Random compound mutagenesis breeding of *Bacillus amyloliquefaciens* X030 with high resistance to pathogenic bacteria on grass carp

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Research

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Abstract

Background: *Bacillus amyloliquefaciens* X030 (BaX030) was obtained by screening from peanut fields in Henan Province, China. It had broad-spectrum antibacterial activity against fish pathogens *Aeromonas hydrophila* and *Aeromonas veronii*. In order to improve its antibacterial effect, BaX030 was carried out the compound mutagenesis that atmospheric and room temperature plasma (ARTP) combined with nitrosoguanidine (NTG).

Results: The result showed the yield of macrolactin A and oxydifficidin of the mutant N-11 were increased by 2.01 times and 3.68 times, respectively. Re-sequencing found that the corresponding 9th and 15th gene clusters had 4 and 6 SNP mutations, respectively, the 15th gene cluster also had 7 InDel mutations. Scanning electron microscopy observed that the N-11 became thin and long. The results of qRT-PCR indicated that feeding the N-11 can increase the expression of immune factors in the liver or kidney tissue of grass carp. It can also significantly reduce the mortality and the surface symptoms of grass carp that was infected by two pathogens through HE staining and protection experiments.

Conclusion: This is the first report that after ARTP-NTG compound mutagenesis, a high-yielding strain of macrolactin A and oxydifficidin were increased by 2.01 times and 3.68 times compared with the original strain, respectively, which laid the foundation for elucidating its biological regulation, they had antibacterial effects on *Aeromonas hydrophila* and *Aeromonas veronii*. We combined re-sequencing to find the mutation sites of gene clusters and. The probiotic strain N-11 can quickly activate the immune protection mechanism of grass carp to resist pathogenic bacteria.

Introduction

The rapid development of aquaculture leads to the disease problem of aquatic animals becoming increasingly prominent. Bacterial infectious diseases that caused by *Aeromonas hydrophila* (*A. hydrophila*) (Singh et al. 2011; Pridgeon and Klesius 2011) and *Aeromonas veronii* (*A. veronii*) (Smyrli et al. 2019; Zhang et al. 2020) had huge harm to fisheries in the whole world. In recent years, biological agents had more attention as the substitute for chemical pesticides. *Bacillus amyloliquefaciens* was a probiotic and can produce a large number of secondary metabolites with a wide range of antibacterial activities, including polyketide compounds and lipopeptide compounds, and polyketide compounds mainly included bacillaene, macrolactin and difficidin. Thus,probiotic bacteria utilization in aquaculture systems has emerged as a solution to prevent pathogens development (Truong et al. 2017; Nandi et al. 2018; Tarnecki et al. 2019).

It was a common method that *Bacillus* and feed were fed to aquatic animals after mixing, which can improve their specific immunity by increasing the expression of immune factors. Some studies had found that feeding *Bacillus* can significantly increase the serum immunoglobulin M (IgM) level of *Sparus aurata* (Bahi et al. 2017). After feeding *Bacillus amyloliquefaciens*, the expression of IgM was also significantly increased, which enhanced the resistance to *A.hydrophila* (Kuebutornye et al. 2020); Zhang DX et al.

discovered that feeding *Bacillus velezensis* can promote the expression of interleukin 1 β (IL-1 β) and protect fish from *A. veronii* infection (Zhang et al. 2019). In addition, common immune factors also included lysozyme (LSZ), complement C3 (C3) and IL8. Therefore, the addition of probiotics can improve the immunity of aquatic animals and prevent disease outbreaks in the process of aquaculture.

Atmospheric and room-temperature plasma (ARTP) method was used to breed high-yielding mutations as a new type of physical method, it accelerated the process of screening mutants of *Bacillus amyloliquefaciens* (Shi et al. 2018). Nitrosoguanidine (NTG) was an efficient chemical mutagen, which can greatly increase the mutation frequency and screen the target strains. Recently, the screening methods that combined chemical mutagenesis and sequencing had become more and more popular to detect essential genes in cells and organisms (Farrell et al. 2014; Li et al. 2016; Forment et al. 2017), these technologies can compensate for the inability to accurately locate single-nucleotide variants (SNV), including single nucleotide polymorphisms (SNPs) and small insertions or deletions (InDels). These technologies can help us screen out mutagenic strains with stronger antibacterial effects.

We found that the yield of antibacterial active substances was increased on a variety of fish pathogens in BaX030, and the structure was identified by nuclear magnetic resonance (NMR). We combined ARTP-NTG and resequencing to sequence the mutant strains and find the mutation sites. The mutagenized strain N-11 has a stronger protective effect on grass carp infected with *Aeromonas hydrophila* X040 (AhX040) and *Aeromonas veronii* X005 (AvX005) in the vivo and vitro experiments. The results also had important production and application value for the prevention and treatment of aquatic fish diseases.

Results

Separation and structural identification of antagonistic active substance

The two peaks we separated from the BaX030 strain by HPLC 1290 had a good antibacterial effect on AhX040 and AvX005 (Fig. 1A). Peak 1 was used to determine the molecular weight of the substance by MALDI-TOF-TOF MS. The result showed that its mass-to-charge ratio was 425.2290 Da $[M + Na]^+$, and its relative molecular mass was determined to 402 Da. The secondary mass spectrum showed that the compound was stable and wasn't easily broken (Supplementary Figure S1A). The molecular structure was obtained by NMR combined with mass spectrometry. The chemical shifts of 1H and ^{13}C have been calculated with ab initio and DFT, all of which were compared with the experimental results (Table S1), and the remaining C was specified by HMBC. We identified its chemical formula as $C_{24}H_{34}O_5$, and the determined molecular structure was a class of twenty-four-membered macrolide compound macrolactin A, which was consistent with the mass spectrometry results (Fig. 1B). The anti-SMASH Version 5.1.0 online bioinformatics platform was used to predict and analyze the genome sequence of BaX030, and the results showed that it contained 15 gene clusters (Table S2). Cluster 9 was the synthetic gene cluster of macrolactin A, which was composed of the *pks2A-H* genes.

The molecular weight of the substance that was identified by mass spectrometry was $[M-H]^- = 559.14\text{Da}$ in peak 2 (Supplementary Figure S1B), the chemical formula was $\text{C}_{31}\text{H}_{44}\text{O}_7\text{P}$ and it was oxydifficidin belonging to polyketides (Fig. 1C). The gene cluster was composed of *difA-O* genes and was located in the fifteenth gene cluster of BaX030 (Table S2).

Determination of ARTP mutagenesis time

The starting strain was increased with the extension of ARTP treatment time by the lethality curve in the first round (Supplementary Figure S2). The mutagenesis time was 45s, the fatality rate was significantly increased to 88.57%; the fatality rate was close to 100% when it was 120s. We finally selected the treatment time of 45s for ARTP mutagenesis.

Screening of highly active mutant strains

The yield results of some mutagenized strains were shown in the figure, and the leftmost strain was the starting strain of this round. The strains with the highest yields of macrolactin A mutagenesis were A-9, A-48 and N-11 in the three rounds, and it were increased by 1.2 times, 1.06 times and 1.09 times compared with the starting strains in this round, respectively (Fig. 2A-C). The yield of macrolactin A in N-11 was increased by 1.38 times (16.34 $\mu\text{g/mL}$), another secondary metabolite of oxydifficidin was also gained by 3.68 times (8.42 $\mu\text{g/mL}$) compared with CK group (macrolactin A 11.84 $\mu\text{g/mL}$; oxydifficidin 2.29 $\mu\text{g/mL}$), and its antibacterial effect was greatly improved (Fig. 2D). After N-11 was continuously subcultured for 5 generations, the yield of antibacterial activity and the antibacterial effect did not decrease, which indicated that the mutant strain can maintain genetic stability.

The growth curve and morphological observation of BaX030 and N-11

The results of growth curve showed that the lag phase of the BaX030 was 0–4 h, the log phase was 4–20 h. But the N-11 grew faster compared with the BaX030, the stable period of the N-11 was extended by 4h (Fig. 3A). We can clearly observe the changes of the bacteria's morphology after mutagenesis through the phase contrast microscope and the scanning electron microscope (Fig. 3B-C). The BaX030 was shorter, while the strain N-11 was slender.

Re-sequencing of mutant strain N-11

The re-sequencing results showed that the coverage of sample N-11 was 99.98% (Table S3), The scatter plot was a shape similar to the Poisson distribution (Supplementary Figure S3A). The sequencing depth was 280, the coverage depth of the bases on the genome was evenly distributed compared to BaX030 as the reference sequence (Supplementary Figure S3B). The results demonstrated that there was no GC bias in resequencing data, and the randomness of sequencing was good.

SNP and InDel annotation of the resequencing genome N-11 compared to the reference genome BaX030

The 117 SNP variants in the CDS region accounted for 86.67% compared to all SNP variants (Table S4). Among the SNP variants of CDS regions, non-synonymous coding mutations accounted for 62.22%. In the 9th gene cluster of BaX030, there were four SNP mutations, and one non-synonymous mutation occurred in the core region gene cluster (gene *pks2F*) (Fig. 4A). Six SNP mutations were in the 15th gene cluster, including 4 mutations in the core gene cluster. There were two and one non-synonymous mutations in *difG* gene and *difK* gene, respectively; the *difJ* gene had a synonymous mutation; a non-synonymous stop codon mutation was present between the core gene clusters *difC* and *difD* (Fig. 4B; Supplementary data 1).

The total number of InDel mutations we detected was 20, the number of insertion was 18, and the number of deletion was 2. In the 15th gene cluster, there were a total of 7 InDel mutations that were single nucleotide insertions, including 4 insertion mutations in the *difD* gene; 3 insertion mutations between the *difD* and *difE* genes. But we did not find any InDel mutations in the 9th gene cluster (Fig. 4B; Supplementary data 2).

Effect of the strain N-11 on the immune gene expression in liver and kidney of grass carp

After grass carp was infected with AhX040, N-11 could promote the significant increase in the expression of C3 and IL-1 β in the liver and kidney. The maximum up-regulation of C3 and IL-1 β were 18.1 and 6.15 times, respectively (Fig. 5A, B). If infected with AvX005, it can stimulate the expression of LSZ and IL8. No matter which pathogen was infected, IgM was up-regulated in the liver and kidney, and the maximum was 9.37 times. The results showed that the strain N-11 can improve the specific immunity of grass carp (Fig. 5C, D).

Protection experiment of the strain N-11 on grass carp

All the grass carp were died in the Ah group. The death rate was 70% in the Av group. The symptoms were mainly abdominal congestion and anal redness. After the 7th day, The cumulative mortality was 60% and 10% in the N-11 + Ah group and N-11 + Av group, which were reduced by 40% and 60% compared to the control group (Table S5). Moreover, the surface symptoms were relieved in the experimental group (Supplementary Figure S4).

We observed that after grass carp was infected with two pathogens, the pathological changes in the liver were that the polygonal structure of cells was destroyed, a large number of cells were broken, and the tightness between cells was reduced through H&E staining (Fig. 6). In the kidney, it is mainly manifested in glomerular atrophy and severe vacuolar degeneration of renal tubular cells. However, the corresponding pathological symptoms of the experimental group were improved. The results showed that feeding N-11 strain can increase the resistance and survival rate of grass carp infected with two pathogens, and it had a stronger antagonistic effect on AvX005.

Materials And Methods

Bacterial strains and culture conditions

AvX005 (Huang et al. 2020), AhX040 (Cao et al. 2019), all laboratory collections. *Bacillus amyloliquefaciens* X030 (CCTCC No. M2014159) was obtained by screening from peanut fields in Henan Province, China. BaX030 and the mutant strain N-11 were cultured overnight at 30°C with agitation at 200 rpm, the overnight culture was transferred into fermentation medium, comprised of sucrose 32 g, polypeptone 10 g, yeast extract 5 g and NaCl 10 g/L (pH 7.0) at the inoculum of 3% vol/vol. They were cultured at 30°C for 60h. Bacterial fermentation broth was collected every 4 h to evaluate the bacterial concentration by detecting optical density at 600 nm (OD₆₀₀), the OD₆₀₀ values were measured by the SmartSpcTM 3000 spectrophotometer, and growth curves were generated by OriginPro 8.5.

Morphological observation of strains

We took 20 hours of strain culture, centrifuged at 10000 rpm for 2 minutes to collect the bacteria, washed 2–3 times with sterile water, dropped about 3 µL on the glass slide and observed them by AXIO Abserver A1 upright optical microscope (Zeiss, Germany).

The above-mentioned cells were washed again with sterile water for 10 times, the supernatant was discarded, and 600 µL of 2.5% glutaraldehyde fixative was added to the precipitate, and fixed at 4°C in the dark for 12 h, the fixative was discarded by centrifugation, 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol were dehydrated step by step, resuspended the bacteria in absolute ethanol, and sucked 3µL on the glass slide. After the samples were naturally air-dried and electroplated, they were observed by the scanning electron microscope (HITACHI SU8000, Japan).

Separation and purification of antagonistic antibacterial active substances

After culturing for 48h, the fermentation broth of the strain was centrifuged at 8500 rpm for 15 min, the supernatant was retained, and the equal volume of ethyl acetate was added for overnight extraction. We collected the organic phases and concentrated them in the vacuum concentrator (SPD121P, Thermo Fisher, USA).

The concentrated crude extract was dissolved in 100% methanol and filtered with the 0.22 µm filter. the sample was loaded on a reverse-phase column ZORBAX SB-C18 (15 µm, 4.6250 mm, Agilent) for HPLC 1290, the ultraviolet monitoring wavelength was 280 nm. Mobile phase A was ultrapure water and phase B was 100% acetonitrile with the flow rate of 1 mL/min. Each peak that collected by freeze-drying (vacuum concentrator, SPD121P, Thermo Fisher, USA) was dissolved in 50% methanol. 10 µL was added on the filter paper of LB solid medium that coated with two kinds of fish pathogens and the bacteriostasis experiment was carried out, incubated at 30°C for 12 h, and selected the peak with the best antagonistic effect as the crude active production. The sample after purification was dried and sent to Shenzhen Weinafei Biotechnology Co., Ltd. for mass spectrometry identification, the Beijing Institute of Biophysics for nuclear magnetic resonance detection.

The program was also used for the determination of the standard curve and the compound concentration. The concentration of macrolactin A and oxydifficidin (the standard curve was drawn after quantification by HPLC 1290) was determined using the following formula: macrolactin A: $y = 1801.1x + 1.23$ ($R^2 = 0.9989$); oxydifficidin: $y = 1976.8x + 1645.3$ ($R^2 = 0.99$). Where y is the peak area and x is the macrolactin A/oxydifficidin concentration.

Mass spectrometry identification and nuclear magnetic resonance analysis of antibacterial active production

The sample was diluted with LC-MS/MS solution and centrifuged for 15 min (13000 rpm, 4°C), then 4 μ L was injected for LC-MS analysis. The molecular weight of the antimicrobial substance was determined as using the LC/MS system that composed of UHPLC (Thermo UltiMate 3000) and high resolution mass spectrometer (Q Exactive). Mobile phase: A-water (containing 0.1% formic acid), B-acetonitrile (containing 0.1% formic acid); flow rate: 0.35 mL/min; elution gradient: phase B (5% (1min)-80% (12min)). The ion source was HESI, the sample mass spectrum signal acquisition adopted positive ion scanning mode.

All NMR spectra were acquired at 25°C on an Agilent DD2 500MHz spectrometer equipped with broad-band ONENMR probe. Compound macrolactin A of 5mg was dissolved in 500 μ L DMSO- d_6 to make the NMR sample. A series of 1D and 2D spectra, including ^1H , ^{13}C , ^1H - ^1H COSY, ^1H - ^1H TOCSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC, were acquired for the structure elucidation. All NMR data were processed and analyzed with MestReNova. The chemical shifts were referenced to the TMS peak at 0.00 ppm for proton and auto-calibrated for carbon. The carbon types (C, CH, CH_2 and CH_3) were assigned by the help of ^{13}C -edited (CH_2 negative, CH_3 , CH positive, C none) HSQC spectrum and the fragments of the compound was elucidated by analyzing the COSY and TOCSY experiments. The HMBC experiment give the key information to connect all the fragments. All other signals of the HMBC spectrum obeyed the determined structure of the compound.

ARTP mutagenesis of BaX030

ARTP mutation operational procedure was conducted following the method described by Ma et al (Ma et al. 2015). We collected the cells that cultured the BaX030 to the logarithmic phase, washed it three times with normal saline, and diluted it into the bacterial suspension that the OD_{600} (nm) value was 0.6–0.8. We took 10 μ L of bacterial suspension and spread it on a sterilized metal slide, and placed the metal slide on a mutation machine named as ARTP (ARTP-IIS; Wuxi Tmaxtree Biotechnology Co., Ltd.). The instrument parameters were set as follows: helium content, the working radio-frequency power input, treatment distance and gas flow was set to 99.99%, 100W, 2.0 mm and 8.0 SLM, respectively. Mutagenesis time was 0 s (control), 15 s, 30 s, 45 s, 60 s, 100 s, 120s. After ARTP mutagenesis, the sample was quickly placed in EP tubes containing 1 mL of physiological saline, and shaken in vortex shaker for 3 minutes. The new bacterial suspension was diluted in a gradient and spread on the LB solid plate, and incubated it upside down at 30°C to grow the single clone. We draw the lethality curve by calculating the lethality of different mutagenesis treatment time. We draw the lethality curve by

calculating the lethality of different mutagenesis treatment time. The lethality rate (%)=(A-B)/A×100%, A was the number of colonies before mutagenesis treatment; B was the number of colonies after mutagenesis treatment. We selected the time when the lethality rate was around 90% as the treatment time for subsequent mutagenesis (Li et al. 2014), and repeated the above steps. After the monoclonal were picked, they were all cultured in fermentation medium for 48h. The extracted samples were loaded on Agilent 1290 to detect yield. The extraction method and elution procedure were the same as the described in 3.2. The corresponding mutant strains were selected based on the increase yield of macrolactin A in the whole compound mutagenesis process, 100 strains were selected in each round.

ARTP-NTG compound mutagenesis of strains

After two rounds of ARTP mutagenesis, the new bacterial suspensions were transferred to fermentation medium for overnight recovery. We washed the bacteria three times with PBS buffer, suspended the bacteria in 10 mL PBS and added NTG to the final concentration of 300 µg/mL, The bacterial solution was cultured in the dark for 1h at 30°C, 220 rpm. The cells were collected, washed with buffer solution to stop the reaction, diluted gradually and cultured upside down on the LB plate to grow the single clone. The subsequent extraction and detection methods were the same as above 3.6.

Genome re-sequencing and assembly

The kit method (Sangon, China) was used to extract the genomic DNA of the mutant strain N-11. The genome of N-11 was sequenced using an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute (Shenzhen, China). Genomic DNA was sheared randomly to construct three read libraries with lengths of 3,979,503 by a Bioruptor ultrasonicator (Diagenode, Denville, NJ, USA) and physico- chemical methods. The paired-end fragment libraries were sequenced according to the Illumina HiSeq 4000 system's protocol. Raw reads of low quality from paired-end sequencing (those with consecutive bases covered by fewer than five reads) were discarded. The sequenced reads were assembled using SOAP denovo v1.05 software.

SNP

With alignment software MUMmer (<http://mummer.sourceforge.net/>), each query sequence is aligned with reference sequence. The variation sites between the query sequence and reference sequence are found out and filtered preliminarily to detect potential SNP sites. The sequences with the length of 100 bp at both sides of SNP in the reference sequence are extracted and aligned with assembly results to verify SNP sites by using BLAT. If the length of aligned sequence is shorter than 101 bp, this SNP is considered as incredible and it will be removed; if the extracted sequence can be aligned with the assembly results several times, this SNP is considered locate in repeat region and it will also be removed. Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), TRF (<http://tandem.bu.edu/trf/trf.html>), Repeatmask (<http://www.repeatmasker.org/>) software are used to predict SNP in repeat regions. The credible SNP can be obtained through filtering SNP located in repeat regions.

InDel

With LASTZ (http://www.bx.psu.edu/miller_lab/dist/README.lastz-1.02.00/) software, the reference sequence and query sequence are aligned to get the alignment results. Through a series of treatment with axt_correction, axtSort and axtBest, the best alignment results are chosen and the InDel results are preliminarily obtained. 150bp (3*SD) in the upstream and downstream of InDel site in the reference sequence are extracted and then aligned with the query reads. The alignment results are verified with BWA (<http://bio-bwa.sourceforge.net/>) and samtools (<http://samtools.sourceforge.net/>).

The Effect of the strain N-11 on the expression of immune-related genes in grass carp

The grass carp (body weight 5 ± 1 g, body length 6.5 ± 1 cm) were divided into 9 groups, 3 groups were control groups (WT: fed the feed without the strain N-11), 6 groups were experimental groups (N-11: fed the feed that supplemented with the strain N-11 at a concentration of 1×10^9 CFU/g). After feeding for 30 days, the remaining grass carp was injected intraperitoneally with 100 μ L of AhX040 with a concentration of 1×10^6 cfu/mL or 150 μ L of AvX005 with a concentration of 1×10^9 cfu/mL. The control group was injected with the same amount of sterile saline. After 12 hours of infection, the liver and spleen of 3 fish in each group were taken for qRT-PCR and H&E staining experiments.

RNA extraction by Trizol method: the liver and kidney of grass carp were thoroughly grinding in liquid nitrogen and transferred to a 1.5 ml centrifuge tube with 1 ml pre-cooled Trizo, placed at room temperature for 5 min; centrifuged at 13000 g at 4°C for 5 min; aspirated the upper liquid to another centrifuge tube, added chloroform in the amount of 0.2 ml chloroform/1ml Trizol; left it at room temperature for 5 minutes, centrifuged at 13000 g at 4°C for 15 minutes; pipetted the upper aqueous phase into another centrifuge tube and added the equal volume of isopropanol at room temperature for 10 minutes, centrifuged at 13000g for 10 minutes at 4°C; discarded the supernatant and added 1 ml of pre-cooled 75% ethanol to suspend the precipitate; removed the supernatant and added 50 μ L DEPC water to dissolve after centrifugation.

Synthesis of cDNA: We used NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) to determine the RNA concentration by measuring the ratio of OD_{260}/OD_{280} , and reversed transcription to synthesize cDNA by using PrimeScript™ reagent Kit with gDNA Eraser kit.

qRT-PCR: We used the SYBR Green II dye method, the reaction system was 10 μ L: SYBR® Green Master Mixes 5 μ L; Forward primer 0.5 μ L; Reverse primer 0.5 μ L; cDNA 1 μ L; sterile water 3 μ L. Reaction procedure: 50°C 2 min; 95°C 10 min, 95°C 15 sec, 60°C 1 min, 40 cycles. Finally, the $2^{-\Delta\Delta Ct}$ relative quantitative method was used to compare and analyze the expression levels of gene mRNA in the samples.

Protective experiment of grass carp with the antagonistic strain N-11

120 healthy grass carp were randomly divided into six groups. After one week of adaptation to the environment, the added amount of N-11 was 1×10^9 CFU/g and mixed into the feed. The control group was given the same amount of feed. During the experiment, the water temperature was controlled at about 25°C, and the volume of each fish tank was 20L. After 30 days, we observed for 7 consecutive days and counted the cumulative mortality by intraperitoneal injection (the described in 3.9).

Discussion

Bacillus has the potential to produce antibacterial metabolites of various structures (Grubbs et al. 2017). In addition to the identified substances, we also found that there were many gene clusters related to antibiotic synthesis in BaX030, which may also affect the growth of fish pathogens. At the same time, the function of 7 gene clusters was unknown in 15 gene clusters, which indicated that the bacteria had great development potential. This study highlights the power of genome mining technologies based on biosynthetic knowledge in natural products discovery.

Macrolactins were the class of twenty-four-membered macrolide compounds and an important natural secondary metabolite that produced by microorganisms (Ortiz and Sansinenea 2020). From the perspective of biosynthesis, the generalized lactone ring was first formed by the polyketone skeleton, and then it is continuously decarboxylated and condensed under the action of PKS to shape the macrolides antibiotics (Kharel et al. 2012; Das and Khosla 2009). At present, macrolactins had 26 family members (macrolactin A-R) by isolating from *Bacillus* and had a wide range of antibacterial activities. The most studied was macrolactin A, which was first discovered from the metabolites of some unclassified deep-sea bacteria (Gustafson et al. 1989) and showed antibacterial activity against bacteria and fungi (Elkahoui et al. 2013; Li et al. 2016). Someone isolated macrolactin A from *Bacillus amyloliquefaciens* FZB42 (Chen XH et al. 2007), we performed the genome-wide sequence alignment by comparing BaX030 with *Bacillus amyloliquefaciens* FZB42, the homology was 98.17% and there were differences. Oxydifficidin was a derivative of difficidin, which showed a broad activity against plant pathogens and bacteria (Zimmerman et al. 1987; Im et al. 2020).

After ARTP-NTG mutagenesis, the 2409950 base of the *pkc2F* gene on the core gene cluster 9 has a mutation A > G, it caused the mutation of glycine to arginine (glycine > arginine), which may have the increase in the content of macrolactin A. The 15th gene cluster has the largest variation, and the SNP variation was located at No. 3364431 (C > G; stop codon > tyrosine), 3344017 (T > G; glycine > valine), 3344012 (C > T; tyrosine > histidine), 3316851 (C > G; glycine > arginine) bases on the core gene cluster. Their common point was the conversion of glycine to arginine, which may be a strategy to improve polyketides. Yi et al. (Yi et al. 2020) obtained the high-yielding *Bacillus* sp. A29 and A72 through ARTP, and their macroactins production was 35.2% and 52.8% higher than the parent strain, respectively. The result showed that the homology of BaX030 and it was 99.58% through 16s rRNA comparison. NTG mainly induced frameshift mutations, etc., which had a huge impact on the function of strains, so it was widely used. Duan et al. (Duan et al. 2020) isolated a new compound peniterester that had antibacterial activity against a variety of bacteria through NTG induction. Ega et al. (Ega et al. 2020) created the

mutant strain through NTG, its cellulase activity was increased by 148%. Moreover, the fifteenth gene cluster of the strain N-11 in the study had the most InDel mutations in all gene clusters, which may lead to significantly increase in oxydifficidin. But the high concentration of NTG can prevent our strain from growing. The strain will be genetically modified through homologous recombination technology to increase the yield in the future.

IgM that was produced by plasma cells was an important indicator of fish-specific immunity, it participated in the immune cycle of the fish and played a vital role in the process (Zhang et al. 2019), the increased expression of IgM indicated that the grass carp's immune system was activated (Bilal et al. 2016; Kole et al. 2019). IL-1 β and IL8 were the two main pro-inflammatory cytokines, which played an important role in triggering the inflammatory response against bacterial and viral infections (Reis et al. 2012; Wang et al. 2017). Their up-regulation helped to activate macrophages and resist pathogenic bacteria's damage to the body (Jantrakajorn and Wongtavatchai 2016; Nguyen et al. 2017). LSZ was mainly involved in the hydrolysis of phagocytes. The fish usually secreted a large amount of LSZ to dissolve pathogens and greatly increase its activity (Mohammadi et al. 2020). C3 was a key component of the complement system and participated in the classical pathway, lectin pathway and bypass pathway of complement activation. It can form a membrane attack complex on the surface of pathogenic bacteria to remove pathogenic bacteria in the body in time (Sunyer et al. 1997).

Bacillus usually colonized the intestines of fish (Thankappan et al. 2015), they were biocompounds optimizing the colonization of intestine microbiota in fish and enhancing immunity (Standen et al. 2013). Adhesion was a key step that made fish sick for pathogen infection. Certain antibiotic-producing bacteria have a strong competitive advantage over sensitive pathogenic strains in the same environment (Dobson et al. 2012). It seemed that there was a competition between *Bacillus* and harmful bacteria (Kesarcodi-Watson et al. 2008). After *Bacillus* colonization in the intestines, it killed harmful bacteria or reduced the adhesion of pathogenic bacteria in fish by producing bacteriocin components, which made pathogens lose their intestinal ecology. It also improved the production of T cells and the intestinal immune system and ultimately protected fish from pathogens (Picchietti et al. 2009; Ahmadifard et al. 2013).

We found for the first time that a high-yield strain of macrolactin A and oxydifficidin through compound mutagenesis, which can inhibit fish pathogens including AhX040 and AvX005. Our study can further ameliorate the production of secondary metabolites by improving the relevant genes for the synthesis of antibacterial substances or using highly expressed host bacteria for efficient expression of antibacterial substances, it laid the foundation for further determining the interaction among antagonistic bacteria, pathogenic bacteria and fish in vivo experiment; Furthermore, ARTP-NTG and resequencing technologies were relatively simple, it can be applied to different *Bacillus* sp. to obtain general resistance mechanisms and can also be used to test whether species-specific mechanisms exist. We hope to develop the bacteria into aquatic microecological preparations to promote the healthy development of aquaculture.

Conclusion

In this study, we separated and identified two secondary metabolites of BaX030, macrolactin A and oxydiffidin. BaX030 was used as the starting strain and used a new ARTP mutagenesis breeding technology combined with nitrosoguanidine chemical mutagenesis method to select a strain with high antibacterial activity. Its bacterial morphology becomes longer. The mutagenic strain N-11 hadn't effects on the liver and kidney, and it had a certain protective effect on grass carp. The study laid the foundation to excavate natural products with higher antibacterial biological activity, the probiotic strain had potential application value in aquaculture production.

Declarations

Ethics approval and consent to participate

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Hunan Normal University Ethics Committee. All animal experiment complied with the guidelines of the Animal Welfare Council of China.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PZ wrote the manuscript. HH, JL, ZZ and SL conducted the experiments. LX analyzed data. JX, KZ and WC participated in sample processing. XD conceived the research and completed the revision of the manuscript. All authors read and approved the manuscript.

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Not applicable.

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Figures

Figure 1

The separation chromatogram and chemical structure of antibacterial substances from BaX030. (A) HPLC chromatogram of the strain N-11 with the yield of macrolactin A and oxydifficidin compared with BaX030. CK—BaX030; 1: macrolactin A; 2: oxydifficidin; (B) The compound of macrolactin A; (C) The compound of oxydifficidin

Figure 2

The yield and antibacterial effect of strains screened by ARTP-NTG compound mutagenesis. (A-B) The yield of representative strains of the first and second rounds of ARTP mutagenesis, CK: The BaX030 was cultured for 48h; (C) The yield of representative strains of the third round of NTG mutagenesis; (D) Comparison of the antibacterial effect of the active substances that isolated from the N-11 and CK strains. WT: 50% methanol; 1: macrolactin A of the BaX030; 2: macrolactin A of the N-11; 3: oxydifficidin of the BaX030; 4: oxydifficidin of the N-11. Datas were mean±SEM for n=3 biologically independent experiments. Statistical analysis were performed using one-way ANOVA. ***P <0.001; **P <0.01; *P <0.05

Figure 3

The Growth curve and morphology of the BaX030 and N-11. (A) The growth curve of BaX030 and N-11 strains. Datas were mean $\pm$ SEM for n=3 biologically independent experiments; (B) Observation the strain morphology at 20h by the phase contrast microscope (100 $\times$); (C) Observation the strain morphology at 20h by the scanning electron microscope (10000 $\times$). CK: BaX030

Figure 4

The mutation site on 9th and 15th gene cluster of BaX030. (A) The mutation sites of SNP on 9th gene cluster; (B) The mutation sites of SNP and InDel on 15th gene cluster. Sn: stop codon non-synonymous mutation; Nn: non-synonymous mutation in the gene region; Sy: synonymous mutations in the gene region

Figure 5

Analysis of immune-related gene expression in grass carp that fed with N-11 strain after infection with AhX040 and AvX005. (A-B) Expression of immune-related genes in the liver and kidney after infection with AhX040 at 12 hours; (C-D) The gene expression levels of immune-related cytokines in liver and kidney from grass carp at 12 hours post-infection AvX005. WT+Ah: feed + injection of AhX040; N-11+Ah: The strain N-11 mixed with feed + injection AhX040; WT+Av: feed + injection of AvX005; N-11+Av: The strain N-11 mixed with feed + injection of AvX005. Datas were mean±SEM for n=4 biologically independent experiments. Statistical analysis were performed using one-way ANOVA. ***P <0.001; **P <0.01; *P <0.05

Figure 6

The pathological changes of the liver and kidney in grass carp were observed by H&E staining (400×)

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