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Regulatory mechanisms of acetic acid, ethanol and high temperature tolerances of acetic acid bacteria during vinegar production

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Abstract
Acetic acid bacteria (AAB) play a pivotal role in the food fermentation industry, especially in vinegar production, due to their ability to partially oxidize alcohols to acetic acid. However, economic bioproduction using AAB is challenged by harsh environments during acetic acid fermentation, among which initial ethanol pressure, subsequent acetic acid pressure, and consistently high temperatures are common experiences. Understanding the stress-responsive mechanisms is essential to developing robust AAB strains. Here, we review recent progress in mechanisms underlying AAB stress response, including changes in cell membrane composition, increased activity of membrane-bound enzymes, activation of efflux systems, and the upregulation of stress response molecular chaperones. We also discuss the potential of advanced technologies, such as global transcription machinery engineering (gTME) and Design-Build-Test-Learn (DBTL) approach, to enhance the stress tolerance of AAB, aiming to improve vinegar production.

Keywords Acetic acid bacteria, Vinegar, Stress-responsive mechanisms, Acetic acid, Ethanol, Thermotolerant

Introduction

Acetic acid bacteria (AAB) are Gram-negative, strictly aerobic bacteria that exhibit diverse cellular morphologies [1, 2] and are found in orchard soil, fruit juices, insect intestinal tracts, and on the surfaces of spoiled food [3]. As of June 2024, this group includes 22 genera and 122 species (data from LPSN, https://lpsn.dsmz.de/) (Fig. 1). The most distinctive feature of AAB is the ability to incompletely oxidize ethanol into acetic acid, the principal component of vinegar [4–7]. In addition to vinegar production, AAB also contribute to the fermentation of other foods, including kefir grains, kombucha and cocoa beans [8–11]. Acetic acid fermentation in AAB is mediated by membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which catalyze the transformation of ethanol to acetic acid while concurrently reducing ubiquinone to ubiquinol. This process funnels electrons from ethanol oxidation directly into the electron transport chain, culminating in oxygen reduction to water and ATP generation [12]. Currently, the efficient production of acetic acid is limited by harsh environmental conditions during fermentation, particularly the stresses caused by acetic acid, ethanol, and elevated temperatures.

Acetic acid is highly toxic to microorganisms; concentrations above 5 g/L can significantly inhibit microbial growth and metabolism, and may even be lethal to most

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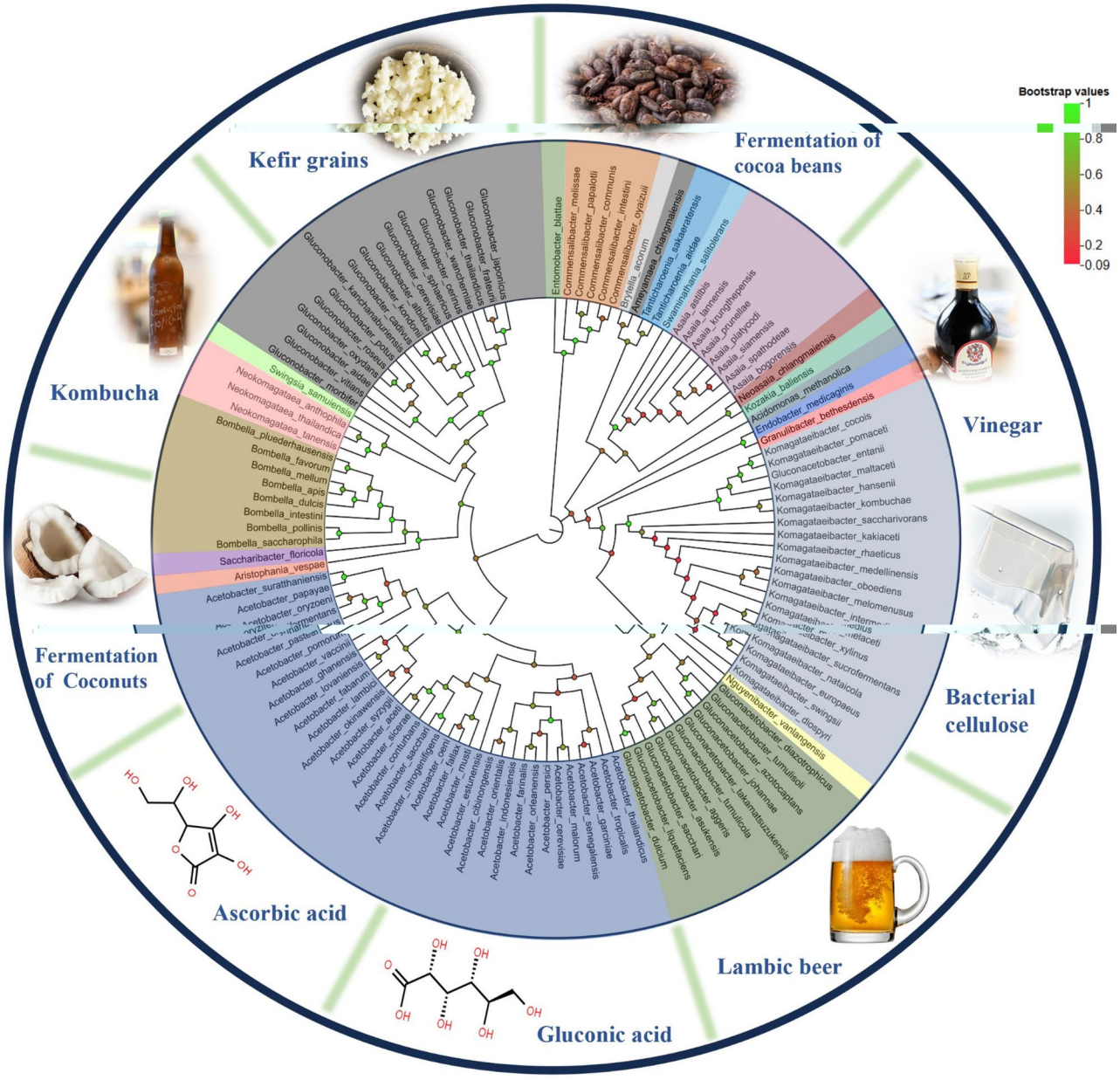

Fig. 1 Phylogenetic tree of AAB based on 16 S rRNA gene sequences and their derived products

microorganisms [13]. Its toxic effect on microorganisms primarily results from its ability to penetrate the cell membrane and damage intracellular macromolecules. Research on acid tolerance mechanisms in bacteria has been more thoroughly studied in *Escherichia coli* and lactic acid bacteria [14, 15], with most studies predominantly addressing the toxicity of inorganic acids, such as hydrochloric acid. In contrast, AAB often face adverse effects from organic acids such as acetic acid. Because of the unique physicochemical properties of organic acids, with a low dissociation constant (Ka), acetic acid largely exists in its undissociated form, allowing it to pass through the cell membrane and into the cell [16], thereby severely inhibiting cellular growth and metabolic activities.

AAB utilize ethanol as a substrate to produce acetic acid. The concentration of ethanol can significantly influence the rate of product synthesis. Higher ethanol concentrations tend to accelerate the production of acetic acid, thereby enhancing the overall efficiency of the fermentation process. However, the beneficial effects of increased ethanol concentrations have their limitations. Excessive ethanol levels can inhibit AAB growth, challenging the maintenance of a stable and productive fermentation process. When ethanol concentrations exceed 4%, the inhibitory effect significantly reduces AAB

growth [17]. Therefore, understanding the mechanisms of ethanol tolerance in AAB is crucial for improving industrial fermentation processes.

Most AAB thrive at an optimal growth temperature of around 30 °C. When the fermentation temperature exceeds 34 °C, the growth and fermentation of most AAB are severely affected, whereas thermotolerant strains can grow normally at 37 °C, with some even maintaining good growth capability at temperatures up to 42 °C [18, 19]. Acetic acid fermentation, similar to other oxidative fermentation processes, is exothermic, causing fermentation temperatures to frequently rise above 40 °C. Without artificial cooling methods, AAB would gradually die at such high temperatures. Thermotolerant AAB can significantly improve production efficiency and reduce costs [19]. Typically, the thermotolerance of AAB is achieved through directed evolution, resulting in strains with enhanced heat resistance [20, 21]. Most research on the thermotolerance mechanisms of AAB has focused on *Acetobacter pasteurianus*, mainly through genomic sequencing and comparative analysis. However, the thermotolerance mechanisms of AAB require further in-depth study.

Understanding the response mechanisms of AAB to harsh conditions not only facilitates the development of more robust strains, reducing the need for stringent control of fermentation parameters and potentially lowering production costs, but also supports the optimization of industrial fermentation processes, thereby improving efficiency and product quality. However, there is a significant gap in our understanding of how AAB adapt physiologically to various stresses and the genetic factors that enable them to survive under extreme conditions. This review seeks to address this gap by compiling, analyzing, and summarizing the stress responses of AAB during vinegar production.

Mechanisms of acetic acid tolerance

Acetic acid fermentation is a typical case in which product toxicity conflicts with cell growth [12]. High concentrations of acetic acid inhibit cell growth, which in turn affects product synthesis. Therefore, to improve the production of acetic acid, researchers have conducted numerous studies on the mechanisms of acetic acid tolerance in AAB. AAB responds to acetic acid stress through several key mechanisms: (1) alteration of capsule and cell membrane components, (2) enhancement of membrane-bound enzyme activity, particularly PQQ-ADH, (3) acceleration of acetic acid efflux efficiency, (4) enhancement of acetic acid peroxidation in the cytoplasm, (5) upregulation of stress response molecular chaperones, (6) activation of the quorum sensing system, and (7) reliance on type II toxin-antitoxin systems (Fig. 2).

Biofilm and plasma membrane

Biofilm, primarily composed of extracellular polysaccharides (EPS), serves as a critical barrier that prevents the influx of unfavorable compounds into the cell. In *Acetobacter* spp., the EPS components—primarily capsular polysaccharides (CPS) and pellicle polysaccharides (PPS)—confer resistance against exogenous acetic acid stress [22–24]. During acetic acid fermentation, CPS concentration increases significantly as the process reaches the stationary growth phase. CPS serves as a protective barrier that prevents the diffusion of acetic acid across the cell membrane [25]. Compared to non-PPS-forming *A. pasteurianus* strains, PPS-forming *A. pasteurianus* strains exhibit significantly greater resistance to acetic acid. Transport assays indicate that PPS functions as a biofilm-like barrier, restricting passive acetic acid diffusion into cells and thereby enhancing resistance to acetic acid stress [24].

Regulation of cell membrane fatty acid composition is a crucial strategy that bacteria use to counteract environmental stress [26]. Under acetic acid stress, AAB alter their fatty acid profiles by increasing the proportion of unsaturated fatty acids (UFAs), the chain length of fatty acids (FAs), and levels of lysophospholipids (LPLs) and cyclopropane fatty acids (CFAs). These modifications decrease membrane fluidity and strengthen membrane integrity, forming a more stable barrier that protects the cell from the harmful effects of acetic acid accumulation [27–29]. Additionally, AAB adjust the head group composition of membrane phospholipids by increasing phosphatidylcholine (PC) and phosphatidylglycerol (PG) content, while reducing phosphatidylethanolamine (PE). This adjustment increases membrane hydrophilicity, limiting passive transport of lipophilic molecules like acetic acid and thereby preventing their entry into the cell [27, 30–32].

Acetic acid transport systems

Acid-tolerant AAB exhibit significantly lower intracellular acetic acid concentrations compared to non-tolerant strains. In addition to the biofilm and cell membrane limiting acetic acid entry, acetic acid efflux systems in the cell membrane function to further reduce intracellular acetic acid concentration. In AAB, two primary mechanisms for acetic acid efflux have been identified: a transporter (PMF-efflux pump) dependent on proton motive force, and an ATP-binding cassette transporter, known as AatA [33].

When proton uncoupling agents and cyanide, which inhibit respiration, are added to the culture medium, acetic acid accumulates within AAB cells and cannot be expelled. This acetic acid efflux system, termed the PMF-efflux pump, functions via a proton motive force-driven mechanism and operates independently of ATP [25].

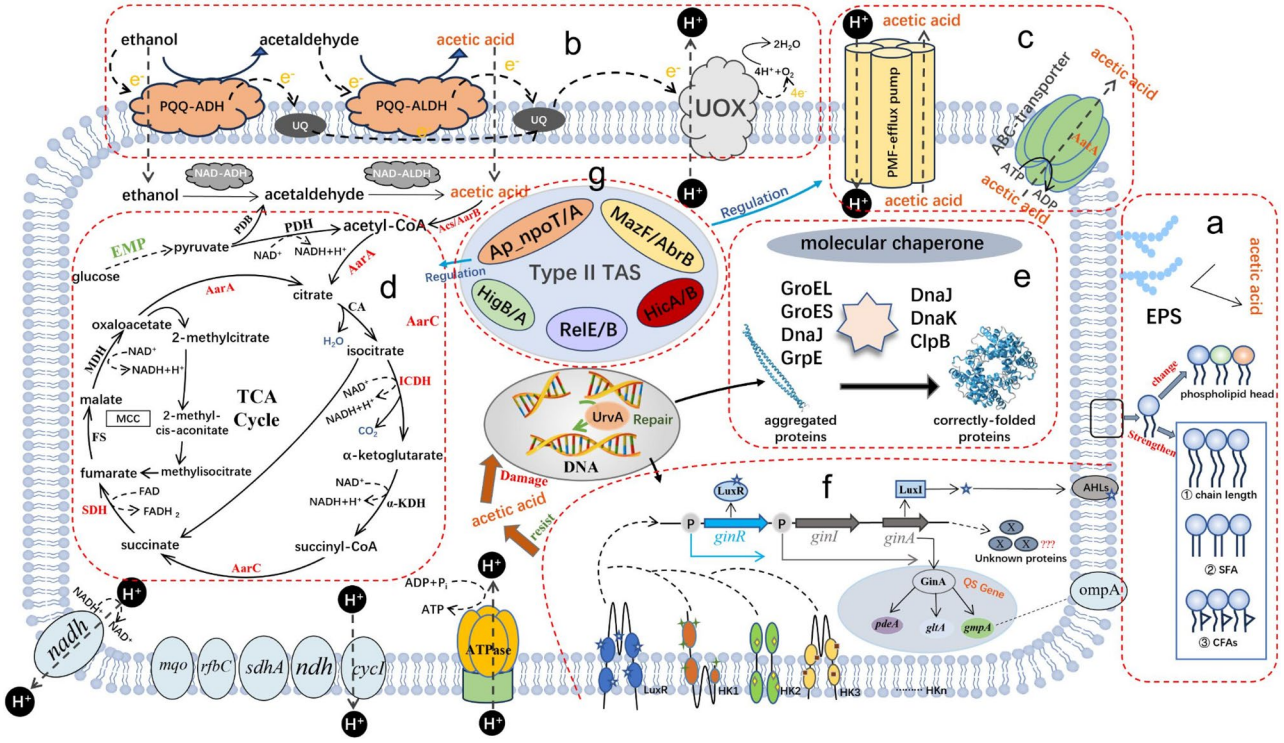

Fig. 2 Overview of acetic acid tolerance mechanisms of AAB. (**a**) Alteration of capsule and cell membrane components prevents the influx of acetic acid. (**b**) Enhancement of membrane-bound enzyme activity, particularly PQQ-ADH. (**c**) Acceleration of acetic acid efflux efficiency by PMF- efflux pump and ABC-transporter. (**d**) Enhancement of acetic acid peroxidation in the cytoplasm. (**e**) Upregulation of stress response molecular chaperones. (**f**) Activation of the quorum sensing system. (**g**) Reliance on type II toxin-antitoxin systems

AAB converts ethanol to acetic acid via ADH and ALDH, generating electrons in the process. Ubiquinone oxidase (UOX) uses these electrons to reduce intracellular oxygen to water. Simultaneously, a proton gradient is established across the cell membrane, enabling the expulsion of acetic acid to mitigate its cytotoxic effects.

The ATP-binding cassette transporter, commonly known as the ABC transporter, is ubiquitously present in both prokaryotic and eukaryotic cell membranes. Its primary function is to use energy from ATP hydrolysis to transport substrates across the cell membrane against their concentration gradients. These substrates include sugars, metal ions, and amino acids [34]. The ABC transporter also plays a critical role in facilitating acetic acid efflux from the cell. Nakano S et al. [35] identified several membrane proteins responsive to acetic acid. Among these, AatA, a protein in the ABC transporter superfamily, was most significantly induced by acetic acid. Mutations in the gene encoding AatA led to a significant reduction in acetic acid resistance. Introducing a plasmid containing the *aatA* gene into the AatA mutant restored acetic acid resistance, highlighting the crucial role of AatA in acetic acid tolerance. This specific ABC transporter is present in several *Acetobacter* spp. and *Gluconacetobacter* spp [35, 36]. In addition, comparative genomics revealed that the acetic acid-resistant *Komagataeibacter* spp. contains more genes encoding ABC transporter proteins compared to the acid-nontolerant AAB [37], which indicated that the number of ABC transporters is positively correlated with the acid-resistant properties of AAB. Research on acetic acid membrane transport proteins in AAB remains challenging. Although two-dimensional gel electrophoresis is effective in proteomics, the lipophilic nature of membrane proteins limits its application for membrane analysis [38]. As a result, there are few reports on acetic acid efflux proteins in AAB membranes. Advances in biotechnology, including ultra-high-speed centrifugation [39], cryo-electron microscopy [40], and mass spectrometry [41], are useful to enrich the understanding of the acetic acid exocytosis system in AAB.

Membrane-bound pyrroloquinoline quinone dependent ethanol dehydrogenase (PQQ-ADH)

Membrane-bound ADH is a key enzyme in the oxidative fermentation of acetic acid and plays a crucial role in enhancing the acid resistance of AAB. PQQ, an essential coenzyme predominantly found in Gram-negative bacteria, enhances bacterial tolerance to harsh environments, including strong acids and high temperatures. In AAB, PQQ-ADH and PQQ-ALDH are membrane-bound enzymes responsible for catalyzing the oxidation

of alcohol to aldehyde and the subsequent conversion of aldehyde to acetic acid, respectively. The synergistic activity of these enzymes enables AAB to produce significant amounts of acetic acid extracellularly [42]. Moreover, PQQ-ADH has been identified in some non-fermentative Gram-negative bacteria, such as *Frateuria aurantia* [43].

The activity and stability of PQQ-ADH are crucial for acid tolerance in AAB. In comparison to *A. pasteurianus* KKP 584, *Komagataeibacter europaeus* V3 exhibited greater resistance to high concentrations of acetic acid, a trait primarily attributed to the enhanced activity and stability of PQQ-ADH in strain V3. Notably, the PQQ-ADH activity in strain V3 is nearly twice that observed in strain KKP 584. Additionally, ADH from strain V3 retains approximately 70% of its initial activity in a 10% acetic acid solution, whereas the ADH activity in strain KKP 584 drastically decreases under the same condition, retaining only 2.3% of its original activity [44]. These results underscore the crucial roles of PQQ-ADH enzyme activity and stability in the acid tolerance of AAB.

Omics technologies offer valuable insights into the complex relationship between the abundance of ADH and acetic acid tolerance. *Komagataeibacter europaeus* is capable of producing acetic acid concentrations as high as 15–20%, significantly exceeding the acid-producing capabilities of *Komagataeibacter oboediens* and *A. pasteurianus* [45]. High-throughput sequencing revealed that the gene copy number encoding PQQ-ADH in *Komagataeibacter europaeus* 5P3 is more than three times that of other strains of *A. pasteurianus*. This high level of PQQ-ADH gene copies is a key factor enabling strain 5P3 to accumulate high concentrations of acetic acid [46]. Overexpression of PQQ-ADH enhanced the ethanol oxidative pathway, thereby improving acid production and acid tolerance in AAB [47]. When the ADH gene is lacking in AAB, its acid tolerance is also lost [48]. Therefore, increasing the PQQ-ADH gene copy number, either through artificial selection of AAB with enhanced PQQ-ADH activity or via genetic engineering techniques, is an effective strategy to enhance the acid tolerance of AAB, thereby improving the efficiency and yield of acetic acid.

Acetic acid peroxidation

Acetic acid fermentation and peroxidation represent two distinct metabolic phases in AAB [49]. The fermentation phase occurs at the cell membrane and predominantly takes place during the logarithmic growth phase. Conversely, acetic acid peroxidation occurs in the cytoplasm once the substrate is exhausted. During this phase, acetyl-CoA synthase (Acs) catalyzes the conversion of acetic acid into acetyl-CoA. Acetyl-CoA is then directed into the tricarboxylic acid cycle (TCA), promoting rapid acetic acid oxidation [50]. Finally, AAB completely oxidizes intracellular acetic acid to carbon dioxide and water, generating ATP for secondary growth and enhancing the strain's growth activity [20, 51]. Moreover, this peroxidation process reduces the cytotoxic impact of acetic acid on the strain's intracellular components and enhances its tolerance to acetic acid.

Using a plasmid-based genomic library screening technique, Fukaya et al. [52] identified a crucial gene cluster—*aarA*, *aarB*, and *aarC*—that enhances acetic acid tolerance in *Acetobacter aceti*. The *aarA* gene encodes citrate synthase, a key enzyme in the TCA cycle, while *aarB* encodes acetate kinase (AckA), which converts intracellular acetic acid into acetyl-CoA for entry into the TCA cycle. Additionally, *aarC* encodes succinyl-CoA-acetyl-CoA transferase, an enzyme that substitutes for succinyl-CoA synthetase in the TCA cycle. This enzyme catalyzes the conversion of succinyl-CoA and some intracellular acetic acid into succinate and acetyl-CoA. Both products then re-enter the TCA cycle [36, 53–55]. The enhanced TCA cycle accelerates acetic acid peroxidation, maintaining its intracellular concentration at a low level.

There are also other enzymes in the TCA cycle that contribute to the acid resistance of AAB. Under high acidity conditions, the expression levels of several enzymes, including phosphate acetyltransferase, isocitrate dehydrogenase, and succinate dehydrogenase, were significantly increased in *A. pasteurianus* Ab3 and LMG 1262^{T}. Overexpression of these enzymes improved the bacteria's resistance to high concentrations of acetic acid [56, 57]. Nakano et al. [58] utilized a multicopy plasmid to enhance the expression of the cis-aconitase (AcnB) gene in *Acetobacter aceti*. Strains with enhanced AcnB expression exhibited significant improvements in acid tolerance and acetic acid production. Although acetic acid peroxidation is a process of acetic acid degradation and consumption by AAB, it does not favor acetic acid accumulation. However, intracellular peroxidation reduces acetic acid levels, preventing significant cellular damage. This reduction stimulates cellular activity, ultimately enhancing extracellular acetic acid production.

Stress response molecular chaperones

Molecular chaperones are proteins that aid in the proper folding and assembly of other proteins, ensuring correct structure without integrating into the final product. Additionally, these chaperones prevent the aggregation of highly denatured proteins and assist in their degradation [59]. The molecular chaperones identified to date are predominantly stressed proteins, with heat shock proteins (HSPs) being a notable example. HSPs play a crucial role in enhancing acetic acid tolerance in AAB [60, 61]. In *A. pasteurianus* NBRC 3283, GroES and GroEL contribute not only to acetic acid fermentation

and tolerance but also to the strain's response to various adverse environmental conditions, including ethanol and high temperature stresses [48, 62]. The expression of the *groEL* and *groES* genes was significantly up-regulated in *A. pasteurianus* NBRC 3283 when treated by ethanol and acetic acid. Overexpression of these genes enhanced the resistance of strains to adverse conditions including acetic acid, ethanol, and high temperatures [48]. GroEL and GroES also play critical roles in acetic acid fermentation and tolerance in both *A. pasteurianus* LMG 1262 and *A. aceti* DSMZ 2002 [51]. The *dnaK*, *dnaJ*, *grpE* and *clpB* genes are present in the genome of *A. pasteurianus* NBRC 3283, and the first three genes are present in tandem, mainly acting as auxiliary molecular chaperones. During acetic acid fermentation, the expression of *dnaK-dnaJ-grpE* and *clpB* genes were significantly upregulated [51, 63, 64], enhancing the strains' activity under acid stress.

In AAB, the nucleotide excision repair protein UvrA, which is induced by acetic acid, is responsible for repairing DNA damage resulting from acetic acid exposure [65]. Overexpression of the UvrA protein in *A. pasteurianus* AC2005 significantly enhanced acetic acid tolerance. Under 6% acetic acid for 20 and 40 min, the survival rates of the UvrA overexpressing strain were 2% and 0.12%, respectively, compared to 1.5% and 0.06% in the control strain [66]. Additionally, UvrA protein in *Lactobacillus helveticus* CNBL1156 and *Streptococcus mutans* has also been identified, and its role in maintaining genomic DNA integrity and enhancing acid tolerance has been confirmed by analyzing the changes in UvrA protein activity under acidic conditions [67, 68].

Quorum sensing (QS)

QS is a collective behavior in microorganisms that involves the autonomous production and release of specific signal molecules in response to changes in the external environment, and the perception of these molecules' concentration variations for intercellular communication, thereby regulating microbial growth and metabolism [69–71]. This QS phenomenon occurs only when bacterial populations reach a certain density and plays a crucial role in controlling microbial interactions, biofilm formation, synthesis of virulence factors, and stress responses [72, 73]. The primary QS signal molecules identified to date include N-acyl-homoserine lactones (AHLs), 4-hydroxy-2-alkylquinolines (HAQ), autoinducer-2 (AI-2), diketopiperazines (DKPs), and diffusible signal factors (DSFs). QS systems are commonly found in opportunistic pathogens such as *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Streptococcus* mutans, *Helicobacter pylori*, and *Staphylococcus aureus* [70, 74–77]. Research on QS in industrial microorganisms has primarily focused on lactic acid bacteria [76, 78, 79]. Iida et al. [80] first demonstrated the existence of a QS system in AAB (*Komagataeibacter intermedius*), where *ginI* and *ginR* are two key regulatory genes. The *ginI* gene encodes a signal molecule synthase, while the *ginR* gene encodes a signal molecule receptor protein. AHLs can bind to the GinR protein to form a complex that activates transcription of the *ginR* gene, thereby regulating the transcription of target genes [80]. The GinI/GinR quorum-sensing system suppresses acetic acid fermentation through the activation of GinA. GinA is an 89-amino-acid protein that can induce the expression of the *gltA*, *pdeA*, and *gmpA* genes [81]. Disruption of these genes leads to higher rates and increased final yields of acetic acid production. GmpA is located in the outer membrane, and its knockout facilitates the entry of the substrate ethanol into the periplasm, where ADH and ALDH convert ethanol, leading to enhanced production of acetic acid [81, 82]. QS signaling molecules and associated regulatory genes were also detected in various AAB, including *Gluconacetobacter diazotrophicus* and *Komagataeibacter xylinus* [83–85]. Although the QS systems and their downstream regulatory genes have been confirmed to be associated with acid production in AAB, the regulatory mechanisms, such as how GinA regulates the expression of *gmpA*, require further research to elucidate [86].

Type II toxin-antitoxin systems (TAS)

TAS plays a pivotal role in microbial responses to environmental stresses. This system usually consists of a pair of genes encoding a stable toxin and an unstable antitoxin [87]. Toxins can modulate the stress response by cleaving sequence-specific RNAs or inhibiting protein synthesis, while antitoxins neutralize toxicity through direct interaction with the corresponding toxins [88]. Eight distinct types of TAS (types I to VIII) have been identified in bacteria [89]. Bioinformatics analysis suggested that the genome of AAB predominantly contains type II TAS, which include the gene pairs Ap_npoT/Ap_npoA, HicA/HicB, HigB/HigA, VapC/VapB, MazF/AbrB, HEPN/MNT, RelE/RelB, VapC/Phd, ParE/ParD, RelE/Xre, and Fic/Phd [89]. The first three TAS pairs have been experimentally identified in *A. pasteurianus* Ab3 based on genomic structure and activity assays. Heterologous expression of these gene pairs in *E. coli* significantly enhanced bacterial resistance to acetic acid stress [90], indicating that TAS is effective in improving acetic acid tolerance.

Further studies by Xia et al. [91] revealed that *hicAB* positively modulates *A. pasteurianus* Ab3's resistance to acetic acid stress, maintenance of acetic acid production, and the formation of persistent cells. Under 5% acetic acid stress, knockout of *hicAB* was found to significantly impair *A. pasteurianus* Ab3 viability. In contrast, when *hicAB* was restored or overexpressed, the survival

ratio of the cells under acetic acid stress was significantly improved, indicating that *hicAB* plays a crucial role in *A. pasteurianus* Ab3's resistance to acetic acid stress. Furthermore, the deletion of *hicAB* significantly reduced cell persistence during high-acid vinegar fermentation. The formation of acid-tolerant persister cells is an important way by which the strain resists high acetic acid stress. Consequently, the reduction in cell persistence leads to decreased strain activity, ultimately resulting in lower acetic acid production [89, 91].

Type II TAS also function as regulatory factors involved in the complex mechanisms of AAR [89]. Under acid stress, the expression of genes encoding type II TAS in AAB is significantly altered. For instance, in *A. pasteurianus* Ab3, the expression levels of the antitoxin proteins AbrB and RelB were significantly upregulated at higher acetic acid concentrations (93 g/L) compared to lower concentrations (36 g/L) [92]. TAS also contribute to the adaptation of AAB to acidic environments by modulating intracellular metabolic pathways. Deletion of the *hicA/hicB* significantly reduced the mRNA levels of genes involved in energy metabolism, including those associated with acetic acid overoxidation, 2-methylcitrate cycle (MCC), and oxidative phosphorylation [91]. Additionally, *hicA/hicB* regulated the expression of genes related to transport systems, such as ABC and MFS transporters [91], which are essential for nutrient uptake and the elimination of toxic substances in bacteria [93]. In summary, type II TAS provide valuable insights into the molecular mechanisms underlying acetic acid resistance in AAB.

Mechanisms of ethanol tolerance

Ethanol serves as a substrate for acetic acid fermentation, providing essential feedstock for the growth and metabolism of AAB. However, when ethanol concentration exceeds 4%, it inhibits the growth of AAB, thereby affecting the production of acetic acid during fermentation [17]. Therefore, AAB capable of tolerating high ethanol concentrations are more suitable for vinegar production. Understanding the ethanol tolerance mechanisms in AAB is crucial for the targeted development of strains that can withstand high ethanol concentrations [94]. Currently, research on microbial ethanol tolerance mechanisms has primarily focused on yeast, but the ethanol tolerance mechanisms of AAB are equally significant. According to existing studies, the ethanol tolerance mechanisms in AAB primarily involve in cell membrane structure, ADH and ALDH enzyme activity, and intracellular metabolites (Fig. 3).

Cell membrane fatty acid composition

Alterations in the fatty acid composition of the cell membrane are a key cellular response to environmental stress, influencing membrane permeability and fluidity [26]. The ratio of saturated fatty acids (SFAs) to unsaturated fatty acids (UFAs) indirectly influences membrane fluidity.

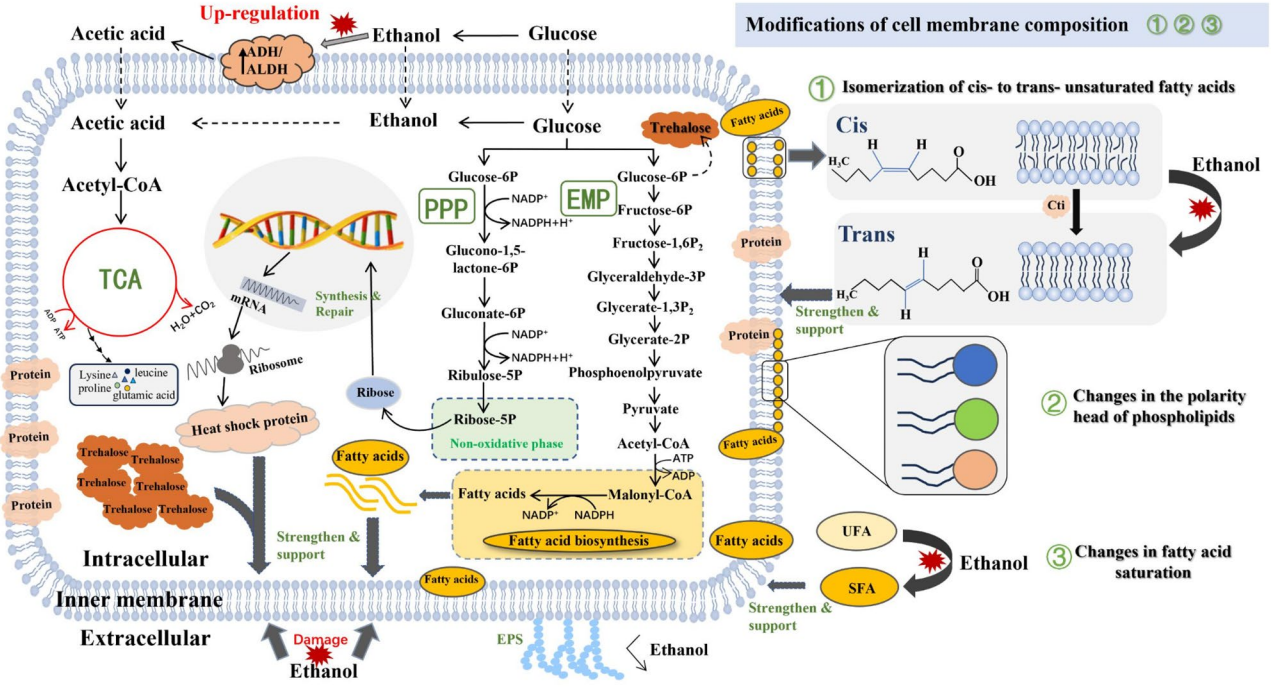

Fig. 3 Overview of ethanol tolerance mechanisms of AAB. AAB mitigate ethanol stress through multiple mechanisms, including structural adjustments of cell membranes, enhanced enzymatic activity for ethanol metabolism, accumulation of protective intracellular metabolites (such as amino acids and trehalose), and regulation of vital metabolic pathways

Short-chain alcohols, such as ethanol, can integrate into the outer phospholipid bilayer of the cell membrane and penetrate the hydrophobic core. This process inhibits the synthesis of SFAs and increases UFAs content, thereby enhancing membrane fluidity [95]. To counteract the membrane fluidity caused by organic solvents, microorganisms gradually increase the proportion of SFAs, thereby reducing the damage inflicted by organic solvents on the cell membrane [96, 97]. Furthermore, alterations in the saturation of membrane fatty acids are energy-intensive, requiring de novo synthesis of fatty acids [98].

The effect of changes in SFAs and UFAs on microbial ethanol tolerance is complex. Under 11% ethanol stress, the SFAs content in ethanol-tolerant *A. pasteurianus* T3-06 initially decreased and then increased, whereas the UFAs content showed the opposite trend. After 72 h of incubation, the SFA content reached 57.29%, while the UFA content reached 42.04%. The cell membrane fluidity of strain T3-06 correlated with the fatty acid composition analysis, showing an initial increase followed by a decrease under ethanol stress [99]. Subsequently, as the strain upregulated SFAs synthesis, membrane fluidity decreased. Reduced cell membrane fluidity helps the strain resist ethanol-induced damage, playing a crucial role in maintaining intracellular stability [99].

Cell membrane phospholipid head

Cell membranes play an important role in maintaining intracellular homeostasis [100]. In response to stress induced by organic solvents like ethanol, microorganisms adjust the fatty acid composition of their cell membranes to maintain stability and fluidity. Additionally, they mitigate solvent-induced damage by modifying the phospholipid heads of the cell membranes [101, 102]. *Pseudomonas putida* S12 exhibited robust survival in supersaturated toluene solutions. The key mechanism underlying this tolerance involves a reduction in phosphatidylethanolamine (PE) and an increase in phosphatidylglycerol (PG) and cardiolipin (CL) under toluene stress. This adjustment helps the strain counteract the sudden increase in the acyl chain length of the bilayer, thereby maintaining cell membrane stability [103]. Trček et al. [30] demonstrated that a decrease in PE content and an increase in PG content enhanced the tolerance of AAB to ethanol and acetic acid. Despite differences in genus classification, AAB and *Pseudomonas putida* exhibit similar stress responses to ethanol and other organic solvents. This indicates that changes in cell membrane phospholipids head are a universal mechanism by which bacteria defend against the toxicity of organic solvents.

Cis-trans isomerization of unsaturated fatty acids

Some bacteria have evolved a mechanism to convert cis-unsaturated fatty acids (cis-UFAs) into trans-unsaturated fatty acids (trans-UFAs) as a response to environmental challenges [104]. For cell membrane fatty acids, trans-UFA structures are more stable than cis-UFA structures, increasing membrane density, resulting in a more tightly ordered membrane with reduced fluidity [105, 106]. Generally, the cis conformation accounts for the majority of UFA configurations. When bacteria are exposed to organic solvents, membrane fluidity increases, exposing the cis-UFA double bonds. The enzyme cis-trans isomerase (Cti) rapidly catalyzes the conversion of cis-UFAs to trans-UFAs upon encountering the double bonds. This rapid reaction results in a tightly packed arrangement of fatty acids along the carbon chains, thereby enhancing the stability of the cell membrane [107].

At the beginning of ethanol stress, the cell membrane of *Pseudomonas putida* S12 exhibited a significant increase in trans-UFAs and a decrease in cis-UFAs, which enhanced the robustness of cell membrane [108]. This process serves as a short-term adaptive response, providing additional time for two long-term mechanisms: changes in the fatty acid saturation of the cell membrane and alterations in the phospholipid head groups. The cis-trans isomerization of unsaturated fatty acids is not unique to the genus *Pseudomonas*, but is also observed in other Gram-negative bacteria, such as *Alcanivorax borkumensis* SK2 and *Methylococcus capsulatus* [109, 110]. A similar mechanism may exist in AAB, playing a critical role when these bacteria are exposed to organic solvents, such as ethanol and acetic acid.

Amino acid-related metabolites

Ethanol causes oxidative damage to proteins and DNA upon entering the cell [111]. In response to ethanol stress, AAB produces protective substances through a series of intracellular metabolic processes to sustain normal cell growth. Amino acids constitute a crucial class of cytoprotective substances [99]. Notably, glutamate and proline are the most common amino acid-derived protective metabolites. Both share the precursor α-ketoglutarate; glutamate can either synthesize proline or generate proline through its own degradation. These amino acids are involved in various cellular metabolic responses under stress conditions, thus maintaining cellular stability [112, 113].

The proline content in ethanol-resistant *A. pasteurianus* T3-06 increased significantly under 11% ethanol stress, reaching levels 8.56 times higher than those in the control strain AP1.01 after 72 h. Additionally, the glutamate content in strain T3-06 was initially much higher than in the control strain AP1.01 at the beginning of ethanol stress. However, as the ethanol stress persisted, glutamate levels in strain T3-06 decreased significantly. This decline may be due to the utilization of synthesized glutamate for the production of other cellular components

and energy metabolites. Under ethanol stress, the tolerant strain T3-06 exhibited upregulation not only of glutamate and proline but also of other amino acids, such as leucine, isoleucine, and lysine [99]. Microorganisms modify amino acid profiles in response to environmental stresses, such as heat, ethanol, and freezing [114, 115]. In *S. cerevisiae*, increased levels of proline, threonine, glutamate, leucine, and histidine enhance resistance to ethanol damage [116]. Similarly, AAB increase amino acid levels as a protective mechanism against ethanol stress.

Carbohydrate-related metabolites

Trehalose is necessary for maintaining cell membrane stability, reducing oxidative damage, and ensuring proper protein folding [117]. Bacteria and yeast protect themselves from osmotic pressure, high temperatures, and organic solvents by accumulating trehalose [118, 119]. After 24 h of exposure to ethanol stress, the trehalose content in the ethanol-tolerant *A. pasteurianus* T3-06 was approximately ten times higher than that in the control strain AP1.01. The higher trehalose content provided better protection for the strain [99]. Some sugar alcohols, such as mannitol, exhibited changes in content in strain T3-06 similar to those of glutamate. These changes involved an initial increase upon ethanol addition, followed by a decrease as mannitol was consumed for synthesizing other protective substances [99]. Under ethanol stress conditions, the expression of genes encoding trehalose in *S. cerevisiae* is upregulated, resulting in the accumulation of trehalose [120]. The higher intracellular trehalose levels enhance ethanol tolerance in yeast. The ethanol stress response of trehalose in yeast is similar to that in *A. pasteurianus* T3-06, suggesting that trehalose-mediated protection may represent a common mechanism among microorganisms in response to ethanol stress.

Enzymatic activities of ADH and ALDH

ADH and ALDH are two important key enzymes in the oxidation of ethanol, with the former converting ethanol to acetaldehyde and the latter converting acetaldehyde to acetic acid [121]. To investigate the relationship between enzyme activities and ethanol tolerance in AAB, the activities of ADH and ALDH were tested in the ethanol-tolerant *A. pasteurianus* FY-24 and DY-5, as well as the control strain AS1.41, under different ethanol concentrations. At lower ethanol concentrations (4-6%), ethanol's inhibitory effect on growth of the three strains was not significant, and the activities of ADH and ALDH remained high. With increasing ethanol concentrations (8-11%), the growth of the control strain AS1.41 was significantly inhibited, and the activities of ADH and ALDH decreased. In contrast, the *A. pasteurianus* FY-24 and DY-5 continued to grow well, with ADH and ALDH activities remaining high, efficiently converting ethanol to acetic acid and reducing ethanol toxicity to the strains [122]. Similarly, ethanol-resistant *A. pasteurianus* JZ1601 had high ADH activity at high ethanol concentrations to reduce ethanol damage to cells compared to the control strain AS1.41 [94]. These studies showed that ethanol tolerance in AAB is positively correlated with the enzyme activities of ADH and ALDH.

Central metabolic pathway

The activities of isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH) and isocitrate lyase (ICL) were enhanced in ethanol-tolerant *A. pasteurianus* JZ1601 in response to ethanol stress [94, 123]. The increased activity of the TCA cycle generates more ATP and metabolic intermediates essential for synthesizing molecules involved in cellular repair and detoxification to counter ethanol stress. The pentose phosphate pathway (PPP) primarily provides NADPH for reductive biosynthesis reactions within cells. However, the enhanced PPP may consume energy through gluconeogenesis [121]. Under ethanol stress, the ethanol-tolerant *A. pasteurianus* JZ1601 downregulated G6PDH activity to conserve energy for cell growth [94]. The glycolytic pathway also plays a crucial role in ethanol resistance in yeast, and its involvement in ethanol tolerance in AAB requires further investigation [124].

Mechanisms of thermotolerance

During the fermentation process, heat production such as bacterial metabolism often causes high temperature stress. In China, vinegar is produced using the traditional solid-state fermentation method, which has poor heat dissipation. This results in high-temperature conditions, often raising fermentation temperatures above 45 °C [125]. However, the optimal growth temperature for most AAB is below 34 °C. High temperatures can significantly reduce the activity of AAB, thus reducing the efficiency of acetic acid synthesis [126, 127]. Therefore, understanding the regulatory mechanisms of heat tolerance in thermotolerant AAB is critical for improving product synthesis under high-temperature conditions. Current research on the heat resistance mechanisms of AAB primarily focuses on the following aspects: extracellular membrane system, genetic variation and molecular chaperone (Fig. 4).

Extracellular membrane system

EPS also play a pivotal role in the microorganisms to resist high temperatures. For instance, the thermotolerant *A. pasteurianus* SKU1100 produces PPS. These polysaccharides enable the strain to float on the surface of the growth medium under high-temperature conditions, thereby minimizing its exposure to the heat source and reducing thermal damage to the strain [23, 24]. The

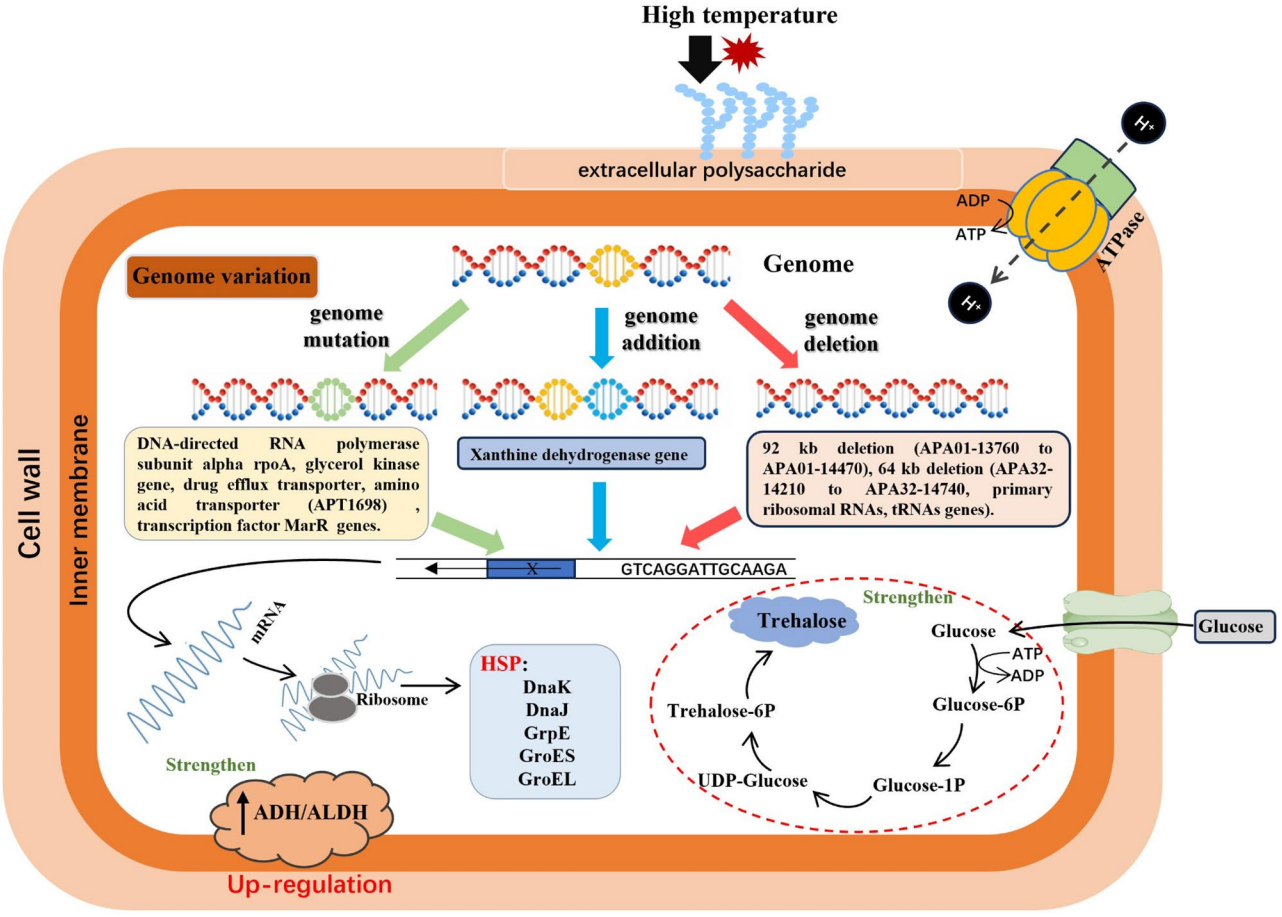

Fig. 4 Overview of thermotolerant mechanisms of AAB. The thermotolerance mechanism of AAB is related to heat shock proteins, trehalose and polysaccharide layer on the cell surface. Additionally, during a prolonged evolutionary process, the genome of AAB has undergone numerous changes to withstand high-temperature stress, including gene mutations (such as MarR, APT1698, etc.), gene insertions (such as *xdh*A), and large-scale deletions of genes (such as tRNA genes)

composition of PPS varies among different AAB. The PPS of the heat-resistant *A. pasteurianus* SL13E-3 and SL13E-4 contains higher amounts of xylose and galactose compared to the mesophilic strain SL13E-2, which enhances the stability of PPS and improves heat resistance [128].

Membrane proteins of thermotolerant strains showed enhanced enzymatic stability at high temperatures, thereby enhancing acetic acid production. A comparative analysis of ADH thermal stability in thermotolerant *A. pasteurianus* MSU10, SKU1108, and the mesophilic strain IFO3191 demonstrated that the ADH of thermotolerant strains had higher optimal temperatures and superior thermal stability compared to that of the mesophilic strain IFO3191. Additionally, under high-temperature conditions, the ADH of thermotolerant strains showed greater resistance to ethanol and acetic acid compared to mesophilic strains [129]. Similarly, Perumpuli et al. [130] reported that the activities of ADH and ALDH in the thermotolerant strains SL13E-2 and SL13E-4 were more stable than those in the mesophilic strain. Therefore, the activity and stability of membrane proteins contributes significantly to the thermotolerance of AAB.

Genome variation

The deletion or mutation of specific genes is strongly correlated with heat tolerance in AAB. Adaptive laboratory evolution (ALE) experiments involve prolonged culturing of cells in stressful conditions to naturally select cells that acquire beneficial mutations [131]. ALE not only facilitates the generation of tolerant strains but also elucidates the genetic mechanisms underlying tolerance [132]. After a gradual domestication process at temperatures of 40 to 42 °C for 72 days, the heat-tolerant *A. pasteurianus* IFO 3283-01-42 C was developed. When compared with the original strain's genome, this heat-tolerant strain exhibited a deletion of approximately 92 kb of DNA, alongside three single-nucleotide mutations located in the *rpoA* gene, the glycolytic genes, and a putative gene [133]. Similarly, in the heat-tolerant strain NM-6 derived from

the domestication of *A. pasteurianus* IFO 3283-32, 11 mutations, a single large 64-kb deletion and a single plasmid loss were observed. Comparative phenotypic analysis showed that deletion of ribosomal RNA and tRNA genes, along with a mutation in DNA polymerase, critically contributed to thermotolerance. The loss or mutation of these genes resulted in reduced DNA replication and protein translation capabilities, but it also resulted in energy saving, potentially enhancing survival under high-temperature conditions [134]. In the heat-tolerant *Gluconobacter frateurii*, a frameshift mutation caused by the insertion of a G base in the sequence coding for a drug efflux transporter was identified. Further domestication and UV induction of the second-generation heat-tolerant strains displayed the same genetic mutation. Introducing the mutated base into the drug efflux transporter genes of the original strain improved its heat tolerance, indicating that mutation in this transporter gene plays a crucial role in enhancing heat tolerance [135]. Genome sequencing of heat-tolerant strains TI and TH3 derived from *A. pasteurianus* SKU 1108 revealed mutations in the genes encoding the amino acid transporter (APT1698) and the transcription factor MarR, including single nucleotide mutations or deletions, frameshifts, and transposon insertions. Deletion and mutation introduction experiments in the original strain SKU 1108 demonstrated that mutations in the *marR* and *APT1698* genes could enhance the original strain's heat tolerance and acetic acid production at 40 °C [136].

These research findings suggest that drug efflux transporters, ribosomal RNAs, tRNAs, DNA polymerase, amino acid transporter and the transcription factor MarR may be associated with the thermotolerance of AAB. The partial gene deletions or mutations in the heat-tolerant AAB, resulting from domestication, play a crucial role in enhancing the high-temperature resistance. This has significant implications for elucidating the mechanisms of thermotolerance in AAB.

Molecular chaperone

The molecular chaperones identified to date that are associated with the thermotolerance of AAB predominantly belong to the heat shock protein family. These include DnaK, DnaJ, GrpE, GroES, and GroEL, which not only enhance the strain's heat tolerance but also improve its resistance to acetic acid [137]. In *A. pasteurianus* NBRC 3283, overexpression of *groES/L* and *grpE-dnaK-dnaJ* genes exhibited significantly enhanced growth activity at 42 °C. Conversely, *clpB* knockout strains lost the ability to grow at high temperature. The expression of *groEL*, *dnaKJ*, *grpE*, and *clpB* were regulated by the sigma factor for RNA polymerase RpoH, whose deletion led to heat sensitivity [138]. These findings indicated the critical role of molecular chaperones in enhancing the heat resistance of AAB.

Other factors

Genes related to stress response, cell division, cell wall and membrane biosynthesis, transport systems, and genome stability influence AAB's high temperature tolerance [139–141]. Notably, the heat sensitivity of *A. pasteurianus* IFO 3191 is associated with mutations in genes related to stress response and cell wall and membrane biosynthesis, in contrast to heat-tolerant strains [139]. Furthermore, random insertion of a transposon (Tn10) into DNA of *A. tropicalis* SKU 1100 identified 24 genes that not only confer heat tolerance but also enhance acetic acid resistance [18]. Overexpression of the gene encoding acyl-CoA dehydrogenase (ACDH) in the *Komagataeibacter medellinensis* NBRC 3288 not only improved heat tolerance but also increased acid production, suggesting a potential role for ACDH in the thermal resistance of AAB [142]. Additionally, *A. pasteurianus* SKU 1108, which exhibits superior heat resistance, possesses an extra gene encoding xanthine dehydrogenase (*xdhA*) and three specific genomic regions, suggesting a potential link between *xdhA* and these regions to heat resistance [141]. In our study, the thermotolerant *A. pasteurianus* TCBRC 103 was isolated from solid-state fermentation substrates (vinegar *Pei*), exhibiting robust growth at 42 °C. In contrast, *A. pasteurianus* Huniang 1.01, a strain commonly used in industrial vinegar fermentation in China, was unable to grow at this temperature. Under heat stress, *A. pasteurianus* TCBRC 103 showed lower levels of intracellular reactive oxygen species (ROS) compared to *A. pasteurianus* Huniang 1.01 (unpublished results). ROS can damage various cellular components, leading to lipid peroxidation, protein oxidation, and genetic damage through DNA modification, ultimately resulting in cell damage or death [143]. Genetic engineering of antioxidant defense system-related genes has been shown to significantly enhance strain resistance to environmental stresses [144, 145]. Transcriptomic analysis revealed that under heat stress, genes associated with antioxidant defense, such as superoxide dismutases, glutathione peroxidases, and thioredoxin reductases, were significantly upregulated in *A. pasteurianus* TCBRC 103, suggesting that the strain can mitigate heat-induced oxidative damage by enhancing its antioxidant system (unpublished results). The application of genetic engineering techniques to elevate the expression of antioxidant genes offers potential for improving the heat tolerance of AAB.

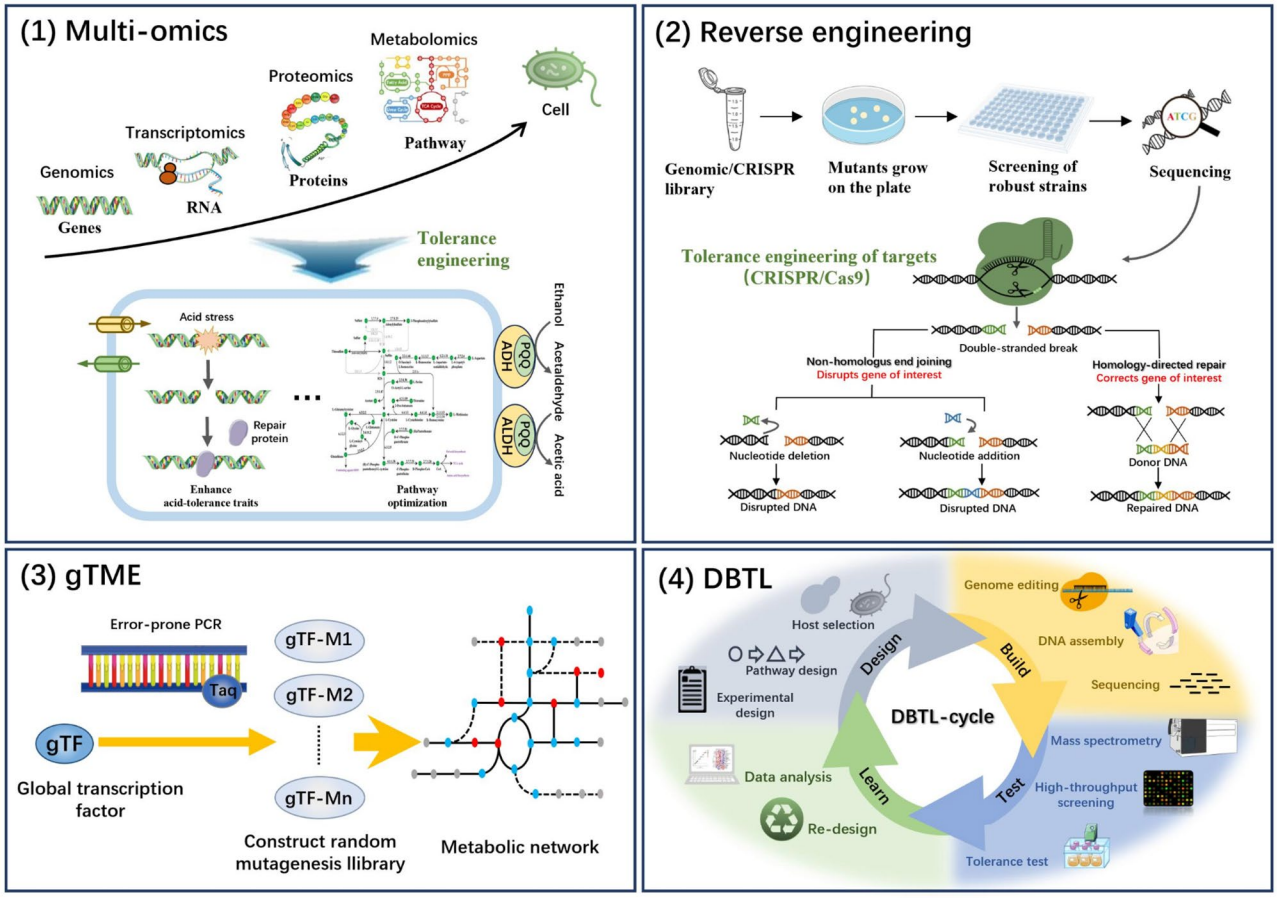

Fig. 5 Strategies for increasing stress tolerance in AAB. (**1**) Multi-omics technology combined with genetic engineering provides more opportunities to rationally manipulate microorganism with desired properties. (**2**) Reverse engineering is a powerful tool for identifying genes and genetic pathways involved in stress tolerance. By screening a genomic/CRISPR library for stress tolerance traits, researchers can discover new genes that confer resistance to various stressors. These genes can then be further studied and potentially used to enhance the stress tolerance of strains through genetic engineering. (**3**) gTME involves modifying the components of the global transcription factors to globally alter gene expression patterns, leading to more extensive phenotypic changes. (**4**) DBTL cycle integrates engineering principles with biological research to accelerate the development of genetically engineered organisms with desired traits

Conclusions and future prospects

AAB have evolved protective mechanisms to withstand various stress conditions during vinegar fermentation progresses. In the early stage of acetic acid fermentation, AAB first encounter the fermentation substrate ethanol. Some AAB with stronger ethanol tolerance increase the activity of membrane-bound and intracellular enzymes to resist ethanol stress. They adjust their cell membrane structure through changes in fatty acid saturation, and modifications in the heads of phospholipids. Additionally, elevated levels of amino acids and sugar metabolites contribute to the ethanol tolerance of AAB.

As the fermentation progresses, the acetic acid content in the fermentation broth gradually increases, prompting AAB to resist acetic acid stress. Acetic acid first contacts the cell wall, where the lipopolysaccharide component acts as the initial barrier to prevent its entry into the cell. Once it passes through the cell wall, the cell membrane initiates a series of stress responses, including alterations in membrane components, enhancement of PQQ-ADH/ALDH enzyme activity, and activation of acetic acid transport systems (PMF-efflux pump and ATP-binding cassette transporter). The entry of acetic acid into the cell enhances the expression of acetic acid peroxidation related enzymes (e.g., AarC, CS), leading to the rapid consumption and decomposition of intracellular acetic acid. Additionally, molecular chaperone-mediated stress mechanisms, such as GroES and GroEL, enhance the resistance of AAB to the acetic acid stress. Furthermore, quorum sensing systems and toxin-antitoxin system provide new insights into the acid resistance mechanisms of AAB.

The high temperatures during fermentation persist throughout almost the entire fermentation stage. The thermotolerance mechanism of AAB is strongly associated with heat shock proteins and genetic variations in

the strains. Additionally, some strains can be triggered by high temperatures to produce a thin polysaccharide layer composed of galactose and xylose on the cell surface, enhancing resistance to the high temperatures.

The elucidation of tolerance mechanisms in AAB is challenging due to the inherent complexity of the strains and the cross-regulation of multiple genes. ALE and artificial mutagenesis methods, such as ultraviolet (UV), chemical, and atmospheric and room temperature plasma (ARTP) mutagenesis, tend to be time-consuming, result in low mutation rates, and rarely lead to beneficial mutations. With the advancement of systems biology and gene editing technologies, many efficient techniques have emerged to enhance strain tolerance. (1) Multi-omics analyses are widely used to identify gene targets for genetic engineering aimed at improving stress tolerance. (2) Reverse engineering techniques, such as plasmid-based genomic libraries and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) libraries, are employed to screen for robust mutants and identify targets for engineering stress tolerance. (3) Global transcription machinery engineering (gTME) is a directed evolution strategy that identifies phenotypes with enhanced tolerance properties by creating and screening mutant libraries of transcription factors. (4) Design-Build-Test-Learn (DBTL) technology enables precise and efficient enhancement of microbial tolerance to a variety of stress conditions by systematically designing, building, testing, and learning from each cycle (Fig. 5). These advanced approaches provide a more efficient process for developing desired microbial strains [146]. However, gene editing tools are significant lack in AAB, and the highly efficient CRISPR gene editing tools have not yet been successfully applied to AAB. Therefore, the development of CRISPR technology is essential to improve the applicability and performance of AAB.

Overall, numerous factors influence the stress tolerance of AAB. The mechanisms responsible for their resistance to ethanol, acetic acid, and high temperatures are complex and require further exploration. These tolerance mechanisms are vital for the growth and metabolic processes of AAB, playing a key role in their stable production of acetic acid and other valuable products.

Abbreviations

AAB	Acetic acid bacteria
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
PQQ-ADH	Pyrroloquinoline quinone dependent ethanol dehydrogenase
UFAs	Unsaturated fatty acids
SFAs	Saturated fatty acids
FAs	Fatty acids
LPLs	Lys phospholipids
CFAs	Cyclopropane fatty acids
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
CL	Cardiolipin
ICDH	Isocitrate dehydrogenase
ACDH	Acyl-CoA dehydrogenase
MDH	Dehydrogenase
ICL	Isocitrate lyase
PPP	Pentose phosphate pathway
NADPH	Nicotinamide adenine dinucleotide phosphate
AHLs	N-acyl-homoserine lactones
HAQ	4-hydroxy-2-alkylquinolines
AI-2	Autoinducer-2
DKPs	Diketopiperazines
DSFs	Diffusible signal factors
UOX	Ubiquinone oxidase
ABC Transporter	ATP-binding cassette transporter
TCA	Tricarboxylic acid cycle; Acs: acetyl-CoA synthase
AckA	Acetate kinase
QS	Quorum sensing
TAS	Toxin-antitoxin systems
MCC	2-methylcitrate cycle
AAR	Acetic acid resistance
ALE	Adaptive laboratory evolution
UV	Ultraviolet
ARTP	Atmospheric and Room Temperature Plasma
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DBTL	Design-Build-Test-Learn
gTME	Global transcription machinery engineering

Author contributions

SKH wrote the original draft. YQW wrote, reviewed, edited, and supervised the manuscript. LYW and QXZ wrote and prepared all the graphical elements. ZTL, PL and KW conceived and designed the work. YYZ and DH collected the literature. YJY reviewed and supervision the manuscript. All authors reviewed and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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