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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Characterization and comparative analysis of toxin–antitoxin systems in *Acetobacter pasteurianus*

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Abstract

Bacterial toxin–antitoxin (TA) systems play important roles in diverse cellular regulatory processes. Here, we characterize three putative type II TA candidates from *Acetobacter pasteurianus* and investigate the profile of type II TA systems in the genus *Acetobacter*. Based on the gene structure and activity detection, two-pairs loci were identified as the canonical *hicAB* and *higAB* TA systems, respectively, and *DB34_01190–DB34_01195* as a putative new one without a canonical TA architecture. Physiologically, the expression of the three pairs conferred *E. coli* with additional plasmid maintenance and survival when under acetic acid stress. Chromosomal TA systems can be horizontally transferred within an ecological vinegar microbiota by co-option, and there was a tendency for toxin module loss. The antitoxin retention in the genome is suggested to have a broad role in bacterial physiology. Furthermore, *A. pasteurianus* strains, universally domesticated and used for industrial vinegar fermentation, showed a higher number of type II TA loci compared to the host-associated ones. The amount of TA loci per genome showed little positive relationship to insertion sequences, although its prevalence was species-associated, to the extent of even being strain-associated. The TA system is a candidate of studying the resistant mechanistic network, the TAs-dependent translatome affords a real-time profile to explore stress adaptation of *A. pasteurianus*, promoting industrial development.

Keywords *Acetobacter pasteurianus* · AAB · TA systems · Vinegar · Resistance mechanism

Introduction

Acetic acid bacteria (AAB) consist of a group of Gram-negative and rod-shaped bacteria that are widespread in nature. Their remarkable abilities to oxidize ethanol into acetic acid and survive stress have resulted in *Acetobacter* and *Komagataeibacter* strains being widely used in the manufacture of vinegar [4, 53]. In contrast to the use of a pure starter as seed in a submerged fermentation, static surface fermentation and solid-state fermentation without any artificial inoculum are usually relied on for traditional vinegar production such as Meigui (China), Komesu (Japan), and Balsamic vinegar (Europe). The diversity of microbes not only provides specific quality but also increases the challenge for maintaining acetic acid bacteria against ecological biotic or abiotic forces [20, 64, 70]. Comparative genomic analysis has shown that *Acetobacter* and *Komagataeibacter* contain numerous transferable genetic factors (TGFs) [3, 7, 65]. The existence of TGFs inevitably result in genome instabilities and also provides regulatory modules in response to environmental

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stimuli, as is similarly observed in the toxin–antitoxin (TA) systems of *E. coli* [44]. While *A. pasteurianus* seems gifted with a plastic genome with typically more than 290 transposons detected and numerous insertion sequences and phage related genes, which facilitates the acquirement of novel mechanisms involved in ecological adaptation [7, 42]. The currently available data from genomics and proteomics studies have unraveled the adaptive responses in cell structure and metabolism processes for resistance mechanisms in AAB [4, 56, 67]. Nevertheless, the regulatory networks involved require more investigation. Three modulatory circuits, two-component systems (TCS), quorum sensing (QS), and toxin–antitoxin (TA) systems, are implicated in a variety of physiological signaling by bacteria [16, 29, 63]. Currently QS has been confirmed experimentally only in *Gluconacetobacter* and involved in stress survival [23]. Genome in silico analysis by our lab suggested that most of the AAB, including *Acetobacter pasteurianus*, had missed the homologous QS gene cluster (data not shown), which promoted us to shift our attentions more on TAS regulatory networks as the potential acid signaling pathway.

TA systems comprise a diverse range of genetic elements, which can be found both on the chromosomes and plasmids of bacteria and *Archaea*, usually involve a pair of genes encoding a stable toxin and a co-transcribed liable antitoxin [21]. Of the six types of TA systems, type II is the most dominant and well characterized in bacterial genomes and plasmids, generally characterized as a bicistronic operon able to produce a small sized antitoxin and the cognate toxin. Toxin can block protein synthesis by either cleaving a sequence-specific RNA or inactivating the essential factors involved in translation process, thereby affecting cellular metabolism in response to stressful stimuli [25]. Antitoxin directly interacts with the cognate toxin and neutralizes its toxicity. Type II antitoxins usually possess two domains: an N-terminal DNA-binding domain (DBD) and a C-terminal region involved in DNA binding and toxin binding, respectively [15]. The DBD provides the antitoxin with roles not only in the regulation of its own promoter, but promoters containing recognized palindromes of other genes, which drives the antitoxin to be a regulator upon stressful inches [22, 57]. In addition to their specific roles in plasmid maintenance, global metabolic stress management, and maintaining a persistent phenotype, chromosomal TA systems can perform additional roles in stabilizing large genomic fragments, integrative-conjugative elements and adjacent genes [21, 43]. For example, MqsR/MqsA TA systems mediate *E. coli* resistance to bile acids [29], and MazEF modulate *Bifidobacterium longum* in cell growth and resistance to acid stress [59], *mosAT* stabilizes genomic fragments SXT (an integrative and conjugative element (ICE) that confers resistance to various antibiotics) [62]. Trcek hypothesized the possible involvement of toxin–antitoxin module (Dinj1–Dinj2), presented on plasmid of *K. europaeus*, in acetic acid resistance [52]. Mirroring the above roles, we can rationalize that TA systems of AAB may function in genetic element stability and physiological resistance in response to environmental stresses (e.g., high concentration of acetic acid and/or ethanol, nutrition starvation, etc.) the expansion in number and types of TA systems may be of great interest for deciphering high acid resistance mechanisms.

Here, we carry out a characteristic analysis of type II TA systems in *A. pasteurianus*. Three putative chromosomal TA candidates (*DB34_03205–DB34_03210, DB34_04120–DB34_04125*, and *DB34_01190–DB34_01195*) of *A. pasteurianus* Ab3 were identified based on a high bioinformatics prediction score (score > 65). Furthermore, in silico analysis of 65 sequenced and annotated genomes of *Acetobacter,* as well as 56 genomes of other acetic acid bacteria were performed to profile their TA systems in terms of diversity, quantity, and genome context. A possible link between physiological phenotype, such as acetic acid resistance, and TA system modules is suggested, which will help pave the way for the further exploration of the TA system function in acetic acid bacteria.

Materials and methods

Bacterial strains and growth conditions

Acetobacter pasteurianus Ab3 was isolated from the traditional rice vinegar in Zhejiang [67]. Strain Ab3 was grown in YPD medium (10 g/L yeast extract, 5 g/L peptone, 10 g/L D-glucose, pH 6.5) containing 3% ethanol (v/v). *E. coli* DH5α and *E. coli* BL21 (DE3) were used for cloning and plasmid maintenance, and recombinant protein expression, respectively. All the strains used in this study are listed in Table 1. The strains containing the plasmid pETDuet-1 (Novagen) or recombinant plasmid were cultured in media LB with (50 μg/mL) ampicillin. Using the genome of *A. pasteurianus* Ab3 as template (NCBI accession: CP012111), three TA system candidates were cloned using PrimeSTAR HS DNA polymerase (TaKaRa, Japan). All primers used in gene cloning are listed in Table S1. The pETDuet-1 expression vector containing two multiple cloning sites (MCS1 and MCS2) was used for cloning. The positive transformants were obtained by colony PCR using primers listed in Table S1, and the recombinant plasmids were verified by sequencing.

Characterizing the putative TA systems in *E. coli*

Cell growth or inhibition depicts the characteristics of putative TA systems when expressed in *E. coli* [21]. After

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description or characteristics	Source
Strains		
A. pasteurianus Ab3	Isolated from Zhejiang traditional vinegar Meigui Chu. Its genome was used as the template for cloning the putative toxin–antitoxin systems	63
E. coli DH5α	DH5α strain was used for cloning and plasmid maintenance	TaKaRa
E. coli BL21 (DE3)	BL21 (DE3) strain was used for recombinant protein expression and plasmid maintenance tests	Novagen
Plasmids		
pETDuet-1	*E. coli* expression vector. Ampicillin resistance. This vector is designed for the co-expression of two target genes. The vector contains two multiple cloning site (MCS1 and MCS2), each of which is preceded by a T7 promoter/lac operator and a ribosome binding site. ColE1 replicon	Novagen
pET-DB34_03210	pETDuet-1 derivative. Expressing the toxin gene from TA system *DB34_03205-DB34_03210* with a His_6 tag at the N-terminal	In this study
pET-DB34_03205	pETDuet-1 derivative. Expressing the antitoxin gene from TA system *DB34_03205-DB34_03210*	In this study
pET-DB34_03205-03210	pETDuet-1 derivative. Expressing the *DB34_03205-DB34_03210* system. The toxin gene has a His_6 tag at the N-terminal	In this study
pET-DB34_04125	pETDuet-1 derivative. Expressing the antitoxin gene from TA system *DB34_04120-DB34_04125*	In this study
pET-DB34_04120	pETDuet-1 derivative. Expressing the toxin gene from TA system *DB34_04120-DB34_04125* with a His_6 tag at the N-terminal	In this study
pET-DB34_04120-04125	pETDuet-1 derivative. Expressing the *DB34_04120–DB34_04125* system. The toxin gene has a His_6 tag at the N-terminal	In this study
pET-DB34_01195	pETDuet-1 derivative. Expressing the antitoxin gene from TA system *DB34_01190-DB34_01195*	In this study
pET-DB34_01190	pETDuet-1 derivative. Expressing the toxin gene from TA system *DB34_01190-DB34_01195* with a His_6 tag at the N-terminal	In this study
pET-DB34_01190-01195	pETDuet-1 derivative. Expressing the *DB34_01190–DB34_01195* system. The toxin gene has a His_6 tag at the N-terminal	In this study

overnight culturing of BL21, cells were diluted by 100-fold and transferred into fresh LB medium and grown until a ~ 0.2 OD_{600}. IPTG (1 mM) was added and growth was monitored every 0.5 h by culture turbidity at OD_{600}. Aliquots (3 µL) of each culture were spotted on LB agar without IPTG and grown at 37 °C for 12 h to observe cell growth.

The MIC (minimal inhibitory concentration) of acetic acid towards *E. coli* was measured as previously described [60]. Briefly, the overnight culture of *E. coli* strains containing different plasmids were inoculated to fresh LB medium and grown to the mid-log phase (~ 10^8 cfu/mL). The cell culture was suspended in the 96 well plates to obtain the cell numbers at ~ 10^6 cfu/mL. Then a series of acetic acid at a final concentrations ranging from 0 to 1% (v/v) was added to each well in 200 µL final volumes. The growth was determined by detecting the optical density at OD_{600}, and the MIC was defined as no visible cell growth (growth was defined as an increase in OD_{600} greater than 0.05).

Acetic acid concentrations of 0.1, 0.25 and 0.5% (v/v) were selected for a survival test on *E. coli* harboring different toxin–antitoxin gene plasmids. Briefly, the overnight cultures of *E. coli* BL21 cells were diluted by 100-fold to new fresh LB culture and grown until an OD600 reach to ~ 0.2, then 1 mM IPTG was added (in control group IPTG was not added) and induced for 2 h, and acetic acid was added with a subsequent treatment for 4 h after the induction. After treatment, culture samples were collected and diluted by tenfold and 10 µL aliquots were spread on LB agar plate to determine the number of colony-forming units (CFU/mL).The cell survival (%) was obtained using CFU/mL counted after the incubation with acetic acid divides CFU/mL counted before the treatment of acetic acid.

Plasmid stability assay

A plasmid stability assay was performed based on a previously reported method [69]. Overnight cultures of recombinant *E. coli* BL21 strains harboring TA plasmids were diluted to 1% (v/v) in fresh LB medium without ampicillin, and then incubated at 37 °C for 12 h, this process was repeated every 12 h and continued from days 1 to 7. The cultures were diluted by tenfold, and 10 µL was spread on LB plates with and without ampicillin (50 µg/mL), the plates were incubated at 37 °C for 12 h and the CFU were obtained. CFU assay was conducted every day up to 7 days. A correlation test was performed using Pearson type involved in Origin 9.0.

RT-PCR and protein extraction

RT-PCR detection was conducted as TaKaRa MiniBEST Universal RNA extraction Kit (TaKaRa, Japan). The RNA

yield and quality were evaluated with a NanoDrop UV spectrometer (Thermo Scientific, USA). The first strand cDNA was obtained using the PrimeScript™II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). gDNA and cDNA were used as templates for PCR amplification and coupling transcriptional analysis of the different TAS locus using the primers listed in Table S1. Toxin proteins were purified with Ni^{2+}-affinity chromatography using the Ni-IDA-Sefinose Column (Sangon, Shanghai, cat.no. C600811) according to the manufacturer's instructions. All proteins were quantified by the method of Bradford and visualized by staining with Coomassie brilliant blue.

Bioinformatics analysis

In silico analysis of potential Type II TA systems in the genome sequences of *Acetobacter* spp. strains relied on 65 genomes (draft or complete) cited in the NCBI public database. Simultaneously, 56 genomes of other acetic acid bacteria were analyzed for the prevalence of TA (Table S2). The putative type II toxin–antitoxin systems in each genome were predicted using a web-based search tool TADB (http://202.120.12.135/TADB2/index.php) (with default parameter) [48]. Toxin and antitoxin domain analysis was based on the Conserved Domains Database (CDD) [34]. The prevalence of TA domains determined in CDD analysis was tabulated and visualized by the web-based software "CIRCOS" (http://www.circos.ca) [28]. Next, the original sequences of the predicted TA sequences in genome of *A. pasteurianus* Ab3 were determined. A 'microbial tBLASTn' tool for each amino acid sequence from the putative three TA systems were compared, against databases of completely or incompletely (draft) sequenced plasmids/genomes in *Acetobacteraceae* or out of *Acetobacteraceae*, and the target sequences were collected and screened for further comparative analysis (query cover > 50%, $E < 1e^{-4}$). Multi-alignment between amino acid sequences was performed using ClustalW. Secondary structure prediction of selected toxins and antitoxins of *A. pasteurianus* Ab3 were performed with SOPMA [14]. The three-dimensional structures of selected TA proteins were constructed and predicted by SWISS-MODEL [6]. The chromosomal synteny analysis of TA systems and the genetic structure analysis of the three TA candidates were performed with SyntTax (http://archaea.u-psud.fr/SyntTax/) [40]. For the draft genomes, chromosomal synteny analysis was conducted manually and the genes located upstream and downstream of the target sequence were determined. The putative promoter was predicted by BPROM (http://linux1.softberry.com/berry.phtml). The putative insertion sequences (IS) in each genome was predicted by ISfinder (https://www-is.biotoul.fr/, *E* value = $1e^{-4}$).

Statistical analysis

All statistical analyses were performed using Origin software (version 9.0). Where appropriate, the data were analyzed using Student's *t* test. Differences were considered statistically significant at $p < 0.05$. The correlation coefficient analysis was performed using Pearson type.

Results and discussion

Three putative TA pairs were identified with active biological activities

Based on the sequence prediction score, three putative TA pairs, *DB34_03205–DB34_03210*, *DB34_04120–DB34_04125*, and *DB34_01190–DB34_01195* in the *A. pasteurianus* Ab3 genome, were screened for further analysis. Of these, *DB34_03210*, *DB34_04120*, and part of *DB34_01190* (263195–263455) were predicted as the possible toxin genes, while *DB34_03205*, *DB34_04125* and part of the *DB34_01195* (263464–263742) as the cognate antitoxin genes (Table S3).

Genetic organization analysis revealed that *DB34_03205* was located upstream of *DB34_03210* with 4 bases overlapped and encoded on the opposite strand (Fig. 1a), which is usually described in type II TA systems [61]. RT-PCR products of *DB34_03210* (106 bp), *DB34_03205* (134 bp), and *DB34_03205-03210* (328 bp) matched consistently with that of genomic DNA (gDNA) as PCR template, testifying the coupled transcription of *DB34_03205* and *DB34_03210* in a bicistronic operon (Fig. 1b). Additionally, a characteristic *α-β(3)-α* structural arrangement of the double stranded RNA (dsRNA)-binding domain was found in DB34_03210 (from loci 30 to 80) (Fig. S1a), which is a highly conserved toxin in the HicA superfamily [31, 32]. While DB34_03205 contained three β-strands and an α-helix at its *N*-terminal domain (*β(3)-α-β* arrangement), in accordance with the conserved structure in HicB superfamily (Fig. S1b). Functionally, the toxin expressed by pET-DB34_03210 in *E. coli* significantly inhibited the bacteria growth (Fig. 1c), and *DB34_03205* product clearly neutralized the toxicity and maintained cell growth. The growth was not restored when the cells were transferred to a LB plate without inducer IPTG (Fig. 1d). Pull-down assays further provided the toxin–antitoxin complex due to toxin insufficient purification (Fig. 1e, lane 1 and 2), while the co-expression produced clear bands, suggesting that the pair form a complex in vivo (Fig. 1e, lane 3 and 4). Collectively, the *DB34_03205–DB34_03210* is identified and named as *Ap_hicAB*. Similarly, *DB34_04120–DB34_*04125 was clarified as *higBA* supported by its gene architecture and biological activities shown in Fig. 2a–e and Fig. S1c–d. The expression

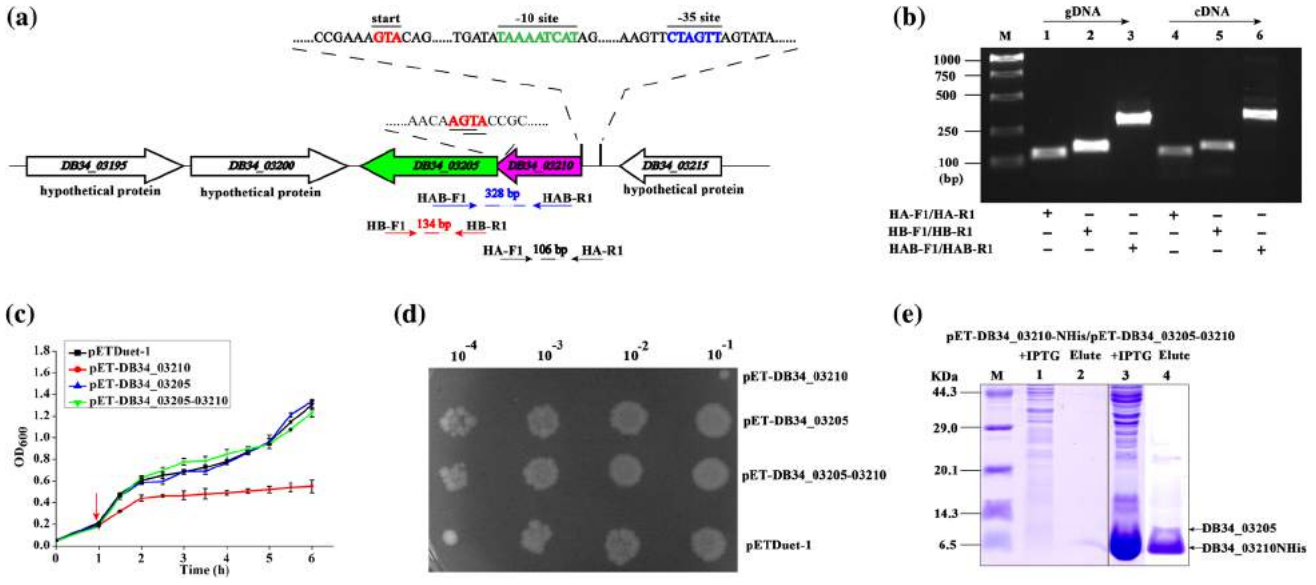

Fig. 1 Identification of putative TA system *DB34_03205–DB34_03210*. **a** Genetic structure analysis. **b** Transcriptional analysis of *DB34_03205–DB34_03210* ("+" and "−" represent the primer was added or not, respectively). **c** Effect of the expression of *DB34_03205* and/or *DB34_03210* on *E. coli* growth. The red arrow indicates that the IPTG (1 mM) was added. **d** Growth of cells after induction on LB plates. **e** SDS-PAGE analysis of toxin and antitoxin proteins. Toxin DB34_03210 with an N-terminal His-tag was constructed alone (pET-DB34_03210) or together with untagged antitoxin DB34_03205 (pET-DB34_03205-03210). After induction with 1 mM IPTG, the 6.5 kDa DB34_03210-NHis and 10.2 kDa DB34_03205 were induced (lane 3). During purification, DB34_03210-NHis and DB34_03205 were co-purified (lane 4). When expressed alone, no sufficient protein of DB34_03210 was obtained and purified (lane 1 and 2)

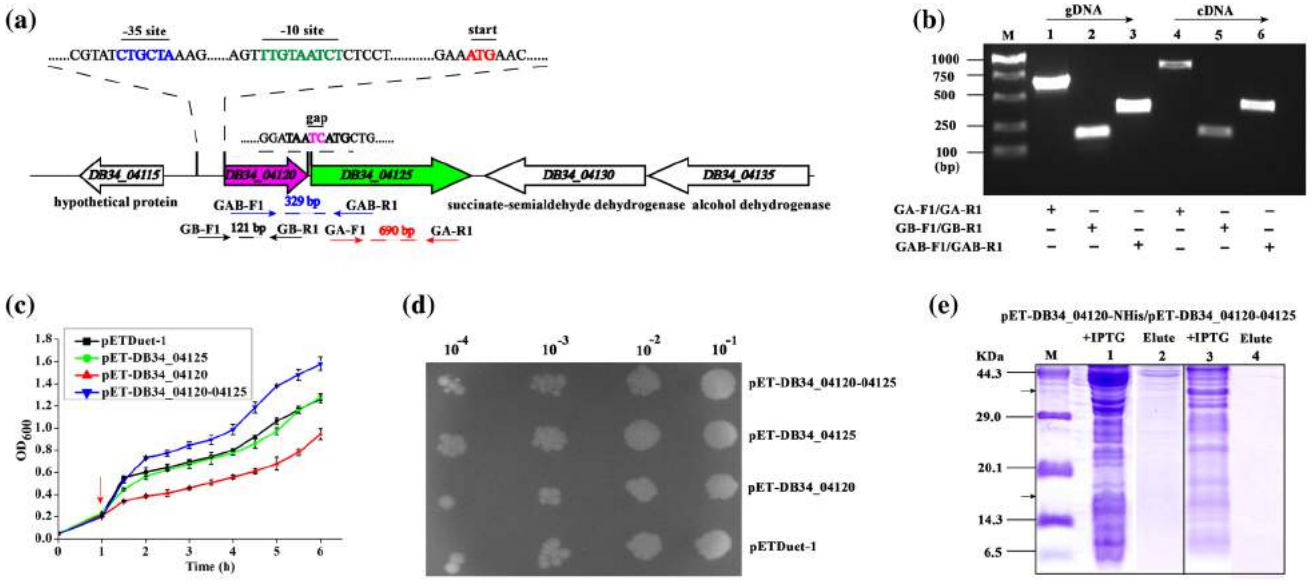

Fig. 2 Identification of putative TA system *DB34_04120–DB34_04125*. **a** Genetic structure analysis. **b** Transcriptional analysis of *DB34_04120–DB34_04125* ("+" and "-" represent the primer was added or not, respectively). **c** Effect of the expression of *DB34_04120* and/or *DB34_04125* on *E. coli* growth. The red arrow indicates that the IPTG (1 mM) was added. **d** Growth of cells after induction on LB plates. **e** SDS-PAGE analysis of toxin and antitoxin proteins. Toxin DB34_04120 with an N-terminal His-tag was constructed alone (pET-DB34_04120) or together with untagged antitoxin DB34_04125 (pET-DB34_04120-04125). After induction with 1 mM IPTG, no sufficient protein of DB34_04120 was induced either by expressing alone (lane 1) or with DB34_04125 (lane 3). During purification, the interest proteins were not obtained (lane 2 and 4)

of *DB34_04120* resulted in an obvious growth inhibition (Fig. 2c), and the cell restored growth was influenced with a decrease in about 2 log units (Fig. 2d). Induction of *DB34_04125* did not influence the cell growth, and could alleviate the growth inhibition caused by the expression of *DB34_04120* (Fig. 2c). In addition, it is interesting to find that the co-expression of *DB34_04120* and *DB34_04125* increased the cell growth even higher than the control group (cell contains empty vector). It is still unclear if it is due to that the expression of this TA system can benefit cell growth, but the similar results were also observed in previous studies that cell was grown better when the toxin and antitoxin were co-expressed [21, 49], which need further study. In contrast, we did not succeed in purifying the toxin Ap_HigB protein, neither expressed alone nor co-expressed with its antitoxin Ap_HigA in *E. coli* (Fig. 2e), the high toxicity of the toxin and the susceptibility of the antitoxin to cleavage or its heterologous expression seemed responsible.

In contrast to the two canonical TA systems above, *DB34_01190–DB34_01195* is proposed to be a novel TA system. The loci were not co-transcribed in one unit but under two different promoters (Fig. 3a, b), and the well-studied toxin/antitoxin domains were not found in this TA pair. However, the expression of *DB34_01190* arrested cell growth and its toxicity was attenuated by the product of *DB34_01195* (Fig. 3c). Specifically, the derived growth arrest was not restored on an LB plate as the same as HicA (Fig. 3d). The putative toxin DB34_01190 was successfully purified when expressed alone or when co-expressed with the DB34_01195 (Fig. 3e, lane 1–4), indicating that these two proteins interact with one another and form a complex in vivo. Recently, some atypical TA arrangements have been observed beyond the canonical structure of TA systems, e.g., tripartite toxin–antitoxin–chaperone (TAC) modules in *M. tuberculosis* [8] and an oxygen-dependent Hha and TomB proteins in *E. coli* [35]. Even Cas1 and Cas2 of CRISPR-Cas have been proposed to be toxin–antitoxin-like [27]. Moreover, RNase activity does not seem necessary to the so-called toxins, GmvT toxin of *S. sonnei* shows acetyltransferase activity not RNase activity [37], and Rv0910 of *M. tuberculosis* lacks of RNase activity but exhibits toxin activity [43]. Here, CDD analysis indicated that DB34_01190 does not possess a conserved domain containing RNase activity, but as a plasmid stabilization protein (AKR47726). Additionally, we found DB34_01195 does not contain a DNA binding region in its *N*-terminal domain, similar to that of antitoxin FicA without a DNA-binding domain [19]. However DB34_01195 contains a domain belonging to the short-chain dehydrogenases/reductase (SDR) superfamily (cl25409), a functionally diverse family of oxidoreductases [24]. Thus, it seems rational to extend the concept of TA systems. *DB34_01190* and *DB34_01195* do not have typical type II TA system structures, yet they can interact with each other as the same as the typical TA systems. Here,

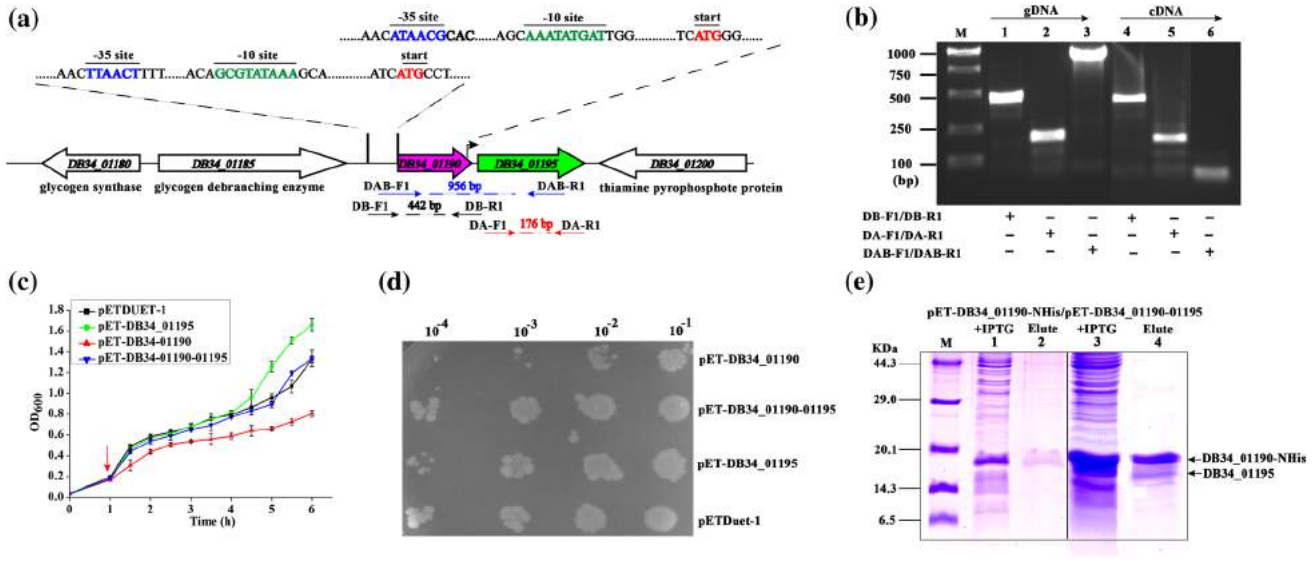

Fig. 3 Identification of putative TA system *DB34_01190–DB34_01195*. **a** Genetic structure analysis. **b** Transcriptional analysis of *DB34_01190–DB34_01195* ("+" and "−" represent the primer was added or not, respectively). **c** Effect of the expression of *DB34_01190* and/or *DB34_01195* on *E. coli* growth. The red arrow indicates that the IPTG (1 mM) was added. **d** Growth of cells after induction on LB plates. **e** SDS-PAGE analysis of toxin and antitoxin proteins. Toxin DB34_01190 with an N-terminal His-tag was constructed alone (pET-DB34_01190) or together with untagged antitoxin DB34_01195 (pET-DB34_01190-01195). After induction with 1 mM IPTG, the 17.5 kDa DB34_01190-NHis was obtained either by expressing alone (lane 1) or with the 16.5 kDa DB34_01195 (lane 3). During purification, DB34_03210-NHis can be purified alone (lane 3) or co-purified with DB34_01195 (lane 4)

DB34_01190–DB34_01195 is supposed a novel TA system, named as *Ap_npoTA*.

TAs heterologous expression in *E. coli* confers plasmid stability and acid resistance

Initially, the role of TA systems was proposed to maintain extrachromosomal elements such as plasmids [41]. Strains harboring heterologous TA systems plasmids such as *virTA* (*Campylobacter jejuni*) and *vapBC* (*E. coli*) show plasmid maintenance ranging from 60 to 100% within 7 days [18, 49, 69]. Here, we observed a similar phenomenon in *E. coli* hosting the *Acetobacter* TA pairs, more than 80% of pET-DB34_03205-03210 (harboring *Ap_hicAB*) was retained (Fig. 4a) after a 7-day. However, pET_DB34_04120–DB34_04125 (harboring *Ap_higBA*) and pET_DB34_01190–DB34_031195 (harboring *Ap_npoTA*) did not produce the same results in *E. coli* (Fig. 4b, c), implying their different biological effects. In acetic acid challenge tests, the expression of the *Ap_hicAB* conferred a 10.6-fold improvement to *E. coli* survival against 0.1% (w/v) acetic acid (Fig. 4d, Fig. S2). Moreover, under a higher acetic acid stress (0.25%, w/v), each induction of the three TA pairs significantly increased cell survivals by 45–150-fold compared to control, particularly in case of *Ap_npoTA* and *Ap_higBA*, suggesting that these TA loci may be stress-response elements conferring stress adaptation [61]. Although polar expression is one main approach of TAs to influence up-or downstream loci, our results seem to support the combination of TA systems with stringent response, like that the suppression of toxin HicA on *E. coli* cells was reported through δ^E factor loss [12]. The details of which are waiting for an answer. Further function annotation of TAs in strain Ab3 will be conducted next step.

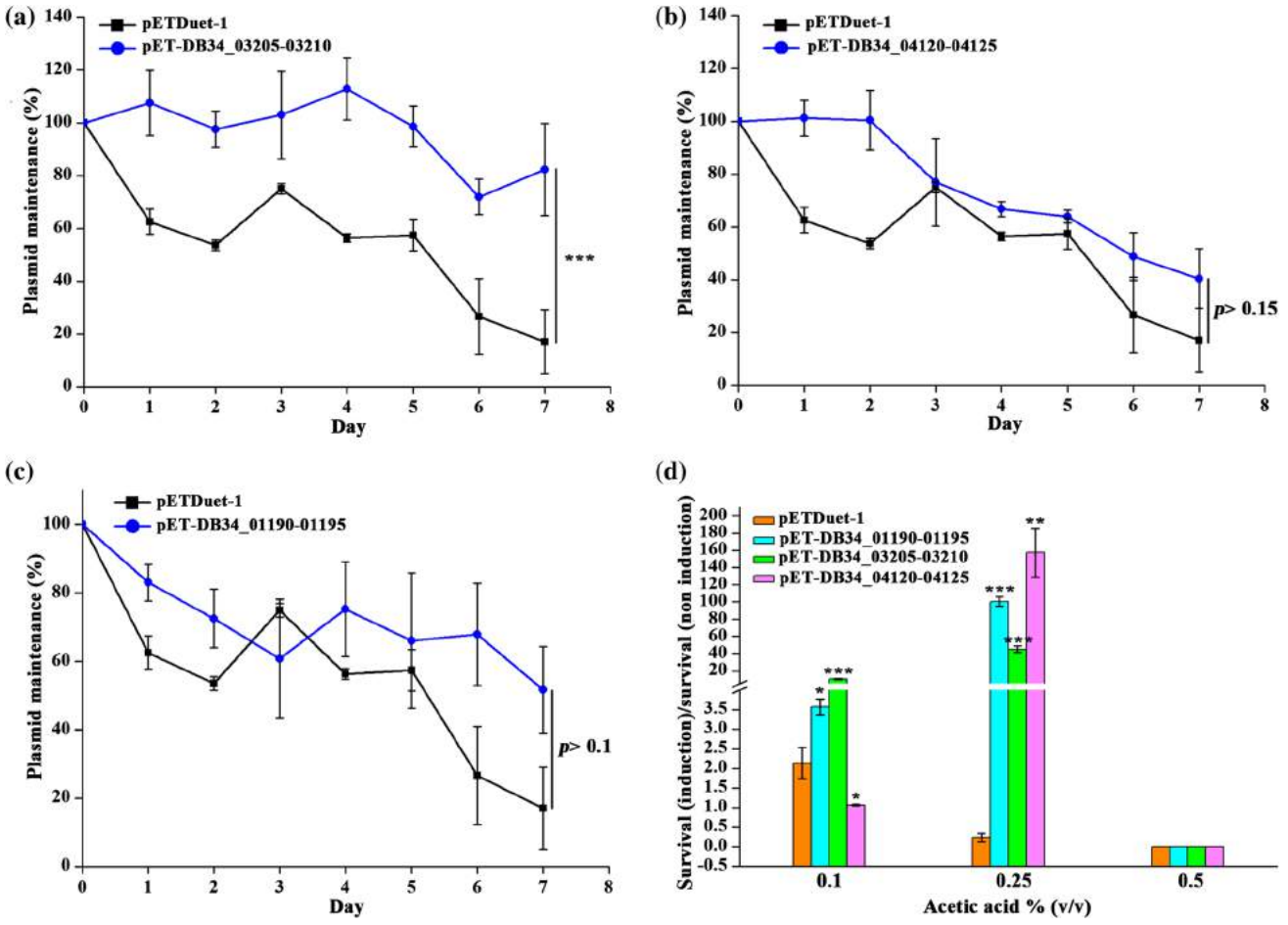

Fig. 4 Influences of *Ap_hicAB* (**a**), *Ap_higBA* (**b**), and *Ap_npoTA* (**c**) expressions on plasmid stability and growth under acetic acid stress (**d**) of *E. coli* cells. The data were shown as mean ± standard deviations. "*" represents that there is significant difference between two studied groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Evolution of the three TA gene pairs in *A. pasteurianus*

Considering the potential relationships between *Ap_hicAB*, *Ap_higBA*, and *Ap_npoTA* with cellular stress responses, we traced their evolution in *A. pasteurianus*. First, sequence analysis revealed that *Ap_hicAB, Ap_higBA* and *Ap_npoTA* found their origins in other bacteria co-existing with *A. pasteurianus* in vinegar fermentation niches, including *Proteobacteria* (e.g., the genera *Pseudomonas* and *Rhizobium*) and *Firmicutes* (e.g., *Lactobacillus*, *Bacillus* and *Lactococcus*) (Fig. 5a–d) [20, 64, 68]. Specifically, *Lactobacillus* spp. are universally found to symbiotically grow with *A. pasteurianus* in the Balsamic vinegar fermentations performed in Europe, rice vinegar and "Shanxi Chenchu" in China, and Komesu vinegar in Japan [33, 39]. Specifically, the complete *Ap_npoTA* was not detected in the chromosomes of these out groups due to toxin deletion though the single *Ap_npoA* was widely present. The co-occurrence implies the co-option of *Acetobacter* together with *Lactobacillus* and/or other bacteria within the natural vinegar fermentation and promises AAB to be selected as the dominant species.

Second, at the species level of *A. pasteurianus*, *Ap_hicAB* and *Ap_npoTA* were widely found in genomes and highly conserved (sequence identity > 90%) (Table S4), indicating their critical roles and the intra-species transfer during propagation processes since they were usually adjacent to transposons (Fig. 5a, d) [36]. The flanked genes of *Ap_npoTA* and *Ap_higBA* were prone be conserved within *A. pasteurianus* (Fig. 5c, d), which may result from the output of toxin activation against stressors and/or regulation. These flanked genes encode enzymes involved in a variety of metabolic processes, including succinate-semialdehyde dehydrogenase, alcohol dehydrogenase, and thiamine pyrophosphate-binding protein. These enzymes and/or bioprocesses are linked with the growth and acid resistance of *Acetobacter* strains through previous proteomics investigations [5, 58, 67]. In terms of the flanked genes of *Ap_hicAB* pair, either transposase or integrase seem to be required. While in the incomplete *Ap_hicB,* the conserved flanking gene *alkB* was found, which encodes alpha-ketoglutarate dependent dioxygenase (AlkB) and acetate permease (ActP) (Fig. 5b). AlkB is induced to specifically remove methyl groups from 1-methyl adenine in single stranded DNA damaged by chemical agents during an adaptive response [1], suggesting *Ap_hicB* might be involved in the recovery of nucleotide damage by *Acetobacter*. ActP is a monocarboxylate transporter and also contributes calcium-irreparable acid-sensitive phenotype [46]. This implies that *Ap_hicB* may transcriptionally regulate *alkB* and *actP* upon external stress, coinciding with the acetic acid challenge tests. Therefore, at the species level in *A. pasteurianus,* these three TA pair locations on the chromosome appear to be specific.

Third, toxin gene loss is observed not only in most of *Acetobacter* strains (Table S4), but also in genera such as *Komagataeibacter*, *Gluconobacter,* and *Kozakia,* and seems to be universal in AAB (Fig. 5b–d). The study of *Xanthomonas* demonstrated that the toxins are under positive selection during an evolution process since their sequences have a much higher diversity than antitoxin sequences (e.g. HipA) [36]. In the evolution of *Shigella sonnei,* TAs from plasmid pINV were gradually deleted allowing the pathogen to better adapt to the host lifestyle [37]. The similar situation also occurred in *A. pasteurianus* in response to multiple adaptive stresses, its toxin sequences showed more divergent than antitoxin sequences (Table S4). Furthermore, the existence of numerous IS in the genome may be another cause of toxin loss [44]. TA gene pairs may share common hotspots

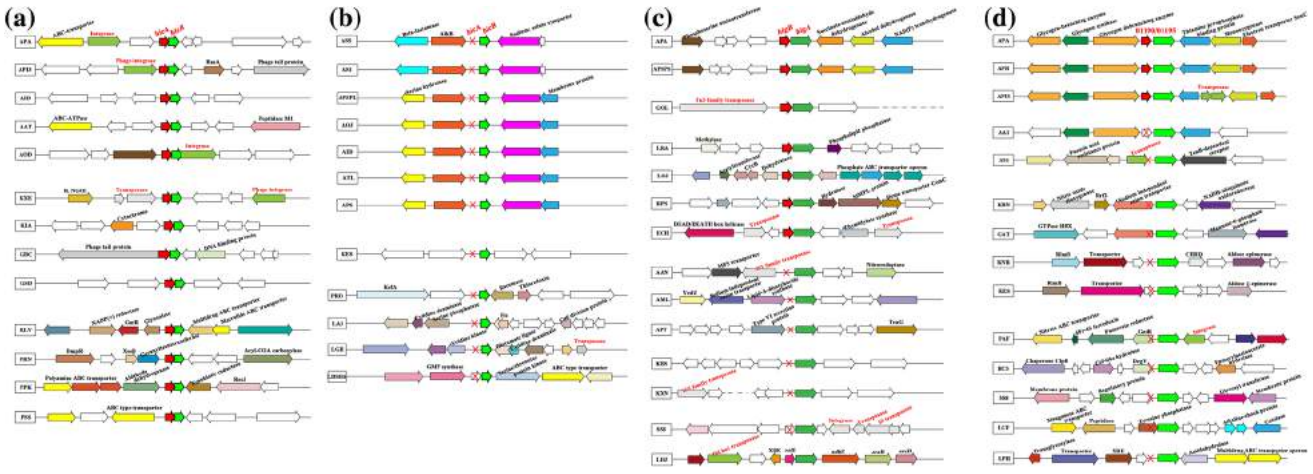

Fig. 5 Chromosomal synteny analysis of *Ap_hicAB, Ap_higBA*, and *Ap_npoTA*. **a**, **b** Synteny of *Ap_hicAB*. **c** Synteny of *Ap_higBA*. **d** Synteny of *Ap_npoTA*. Arrows with the same color represent the homologous sequences. Arrows in white in all parts stand for hypothetical proteins. The cross in each synteny represents the toxin loss, and strain abbreviations displayed in the left were denoted in Table S6

for integration as observed in the *ccdAB* system [38]. Thus, toxin deletion may also take place since they fail to co-opt important cellular functions; but retained antitoxin may be co-opted to other functions or function independently. An anti-addiction role of antitoxin retentions produces a positive effect on their fixation within populations and promotes the directional selection of natural variants of these plasmid encoded systems [13, 45]. Also, antitoxin *MqsA* directly inhibits sigma factor *rpoS* of *E. coli*, thereby regulating general stress response and biofilm formation [50]. DinJ is involved in general stress response through RpoS level [22]. In *Acetobacter* the retained antitoxins *Ap_hicB, Ap_higA* and *Ap_npoA* may also function a broad role in bacterial physiology. Collectively, the TA pairs evolution suggests that an ecological community may provide a more diverse gene space, promoting the accumulation of various interesting phenotypes in industrial strain domestication when compared to pure colony cultivation.

The distribution profile of total TAs in *Acetobacter*

A total of 741 TA systems were predicted in 65 *Acetobacter* genomes with a mean value ranged from 2 to 19.3 per species (including 223 TA pair in plasmids, Table S2 and S5). *A. pasteurianus, A. senegalensis*, *A. pomorum*, *A. cerevisiae,* and *A. aceti* showed higher numbers of complete TA pairs with mean values of 19.3, 15, 13.5, 11.3, and 11.3 per genome, respectively, than the average of 3.8 for bacterial chromosomes reported [13]. *A. pasteurianus subsp. paradoxus* LMG 1591 had the highest number of TA operons (52 per genome), and no TA system was found in *A. malorum* LMG 1746. Thus, the prevalence of TA systems appears to be species-dependent in the genus *Acetobacter*. Previous studies showed that the amounts of type II TA systems neither correlated with genome size nor the total amounts of protein coding sequences (CDS) [30], and the same was observed in *Acetobacter* (Fig. 6a, b). While, the occurrence

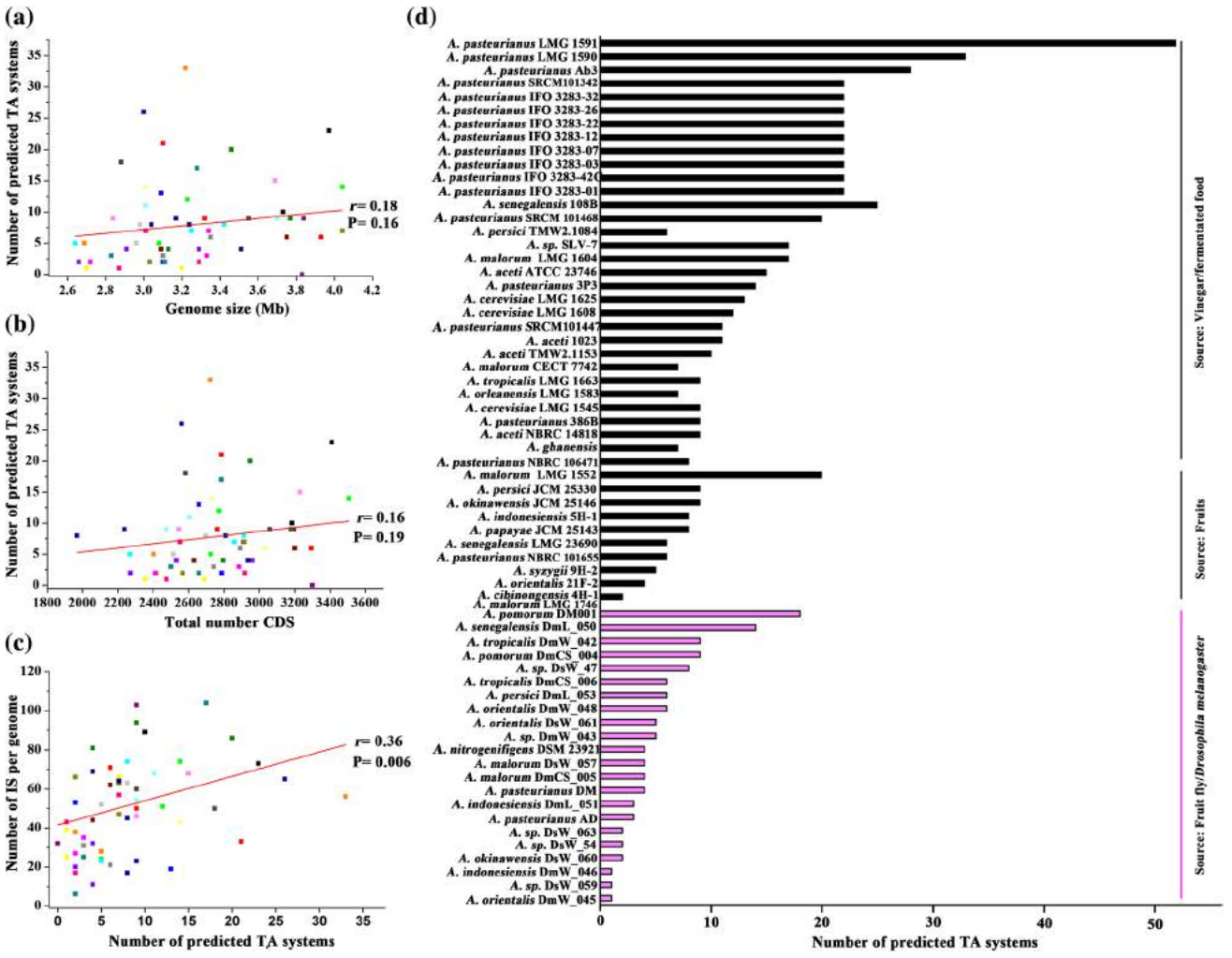

Fig. 6 Correlation analysis between the predicted complete TA pairs and the genome size (**a**), the total number of CDS (**b**), insertion sequences (**c**), physiological stress (**d**) (color figure online)

of IS on the *Acetobacter* chromosome may confer various hotspots to its TA systems supported by a positive correlation between the number of IS elements and the amount of TA systems (Fig. 6c) [44]. In addition, no correlation between CRISPR systems and the amount of TA systems was found here, though CRISPR system is an immune system for protecting bacteria in response to environmental stresses [27].

In parallel to the genomic features, diverse habitats may also be associated with the prevalence of TA loci. *E. coli* strains with greater virulence properties, genetic instability, and adaptations contain more TA systems [13]. *Xanthomonas* strains, which are less host-associated but more exposed to the environmental stresses, contain more TA loci [36]. In *Acetobacter,* we observed that one-third of 65 strains, isolated from *Drosophila melanogaster* (Fig. 6d, in pink), showed a mean value of 5.32 TA systems which is much lower than the average number (14.51) in the *Acetobacter* strains isolated from vinegar, fruit and flowers. Strains of genera *Asaia, Saccharibacter* and *Commensalibacter,* symbiotically associated with their *Drosophila* and other insect hosts [10, 11], showed a lower number of TA systems (mean value lower < 3). Some species, often described as the opportunistic human pathogens, including *Gluconobacter morbifer* [26], *Asaia bogoriensis* and *Granulibacter bethesdensis* [17], also contained fewer TA systems. Thus, we conclude for *Acetobacter* and other AAB strains, the more host-associated the species does the lower the number of TA systems. The long industrial domestication under stresses may promote the accumulation of TA systems in industrial *Acetobacter* and *Komagataeibacter* strains.

Beyond the amounts, all TA systems can be clustered into 11 toxin superfamilies and 14 antitoxin superfamilies as shown in Fig. 7a (chromosomal) and b (plasmidic), showing a conserved distribution among *Acetobacter* strains. Of which, the principal hubs of the TA system network are toxins RelE and PIN_VapC, and antitoxins RHH and AbrB domains, corresponding to 85% of all the TA systems (Fig. 7c). HEPN–MNT, HicA–HicB and COG5654–COG5642 pairs occurred separately as unique pairs and were not involved in the other TA pairs. However, HipA and domain 09812 only occurred in *A. malorum* LMG 1552 and *A. syzygii* 9H-2, respectively. At the species level, *A. pasteurianus* contained the highest diversity of TA systems, while *A. cibinongensis* and *A. ghanensis* contained only two types of TA systems. In terms of plasmidic TA systems, there were five types of toxin super-families found in the genus *Acetobacter*, with each plasmid including at least two TA systems. The various distribution and amount of TAs superfamilies among *Acetobacter* species implies their diverse roles in biological functions in evolution, and adaption.

TAs modules, a promising network to study resistant mechanisms in Acetobacter?

Endoribonuclease activities are the well-known and predominant characteristics of type II TAs. It is rational for *Acetobacter* strain that the cleavage selectivity of toxins allows targeting RNA pools and produces diverse and real-time ribosomal profiles to perform complex and nuanced regulations in response to stresses [55]. This is in line with the previous studies, i.e., ribosome and protein metabolisms are the core bioprocess involved in resistant mechanism in *Acetobacter* [5, 67]. Therefore, it is worthy that integrating TAs-derived translatome with proteome will promote the accurately deciphering resistant mechanistic network. TAs-associated persister formation among *Acetobacter* populations should be the second open question. Though *Acetobacter* persister has not been reported currently (data no published), persistence-related TA systems, e.g. MazF and Fic, were abundant in *Acetobacter* (Table S2), while it is supposed to benefit *Acetobacter* survival to vinegar stress. Actually, the activation of *mazEF* is observed in *Bifidobacterium longum*, *E. coli* and *M. tuberculosis* against the acid stress either [9, 51, 59]. Intriguingly, a subset of pathogenic bacteria (*Vibrio, Legionella, Xanthomonas, and Pseudomonas*) use Fic protein to rewire cellular signaling network to maximize pathogen survival and proliferation in the eukaryotic host [54], what fitness its specific occurrence not in the host-associated *Acetobacter* strains but in industrial ones is elusive. In case of HipA with high persistence formation in *E. coli* [47], it was seldom found in most *Acetobacter* strains, indicating that its function may be complemented by other TA modules. Plasmid and/or genetic stability of *Acetobacter* complicated into TA systems is the third question. Beside the mentioned as in *hicAB*, HEPN toxins are universal immunity-related and complicated in multi-pronged defense strategies against bacteriophage in parallel with the CRISPR and restriction-modification systems [2]. Especially in the case of small CRISPR systems were just found in *Acetobacter* [66], the effects of TA systems on bacterial immune need to be addressed further.

In conclusion, TA systems performs an alternative signaling layer onto modulatory network in *A. pasteurianus*, the translatome profile will be another promising key to unlock resistant mechanism. Co-option with vinegar microbiota provides *Acetobacter* a great gene space benefits resistant revolution. Persistence, plasmid addiction, and anti-phage activity are reciprocally regulated by TA systems. Further study is needed to establish TAs direct involvement in stress signaling, specifically about the hypothesis that longer domestication under external stress may introduce more TA systems into industrial strains due to its potential application in the development of commercial process strains.

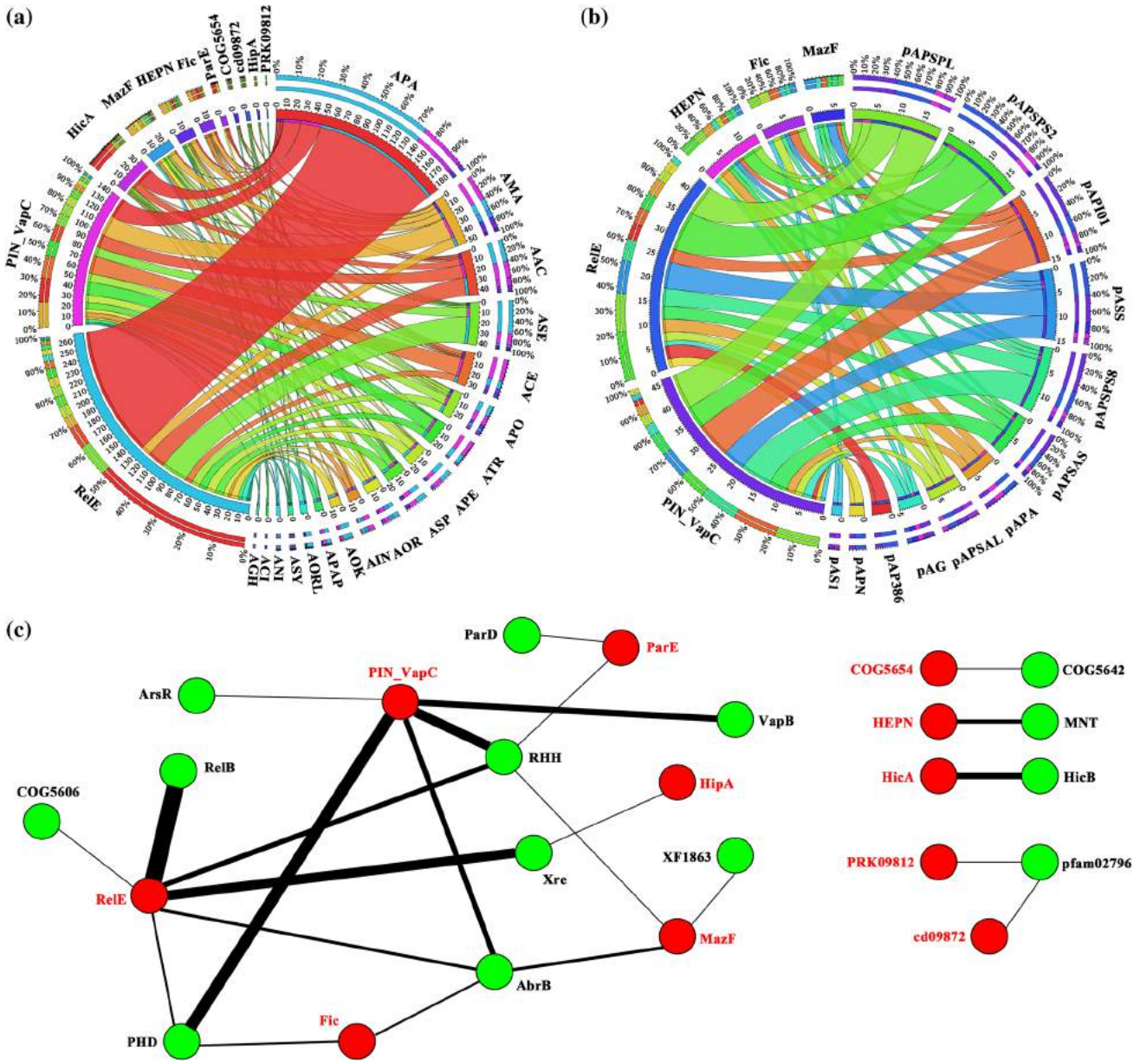

Fig. 7 The prevalence of TA super-families in genus *Acetobacter*. **a** The visual representation of the toxin super-families detected (on the left) among 18 selected genus. Each color represents one superfamily and the wider the ribbons, the higher the number of toxins contained. **b** The prevalence of TA super-families on plasmids. **c** Relationships between different super-families of toxins and antitoxins. Toxins (red circle) and antitoxins (green circle) were connected with the predicted TA operon organizations, thickness of a line is proportional to the frequency of each operon. All abbreviations were denoted in Table S6 (color figure online)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This paper does not contain any studies with human participants or animals.

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