Ampicillin used in aseptic processing influences the production of pigments and fatty acids in *Chlorella sorokiniana*

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Abstract

Ampicillin sodium salt (AMP) is commonly and effectively used to prevent bacterial infection in algal culture, but the response of algal strains to AMP has not been investigated. In this study, *Chlorella sorokiniana* was selected to evaluate the influence of AMP on algae. AMP enhanced the contents of chlorophyll and two fatty acids, myristic acid (C22:1N9) and tetracosanoic acid (C6:0), but inhibited the growth, carotenoid production, and contents of 16 fatty acids in *C. sorokiniana*. A global transcriptome analysis from experimental data identified 3 825 upregulated and 1 432 downregulated differentially expressed genes (DEGs) in *C. sorokiniana*. The upregulated DEGs, such as hemBlalaD, mmaB/pduO, cox15/ctaA, fxF, cpoX/hemF, and earSlghtX, were enriched in the porphyrin and chlorophyll metabolism pathways, whereas the downregulated DEGs, including lcyB (crl1), crtY (lcyE, crtl2), lut1 (CYP97C1), z-isO, crtZ and crtsO (crtH), were enriched in the carotenoid biosynthesis pathway, and the downregulated DEGs, abH, fadD, fabF, acsL, fabG, and accD were enriched in the fatty acid biosynthesis pathway. Thus, the use of AMP to obtain an axenic strain revealed that AMP might affect the regulatory dynamics and the results of the metabolic process in *C. sorokiniana*. The data obtained in the study provide foundational information for algal purification and aseptic processing.

Supplementary Information

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Introduction

Algae, which have a relatively simple structure and the capability of photosynthesis, have attracted considerable attention worldwide as a source of clean energy (Li et al. 2013; Zhou et al. 2020). Algae are promising resources for a wide range of product applications, including nutraceuticals and pharmaceuticals, and industrial applications, such as in the fields of bioenergy and aquaculture (Mata et al. 2010; Xiao and Zheng 2016). The economic feasibility of algal culture for biodiesel production greatly depends on the high biomass productivity, carotenoid contents and appreciable fatty acid yields of the culture (Amir and Singh 2018; Novoveská et al. 2019). Currently, the production of carotenoids has become one of the most successful activities in algal biotechnology due to the demand for natural products instead of chemically synthesised products, even though the chemical synthesis process for carotenoids has a lower cost (de Carvalho and Caramujo 2017). In addition, fatty acids are organic acids that contain a carboxylic functional group with an aliphatic chain that can be saturated, mono-unsaturated or poly-unsaturated. The number of carbon atoms can vary, which results in the generation of short-chain, medium-chain, or long-chain fatty acids in microalgae depending on the taxonomic group and growth conditions (Bilbao et al. 2017). Thus, the algae biomass and its inclusions are of great interest and are expected to play a crucial role in the global energy infrastructure in the future.

Axenic algal cultures are always required for applications, such as genome sequencing (Metzker 2010; Alvarenga et al. 2017), identifying a biological producer of
a bioactive compound prior to large-scale production (Borowitzka 2013), constructing a bioremediation artificial consortia (Subashchandrabose et al. 2011; Olguín 2012), and elucidating the relationship between algae and other organisms using omics tools (Schonknecht et al. 2013; Ramanan et al. 2016). The establishment of an aseptic algae culture system would be helpful for determining the activity of algae in ecological, environmental and bioenergy applications. However, algae easily form a symbiotic microenvironment with bacteria during the process of algae cultivation, particularly large-scale cultivation (Ramanan et al. 2016). Even in the laboratory, bacteria exist in culture and affect the experimental results obtained with algae (Vu et al. 2018; Zhou et al. 2020). The various interactions among algae and bacteria seriously affect algal growth and its use in commercial development (Ramanan et al. 2016; Gonzalez-Camejo et al. 2020).

At present, centrifugal cleaning, dilution filtration and antibiotic addition are the main methods used for the purification of algae (Brown 1982; Ugwu et al. 2008). The addition of antibiotics to a culture of algae is a convenient method for controlling bacteria. However, the effects of antibiotics on algae have frequently been reported (Guo et al. 2016; Wang and Sheng 2020). Antibiotics can present a serious environmental risk to the environment. Eight antibiotics, namely, erythromycin, trimethoprim, sulfamethoxazole, tetracycline, oxytetracycline, ofloxacin, ciprofloxacin, and amoxicillin, were examined to analyse their toxicity on algae, and the results indicate that the selected antibiotics might pose a threat to aquatic environments (Kovalakova et al. 2020). Three antibiotics, tylosin, lincomycin and trimethoprim, can significantly influence photosynthesis but not the growth endpoint in chlorophytes, whereas the reverse is observed in cyanobacteria and diatoms (Guo et al. 2016). Over the range of concentrations from 0.1 to 10 mg L⁻¹, three veterinary antibiotics (chlorotetraacycline, oxytetracycline and enrofloxacin) alone or in combinations display various toxicities (inhibition or no effect) on the growth rates of the green algae Pseudokichneriella subacapitata and Ankistrodesmus fusciformis (Carusso et al. 2018). Due to these findings, the direct effects of antibiotics on algae vary, and algal species have their different responses to the various antibiotics (Kovalakova et al. 2020).

AMP is most used for eliminating bacteria from algae cultures (Chen et al. 2013). AMP was the first broad-spectrum penicillin and has been widely used since the 1960s. AMP, as a lactam antibiotic, exerts effects against gram-negative and gram-positive bacteria by inhibiting cytoderm synthesis enzymes and cell wall synthesis on the cytomembrane (Baselga-Cervera et al. 2019). Baselga-Cervera et al. (2019) showed that AMP has no effects in chlorophytes, whereas cyanobacteria show susceptibility to AMP over time. Previous studies have examined the influences of AMP on physiological changes in algae, but the influences of AMP on the global gene expression level of algae remain unknown.

Chlorella sorokiniana (Chlorophyta), a new alternative source of carotenoids for food applications, contains large amounts of useful metabolites and is considered a clean source of bioenergy (Diprat et al. 2020). Algae might be vulnerable to bacterial contaminants, and the addition of antibiotics to cultures of C. sorokiniana becomes crucial for obtaining and maintaining axenic algal strains. In fact, there is some concern that algae are sensitive to antibiotics, and the physiological changes induced by AMP should not be neglected during the maintenance of algal species. However, the exposure of algae to AMP has not been studied in detail. This study aimed to investigate the effects of AMP on global gene transcription and fatty acid biosynthesis in algae.

Materials and methods

Algal culture conditions

The algal strain C. sorokiniana SK used in this study was isolated and purified from a reservoir in Yantai City, China. The algal species was identified according to its phenotype structure and further verified by total DNA extraction, amplification of the 18S rRNA gene sequence using 18S rRNA gene primers (Table S1) and sequencing. The obtained sequence was blasted against the database of the National Center for Biotechnology Information. C. sorokiniana SK exhibited 99% similarity to C. sorokiniana IPPAS C-1 (MN160398.1). C. sorokiniana NZmm3W1 (KY054944.1) and C. sorokiniana NKH6 (LC505542.1) (Fig. S1). The BG-11 medium was prepared with deionized water, and the pH value was adjusted to 7.0 ± 0.1 (Singh et al. 2020). The medium and all the flasks were maintained at 121 °C for 20 min to ensure that they were sterile before the experiment. All culture flasks were cultured at 28 ± 1 °C in an incubator with 150 µmol photons m⁻² s⁻¹ and shaken in the shaker with 150 rpm and their positions were changed randomly to minimize the light-intensity effects. Algal cells were collected from 5-L cultures and centrifuged at 5000 g for 15 min. Chlorella cells were added to flasks containing 800 mL of culture on average. In this study, the response of C. sorokiniana to AMP at concentrations ranging from 20 to 100 mg L⁻¹, which are common concentrations used to obtain or store axenic algal cultures (Bhattarai et al. 2007; Chen et al. 2013), was studied. Four experimental groups were designed based on four concentrations of AMP (0 mg L⁻¹, 20 mg L⁻¹, 50 mg L⁻¹, and 100 mg L⁻¹). The group of 0 mg L⁻¹ concentration AMP was considered as the control.
group (CK). Three replicates of each group were included in the study. Samples were collected every 24 h over 6 days. The bacteria in algal culture were analysed after 1-mL culture of each sample was stained with 4′,6-diamidino-2-phenylindole (DAPI, Roth, Karlsruhe, Germany) solution. The DAPI-stained bacteria were filtered through Irgalan black-stained 0.2-µm Isopore™ polycarbonate membranes (Whatman, Meterstone, England) by the 25 mm diameter filter (Porter and Feig 1980; Niu et al. 2011). All bacterial cells on the Irgalan black-stained 0.2-µm Isopore™ polycarbonate membranes were embedded in immersion oil (Olympus, Tokyo, Japan) and counted under an epifluorescence microscope at ×1000 magnification (Zeiss Axioskop 20, Oberkochen, Germany). From 1-mL culture of each sample, at least 1000 cells and an average of 20 fields of view were analysed.

**Algal growth and pigment analysis**

*Chlorella* cells were fixed with glutaraldehyde (1% final concentration). The *Chlorella* cell count was directly determined with a 0.1-mL haemocytometer at an appropriate magnification using an Olympus BX50 microscope (Tokyo, Japan). Every sample was observed three times. The 10-mL *Chlorella* samples were placed into a 10-mL tube and centrifuged at 5000g for 15 min to collect the *Chlorella* cells. The *Chlorella* cells were stored at −80°C and thawed at room temperature before the determination. The samples were resuspended using 10-mL 95% ethanol solution for 30 min at room temperature to extract carotenoids and chlorophylls (Kulkarni and Nikolov 2018). Ethanol was recovered by centrifugation at 7500g for 10 min. The absorbances at wavelengths of 470 nm, 649 nm, and 664 nm were detected with a spectrophotometer (Persee, China). The chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid contents in the supernatants were calculated according to Lichtenthaler (1987).

**GC/MS determination of the fatty acid concentration**

After 6 days of, *Chlorella* cells were collected from the algal cultures, washed three times with potassium phosphate buffer (pH 7) and frozen in liquid nitrogen. For fatty acid profiling analysis, 1-mL of a methanol:acetonitrile:water (2:2:1, v/v/v) solution was added to each sample, and the mixture was homogenized and subjected to ultrasound for 20 min. The proteins were then precipitated at −20°C for 1 h. After centrifugation at 12,000g and 4°C for 20 min, the supernatant was collected and dried under vacuum. The sample was dissolved again with 100 µL of acetonitrile:water (1:1, v/v) solution and centrifuged at 12,000g and 4°C for 20 min. The supernatant was used for further analysis with an Agilent 1290 Infinity LC coupled to a 5500 QTRAP MS system (AB SCIEX, Darmstadt, Germany). The detection conditions used in the chromatography-mass spectrometry analysis were the same as those reported by Bai et al. (2018). The chromatographic peak area and retention time were extracted using MultiQuant software (AB Sciex, High-Tech, Life Sciences, MA, USA).

**RNA extraction, library preparation and transcriptome sequencing**

At the end of the experiment, samples were collected by centrifugation at 4°C and 3500g for 15 min and washed three times with 20 mM potassium phosphate buffer (pH 7). The samples were disrupted with mini glass beads under a Mini-Beadbeater (Biospec, USA). Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) and further purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA purity was measured with a spectrophotometer (Implen, CA, USA), and the RNA integrity and contamination were checked by 1% agarose gel electrophoresis. The RNA concentration was monitored using a Qubit RNA Assay Kit with a Qubit 2.0 fluorometer (Life Technologies, CA, USA).

A total of 1.5 µg of RNA per sample was used to analyse the transcription levels. According to the manufacturer’s recommendations for the NEBNext Ultra™ RNA Library Prep Kit (Neb, USA), sequencing libraries were generated for Illumina (Neb, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation buffer can disrupt mRNA into small fragments. First-strand cDNA was synthesized with random hexamers using mRNA as the templates. Second-strand cDNA was completed with DNA Polymerase I and RNase H and purified with AMPure XP beads. The purified second-strand cDNA was performed by terminal repair and adding a tail to connect the sequencing connector. After selecting cDNA fragments of 250–350 bp in length with AMPure XP beads, PCR amplification was performed. The PCR products were purified with an AMPure XP system (Beckman Coulter, Beverly, MA, USA) to obtain the final DNA library. The library quality was assessed with an Agilent Bioanalyzer 2100 system. The purified PCR products were sent to Novogene Experimental Departments (Novogene Co., Beijing, China) for clustering and sequencing with a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot- HS (Illumina). The raw data, including biological replicates, were submitted to the Sequence Read Archive of the NCBI database (SUB5945213).

**Data analysis for sequences**

The raw reads in fastq format were first processed by CASAVA base calling with the original image files. Clean
data were obtained by removing reads containing adaptors, reads containing poly-N and low-quality reads. Transcriptome assembly was accomplished using the method described by Grabherr et al. (2011). The gene functions were annotated based on the KO (KEGG orthologue), KOG (EuKaryotic Orthologous Groups), GO (Gene Ontology), Nr (NCBI nonredundant nucleotide sequences), Swiss-Prot and Nt (nonredundant nucleotide sequences) databases. The gene expression levels were estimated by RSEM (Li and Dewey 2011). Differential expression analysis between the experimental and control groups was performed using the DESeq R package (1.10.1). The P values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Differentially expressed genes (DEGs) with an adjusted P-value < 0.05 & |log2foldchange| > 1.5 were defined as differentially expressed.

Quantitative reverse transcription PCR (RT-qPCR)

The gene expression levels of the 18S rRNA, csaB, capD, mcvB, psaB and rbcL, genes were quantified by RT-qPCR (Table S1). RT-qPCR analyses were performed with a CFX 96 C 1000™ Thermal cycler (Bio-Rad, Hercules, CA, USA) and 10-µL reaction mixtures containing 5 µL of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA), 0.4 µL of each forward and reverse primer (10 pmol), 1 µL of the template DNA and 0.4 µL of bovine serum albumin (100 ng L⁻¹). The initial denaturation step (4 min at 95 °C) was followed by 40 PCR cycles consisting of 15 s at 95 °C, 30 s at a specified temperature and 30 s at 72 °C and then by a final elongation step of 5 min at 72 °C. A melting curve analysis of all the samples was performed by increasing the temperature from 70 to 90 °C at increments of 0.5 °C. All RT-qPCR assays were performed with six replicates. Negative controls lacking the cDNA template were analysed to monitor any potential DNA contamination. The 18S rRNA gene was selected as the housekeeping transcript to normalize the expression levels of the target gene transcripts. The data presented in this manuscript are the relative mRNA levels normalized against the 18S rRNA transcript levels, and the value of untreated *Chlorella* was set to 1. All the experiments described in this section were performed in triplicate to obtain the means and SDs.

Statistical analysis

All the data were analysed using OriginPro 9.1 and SPSS for Windows Version 22 and are reported as the means ± SDs. The data were statistically analysed by one-way ANOVA with post hoc test to determine the significant differences of bacterial cell density, algal cell density, and fatty acid between the control and AMP-treated groups, respectively. P values of < 0.05 were considered to indicate statistical significance in this study.

Results

Effects of AMP on symbiotic bacteria

The inhibitory effects of AMP on bacteria are dependent on the AMP concentration. The bacterial abundance after treatment with 50 mg L⁻¹ or 100 mg L⁻¹ AMP was notably lower than that obtained with the CK treatment (p < 0.05) (Fig. 1a). The abundance and composition of the bacterial community in algal culture were determined using high-throughput sequencing technology. Sequence clustering of bacteria was performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/), and clustered into operational taxonomic units (OTUs). Taxonomy of each OTU was assigned by blasting the representative sequence against Silva Database (https://www.arb-silva.de/) (Fig. S2). The abundance of *Bacteroidetes* declined from 41.55 to 12.73% after treatment with 100 mg L⁻¹ AMP. The 20 mg L⁻¹ AMP treatment increased the abundances of *Proteobacteria* and *Cyanobacteria* from 30.17 to 37.89% and from 4.61 to 19.67%, respectively (Fig. S2). The relative abundances of *Acetobacteraceae*, *Cyanobacteria*, and *Sphingomonadaceae* were enhanced by AMP, but the AMP treatments decreased those of *Cyclobacteriaceae* and *Rhodobacteraceae*. The richness and diversity of the symbiotic bacterial community changed depending on the AMP concentration (Table S2). Thus, the highest concentration of AMP exerted the highest inhibitory effects on the growth of bacteria, particularly *Bacteroidetes*.

Effects of AMP on *C. sorokiniana* growth

The addition of AMP to the culture exerted a concentration-dependent inhibitory effect on the algal cell density (Fig. 1b). Specifically, the algal cell densities obtained with the 50 mg L⁻¹ and 100 mg L⁻¹ AMP treatments were remarkable lower than that obtained with the control treatment (p < 0.05). Among these experimental groups, the algae treated with 20 mg L⁻¹ AMP presented the highest cell densities, whereas the lowest cell density was obtained with the 100 mg L⁻¹ AMP treatment. The concentrations of Chl a obtained with the CK, 20 mg L⁻¹, 50 mg L⁻¹, and 100 mg L⁻¹ AMP treatments were 0.03 ± 0.0034 pg cell⁻¹, 0.06 ± 0.0024 pg cell⁻¹, 0.08 ± 0.0071 pg cell⁻¹, and 0.07 ± 0.0053 pg cell⁻¹, respectively (Fig. 1c), and those of Chl b were 0.04 ± 0.0011 pg cell⁻¹, 0.05 ± 0.0015 pg cell⁻¹, 0.08 ± 0.0013 pg cell⁻¹, and 0.09 ± 0.0025 pg cell⁻¹, respectively (Fig. 1d). In
addition, the concentrations of carotenoids after the CK, 20 mg L⁻¹, 50 mg L⁻¹, and 100 mg L⁻¹ AMP treatments were 0.04 ± 0.0024 pg cell⁻¹, 0.03 ± 0.0024 pg cell⁻¹, 0.02 ± 0.0014 pg cell⁻¹, and 0.02 ± 0.0012 pg cell⁻¹, respectively (Fig. 1e).

**Fatty acid determination**

Nineteen fatty acids, namely, palmitic acid (C16:0), stearic acid (C18:0), α-linolenic acid (C18:3N3), linoleic acid (C18:2N6), myristoleic acid (C14:1), oleic acid (C18:1N9), behenic acid (C22:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), heptadecenoic acid (cis-10-C17:1), arachidic acid, (C20:0), myristic acid (C14:0), erucic acid (C22:1N9), tetracosanoic acid (C24:0), hexanoic acid (C6:0), heicosenoic acid (C21:0), tricosanoic acid (C23:0), arachidonic acid (C20:4N6), and cis-11-eicosanoic acid (C20:1), were analysed by GC/MS. These 19 fatty acids were identified by GC/MS, and the results showed that the contents of 16 fatty acids were decreased by the 20 mg L⁻¹, 50 mg L⁻¹ and 100 mg L⁻¹ AMP treatments (Fig. 1f). The highest concentration measured (0.32 ± 0.0144 pg cell⁻¹) was found for palmitic acid (C16:0), and the content of heptadecanoic acid (C17:0), which was the second most abundant fatty acids in algal cells, were 0.30 ± 0.0124 pg cell⁻¹. In addition, three fatty acids, erucic acid (C22:1N9), hexanoic acid (C6:0), and cis-11-eicosanoic acid (C20:1), were markedly enhanced by treatment with AMP (p < 0.05). The levels of these fatty acids in the 100 mg L⁻¹ AMP-treated group were 2- to 5-fold higher than those in the control group. Cis-11-eicosanoic acid (C20:1) was not detected in the control group but was detected at levels of 0.1–0.2 pg cell⁻¹ after treatment with 20 mg L⁻¹, 50 mg L⁻¹, and 100 mg L⁻¹ AMP.

**Global transcription assembly and functional annotation**

A whole-genome shotgun strategy was utilised to sequence the transcript level of *C. sorokiniana*. A total of 262 346
492 bp raw reads and 251 298 331 bp clean reads were obtained from all the samples (Table 1). Among the clean reads, more than 97% of the bases obtained from all the treatment groups had a Q value > 20, and a Q value of 30% was obtained for more than 92% of the bases. An overview of the transcript length distribution in the transcriptome is shown in Table 2. The de novo assembly of the sequences generated a total transcriptome of 160 302 transcripts with a mean length of 2 027 bp. The analysis yielded a total of 58 415 unigenes with an average length and N50 value of 2 256 bp and 2 256 bp, respectively.

For identification of the unigene functions, functional annotation was performed with various databases (Nr, the Swiss-Prot database, Nt, KO, KOG, and GO). Based on the KEGG orthologue database, all the unigenes were annotated into 19 processes: transport and catabolism (7.55%), membrane transport (1.09%), signal transduction (3.07%), folding, sorting and degradation (8.65%), replication and repair (4.86%), transcription (4.62%), translation (11.02%), amino acid metabolism (8.53%), biosynthesis of other secondary metabolites (1.23%), carbohydrate metabolism (12.61%), energy metabolism (4.95%), glycan biosynthesis and metabolism (1.89%), lipid metabolism (6.88%), metabolism of cofactors and vitamins (3.74%), metabolism of other amino acids (2.40%), metabolism of terpenoids and polyketides (1.72%), nucleotide metabolism (4.91%), overview (9.13%), and environmental adaptation (1.14%) (Fig. 2). The percentage of homologous sequences in the Nt database that showed homology between Chlorella sp. and closely related algae is shown in Fig. 3. The top-hit species included C. sorokiniana, Chlorella variabilis, Coccomyxa subellipsoidea, Chlamydomonas reinhardtii and Volvox carteri.

**EG analyses**

The DEGs were differentially expressed genes with $P$ value < 0.05 & $|\log2\text{foldchange}| > 1.5$ between the control and AMP-treated groups. A total of 3 825 upregulated and 1 432 downregulated DEGs were detected in all the experimental groups, and 1 933, 1 043, and 849 upregulated genes and 2 532, 4 202, and 4 700 downregulated genes were identified in the 20 mg L$^{-1}$, 50 mg L$^{-1}$, and 100 mg L$^{-1}$ AMP-treated groups, respectively. The heat map of DEGs is shown in Fig. 4, and as shown by the

| Table 2 | Overview of transcript length distribution of transcriptome |
|---------|----------------------------------------------------------|
| Transcript length | 301–500 | 501–1000 | 1001–2000 | > 2000 | Mean length | N50 | N90 |
| Transcript | 25 504 | 30 270 | 43 200 | 61 328 | 2 027 | 3 026 | 1 010 |
| Unigene | 15 118 | 14 537 | 15 114 | 13 646 | 1 469 | 2 256 | 621 |

Fig. 2 KEGG functional classification of assembled unigenes. a Organismal systems. b Metabolism. c Genetic information processing. d Environmental information processing. e Cellular processes
different colours, the samples treated with AMP showed obvious changes in the algal metabolism compared with that the control samples. A KEGG analysis was performed to further explore the functional classification and pathway assignments of the DEGs identified in the 20 mg L\(^{-1}\), 50 mg L\(^{-1}\), and 100 mg L\(^{-1}\) AMP-treated groups. The enriched pathways obtained from the KEGG analysis were statistically analysed (Figs. 5 and S3). The upregulated genes in the 20 mg L\(^{-1}\) AMP-treated group were enriched in the following pathways: vitamin B6 metabolism, ubiquitin-mediated proteolysis, sulphur metabolism, starch and sucrose metabolism, ribosome biogenesis in eukaryotes, riboflavin metabolism, regulation of autophagy, protein processing in endoplasmic reticulum, porphyrin and chlorophyll metabolism, plant hormone signal transduction, other types of O-glycan biosynthesis, nucleotide excision repair, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, fructose and mannose metabolism, fatty acid elongation, endocytosis, DNA replication, cysteine and methionine metabolism, caffeine metabolism and amino sugar and nucleotide sugar metabolism (Fig. 5a). The downregulated genes in the 20 mg L\(^{-1}\) AMP-treated group were enriched in the following pathways: valine, leucine and isoleucine degradation, tryptophan metabolism, synthesis and degradation of ketone bodies, steroid biosynthesis, RNA polymerase, regulation of autophagy, propanoate metabolism, phagosome, pentose and glucuronate interconversions, oxidative phosphorylation, one carbon pool by folate, RNA surveillance pathway, lysine degradation, glyoxylate and dicarboxylate metabolism, glycosphingolipid biosynthesis-ganglio series, endocytosis, cyanoamino acid metabolism, citrate cycle (TCA cycle), carotenoid biosynthesis, and arginine and proline metabolism (Fig. 5b). Most notably, the upregulated DEGs were enriched in the porphyrin and chlorophyll metabolism pathways, whereas the downregulated DEGs were enriched in the carotenoid biosynthesis pathway. The most enriched GO terms of the upregulated and downregulated genes in the 100 mg L\(^{-1}\) AMP-treated group were classified into the top 30 pathways belonging to three terms: biological process, cellular component, and molecular function (Fig. 5c, d). The most enriched GO terms related to the downregulated genes were GTPase-mediated signal transduction, intracellular signal transduction, cell-matrix adhesion, cell-substrate adhesion, G-protein coupled receptor signalling pathway, G-protein beta/gamma-subunit complex binding, signal transducer activity, guanyl ribonucleotide binding, GTP binding, guanyl nucleotide binding, and molecular transducer activity.

**DEGs in the porphyrin and chlorophyll metabolism pathways**

A total of 113 genes related to the porphyrin and chlorophyll metabolism pathways were identified from the KEGG analysis (Table 3; Fig. 6). The statistical analysis of the enriched pathways showed that the upregulated DEGs were enriched in the porphyrin and chlorophyll metabolism pathways. Ten DEGs related to the porphyrin and chlorophyll pathways were identified, and these are shown in Table 3. The *mmaB* and *pduO* genes encoding cob(I) alamin adenosyltransferase, the *cpoX* and *hemF* genes, which encode coproporphyrinogen III oxidase, and the *hemB* and *alaD* genes, which are related to the production of porphobilinogen synthase, were upregulated with a log\(_2\) fold change > 0 in all experimental
Fig. 4  Heat map of DEGs in C. sorokiniana
groups compared with the control group. Another seven genes related to the porphyrin and chlorophyll metabolism pathway were downregulated with a log2 fold change < 0 in all AMP-treated groups compared with the control group.

DEGs in the carotenoid biosynthesis pathway

Eighty-five genes associated with the carotenoid biosynthesis pathway were identified by the KEGG analyses, and 10 of these DEGs were classified in this study (Table 3; Fig. 6). Eight DEGs, namely, *aldH* (aldehyde dehydrogenase (NAD+)), *z-iso* (zeta-carotene isomerase), *lcyE/crtL2* (lycopene epsilon-cyclase), *crtZ* (beta-carotene 3-hydroxy-lase), *tktA/tktB* (transketolase), *lcyB/crtL1/crtY* (lycopene beta-cyclase), *accD* (acetyl-CoA carboxylase carboxyl transferase subunit beta), and *lut1/cyp97c1* (carotenoid epsilon hydroxylase), were downregulated by AMP. Compared with that in the control group, the expression of two other DEGs was enhanced after treatment with AMP.

AMP decreased fatty acid biosynthesis

A total of 75 genes in the algal strain were classified, and the downregulated DEGs were enriched in the fatty acid biosynthesis pathway (Figs. 6 and S4). The expression levels of six DEGs were downregulated after treatment with AMP (Table 3), and the lowest expression levels of these DEGs were found after treatment with 100 mg L^{-1} AMP. The log2 fold change values of these six DEGs, namely, *acsL/fadD* (long-chain acyl-CoA synthetase), *fabI* (enoyl-), *fabF* (3-oxoacyl-), *fabG* (3-oxoacyl-), and *accD* (acetyl-CoA carboxylase carboxyl transferase subunit beta), were < 0.

qRT-PCR validation

To confirm the RNA-seq results, several genes were selected for qRT-PCR analysis of their relative expression levels in the experimental groups of algae compared with the control group (Fig. 7). Three unigenes related to chlorophyll synthesis (*mmaB*, *dvR*, and *chI*)CyD), three unigenes related to carotenoid biosynthesis (*crtZ*, *z-iso*, and *crtisO*) and three unigenes related to fatty acid biosynthesis (*fabF*, *fabH*, and *accD*) were selected as the representative genes. The relative expression levels of *mmaB* were enhanced by AMP in the experimental groups compared with the control group. The *dvR*, *chI*, *crtZ*, *z-iso*, *crtisO*, *fabF*, *fabH*, and *accD* genes were downregulated by AMP. Although only eight genes were selected for qRT-PCR analysis, the trends observed in the RNA-seq and qRT-PCR results were consistent, which suggested the reliability of the gene expression profiles obtained from the RNA-seq analysis.
Discussion

Although no obvious effects of AMP were observed in the physiologies of *Chlorophytes* *Chlamydomonas reinhardtii* Cr1D, *Dictyosphaerium chlorelloides* Dc1M and *Dunaliella* sp. (Baselga-Cervera et al. 2019), the results of this study demonstrated that AMP exerts a slight inhibitory effect on the growth of *C. sorokiniana*, but the transcription of many genes related to chlorophyll, carotenoids and fatty acids were markedly influenced. In this study, 20 mg L$^{-1}$ AMP resulted in the best inhibition of bacteria in the algal culture, which was consistent with the results from a previous study that showed that AMP concentration of 10 mg L$^{-1}$–100 mg L$^{-1}$ was always used in algal culture (Chen et al. 2013). However, although AMP plays a role in protecting against bacterial contamination and maintaining the axenic purification of algae, the expression levels of some functional genes in *C. sorokiniana* might be upregulated or downregulated.

AMP increased porphyrin and chlorophyll metabolism

A comparative transcriptome analysis between the control and experimental groups revealed that the DEGs upregulated by AMP were enriched in the porphyrin and chlorophyll metabolic pathways. The upregulated DEGs were enriched in the KEGG pathway of porphyrin and chlorophyll metabolism. These upregulated genes performed their specific function in porphyrin and chlorophyll metabolism. For example, the nucleus-encoded locus *hemB*, which encodes aminolaevulinic acid dehydratase, is related to the production of porphyrin (Piao et al. 2004). The *hemF* gene, which encodes coproporphyrinogen III oxidase, plays important roles in the regulation of porphobilinogen and in the growth of *Rhodobacter sphaeroides* (Zeilstra-Ryalls and Schornberg 2006). Cox15 controls the synthesis of cytochrome c oxidase assembly protein subunit.

### Table 3 DEGs related to the pathways of porphyrin and chlorophyll metabolism, carotenoid biosynthesis and fatty acid biosynthesis

| Gene ID | log$_2$Fold change | KO Name | KO description |
|---------|--------------------|---------|----------------|
| **Porphyrin and chlorophyll metabolism** | | | |
| Cluster-10635.0 | 6.56 | 6.57 | 7.03 | mmaB/pduO | Cob(I)alamin adenosyltransferase |
| Cluster-16222.0 | −1.93 | −3.20 | −4.90 | hemE/uroD | Uroporphyrinogen decarboxylase |
| Cluster-18904.10236 | −1.51 | −1.86 | −2.64 | cobA | Uroporphyrin-III C-methyltransferase |
| Cluster-18904.18666 | −0.47 | −1.59 | −2.64 | chlD/bchD | Magnesium chelatase subunit D |
| Cluster-18904.19024 | 2.55 | 4.29 | 6.87 | cpoX/hemF | Coproporphyrinogen III oxidase |
| Cluster-18904.7241 | −0.46 | −1.50 | −4.45 | hepH | Hephæsin |
| Cluster-18904.4585 | −2.48 | −3.18 | −3.46 | earS/gltX | Glutamyl-tRNA synthetase |
| Cluster-18904.664 | 2.06 | 2.35 | 2.55 | hemB/alaD | Porphobilinogen synthase |
| Cluster-18904.861 | −1.84 | −1.88 | −1.97 | chlN | Light-independent protochlorophyllide reductase subunit N |
| Cluster-18904.9762 | −1.77 | −1.75 | −1.72 | dvR | Divinyl chlorophyllide a 8-vinyl-reductase |
| **Carotenoid biosynthesis** | | | |
| Cluster-10324.0 | −2.63 | −2.95 | −3.61 | aldH | Aldehyde dehydrogenase |
| Cluster-17234.0 | −3.52 | −3.60 | −6.61 | z-isO | Zeta-carotene isomerase |
| Cluster-18904.1513 | −1.63 | −2.64 | −3.94 | lcyE/crtL2 | Lycopene epsilon-cyclase |
| Cluster-18904.9924 | −1.53 | −1.69 | −1.82 | criZ | Beta-carotene 3-hydroxylase |
| Cluster-18904.3436 | −1.24 | −1.58 | −1.60 | crisO/crtH | Prolycopene isomerase |
| Cluster-18904.3570 | −1.46 | −1.66 | −1.80 | tktA/tktB | Transketolase |
| Cluster-18904.25006 | −2.21 | −2.22 | −2.35 | lcyB/crtL1, crtY | Lycopene beta-cyclase |
| Cluster-18904.2539 | −1.53 | −5.86 | −5.87 | accD | Acetyl-CoA carboxylase carboxyl transferase subunit beta |
| Cluster-18904.9148 | −1.54 | −1.82 | −2.04 | lut1/cyp97c1 | Carotenoid epsilon hydroxylase |
| **Fatty acid biosynthesis** | | | |
| Cluster-17471.0 | −1.63 | −2.51 | −2.59 | acsL/fadD | Long-chain acyl-CoA synthetase |
| Cluster-18904.25659 | −1.68 | −1.97 | −3.16 | fabI | Enoyl- |
| Cluster-18904.2417 | −1.79 | −1.82 | −2.13 | fabF | 3-oxoacyl- |
| Cluster-18904.5925 | −1.57 | −4.91 | −6.31 | fabG | 3-oxoacyl- |
| Cluster-20978.0 | −2.24 | −3.42 | −3.26 | fabH | 3-oxoacyl- |
| Cluster-18904.2539 | −0.21 | −0.48 | −1.53 | accD | Acetyl-CoA carboxylase carboxyl transferase subunit beta |
15 to influence the content of HemeA (He et al. 2016). The mmaB gene helps produce an enzyme involved in the formation of adenosylcobalamin, which is a derivative of vitamin B12 (cobalamin). The enhancement of the hemB, hemF, and hemeA genes, which encode proteins involved in the pigment production (Kwon et al. 2003; Mukougawa et al. 2006; Ge et al. 2018), increases the concentrations of Chl a and Chl b. RT-qPCR analysis performed in this study showed that the higher relative expression level of the mmaB gene accelerated the production of chlorophyll. In this study, the concentrations of Chl a and Chl b were enhanced by AMP, and the upregulated genes related to porphyrin and chlorophyll metabolism in the AMP-treated group might synthesize more enzymes related to the formation of chlorophyll.

AMP downregulated carotenoid biosynthesis

Antibiotic exposure decreased the concentration of carotenoids in Desmodesmus subspicatus (chlorophytes) (Guo et al. 2016), and the inhibitory effect of AMP on the production of carotenoids was also observed in this study. Furthermore, five downregulated genes, namely, the lcyB (crtL1), lcyY (icyE, crtL2), lut1 (cyp97C1), z-isO, crtZ and.crtisO (crtH) genes, were enriched in the carotenoid biosynthesis pathway, as determined by KEGG enrichment analysis. Among these downregulated genes, the gene cluster 5301.0 was annotated as lcyE, which encodes lycopene epsilon-cyclase and is related to the production of δ-carotene or ε-carotene (Cui et al. 2011). crtL-b exhibits lycopene β-cyclase activity, whereas crtL-e is a bifunctional enzyme with both lycopene ε-cyclase and lycopene β-cyclase activity for 7,8-dihydro-β-carotene (Takaichi 2011). The gene lut1, which encodes a cytochrome p450-type monooxygenase CYP97C1, catalyses the hydroxylation of the ε-ring of β,ε-carotenoids to influence the formation of lycopene and lutein (Kim and DellaPenna 2006). The 1.141427 gene cluster encoding the enzymes crtZ, cyp175A, and crtS influences the synthesis of astaxanthin. In addition, AMP
downregulated the expression profiles of DEGs enriched in carotenoid biosynthesis and decreased the carotenoid concentration in *C. sorokiniana*. In addition, the *crtZ*, *z-iso*, and *crtisO* genes showed decreased transcriptional abundance in the experimental groups, which verified that AMP inhibited carotenoid biosynthesis.

**AMP inhibited fatty acid biosynthesis**

Fatty acids are important metabolites and are considered chemotaxonomic markers for defining groups of various taxonomic ranks (Stamenković et al. 2020). The production efficiency of fatty acids in algal cells can estimate the latent capacity of *C. sorokiniana* to serve as a clean source of bioenergy. As demonstrated by the statistical analysis of the enriched pathways, the downregulated genes *fabH*, *fadD*, *fabF*, *acsL*, *fabG*, and *accD* were enriched in the fatty acid biosynthesis pathway. The *accD* gene, which encodes a subunit of the acetyl-CoA carboxylase (ACCase) enzyme, regulates the translation of acetyl-CoA to malonyl-CoA (Cui et al. 2011). The *fabH* gene encoding β-ketoacyl-ACP synthase III affects the formation of acetoacetyl-[ACP]. The *fabF* and *fabG* genes, which encode 3-oxoacyl-[acyl-carrier-protein] synthase 2 and 3-oxoacyl-[acyl-carrier-protein] reductase in *Escherichia coli* (strain K12), respectively, influence the production of octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, hexadecenoic acid and octadecanoic acid. The *acsL* gene, which encodes acyl-CoA synthetase long-chain family member 4, affects the production of hexadecanoyl-CoA (Tan and Lee 2016). Hexadecanoyl-CoA serves as a link between fatty acid biosynthesis and fatty acid elongation. These downregulated genes were enriched in the fatty acid biosynthesis pathway. With respect to fatty acid metabolism, all detected fatty acids except for two fatty acids, myristic acid (C22:1N9) and tetracosanoic acid (C6:0), showed decreased expression after treatment with AMP. In addition, 12 key DEGs related to fatty acid biosynthesis were identified among the annotated genes, and these genes encoded long-chain acyl-CoA synthetase, fatty acyl-ACP thioesterase A, enoyl-[acyl-carrier protein] reductase I, acyl-[acyl-carrier protein] desaturase, acetyl-CoA carboxylase, biotin carboxylase subunit, acetyl-CoA carboxylase carboxyl transferase subunit alpha, acetyl-CoA carboxylase/biotin carboxyl carrier protein, acetyl-CoA carboxylase/biotin carboxylase subunit alpha. In summary, AMP inhibited the transcript levels of various genes involved in fatty acid biosynthesis, such as *fabF*, *fabH*, and *accD*, and thereby led to a decline in the production of fatty acids.

Consequently, exposure to AMP at certain concentrations influences algal proliferation. AMP significantly upregulated the expression of the *hemB*, *hemF*, *mmaB*, and *HemeA* genes, which participate in the porphyrin and chlorophyll metabolism pathways, and downregulated the expression

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**Fig. 7** Gene expression levels determined by RNA-seq and qRT-PCR
levels of the lcyB (crtL1), crtY (crtE, crtL2), lut1 (cyp97C1), z-isO, crtZ and crtsisO (crtH) genes in the carotenoid biosynthesis pathway and the fabH, fabD, fabF, acsL, fabG, and accD genes in the fatty acid biosynthesis pathway. Because AMP is used for obtaining axenic C. sorokiniana, the recovery of the physiological metabolism of algal strains should not be neglected. The data obtained in this study provide critical information for understanding the effects of AMP on pigment production and fatty acid metabolites during aseptic processing and serve as an important reference for the application of C. sorokiniana in commercial applications.

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Author contributions Wenjing Wang performed the experiments and wrote the manuscript; Yanqing Sheng revised the manuscript.

Availability of data and material The datasets analysed in the current study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

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

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