Unveiling the mechanisms of black phosphorus nanosheets-induced viable but non-culturable state in *Bacillus tropicus*

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1. Materials and methods

1.1 Materials

BP crystals were purchased from XFNANO Materials Tech Co., Ltd. (Nanjing), exfoliated to obtain the suspension of BP nanosheets at a concentration of 502 ± 28 μg mL^{-1} according to our published research.[1] The average hydrodynamic size of BP nanosheets was 155 ± 15.13 nm and the zeta potential was -32.1 ± 3.51 mV. *B. tropicus* was provided by Heilongjiang Provincial Key Laboratory of Environmental Microbiology and Recycling of Argo-Waste in Cold Region (Daqing, China) and cultured in our laboratory. The obtained bacteria strain was further identified by Personalbio Co., Ltd. (Shanghai, China) using 16S rRNA sequencing. Kanamycin was purchased from Harveybio Gene Technology Co., Ltd. (Harveybio, China). SYTO 9 and PI were bought from Invitrogen (Thermo, USA) and Solarbio Science & Technology Co., Ltd. (Solarbio, China), respectively. The PrimeScript™ RT Reagent Kit and TB Green® Premix Ex Taq™ (Tli RNaseH Plus) were purchased from Takara Biomedical Technology Co., Ltd. (TaRaKa, Japan). Cell-Tak was bought from Corning Biotech Co., Ltd. (Corning, USA). The BacTiter-Glo microbial cell viability assay reagent was purchased from Promega Biotech Co., Ltd. (Promega, China). The cell mitochondrial stress test kit for cell respiration measurement, DEME medium (pH = 7.4), 1.0 M glucose, 200 mM glutamine, and 100 mM pyruvate were purchased from Agilent Technologies Co., Ltd. (Agilent, USA).

1.2 Determination of suspension concentration BP nanosheets

The concentration of BP nanosheets was measurement according to our published research.[1] Briefly, BP nanosheets were exfoliated in oxygen-free water by ultrasonication. Subsequently, oxygen gas was introduced into the suspension of BP nanosheets for 5 min every 12 h to induce the degradation into phosphates. When the solution became clear and no absorbance can be detected, the concentration of BP nanosheets was determined to be 502 ± 28 μg mL^{-1} by inductively coupled plasma optical emission spectrometer (ICP-OES).

1.3 SYTO/PI staining and flow cytometry analysis

To visualize the bactericidal effect of BP nanosheets on *B. tropicus*, SYTO/PI staining was performed. After 12 hours of BP nanosheets (0, 1, 10, 50 and 100 μg mL^{-1}) exposed, the bacterial cells were centrifuged at 800 rpm to remove the nanoflakes, followed by centrifugation at 10000 rpm for collection. After rinsing with PBS for 2-3 times, the bacterial cells were stained with an SYTO/PI staining kit for 15 min. Subsequently, 20 μL of the bacterial suspension was dropped onto a glass slide and visualized by a laser scanning confocal microscopy (LSCM, FV1200, Olympus, Japan). The bacterial cells exposed to 10% H_2O_2 for 4 h were set as the positive control, following the same experimental protocol. Visualization was performed by LSCM and the fluorescence intensity of SYTP of *B. tropicus*$_{control}$, *B. tropicus*$_{BP100}$, and the positive control group was detected by flow cytometry (Guava EasyCyte, Luminex, USA). The percentage of dead cells (%) was calculated as follows:

$$\text{Percentage of dead cells}\,(\%) = \left(\frac{\text{Dead cells}}{\text{Live cells} + \text{Dead cells}}\right) * 100\%$$

1.4 Membrane integrity

After 12 hours of BP nanosheets (100 μg mL^{-1}) exposed, the mixture was centrifuged at 800 rpm for 10 min to remove most of the BP nanosheets in the suspension. Subsequently, the suspension was further centrifuged at 10000 rpm for 10 min to collect the bacterial cells. Afterwards, the bacterial cells were fixed with 2.5% glutaraldehyde overnight at 4 °C, followed by washing with PBS (pH 7.02) and gradually dehydrated with a series concentrations of ethyl alcohol (50%, 70%, 90%, 100%, and 100%) for 10 min at 4 °C. Similarly, bacterial cells without BP nanosheet treatment underwent the same procedure and set as control. The samples were visualized by a field emission scanning electron microscopy SU8010 (FE-SEM SU8010, Hitachi, Japan) after lyophilization.

1.5 Calculation of the percentage of VBNC cells

To clarify the calculation process, the total cell number in control group was set to 10000 (Table S2). After treatment with BP nanosheets at 100 μg mL^{-1}, the total bacterial count was approximately 8468 cells, as indicated by the OD measurement at 600 nm. The amount of viable cells, as determined by ATP production, and the

number of culturable cells, as determined by CFU counts, were approximately 6093 and 343 , respectively. Therefore, the percentage of VBNC cells was calculated to be 60.90%.

1.6 Transcriptome analysis

B. tropicus cells were exposed to BP nanosheets (100 μg mL^{-1}) for 12 h, and the mixture was centrifuged at 800 and 12000 rpm to remove the nanoflakes and collect the bacterial cells, respectively. These collected bacterial cells were referred to as *B. tropicus*$_{BP100}$ and were stored at -80 °C after freezing with liquid nitrogen. *B. tropicus* cells without BP nanosheet treatment served as the control group and were named as *B. tropicus*$_{control}$. Each group consisted of three biological replicates. Subsequently, the samples were sent to Personalbio Co., Ltd. (Shanghai, China) for prokaryotic transcriptome sequencing. The libraries were sequenced on the Illumina MiSeq platform, generating paired-end reads. After sequencing, the raw reads were deposited in the NCBI Sequence Read Archive (SRA, https://submit.ncbi.nlm.nih.gov/subs/sra/) database (Accession number: PRJNA874463). DESeq (v1.30.0) was employed to analyze the differential expression of mRNA, and transcripts with |log2FoldChange| > 1 and $p < 0.05$ were considered as differentially expressed mRNA.

1.7 Sequencing data validation by qRT-PCR

To validate the transcriptome sequencing data, we conducted quantitative real-time polymerase chain reaction (qRT-PCR) on several randomly chosen genes. The gene primers were designed by SBS genetech Co., Ltd. The qRT-PCR assays were performed using the LightCycler 96 System (Roche, Switzerland). PCR amplification followed the following conditions: 95 °C for 120 s, followed by 95 °C for 15 s, 55 °C for 10 s, and 72 °C for 30 s (40 cycles) for the *argC*, *patB*, *pstS*, and *motA* genes; 95 °C for 120 s, followed by 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s (40 cycles) for the *cat1 astE*, *narG*, and *MDA* genes; and 95 °C for 30 s, followed by 95 °C for 5 s, and 58 °C for 30 s (40 cycles) for the *qoxA*, *qoxB*, *cydB*, and *ridB* genes. The 16S rRNA gene was used as a house-keeping gene. The gene-specific primers for qRT-PCR were shown in Table S1. Based on the $2^{-\Delta\Delta Ct}$ method, the relative expression levels of the selected genes were analyzed and all qRT-PCR analyses were repeated in three biological replicates.

1.8 Bacterial respiration

The Seahorse XFe24 Extracellular Flux Analyzer (Bioscience) was used to measure the oxygen consumption rates (OCRs) of *B. tropicus* in the presence of BP nansoheets.[2] *B. tropicus* cells were harvested after overnight culture and added into 10 mL of fresh LB media containing BP nanosheets at the final concentrations of 10^6 CFU mL^{-1} and 100 μg mL^{-1}, respectively. After exposured with BP nanosheets for 4 h, the mixture was diluted 1:100 in fresh LB media, and 50 μL of the diluted cells were added to each well of the XF cell culture microplates pre-coated with Cell-Tak. Subsequently, the microplates were centrifuged for 2 min at 200 rpm to facilitate cell attachment to the pre-coated plates. After centrifugation, an additional 50 μL of fresh LB media was added to each well. The OCR values were measured every 7 min using the instrument. The OCR value obtained from *B. tropicus* in LB medium was normalized to the number of viable cells using ATP measurement.

1.9 Protein aggregation observation by TEM

To analyze the protein aggregation of bacterial cells in different states, the morphology of cells after BP nanosheet treatment was observed by transmission electron microscopy (TEM). Once the count of culturable cells dropped below 1 CFU mL^{-1}, 1 mL of the suspension was added to 10 mL LB medium and further cultured at 37 °C for another 12 h with shaking at 120 rpm. The state of protein aggregation in the bacterial cells was visualized by TEM (HT7800, Hitachi, Japan).

1.10 Antibiotic susceptibility

The antimicrobial susceptibility profiles of *B. tropicus* were evaluated by measuring the absorbance at 600 nm after treatment with different types of antibiotics, including Ampicillin, Vancomycin, Cefuroximem, Kanamycin, Gentamicin, and Ciprofloxacin. Briefly, *B. tropicus* cells were pretreated with BP nanosheets at a dose of 100 μg mL^{-1}. Subsequently, both *B. tropicus*$_{control}$ and *B. tropicus*$_{BP100}$ cells were collected and resuspended in PBS. Furthermore, these cells were inoculated into 10 mL of fresh LB medium containing different concentrations of antibiotics (0, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 μg mL^{-1}) to reach the final bacterial density of

10^6 CFU mL^{-1}. After a 12-hour incubation in a shaking incubator at 120 rpm and 37 °C, the optical density at 600 nm was recorded.

In addition, to determine the short-term susceptibility of *B. tropicus* cells to antibiotics post-BP nanosheet treatment, both *B. tropicus*$_{BP100}$ and *B. tropicus*$_{control}$ cells were resuspended in PBS. Subsequently, an equal number of viable cells from each group were exposed to various concentrations of kanamycin (0, 1, 10, 25 and 50 μg mL^{-1}). After 60 minutes of exposure, the bacterial viability was quantified by the ATP assay.

For real-time monitoring of bacterial resistance to antibiotics, the OCRs of *B. tropicus*$_{BP100}$ and *B. tropicus*$_{control}$ cells were measured. After rinsing the bacterial cells with PBS for 2-3 times, they were diluted 1:100 in fresh LB medium. The subsequent procedures were performed as described in Section 1.7. To ensure consistent cellular seeding, the initial OCR was measured for three cycles (7 min per cycle) before the addition of antibiotics (50 μg mL^{-1} kanamycin).

1.11 Statistical analysis

The significant difference between different treatments was analyzed by an independent *t* test or one-way analysis of variance (ANOVA) at a significant level of $P < 0.05$. All data represented were the means ± standard deviations (SD) of three replicates for each treatment.

2. Results and discussion

2.1 Transcriptome analysis

A total of 4758 genes were identified, with quantitative information available for 4745 of these genes (Table S3). Among these genes, 406 differentially expressed genes (DEGs) were identified, with 107 genes up-regulated and 299 genes down-regulated (Figure S2a). To further validate the accuracy of the transcriptome data, 11 DEGs were randomly selected and analyzed by qRT-PCR. The expression patterns of these genes were found to be consistent with the transcriptome data, providing additional support for the reliability of the findings (Figure S3).

The DEGs identified in this study were functionally annotated by gene ontology

(GO) analysis, which categorized the genes into biological process, cellular component, and molecular function. The significantly enriched terms in each category were identified and shown in Table S3. Among the biological processes, the top five enriched terms were transport (GO:0006810), establishment of localization (GO:0051234), localization (GO:0051179), ion transport (GO:0006811), and transmembrane transport (GO:0055085). In terms of cellular components, the primary enriched GO terms were repair complex (GO:0009380, GO:1990391, GO:1905348) and plasma membrane (GO:0005886, GO:0005887, GO:0031226). The molecular function category was enriched in transporter activity (GO:0022857, GO:0015144, GO:0015075, GO:0015144, GO:0042626), oxidoreductase activity (GO:0016679, GO:0016682), and electron transfer activity (GO:0009055). It is worth noting that most of the genes associated with these enriched terms were down-regulated under BP nanosheet stress (Figure S2, Table S4). KEGG pathway analysis revealed that 73 DEGs were significantly enriched in 10 pathways under BP nanosheet stress compared to untreated *B. tropicus* (Table S5). Among them, 6 pathways were related to metabolism, including butanoate metabolism (ko00650), riboflavin metabolism (ko00740), propanoate metabolism (ko00640), nitrogen metabolism (ko00910), glyoxylate and dicarboxylate metabolism (ko00630), and valine, leucine and isoleucine degradation (ko00280). Interestingly, most of the genes involved in these metabolic pathways were down-regulated under BP nanosheet stress (Figure S2c, d).

Table S1. Sequences of qRT-PCR primers used in the study.

Gene	Forward primer sequence	Reverse primer sequence	Ref.
MDH	GGTATGGATCGTTCCGACCT	GGCAGAATGGTAACACCAGAGT	[3, 4]
ArgC	AAAGAGCCTGCTGAAGACCA	AGGGAATCAAGGCCAACTCT	[5]
motA	TGAACGACCCCCATTACAGC	AGCGGTCACATGAACACCTT	[6]
pstS	TTGTGCAACTCAACACAGCA	TTGGGATCATTCCAGTTG GT	[7]
16S *rRNA*	AGACACGGCCCAGACTCCTAC	TCACACCCGTTCTTCTCTTACAA	[5]
qoxA	TCGCATCTATCGTATTAACGC	ACGAATGCAAAGCCAAAGAT	[8]
qoxB	AGAAGGCGTTAACCCAACAA	AATGGCCCAAACGATACCTA	[8]
cydB	AACAGCGAGCGGAATGGTAA	CCGGAAAATGGCGCAAAAGA	[9]
ridB	TTCGATTGTGCCTGTTTCTTC	ATCAAACTGACGGCAAAACC	[10]
patB	GCCAGGCTATGCGATTGAGT	GCTGGAACCCTGTCCATTGT	[11]
narG	TAGTGGGCAGGAAAACTG	CGTAGAAGAAGCTGGTGCTGTT	[12]
cat1	ACAGCGATGAGTTGTGACGT	ATCCTTGCTGCATCTGTCCG	[13]

Table S2. Percentage of bacterial cells in each state under the treatment of different concentrations of BP nanosheets.

	Cell state	BP concentration (μg mL^{-1})			
		1	10	50	100
Percentage of control (%) [a]	Growth activity	100.43	96.91	94.32	84.68
	ATP concentration	99.72	95.03	78.08	60.93
	Cell viability	76.04	47.66	25.07	3.43
Bacterial cell number	Total bacterial count	10043	9691	9432	8468
	Viable of the cells	9972	9503	7808	6093
	Culturable cells	7604	4766	2507	343
	VBNC cells	2368	4737	5301	5751
	Dead cells	71	188	1624	2375
Percentage in different treatments (%)	Viable of the cells	99.29	98.06	82.78	71.95
	Culturable cells	75.71	49.18	26.58	4.05
	VBNC cells	23.58	48.88	56.20	67.91
	Dead cells	0.71	1.94	17.22	28.05

a: These data were measured by OD 600 nm, ATP concentration, and CFU count in Figure 1b, figure 1d, and figure 2f, respectively.

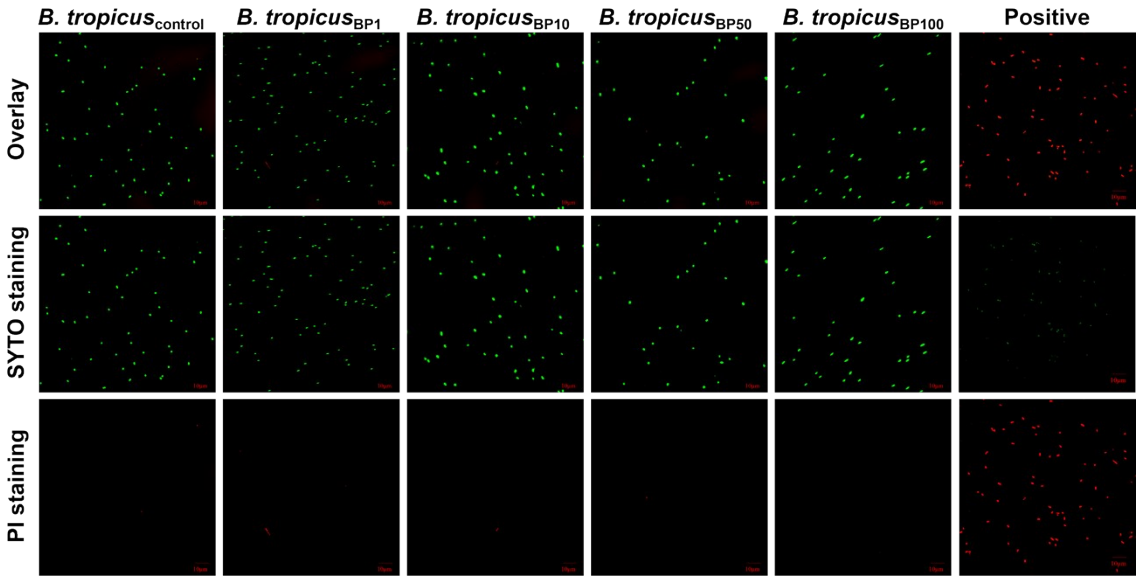

Figure S1. Fluorescence images of *B. tropicus* cells treatment with different concentration of BP nanosheets (0, 1, 10, 50 and 100 μg mL^{-1}) for 12 h, visualized by SYTO/PI staining. 10% H_2O_2 exposed for 4 h was set up as positive control. Scale bars in the images and enlarged views were 10 μm.

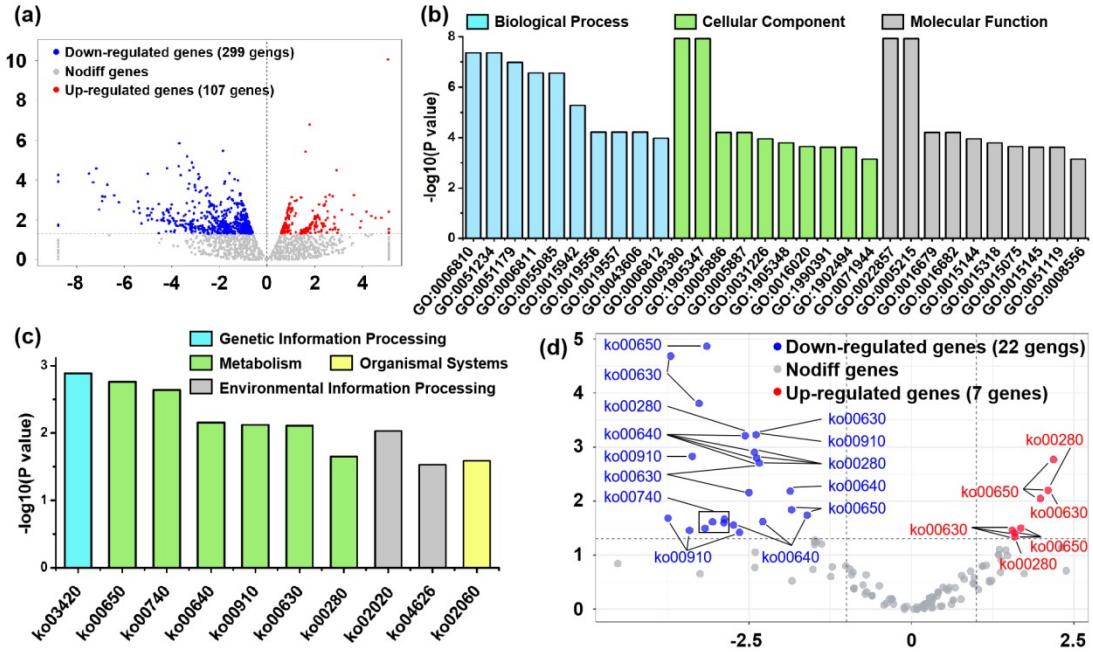

Figure S2. Transcriptome analysis and enrichment analysis of *B. tropicus* cells under the stress of BP nanosheets. Volcano plot of up- and down-regulated DEGs (a), GO enrichment analysis (b), KEGG pathway enrichment analysis (c), and a volcano plot of up- and down-regulated DEGs related to metabolism pathways (d) in *B. tropicus* cells under BP nanosheet stress.

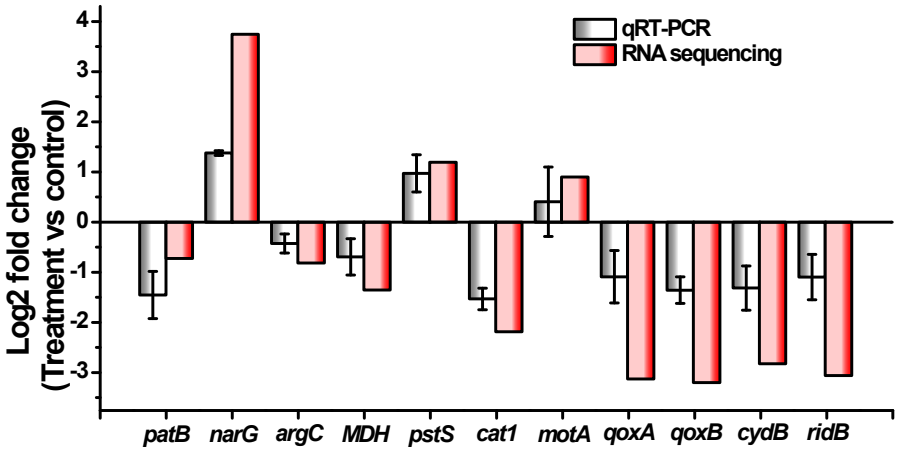

Figure S3. Comparison of gene expression data between qRT-PCR and transcriptome sequencing. Data presented as mean ± S.D. of three replicates (n = 3).

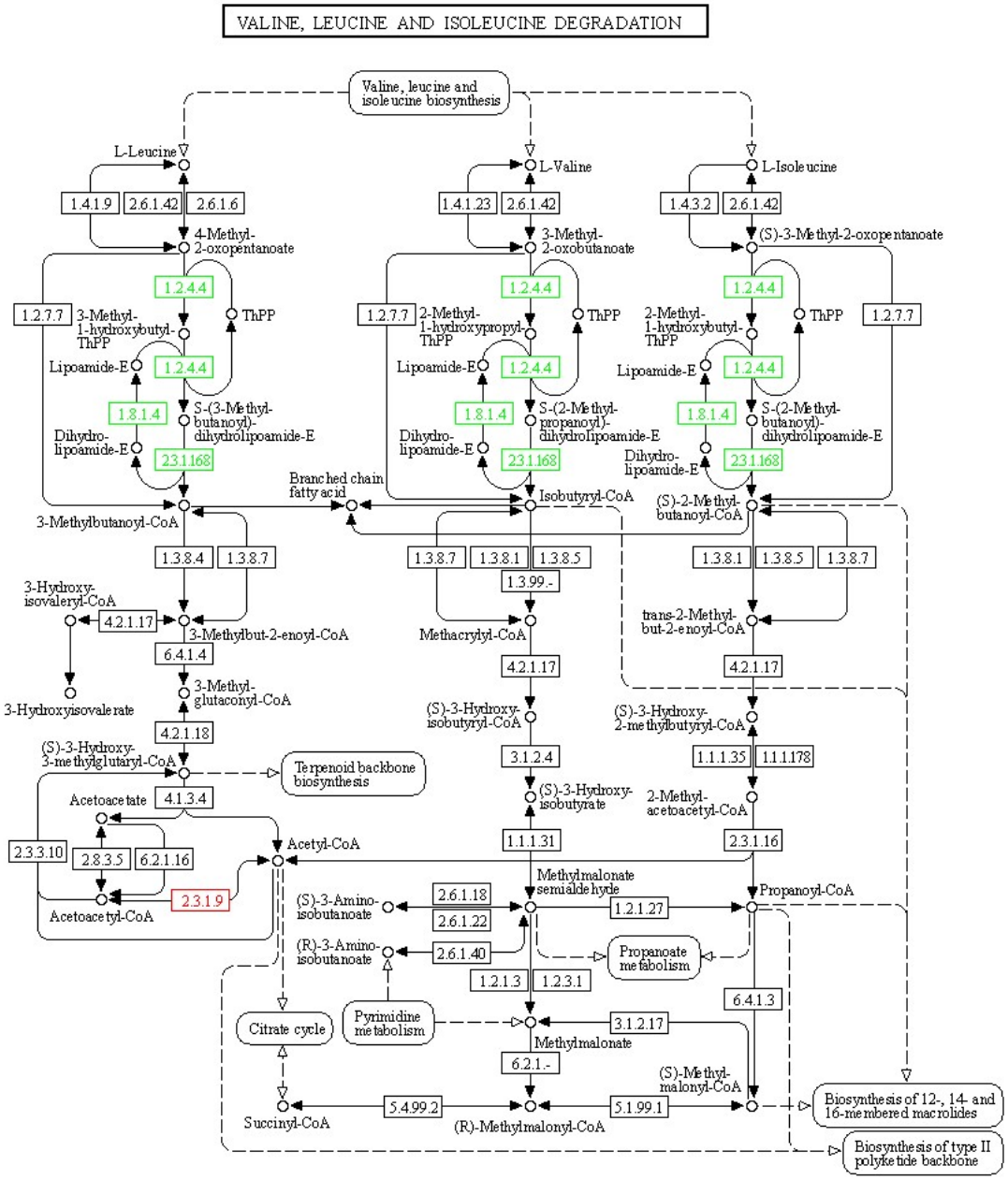

Figure S4. Diagram of correlation of DEGs involved in valine, leucine, and isoleucine degradation in *B. tropicus* under BP nanosheet stress. Rectangles represent genes and circles represent metabolites. Genes with a red (up-regulated) or green (down-regulated) background belong to the DEGs identified under BP nanosheet stress.

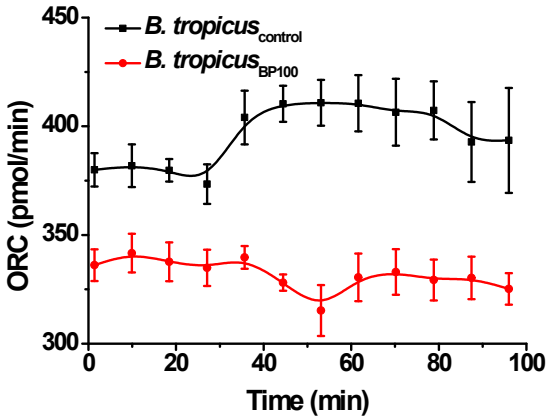

Figure S5. Changes of OCRs over time in response to BP nanosheet treatment in *B. tropicus*.

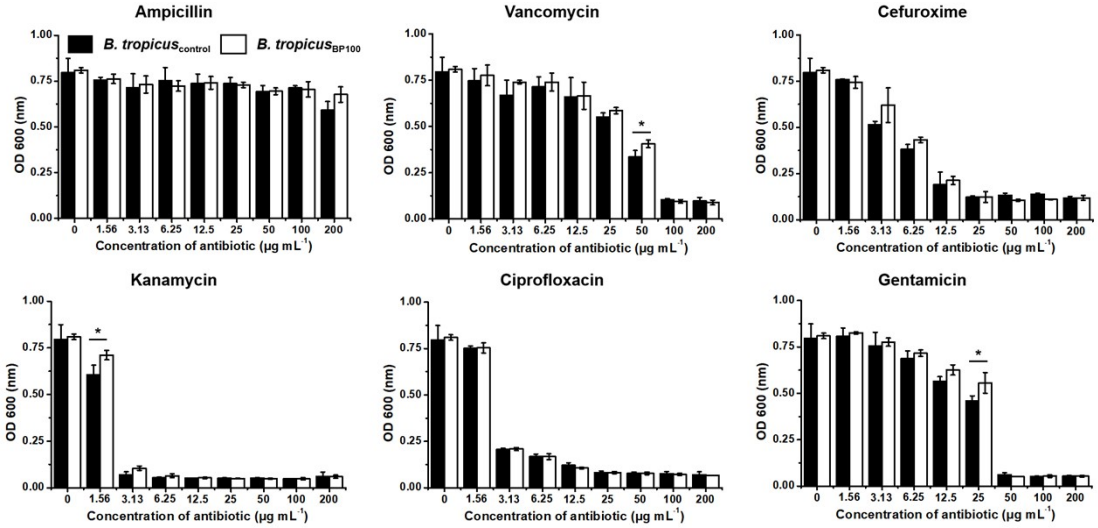

Figure S6. The antimicrobial susceptibility profiles of *B. tropicus* to different types of antibiotics, including Ampicillin (Penicillins), Vancomycin (Glycopeptides), Cefuroxime (Cephalosporins), Kanamycin and Gentamicin (Aminoglycosides), and Ciprofloxacin (Fluoroquinolones).

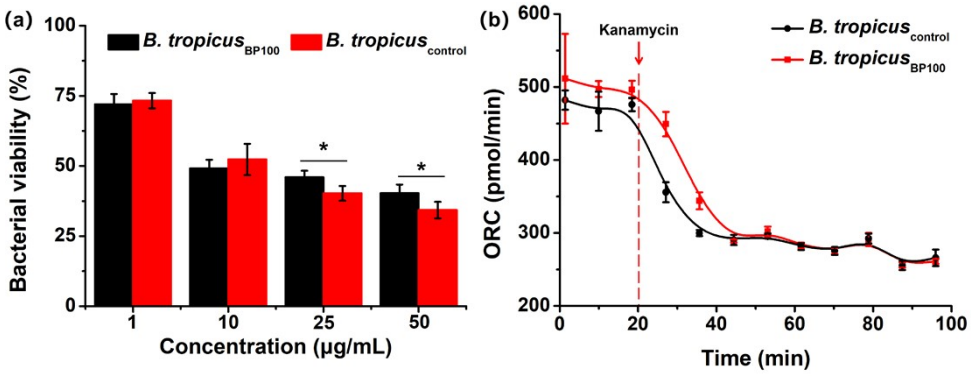

Figure S7. The response of *B. tropicus*$_{control}$ and *B. tropicus*$_{BP100}$ to kanamycin. (a) Bacterial viability of *B. tropicus*$_{control}$ and *B. tropicus*$_{BP100}$ after 60 minutes of kanamycin stress. (b) Real-time monitoring of OCR under kanamycin stress using Seahorse XF24. To ensure consistent cell seeding, the initial OCR was measured for three cycles (7 min per cycle) prior to the addition of kanamycin (50 μg mL^{-1}).

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