Fine-tuning ethanol oxidation pathway enzymes and cofactor PQQ coordinates the conflict between fitness and acetic acid production by *Acetobacter pasteurianus*

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Summary  
The very high concentrations required for industrial production of free acetic acid create toxicity and low pH values, which usually conflict with the host cell growth, leading to a poor productivity. Achieving a balance between cell fitness and product synthesis is the key challenge to improving acetic acid production efficiency in metabolic engineering. Here, we show that the synergistic regulation of alcohol/aldehyde dehydrogenase expression and cofactor PQQ level could not only efficiently relieve conflict between increased acetic acid production and compromised cell fitness, but also greatly enhance acetic acid tolerance of *Acetobacter pasteurianus* to a high initial concentration (3% v/v) of acetic acid. Combinatorial expression of *adhA* and *pqqABCDE* greatly shortens the duration of starting-up process from 116 to 99 h, leading to a yield of 69 g l⁻¹ acetic acid in semi-continuous fermentation. As a final result, average acetic acid productivity has been raised to 0.99 g l⁻¹ h⁻¹, which was 32% higher than the parental *A. pasteurianus*. This study is of great significance for decreasing cost of semi-continuous fermentation for producing high-strength acetic acid industrially. We envisioned that this strategy will be useful for production of many other desired organic acids, especially those involving cofactor reactions.

Introduction  
Metabolic engineering manipulates the cellular metabolism of microbes to maximize metabolic flux towards a desired product formation pathway (Smanski, *et al.*, 2016; He, *et al.*, 2017). Since cellular metabolism is strictly regulated to produce metabolites required for cell growth, overexpression of endogenous/heterologous pathways often leads to competition between cell growth and the desired product formation ([Wu, *et al.*, 2016; Soma, *et al.*, 2017; Tsoi, *et al.*, 2018]). In particular, when the heterologous proteins or pathway intermediates/products are toxic to host, overproduction of toxic proteins or intermediates/products leads to growth retardation or adaptive responses that reduce productivity ([Chubukov, *et al.*, 2016; Wu, *et al.*, 2016; Davy, *et al.*, 2017]). To obtain economically viable fermentation and further improvements in chemical production, it is essential to manage this trade-off phenomenon.

Acetic acid is a weak organic acid that exerts a toxic effect on most microorganisms at concentrations as low as 0.5%(w/w), and it usually serves as an effective food preservative to prevent the growth of pathogenic and spoilage organisms in fermented foods (Gullo, *et al.*, 2014). Acetic acid bacteria (AAB), especially the *Acetobacter* and *Komagataeibacter* species, are used industrially as acetic acid producers, owing to their high resistance to acetic acid. Acetic acid fermentation is a typical case in which product toxicity conflicts with cell growth. On the one hand, acetic acid fermentation is performed by membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in acetic
acid bacteria (AAB) (Lynch, et al., 2019). As shown in Fig. 1A, membrane-bound ADH and ALDH not only catalyze the conversion of ethanol substrate to acetic acid but also couple the respiration chain through reduction of ubiquinone to ubiquinol. The electrons released by oxidation from ethanol substrate flow directly into electron transport chain to reduce oxygen to water, which is coupled to the production of ATP (Matsushita, et al., 2016). Thus, acetic acid accumulates outside the cells with production of energy inside the cells, and the ethanol is primarily used as an energy source to support cell growth (Qi, et al., 2014a; Zheng, et al., 2017). On the other hand, rapid acetic acid accumulation generally causes severe acid stress, and this stress could harm cell fitness and inhibit the initiation of cell cultures by decreasing intracellular pH and disturbing energy metabolism (Trcek, et al., 2015; Xia, et al., 2015). Semi-continuous fermentation mode is reported to be the most advantageous for high-titre acetic acid production. An initial acetic acid concentration of 35–40 g l⁻¹ is usually retained for semi-continuous fermentation, until the final acetic acid content increases to 70–100 g l⁻¹ (Qi, et al., 2014a; Krusong, et al., 2015). For example, Krusong, et al. (2014) assessed the effect of a stepwise increment of initial acetic acid concentration in fermentation by high acid-tolerant strain of A. aceti WK. Although their total
acid concentration was even increased to 100 g l⁻¹, the average productivity still is very low. Growth retardation caused by high concentration acetic acid is a bottleneck that reduced productivity of semi-continuous fermentation. Therefore, exploring the trade-off relationship and further obtaining an optimum balance between improved acetic acid production and cell fitness is a vital step in the strain engineering process.

Previous studies on strain improvement were mostly focused on enhancing ethanol oxidation to increase acetic acid production, using measures such as overexpressing membrane-bound ADH or ALDH (Fukaya, et al., 1989; Wu, et al., 2017). Several fermentation engineering optimization strategies showed that enhancing energy generation by facilitating the electron transfer and oxidative phosphorylation could not only promote cell growth but also increase acetic acid production, through actions such as supplementing precursors of the electron transfer carrier coenzyme Q and oxygen electron acceptor (Qi, et al., 2014b; Xia, et al., 2015). But, these precursors, such as haem, coenzyme Q, isopenetyl alcohol and β-hydroxybenzoic acid, are very expensive, which greatly increase the cost of product acetic acid. In fact, engineering cofactor by manipulating availability of cofactors such as NADH, NADPH and ATP proved to be an effective strategy for coordinating material and energy needs, and it substantially improved the yields of target products (Wang, et al., 2017). NADH or ATP-driven systems (Lan and Liao, 2012; Ji, et al., 2013) have been devised as a potential solution for dilemmas related to cell growth and target chemical production. Pyrroloquinoline quinone (PQQ) has been determined to be another important cofactor, after NADH and NADPH, serving as a redox cofactor for a large number of dehydrogenases known as quinoproteins (Misra, et al., 2012). It has been shown to improve the adaptability of microorganisms to extreme environments, to factors such as radiation, high acid concentrations and high temperatures (Choi, et al., 2008). Various levels of PQQ in Gluconobacter oxydans could be generated by overexpressing various parts of the PQQ biosynthesis gene clusters, such as pqqA, pqqABCDE and pqqABCDE (Du, et al., 2013; Gao, et al., 2014; Wang, et al., 2016). Moreover, the balanced coexpression of sorbose/sorbosone dehydrogenase and cofactor PQQ could increase the 2-keto-L-gulonic acid production and maintain specific growth rate of Ketogulonigenium vulgare–Bacillus cereus consortium (Du, et al., 2013; Gao, et al., 2014). In A. pasteurianus, PQQ, served as cofactor of membrane-bound ADH, is involved in electron transfer of ethanol respiration chain (Matsutani and Yakushi, 2018), but it remains vague how the growth rate and acetic acid production would be affected by PQQ level generation. And the manipulation of expression level of PQQ syntheses was also not examined in A. pasteurianus. The availability of cofactor PQQ and its balance in relation to expression of ethanol oxidation pathway enzymes have also generally been neglected.

Therefore, in this study, we manipulated the expression of ethanol respiration chain-dehydrogenase module, cofactor PQQ biosynthesis module and various combinations of the two modules to coordinate the conflict between cell fitness and acetic acid production. The concentration of PQQ, cell fitness and acetic acid production were detected in wild-type A. pasteurianus and engineered strains in fermentation medium supplemented with 0, 1% (v/v) and 3% (v/v) initial acetic acid concentrations. Fine-tuning the balance between ethanol oxidation pathway enzymes and PQQ regeneration level not only relieves the conflicts between fitness and acetic acid production but also enables A. pasteurianus cells to grow in environments with very high acetic acid concentrations.

**Results**

**Effect of overexpressing membrane-bound ADH/ALDH on A. pasteurianus fitness and acetic acid biosynthesis**

Our previous study showed that promoter P<sub>tuf</sub> is 1.8-fold stronger than P<sub>adhA</sub> in the overexpression of green fluorescent protein (GFP) in A. pasteurianus (see Fig. S2). To enhance acetic acid production, we overexpressed adhA and aldhl genes with the strong promoter P<sub>tuf</sub> in a broad-host vector pBBR1MCS-2 in A. pasteurianus. The growth rate (μ) was used as a proxy for strain’s fitness (Brauer, et al., 2008; Bershtein, et al., 2015). As shown in Fig. 2 and Table S2, the wild-type A. pasteurianus B7003 produced 26.89 ± 0.93 g l⁻¹ acetic acid after 24 h of incubation (μ = 0.17 ± 0.01 h⁻¹, W = 1.00). A. pasteurianus strain with control plasmid showed the same acetic acid yield and growth rate as wild type, which indicated that the exogenous blank plasmid did not place a metabolic burden on cells. As expected, three mutants with adhA, aldhl and adhA-aldhl overexpression all showed increases in acetic acid yields (Fig. 2). In particular, the coexpression sample (A. pasteurianus/pT-aa) displayed a highest level of acetic acid, at 38.86 ± 1.80 g l⁻¹ and simultaneously kept similar growth to wild type. However, the mutants A. pasteurianus/pT-adhA and A. pasteurianus/pT-aldhl both showed a moderate drop in cell growth (P < 0.05), which indicated that their fitness was threatened.

**Effect of endogenous PQQ biosynthesis pathway overexpression**

PQQ serves as cofactor of membrane-bound ADH/ALDH that catalyses redox reaction (and electron transfer) from
ethanol to acetic acid (Sengun, et al., 2017). The concentrations of PQQ were analysed in wild-type and engineered strains. As shown in Fig. 2 and Table S2, PQQ level was decreased in all mutants with dehydrogenase module overexpression (\( P < 0.01 \)). In particular, PQQ level in coexpression strain A. pasteurianus/pT-aal was seriously decreased to \( 80.75 \pm 6.82 \mu g \) at approximately 24 h (wild type: \( 145.25 \pm 5.42 \mu g \)). We hypothesized that the imbalanced concentration between PQQ and dehydrogenases might be a bottleneck that inhibited further improvement of acetic acid production.

To explore whether PQQ could enhance acetic acid production in engineered strains, we first determined the effects of \( pqqAB \) and \( pqqABCDE \) gene overexpression on PQQ regeneration, cell growth and acetic acid production in A. pasteurianus (Fig. 2 and Table S2). As expected, mutant strain A. pasteurianus/pT-pqqAB enhanced PQQ level by 29% (187.00 \( \pm 2.97 \mu g \)) and A. pasteurianus mutant harbouring a plasmid bearing the complete \( pqqABCDE \) cluster (pT-pqqABCDE) achieved an eightfold higher level of PQQ biosynthesis (1181.25 \( \pm 7.36 \mu g \)) than wild type (145.3 \( \pm 5.42 \mu g \)). This result confirmed that overexpression of \( pqqAB \) and \( pqqABCDE \) in A. pasteurianus enabled differential increase in PQQ level. Moreover, A. pasteurianus mutants with overexpression of \( pqqAB \) and \( pqqABCDE \) both increased acetic acid yield by 12% (30.00 \( \pm 1.50 g \)) than wild type, which suggested that increasing PQQ regeneration has a positive effect on acetic acid production. However, the growth rates of mutants (A. pasteurianus/pT-pqqAB and A. pasteurianus/pT-pqqABCDE) were lower than that of wild type (\( P < 0.05 \)). And strain A. pasteurianus/pT-pqqABCDE displayed a slower growth rate than A. pasteurianus/pT-pqqAB (Fig. 2). These results indicated that overexpression of the endogenous PQQ biosynthesis pathway genes also resulted in a trade-off between improved acetic acid production and compromised cell fitness.

Effect of co-overexpressing membrane-bound ADH/ALDH and PQQ biosynthesis pathway

Subsequently, to investigate the balancing relationship between dehydrogenase module and cofactor PQQ module on cell fitness and acetic acid production, six expression patterns in the combined genes from acetic acid biosynthesis and PQQ biosynthesis pathways were constructed and determined in A. pasteurianus (as shown in Fig. 1 B). The growth rate and acetic acid production are summarized in Fig. 3 and Table S2. In comparison with A. pasteurianus/pT-adhA, coexpression of \( adhA \) with \( pqqAB \) increased growth rates by 49% and 56% in the A. pasteurianus/pT-adhA-pqqAB and A. pasteurianus/pT-adhA-pqqABCDE strains, simultaneously increased acetic acid yields from 32.71 \( \pm 1.99 g \) to 35.44 \( \pm 0.44 g \) and 38.01 \( \pm 0.45 g \) (Fig. 3). Similarly, coexpression of \( aldh \) and \( pqqAB/pqqABCDE \) both had a positive effect on growth rate and increased acetic acid yields from 34.20 \( \pm 1.57 g \) to 37.05 \( \pm 0.37 g \) (Table S2). These results indicated that the synergistic improvement of membrane-bound ADH/ALDH and cofactor PQQ modules could relieve conflict between acetic acid production and compromised cell growth, thereby maintaining acetic acid production and simultaneously improving cell fitness. However, the coexpression of \( pqqAB \) had little

**Fig. 2.** Effects of overexpression of dehydrogenase module and cofactor PQQ module on A. pasteurianus. Error bars represent the standard deviation of three biological replicates. Comparison was performed with wild-type A. pasteurianus B7003 (* and ** \( P < 0.05 \) and 0.01 respectively).
Effect on growth rate and acetic acid yield in strain A. pasteurianus/pT-aal, leading to a highest production of acetic acid (39.14 ± 1.58 g l⁻¹). The expression of pqqABCDE in strain A. pasteurianus/pT-aal led to 38% decrease of growth rate and marginally decreased acetic acid from 38.86 ± 1.80 to 35.05 ± 0.57 g l⁻¹ (Fig. 3), suggesting that intracellular PQQ level achieved by pqqABCDE was not optimal level for improving acetic acid yield in strain A. pasteurianus/pT-aal.

Effect of initial acetic acid on fitness and acetic acid fermentation by recombinants

The high acetic acid tolerance of A. pasteurianus is important for semi-continuous fermentation for producing high-strength acetic acid industrially. It is commonly accepted that good determination and control of the starting-up process will determine productivity of a semi-continuous fermentation process (Charles, et al., 2009). In previous work, we proposed a two-stage starting-up protocol to achieve high acid production, in which 10 g l⁻¹ of initial acetic acid was used to promote acetic acid production, and then, acetic acid from 35 to 40 g l⁻¹ was retained for the second starting-up fermentation, until the final acetic acid content increased to approximately 70 g l⁻¹ (Xia, et al., 2015). However, a high initial concentration of acetic acid at 35–40 g l⁻¹ often results in a long lag phase during the second starting-up process.

In our study, we simulated the acidic environment over the entire starting-up stage to determine acetic acid production and tolerance of engineered strains. Concentrations of 1% (v/v) and 3% (v/v) acetic acid were added to fermentation medium from the beginning. As expected, acetic acid inhibited cell growth in a concentration-dependent manner, including that of all the over-expression strains (Fig. 3A, Fig. 4A and B). Similar to reduced growth rate, PQQ regeneration level also decreased with the increased initial acetic acid concentration (Table S2, S3 and S4). Differently, acetic acid production of all strains reached a maximum value in the presence of 1% (v/v) initial acetic acid (Figs 3 and 4). This result is consistent with previous study in which a low concentration of initial acetic acid had a positive effect on acetic acid production (Wang, et al., 2015).

Among all strains, A. pasteurianus strains harbouring adhA-aldh genes (i.e. pT-aal, pT-aal-pqqAB and pT-aal-pqqABCDE) produced the highest acetic acid, with a yield of 41.21 ± 0.83, 41.75 ± 0.95 and 41.69 ± 0.82 g l⁻¹, respectively, in the presence of 1% initial acetic acid (Table S3). Their productivities (1.61 ± 0.07, 1.65 ± 0.09 and 1.67 ± 0.04 g l⁻¹ h⁻¹) were also significantly higher than from A. pasteurianus JST-S/pBBR-adhA-adhB (Wu, et al., 2017) and A. pasteurianus/pMV24-uvRA (Zheng, et al., 2018), under the condition of shake flask with 1% initial acetic acid at 30° (Table 1). However, in the presence of 3% (v/v) initial acetic acid, acetic acid production of all engineered strains showed a significant drop compared to wild type (Fig. 4 D). This decrease was most likely caused by metabolic flux change induced by the high initial concentration of acetic acid.

Fig. 3. Growth rate (A) and acetic acid production (B) of the wild-type A. pasteurianus and all engineered strains in the fermentation medium supplemented without initial acetic acid. The grey columns represented wild-strain A. pasteurianus and A. pasteurianus/pBBR1MCS-2. The blue and red columns represented growth rate and acetic acid production respectively. Error bars showed the standard deviation of three biological replicates. **P < 0.01.
The TCA cycle, which assimilates intracellular acetic acid, was upregulated to produce more energy in response to high acetic acid stress, which led to decreased acetic acid production in all the recombinants. Interestingly, fine tuning PQQ regeneration level by PQQ biosynthesis genes could also effectively improve growth rate of engineered strains with dehydrogenase expression module (i.e. A. pasteurianus/pT-adhA, A. pasteurianus/pT-adh and A. pasteurianus/pT-aal) in the presence of initial acetic acid (Fig. 4A and B). In particular, the growth rates of engineered strains A. pasteurianus/pT-adhA-pqqABCDE and A. pasteurianus/pT-adhA-pqqAB, respectively, showed a significant increased in presence of 1% (v/v) or 3% (v/v) acetic acid, which indicated that they are likely to be more resistant to acetic acid than wild-type. Thus, a spot assay experiment was performed to detect two strains’ acetic acid tolerance (Fig. S3). Obviously, above two strains grew better than...
wild type on fermentation medium plates containing 2 and 3 g l⁻¹ acetic acid. These results further demonstrated that balanced relationship between dehydrogenase and cofactors played an important role on cell growth and tolerance in *A. pasteurianus*.

**Semi-continuous fermentation of engineered strains for high-strength acetic acid production**

Taking consideration of acetic acid production and growth rate, engineered strains *A. pasteurianus/pT-adhA-pqqABCDE*, *A. pasteurianus/pT-aal*, *A. pasteurianus/pT-aal-pqqAB* and *A. pasteurianus/pT-aal-pqqABCDE* were presumed to be a good starter culture candidate for high-strength acetic acid production. We further performed semi-continuous culture experiments of these four engineered strains and original strain *A. pasteurianus B7003* in a well-controlled 7.5 L fermenter with a working volume of 4 L to produce high-strength acetic acid (Fig. 5). For *A. pasteurianus* B7003, it took 116 h to complete starting-up, leading to a yield of 70 g l⁻¹ acetic acid (Fig. 5A). Subsequently, two repeated batches of fermentation were performed. The period of first fermentation batch was 65 and 37 h for second batch. As a result, the average acetic acid productivity of whole process is 0.75 g l⁻¹ h⁻¹ in original strain *A. pasteurianus* B7003. In comparison, semi-continuous culture of the engineered strain *A. pasteurianus/pT-adhA-pqqABCDE* displayed the best performance, which resulted in 99 h of staring-up with average productivity of 0.99 g l⁻¹ h⁻¹. The period of each fermentation batch was 34–35 h (Fig. 5 B). Obviously, cell growth and acetic acid accumulation of engineered strain *A. pasteurianus/pT-adhA-pqqABCDE* were faster than *A. pasteurianus* B7003 in the starting-up phase due to the synergistic expression of ADH and cofactor PQQ. The result is also better than from *A. acetic WK* (Krusong, *et al*., 2015) and *A. pasteurianus* UMCC 2951 (Ruttipron, *et al*., 2020), under the condition of semi-continuous fermentation (Table 1). Similar to flask experiments, the performance of strain *A. pasteurianus/pT-aal* with overexpression of PQQ both are superior to *A. pasteurianus/pT-aal*, owing to their better growth on fermentation medium with initial acetic acid. In general, higher acetic acid productivity and cell fitness of *A. pasteurianus/pT-aal-pqqABCDE* and *A. pasteurianus/pT-aal-pqqAB* and *A. pasteurianus/pT-aal-pqqABCDE* demonstrated the effectiveness of strategy of balanced coexpressed pathway enzymes and cofactor.

**Discussion**

The redox cofactor PQQ is a member of secondary metabolites annotated as ribosomally synthesized and post-translationally modified peptides and acts as a prothet group of alcohol and/or sugar dehydrogenases (Koehn, *et al*., 2019). The presence of this molecule has been shown to enhance cell growth rate (Ke, *et al*., 2019). Gene knockout and bioinformatics studies have identified that PQQ biosynthesis is accomplished by the gene products of a specific *pqq* operon (i.e. *pqqA-E*), but a detail account of PQQ biosynthesis has remained

### Table 1. Comparison of acetic acid production in engineered *A. pasteurianus* and *A. aceti* strains.

| Microorganism hosts | Fermentation temperature (°C) | Carboxyl source | Fermentation mode | References |
|---------------------|-------------------------------|-----------------|-------------------|------------|
| *A. pasteurianus* JST-S/pBBR-adhA-adhB | 30 | Ethanol | Shake flask | (Wu, *et al*., 2017) |
| *A. pasteurianus* pMV24-uvrA | 30 | Ethanol | Shake flask | (Zheng, *et al*., 2018) |
| *A. aceti* 10-8S2/pACO300 | 30 | Ethanol | Batch | (Nakano, *et al*., 2004) |
| *A. aceti* 10-8S2/pABC101 | 30 | Ethanol | Shake flask | (Nakano, *et al*., 2006) |
| *A. aceticum* | 30 | Ethanol | Semi-continuous (nine repeat batches) | (Krusong, *et al*., 2015) |
| *A. pasteurianus* UMCC 2951 | 30 | Ethanol | Semi-continuous (five repeat batches) | (Ruttipron, *et al*., 2020) |
| *A. pasteurianus* CV01 | 38 | Ethanol | Semi-continuous (one repeat batch) | (Majid, *et al*., 2016) |
| *A. pasteurianus* CICIM B7003-02 | 30 | Ethanol | Semi-continuous (discharge/charge ratio optimization and aeration strategy, four repeat batches) | (Qi, *et al*., 2014a) |
| *A. pasteurianus* pT-adhA-pqqABCDE | 30 | Ethanol | Semi-continuous (two repeat batches) | This study |
unresolved (Holscher and Gorisch, 2006; Shen et al., 2012). Several recombinant strains have been developed to enhance PQQ production in *G. oxydans* by overexpression of each individual gene, and the PQQ levels followed the order: \( pqqB > pqqA > pqqD > pqqC > pqqE \) (Wang, et al., 2016). It has been reported that PQQ backbone (glutamate and tyrosine) is probably derived from PqqA peptide (Goosen, et al., 1992). Supplementation of amino acids involved in the biosynthesis of PQQ precursor peptide PqqA can effectively increase accumulation of PQQ in cells (Ke, et al., 2019). The PqqB protein takes part in transporting PQQ across the membrane and produces quinone moiety of the mature PQQ cofactor (Velterop, et al., 1995; Koehn, et al., 2019). The orthoquinone structure in PQQ is directly responsible for oxidoreduction, essential for the primary oxidation step of non-phosphorylated substrates, such as alcohols, aldehydes or aldoses (Minenosuke and Toshiharu, 2018). PqqC catalyses the final step in PQQ formation (Magnusson, et al., 2004). PqqD as a novel peptide chaperone forms a ternary complex with the radical S-adenosylmethionine protein PqqE (Latham, et al., 2015). PqqE catalyses de novo carbon–carbon cross-linking within a peptide substrate PqqA in the presence of the peptide chaperone PqqD (Barr, et al., 2016). In our study, considering that ethanol oxidation occurs on cytoplasmic membrane, part of genes in *pqq* operon (i.e. *pqqAB*) and a full-length *pqqABCDE* cluster were independently overexpressed to determine their impacts on PQQ biosynthesis and acetic acid production. Similar to previous report, overexpression of part of genes in PQQ biosynthetic gene cluster also can promote PQQ regeneration (Wang, et al., 2016), and the PQQ production followed order: \( pqqAB < pqqABCDE \) (Fig. 2). Although PQQ levels in *A. pasteurianus* were found to have a positive correlation with the conversion efficiency of ethanol to acetic acid, direct recombinant expression of PQQ biosynthetic gene cluster probably competes with precursor amino acids and energy required for cell growth, resulting in poor cell growth (Fig. 2).

In Asian countries (i.e. China and Japan), *A. pasteurianus* is commonly used for the industrial production of acetic acid, which could resist up to 6% (v/v) acetic acid. However, in European countries (i.e. Germany), *Komagataeibacter europaeus* is the main species used for the same purpose, which has been reported to exhibit 10% (v/v) acetic acid tolerance (Trcek, et al., 2006). Genomic analysis showed that there were significant intergeneric differences in the number of genes encoding PQQ-ADH; *K. europaeus SP3* contained up to 6 copies of PQQ-ADH gene, while *A. pasteurianus* harboured only two copies (Wang, et al., 2015; Xia, et al., 2017). Our results demonstrated that increasing the number of copies of membrane-bound ADH genes indeed enable *A. pasteurianus* stronger acetic acid production (Fig. 2). However, cell growth rate in strain *A. pasteurianus/pT-adhA*
slowed down comparing with original strain, which is consistent with previous reports in *A. pasteurianus* JST-S (Wu, et al., 2017). Further study suggested that the synergistic regulation of cofactor PQQ and dehydrogenase plays an important role in coordinating conflict between acetic acid production and compromised growth rate. Especially in the semi-continuous fermentation process, proper PQQ regeneration effectively shortened the lag phase of engineered strain *A. pasteurianus*/pT-aa, leading to a higher productivity (Fig. 5). Cofactor PQQ generation, as an electron carrier, on one hand, accelerates the electron transfer in ethanol respiration chain, thus ensuring energy supply needed for cell growth and resistance to acetic acid stress. On the other hand, it may directly improve the catalytic activity and acetic acid stability of PQQ-ADH that enables *A. pasteurianus* to grow and stay metabolically active at extremely high concentrations of acetic acid (Trecek, et al., 2006). In present study, *A. pasteurianus* strains harbouring *adhA* as the acetic acid biosynthesis module (i.e. pT-adhA, pT-adhA-pqqAB and pT-adhA-pqqABCD) displayed an increasing growth rate and increasing acetic acid production with the increasing PQQ level in presence of 0 or 1%(v/v) initial acetic acid (Figs 3 and 4). This result suggested that cofactor PQQ levels were positively correlated to PQQ-ADH activity. Over the past few years, the cofactor of membrane-bound ALDH remains unclear in acetic acid bacteria. Molybdopterin is presumed to be the putative prosthetic group of membrane-bound ALDH in *K. europaeus* (Thurner, et al., 1997), whereas in *Ga. diazotrophicus*, PQQ is considered the prosthetic group (Gomez-Manzo, et al., 2010). Different to co-over-expression of ADH and PQQ, *A. pasteurianus*/pT-aldh-pqqABCDE with more PQQ level displayed a slower growth rate than *A. pasteurianus*/pT-aldh-pqqAB and they produced similar amounts of acetic acid (Figs 3 and 4). Thus, PQQ is probably not the cofactor of membrane-bound ALDH in *A. pasteurianus*.

In recent years, with in-depth study of the acetic acid-tolerance mechanisms of AAB, many genes and proteins related to stress response and tolerance, such as membrane-bound ADH (Wu, et al., 2017), nucleic acid repair enzyme UvrA (Zheng, et al., 2018), aconitase (Nakano, et al., 2004) and ABC transporter (Nakano, et al., 2006), have been successfully identified, facilitating the application of genetic improvement to improve acetic acid tolerance and product yield. A comparative of acetic acid productivity in engineered *A. pasteurianus* and *A. aceti* strains, was summarized in Table 1. Overall, the acidification rate of these engineered strains still stayed at flask level, and is lower than our work in presence of 1% initial acetic acid (Table S3). Under industrial conditions, although the high-acid-tolerant strain of *A. aceti* WK, and *A. pasteurianus* UMCC 2951 produced a total acid to 80–100 g l⁻¹, the period of each fermentation batch and aceticification rate both were lower than our engineered strains (Fig. 5). Moreover, strains exhibit different degrees of resistance of acetic acid with the different fermentation phases of semi-continuous. This resistance is affected by initiation of cell cultures, aeration rate, discharge/charge ratio, the number and modality of recursive cultivations in acetic acid media (Gullo, et al., 2014). For example, *A. pasteurianus* CICIM B7003-02, an ultraviolet mutant from wild-type strain *A. pasteurianus* CICIM B7003, produces a high acidity vinegar with an acetic acid concentration that reached up to 93.1 g l⁻¹ in the semi-continuous mode via optimization of discharge/charge rate and aeration strategy (Qi, et al., 2014a). It should be noted that we just strengthened the ethanol respiratory chain from ethanol to acetic acid at the production stage. Further improvement in acetic acid resistance and productivity might be attained through optimizing process control.

In summary, this work represented the effectiveness of combining modular biological parts, in enhancing acetic acid production and tolerance, by constructing genes in ethanol oxidation pathway and in PQQ synthesis pathway as two modules. The engineered strain *A. pasteurianus*/pT-adhA-pqqABCDE with high cell fitness was obtained and serves as a good starter culture candidate for semi-continuous fermentation, which is of great significance for decreasing cost of semi-continuous fermentation for producing high-strength acetic acid industrially. In the future, the engineering strategies can also be used to engineer cell factories for production of other organic acid.

**Experimental procedures**

**Strains, media and culture conditions**

*Acetobacter pasteurianus* CICIM B7003 isolated from a brewing factory (Hengshun Wantong Food Brewing Co., Ltd., Xuzhou, China) was used in this study. *Escherichia coli* JM109 used for general cloning was grown under routine conditions, on Luria–Bertani (LB) agar plates or in LB broth at 37°C. All the bacterial strains used in this study are shown in Table 2. The seed medium contained 10 g l⁻¹ glucose, 10 g l⁻¹ yeast extract and 3% (v/v) ethanol. The fermentation medium contained 10 g l⁻¹ glucose, 10 g l⁻¹ yeast extract, 0.6 g l⁻¹ KH₂PO₄, 0.4 g l⁻¹ MgSO₄ and 4% (v/v) ethanol. When required, kanamycin (50 µg ml⁻¹ for *E. coli* or 25 µg ml⁻¹ for *A. pasteurianus*) was added to the culture medium. Cells from cryovials were incubated in 50 ml of seed medium in 250 ml Erlenmeyer flasks, and they were cultured at 30°C for 24 h at 170 rpm. Fermentations were performed in fermentation medium at 30 °C at 220 rpm. Different initial concentrations of acetic acid were added to
fermentation medium for detection of growth and production in *A. pasteurianus* and mutations.

**Plasmid construction**

All the plasmids used in this study are listed in Table 2. Plasmid construction and DNA manipulations were performed by following standard molecular biology techniques. All the primers used for PCR amplification are listed in Supplementary Table S1. Schematic diagrams of genetic constructs containing the enzyme genes from acetic acid biosynthesis pathway, PQQ biosynthetic genes and their various combinations are shown in Fig. 1.

The open reading frames (ORFs) of *adhA, aldh* and promoter of elongation factor TU (Gene ID: 8435080) as well as the *ppqAB* and *ppqABCDE* genes were amplified separately using genomic DNA of *A. pasteurianus*. The promoter of elongation factor TU was ligated with different *adhA, aldh, ppqAB* and *ppqABCDE* genes using SOE-PCR. Subsequently, the resulting fragments *P*₅*₆*-*adhA* and *P*₅*₆*-*aldh* were inserted into *KpnI*-*BamHI* sites of the pBBR1MCS-2 plasmid using In-Fusion Cloning, resulting in plasmids pT-*adhA*, pT-*aldh* and pT-*aal*. The fragments *P*₅*₆*-*ppqAB* and *P*₅*₆*-*ppqABCDE* were digested and inserted at *SpeI*-*PvuI* sites of the pBBR1MCS-2 plasmid to produce pT-*ppqAB* and pT-*ABCDE*, and they were separately inserted into pT-*adhA*, pT-*aldh* or pT-*aal* plasmids, generating six plasmids with different gene combinations (listed in Table 2). All the constructs were transformed into *A. pasteurianus* by electroporation (Zhang, *et al.*, 2010).
of enzyme solution, 0.20 M substrate glucose, 0.67 mM phenazine methosulfate (PMS) and 0.1 mM 2,6-dichlorophenolindophenol (DCIP) in 1.0 ml of phosphate buffer pH 7.0 at 30 °C for 5 min. The absorbance changes in the reaction mixture were measured at 600 nm once the D-glucose was added. The protein concentrations were measured using a Bradford Protein Assay kit (purchased from Sangon Biotech, Shanghai, China).

Semi-continuous fermentation

Semi-continuous acetic acid fermentation was performed in a 7.5 l fermentor like our previous work (Qi, et al., 2014a). For starting-up process, 3.16 l fermentation medium containing 10 g l⁻¹ acetic acid was poured into fermentor and mixed adequately with 0.3 l seeds. Aeration rate was set at 0.865 l min⁻¹ (0.25 vvm). When the residual ethanol concentration was below 5 g l⁻¹, 0.54 l fermentation medium with 260 g l⁻¹ ethanol was supplemented into fermentor to continue the starting-up process. Simultaneously, aeration rate was set at 1.2 l min⁻¹ (0.3 vvm). Temperature was set at 30 °C for whole process. Starting-up process was completed when the acetic acid content increased to about 70 g l⁻¹ with less than 5 g l⁻¹ residual ethanol. Subsequently, a new repeated batch was operated with discharging 43% (v/v) of total working volume (4 L) and then feeding the same volume of fresh fermentation medium containing 81.4 g l⁻¹ ethanol. Then, an acetylation process was occurred as the previous one.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** The relationship between the OD$_{600}$ and the number of living bacteria (N, CFU/mL) in *A. pasteurianus* ($N = 10^{3.1666x + 7.0226}$).

**Fig. S2.** The whole cell fluorescence intensities of strains with $P_{adhA}$-GFP (A) and $P_{tuf}$-GFP (B). The promoter strength was defined as RFU/OD$_{600}$ (relative fluorescence unit divided by the corresponding OD$_{600}$).

**Fig. S3.** Acetic acid tolerance of wild-type strain *A. pasteurianus* B7003, *A. pasteurianus/pT-adhA-pqqAB* and *A. pasteurianus/pT-adhA-pqqABCDE*. Cells were spotted in serial dilutions (diluted by a factor 10) and grown on fermentation medium agar plates amended with various concentrations of acetic acid.

**Tables S1.** PCR primers used for genetic constructs.

**Table S2.** Summary of growth and production characteristics of *A. pasteurianus* B7003 and recombinants in fermentation medium (containing 4% (v/v) ethanol) without initial acetic acid.

**Table S3.** Summary of growth and production characteristics of *A. pasteurianus* B7003 and recombinants in fermentation medium (containing 4% (v/v) ethanol) supplemented with 1% (v/v) initial acetic acid. (The acetic acid yield has been subtracted from the initial acetic acid).

**Table S4.** Summary of growth and production characteristics of *A. pasteurianus* B7003 and recombinants in fermentation medium (containing 4% (v/v) ethanol) supplemented with 3% (v/v) initial acetic acid. (The acetic acid yield has been subtracted from the initial acetic acid).