Screening of associated bacteria of *Alexandrium catenella* on affecting PSTs production and influence mechanism

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Abstract

Alexandrium causes serious food safety and human death due to paralytic shellfish toxins (PSTs) production. The associated bacteria can affect PSTs production of *Alexandrium*. However, the influencing mechanism is still unclear. Here we firstly screened functional associated bacteria for affecting PSTs production of *Alexandrium catenella* in Yangtze Estuary and further studied their influence on physiological process and molecular regulation of *A. catenella*. Thirteen bacteria strains for affecting PSTs production of *A. catenella* were selected. The *A. catenella* strains co-cultured with different functional associated bacteria all produced more PSTs than axenic strain with antibiotic treatment. Compared with axenic *A. catenella*, the non-axenic *A. catenella* produced more algal cells, soluble sugar, soluble protein and neutral lipid. By RNA-seq, it was found that non-axenic *A. catenella* produced more upregulated functional genes than axenic *A. catenella*. The biosynthesis of cofactors and spliceosome were the dominant different pathways between axenic and non-axenic *A. catenella* strains. The sxtA expression was closely related with Arginine and proline metabolism, Arginine biosynthesis, Fatty acid biosynthesis, TCA cycle and Glutathione metabolism, which were all downregulated in axenic *A. catenella*. Meantime, the non-axenic *A. catenella* under nitrogen deprivation produced less PSTs and functional genes than non-axenic strain under common culture condition, indicating the nitrogen significance for PSTs production. The detailed signal molecular released by associated bacteria for regulating PSTs of *A. catenella* needs to be further studied.

Keywords: *Alexandrium catenella*; associated bacteria; PSTs; physiological process; molecular regulation

1. Introduction

Harmful algal blooms (HABs) occur globally and have produced severe effects on fisheries, aquaculture, tourism and recreation[1, 2]. Dinoflagellates, a large group of phytoplanktonic organisms during marine HABs, play a crucial role in food webs and in balancing ecosystem energy fluxes [3-8]. Many marine dinoflagellates can cause food safety and human fatalities through their production of paralytic shellfish toxins (PSTs) which accumulate in shellfish through the food chain when contaminated seafood is consumed [2, 9-13]. The most widespread dinoflagellates species producing PSTs are in the genus *Alexandrium* [14, 15].

PSTs, mainly produced from dinoflagellate and cyanobacteria, is one of the most powerful neurotoxins such as TTX [16]. Currently, the regulation mechanism for PSTs production in cyanobacteria is clear, which mainly refers to 26 functional genes for PSTs biosynthesis [17]. However, the regulation mechanism for PSTs production of harmful dinoflagellates remains poorly understood due to the exceptionally large genome size and high gene copy number of dinoflagellates [18]. Thus, uncovering the factors that influence the biosynthesis of PSTs toxins in dinoflagellates is not only important for understanding PSTs biosynthesis mechanism but also crucial for seafood safety and human health assurance worldwide [19-23].

The environmental factors, such as light, temperature, salinity, nitrogen or phosphorous, have been shown to affect PSTs content for several *Alexandrium*

species[24]. For example, while nitrogen limitation causes the toxin content to decrease phosphorous limitation often results in higher toxin cell quotas [25-32].But the salinity and temperature just have limited effects on toxin composition [28, 33].

Bacteria directly or indirectly affect the growth and toxin production of *Alexandrium* species [34-40]. Originally it was considered that the PSTs in dinoflagellate was produced by their associated bacteria, e.g., *protogonyaulax* and *Alexandrium* [37, 41, 42]. However, the subsequent research has shown that the "PSTs" produced by bacteria was actually the analogues of PSTs [43, 44]. The false positive for PSTs detection in bacteria is mainly because of the low concentration. Subsequently many studies indicated that the PSTs in dinoflagellate was produced by dinoflagellates alone, but their associated bacteria could affect their PST production [45-48]. Different bacteria community had different capacity for affecting PSTs production of dinoflagellate [45]. It has been suggested that the effects of bacteria on PSTs production of dinoflagellate can be explained by altered toxin synthesis pathways through algal-bacterium interactions [40]. But the detailed influencing mechanism is still unclear. It is hypothesized that the associated bacteria of dinoflagellate affect the PSTs production by releasing some signals or metabolites that regulate the growth and physiological process of dinoflagellate [45-47]. Thus, understanding the influencing mechanism of associated bacteria for PSTs production of dinoflagellate is not only helpful for

underlying the PSTs biosynthesis in dinoflagellate but also useful for controlling the PSTs production by microbial ways.

Yangtze Estuary is the largest estuary in China, which plays an important role for industry, agriculture, aquaculture and marine ecology. However, HABs often happen in Yangtze Estuary due to the environmental pollution and eutrophication, especially for dinoflagellate. Therefore, the production of PST from dinoflagellates, especially from *Alexandrium* species, can cause severe shellfish food poisoning [49]. Due to the difficulty in identifying dinoflagellate by morphological characters, in this study, the eDNA metabarcoding [50] was employed to reveal the community composition of dinoflagellate in Yangtze Estuary. The associated bacteria community diversity of *A. catenella* from Yangtze Estuary was revealed and the functional bacteria strains for affecting PSTs production were screened. Then the physiological process and molecular regulation of *A. catenella* under influence of associated bacteria on PSTs production were further evaluated.

2. Materials and methods

2.1 Sample collection

A. catenella seawater samples collected from Shanghai (*A. catenella*-C, 121.92348°E, 30.862512°N), Shengsi (*A. catenella*-S, 122.454838°E, 30.700638°N) and Zhoushan (*A. catenella*-Z, 122.413229°E, 29.92582°N) in Yangtze Estuary of China were firstly pre-filtered through a 200μm pore-size sieve to remove debris, mesoplankton, and macroplankton. Three subsamples of

equal volume from 0.5 m, 1.0 m and 2.0 m depths were collected and mixed as one sample from each plot. Then each water sample (~500 ml) was subsequently filtered through a 0.22μm pore-size polycarbonate membrane (Millipore, Billerica, MA, USA). The membranes were stored at -80 °C until DNA extraction. The toxicity of *A. catenella* strains from different locations was detected, where the stains with the highest toxicity was selected for experiment analysis.

2.2 Dinoflagellate community diversity

The 18S-v4-v5 and 28S primers (Table 1) were used for metabarcoding eukaryotic plankton and dinoflagellates by Illumina-miseq high throughput sequencing. DNA was extracted by OMEGA E.Z.N.ATM Mag-Bind Soil DNA Kit for each sample. PCR amplification of all samples was performed in triplicate. After sequencing, the fastq files were demultiplexed to separate the samples based on the indexes. Reads were then analyzed and assigned using QIIME2, where the PCR primers were removed by cut adapt package and Dada2 was applied for denoising, dereplicate-sequence, filtering chimera and merging the paired reads [51]. Taxonomic classification was performed by feature-classifier module with Naive Bayes [51]. The reference library for ASV classification was referred from Zou et al [52].

2.3 A. catenella isolation, culture and antibiotic treatment

Vegetative cells preliminarily identified as *A. catenella* based on morphological characteristics were isolated by pipette under a microscope (40× zoom), and transferred to a 24-well plates containing culture f/2 medium. These

strains were cultured at 19-26°C, 3000-6000lux light and light to dark ratio of 12D: 12N. Then the cultures were expanded from orifices to 250ml triangulated vials and maintained in the laboratory using f/2 medium under the same conditions. The obtained strains were then further detected by 18S sequencing. A set of publicly available 18S sequences of *Alexandrium* downloaded from GenBank was added to the new sequences to build phylogenetic trees by the maximum-likelihood method.

For obtaining axenic strains, cefotaxime sodium and vancomycin hydrochloride were selected as the antibacterial antibiotics after testing with different antibiotic mixtures. 30μg/ml cefotaxime sodium and 50μg/ml vancomycin hydrochloride were added to the sterile f/2 medium for producing axenic *A. catenella* cultures, which was conducted for triple replicates. Tow replicates were used to assess the axenic state of the cultures, while the remainder was used to determine the toxicity. For non-axenic culture with nitrogen deprivation (non-axenic-ndep) the concentration of NaNO3 was set at 1/4 of the control group (2.94 mmol/liter).

2.4 Associated bacteria community diversity of A. catenella

The associated bacteria community diversity of *A. catenella* under laboratory culture condition was analyzed by 16Sv3v4 and 16S metabarcoding respectively (Table 1). *A. catenella* culture was filtered with a 0.22μm pore-size polycarbonate membrane to obtain the associated bacteria (Millipore, Billerica, MA, USA). DNA was extracted by OMEGA E.Z.N.ATM Mag-Bind Soil DNA Kit. PCR

amplification of all samples were triple. The QIIME2 was used for bioinformatics analysis as above [51].

2.5 Isolation and reseeding of associated bacteria

The 2216E medium was used for isolation of associated bacteria of *A. catenella* [53]. Each sample was plated in three plates that were incubated at 20°C, 30°C and 37°C respectively, which were then checked for colonies after 24h, 48h and 72h. Individual bacterial colonies showing different colour, colony size or morphology were re-isolated on the same medium and further characterized morphologically and biochemically with microscope and Gram staining method. Finally, the single clones were further identified by 16Sv3v4 sanger sequencing.

The isolated bacteria were then reseeded to axenic *A. catenella* with antibiotic treatment, through ways of separate strain and different combined strains. While *A. catenella* was cultured to logarithmic phase antibiotic treatment was carried out to get axenic algal solution. The isolated bacterial strains were cultured to logarithmic phase and then added to the algal solution.

2.6 Toxicity Type and content test

Taking the algal sap from the logarithmic phase, the algal cells were collected by centrifugation at 6000 rpm·min^{-1} into 50mL polypropylene centrifuge tubes, which were washed and centrifuged several times using ultrapure water to ensure that the culture fluid was thoroughly removed. To the washed algal cells, 20mL of acetic acid solution at a concentration of 0.05 mol/L was added. The algal cells were sonicated under ice bath conditions using an ultrasonic cell

disruptor with the power set at 200W and 20s of sonication paused for 40s, for a total of 5min. After sonication, the extraction was continued using ultrasonic assisted extraction for 15 min. the cells were centrifuged at 4000 rpm·min^{-1} for 10 min at 4 °C, and then 1 mL of the supernatant was removed from the centrifuge tube. The supernatant was transferred to an ultrafiltration centrifuge tube (MWCO of 10,000u) and centrifuged at 10,000 rpm·min^{-1} for 10min at 4°C. Finally, the ultrafiltrate was collected into an injection vial and stored at 4°C for subsequent analysis.

The type and content test of PSTs potentially produced by *A. catenella* and associated bacteria was detected by liquid chromatography-mass spectrometry (LC-MS). Samples were analyzed on an Ultimate 3000 ultra-high-pressure liquid chromatography-Q-Exactive electrostatic field Orbitrap high-resolution mass spectrometer system. The column was TSK-gel Amide-80 (3μm, 2mm×15cm). Algal toxin extraction was performed using acetic acid and ultrasound. The instrumental conditions were determined with reference to the method of Zhang et al.[54].

2.7 Measurements of growth and biochemical composition

The biomass was detected based on cell number using a hemacytometer (improved double-Neubauer). Soluble sugar was determined by phenol-sulfuric acid method. Soluble proteins were determined using the Thomas Brilliant Blue G-250 staining method. The neutral lipid content was determined by Nile red fluorescence method.

2.8 RNA isolation, library construction and transcriptome sequencing

The axenic, non-axenic and non-axenic-ndep *A. catenella* strains were selected for RNA sequencing (RNA-Seq) respectively. Triple replicates of each strain were cultured under the same conditions and were mixed for RNA extraction and sequencing. Total RNA was firstly extracted and qualified by ethanol precipitation protocol and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA) respectively. Then the mRNA was purified, fragmented and cDNA produced. The cDNA was subsequently index ligated and enriched. The product was validated on the Agilent Technologies 2100 bioanalyzer for quality control. Finally, the qualified library was sequenced on the Illumina HiSeq platform. Clean reads were mapped to the assembled unique genes by Bowtie2 (v2.2.5) [55] and the expression levels of genes was calculated by RSEM (v1.2.8) and normalized to the FPKM [56].

Functional annotation of genes was achieved by mapping genes to different databases (NT, NR, GO, COG, KEGG, Swiss-Prot, and InterPro) using the software BLAST (v2.2.23) [57]. GO annotation was performed by Blast2GO (v 2.5.0) with NR annotations. PossionDis [58] was used to detect different expression genes (DEGs), and DEGs with a fold change of >2 or <-2 and a false-discovery rate (FDR) of <0.001 were considered significantly differentially expressed genes. GO and KEGG enrichment analysis were performed using Phyper, a function of R. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q <0.05).

3. Results

3.1 The dinoflagellate community diversity

From the relative abundance of 18S-v4 and 28S ASVs (Figure 1A), it was shown that the Pyrrophyta accounted for a considerable proportion compared to another microalgae phylum, especially higher than Bacillariophyta (Figure 1A). The zooplankton was revealed with a high proportion but the Chlorophyta was uncovered with a low proportion. The dominant dinoflagellate species were mainly included in *Alexandrium*, *Karlodinium*, *Prorocentrum*, Dinophyceae, etc. Thereinto, the *Alexandrium fundyense*, *Alexandrium* sp., *A. catenella* and *Alexandrium tamarense* dominated high abundance (Figure 1B). The phylogenetic tree showed that the *A. catenella* sequences from Shanghai, Shengsi and Zhoushan (Genbank: OR357631-OR357633) clustered as one clade (Figure 2A). The toxin content of *A. catenella* samples from the three locations was different (Figure 2B), consistent with the difference in bacterioplankton (Figure 2C). The *A. catenella* strain with the highest toxin content from Shengsi was selected for detailed experimental analysis.

3.2 The associated bacteria composition of A. catenella

Here the 16S and 16Sv3v4 assignments were combined together to reveal the associated bacteria composition of *A. catenella*. Six phyla, Bacteroidetes, Proteobacteria, Steptophyta, Firmicutes, Bacteroidota and Pseudomonadota, were detected. The dominant classes were Cyanbacteria, Cytophagia, Deltaproteobacteria, Flavobacteriia, Bacilli, Gammaproteobacteria,

Alphaproteobacteria and Bacteroidia. The main orders included Alteromonadales, Flavobacteriales, Cytophagales, Rhodobacterales etc. (Figure 3). The dominant associated bacteria species included *Paenibacillus* sp., *Yangia pacifica*, *Formosa* sp., *Bacillus humi*, *Alteromonas macleodii*, *Marinobacter* sp., *Cesiribacter* sp., *Flavobacteriales bacterium* etc. Among them, *Marinobacter* accounted for the highest proportion in 16S and *Paenibacillus* accounted for the highest proportion in 16Sv3v4 (Figure 4).

3.3 Associated bacteria isolation and capacity of affecting toxin production test

A total of 31 associated bacteria of *A. catenella* were isolated, which were identified to 13 different bacteria species by 16Sv3v4 sequences (Figure 5A). These were *A. macleodii*, *Alteromonas gracilis*, *Alteromonas marina*, *Thalassospira marina*, *Alteromonas* sp., *Exiguobacterium acetylicum*, *Marinobacter* sp., *Cellvibrio* sp., *Spongiibacter* sp., *Saccharospirillum* sp., *Saccharospirillum alexandrill*, *Marinobacter confluentis*, *Marinobacterium rambilcola* (Genbank: OR958743-OR958746, OR960642-OR960647, OR976523-OR976525). The 13 bacteria were assigned to four classes, six orders and eight families (Figure S1).

With LC-MS analysis, it was showed that no PSTs was detected in the mixed associated bacterial culture since there was no same molecular weight with PSTs component was found (Figure 6A). On the other hand, a molecular weight of 395.0878 was detected in the algal culture, which was the same as GTX toxin at the peak time of 6.475min (Figure 6B, C).

The capacity of isolated bacteria for affecting PSTs production of *A. catenella* was tested separately, together with different combined bacteria strains (Figure 5B, C). Under normal cultivation conditions, the non-axenic *A. catenella* strains without antibiotic treatment apparently produced more PSTs than strains with antibiotic treatment (Figure 5B). Generally, the non-axenic *A. catenella* strains with separate and combined isolated bacteria all produced more PSTs than axenic strain, especially for stains with combined bacteria strains (Figure 5B). Moreover, the capacity of isolated bacteria for affecting PSTs production was detected under nitrogen deprivation, to select typical bacteria that affects PSTs production pronouncedly (Figure 5C). It was apparent that the toxin content decreased for almost all treatments under nitrogen deprivation (Figure 5C). Among the 13 bacterial strains, it was showed that *A. catenella* produced more PSTs with five associated bacteria strains, e.g. *E. acetylicum*, *A.* sp., *A. macleodii*, *S. alexandrill and T. marina* (Figure 5B, C).

3.4 The growth and biochemical composition for axenic and non-axenic A. catenella strains

It was shown that the axenic *A. catenella* strain with antibiotic treatment grew slower than non-axenic *A. catenella* strain without antibiotic treatment during the whole growth cycle under normal cultivation conditions (Figure 7A). Thereinto, the non-axenic *A. catenella* strain with nitrogen deprivation (non-axenic-ndep) grew the slowest (Figure 7A). The non-axenic *A. catenella* strain produced more soluble protein, sugar and neutral lipid than axenic *A.*

catenella strain with antibiotic treatment normal cultivation conditions (Figure 7B). On the other hand, the soluble sugar and protein content decreased and the neutral lipid content increased in the non-axenic *A. catenella* strains with nitrogen deprivation (non-axenic-ndep), compared with both axenic and non-axenic *A. catenella* strains under normal cultivation conditions (Figure 7B).

3.5 Genetic regulation for PSTs production with influence of associated bacteria

A total of 45,778,990, 48,518,624 and 43,035,798 reads were generated for each treatment of axenic, non-axenic and non-axenic-ndep *A. catenella* strains respectively (Table 2). The clean read ratio of all samples was approximately 97%. The total number of transcripts for three treatments were 145,580, with a mean length 923 bp and 1382 N50 length. A total of 126,626 unigenes were obtained for three treatments, with a mean length of 879 bp and an N50 length of 1327. Thus, the reads and assembly quality showed a good preformation of transcriptome sequencing for functional analysis.

Among the annotations of the six databases, the Pfam database showed higher annotation proportion and more unique annotation (Figure 8A). Among the transcription factors, the vast majority of unigenes were assigned to C3H, followed by MYB_superfamily (Figure 8B). The axenic, non-axenic and non-axenic-ndep strains all produced abundant common and distinct unigenes (Figure 8C). The up-regulated and down-regulated genes among three *A. catenella* treatments were different (Figure 8D). The GO functional annotations was performed for all unigenes where the membrane part, cell part, cellular progress,

metabolic process, binding and catalytic activity accounted for the top classification categories (Figure S2). The KEGG database assigned the Carbohydrate metabolism, Amino acid metabolism, Translation and Folding, sorting and degradation as the top annotation (Figure S3). It was indicated that the biosynthesis of cofactors was the dominant KEGG pathway annotated between axenic and non-axenic strains (Figure 9A). The dominant DEGs included Glutathione metabolism, Plant-pathogen interaction, Arginine and proline metabolism, Tryptophan metabolism, and spliceosome, etc. (Figure 9B). The PSTs synthesis genes sxtA was found in *A. catenella* strains of all three treatments, which were expressed highly in non-axenic strain (Figure 9B). The main pathways enriched between non-axenic and non-axenic-ndep strains were Phenylpropanoid biosynthesis, Spliceosome, etc. (Figure S4).

The pathway network related with PSTs production was further constructed among axenic, non-axenic and non-axenic-ndep strains, including variable physiological metabolism and PSTs production genes (Figure 10). The pathways Arginine and proline metabolism, Arginine biosynthesis, Fatty acid biosynthesis, TCA cycle and Glutathione metabolism were closely related with sxtA expression, which were all downregulated in axenic strain (Figure 10A). As expected, the plant hormone signal transduction and Indole-3-acetic acid-amido synthetase were up-regulated in non-axenic strain (Figure 10A). Similar with non-axenic vs axenic comparison, generally the non-axenic *A. catenella* strains with nitrogen deprivation produced more downregulated genes than non-axenic *A. catenella*

strains under normal cultivation conditions in the various pathways of Glycolysis, TCA cycle, Arginine biosynthesis, Fatty acid biosynthesis, etc. (Figure 10B). Some potential functional genes connecting various pathways related with PSTs production were found, like arginine deiminase and ornithine decarboxylase among Arginine biosynthesis, Arginine and proline metabolism and Glutathione metabolism.

4. Discussion

The PSTs production by dinoflagellate is associated with bacteria [59, 60]. The PSTs control by microbes is becoming important since the microbes play a key role in microalgae' growth, metabolism and toxin production [61-63]. Elucidating the associative mechanism between dinoflagellate and associated bacteria can control the PSTs production of dinoflagellate in environmental ways.

The metabarcoding results here indicated that the dinoflagellate and *Alexandrium* were dominant microalgae phylum and species in Yangtze Estuary, suggesting that the PSTs produced by dinoflagellate in China coast is becoming serious. Actually, the dominant phytoplankton in Yangtze Estuary has been transferred from diatom to dinoflagellates in recent years [64]. The toxin-producing capacity and associated bacteria community diversity of *A. catenella* from three Yangtze Estuary locations both differed from each other, indicating that the associated bacteria possibly affect the PSTs production of *A. catenella*. Green et al. (2010) also found that the associated bacteria diversity of *G. catenatum* was related with environmental factors and speculated that the

associated bacteria regulated the physiological ecological progress to affect PSTs production.

The LC-MS detection showed that the PSTs produced by *A. catenella* was GTX and the associated bacteria did not produce any component of PSTs. This is consistent with previous reports that the associated bacteria did not produce PSTs and the "PSTs" detected from bacteria may be the analogues of PSTs or false positive [43, 44]. Apparently, the PSTs production capacity of axenic *A. catenella* strains with antibiotic treatment decreased compared with non-axenic strains, which is consistent with previous reports that the associated bacteria affected the PSTs production of dinoflagellate [45-48].

The capacity of associated bacteria for affecting PSTs production was further detected. The *A. catenella* stains co-cultured with separate and combined associated bacteria both produced higher levels of PSTs than axenic *A. catenella* stains, especially with combined associated bacteria. Albinsson et al. [45] also demonstrated that different bacterial communities had different effects on *G.catenatum* toxin production. These further confirm that the associated bacteria affect the PSTs production of dinoflagellate. In this case, it is important to untangle the regulation mechanism of bacteria for PSTs production of dinoflagellate.

The axenic *A. catenella* strains grew slowly compared with non-axenic strains, indicating that the associated bacteria also affect the growth of dinoflagellate. Likewise, the bacterial associates can modify growth dynamics of

Gymnodinium catenatum [65]. These support the concept that algal–bacterial interaction is an important structuring mechanism in phytoplankton communities. Also the contents of soluble sugar, soluble protein and neutral lipid in non-axenic *A. catenella* strains were somewhat higher than that in axenic strains. All these suggest that the associated bacteria affect the PSTs production of dinoflagellate by regulating growth and physiological metabolism [38-40]. On the other hand, the PST production capacity of non-axenic *A. catenella* strains under nitrogen deprivation were both decreased compared to axenic and non-axenic strains under normal culture conditions, indicating that nitrogen is an important factor for toxin production of dinoflagellate since it is the main component of PSTs [28-30, 32].

The sxt gene cluster for STX synthesis has been elucidated in cyanobacteria [17, 66-68]. Among the 26 sxt genes revealed in cyanobacteria, only several sxt genes were found in dinoflagellate, including sxtA, sxtG and sxtO [69]. The biosynthesis of PSTs starts with sxtA [66, 69]. However, the functional mechanism of sxt for PSTs production is still unclear in dinoflagellate. The polyketide synthase-like (PKS) is another important catalyzing enzyme for carbon skeleton formation of algae toxins [70]. Here, both sxtA and PKS were both included in the DEGs. We found that the sxtA expression was related with TCA cycle, Arginine biosynthesis, proline metabolism, Glutathione metabolism, and Fatty acid biosynthesis, which were all downregulated in axenic strains. Also, Arginine is the main elements of PSTs. Thus, here it is clear that the associated bacteria enhance PSTs production of *A. catenella* by upregulating the expression

of sxtA genes and their associated physiological pathways. Other sxt genes, such as sxtG and sxtO, were not detected in this study, which may be related with the intraspecific difference that different strains within one species have different toxicity and possible regulation mechanism [59, 71]. Consistent with the physiological analysis, the genetic expression related with PSTs production was generally downregulated in *A. catenella* strain with nitrogen deprivation, confirming the nitrogen importance in molecular regulation.

Compared with axenic strain, the Tryptophan metabolism and Plant hormone signal transduction were also upregulated in non-axenic *A. catenella* strain, consistent with their growth tendency. This indicates that the associated bacteria can also promote the growth of *A. catenella* except the toxin production. It has already been reported that the associated bacteria advanced the growth of diatom by releasing signal molecules [72, 73]. Finally, some functional genes that were possibly related with the PSTs production were revealed. So, the future studies will focus on uncovering the determinant signal molecular released by associated bacteria and their related regulation pathways for affecting PSTs production of dinoflagellates.

5. Conclusion

The toxin production capacity, physiological process and molecular regulation among *A. catenella* strains of axenic, non-axenic and non-axenic with nitrogen deprivation were compared. Thirteen associated bacteria for affecting PSTs production of *A. catenella* were selected. Compared with axenic strain with

antibiotic treatment, the non-axenic strain with associated bacteria produced more PSTs contents, soluble sugar, soluble protein and neutral lipid. The biosynthesis of cofactors and spliceosome were the dominant pathways between axenic and non-axenic strains. The pathways Arginine and proline metabolism, Arginine biosynthesis, Fatty acid biosynthesis, TCA cycle and Glutathione metabolism were closely related with sxtA expression. The nitrogen plays key role in PSTs production of *A. catenella* through affecting physiological and molecular regulation. These findings highlight the importance of associated bacteria in affecting PSTs production of dinoflagellate through physiological and molecular regulation.

Credit authorship contribution statement

Shanmei Zou conceived the design and wrote the manuscript. Xinke Yu conducted the experiments. Shanmei Zou, Xinke Yu, Tiantian Sun analyzed the data. Xinke Yu and Xuemin Wu collected the samples.

Declaration of competing interest

The authors have no conflict of interest.

Data availability

The NGS sequence data of all samples obtained in this study are deposited in NCBI. The SRA accession for the NGS sequences is PRJNA992270, PRJNA998555. The submission information for each sample mainly includes the reads sequence in fastq form, the sampling location, the sequence platform.

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659
660

Figure legends

Figure 1. The community diversity of dinoflagellate in Yangtze Estuary. (A): ASV mean relative abundance; (B): Dominant dinoflagellate species.

Figure 2. (A): Maximum-likelihood phylogenetic tree of *A. catenella* from 18S. (B): The toxin content of *A. catenella* samples from three locations; (C): The associated bacteria difference of *A. catenella* samples from three locations. The points represent means ± SD of triplicate samples.

Figure 3. Associated bacteria composition of *A. catenella* from 16S and 16Sv3v4 at phylum, class, order taxonomic level.

Figure 4. Associated bacteria composition of *A. catenella* from 16S and 16Sv3v4 at family, genus, species taxonomic level.

Figure 5. (A): Maximum-likelihood phylogenetic tree of isolated associated bacteria of toxic *A. catenella* strain. (B): The influence of isolated bacteria on toxin production of *A. catenella* under normal culture conditions. (C): The influence of isolated bacteria on toxin production of *A. catenella* under nitrogen deprivation conditions. 1-13 indicates the bacteria serial number for reseeding with *A. catenella*.

Figure 6. (A): LC-MS mass spectral peaks for mixed bacterial solution. Vertical coordinate is mean relative abundance, horizontal coordinate is time. (B): LC-MS mass spectral peaks of the toxic *A. catenella* strain; (C): The molecular weight of detected GTX toxins (395.0878) from LC-MS peaks of toxic *A. catenella* strain.

Figure 7. (A): Growth of axenic, non-axenic and non-axenic-ndep *A. catenella* strains; (B): The content of soluble protein, sugar and neutral lipid from non-axenic and non-axenic-ndep *A. catenella* strains. The points represent means ± SD of triplicate samples. Different letters above the bars indicate significant differences ($P < 0.05$) between growth condition.

Figure 8. (A): Annotation difference of six databases; (B): Transcription factors annotated from unigenes; (C): Shared and distinct unigenes from axenic, non-axenic and non-axenic-ndep *A. catenella* strains; (D): Up and down-regulated genes among axenic, non-axenic and non-axenic-ndep *A. catenella* strains. For example, non-axenic vs axenic comparison shows that non-axenic strains generated more downregulated genes (green) than upregulated genes (orange) in comparison with axenic strain.

Figure 9. (A): Biosynthesis of cofactors annotated between axenic and non-axenic *A. catenella* strains from KEGG pathway; (B): The dominant different expression genes (DEGs) among axenic, non-axenic and non-axenic-ndep *A. catenella* strains.

Figure 10. The pathway network related with genetic expression of PSTs production. Red box and circle: upregulated genes, green box and circle: downregulated genes. For example, “non-axenic vs axenic” means the upregulated and downregulated genes non-axenic strains produced compared with axenic strain. The size of box and circle indicates the amount of upregulated and downregulated genes.

Figure S1. Assignments of isolated associated bacteria at each taxonomic level.

Figure S2. The GO functional annotation performed for all unigenes.

Figure S3. The KEGG pathways assigned.

Figure S4. The main pathways enriched between non-axenic and non-axenic-ndep *A. catenella* strains.

Table 1 Primers for PCR amplification

Fragment	Primer Name	Sequence (5'-3')	Reference	Fragment size (bp)
16S-v3v4	16Sv3v4F	CCTACGGGNGGCWGCAG	Klindworth et al., 2012	450-500
	16Sv3v4R	GACTACHVGGGTATCTAATCC	Klindworth et al., 2013	
16S	16SF174F	CGGTAAGACRGRGGATGCAA	Zou et al., 2020	450
	16SR316R	CRGCTARGACTACTGGGGTA	Zou et al., 2020	
18S-v4-5	v4-387F	GYTGCAGTTAAAAMGCTCGT	Zou et al., 2020	500-520
	v4-730R	TTCAGNCTTGCGACCATACT	Zou et al., 2020	
28S	1FC1RF272F	GAGACCGATAGCRMACAAGT	Zou et al., 2020	400-500
	1FC1RF593R	CTYGGTCCGTGTTTCWAGAC	Zou et al., 2020	

Table 2 Sequencing data statistics table

Sample	Raw reads	Clean reads	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
non-axenic-ndep	45778990	45067136	0.0234	98.68	95.83	56.25
non-axenic	48518624	48028712	0.024	98.44	95.15	58.51
axenic	43035798	42337016	0.0245	98.25	94.76	56.55

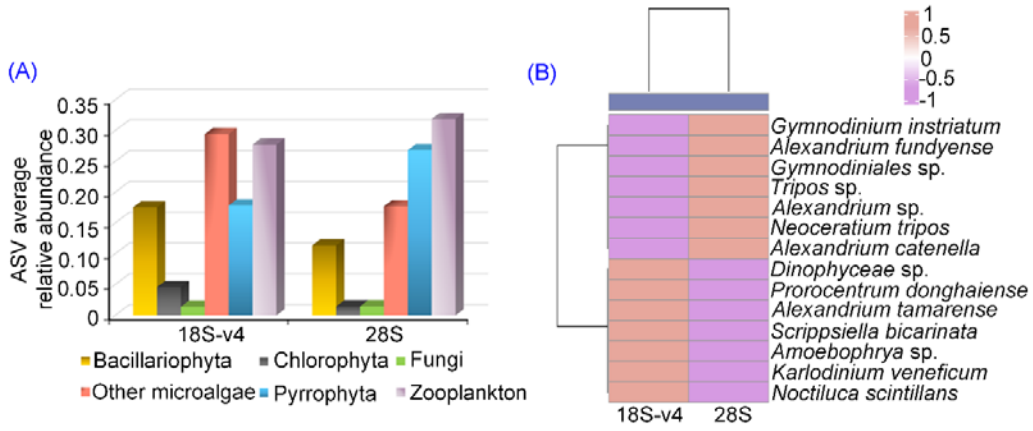
(A)
ASV average relative abundance
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
18S-v4
28S
Bacillariophyta
Chlorophyta
Fungi
Other microalgae
Pyrrophyta
Zooplankton
(B)
1
0.5
0
-0.5
-1
Gymnodinium instriatum
Alexandrium fundyense
Gymnodiniales sp.
Tripos sp.
Alexandrium sp.
Neoceratium tripos
Alexandrium catenella
Dinophyceae sp.
Prorocentrum donghaiense
Alexandrium tamarense
Scrippsiella bicarinata
Amoebophrya sp.
Karlodinium veneficum
Noctiluca scintillans
18S-v4
28S

Figure 1

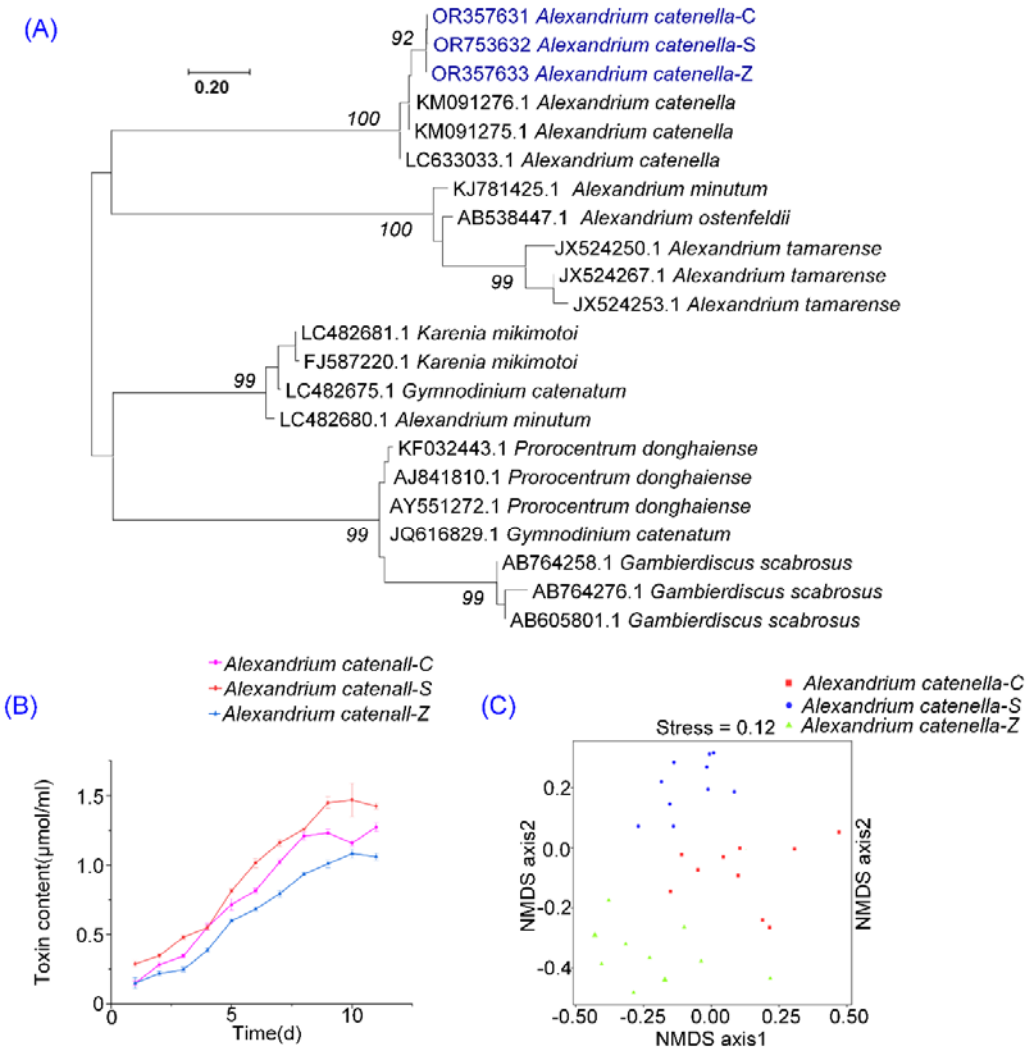
(A)
0.20
OR357631 Alexandrium catenella-C
OR753632 Alexandrium catenella-S
OR357633 Alexandrium catenella-Z
KM091276.1 Alexandrium catenella
KM091275.1 Alexandrium catenella
LC633033.1 Alexandrium catenella
KJ781425.1 Alexandrium minutum
AB538447.1 Alexandrium ostenfeldii
JX524250.1 Alexandrium tamarense
JX524267.1 Alexandrium tamarense
JX524253.1 Alexandrium tamarense
LC482681.1 Karenia mikimotoi
FJ587220.1 Karenia mikimotoi
LC482675.1 Gymnodinium catenatum
LC482680.1 Alexandrium minutum
KF032443.1 Prorocentrum donghaiense
AJ841810.1 Prorocentrum donghaiense
AY551272.1 Prorocentrum donghaiense
JQ616829.1 Gymnodinium catenatum
AB764258.1 Gambierdiscus scabrosus
AB764276.1 Gambierdiscus scabrosus
AB605801.1 Gambierdiscus scabrosus
92
100
100
99
99
99
99
(B)
Alexandrium catenall-C
Alexandrium catenall-S
Alexandrium catenall-Z
Toxin content(μmol/ml)
Time(d)
(C)
Alexandrium catenella-C
Alexandrium catenella-S
Alexandrium catenella-Z
Stress = 0.12
NMDS axis2
NMDS axis1

Figure 2

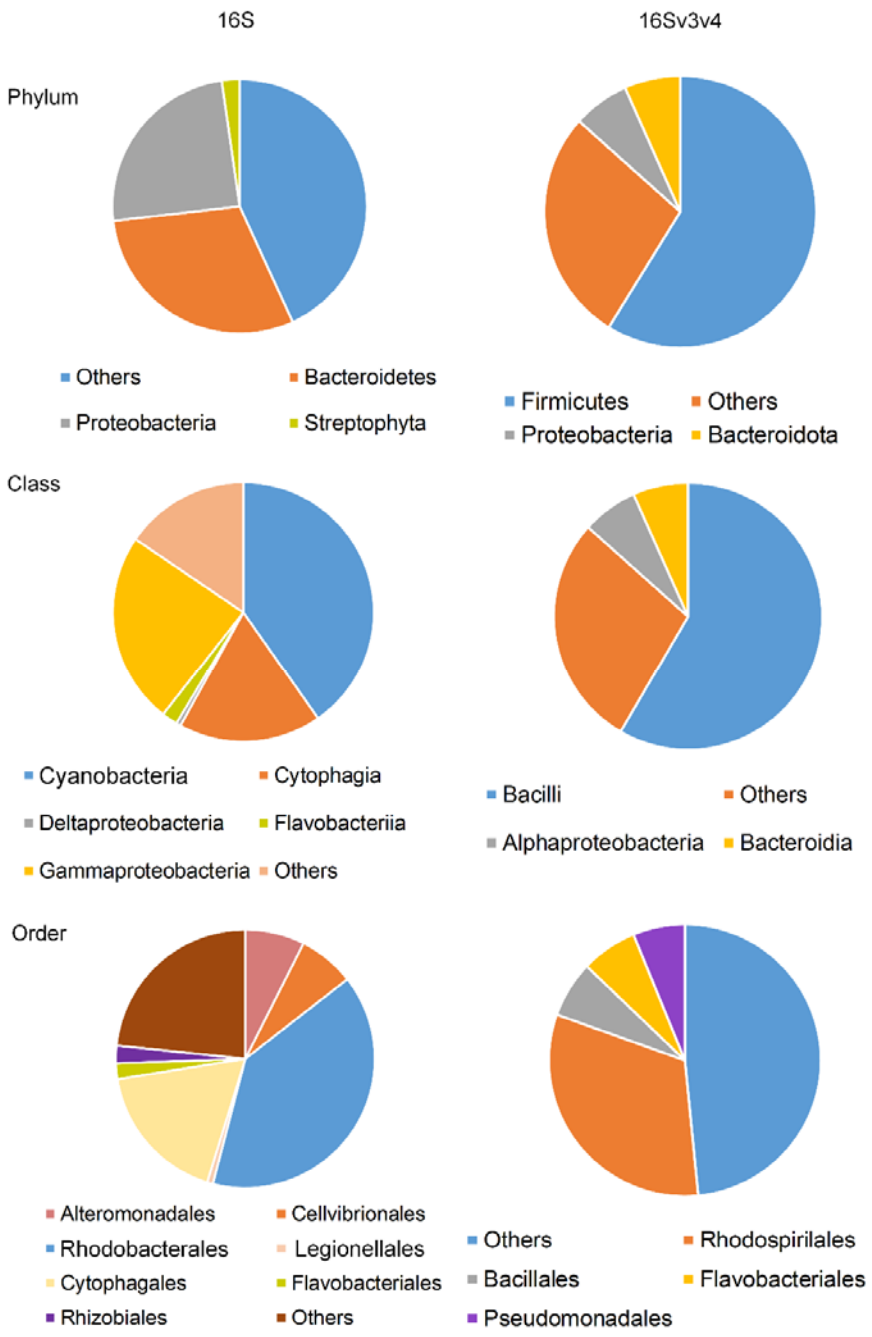
16S
16Sv3v4
Phylum
Others
Bacteroidetes
Proteobacteria
Streptophyta
Firmicutes
Others
Proteobacteria
Bacteroidota
Class
Cyanobacteria
Cytophagia
Deltaproteobacteria
Flavobacteriia
Gammaproteobacteria
Others
Bacilli
Others
Alphaproteobacteria
Bacteroidia
Order
Alteromonadales
Cellvibrionales
Rhodobacterales
Legionellales
Cytophagales
Flavobacteriales
Rhizobiales
Others
Others
Rhodospirilales
Bacillales
Flavobacteriales
Pseudomonadales

Figure 3

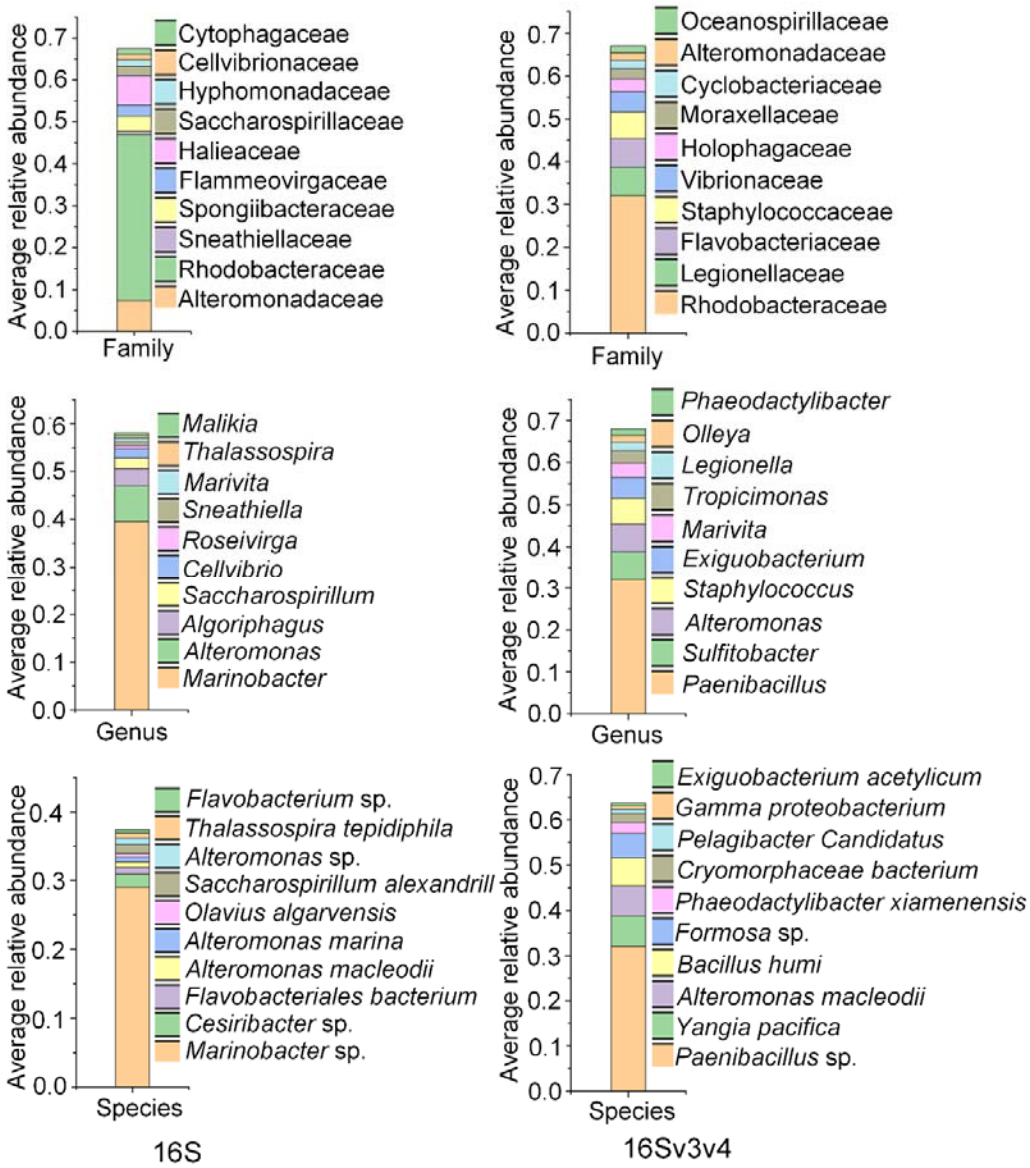
Average relative abundance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Family
Cytophagaceae
Cellvibrionaceae
Hyphomonadaceae
Saccharospirillaceae
Halieaceae
Flammeovirgaceae
Spongiibacteraceae
Sneathiellaceae
Rhodobacteraceae
Alteromonadaceae
Oceanospirillaceae
Alteromonadaceae
Cyclobacteriaceae
Moraxellaceae
Holophagaceae
Vibrionaceae
Staphylococcaceae
Flavobacteriaceae
Legionellaceae
Rhodobacteraceae
Genus
Malikia
Thalassospira
Marivita
Sneathiella
Roseivirga
Cellvibrio
Saccharospirillum
Algoriphagus
Alteromonas
Marinobacter
Phaeodactylibacter
Olleya
Legionella
Tropicimonas
Marivita
Exiguobacterium
Staphylococcus
Alteromonas
Sulfitobacter
Paenibacillus
Species
Flavobacterium sp.
Thalassospira tepidiphila
Alteromonas sp.
Saccharospirillum alexandrill
Olavius algarvensis
Alteromonas marina
Alteromonas macleodii
Flavobacteriales bacterium
Cesiribacter sp.
Marinobacter sp.
Exiguobacterium acetylicum
Gamma proteobacterium
Pelagibacter Candidatus
Cryomorphaceae bacterium
Phaeodactylibacter xiamenensis
Formosa sp.
Bacillus humi
Alteromonas macleodii
Yangia pacifica
Paenibacillus sp.
16S
16Sv3v4

Figure 4

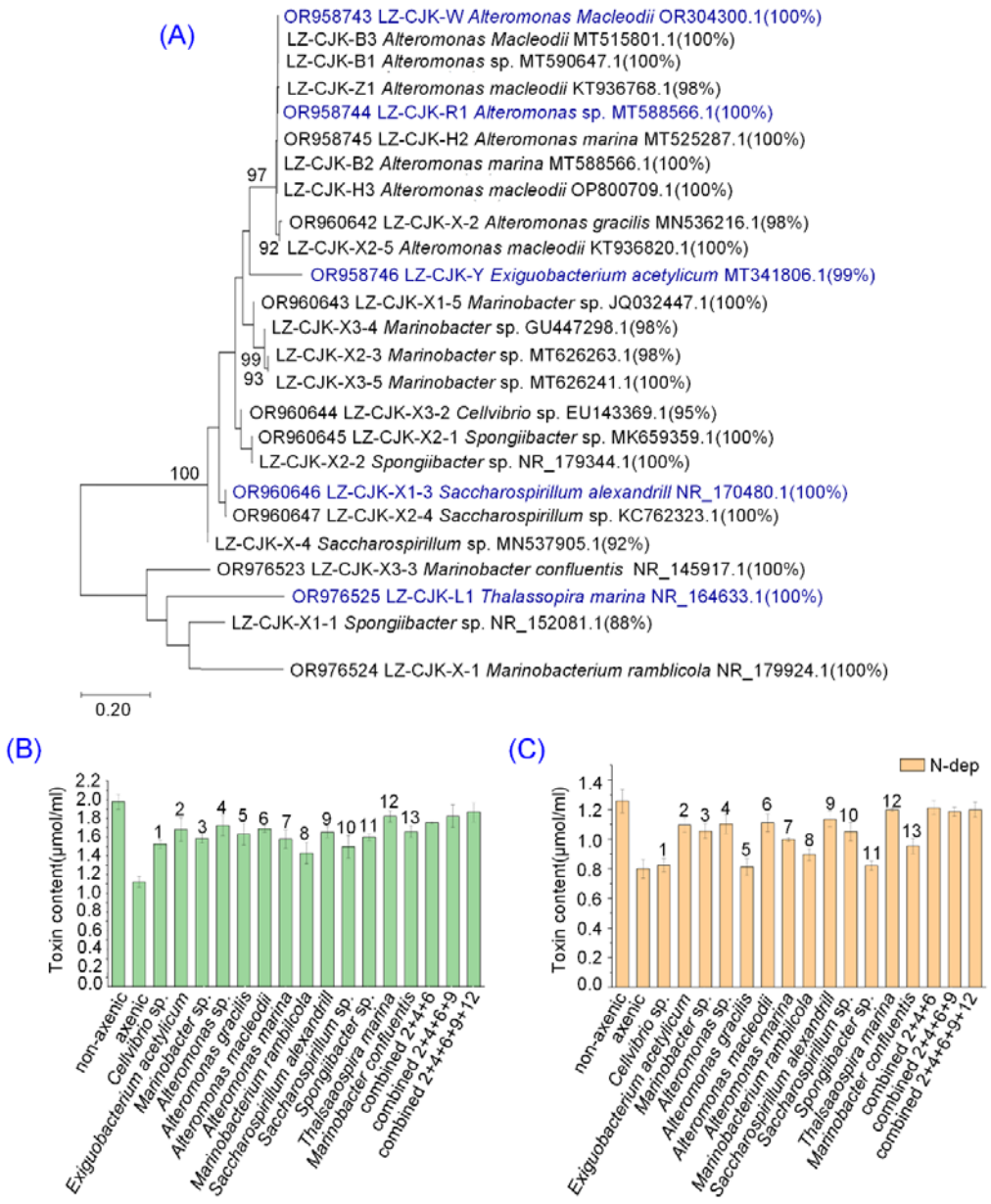
(A)
OR958743 LZ-CJK-W Alteromonas Macleodii OR304300.1(100%)
LZ-CJK-B3 Alteromonas Macleodii MT515801.1(100%)
LZ-CJK-B1 Alteromonas sp. MT590647.1(100%)
LZ-CJK-Z1 Alteromonas macleodii KT936768.1(98%)
OR958744 LZ-CJK-R1 Alteromonas sp. MT588566.1(100%)
OR958745 LZ-CJK-H2 Alteromonas marina MT525287.1(100%)
LZ-CJK-B2 Alteromonas marina MT588566.1(100%)
LZ-CJK-H3 Alteromonas macleodii OP800709.1(100%)
OR960642 LZ-CJK-X-2 Alteromonas gracilis MN536216.1(98%)
LZ-CJK-X2-5 Alteromonas macleodii KT936820.1(100%)
OR958746 LZ-CJK-Y Exiguobacterium acetylicum MT341806.1(99%)
OR960643 LZ-CJK-X1-5 Marinobacter sp. JQ032447.1(100%)
LZ-CJK-X3-4 Marinobacter sp. GU447298.1(98%)
LZ-CJK-X2-3 Marinobacter sp. MT626263.1(98%)
LZ-CJK-X3-5 Marinobacter sp. MT626241.1(100%)
OR960644 LZ-CJK-X3-2 Cellvibrio sp. EU143369.1(95%)
OR960645 LZ-CJK-X2-1 Spongiibacter sp. MK659359.1(100%)
LZ-CJK-X2-2 Spongiibacter sp. NR_179344.1(100%)
OR960646 LZ-CJK-X1-3 Saccharospirillum alexandrill NR_170480.1(100%)
OR960647 LZ-CJK-X2-4 Saccharospirillum sp. KC762323.1(100%)
LZ-CJK-X-4 Saccharospirillum sp. MN537905.1(92%)
OR976523 LZ-CJK-X3-3 Marinobacter confluentis NR_145917.1(100%)
OR976525 LZ-CJK-L1 Thalassopira marina NR_164633.1(100%)
LZ-CJK-X1-1 Spongiibacter sp. NR_152081.1(88%)
OR976524 LZ-CJK-X-1 Marinobacterium ramblicola NR_179924.1(100%)
97
92
99
93
100
0.20
(B)
Toxin content(μmol/ml)
non-axenic
axenic
Cellvibrio sp.
Exiguobacterium acetylicum
Marinobacter sp.
Alteromonas sp.
Alteromonas gracilis
Alteromonas macleodii
Alteromonas marina
Marinobacterium ramblicola
Saccharospirillum alexandrill
Saccharospirillum sp.
Spongiibacter sp.
Thalsaaospira marina
Marinobacter confluentis
combined 2+4+6
combined 2+4+6+9
combined 2+4+6+9+12
(C)
N-dep
Toxin content(μmol/ml)

Figure 5

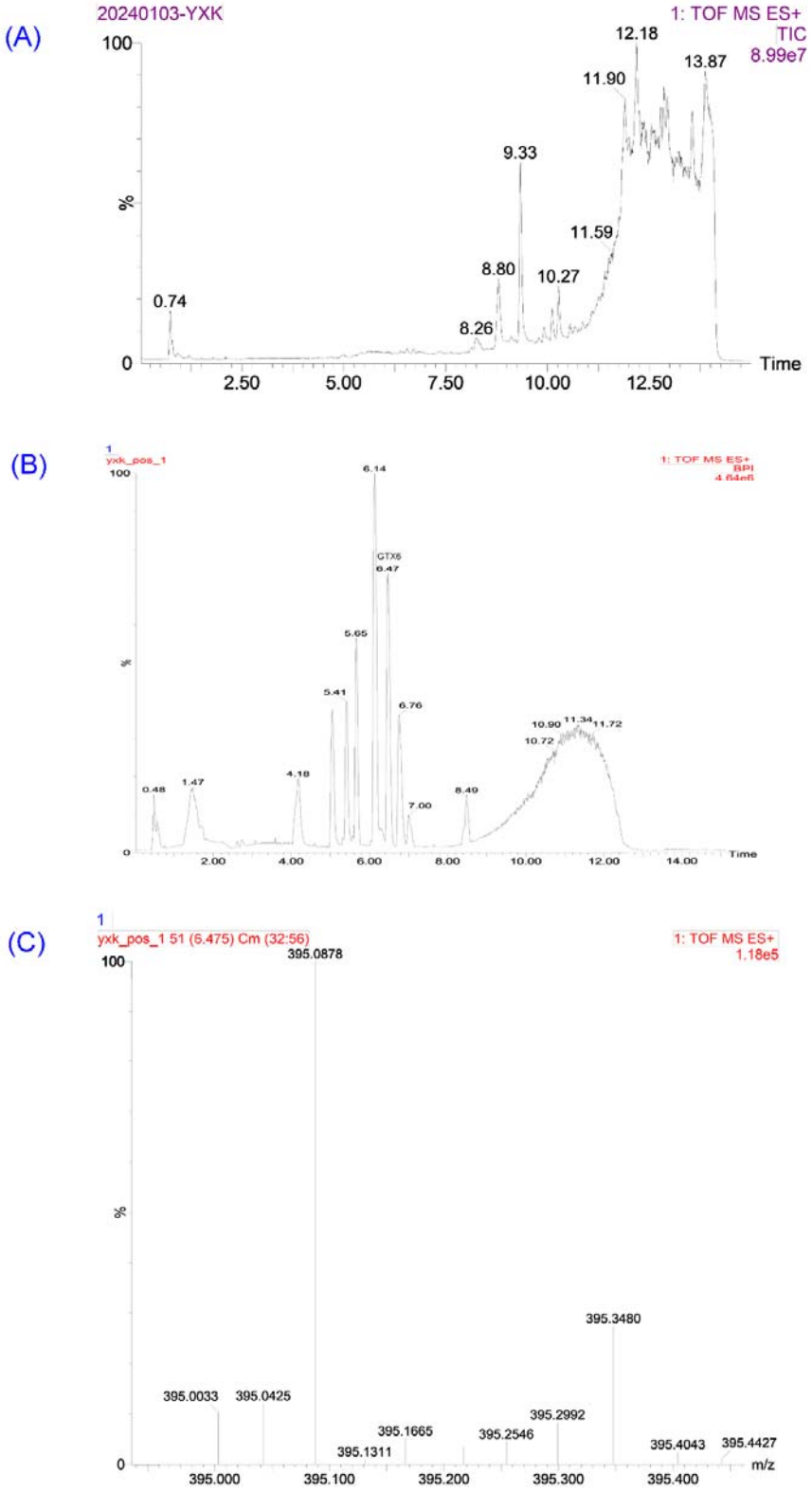
(A)
20240103-YXK
1: TOF MS ES+
TIC
8.99e7
100
%
0
0.74
8.26
8.80
9.33
10.27
11.59
11.90
12.18
13.87
2.50
5.00
7.50
10.00
12.50
Time
(B)
1
yxk_pos_1
1: TOF MS ES+
BPI
4.64e6
100
%
0
0.48
1.47
4.18
5.41
5.65
6.14
GTX6
6.47
6.76
7.00
8.49
10.72
10.90
11.34
11.72
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Time
(C)
1
yxk_pos_1 51 (6.475) Cm (32:56)
1: TOF MS ES+
1.18e5
100
%
0
395.0878
395.0033
395.0425
395.1311
395.1665
395.2546
395.2992
395.3480
395.4043
395.4427
395.000
395.100
395.200
395.300
395.400
m/z

Figure 6

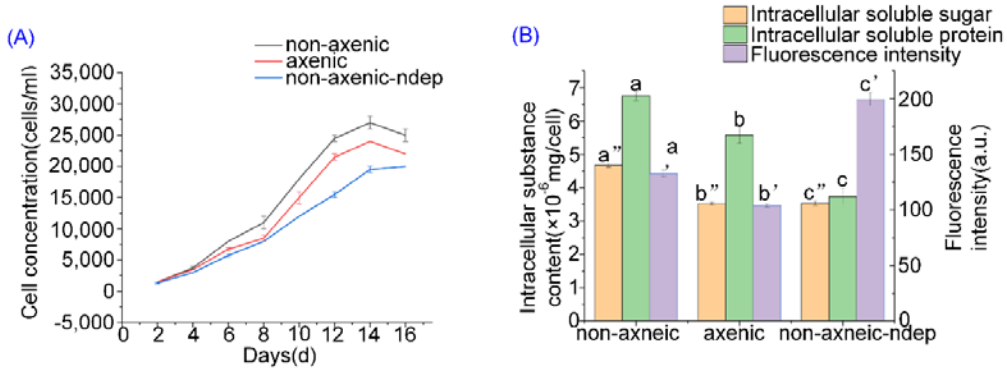
(A)
non-axenic
axenic
non-axenic-ndep
Cell concentration(cells/ml)
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
-5,000
0 2 4 6 8 10 12 14 16
Days(d)
(B)
Intracellular soluble sugar
Intracellular soluble protein
Fluorescence intensity
Intracellular substance content(×10-6 mg/cell)
Fluorescence intensity(a.u.)
a"
a
a'
b"
b
b'
c"
c
c'
non-axneic
axenic
non-axneic-ndep

Figure 7

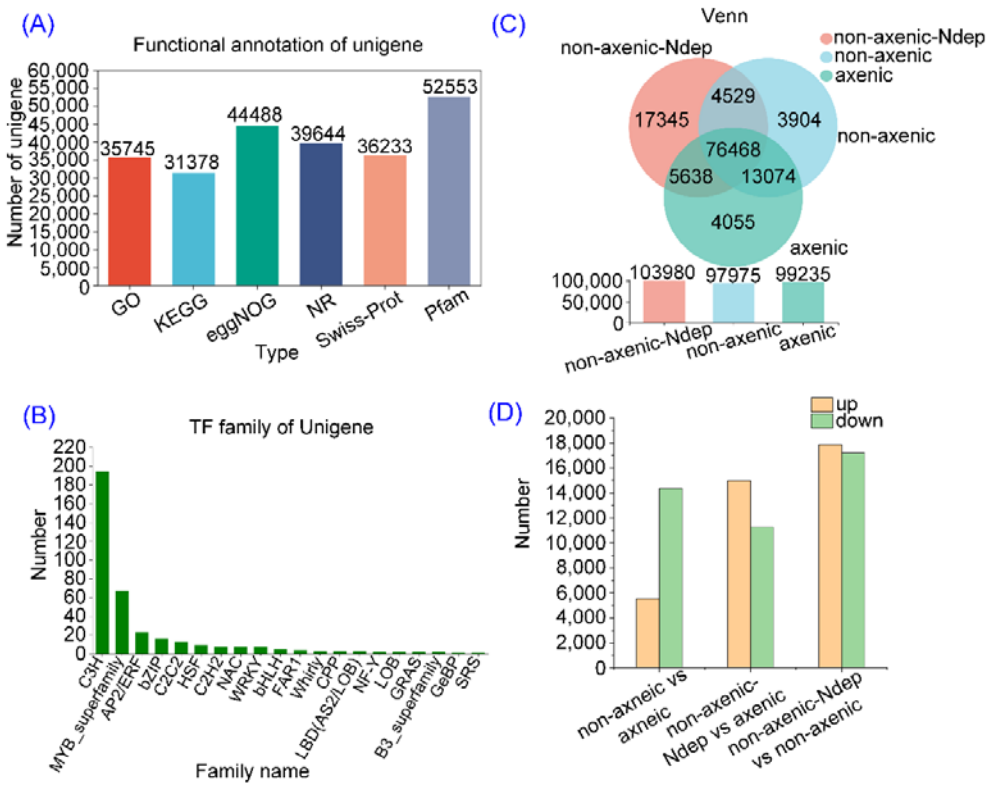
(A)
Functional annotation of unigene
Number of unigene
60,000
55,000
50,000
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
35745
31378
44488
39644
36233
52553
GO
KEGG
eggNOG
NR
Swiss-Prot
Pfam
Type
(C)
Venn
non-axenic-Ndep
non-axenic-Ndep
non-axenic
axenic
17345
4529
3904
non-axenic
76468
5638
13074
4055
axenic
100,000
50,000
0
103980
97975
99235
non-axenic-Ndep
non-axenic
axenic
(B)
TF family of Unigene
Number
220
200
180
160
140
120
100
80
60
40
20
0
C3H
MYB_superfamily
AP2/ERF
bZIP
C2C2
HSF
C2H2
NAC
WRKY
bHLH
FAR1
Whirly
CPP
LBD(AS2/LOB)
NF-Y
LOB
GRAS
B3_superfamily
GeBP
SRS
Family name
(D)
up
down
Number
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
non-axneic vs axneic
non-axenic-Ndep vs axenic
non-axenic-Ndep vs non-axenic

Figure 8

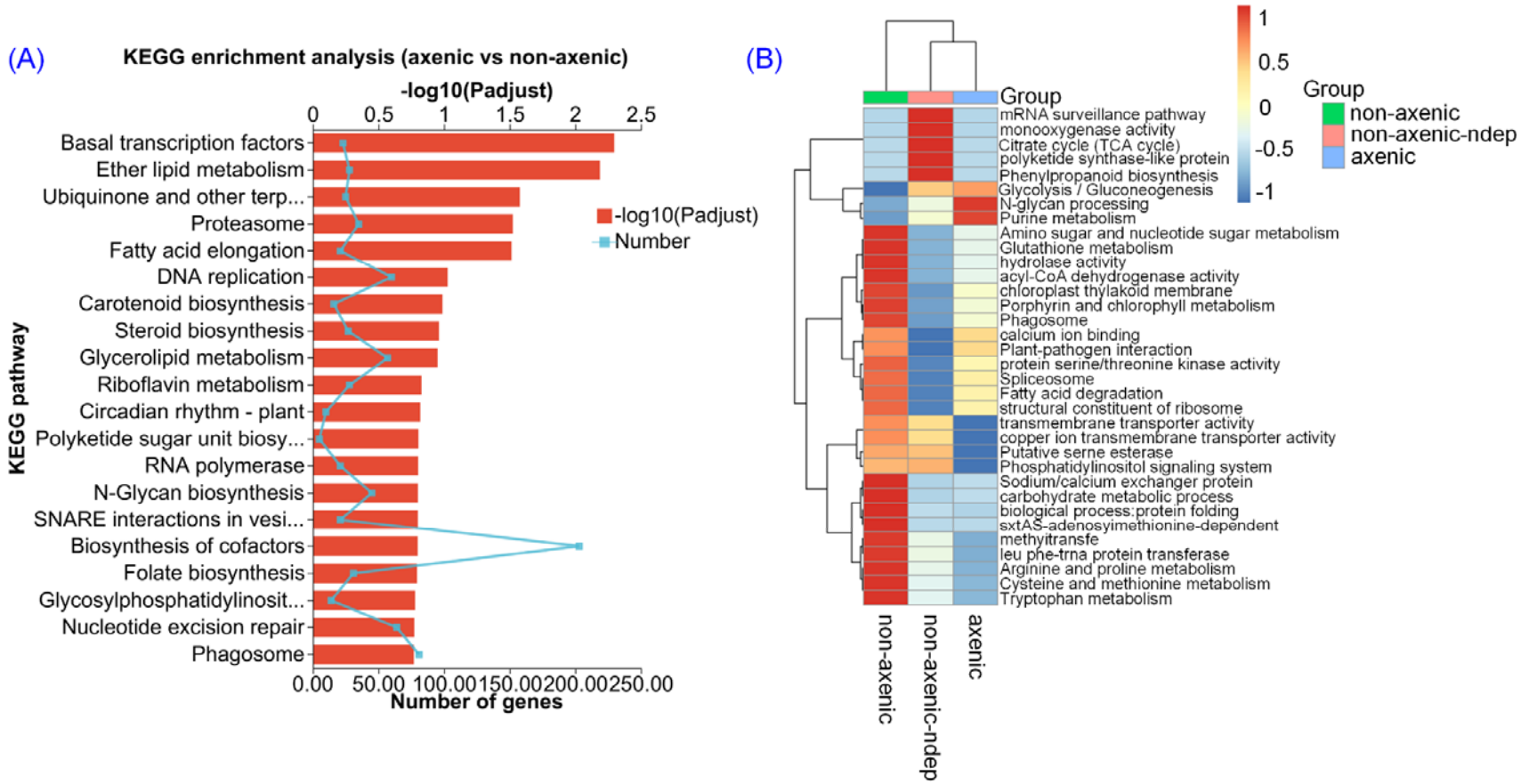
(A)
KEGG enrichment analysis (axenic vs non-axenic)
-log10(Padjust)
0
0.5
1
1.5
2
2.5
Basal transcription factors
Ether lipid metabolism
Ubiquinone and other terp...
Proteasome
Fatty acid elongation
DNA replication
Carotenoid biosynthesis
Steroid biosynthesis
Glycerolipid metabolism
Riboflavin metabolism
Circadian rhythm - plant
Polyketide sugar unit biosy...
RNA polymerase
N-Glycan biosynthesis
SNARE interactions in vesi...
Biosynthesis of cofactors
Folate biosynthesis
Glycosylphosphatidylinosit...
Nucleotide excision repair
Phagosome
KEGG pathway
-log10(Padjust)
Number
0.00
50.00
100.00
150.00
200.00
250.00
Number of genes
(B)
Group
mRNA surveillance pathway
monooxygenase activity
Citrate cycle (TCA cycle)
polyketide synthase-like protein
Phenylpropanoid biosynthesis
Glycolysis / Gluconeogenesis
N-glycan processing
Purine metabolism
Amino sugar and nucleotide sugar metabolism
Glutathione metabolism
hydrolase activity
acyl-CoA dehydrogenase activity
chloroplast thylakoid membrane
Porphyrin and chlorophyll metabolism
Phagosome
calcium ion binding
Plant-pathogen interaction
protein serine/threonine kinase activity
Spliceosome
Fatty acid degradation
structural constituent of ribosome
transmembrane transporter activity
copper ion transmembrane transporter activity
Putative serne esterase
Phosphatidylinositol signaling system
Sodium/calcium exchanger protein
carbohydrate metabolic process
biological process:protein folding
sxtAS-adenosyimethionine-dependent
methyitransfe
leu phe-trna protein transferase
Arginine and proline metabolism
Cysteine and methionine metabolism
Tryptophan metabolism
non-axenic
non-axenic-ndep
axenic
1
0.5
0
-0.5
-1
Group
non-axenic
non-axenic-ndep
axenic

Figure 9

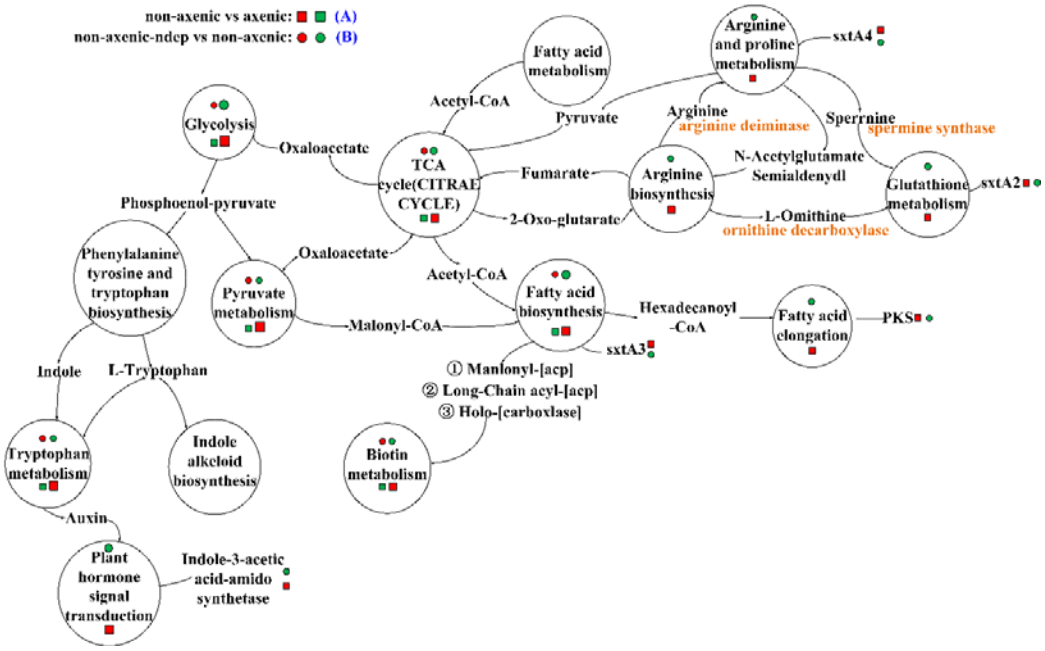
non-axenic vs axenic: (A)
non-axenic-ndep vs non-axenic: (B)
Fatty acid metabolism
Arginine and proline metabolism
sxtA4
Acetyl-CoA
Pyruvate
Arginine
arginine deiminase
Sperrnine
spermine synthase
Glycolysis
Oxaloacetate
TCA cycle(CITRAE CYCLE)
Fumarate
Arginine biosynthesis
N-Acetylglutamate Semialdenydl
Glutathione metabolism
sxtA2
Phosphoenol-pyruvate
2-Oxo-glutarate
L-Omithine
ornithine decarboxylase
Phenylalanine tyrosine and tryptophan biosynthesis
Oxaloacetate
Acetyl-CoA
Pyruvate metabolism
Malonyl-CoA
Fatty acid biosynthesis
Hexadecanoyl -CoA
Fatty acid elongation
PKS
sxtA3
Indole
L-Tryptophan
① Manlonyl-[acp]
② Long-Chain acyl-[acp]
③ Holo-[carboxlase]
Tryptophan metabolism
Indole alkcloid biosynthesis
Biotin metabolism
Auxin
Plant hormone signal transduction
Indole-3-acetic acid-amido synthetase

Figure 10

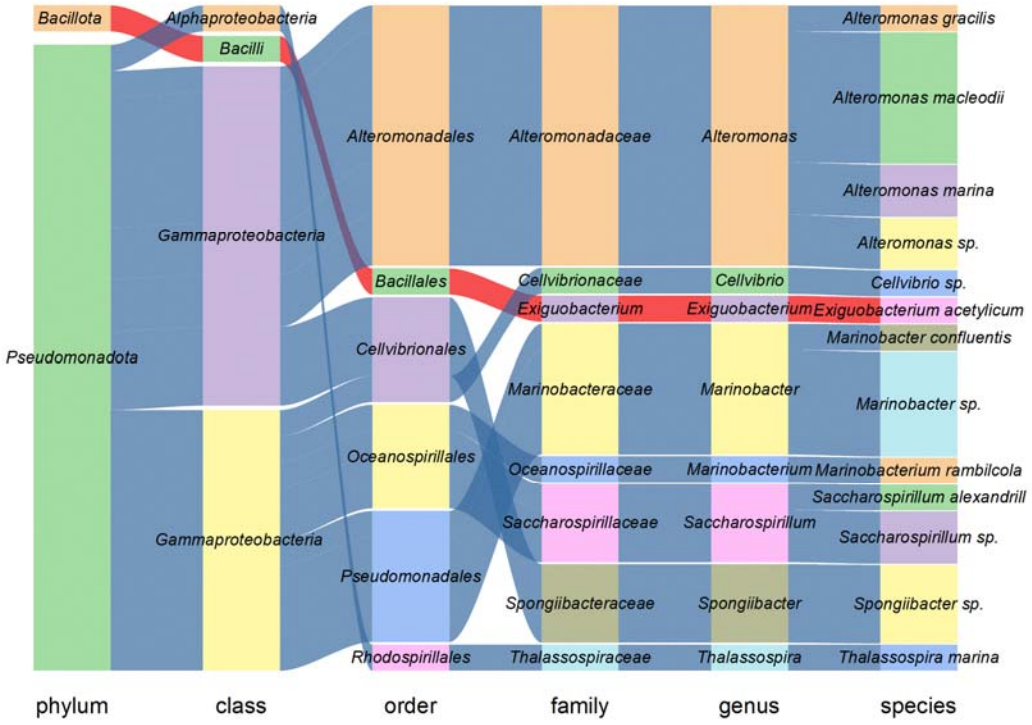
Bacillota
Pseudomonadota
Alphaproteobacteria
Bacilli
Gammaproteobacteria
Gammaproteobacteria
Alteromonadales
Bacillales
Cellvibrionales
Oceanospirillales
Pseudomonadales
Rhodospirillales
Alteromonadaceae
Cellvibrionaceae
Exiguobacterium
Marinobacteraceae
Oceanospirillaceae
Saccharospirillaceae
Spongiibacteraceae
Thalassospiraceae
Alteromonas
Cellvibrio
Exiguobacterium
Marinobacter
Marinobacterium
Saccharospirillum
Spongiibacter
Thalassospira
Alteromonas gracilis
Alteromonas macleodii
Alteromonas marina
Alteromonas sp.
Cellvibrio sp.
Exiguobacterium acetylicum
Marinobacter confluentis
Marinobacter sp.
Marinobacterium rambilcola
Saccharospirillum alexandrill
Saccharospirillum sp.
Spongiibacter sp.
Thalassospira marina
phylum
class
order
family
genus
species

Figure S1

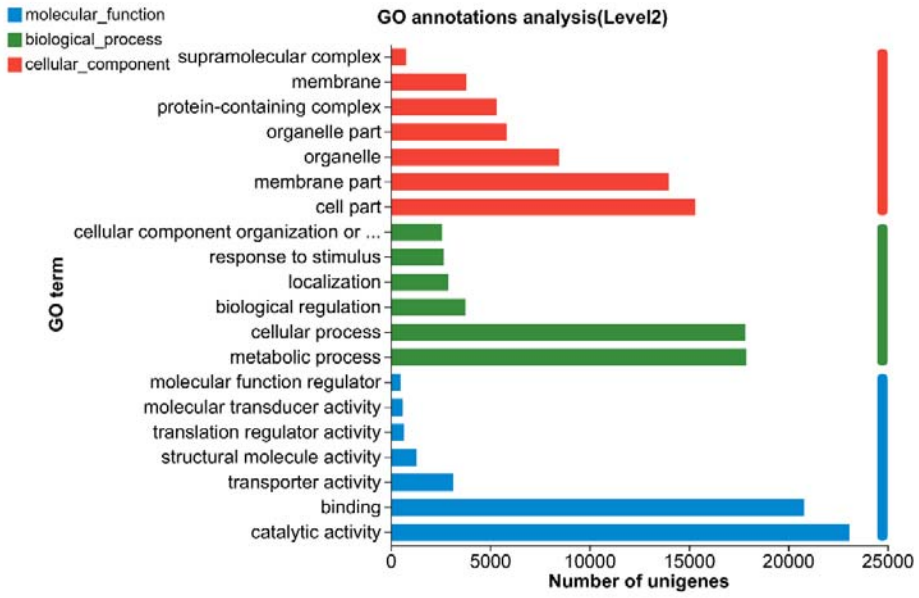
molecular_function
biological_process
cellular_component
GO annotations analysis(Level2)
supramolecular complex
membrane
protein-containing complex
organelle part
organelle
membrane part
cell part
cellular component organization or ...
response to stimulus
localization
biological regulation
cellular process
metabolic process
molecular function regulator
molecular transducer activity
translation regulator activity
structural molecule activity
transporter activity
binding
catalytic activity
GO term
0
5000
10000
15000
20000
25000
Number of unigenes

Figure S2

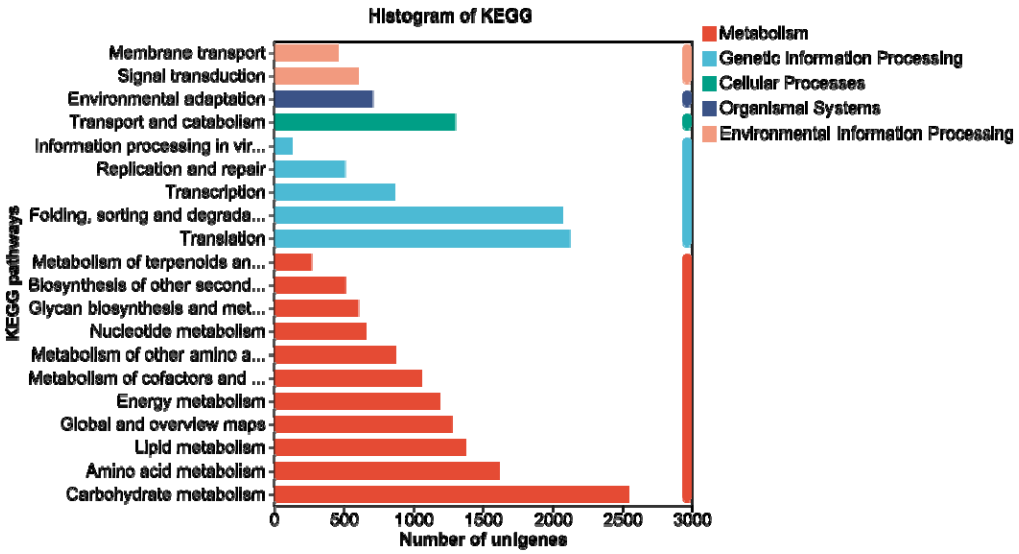
Histogram of KEGG
Membrane transport
Signal transduction
Environmental adaptation
Transport and catabolism
Information processing in vir...
Replication and repair
Transcription
Folding, sorting and degrada...
Translation
Metabolism of terpenoids an...
Biosynthesis of other second...
Glycan biosynthesis and met...
Nucleotide metabolism
Metabolism of other amino a...
Metabolism of cofactors and ...
Energy metabolism
Global and overview maps
Lipid metabolism
Amino acid metabolism
Carbohydrate metabolism
KEGG pathways
0
500
1000
1500
2000
2500
3000
Number of unigenes
Metabolism
Genetic Information Processing
Cellular Processes
Organismal Systems
Environmental Information Processing

Figure S3

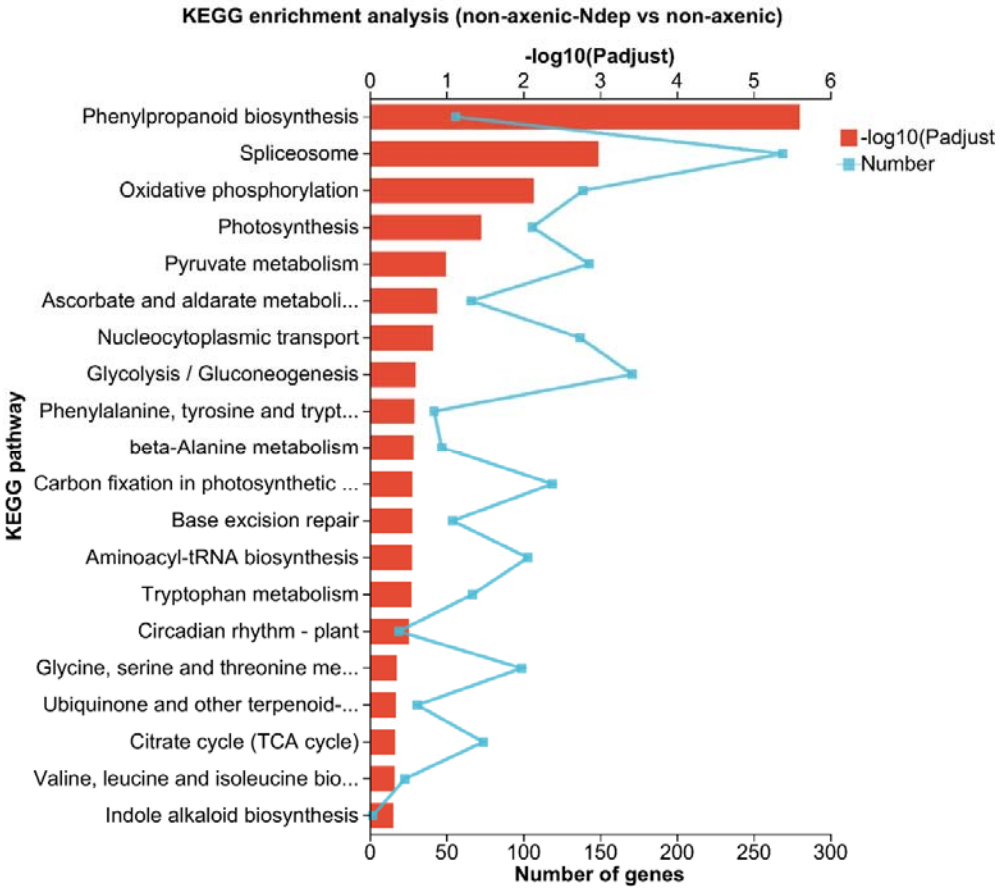
KEGG enrichment analysis (non-axenic-Ndep vs non-axenic)
-log10(Padjust)
0
1
2
3
4
5
6
Phenylpropanoid biosynthesis
Spliceosome
Oxidative phosphorylation
Photosynthesis
Pyruvate metabolism
Ascorbate and aldarate metaboli...
Nucleocytoplasmic transport
Glycolysis / Gluconeogenesis
Phenylalanine, tyrosine and trypt...
beta-Alanine metabolism
Carbon fixation in photosynthetic ...
Base excision repair
Aminoacyl-tRNA biosynthesis
Tryptophan metabolism
Circadian rhythm - plant
Glycine, serine and threonine me...
Ubiquinone and other terpenoid-...
Citrate cycle (TCA cycle)
Valine, leucine and isoleucine bio...
Indole alkaloid biosynthesis
KEGG pathway
-log10(Padjust
Number
0
50
100
150
200
250
300
Number of genes

Figure S4