Applied Microbiology and Biotechnology (2024) 108:17
https://doi.org/10.1007/s00253-023-12916-3

ENVIRONMENTAL BIOTECHNOLOGY

Response of *Paenibacillus polymyxa* SC2 to the stress of polymyxin B and a key ABC transporter YwjA involved

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Received: 25 July 2023 / Revised: 24 September 2023 / Accepted: 4 October 2023
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Abstract

Polymyxins are cationic peptide antibiotics and regarded as the "final line of defense" against multidrug-resistant bacterial infections. Meanwhile, some polymyxin-resistant strains and the corresponding resistance mechanisms have also been reported. However, the response of the polymyxin-producing strain *Paenibacillus polymyxa* to polymyxin stress remains unclear. The purpose of this study was to investigate the stress response of gram-positive *P. polymyxa* SC2 to polymyxin B and to identify functional genes involved in the stress response process. Polymyxin B treatment upregulated the expression of genes related to basal metabolism, transcriptional regulation, transport, and flagella formation and increased intracellular ROS levels, flagellar motility, and biofilm formation in *P. polymyxa* SC2. Adding magnesium, calcium, and iron alleviated the stress of polymyxin B on *P. polymyxa* SC2, furthermore, magnesium and calcium could improve the resistance of *P. polymyxa* SC2 to polymyxin B by promoting biofilm formation. Meanwhile, functional identification of differentially expressed genes indicated that an ABC superfamily transporter YwjA was involved in the stress response to polymyxin B of *P. polymyxa* SC2. This study provides an important reference for improving the resistance of *P. polymyxa* to polymyxins and increasing the yield of polymyxins.

Key points

- *Phenotypic responses of P. polymyxa to polymyxin B was performed and indicated by RNA-seq*
- *Forming biofilm was a key strategy of P. polymyxa to alleviate polymyxin stress*
- *ABC transporter YwjA was involved in the stress resistance of P. polymyxa to polymyxin B*

Keywords *Paenibacillus polymyxa* · Polymyxin · Stress response · ABC transporter

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Published online: 03 January 2024

Introduction

The overuse of antibiotics has resulted in an enormous rise in antibiotic resistance in bacteria (Kumar et al. 2018). Moreover, the emergence of multidrug-resistant bacteria poses a major threat to public health and causes huge economic losses (Hofer 2019; Dokla et al. 2020). Since polymyxins have significant indigenous effects on inhibiting multidrug-resistant bacteria, they are used as an effective therapeutic agent in clinical treatment (Landman et al. 2008). Polymyxins are lipopeptide antibiotics synthesized by a nonribosomal peptide synthase system, which was firstly isolated from *Paenibacillus polymyxa* (Storm et al. 1977; Martin et al. 2003). There are various types of polymyxins, among which polymyxin B and polymyxin E have effective inhibitory effects on multidrug-resistant bacteria (Poirel et al. 2017; Manchandani et al. 2018; Nakwan et al. 2019). Unfortunately, the increased and inappropriate use of polymyxins has led inexorably to the worldwide emergence of polymyxin-resistant bacteria in both veterinary clinics and human medical clinics. For example, Li et al. discovered a polymyxin-resistant *Pseudomonas aeruginosa* during the treatment of cystic fibrosis with polymyxins (Li et al. 2001). Tan et al. found that polymyxin-resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* appeared worldwide and showed an increasing trend (Tan et al. 2007; Olaitan et al. 2014; Parisi et al. 2015). Because of this severe situation, it is important to study the tolerance mechanism of bacteria to polymyxins.

Previous research has established that the premise of the bactericidal mechanism of polymyxins is to destroy the bacterial surface, and oxidative damage plays an auxiliary role in cell death (Yin et al. 2020). Due to the different cell wall structures of gram-negative and gram-positive bacteria, the targets of polymyxins are different. In gram-negative bacteria, the electrostatic interaction between the positive charge of polymyxins and the negatively charged lipid A in lipopolysaccharide makes the cell membrane unstable (Trimble et al. 2016). Recently, the resistance mechanism of gram-negative bacteria to polymyxins has been clearly explained (Yu et al. 2015b), and they are mainly divided into two types. (1) Chemical substances or transcription factors are regulated by two-component PhoP-PhoQ, such as phosphoethanolamine (pEtN), 4-amino-L-arabinose (L-Ara4N), and oprH, which improve the resistance of bacteria to polymyxins by modifying the lipopolysaccharide on the cell surface (Macfarlane et al. 2010; Liu et al. 2016; Moffatt et al. 2019). (2) Multidrug efflux pumps play an important role in the resistance of gram-negative bacteria to polymyxins, such as those reported in *Campylobacter jejuni* (Lin et al. 2002) and *Rhizobium alfalfa* (Eda et al. 2011). Multidrug efflux pumps are a common strategy for antibiotic. There are many types of efflux pumps in both gram-negative and gram-positive bacteria, which can be divided into five different families according to their sequence similarity: the resistance-nodulation-cell division (RND), the major facilitator superfamily (MFS), the ATP-binding cassette (ABC), the multidrug and toxic compound extrusion family (MATE), and the small multidrug resistance (SMR) (Li and Nikaido 2004; Alekshun and Levy 2007). To date, a variety of polymyxin efflux pumps have been found in bacteria, such as the AbcAB efflux pump in *Bifidobacterium*, EmrB of the MFS family in *A. baumannii*, the NorM efflux pump in *Berkholderia*, and the KpnEF efflux pump in *K. pneumoniae* (Fehlner-Gardiner and Valvano 2002; Margolles et al. 2006; Srinivasan and Rajamohan 2013; Lin et al. 2017). Gram-positive bacteria do not have lipopolysaccharide but have teichoic acid targets. As lipopolysaccharide in gram-negative bacteria, teichoic acid is a polyanionic, phosphate-rich linear polymer that requires divalent cations for stability in gram-positive bacteria (Rudilla et al. 2018). Three other resistance mechanisms of gram-positive bacteria to polymyxins have also been reported (Cheung et al. 2014; Otto 2009; Yang et al. 2012; Yin et al. 2020). These include modifications in the membrane/cell wall structure (Vestergaard et al. 2017). Gram-positive bacteria use D-alanineylation of teichoic acids (Collins et al. 2002; Neuhaus and Baddiley 2003) or aminoacylation of phospholipids (Steinbuch and Fridman 2016; Ernst and Peschel 2019) to reduce polymyxin affinity to the cell surface and cell damage. Then, there is the hydrolysis mechanism of polymyxins. The degradation of polymyxins by alkaline protease is a known mechanism of polymyxin hydrolysis (Ito-Kagawa and Koyama 1980; Yin et al. 2019). In addition to the abovementioned bacterial resistance mechanisms to polymyxins, biofilm formation has an important role in the resistance to antibiotics of some bacterial species (Berleman et al. 2016). Biofilms are bacteria that form complex microbial communities by attaching to the extracellular matrix (Hall-Stoodley and Stoodley 2005; Voloshin et al. 2005), which hinders the bactericidal activities of polymyxins and other antibiotics. Moreover, biofilms can enhance the ability of bacterial strains to tolerate polymyxins (Kim et al. 2013).

P. polymyxa mostly exists in the rhizosphere soil of plants, and its growth process can produce a variety of small molecular substances that promote plant growth (Timmusk et al. 1999; Holl and Chanway 1992; Lebuhn et al. 1997), and produce a variety of antibacterial substances, such as fusaricidins and polymyxins (Komura and Kurahashi 1980). *P. polymyxa* is a producer of polymyxins, but polymyxins also have bactericidal activity against *P. polymyxa*. *P. polymyxa* C12 was found to produce polymyxin E, which has

bactericidal activity against the strain itself by inducing oxidative damage and cell membrane damage (Yu et al. 2015b, 2017). At present, there are few studies on the resistance mechanism of *P. polymyxa* to polymyxins. To ensure survival during the biosynthesis of antibiotics, antibiotic-producing bacteria have evolved a variety of self-resistance mechanisms that can cope with antibiotic stress (Hopwood 2010), and the bactericidal activity of antibiotics to their producers may inhibit the accumulation of antibiotics during fermentation. Therefore, it is of great significance to study the response characteristics and mechanism of *P. polymyxa* to polymyxin stress to improve the viability of the strain and increase the yield of polymyxins.

P. polymyxa SC2 has a broad antibacterial spectrum screened by our team in the pepper rhizosphere. Previous research has discovered that strain SC2 can produce polymyxin B and P, which are antagonistic to a wide range of pathogenic bacteria. Since polymyxin B is more widely used, in this study, we aim to obtain a comprehensive view of *P. polymyxa* SC2 in response to the stress of polymyxin B. The transcriptional and phenotypic responses to the treatment with subinhibitory concentrations of polymyxin B were studied. More importantly, we identified some genes involved in the resistance of strain SC2 to polymyxin B. Our results provide a crucial theoretical foundation for improving the ability of *P. polymyxa* to tolerate polymyxins and increasing the production of polymyxins.

Materials and methods

Strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. The primers used in this study are listed in Supplemental Table S1.

Gene deletion mutants of strain SC2 were constructed by homologous recombination (Blancato and Magni 2010). PCR amplification of 900 bp upstream and downstream nucleotide sequences of the target genes (*ywjA*) were used as upstream and downstream homologous arms, respectively. The upstream and downstream homologous arms were fused by overlap extension PCR (Urban et al. 1997) and then cloned into the *Nco* I and *Sac* I sites of the pBVGH plasmid using the Gibson assembly (Gibson 2011), resulting in the *ywjA* knockout vector. Since strain SC2 is recalcitrant to genetic manipulation, the mutant strain SC2-M1 of strain SC2 was selected for gene knockout. The constructed gene knockout vector was transformed into SC2-M1 by the electrotransformation method reported by our research group (Hou et al. 2016), and the mutant strain (SC2-M1 Δ*ywjA*) was screened via colony PCR and sequencing.

To construct the complementation strain, we obtained the promoter sequence and complementary gene sequences (*ywjA*) from strain SC2-M1 (Li et al. 2019; Sun et al. 2022). The above two fragments were fused and then inserted into the *Hin*d III and *Bam*H I sites of plasmid pHY300PLK (contains the tetracycline resistance gene), forming the complementary vector of gene *ywjA*. The complementary vectors were transformed into competent knockout strain cells by the electrotransformation method. In addition, as a control, the

Table 1 List of strains and plasmids

Strains or plasmids	Genotype/properties	Source/reference
Strain		
E. coli (strain DH5α)	*F-φ80 lac ZΔM15 Δ(lacZYA-arg F) U169 endA1 recA1 hsdR17(rk−,mk+) supE44λ- thi-1 gyrA96 relA1 phoA*	Trans Gen Biotech
Paenibacillus polymyxa SC2	Wild type, isolated from the rhizosphere of pepper plants in Guizhou, China	CGMCC 7.523
P. polymyxa SC2-M1	Spontaneous mutant of *P. polymyxa* SC2	This laboratory (Hou et al. 2016)
SC2-M1 Δ*ywjA*	SC2-M1 derivative; *ywjA* in-frame deletion	This work
SC2-M1 Δ*ywjA* + *ywjA*	SC2-M1-Δ*ywjA* derivative; {pHY300PLK-*ywjA*}	This work
SC2-M1 Δ*ywjA* + *vector*	SC2-M1-Δ*ywjA* derivative; {pHY300PLK}	This work
Plasmid		
pHY300PLK	*E. coli* and *B. subtilis* shuttle vector; Ampr, Tetr	This laboratory
pHY300PLK-*promoter*	pHY300PLK-*promoter*	This work
pBVGH	Temperature-sensitive plasmid, Ery^r	This laboratory
pBVGH-*ywjA*	pBVGH-*ywjA* homologous arms	This work
pHY300PLK-*ywjA*	pHY300PLK- *promoter-ywjA*	This work

pHY300PLK empty vector was used to transform competent cells of the knockout strain. We selected the correct positive clones (SC2-M1 Δ*ywjA* + *ywjA*) or the control clones (SC2-M1 Δ*ywjA* + pHY300PLK) by PCR amplification and restriction enzyme digestion of the single colonies grown on LB solid medium containing 15 μg/mL tetracycline. In the construction of the complementation strains, *Escherichia coli* DH5α was used for plasmid amplification and subcloning.

The media used in this study were Luria–Bertani (LB) medium, fermentation medium (sucrose 49.6 g/L, $MgSO_4$ 0.2 g/L, KH_2PO_4 0.2 g/L, NaCl 0.2 g/L, $CaCO_3$ 5.06 g/L, $(NH4)_2SO_4$ 6.94 g/L), and swimming assay medium (peptone 10 g/L, beef extract 3.0 g/L, sodium chloride 5.0 g/L, agar 7 g/L).

Treatment of polymyxin B on P. polymyxa SC2

The growth curves of strain SC2 were monitored by OD_{600} using a spectrophotometer (Eppendorf, Germany) (Li et al. 2019). Strain SC2 was inoculated with an initial OD_{600} of 0.1 and cultivated at 37 °C and 180 rpm, and polymyxin B was added to final concentrations of 0 mg/mL, 0.05 mg/mL, 0.08 mg/mL, and 0.1 mg/mL and 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, and 0.5 mg/mL, respectively.

Genome-wide RNA sequencing and analysis of strain SC2 against polymyxin B

For transcriptomic RNA sequencing (RNA-seq), we chose *P. polymyxa* SC2 with or without 0.08 mg/mL polymyxin B treatment for 5 h. Three biological replicates were assessed for each sample. Total RNA was extracted using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed using DNase I (Takara). Then, RNA quality was determined using a 2100 Bioanalyzer (Agilent) and quantified using an ND-2000 (NanoDrop Technologies). A high-quality RNA sample ($OD_{260/280} = 1.8 \sim 2.2$, $OD_{260/230} \geq 2.0$, RIN ≥ 6.5, 28S:18S ≥ 1.0) was used to construct the sequencing library. RNA-seq strand-specific libraries were prepared following the TruSeq RNA Sample Preparation Kit from Illumina (San Diego, CA).

On 2% Low Range Ultra Agarose, libraries were selected for cDNA target fragments of 200–300 bp, which were then PCR amplified for 15 PCR cycles using Phusion DNA polymerase (NEB). After quantification by TBS380, paired-end libraries were sequenced by Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China) with the Illumina HiSeq PE 2 × 151 bp read length. The raw paired-end reads were trimmed and quality controlled by Trimmomatic with default parameters (http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic). The genome of *P. polymyxa* SC2 was used as the reference sequence and was downloaded from the National Center for Biotechnology Information (GenBank accession number CP002213.2). Then, clean reads were separately aligned to the reference genome with orientation mode using Rockhopper software (http://cs.wellesley.edu/~btjaden/Rockhopper/). This software was used to calculate gene expression levels with default parameters (Tjaden 2015). To identify DEGs (differentially expressed genes) between the two different samples, the expression level for each transcript was calculated using the fragments per kilobase of read per million mapped reads (RPKM) method. The DEGs between two samples were selected using the following criteria: the logarithmic fold change was greater than 2, and the false discovery rate (FDR) was less than 0.05. GO (Conesa et al. 2005) and KEGG (Kanehisa et al. 2014) were used to annotate and classify differentially expressed genes. The raw data was submitted to NCBI-SRA database with the BioProject accession number PRJNA900018.

Quantitative real-time PCR assay for some DEGs in RNA-seq

To confirm the RNA-seq results, ten upregulated or downregulated genes from our RNA-seq analysis were selected, and qRT-PCR was used to confirm the expression changes of these genes with polymyxin B or not. Complementary DNA (cDNA) was obtained by the reverse transcription of 500 ng of total RNA using an Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology (Hunan) Co., Ltd.). PCR primers were designed using beacon Designer 7 and are listed in Supplemental Table S1. PCR reactions used SYBR® Green *Pro Taq* HS (Accurate Biotechnology (Hunan) Co., Ltd.) All reactions were run in triplicate, and the reference gene was the GAPDH gene (encoding glyceraldehyde-3-phosphate dehydrogenase), which was amplified in a parallel reaction for normalization. Relative expression levels were calculated using the delta–delta Ct method.

Measurements of intracellular ROS

ROS can oxidize the dye DCFHDA to DCF-DA. The level of ROS in cells of strain SC2 treated with polymyxin B can be determined by monitoring the fluorescence intensity of DCF-DA. Strain SC2 was cultured in LB medium with or without 0.08 mg/mL polymyxin B for 5 h at 37 °C and 180 rpm, and the OD_{600} of the bacterial suspension was adjusted to 0.8. The bacterial suspension was mixed with DCFH-DA, incubated at 37 °C for 20 min, centrifuged to remove the supernatant, washed three times with PBS, and then adjusted to an OD_{600} of 0.5. Fluorescence was detected on a microplate reader (Berthold technologies) using a

96-well plate with an excitation wavelength of 488 nm and an emission wavelength of 525 nm (Yu et al. 2017).

Swimming and biofilm assays

For swimming assays, strain SC2 was cultured in LB liquid medium with or without 0.08 mg/mL polymyxin B at 37 °C with shaking at 180 rpm for 5 h. The cells were washed with PBS, and the OD_{600} of the bacterial suspension was adjusted to 0.6. A total of 2 μL of bacterial suspension was taken to the surface of the nutrient broth medium and cultured at 37 °C for 12 h, and the movement of the bacterial flora was observed. In this growth medium, strains can swim through the soft agar and produce a halo. The diameter of the halo is a measure of the swim ability of strains.

For the biofilm assay, biofilm formation was evaluated in a crystal violet biofilm assay in 6-well plates (Rose et al. 2009). Bacteria were cultured in LB medium with or without polymyxin B for 5 h, and the OD_{600} of the bacterial suspension was adjusted to 0.1. The bacterial suspensions (1.5 mL) were deposited into 6-well plates and incubated at 37 °C for 72 h. The plates were washed with PBS buffer 3 times and fixed with 2 mL of methanol for 15 min. Then, the methanol in the well was aspirated and dried naturally. Two milliliters of 1% crystal violet solution was added to each well and stained at room temperature for 5 min. The dye in the 6-well plates was discarded, and the cells were dried at room temperature. Then, 2 mL of 33% glacial acetic acid solution was added and placed in a 37 °C incubator for 30 min. The OD_{590} values and fluorescence quantity of the solution in the culture well were measured using a microplate reader (Tecan, Switzerland) (Christensen 1985). Each test was repeated in three wells for each strain, and the test values were average values from three biological repeats.

CFU and cell membrane integrity assay of biofilm cells

We cultured bacteria with glycerol to obtain biofilm cells and washed them 2–3 times using PBS buffer. The biofilm cells and biofilm-free cells were inoculated into LB medium containing 0.08 mg/mL polymyxin B at an initial OD_{600} of 0.1 and incubated at 37 °C for 5 h. The gradient-diluted suspension was spread on LB solid medium and incubated for 24 h at 37 °C in a constant temperature incubator to calculate colony forming units (CFUs).

Propidium iodide (PI) does not penetrate living cell membranes, but can bind to DNA through broken cell membranes and release fluorescence. It is therefore one of the most commonly used dyes for cell membrane integrity assays. Bacteria were washed 2–3 times with PBS buffer; 1 μL PI was added to the bacterial suspension, left for 5 min away from light, washed with PBS, and resuspended. The fluorescence value was measured using a microplate reader (Berthold technologies) set at 536-nm excitation and 618-nm emission wavelengths.

Detection of polymyxin resistance of strain SC2

The OD_{600} values of strain cultures were measured using a spectrophotometer (Eppendorf, Germany), which was used to draw a growth curve (Li et al. 2019). The bacterial suspension was inoculated into fresh LB liquid medium with or without 0.08 mg/mL polymyxin B, and the initial OD_{600} was adjusted to 0.1. Different concentrations of calcium, magnesium, or iron ions were added to the medium, and the OD_{600} was detected every 3 h. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) was used to plot the growth trend of strains under different conditions.

Bioassays for antibacterial activity

Strain SC2-M1 and its derivative strains were cultured in fermentation medium for 36–48 h at 37 °C and 180 rpm for antibiotic production. The supernatants were centrifuged at 12,000 rpm for 10 min, and the antibacterial activities of the supernatants were detected by agar diffusion bioassay (Shaheen et al. 2011). The melted semisolid LB media inoculated with 0.1% *E. coli* DH5α (v/v) was poured into plates that had been placed in 8-mm Oxford cups. After 15 min, the Oxford cups were pulled out, and grooves were formed. A total of 100 μL of supernatant was added to the grooves. After incubation at 37 °C for 12–16 h, the diameters of the inhibition zones on plates were measured using a Vernier caliper.

Results

Polymyxin B has an inhibitory effect on the growth and polymyxin synthesis of *P. polymyxa* SC2

Our previous results showed that the polymyxin production of *P. polymyxa* SC2 could reach up to 0.2–0.3 mg/mL (~ 0.17–0.25 mM). So, we chose the levels of polymyxin B (PB) from 0 to 0.5 mg/mL to clarify the relevance of the concentration of polymyxin B to the growth capacity of strain SC2. The growth inhibitory activity of polymyxin B against *P. polymyxa* SC2 was determined by optical density (OD_{600}) at a wavelength of 600 nm. The results in Fig. 1a showed that polymyxin B inhibited the growth of the gram-positive strain SC2. Treatment with 0.05 mg/mL and 0.08 mg/mL polymyxin B resulted in strain SC2 exhibiting longer lag phases and lower cell growth rates during the exponential phase compared to the control without polymyxin B treatment. However, the maximum cell density in the stationary phase

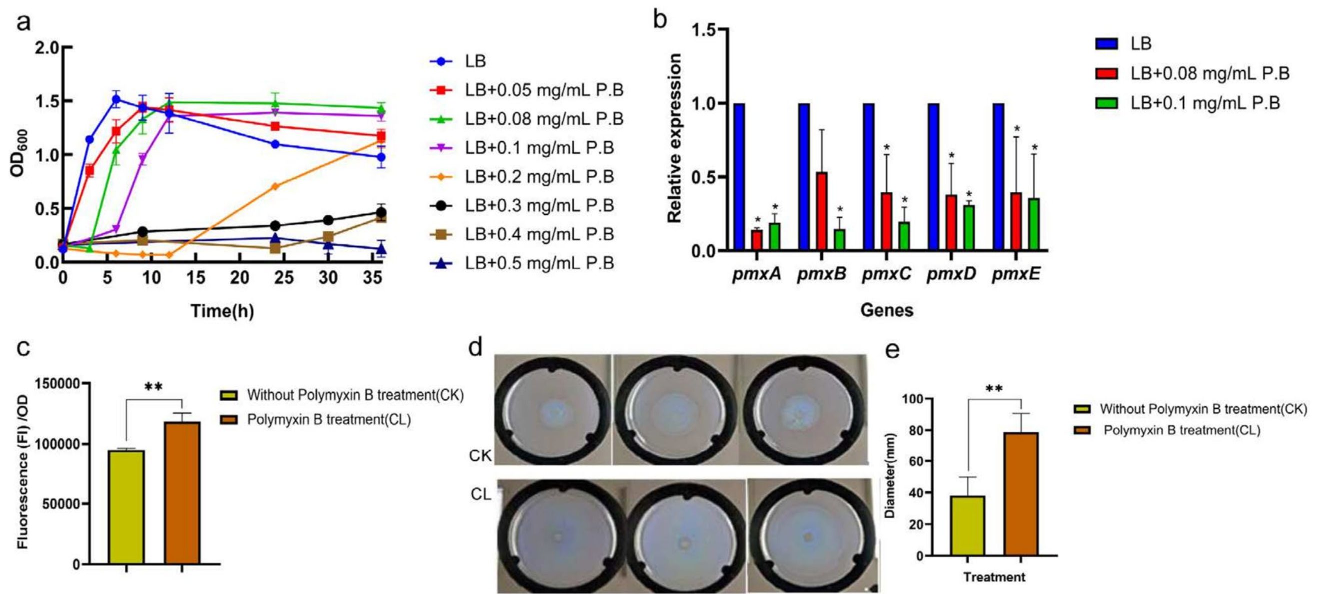

Fig. 1 Effects of polymyxin on physiological levels of *P. polymyxa* SC2. **a** Polymyxin B inhibited the growth of strain SC2. The growth of strain SC2 was measured in Luria–Bertani (LB) medium with a range of concentrations (0 mg/mL, 0.05 mg/mL, 0.08 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL) of polymyxin B (PB). The initial OD_{600} was 0.1, and the growth of strain SC2 was monitored using spectrophotometry. **b** The mRNA levels of genes involved in polymyxin biosynthesis in strain SC2 after 5 h of exposure to 0.08 mg/mL and 0.1 mg/mL PB. **c** ROS levels in SC2 strains with or without polymyxin B treatment. The fluorescence of the reaction was detected on a multiplate reader using a 96-well plate with excitation at 488 nm and emission at 525 nm. The means and standard errors of the means for all three independent experiments are shown in the figure. "*" indicates significance in unpaired *t* tests with *P* values of 0.05. "**" indicates significance in unpaired *t* tests with *P* values of 0.01. **d** The motility with (CL) or without (CK) polymyxin B-treated bacteria was tested on swimming plates containing 0.8% agar. **e** The diameter of the bacterial movement area was measured using a Vernier caliper. The means and standard errors of the means for all three independent experiments are shown. "**" indicates significance in unpaired *t* tests with *P* values of 0.01

did not change significantly. When the polymyxin B concentration was greater than 0.1 mg/mL, the growth of the strain was significantly inhibited compared with that of the control group, and the growth rates during the exponential phase and the maximum densities of cells in the stationary phase of the strain were reduced. The specific growth rates of strain SC2 on LB and with different concentrations of 0.05 mg/mL, 0.08 mg/mL, 0.1 mg/mL, and 0.2 mg/mL polymyxin B were calculated to be 0.42 h^{-1}, 0.37 h^{-1}, 0.32 h^{-1}, 0.14 h^{-1}, and 0.08 h^{-1}, respectively. Furthermore, the growth of strain SC2 was completely inhibited by 0.4 mg/mL and 0.5 mg/mL polymyxin B treatment. Therefore, within a given concentration range, the polymyxin B concentration was positively correlated with the inhibitory effect on strain SC2. To further test whether polymyxin B has a feedback inhibition on the expression of polymyxin synthetic gene clusters on strain SC2, polymyxin B doses of 0.08 mg/mL and 0.1 mg/mL were applied for 5 h of incubation (Fig. 1b). When the concentration of polymyxin B was 0.08 mg/mL, compared with the control, the transcript levels of *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* were decreased by 86%, 46.7%, 60.4%, 62%, and 60.3%, respectively. When the concentration of polymyxin B was 0.1 mg/mL, compared with the control, the transcript levels of *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* were decreased by 81.2%, 85.3%, 80.3%, 69.2%, and 64.4%, respectively. The quantitative (Q)RT-PCR analysis revealed that the transcript levels of genes involved in polymyxin biosynthesis (*pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE*) in polymyxin-treated strains were considerably lower than those in strains not treated with polymyxin B. As the polymyxin B concentration increased, the transcription levels of the genes were reduced. This result demonstrates that extracellular polymyxin B may inhibit the secondary metabolic process of strain SC2 by reducing the transcription level of genes involved in polymyxin biosynthesis. Therefore, polymyxin B had an inhibitory effect on the growth (Fig. 1a) and metabolic processes (Fig. 1b) of strain SC2.

Whole-cell transcriptional response of *P. polymyxa* SC2 to polymyxin B stress

It has been reported that antibiotics at subinhibitory concentrations can operate as signaling molecules, causing bacteria to exhibit broader physiological and morphological effects, such as pathogenicity, biofilm formation, and gene expression (Lorian 1975; Andersson and Hughes

2014). *P. polymyxa* SC2 has a resistance capacity to low concentrations of polymyxin B, as indicated in Fig. 1a. To fully represent the transcriptional response of strain SC2 to polymyxin B and identify the primary expression profile of antibiotic specificity, 0.08 mg/mL polymyxin B at the subinhibitory concentration (the concentration that hardly inhibits growth) was chosen for transcriptome sequencing. There are 5387 coding genes in the genome of strain SC2. Using the criteria of $|\log 2\ FC| > 1$ and $FDR < 0.05$, 953 genes were identified as differentially expressed genes (DEGs), including 827 (accounting for 15.4% of the total coding genes) upregulated and 126 (accounting for 2.3% of the total coding genes) downregulated genes. Volcano plot diagrams showed up regulated and downregulated DEGs in the polymyxin B treatment group compared to the untreated group, as illustrated in Supplemental Fig.S1a. The complete overview of differentially expressed genes is provided in Supplementary File 1.

GO enrichment analysis of DEGs

GO enrichment analysis was used to classify the DEGs in *P. polymyxa* SC2. The 953 DEGs were assigned to 118 GO terms, including 76 biological process (BP) terms, 39 molecular function (MF) terms, and 3 cellular component (CC) terms. The expression patterns of genes belonging to the same taxon were different (Supplemental Fig.S1b). All DEGs related to the behavior, biological adhesion, cell proliferation, detoxification, growth, immune system process, locomotion, and single organism process were upregulated in the biological process category. In the cell component category, all DEGs related to the membrane-enclosed lumen, nucleoid, and supramolecular complex were upregulated, and all DEGs related to the cell junction and symplast were downregulated. In the molecular function category, the expression levels of DEGs related to antioxidant activity and transcription regulator activity were all upregulated. In addition, GO enrichment analysis revealed that more differentially expressed genes were enriched in the functions of transport, transmembrane transporter activity, localization, and oxidoreductase activity.

KEGG enrichment analysis of DEGs

We further performed enrichment analysis of the DEGs using the KEGG database. To elucidate the functional information and relationships between DEGs (Liu et al. 2020), we categorized these DEGs into distinct pathways. A total of 503 genes were enriched in 83 pathways in the KEGG database. The obviously enriched pathways are shown in Supplemental Fig.S1c. Among the DEGs of strain SC2, 46 genes were enriched in the ABC transporter pathway. There were 29 genes enriched in the two-component system. Up to 26 genes were enriched in the carbohydrate metabolism pathway. Approximately 23 genes were enriched in the biosynthesis pathway of amino acids. Furthermore, there were some differentially expressed genes involved in bacterial chemotaxis, flagella assembly, purine metabolism, repair, drug metabolism, resistance, degradation, and oxidative phosphorylation pathways. As shown in Supplemental Fig.S1c, the results indicated that the ABC transporter pathway was the most enriched in DEGs, and other major pathways included the two-component system, carbohydrate metabolism, biosynthesis of amino acid pathways, etc., indicating that these pathways played important roles in the resistance of strain SC2 to polymyxin B.

Validation of partial DEGs in RNA-seq results by qRT-PCR

Reverse transcription quantitative PCR is an important approach for verifying DEGs in RNA-seq results. To verify the accuracy of the RNA-seq results, 10 differentially expressed genes were selected in strain SC2 for qRT-PCR validation under the same culture conditions (Supplemental Fig.S2a). As shown in Supplemental Fig.S2a and S2b, the qRT-PCR data correlated well with the RNA-seq data ($R^2 = 0.9112$). The mRNA levels of all genes obtained by qRT-PCR were generally consistent with those obtained by RNA-seq analysis, although the specific fold-change values varied. This finding indicates that the RNA-seq results are reliable. The primers used for qRT-PCR are listed in Supplemental Table S1.

Phenotypic responses of *P. polymyxa* SC2 to polymyxin stress as indicated by the RNA-seq results

Some of the genes with changes in their expression levels may play a role in the stress response of strain SC2 to polymyxin B. These genes are mainly involved in the oxidative stress response, metal ion transport, carbon metabolism, cell motility, and biofilm formation. Thus, functional experiments were carried out to determine whether bacterial phenotypes were altered as a result.

Strain SC2 responds to polymyxin-induced oxidative stress by changing its pattern of gene expression

Polymyxin-induced oxidative stress generates intracellular reactive oxygen species (ROS) in gram-negative bacteria (Imlay 2013; Yu et al. 2017). These oxidative substances, such as O_2^-, H_2O_2, and •OH, are known as reactive oxygen species (ROS) (Forooshani et al. 2020). When the ROS concentration reaches uncontrolled levels, oxidative damage to DNA, lipids, and proteins can result in cell death (Pan et al. 2019). To test whether polymyxin B can induce

oxidative stress of strain SC2, we found that the addition of polymyxin B resulted in a 28.6% increase in ROS levels compared to the control without polymyxin B treatment (Fig. 1c). This finding demonstrates that polymyxin B can increase intracellular ROS accumulation in strain SC2. RNA-seq results revealed that the genes involved in the oxidative stress response were upregulated after polymyxin treatment (Supplemental Fig.S3a), among which *bsaA1* and *bsaA3*, encoding glutathione peroxidase, were upregulated by 2- and 3.4-fold, respectively. Glutathione peroxidase is a type of functional protein that is essential in the antioxidant defense system. It can remove various peroxides and protect cells from peroxidation damage (Wang et al. 2021a, b). The *PPSC2_13670*, *PPSC2_04100*, *PPSC2_12325*, *PPSC2_12310*, and *PPSC2_09075* genes encoding thioredoxin were upregulated by 2.7-, 2.3-, 2.3-, 1.8-, and 1.2-fold, respectively. Thioredoxin is an important regulator of cellular redox homeostasis (Ordoñez et al. 2020). In addition, a large number of genes encoding oxidoreductases (with oxidoreductase activity, acting on the CH–OH group of the donor) were significantly upregulated (Supplemental Fig. S3b). Besides, a large number of genes related to Fe–S cluster synthesis were significantly upregulated (Supplemental Fig.S3c). The *PPSC2_08000*, *PPSC2_20965*, and *PPSC2_07985* genes, which encode Fe–S cluster assembly proteins, were upregulated by 1-, 1.25-, and 1.2-fold, respectively. Fe–S clusters are highly vulnerable to oxidative stress (Py and Barras 2010). Therefore, some regulatory proteins recruit Fe–S clusters to respond to superoxide stress.

Strain SC2 escapes unfavorable polymyxin stress through increased motility

Bacterial motility is a crucial physiological process for bacteria to adapt to a new environment, and it plays an important role in bacterial colonization (Hu et al. 2016). RNA-seq results showed that genes related to bacterial flagellar assembly and bacterial chemotaxis were significantly upregulated under polymyxin B stress (Supplemental Fig.S4a and Fig.S4b). For example, genes *PPSC2_09965* and *PPSC2_09960* related to flagellar assembly were upregulated by 3.0-fold and 4.9-fold, respectively. The *PPSC2_09965* gene encodes FlhG, the major protein that determines the number of flagella. *PPSC2_09960* encodes FlhF, which is the major protein that determines the position of flagella. Moreover, FlhG and FlhF are landmark proteins that mediate flagellar localization in a variety of bacteria (Schuhmacher et al. 2015; Arroyo-Pérez and Ringgaard 2021). The genes *PPSC2_09955*, *PPSC2_09950*, *PPSC2_09935*, *PPSC2_09940*, and *PPSC2_09945* were upregulated by 4.4-, 3.9-, 3.7-, 3.6-, and 3.6-fold, respectively. The five genes mentioned above encode five transmembrane proteins that constitute a type III protein export apparatus transport. Type III protein export apparatus transport is a carrier responsible for transporting the hook and filament during flagella assembly and has an important function in flagellar assembly (Minamino 2014). In addition, some genes encoding transcriptional regulators that regulate flagella formation and bacterial motility were also upregulated. Among them, the *cheA*, *cheW1*, *cheW3*, and *PPSC2_12825* genes were upregulated by 2.9-, 3.6-, 3.2-, and 2.9-fold, respectively. CheA and CheW are components of the chemoreceptor signaling core complexes that drive bacterial motility by regulating the switch in bacterial flagellar rotation (Frutos-Grilo et al. 2020).

To investigate whether polymyxin B influences the motility of strain SC2, we examined bacterial motility on swimming agar with and without polymyxin B. The results showed that polymyxin B treatment (Fig. 1d, e) increased the range of bacterial movement by 105% compared with no polymyxin B treatment. The results of the RNA-seq and biological phenotypic experiments both demonstrate that polymyxin B can promote the motility of strain SC2.

Strain SC2 alleviates polymyxin stress by cations (Mg^{2+}, Ca^{2+}, and Fe^{3+})

RNA-seq analysis showed that the genes encoding magnesium ion transporters were significantly upregulated (Supplemental Fig.S5a). The corresponding genes *PPSC2_04450*, *PPSC2_07235*, and *PPSC2_21100*, all encoding magnesium transporters, were upregulated by 11.56-, 1.49-, and 2.85-fold, respectively. Therefore, metal ions may aid in alleviating the cell damage induced by polymyxin B. In the Mg^{2+} addition experiment, we firstly ruled out the interference of $MgCl_2$ on the growth of the strain SC2. As shown in Fig. 2a (blue line and red line), $MgCl_2$ did not have a significant effect on the growth of strain SC2. Then, 20 mM $MgCl_2$ was added to LB medium containing 0.08 mg/mL polymyxin B. The results showed that, compared to the control (LB + PB, green line), the addition of $MgCl_2$ (Fig. 2a, purple line) alleviated polymyxin B-induced growth inhibition (Fig. 2a). The protective effect of $MgCl_2$ on strain SC2 was significant, especially in the logarithmic growth phase.

In the Ca^{2+} addition experiment, it was necessary to exclude the interference of $CaCl_2$ on strain growth, and it was discovered that $CaCl_2$ did not affect strain growth in the logarithmic growth phase but caused strain SC2 to rapidly decline in the stationary phase (Fig. 2b). Then, 10 mM and 20 mM $CaCl_2$ were added to the LB medium containing 0.08 mg/mL polymyxin B (Fig. 2b). The results showed that Ca^{2+} alleviated polymyxin B-induced strain growth inhibition during the logarithmic growth phase. Meanwhile, we discovered that the gene *PPSC2 15555* encoding the calcium/proton exchanger was upregulated by 1.59-fold according to the RNA-seq results (Supplemental Fig.S5a).

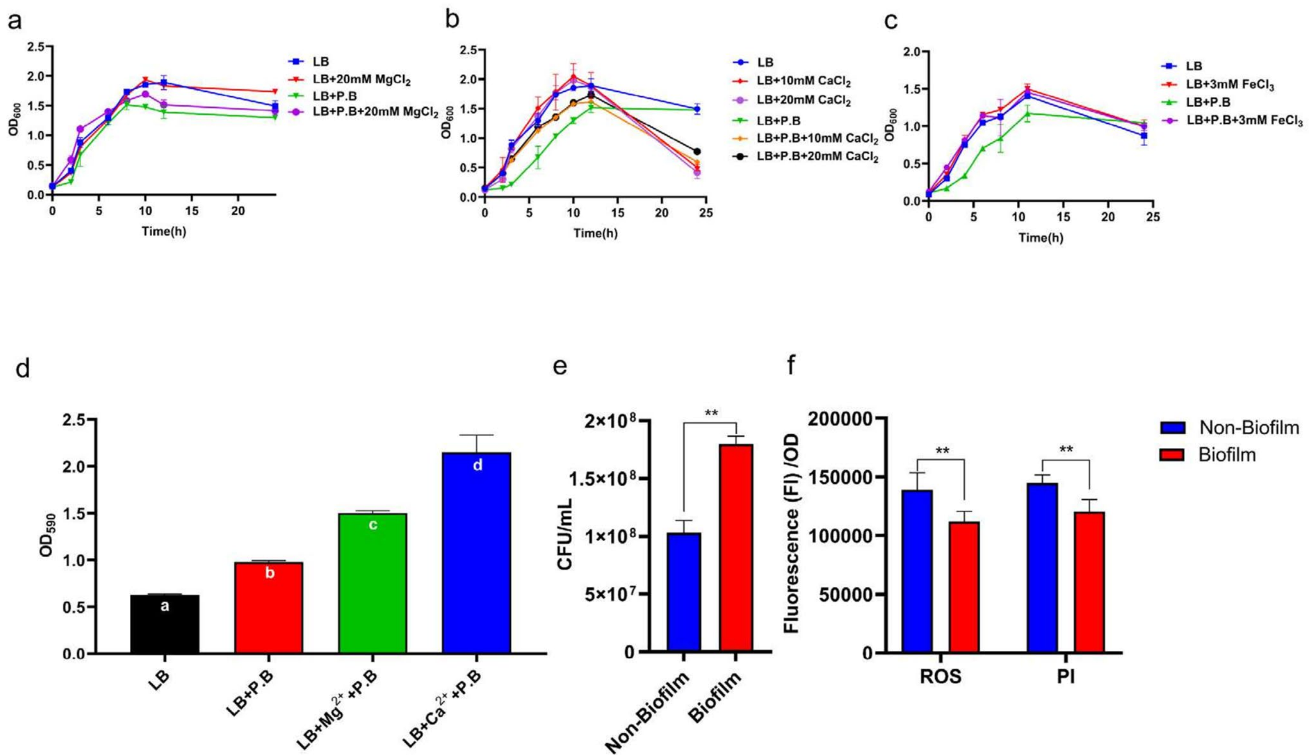

Fig. 2 The addition of exogenous substances and biofilm formation relieve the stress of polymyxin to *P. polymyxa* SC2. **a** Mg^{2+}alleviate polymyxin stress on strain SC2. Growth of strain SC2 in LB containing $MgCl_2$ or polymyxin B. **b** Ca^{2+}alleviate polymyxin stress on strain SC2. Growth of strain SC2 in LB containing different concentrations of $CaCl_2$ or polymyxin B. **c** Fe.$^{3+}$alleviate polymyxin stress on strain SC2. Growth of strain SC2 in LB containing $FeCl_3$ or polymyxin B. **d** Colorimetric detection of biofilm formation. The bacterial suspensions of strain SC2 were grown in tissue culture-treated 6-well plates for 3 days at 30 °C. The effect of polymyxin B or metal ion treatment on biofilm formation of strain SC2 was investigated by detecting OD_{590} using crystal violet staining. **e**, **f** Biofilm cells are more resistant to polymyxin B. Biofilm-forming cells and non-biofilm-forming cells were treated with polymyxin B for 5 h, and CFU assay, intracellular ROS assay, and cell membrane integrity assay were performed (characterized by PI fluorescence intensity, the stronger the fluorescence, the higher the degree of cell membrane damage). The means and standard errors of the means for all three independent experiments are shown. "*" indicates significance in unpaired *t* tests with *P* values of 0.05. "**" indicates significance in unpaired *t* tests with *P* values of 0.01

Among the significantly upregulated genes related to metal ion transport, in addition to the genes associated with Mg^{2+} and Ca^{2+} transport, we also discovered that there were a number of genes associated with iron transport (Supplemental Fig.S5a). Therefore, Fe^{3+} might help alleviate the stress of polymyxin B on strain SC2. The experimental results of iron addition showed that $FeCl_3$ did not have a significant effect on the growth of the strain in LB medium (Fig. 2c, blue line and red line). Then, 3 mM $FeCl_3$ was added to LB medium containing 0.08 mg/mL polymyxin B, and the results showed that strains of the experimental group (LB + P. B + Fe^{3+}, purple line) grew better than the control group (LB + P.B, green line). More importantly, the maximum cell density of strains in the experimental group was similar to that of the control group. This result indicated that Fe^{3+} could alleviate the inhibition of polymyxin B on the growth of strain SC2.

Given the importance of Ca^{2+}, Mg^{2+}, and Fe^{3+} metal ions in strain tolerance to polymyxin stress, the addition of Fe^{3+} can fully abolish the inhibitory effect of polymyxin on the growth of strain SC2 and Fe^{3+} exhibited the most significant protective effect on strain SC2. As reported in *P. polymyxa* C12 (Yu et al. 2017), adding Fe^{3+} can repair the inactivated Fe–S cluster caused by polymyxin, thereby alleviating polymyxin-induced cell damage. In this study, Fe^{3+} may also alleviate polymyxin-induced cell damage by repairing inactivated Fe–S clusters.

Forming biofilm was a key strategy of strain SC2 to alleviate polymyxin stress

Biofilms are microbial communities in which cells are embedded within a self-produced matrix of extracellular polymeric substances (Nobile et al. 2012). Biofilms are

formed by bacteria to protect themselves from external damage. Bacteria living as a mode of biofilms exhibit inherent resistance to antimicrobial compounds in both clinical and natural settings; thus, biofilms play an important role in bacterial resistance (Pamp et al. 2008; Lopez et al. 2011).

RNA-seq analysis of strain SC2 showed that most genes related to biofilm formation were significantly upregulated (Supplemental Fig.S5b). The *PPSC2_27500* gene was upregulated by 4.51-fold and encodes a protein of unknown function in strain SC2, but the majority of its orthologous genes encode the XRE family transcriptional regulator, which is a master regulator of biofilm formation. The *PPSC2_05535* gene was upregulated by 3.01-fold and is responsible for the production of biofilm matrix polymers (Kimura and Kobayashi 2020). The *PPSC2_12785* gene is involved in peptidoglycan biosynthesis and was upregulated by 3.64-fold. The *PPSC2-10230* gene, encoding the YajQ family cyclic di-GMP-binding protein, was upregulated by 2.69-fold.

The relationship between polymyxin B stress and biofilm formation was deeply explored through biological phenotype experiments. Biofilm formation could be assessed by a colorimetric method based on crystal violet staining (Mitrofanova et al. 2017) in the presence or absence of polymyxin B. The results showed that the OD_{590} of polymyxin-treated strains increased by 55.3% compared with that of the strain without polymyxin B treatment (Fig. 2d). This indicates that polymyxin B can promote the formation of biofilms. To demonstrate the protective effect of biofilm on cells, we treated biofilm-forming cells and non-biofilm-forming cells with polymyxin B for 5 h, and performed CFU assay, intracellular ROS assay, and cell membrane integrity assay. The results showed that the biofilm-forming cells had much more CFUs, lower intracellular ROS, and lower PI fluorescence intensity after polymyxin treatment (Fig. 2e, f). This indicates that biofilm has a protective effect on cells, biofilm cells are more resistant to polymyxin B, and biofilm formation may be one of the mechanisms by which strain SC2 resists polymyxin B.

$Mg^{2+}$and Ca^{2+} have been proven to alleviate polymyxin stress on strain SC2 (Fig. 2a, b). To determine whether $Mg^{2+}$and Ca^{2+} are related to the promotion of biofilm formation to alleviate polymyxin stress in strain SC2, biofilm formation experiments were performed, and in the presence of polymyxin B, the addition of $Ca^{2+}$and Mg^{2+} increased the OD_{590} by 204.5% and 53%, respectively, compared to the control group (without the addition of Ca^{2+} and Mg^{2+}) (Fig. 2d). These results indicate that $Ca^{2+}$and Mg^{2+} may indirectly reduce polymyxin B stress on the strain SC2 by promoting biofilm formation. Forming biofilm might be a key strategy of strain SC2 to alleviate polymyxin stress.

The ABC transporter YwjA contributes to polymyxin B resistance in strain SC2

Multidrug transporters (efflux pumps) play an important role in antibiotic resistance in bacteria. By analyzing the RNA-seq results of polymyxin B-treated strain SC2 at subinhibitory concentrations, it was found that transporter proteins played an important role in the resistance of strain SC2 to polymyxin B. Among the 827 DEGs upregulated under polymyxin B treatment, 119 DEGs encode transporters and 60 DEGs encoding ABC transporters. The genes encoding ABC transporters are shown in Supplementary File 2. ABC transporter YwjA was firstly verified to contribute to polymyxin B resistance of strain SC2 in our study.

The mRNA transcription level of *ywjA* was upregulated by 3.05-fold after polymyxin B treatment in strain SC2, according to the RNA-seq results, and a consistent trend was also obtained by qRT-PCR (Supplemental Fig.S2). The *ywjA* gene encodes a multidrug ABC transporter with 35% amino acid sequence similarity to the multidrug ABC transporter Sav1866, which can transport doxorubicin, verapamil, ethidium, tetraphenylphosphonium, vinblastine, and the fluorescent dye (Velamakanni et al. 2008). To reveal the role of *ywjA* in the resistance of strain SC2 to polymyxin B, the function of *ywjA* was further investigated.

The mutant strain SC2-M1 (Hou et al. 2016) of strain SC2 was selected for gene *ywjA* knockout. The results in Fig. 3a reveal that in the presence of 0.08 mg/mL polymyxin B, the *ywjA* deletion mutant strain (SC2-M1 Δ*ywjA*) exhibited a longer logarithmic growth period and a lower maximum cell density than the control strain (SC2-M1). This indicates that deleting *ywjA* makes the strain more susceptible to polymyxin B. Then, the *ywjA* gene was complemented in the strain SC2-M1 Δ*ywjA*. The strain SC2-M1 Δ*ywjA* transformed vector pHY300PLK was used as a control (SC2-M1 Δ*ywjA* + vector). The inhibition of polymyxin B on the growth of the control strain (SC2-M1 Δ*ywjA* + vector) was more significant than that of the *ywjA* complement strains (SC2-M1 Δ*ywjA* + *ywjA*) (Fig. 3b). These findings demonstrated that YwjA conferred resistance to polymyxin B in strain SC2.

The agar diffusion bioassay was further performed to assess the bactericidal activity of the culture supernatant of strain SC2 against *E. coli* DH5α to investigate the effect of *ywjA* deletion on polymyxin yield (Shaheen et al. 2011). The bactericidal activity of the *ywjA* deletion mutant strain (SC2-M1 Δ*ywjA* + vector) against *E. coli* DH5α was much lower than that of the control strain (SC2-M1 + vector) (Fig. 3c). However, the bactericidal activity of the strain (SC2-M1 Δ*ywjA* + *ywjA*) against *E. coli* DH5α was greatly improved with complementation of *ywjA* in the mutant strain (SC2-M1 Δ*ywjA* + vector) (Fig. 3e). It means that *ywjA* has a positive correlation with the synthesis of polymyxins in strain

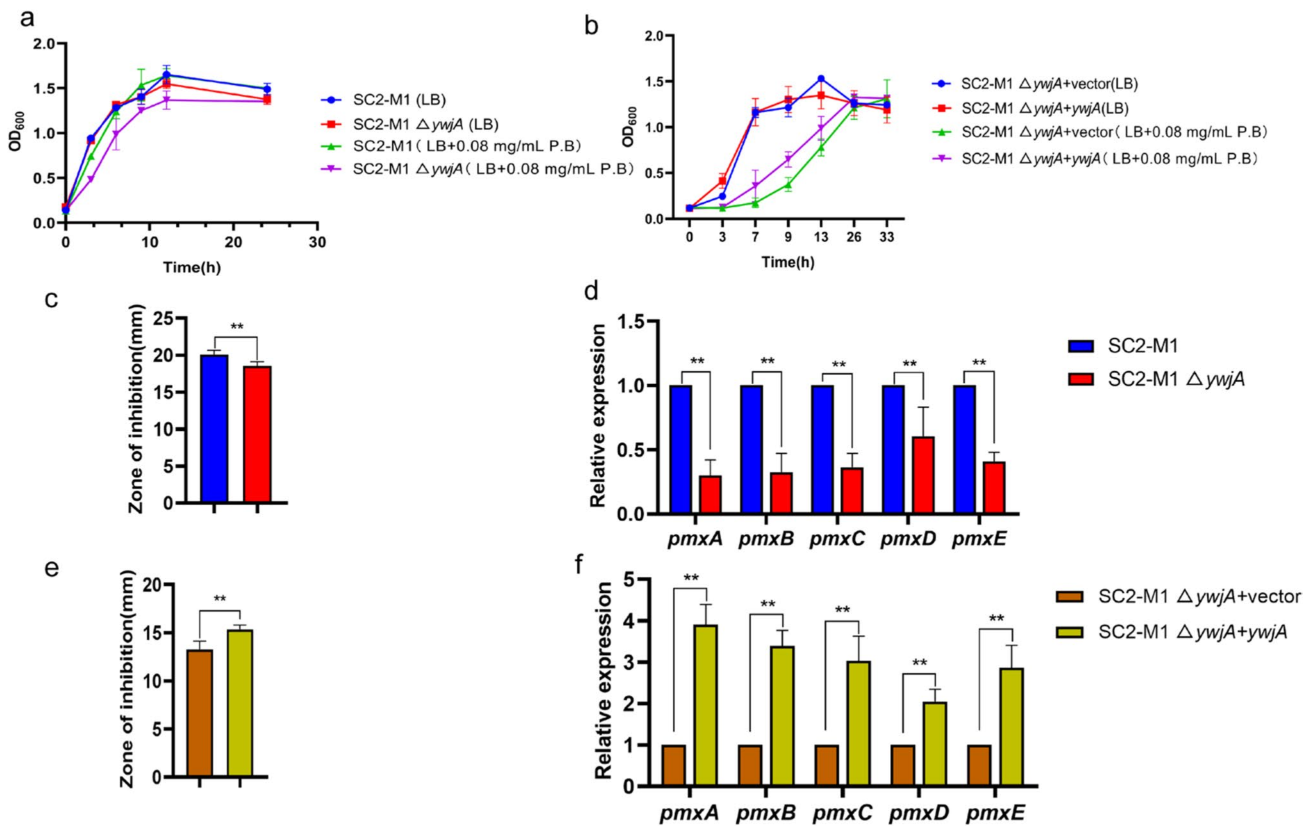

Fig. 3 Functional assay of the *ywjA* mutant strain**. a** Growth and polymyxin B resistance detection of *ywjA* deletion strains. **b** Growth and polymyxin B resistance detection of *ywjA* complementing strains. **c** Bacteriostatic activity test of strains against *E. coli* DH5α. The bacteriostatic activity of the culture supernatant of strains SC2-M1 and SC2-M1 Δ*ywjA* against *E. coli* DH5α was detected by agar diffusion biological assay, and the diameter of the inhibition zone was measured. **d** qRT-PCR results of genes involved in polymyxin biosynthesis in strains SC2-M1 Δ*ywjA* and SC2-M1. **e** Bacteriostatic activity test of SC2-M1 Δ*ywjA* + vector and SC2-M1 Δ*ywjA* + *ywjA* against *E. coli* DH5α. **f** qRT-PCR results of genes involved in polymyxin biosynthesis in strains SC2-M1 Δ*ywjA* + vector and SC2-M1 Δ*ywjA* + *ywjA*. The means and standard errors of the means for all three independent experiments are shown. "*" indicates significance in unpaired *t* tests with *P* values of 0.05. "**" indicates significance in unpaired *t* tests with *P* values of 0.01

SC2. Meanwhile, qRT-PCR results (Fig. 3d) revealed that the mRNA levels of genes (*pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE*) involved in polymyxin biosynthesis were much lower in the *ywjA* deletion mutant strain (SC2-M1 Δ*ywjA*) than in the control strain SC2-M1. The mRNA levels of these genes were greatly increased with the complementation of *ywjA* (Fig. 3f). This suggests that the deletion of *ywjA* may have increased the susceptibility of strain SC2 to polymyxin B, and therefore, the strain survived by decreasing polymyxin biosynthesis.

Discussion

Polymyxin B is known to have strong antibacterial activity against MDR gram-negative bacteria (Speranzini et al. 2016). However, this study found that polymyxin B has antibacterial activity against strain SC2 (gram-positive and polymyxin-producing strain), and within a certain concentration range, the antibacterial activity on strain SC2 is in proportion to the concentration of polymyxin B (Fig. 1a). Previous studies have found that polymyxin E has antibacterial activity against its producer (gram-positive bacteria) and induces bacterial death through cell membrane damage and oxidative damage (Yu et al. 2017). Furthermore, we found that the transcript levels of the polymyxin biosynthesis-related genes *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* were significantly downregulated in the presence of polymyxin B in strain SC2 (Fig. 1b). This indicated that the antibacterial activity of polymyxins to their producers might reduce accumulation during fermentation, resulting in a decrease in polymyxin yield. Therefore, to improve the self-resistance of strain SC2 to polymyxins and increase the yield of polymyxins, we used genome-wide RNA sequencing technology to identify polymyxin B stress-responsive genes in strain SC2.

Genome-wide transcriptional analysis helps characterize the action mechanism of antibiotics and bacterial resistance to antibiotics. Although genome-wide transcriptional

profiling of some bacterial responses to polymyxins has been documented (Loutet et al. 2011; Wang et al. 2021a, b), there has been no report of genome-wide transcriptional profiling of polymyxin producers. Therefore, genome-wide transcriptional analysis of strain SC2 in response to polymyxin B was performed in this study, and this was beneficial for revealing the self-resistance mechanism, increasing polymyxin production, and enhancing dominance in the ecological niche of strain SC2. RNA-seq results showed that polymyxin B treatment resulted in significant transcriptional changes in functional genes related to bacterial motility, transport, carbon source metabolism, trace element metabolism, and so on in strain SC2 (Supplemental Fig.S1b and Fig.S1c).

Polymyxin B treatment resulted in a significant upregulation of oxidative stress-related genes in strain SC2 and a significant increase in intracellular ROS (Supplemental Fig.S3a and Fig.S3b, Fig. 1c). This conclusion is consistent with the results reported in a literature that polymyxin E induces an increase in intracellular ROS content and induces oxidative damage in strains (Yu et al. 2017). Following oxidative damage to cells, genes related to Fe–S cluster synthesis were significantly upregulated (Supplemental Fig.S3c). Fe–S clusters are easily degraded by oxidative stress (Satoshi et al. 2017). However, Fe–S clusters can be resynthesized or repaired, when degraded. Genes related to Fe–S cluster synthesis were significantly upregulated in our study, indicating that polymyxin B may destroy Fe–S by inducing the production of reactive oxygen species, and the Fe–S cluster was involved in the resistance of strain SC2 to polymyxin B. More importantly, we found that Fe^{3+} could completely eliminate the inhibitory effect of polymyxin B on the growth of strain SC2 (Fig. 2c). Polymyxins might oxidize Fe^{2+} to Fe^{3+} by inducing the Fenton reaction to generate –OH (Yu et al. 2017). When –OH exceeds a certain concentration, it will eventually lead to cell death (Kohanski et al. 2007). The addition of Fe^{3+} and the high expression of iron transporters at the transcriptional level can help repair the oxidized and inactivated Fe–S clusters, thereby reducing cell damage. Similarly, as reported in *P. polymyxa* C12, Fe^{3+} could mitigate cell death caused by polymyxin E (Yu et al. 2017). Among the metal ion transporters whose expression levels were upregulated in the RNA-seq results, in addition to Fe^{3+} transporters, there were also Ca^{2+} and Mg^{2+} transporters. The results in Fig. 2a, b show that Ca^{2+} and Mg^{2+} can alleviate the damage caused by polymyxin B in strain SC2. Yu et al. also proved that Ca^{2+} and Mg^{2+} could alleviate the damage of polymyxin E on the cell membrane of *P. polymyxa* C12, and the alleviation effect of Ca^{2+} was the most obvious (Yu et al. 2015a). Furthermore, Ca^{2+} can inhibit the bactericidal activity of polymyxin E in the gram-negative bacterium *P. aeruginosa* (Davis et al. 1971). Therefore, Ca^{2+} and Mg^{2+} may reduce the damage caused by polymyxin B on the cell membrane by reducing the electrostatic interaction between polymyxin B and the bacterial surface. However, an interesting phenomenon appeared in the Ca^{2+} addition experiment; regardless of the presence or absence of polymyxin B, the addition of Ca^{2+} caused strain SC2 to rapidly enter the decline phase (Fig. 2b). As an important second messenger in cells, Ca^{2+} plays an important role in many life processes of cells. However, excessive accumulation of intracellular calcium ions will cause cytotoxicity and cell death. Therefore, the reason for the rapid decline of the strain may be the accumulation of Ca^{2+}, and the addition of Ca^{2+} can only improve the resistance of strain SC2 to polymyxin B in the logarithmic growth phase. Biofilms are bacterial communities that grow on abiotic or biotic surfaces (Niemira 2007). Bacterial biofilms can weaken the bactericidal activity of antibiotics (Kim et al. 2013). Studies have shown that low concentrations of antibiotics can promote biofilm formation in *P. aeruginosa*, *E. coli*, and *Staphylococcus aureus* (Hoffman et al. 2005; O'Neill et al. 2008; Kaplan 2011). The results in Supplemental Fig.S5b showed that polymyxin treatment resulted in significant upregulation of the transcript levels of genes involved in biofilm formation, as well as increased bacterial biofilm formation, implying that strain SC2 may respond to polymyxin stress by increasing biofilm formation. Meanwhile, we discovered that adding Mg^{2+}or Ca^{2+} in the presence of polymyxin B all increased the biofilm formation of strain SC2, leading us to believe that $Mg^{2+}$and Ca^{2+} may alleviate polymyxin stress on strain SC2 by promoting biofilm formation (Fig. 2d). There is now a lack of research on the mechanism by which $Mg^{2+}$and Ca^{2+} promote biofilm formation, and this mechanism needs to be discovered in the future. Flagellar motility plays an important role in the colonization, adhesion, and biofilm formation of bacteria. Bacterial motility is a behavior by which bacteria perceive the surrounding environment and move themselves (via pulling or pushing) or prevent themselves from moving using their flagella components. The addition of polymyxin B promoted the movement of strain SC2 (Fig. 1d, e), which showed the bacterial avoidance movement. It was also reported that citrinin could greatly induce the swarming movement of *P. polymyxa* E681(Park et al. 2008). When bacteria are exposed to an environment that is not conducive to growth, they may escape by increasing their ability to move and explore a space with low concentrations of toxic compounds. This enhancement in mobility may represent the protection of bacteria (Bazzini et al. 2011).

Drug transporters (efflux pumps) also play an important role in bacterial resistance. Among the five drug transporter families, the ABC transporter superfamily is the largest (Geourjon et al. 2001). Many ABC transporters are related to drug resistance (Nishino and Yamaguchi 2001; Verrier et al. 2008; Chen et al. 2016). Moreover, several ABC transporters, such as SapABCDF, AbcAB, and MacAB, have been shown to endow bacteria with resistance to polymyxins

(Margolles et al. 2006; Subashchandrabose et al. 2013; Crow et al. 2017; Hsu et al. 2019). However, in the polymyxin producer *P. polymyxa*, the transporters conferring polymyxin resistance have rarely been studied. In this study, YwjA was predicted to be ABC superfamily transporter. However, we discovered that YwjA conferred resistance of strain SC2 against polymyxin B. Furthermore, deletion of *ywjA* resulted in decreased polymyxin production (Fig. 3c). The deletion of YwjA caused cells to be more sensitive to polymyxin B. To avoid the cell damage caused by excessive polymyxin concentrations, the biosynthesis of polymyxins is reduced. Therefore, we hypothesized that the resistance of strain SC2 to polymyxins limited the biosynthesis of polymyxins itself.

In addition to the above resistance mechanisms of strain SC2 to polymyxin B, RNA-seq results also showed that some other genes were also involved in the self-resistance of strain SC2. For example, the related genes involved in the respiratory chain were significantly changed. Among them, the gene *PPSC2_16985* encoding malate dehydrogenase (oxaloacetate-decarboxylating) was upregulated by 6.3-fold, and the gene *ykwA* encoding malate dehydrogenase was upregulated by 2.7-fold. The gene *citC*, encoding isocitrate dehydrogenase, was upregulated 2.7-fold. This result indicated that polymyxin B could stimulate the respiratory chain-specific genes of strain SC2, and such specific genes could respond to polymyxin stress in different ways. These results are consistent with those of Yu et al., who treated *P. polymyxa C12* with polymyxin E (Yu et al. 2019).

In conclusion, we presented some of the transcriptional and physiological responses of the polymyxin producer *P. polymyxa* SC2 to the stress of polymyxin B (Fig. 4). The resistance of strain SC2 to polymyxin B may limit the biosynthesis of polymyxins. The resistance of strain SC2 to polymyxin B can be enhanced by adding metal ions (Ca^{2+}, Mg^{2+}, and Fe^{3+}), promoting bacterial biofilm formation, reducing oxidative damage to cells, or improving

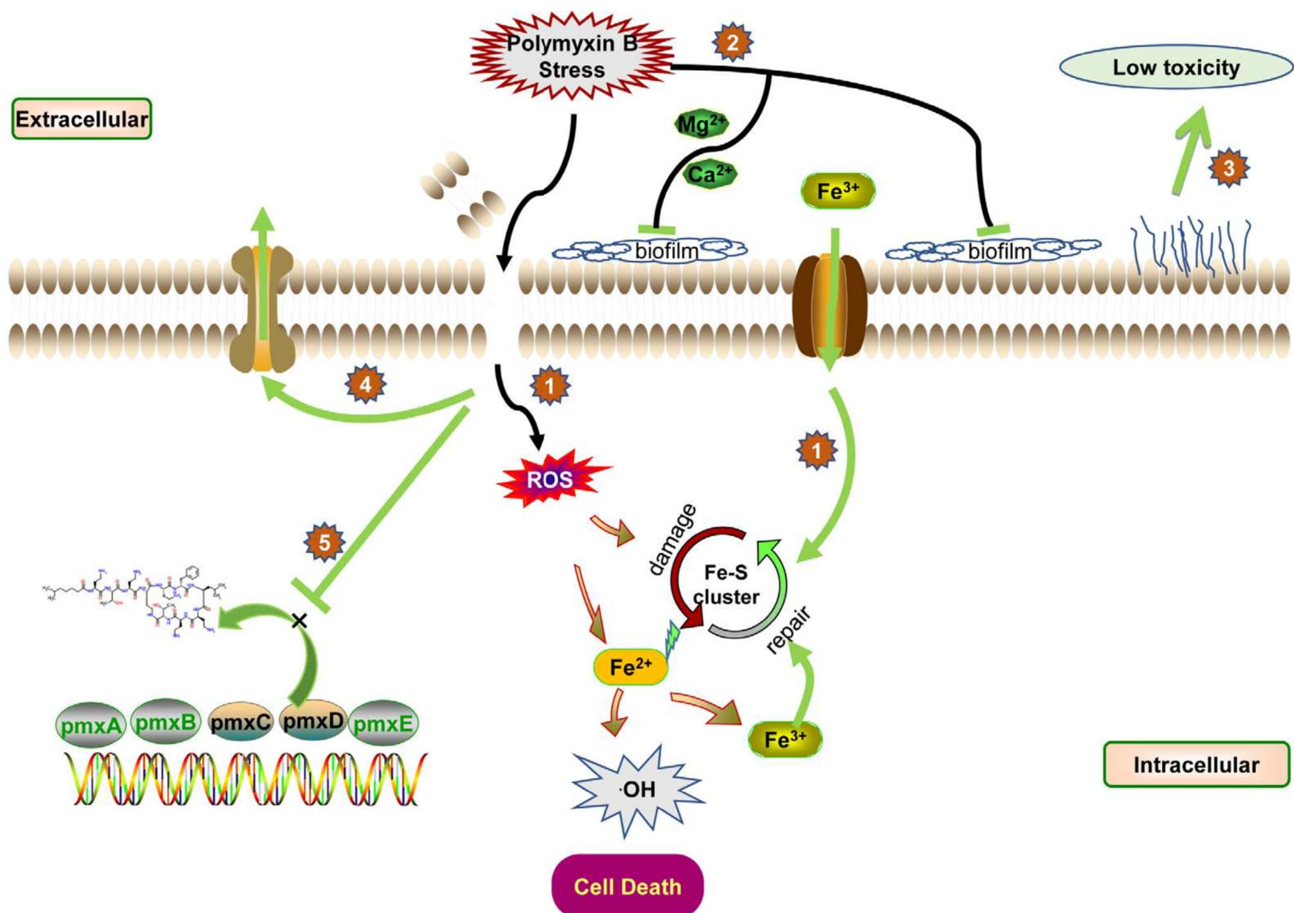

Fig. 4 Stress response of *P. polymyxa* SC2 to polymyxin B. (1) Polymyxin B treatment resulted in the accumulation of intracellular ROS and oxidative damage to Fe-S clusters, while the addition of Fe^{3+} reduced cellular damage by repairing oxidatively inactivated Fe-S clusters. (2) Forming biofilm was a key strategy of strain SC2 to alleviate polymyxin stress: polymyxin B can promote the formation of biofilm; Ca^{2+} and Mg^{2+} may indirectly reduce polymyxin B stress on strain SC2 by promoting biofilm formation. (3) Strain SC2 escapes unfavorable conditions through increased motility. (4) The ABC transporter YwjA contributes to polymyxin B resistance in strain SC2. (5) Strain SC2 responded to polymyxin stress by reducing polymyxin biosynthesis

the transport activity of some ABC transporters and other measures, thereby increasing the yield of polymyxins and improving the biocontrol effect of *P. polymyxa* SC2.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-023-12916-3.

Author contribution CW designed the study. HL and WE performed the laboratory work and analyzed the data. HL and WE wrote the manuscript. DZ, HL, JP, KL, and XZ advised the manuscript. CW and BD supported the study.

Funding This work was funded by the National Natural Science Foundation of China (32170133 and 31700094), Shandong Provincial Key Research and Development Program (Major Science and Technology Innovation Project)-Boost Plan for Rural Vitalization Science and Technology Innovation (No. 2021TZXD001), and State Key Laboratory for Managing Biotic and Chemical Treats to the Quality and Safety of Agro-products (No. 2021DG700024-KF202314).

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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