tolC*, or *macAB-tolC* pumps. Similarly, it was reported that multiple efflux pumps (AcrAB-TolC, EmrAB-TolC, and MdtEF-TolC) were responsible for the efflux of free fatty acids, though they are all RND superfamily-type efflux pumps (Lennen et al., 2013). The capability to recruit multiple transporters to secrete the same toxic or unwanted compounds might endow *E. coli* with better adaptability to harsh environments. The increased AD production with the overexpression of these transporters was likely to be due to the increase in the forward flux in some of these reversible reactions (*dxr* (Hoeffler et al., 2002), *ispG* (Xiao et al., 2011), and *ispH* (Rohdich et al., 2002)) and the reduction in product inhibition (Dunlop, 2011) by shuttling AD out of cells. Interestingly, unlike other *tolC*-related transport system, overexpression of *entS-tolC* pump complex has no effect on AD production, possibly because AD do not interact with EntS protein (involved in enterobactin and arabinose efflux, Supplementary Table SV).

The observation that overexpression of many *tolC*-dependent transporters could enhance AD production suggested that there could be multiple native transporter systems regulating AD efflux instead of a single one. This finding can explain why the single-efflux-gene knockout had no significant effect on AD efflux (Fig. 2), as AD can be exported through other efflux pump systems. We further investigated LPS transporters which are known to export hydrophobic compounds, including LptD, LptCABFG, and MsbA (Fig. 1). LptD is an OMP that is required for LPS transport to the cell surface in *E. coli*. In addition, LptCBFG, an ABC transporter, is responsible for the release of LPS from the inner membrane (Ruiz et al., 2009). LptA is a periplasmic protein which acts as a LPS

chaperone or a bridge between the inner membrane and the outer membrane (Sperandeo et al., 2007). MsbA facilitates flipping of the lipid A-core structure across the inner membrane (Polissi and Georgopoulos, 1996) and exports antibiotics and chemotherapeutic drugs (Eckford and Sharom, 2008). We found that overexpression of LPS transporters *msbA* or *lptCABFG* also increased AD titer noticeably.

Furthermore, we explored combinations of different types of transporters. Our results indicated that combination of multiple useful transporters had synergistic benefits on AD production. Interestingly, hybrid combination of MsbA and TolC enhanced the AD yield and titer synergistically due to unknown mechanisms. However, the AD yield with combination of two OMPs LptD and TolC was lower than that with TolC alone but higher than that with LptD alone, possibly due to the similar role of TolC and LptD. Neither did co-overexpression of LptCABFG and LptD increase AD yield, which could be due to overexpression of too many membrane proteins had adverse effect instead of benefits on AD production. Further study is required to understand the underlying mechanisms. The highest AD yield (25–29 mg/L/OD) here is significantly lower than that in *Saccharomyces cerevisiae* (242 mg/L/ OD) that uses the mevalonate pathway (Westfall et al., 2012). However, it is comparable to those reports using the 1-deoxy-D-xylulose-5-phosphate pathway in *E. coli* (Wang et al., 2013; Zhang et al., 2013, 2015a). To further enhance AD production, fine tuning of the expression levels of transporter systems and AD pathway genes with promoter engineering (Zhang et al., 2015a) or dynamic control (Holtz and Keasling, 2010; Xu et al., 2014) could be explored in the future. Besides AD, we have also overexpressed these transporters in lycopene-producing strains. However, none of these pumps facilitated lycopene secretion (data not shown), further targeted screening of transporter is required to facilitate lycopene secretion.

In conclusion, our study has demonstrated the role of transporter engineering in increasing the production of AD. It is a complementary approach that complements many other approaches such as pathway optimization, gene knockout/ knockdown, combinatorial approaches, and especially the co-culture approach as the production rate could be limited by the efflux of the intermediates (Zhang et al., 2015b; Zhou et al., 2015). More broadly, this strategy may be useful in increasing the productivity of other products, especially hydrophobic compounds.

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