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

Shanmei Zou*, Xinke Yu, Tiantian Sun, Xuemin Wu

College of Resources and Environmental Science, Nanjing Agricultural University, Nanjing 210095, China

* Corresponding authors. E-mail: zousm912@njau.edu.cn

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

Introduction

Harmful algal blooms (HABs) occur globally and have produced severe effects on fisheries, aquaculture, tourism and recreation (Hallegraef 2004; Berdalet et al., 2016). Dinoflagellates, a large group of phytoplanktonic organisms during marine HABs, play a crucial role in food webs and in balancing ecosystem energy fluxes (Totti et al., 2010; Corinaldesi et al., 2011; Tas and Okus 2011; Canini et al., 2013; Rosic et al., 2015; Lu et al., 2016). 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 (Hallegraef 2004; Tsujino and Uchida 2004; Persson et al., 2006; Wang et al., 2011; Meyer et al., 2015; Guo et al., 2016). The most widespread dinoflagellates species producing PSTs are in the genus *Alexandrium* (Cembella 1998; Anderson et al., 2012).

PSTs, mainly produced from dinoflagellate and cyanobacteria, is one of the most powerful neurotoxins such as TTX (Akbar et al., 2020). Currently, the regulation mechanism for PSTs production in cyanobacteria is clear, which mainly refers to 26 functional genes for PSTs biosynthesis (Troco et al., 2009). However, the mechanism regulation for PSTs production of harmful dinoflagellates remains poorly understood due to the exceptionally large genome size and high gene copy number of dinoflagellates (Zhang et al., 2014). 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 (Shumway 1990; Hallegraeff 1993; Scholin and Anderson 1994; Scholin et al., 1994; Su et al., 2004).

The environmental factors, such as light, temperature, salinity, nitrogen or phosphorous, have been shown to affect PSTs content for several *Alexandrium* species (Han et al., 2016). For example, while nitrogen limitation causes the toxin content to decrease phosphorous limitation often results in higher toxin cell quotas (Boyer et al., 1987; Anderson et al., 1990; Matsuda 1996; Marsot 1997; Bechemin et al., 1999; John and Flynn 2000; Maestrini et al., 2000; Etheridge and Roesler

2005; Wang and Hsieh 2005). But the salinity and temperature just have limited effects on toxin composition (Taroncher-Oldenburg et al., 1999; Etheridge and Roesler 2005).

Bacteria directly or indirectly affect the growth and toxin production of *Alexandrium* species (Doucette 1995; Dantzer and Levin 1997; Doucette and Powell 1997; Gallacher et al., 1997; Gallacher and Smith 1999; Maas et al., 2007; Kodama 2010). Originally it was considered that the PSTs in dinoflagellate was produced by their associated bacteria, e.g., *protogonyaulax* and *Alexandrium* (Kodama et al., 1988; Gallacher et al., 1997; Smith et al., 2002). However, the subsequent research has shown that the "PSTs" produced by bacteria was actually the analogues of PSTs (Baker et al., 2003; Martins et al., 2003). 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 (Hold et al., 2001; Uribe and Espejo 2003; Ho et al., 2006; Green et al., 2010; Albinsson et al., 2014). Different bacteria community had different capacity for affecting PSTs production of dinoflagellate (Albinsson et al., 2014). 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 (Maas et al., 2007). 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 (Ho et al., 2006; Green et al., 2010; Albinsson et al., 2014). 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 (Liu 2017). Due to the difficulty in identifying dinoflagellate by morphological characters, in this study, the eDNA metabarcoding (Chariton 2019) 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.

Materials and methods

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.

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 (Bolyen et al., 2019). Taxonomic classification was performed by feature-classifier module with Naive Bayes (Bolyen et al., 2019). The reference library for ASV classification was referred from Zou et al (Zou and Smith 2020).

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).

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 (Bolyen et al., 2019).

Isolation and reseeding of associated bacteria

The 2216E medium was used for isolation of associated bacteria of *A. catenella* (Fidalgo et al., 2016). 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.

Toxicity Type and content test

The type 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. (Zhang et al.).

The PSTs content of different treatments of *A. catenella* and associated bacteria was measured by mouse toxin assay. The algae solution was collected on the 7th day of culture and was centrifuged at 2,500 g for 10 min. After the supernatant was discarded 5 mL of 0.01 mol/L HCl was added into the algae centrifugate and the pH was adjusted to 3 with 1 mol/L NaOH. Then all algae cells were broken with ultrasonic and were placed in a boiling water bath for 5 min with

pH 3 adjusted. The final volume was fixed to 5 mL with distilled water, and its supernatant was taken as the stock solution for algal toxicity test. The Kunming male mice was used for toxicity test. 1 mL toxin supernatant was injected into the abdomen of the mice. The reaction of the mice was observed and the death time was recorded. The Mouse Unit was used for toxicity (1 mouse unit means that the TTX could kill the mouse within 30 minutes).

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.

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) (Langmead and Salzberg 2012) and the expression levels of genes was calculated by RSEM (v1.2.8) and normalized to the FPKM (Dewey and Bo 2011).

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) (Miller et al., 2008). GO annotation was performed by Blast2GO (v 2.5.0) with NR annotations. PossionDis (Audic and Claverie 1997) 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).

Results

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.

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* (Figure 3). Six phyla, Bacteroidetes, Proteobacteria, Steptophyta, Firmicutes, Bacteroidota and Pseudomonadota, were detected. The dominant classes were Chrysophyceae, Cytophagia, Deltaproteobacteria, Flavobacteriia, Bacilli, Gammaproteobacteria, Alphaproteobacteria and Bacteroidia. The main orders included Alteromonadales, Chromulinales, Cytophagales, Rhodobacterales etc. The dominant associated bacteria species included *Paenibacillus* sp., *Yangia pacifica*, *Formosa* sp., *Bacillus humi*, *Alteromonas macleodii*, *Ochromonas* sp., *Cesiribacter* sp., *Flavobacteriales*

bacterium etc. Among them, *Ochromonas* accounted for the highest proportion in 16S and *Paenibacillus* accounted for the highest proportion in 16Sv3v4 (Figure 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 4A). 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 5A). 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 5B, C).

The capacity of isolated bacteria for affecting PSTs production of *A. catenella* was tested separately, together with different combined bacteria strains (Figure 4B, C). Under normal cultivation conditions, the non-axenic *A. catenella* strains without antibiotic treatment apparently produced more PSTs than strains with antibiotic treatment (Figure 4B). 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 4B). 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 4C). It was apparent that the toxin content decreased for almost all treatments under nitrogen deprivation (Figure 4C). 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 4B, C).

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 6A). Thereinto, the non-axenic *A. catenella* strain with nitrogen deprivation (non-axenic-ndep) grew the slowest (Figure 6A). 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 6B). 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 6B).

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 7A). Among the transcription factors, the vast majority of unigenes were assigned to C3H, followed by MYB_superfamily (Figure 7B). The axenic, non-axenic and non-axenic-ndep strains all produced abundant common and distinct unigenes (Figure 7C). The up-regulated and down-regulated genes among three *A. catenella* treatments were different (Figure 7D). The GO functional enrichment 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 8A). The dominant DEGs included Glutathione metabolism, Plant-pathogen interaction, Arginine and proline metabolism, Tryptophan metabolism, and spliceosome, etc. (Figure 8B). The PSTs synthesis genes sxtA was found in *A. catenella* strains of all three treatments, which were expressed highly in non-axenic strain (Figure 8B). 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 9). 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 9A). As expected, the plant hormone signal transduction and Indole-3-acetic acid-amido synthetase were up-regulated in non-axenic strain (Figure 9A). 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. 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.

Discussion

The PSTs production by dinoflagellate is associated with bacteria (Orr et al., 2011; Brandenburg et al., 2018; Zou 2020). The PSTs control by microbes is becoming important since the microbes play a key role in microalgae' growth,

metabolism and toxin production (Croft et al., 2013; Ramanan et al., 2016; Kristy et al., 2017). 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 (Li et al.). 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 (Baker et al., 2003; Martins et al., 2003). 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 (Hold et al., 2001; Uribe and Espejo 2003; Ho et al., 2006; Green et al., 2010; Albinsson et al., 2014).

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. (2014) 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* (Bolch et al., 2017). 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 (Gallacher and Smith 1999; Maas et al., 2007; Kodama 2010). 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 (John and Flynn 2000; Maestrini et al., 2000; Etheridge and Roesler 2005; Wang and Hsieh 2005).

The sxt gene cluster for STX synthesis has been elucidated in cyanobacteria (Kellmann et al., 2008; Troco et al., 2009; Stucken et al., 2010; Mihali et al., 2011). Among 26 sxt gene revealed in cyanobacteria, only several sxt genes were found in dinoflagellate, including sxtA, sxtG and sxtO (Wang et al., 2016). The biosynthesis of PSTs starts with sxtA (Kellmann et al., 2008; Wang et al., 2016). 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 (Snyder et al., 2003). Here the 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, like 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 (Orr et al., 2011; Brandenburg et al., 2018). 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 (Amin et al., 2015; Shibl et al., 2020). 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.

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.

Author 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.

Conflict of Interest

The authors have no conflict of interest.

Availability of data and materials

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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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 *Alexandrium 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 in each taxonomic level.

Figure 4. (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. B1-B13 indicates the bacteria serial number for reseeding with *A. catenella*.

Figure 5. (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 6. (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 7. (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 8. (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 9. 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 enrichment 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

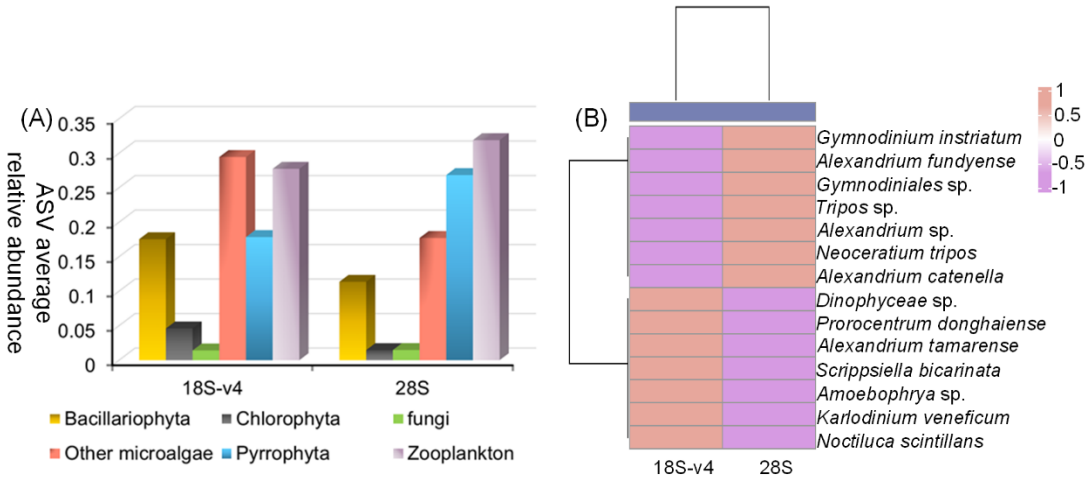
(A)
relative abundance
ASV average
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
18S-v4
28S
Bacillariophyta
Chlorophyta
fungi
Other microalgae
Pyrrophyta
Zooplankton
(B)
Gymnodinium instriatum
Alexandrium fundyense
Gymnodiniales sp.
Tripos sp.
Alexandrium sp.
Neoceratium tripos
Alexandrium catenella
Dinophyceae sp.
Prorocentrum donghaiense
Alexandrium tamarense
Scrippsiella bicarinata
Amoebophrya sp.
Karlodinium veneficum
Noctiluca scintillans
18S-v4
28S
1
0.5
0
-0.5
-1

Figure 1

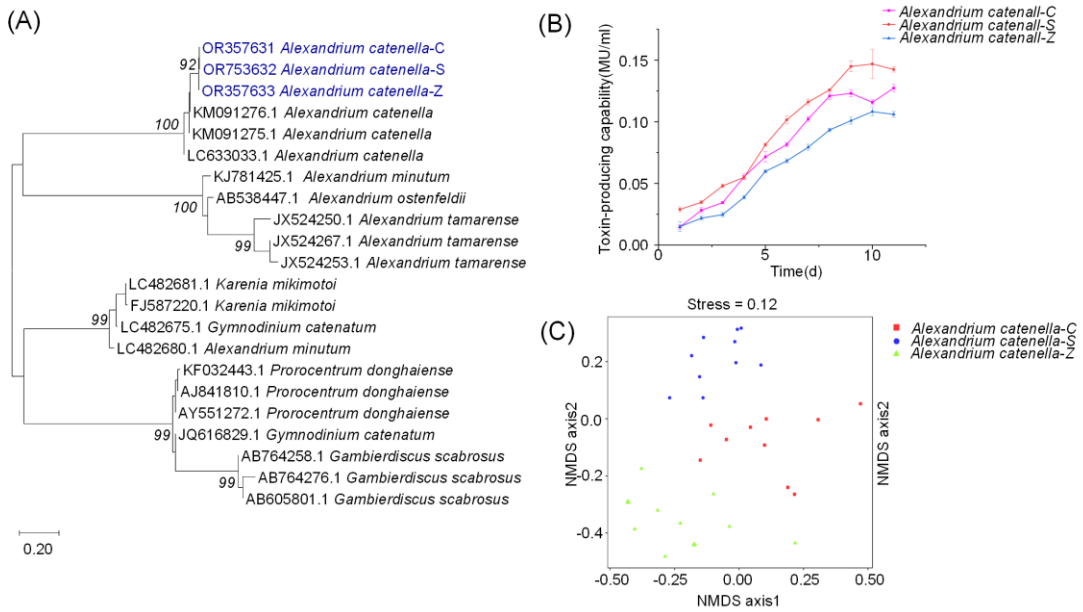
(A)
OR357631 Alexandrium catenella-C
OR753632 Alexandrium catenella-S
OR357633 Alexandrium catenella-Z
KM091276.1 Alexandrium catenella
KM091275.1 Alexandrium catenella
LC633033.1 Alexandrium catenella
KJ781425.1 Alexandrium minutum
AB538447.1 Alexandrium ostenfeldii
JX524250.1 Alexandrium tamarense
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
0.20
(B)
Alexandrium catenall-C
Alexandrium catenall-S
Alexandrium catenall-Z
Toxin-producing capability(MU/ml)
0.15
0.10
0.05
0.00
0
5
10
Time(d)
(C)
Stress = 0.12
Alexandrium catenella-C
Alexandrium catenella-S
Alexandrium catenella-Z
NMDS axis2
0.2
0.0
-0.2
-0.4
-0.50
-0.25
0.00
0.25
0.50
NMDS axis1

Figure 2

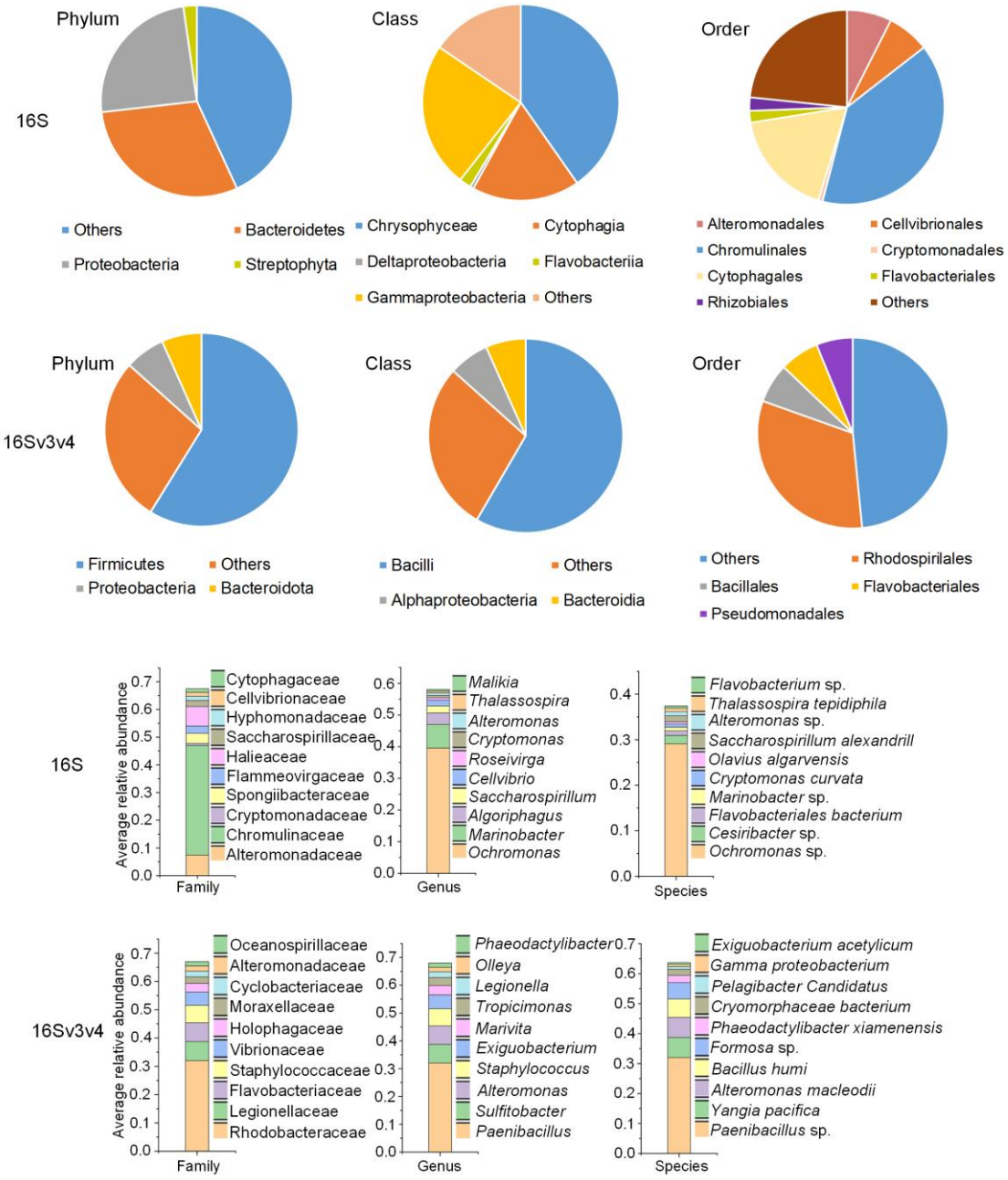
16S
Phylum
Others
Bacteroidetes
Proteobacteria
Streptophyta
Class
Chrysophyceae
Cytophagia
Deltaproteobacteria
Flavobacteriia
Gammaproteobacteria
Others
Order
Alteromonadales
Cellvibrionales
Chromulinales
Cryptomonadales
Cytophagales
Flavobacteriales
Rhizobiales
Others
16Sv3v4
Phylum
Firmicutes
Others
Proteobacteria
Bacteroidota
Class
Bacilli
Others
Alphaproteobacteria
Bacteroidia
Order
Others
Rhodospirilales
Bacillales
Flavobacteriales
Pseudomonadales
16S
Average relative abundance
Family
Cytophagaceae
Cellvibrionaceae
Hyphomonadaceae
Saccharospirillaceae
Halieaceae
Flammeovirgaceae
Spongiibacteraceae
Cryptomonadaceae
Chromulinaceae
Alteromonadaceae
Genus
Malikia
Thalassospira
Alteromonas
Cryptomonas
Roseivirga
Cellvibrio
Saccharospirillum
Algoriphagus
Marinobacter
Ochromonas
Species
Flavobacterium sp.
Thalassospira tepidiphila
Alteromonas sp.
Saccharospirillum alexandrill
Olavius algarvensis
Cryptomonas curvata
Marinobacter sp.
Flavobacteriales bacterium
Cesiribacter sp.
Ochromonas sp.
16Sv3v4
Average relative abundance
Family
Oceanospirillaceae
Alteromonadaceae
Cyclobacteriaceae
Moraxellaceae
Holophagaceae
Vibrionaceae
Staphylococcaceae
Flavobacteriaceae
Legionellaceae
Rhodobacteraceae
Genus
Phaeodactylibacter
Olleya
Legionella
Tropicimonas
Marivita
Exiguobacterium
Staphylococcus
Alteromonas
Sulfitobacter
Paenibacillus
Species
Exiguobacterium acetylicum
Gamma proteobacterium
Pelagibacter Candidatus
Cryomorphaceae bacterium
Phaeodactylibacter xiamenensis
Formosa sp.
Bacillus humi
Alteromonas macleodii
Yangia pacifica
Paenibacillus sp.

Figure 3

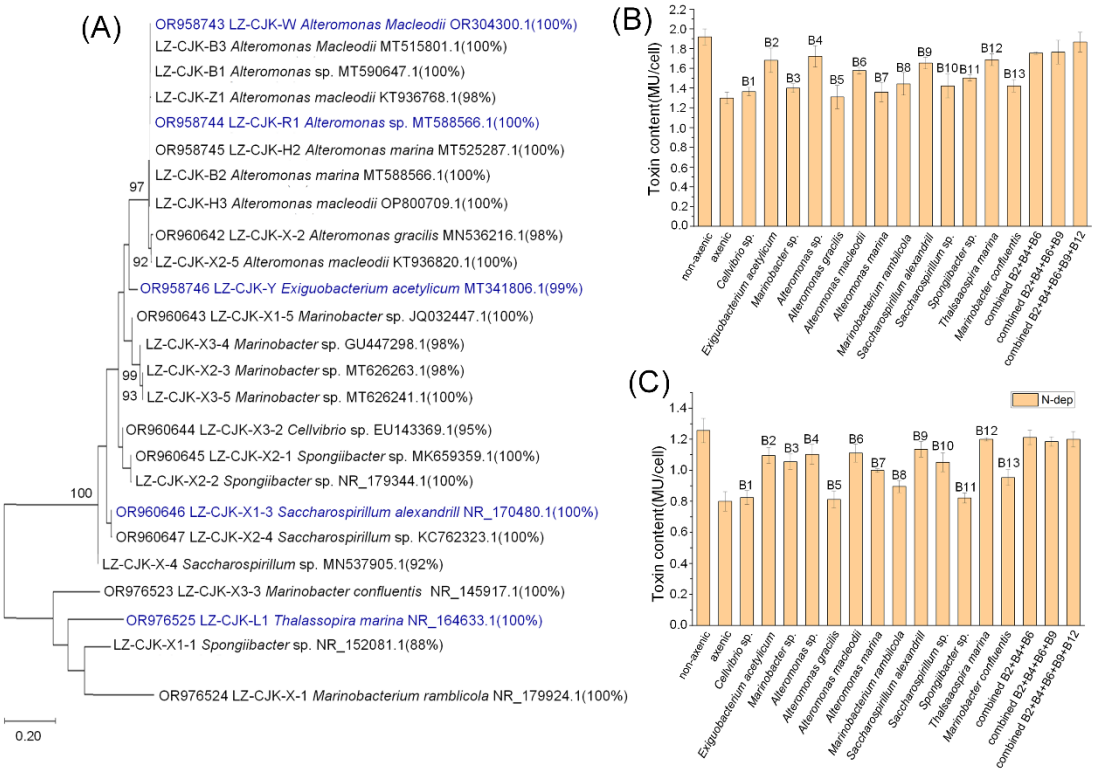
(A)
OR958743 LZ-CJK-W Alteromonas Macleodii OR304300.1(100%)
LZ-CJK-B3 Alteromonas Macleodii MT515801.1(100%)
LZ-CJK-B1 Alteromonas sp. MT590647.1(100%)
LZ-CJK-Z1 Alteromonas macleodii KT936768.1(98%)
OR958744 LZ-CJK-R1 Alteromonas sp. MT588566.1(100%)
OR958745 LZ-CJK-H2 Alteromonas marina MT525287.1(100%)
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(MU/cell)
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 B2+B4+B6
combined B2+B4+B6+B9
combined B2+B4+B6+B9+B12
(C)
N-dep
Toxin content(MU/cell)

Figure 4

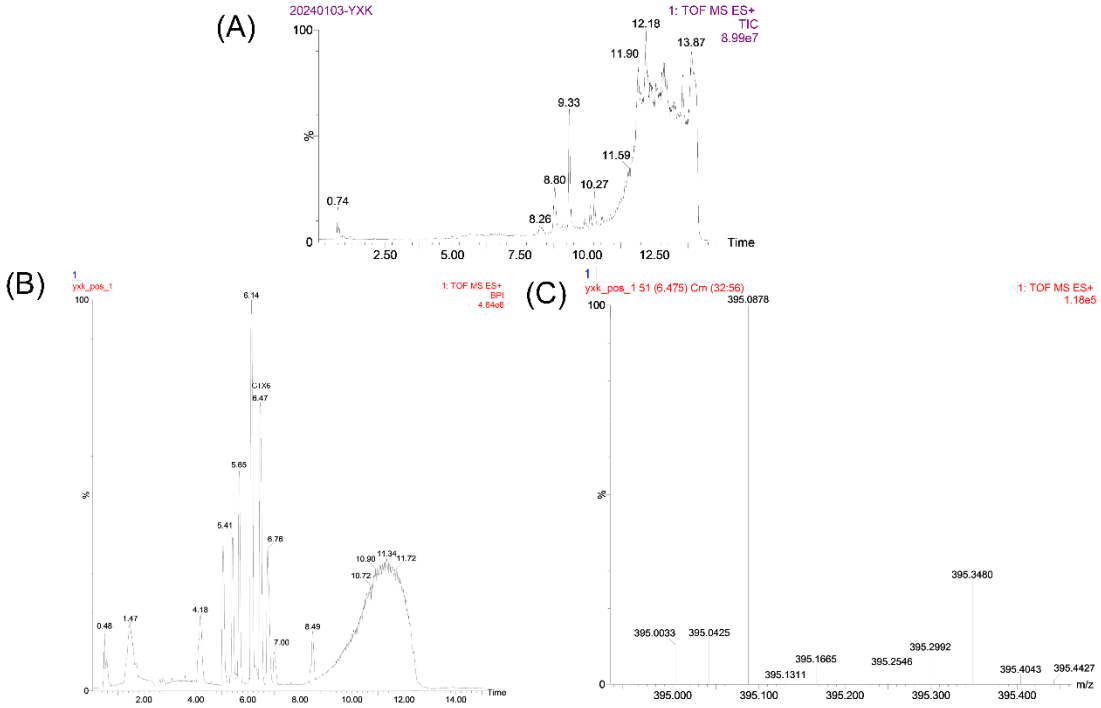
(A)
20240103-YXK
1: TOF MS ES+
TIC
8.99e7
100
%
0
0.74
8.26
8.80
9.33
10.27
11.59
11.90
12.18
13.87
2.50
5.00
7.50
10.00
12.50
Time
(B)
1
yxk_pos_1
1: TOF MS ES+
BPI
4.64e6
100
%
0
0.48
1.47
4.18
5.41
5.65
6.14
6.47
6.78
7.00
8.49
10.72
10.90
11.34
11.72
2.00
4.00
6.00
8.00
10.00
12.00
14.00
Time
(C)
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 5

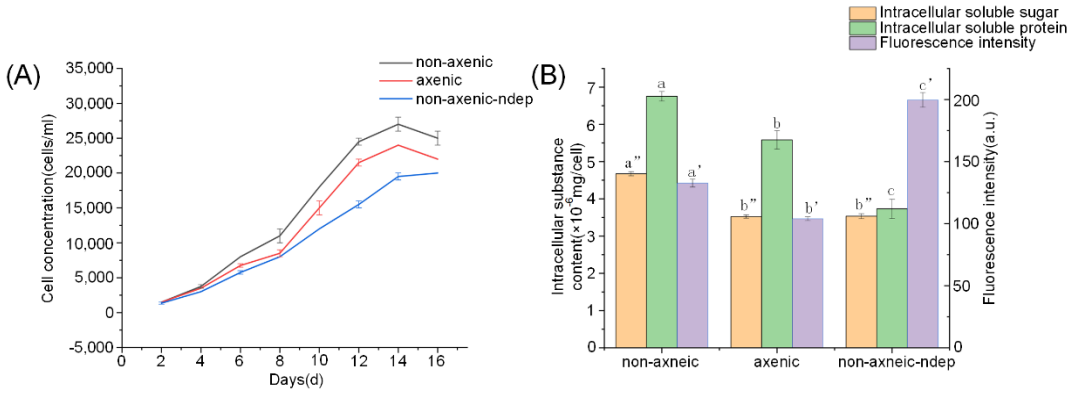
(A)
non-axenic
axenic
non-axenic-ndep
Cell concentration(cells/ml)
Days(d)
(B)
Intracellular soluble sugar
Intracellular soluble protein
Fluorescence intensity
Intracellular substance content(×10⁻⁶mg/cell)
Fluorescence intensity(a.u.)
non-axneic
axenic
non-axneic-ndep

Figure 6

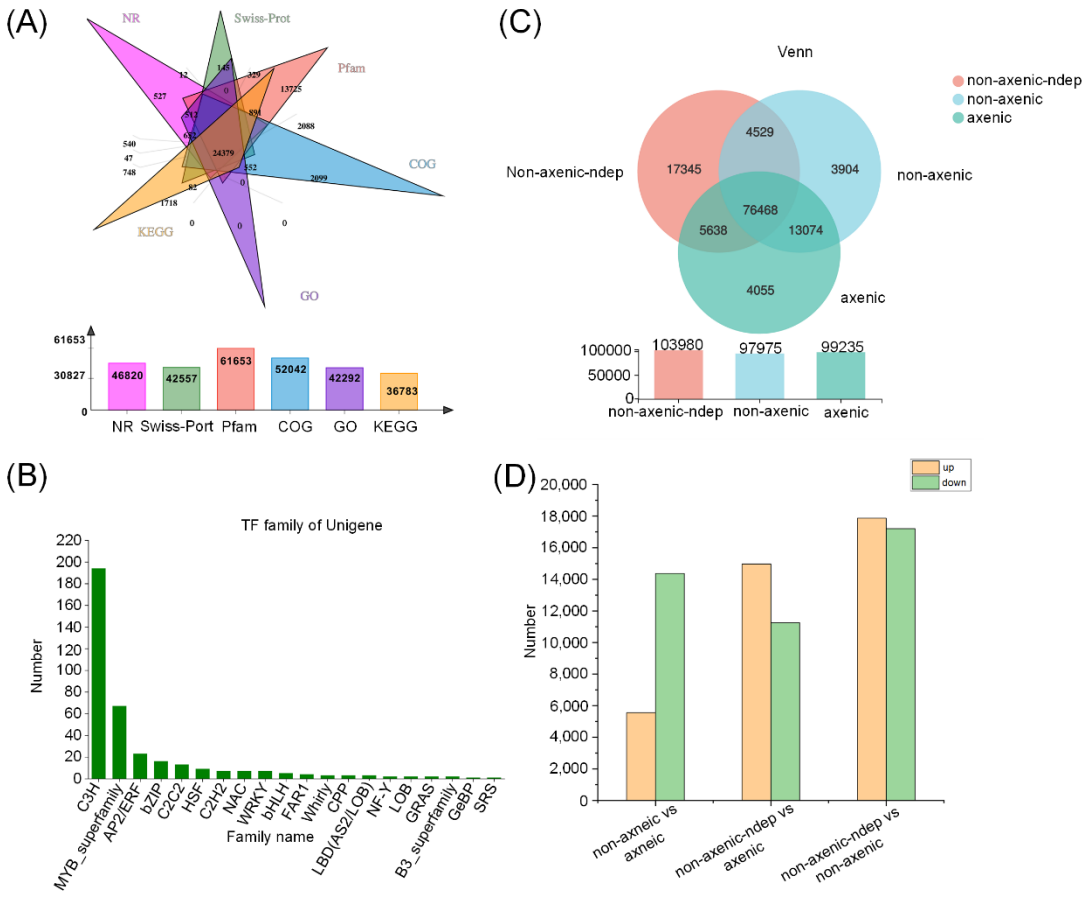
(A)
NR
Swiss-Prot
Pfam
COG
GO
KEGG
61653
30827
0
46820
42557
61653
52042
42292
36783
NR Swiss-Port Pfam COG GO KEGG
(C)
Venn
non-axenic-ndep
non-axenic
axenic
Non-axenic-ndep
non-axenic
axenic
17345
4529
3904
76468
5638
13074
4055
100000
50000
0
103980
97975
99235
non-axenic-ndep non-axenic axenic
(B)
TF family of Unigene
Number
Family name
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
(D)
up
down
Number
non-axneic vs axneic
non-axenic-ndep vs axenic
non-axenic-ndep vs non-axenic

Figure 7

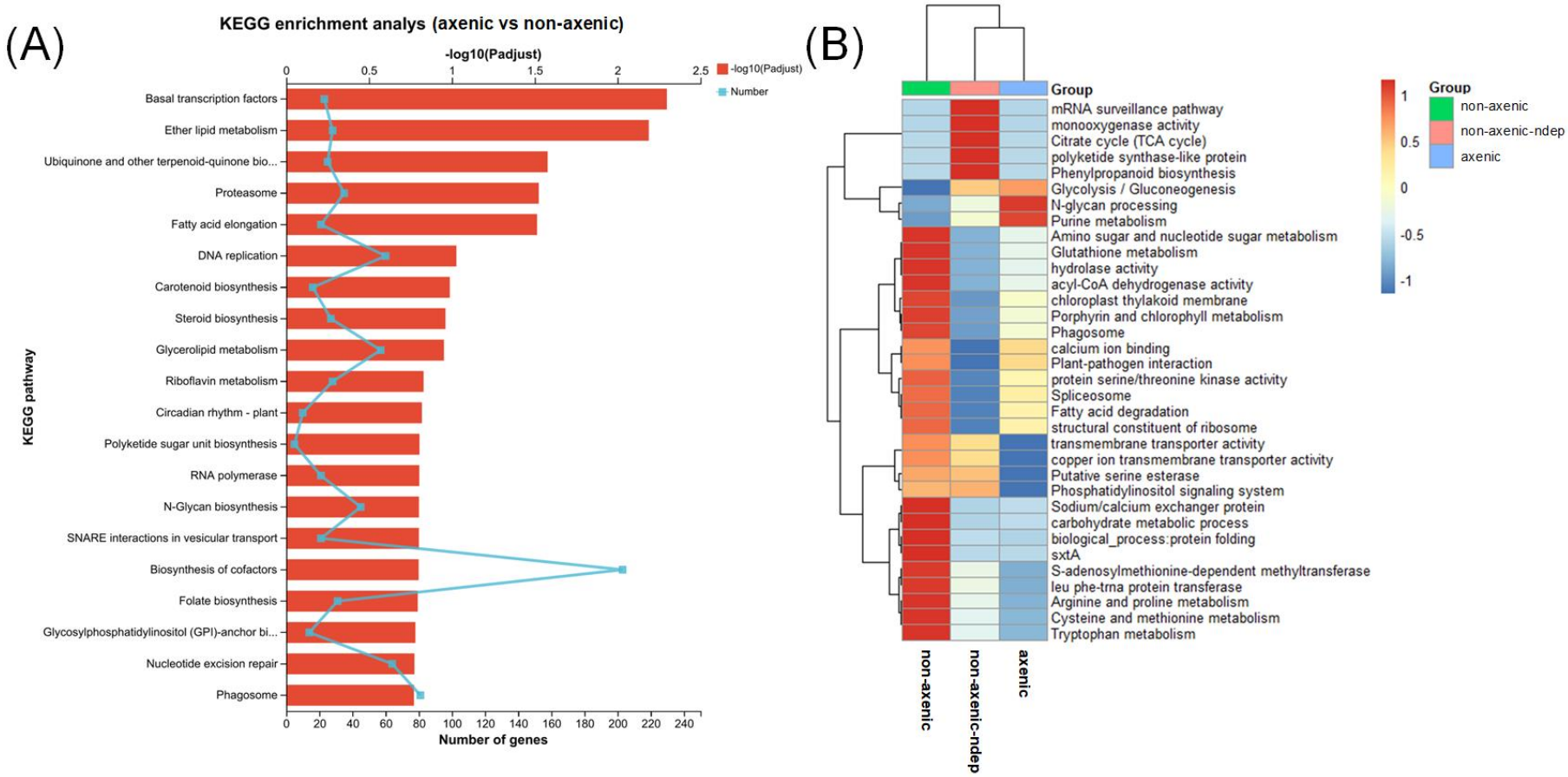
(A)
KEGG enrichment analys (axenic vs non-axenic)
-log10(Padjust)
0
0.5
1
1.5
2
2.5
-log10(Padjust)
Number
KEGG pathway
Basal transcription factors
Ether lipid metabolism
Ubiquinone and other terpenoid-quinone bio...
Proteasome
Fatty acid elongation
DNA replication
Carotenoid biosynthesis
Steroid biosynthesis
Glycerolipid metabolism
Riboflavin metabolism
Circadian rhythm - plant
Polyketide sugar unit biosynthesis
RNA polymerase
N-Glycan biosynthesis
SNARE interactions in vesicular transport
Biosynthesis of cofactors
Folate biosynthesis
Glycosylphosphatidylinositol (GPI)-anchor bi...
Nucleotide excision repair
Phagosome
0
20
40
60
80
100
120
140
160
180
200
220
240
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 serine esterase
Phosphatidylinositol signaling system
Sodium/calcium exchanger protein
carbohydrate metabolic process
biological_process:protein folding
sxtA
S-adenosylmethionine-dependent methyltransferase
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 8

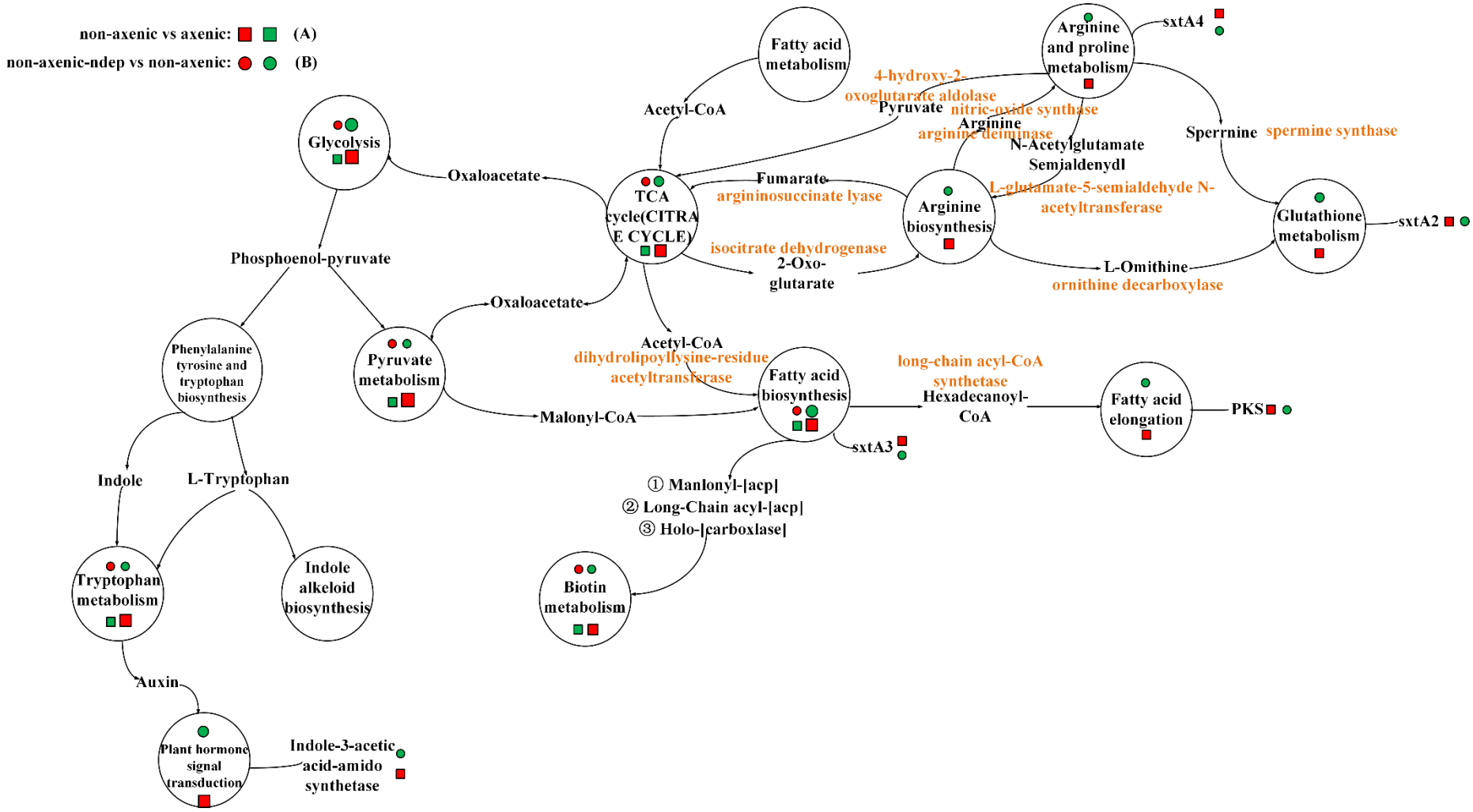
non-axenic vs axenic: (A)
non-axenic-ndep vs non-axenic: (B)
Fatty acid metabolism
Arginine and proline metabolism
sxtA4
4-hydroxy-2-oxoglutarate aldolase
Pyruvate
nitric-oxide synthase
Arginine
arginine deiminase
N-Acetylglutamate Semjaldenydl
Sperrnine
spermine synthase
Acetyl-CoA
Glycolysis
Oxaloacetate
TCA cycle(CITRAE CYCLE)
Fumarate
argininosuccinate lyase
Arginine biosynthesis
L-glutamate-5-semialdehyde N-acetyltransferase
Glutathione metabolism
sxtA2
isocitrate dehydrogenase
2-Oxo-glutarate
L-Omithine
ornithine decarboxylase
Phosphoenol-pyruvate
Oxaloacetate
Phenylalanine tyrosine and tryptophan biosynthesis
Pyruvate metabolism
Acetyl-CoA
dihydrolipoyllysine-residue acetyltransferase
Fatty acid biosynthesis
long-chain acyl-CoA synthetase
Hexadecanoyl-CoA
Fatty acid elongation
PKS
Malonyl-CoA
sxtA3
Indole
L-Tryptophan
① Manlonyl-[acp]
② Long-Chain acyl-[acp]
③ Holo-[carboxlase]
Tryptophan metabolism
Indole alkeloid biosynthesis
Biotin metabolism
Auxin
Plant hormone signal transduction
Indole-3-acetic acid-amido synthetase

Figure 9

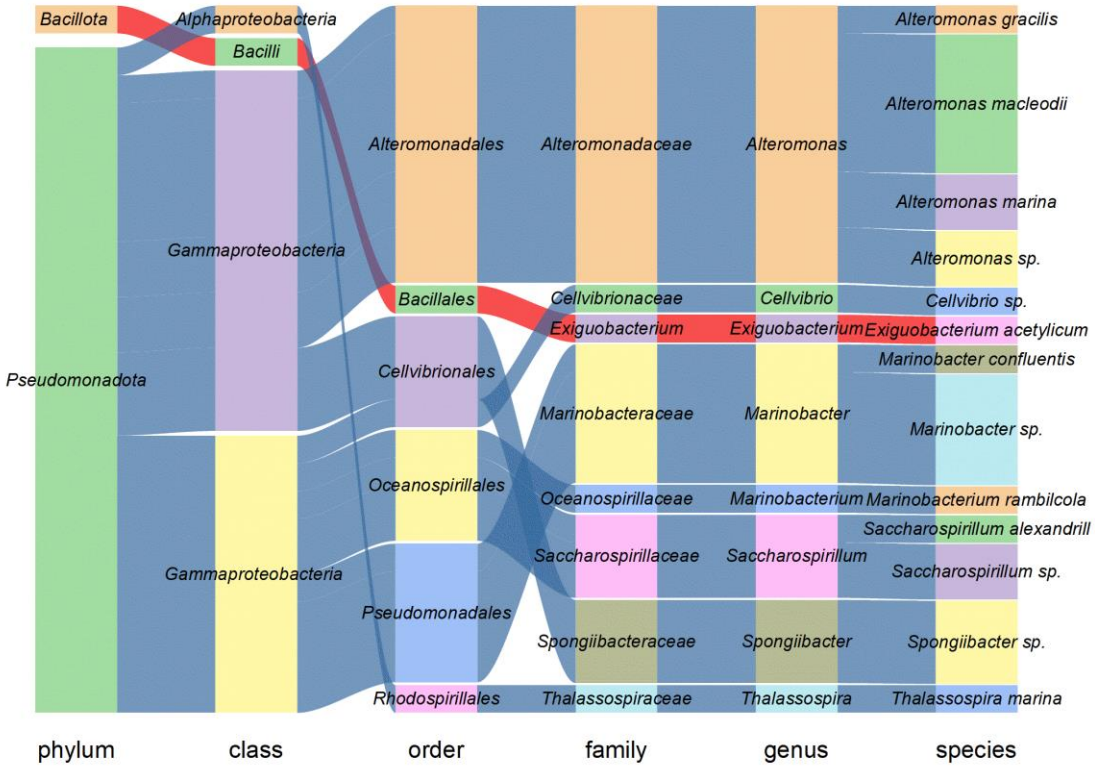
Bacillota
Alphaproteobacteria
Bacilli
Pseudomonadota
Gammaproteobacteria
Gammaproteobacteria
Alteromonadales
Bacillales
Cellvibrionales
Oceanospirillales
Pseudomonadales
Rhodospirillales
Alteromonadaceae
Cellvibrionaceae
Exiguobacterium
Marinobacteraceae
Oceanospirillaceae
Saccharospirillaceae
Spongiibacteraceae
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

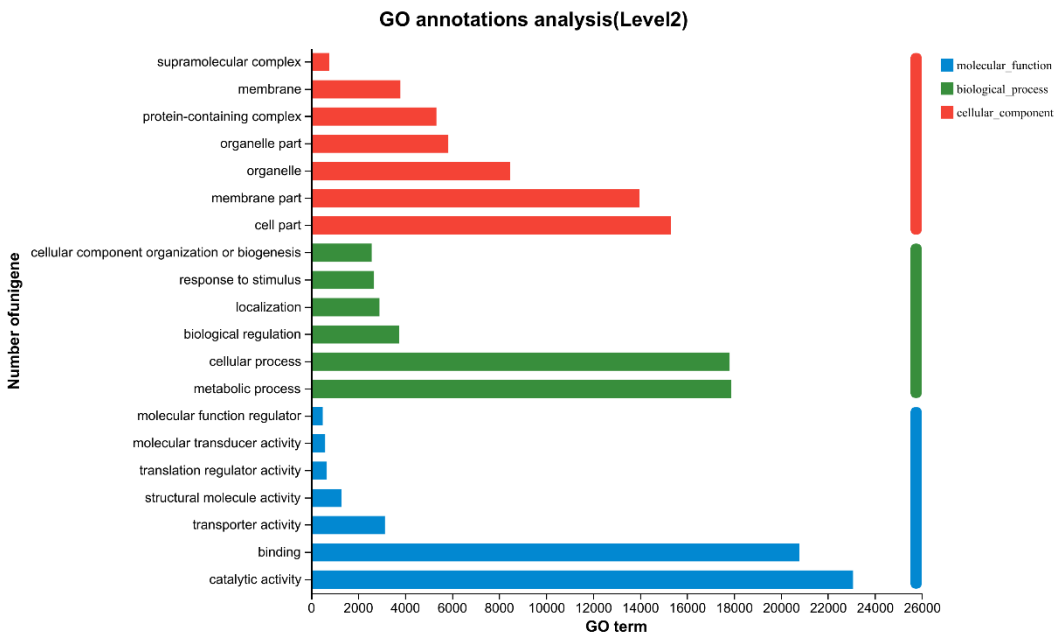
GO annotations analysis(Level2)
supramolecular complex
membrane
protein-containing complex
organelle part
organelle
membrane part
cell part
cellular component organization or biogenesis
response to stimulus
localization
biological regulation
cellular process
metabolic process
molecular function regulator
molecular transducer activity
translation regulator activity
structural molecule activity
transporter activity
binding
catalytic activity
Number ofunigene
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
22000
24000
26000
GO term
molecular_function
biological_process
cellular_component

Figure S2

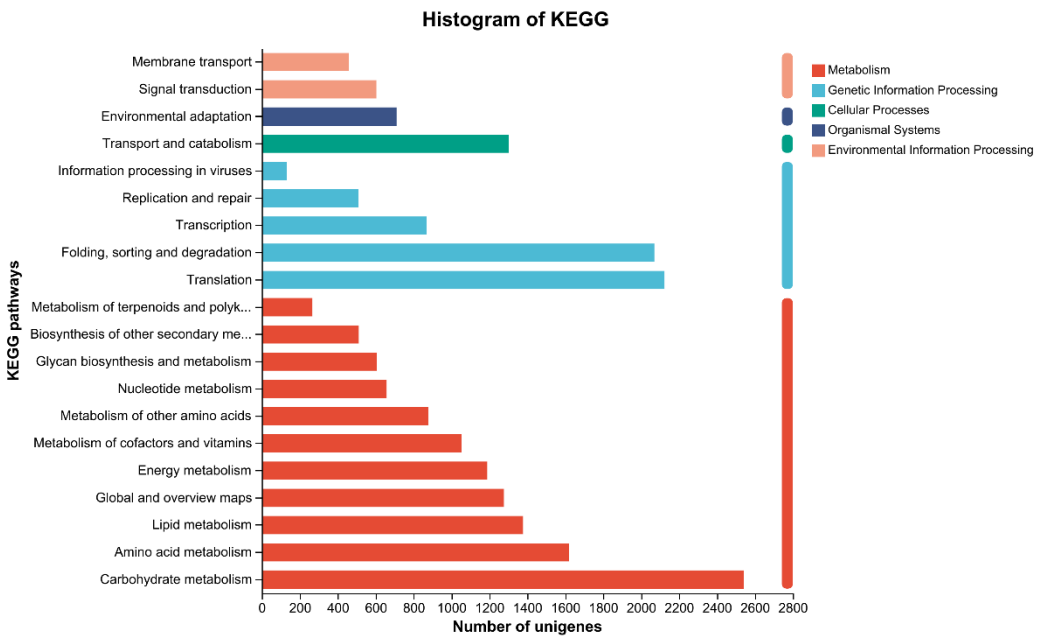
Histogram of KEGG
Membrane transport
Signal transduction
Environmental adaptation
Transport and catabolism
Information processing in viruses
Replication and repair
Transcription
Folding, sorting and degradation
Translation
Metabolism of terpenoids and polyk...
Biosynthesis of other secondary me...
Glycan biosynthesis and metabolism
Nucleotide metabolism
Metabolism of other amino acids
Metabolism of cofactors and vitamins
Energy metabolism
Global and overview maps
Lipid metabolism
Amino acid metabolism
Carbohydrate metabolism
KEGG pathways
0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600 2800
Number of unigenes
Metabolism
Genetic Information Processing
Cellular Processes
Organismal Systems
Environmental Information Processing

Figure S3

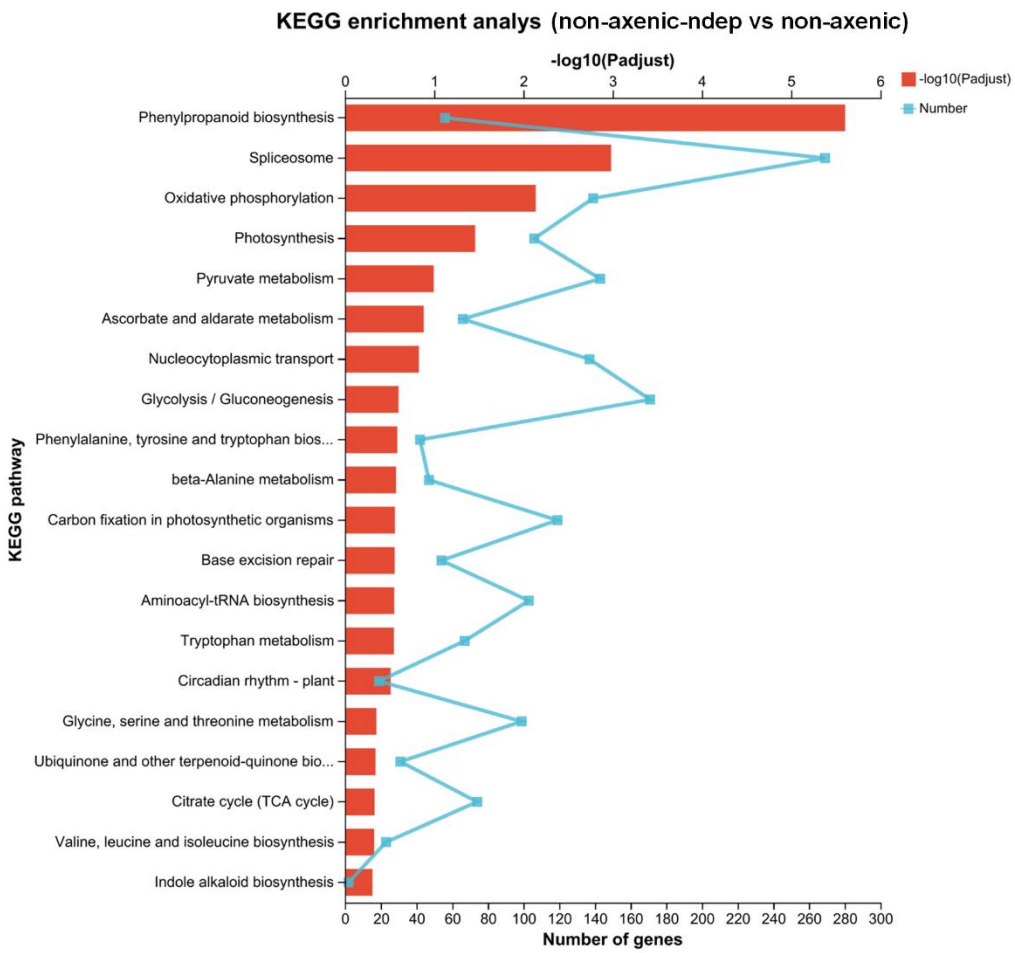
KEGG enrichment analys (non-axenic-ndep vs non-axenic)
-log10(Padjust)
0
1
2
3
4
5
6
-log10(Padjust)
Number
Phenylpropanoid biosynthesis
Spliceosome
Oxidative phosphorylation
Photosynthesis
Pyruvate metabolism
Ascorbate and aldarate metabolism
Nucleocytoplasmic transport
Glycolysis / Gluconeogenesis
Phenylalanine, tyrosine and tryptophan bios...
beta-Alanine metabolism
Carbon fixation in photosynthetic organisms
Base excision repair
Aminoacyl-tRNA biosynthesis
Tryptophan metabolism
Circadian rhythm - plant
Glycine, serine and threonine metabolism
Ubiquinone and other terpenoid-quinone bio...
Citrate cycle (TCA cycle)
Valine, leucine and isoleucine biosynthesis
Indole alkaloid biosynthesis
KEGG pathway
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
Number of genes

Figure S4