Supporting information

Development of bacterial resistance induced by low concentration of two-dimensional black phosphorus via mutagenesis

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1. Cell morphology and ultrastructure observation

The BP-sensitive and BP-resistant bacterial broth was exposed to 500 mg/L 2D-BP nanosheets dispersion for 24 h, and centrifuged at 1000 rpm for 10 min to remove most of the 2D BP nanosheets in the mixed solution. The retained bacteria were washed two times with PBS buffer and immediately fixed in 2.5% glutaraldehyde for 12 h at room temperature. After fixation, the specimens were dehydrated by a graded series of ethanol solutions (50, 70, 90 and 100%) two times each for 10 min, respectively. Subsequently, the dehydrated cells were immersed in 50% tert-butanol and 100% tert-butanol for 20 min, respectively. They were pre-frozen at -20 ℃ for 24 h and then freeze-dried in vacuum. Finally, the bacteria were sputter-coated with gold (20 s, 30 mA) and the morphology of bacterial cell was observed by scanning electron microscope (SEM; SU8020; Hitachi, Japan).

The bacterial cells were fixed with 2.5% glutaraldehyde solution for 12 h (the fixed solution was filled with the centrifuge tube to make the bacteria precipitate completely immersed), then the fixed solution was poured out and the samples were rinsed with PBS buffer for three times, 15 min each time. The sample was fixed with 1% osmium acid solution for 1~2 h. The osmium acid waste solution was carefully removed, and the sample was rinsed with PBS buffer three times, 15 min each time. The samples were dehydrated with ethanol solution of gradient concentration (including 30%, 50%, 70%, 80%, 90% and 95%) for 15 min at each concentration, and then treated with 100% ethanol for 20 min, the treatment was transferred to pure acetone for 20 min. The sample was treated with a mixture of embedding agent and acetone (V/V=1/1) for 1 h. The sample was treated with a mixture of embedding agent

and acetone (V/V=3/1) for 3 h. The samples were treated overnight with pure embedding agent. The embedded sample is obtained by embedding the permeated sample and heating it overnight at 70 ℃. The samples were selected in a Leica EM UC7 ultrafine slicer, and slices at 70~90 nm were obtained. The slices were stained with lead citrate solution and 50% ethanol saturated solution of uranium dioxide acetate for 5~10 min respectively. After drying, the slices were observed under a transmission electron microscope (H-7650, Hitachi, Japan).

2. Lactate dehydrogenase (LDH) assay

Experimental Settings: No bacterial cell group (blank group), without BP treatment of bacterial cells (control group), without BP treatment used for subsequent cracking of bacterial cells group (maximum enzyme activity), black phosphate treatment of bacterial cells group (treatment group), 1 h before reach testing point in time, to add the "largest enzyme activity of samples" group LDH releasing reagent, add 10% of the original medium, mix well and continue to culture. After the culture, the experimental samples were centrifuged at 10000 × g for 10 min, and 120 μL supernatant of each component was added into 96-well plate and mixed with 60 μl LDH detection solution. The mixed solution was incubated at room temperature (25 ℃) for 30 min in dark. The LDH release was quantified by dual wavelength absorbance measurements at 490 nm and 600 nm.

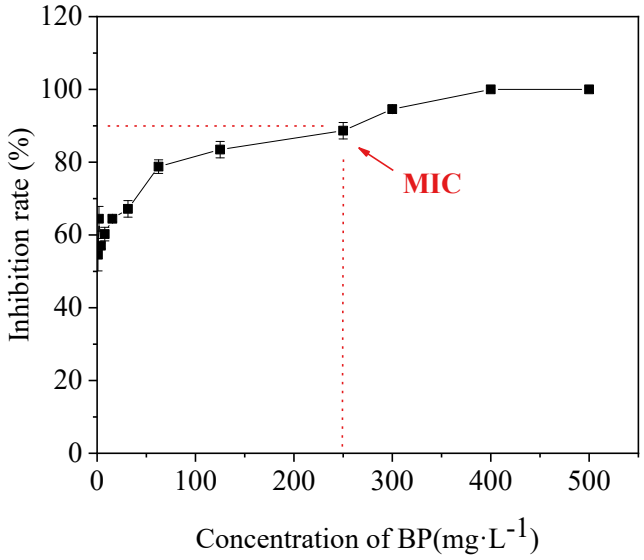

Fig. S1 Effect of different concentrations of 2D-BP nano-material dispersion on the growth inhibition rate of sensitive *E. coli K12*.

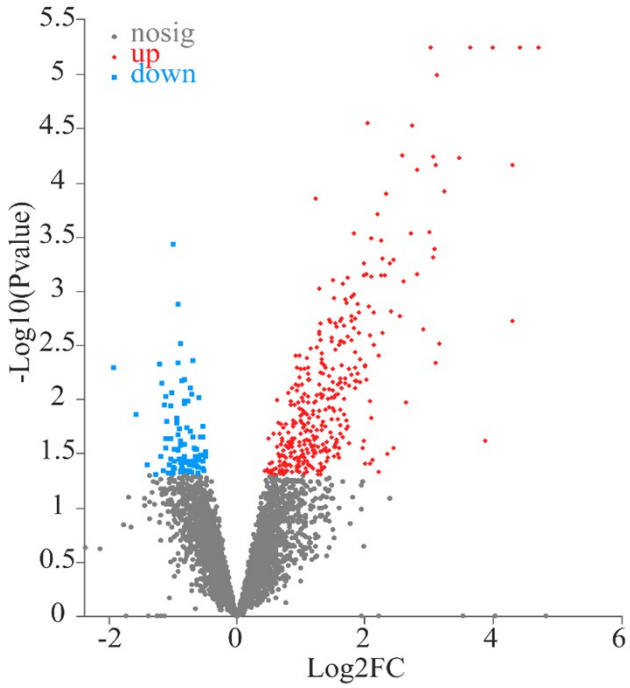

Fig. S2 Sample volcano plot for comparing resistant bacteria with sensitive bacteria of DEGs (|FC|>1, P<0.05). Log_2FC represents the logarithm base 2 of fold change of expression differences.

Table S1

Three genetically changed genes in BP-resistant bacteria.

Site	Ref→Alt	Types	Gene	Amino acid mutants	Function
803662	C→A	SNP	*dmdA*	Arg → Gln	dimethylmalate dehydratase large subunit
1905761	G→A	SNP	*mntP*	Let →Ile	putative manganese efflux pump *MntP*
2339173	G→A	SNP	*gyrA*	Gly →Asp	DNA gyrase subunit A

Table S2

Sequencing and assembly statistics for the transcriptomic data.

Sample	Raw Reads	Clean Reads	Tatal mapped[a]	Uniquely mapped[b]
BP-sensitive bacteria-1	24797288	24463394	23919004 (97.77%)	23012286 (94.07%)
BP-sensitive bacteria-2	24890614	24549818	24222917 (98.67%)	23154051 (94.31%)
BP-sensitive bacteria-3	23004758	22642684	22281213 (98.4%)	21139211 (93.36%)
BP-resistant bacteria-1	22471226	22167898	21926241 (98.91%)	20740174 (93.56%)
BP-resistant bacteria-2	22089754	21757476	21344681 (98.1%)	20426838 (93.88%)
BP-resistant bacteria-3	25137732	24625256	24323742 (98.78%)	22032418 (89.47%)

a Total mapped is the number of clean reads that mapped to the reference genome.

b Uniquely mapped is the number of clean reads that mapped to the reference genome only at one site.

Table S3

The significant up-regulated genes in resistant bacteria compared with sensitive bacteria (p<0.05).

Class	Gene name	Gene description	Log_2FC
Metabolism	*tnaB*	tryptophan: H (+) symporter TnaB	3.47
	tnaA	tryptophanase	3.23
	tdcD	propionate kinase	3.06
	glpC	anaerobic glycerol-3-phosphate dehydrogenase subunit C	3.06
	tnaC	tnaAB operon leader peptide	2.81
	waaU	putative ADP-heptose:LPS heptosyltransferase 4	2.58
	glpB	anaerobic glycerol-3-phosphate dehydrogenase subunit B	2.38
	tdcC	threonine/serine:H(+) symporter	2.26
	fau	putative 5-formyltetrahydrofolate cyclo-ligase	1.99
	tdcA	DNA-binding transcriptional activator TdcA	1.89
	murP	N-acetylmuramic acid-specific PTS enzyme IICB component/anhydro-N-acetylmuramic acid transporter	1.62
	tdcE	2-ketobutyrate formate-lyase/pyruvate formate-lyase 4	1.55
	tdcB	catabolic threonine dehydratase	3.01
Transcription and Translation	*rpsQ*	30S ribosomal subunit protein S17	1.86
	rpoC	RNA polymerase subunit beta'	1.62
	rpmJ	50S ribosomal subunit protein L36	1.45
	rpmF	50S ribosomal subunit protein L32	1.44
	rpmD	50S ribosomal subunit protein L30	1.42
	rplQ	50S ribosomal subunit protein L17	1.31

	rpmC	50S ribosomal subunit protein L29	1.16
	rpoA	RNA polymerase subunit alpha	1.05
Cellular	*isrC*	-	4.71
Processes	*flu*	CP4-44 prophage%3B self recognizing antigen 43 (Ag43) autotransporter	4.00
	mglC	D-galactose/methyl-galactoside ABC transporter membrane subunit	2.42
	fliL	flagellar protein FliL	2.07
	flgD	flagellar biosynthesis%2C initiation of hook assembly	2.01
	gfcC	capsule biosynthesis GfcC family protein	1.99
	mglA	D-galactose/methyl-galactoside ABC transporter ATP binding subunit	1.92
	aroF	3-deoxy-7-phosphoheptulonate synthase%2C Tyr-sensitive	1.84
	gfcB	lipoprotein GfcB	1.79
	mglB	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein	1.69
	fliS	flagellar biosynthesis protein FliS	1.30
	gfcD	putative lipoprotein GfcD	1.28
	aer	aerotaxis sensor receptor%2C flavoprotein	1.11

102 Log_2FC represents the logarithm base 2 of fold change of expression differences.

Table S4

The enriched up-regulated genes in resistant bacteria compared with sensitive bacteria (p<0.05).

KEGG Pathway	Gene Name	Description	FC
Bacterial chemotaxis	*cheA*	chemotaxis protein CheA	1.28
	mglB	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein	3.23
	motB	motility protein B	1.11
	cheZ	chemotaxis protein CheZ	1.09
	cheY	chemotaxis protein CheY	1.26
	cheR	chemotaxis protein methyltransferase	2.11
	tap	methyl-accepting chemotaxis protein Tap	1.61
	rbsB	ribose ABC transporter periplasmic binding protein	1.52
	fliM	flagellar motor switch protein FliM	1.96
	tar	methyl-accepting chemotaxis protein Tar	1.08
	fliN	flagellar motor switch protein FliN	1.25
	trg	methyl-accepting chemotaxis protein Trg	1.35
	dppA	dipeptide ABC transporter periplasmic binding protein	1.31
	lafU	flagellar system protein%2C promoterless fragment	1.29
	motA	motility protein A	1.35
	fliG	flagellar motor switch protein FliG	1.36
Flagellar assembly	*motB*	motility protein B	1.11
	fliM	flagellar motor switch protein FliM	1.96
	flgE	flagellar hook protein FlgE	1.72
	fliN	flagellar motor switch protein FliN	1.25
	lafU	flagellar system protein%2C promoterless	1.29

		fragment	
	flhD	DNA-binding transcriptional dual regulator FlhD	1.15
	flhC	DNA-binding transcriptional dual regulator FlhC	1.68
	motA	motility protein A	1.35
	fliG	flagellar motor switch protein FliG	1.36
	flhB	flagellar biosynthesis protein FlhB	1.52
Oxidative	*frdC*	fumarate reductase membrane protein FrdC	1.47
phosphorylation	*frdA*	fumarate reductase flavoprotein subunit	1.23
	frdB	fumarate reductase iron-sulfur protein	1.61
	nuoM	NADH:quinone oxidoreductase subunit M	1.57
	nuoL	NADH:quinone oxidoreductase subunit L	1.29
	nuoK	NADH:quinone oxidoreductase subunit K	1.88
	atpH	ATP synthase F1 complex subunit delta	2.28
	atpF	ATP synthase Fo complex subunit b	2.19
	atpE	ATP synthase Fo complex subunit c	3.21
	atpD	ATP synthase F1 complex subunit beta	3.59
	atpG	ATP synthase F1 complex subunit gamma	3.34
	atpB	ATP synthase Fo complex subunit a	1.69
	sdhB	succinate:quinone oxidoreductase%2C iron-sulfur cluster binding protein	1.39
	sdhD	succinate:quinone oxidoreductase%2C membrane protein SdhD	1.33
	sdhA	succinate:quinone oxidoreductase%2C FAD binding protein	1.13
Pentose and	*sgbE*	L-ribulose-5-phosphate 4-epimerase SgbE	1.03
glucuronate	*sgbU*	putative L-xylulose 5-phosphate 3-epimerase	1.75
interconversions	*sgbH*	3-keto-L-gulonate-6-phosphate decarboxylase SgbH	1.42
	lyxK	L-xylulose kinase	1.22

	araA	L-arabinose isomerase	2.07
	yagF	CP4-6 prophage%3B D-xylonate dehydratase	1.29
	yagE	CP4-6 prophage%3B putative 2-keto-3-deoxygluconate aldolase	1.47
	araD	L-ribulose-5-phosphate 4-epimerase AraD	1.68
	rhaB	rhamnulokinase	3.02
	kduI	5-dehydro-4-deoxy-D-glucuronate isomerase	1.25
	kduD	putative 2-keto-3-deoxy-D-gluconate dehydrogenase	1.03
	yiaK	2%2C3-diketo-L-gulonate reductase	1.59
	rhaD	rhamnulose-1-phosphate aldolase	1.41
	araB	ribulokinase	1.78
	ulaE	L-ribulose-5-phosphate 3-epimerase UlaE	1.21
	ulaD	3-keto-L-gulonate-6-phosphate decarboxylase UlaD	1.67
	ulaF	L-ribulose-5-phosphate 4-epimerase UlaF	1.20
Thiamine metabolism	*thiC*	phosphomethylpyrimidine synthase	1.72
	rsgA	ribosome small subunit-dependent GTPase A	1.14
	thiL	thiamine monophosphate kinase	1.01
	thiD	bifunctional hydroxymethylpyrimidine kinase/phosphomethylpyrimidine kinase	1.33
	thiM	hydroxyethylthiazole kinase	1.23

Fold changes (FC) of the expression of genes between 2D-BP resistant bacteria and sensitive bacteria *E. coli K12*.