Supplementary material:

Surface Charge Governs Polystyrene Nanoplastics' Influence on Conjugative Transfer of Antibiotic Resistance Genes

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Text S1. Characterization of PSNPs

Three types of polystyrene nanoplastics (PSNPs) were used in this study, including unfunctionalized (PS) and two surface-functionalized (PS-NH_2 and PS-COOH). Transmission electron microscopy (TEM) revealed that all PSNPs were spherical with uniform particle size distribution (Fig. S1a-c). The average radius of PSNPs measured by dynamic light scattering (DLS) in the mating buffer for gene transfer (PBS), were 43.74 ± 8.29 nm for PS, 39.60 ± 8.36 nm for PS-NH_2, and 55.10 ± 10.92 nm for PS-COOH, which were generally consistent with their nominal sizes (Fig. S1d). Surface charges measured by a zeta potential measurement showed average zeta potentials of -33.80 ± 0.78 mV (PS), $+33.83 \pm 0.42$ mV (PS-NH_2), and -41.27 ± 0.58 mV (PS-COOH) in PBS (Fig. S1e). Although nanoparticles are prone to aggregation in aqueous environments, the commercial PSNPs used in this study contained trace amounts of sodium dodecyl sulfate (SDS), which effectively prevented particle aggregation during the conjugation assays.

Text S2. Bacterial growth inhibition

To evaluate the inhibitory effects of PSNPs with different surface functional groups on bacterial growth, diluted starting cultures were added into fresh medium and incubated with PSNPs at final concentrations of 0.1, 0.8, 1.5, 3.1, 6.3, 12.5, 25, 50, 100, and 200 mg/L. Control experiments were performed under identical conditions without PSNPs treatment. And the optical density at 600 nm (OD_{600}) values were recorded hourly to monitor growth kinetics, and relative bacterial viability was calculated after correction

for optical interference using nanoparticle-only blanks. The viability of the donor and recipient strains at the final time point (14 h) was recorded in Fig. S2a - c following exposure to the various PSNPs concentrations. In addition, the time-dependent growth measurements for bacteria exposed to 100 mg/L PSNPs were presented in Fig. S2d - f.

Text S3. Influence of ROS scavenger on bacteria growth

The donor bacteria *E. coli* DH5α containing RP4 plasmid was cultured in Luria-Bertani (LB) medium supplemented with 100 mg/L ampicillin (Amp), 50mg/L kanamycin (km) and 10mg/L tetracycline (TC). The recipient bacteria *E. coli* Rosetta (DE3) was cultured in LB medium supplemented with 25 mg/L chloramphenicol (Chl). After 16 h of overnight incubation, bacteria were collected and resuspended in PBS to reach an optical density (OD) measured with the absorption wavelength of 600 nm (OD600) of 0.5. The reactive ROS scavenger N-acetyl-L-cysteine was supplemented into the LB medium to reach a final concentration of 10mg/L. LB medium without NAC was used as a control. The medium was cultured in 96-well plates at 37 °C for 6 h. Bacterial survivals were monitored by measuring the optical density at 600 nm (OD600) using a multimode microplate reader (Thermo Scentific, USA), and relative bacterial viability was calculated accordingly. The result was shown in the Fig. S3.

Text S4. Transmission Electron Microscopy Sample Preparation and Imaging

Biological samples (approximately 10^7 cells, equivalent to a pellet between the size of a rice grain and a soybean) were harvested and immediately fixed in 2.5%

glutaraldehyde in a 1.5mL (2 mL) centrifuge tube at 4 °C for 12–24 hours. After primary fixation, the fixative was discarded, and the samples were rinsed three times with PBS (pH 7.4) for 15 minutes each. Post-fixation was performed with 1% osmium tetroxide (OsO_4) for 1–2 hours, followed by three additional rinses in 0.1 M PBS (pH 7.4) for 15 minutes each. The samples were then soaked in 2% aqueous uranyl acetate for 30 minutes. Dehydration was carried out at room temperature through a graded ethanol series (50%, 70%, 80%, 90% for 15 minutes each, and 100% for 20 minutes), followed by two washes with 100% acetone for 20 minutes. For resin embedding, samples were infiltrated and embedded in pure EMBed 812 epoxy resin, placed in embedding molds, and polymerized in a 60 °C oven for over 48 hours. Ultrathin sections (70–90 nm) were cut using an ultramicrotome and mounted onto copper grids. The sections were stained with 4% uranium acetate for 30 minutes, wash the slice using water, followed by lead citrate staining for minutes, then wash the slice using water. After drying, the sample is ready for examination. The samples are examined for CCD imaging in transmission electron microscopy (TEM) in the voltage 80 kV.

Text S5. Extraction and concentration determination of extracellular DNA

To quantify extracellular plasmids from antibiotic-resistant bacteria, extracellular plasmids were initially extracted and concentrated. Samples (1.5 mL) from various exposure groups of antibiotic-resistant bacteria were collected, followed by centrifugation at 8,000 rpm for 2 minutes, repeated twice. The supernatant was then collected, and the beads were washed twice using a sterile filter membrane with a pore

size of 0.22 μm (Sangon, China). Subsequently, plasmids were concentrated following the protocol provided in the High Pure Plasmid DNA Mini Extraction Kit (Tsingke Biotechnology, China). The quality of extracted DNA was determined by a micro-volume UV-Vis spectrophotomete (NanoDrop2000, Thermo Scentific, USA). All the experiments were conducted in triplicate.

Text S6 Influence of SDS on gene transfer efficiency and bacteria viability

Finally, SDS (sodium dodecyl sulfate) at concentrations of 4 and 40 μg/L was selected to evaluate its potential effects on the bacterial conjugation system as well as on the viability of donor and recipient strains. These two concentrations were chosen based on calculations derived from the residual SDS content in the PSNPs stock suspension (<0.05%) and the corresponding dilution factors, representing the estimated maximum and secondary SDS levels that could enter the experimental system following particle pretreatment under PSNPs exposure conditions of 100 mg/L and 10 mg/L, respectively. E. coli DH5α containing RP4 plasmid (donor bacteria) was cultured in Luria-Bertani (LB) medium (10 g/L NaCl, 5 g/L yeast extract and 10 g/L tryptone) supplemented with 100 mg/L ampicillin (Amp), 50mg/L kanamycin (Km) and 10mg/L tetracycline (TC). E. coli Rosetta (DE3) (recipient bacteria) was cultured in LB medium supplemented with 25 mg/L chloramphenicol (Chl). After 16 h of overnight incubation, bacteria were collected and resuspended in PBS to reach an optical density (OD) measured with the absorption wavelength of 600 nm (OD600) of 0.5. The donor and recipient bacteria were mixed at the ratio (v/v) of 3:1, followed by addition of SDS with different final

concentrations (4 and 40 μg / L), respectively. The samples were incubated at 37 °C for 6 h according to previously established conjugation protocols, which have been shown to provide reliable cell-to-cell contact and stable conjugative transfer in E. coli systems. Control experiments were carried out under the same conditions except for SDS treatment. After that, 100 μL of the mixture was serially diluted, coated onto LB plates containing the selective antibiotics, and incubated. The LB plates containing Amp (100 mg / L), Km (50 mg/L), TC (10 mg/ L), and Chl (25 mg /L) were used to select the transconjugants. The recipient cells after exposure to the various treatments were selected using the LB plates that contained 25 mg/L Chl. After 16 - 18 h of incubation at 37 °C, the transconjugants and recipient bacteria were counted (Fig. 1a), and then the conjugative transfer frequency was calculated as follows:

$$\text{Gene transfer efficiency} = \frac{\text{Transconjugants (recipient bacteria with RP4 plasmid)}}{\text{Recipient bacteria}} \quad (1)$$

All experiments were repeated for three times.

To evaluate the inhibitory effects of SDS during the 6h conjugation period, donor and recipient strains were incubated with SDS at final concentrations of 4 and 40 μg / L. Overnight-grown cultures of the donor and recipient strains were exposed to different treatments for 6 h. Bacteria without any treatment (incubation in the LB medium only) were used as a control. After 6h of exposure, bacterial viability was assessed by measuring the optical density at 600 nm (OD600), and relative bacterial viability was calculated. All experiments were repeated for three times.

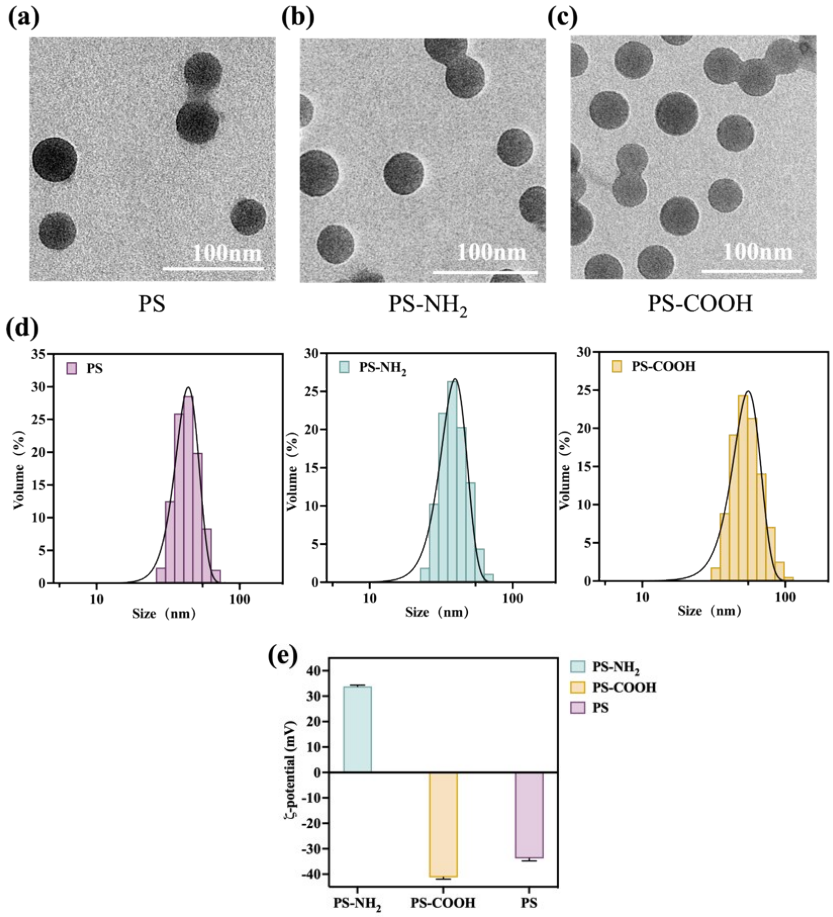

Fig. S1. Physicochemical characterizations of polystyrene nanoparticles (PSNPs). Representative TEM images of PS (a), PS-NH_2 (b), and PS-COOH (c). (d) Size distributions of PS, PS-NH_2, and PS-COOH measured by dynamic light scattering (DLS). (e). Surface charge of different PSNPs dispersed in aqueous solution.

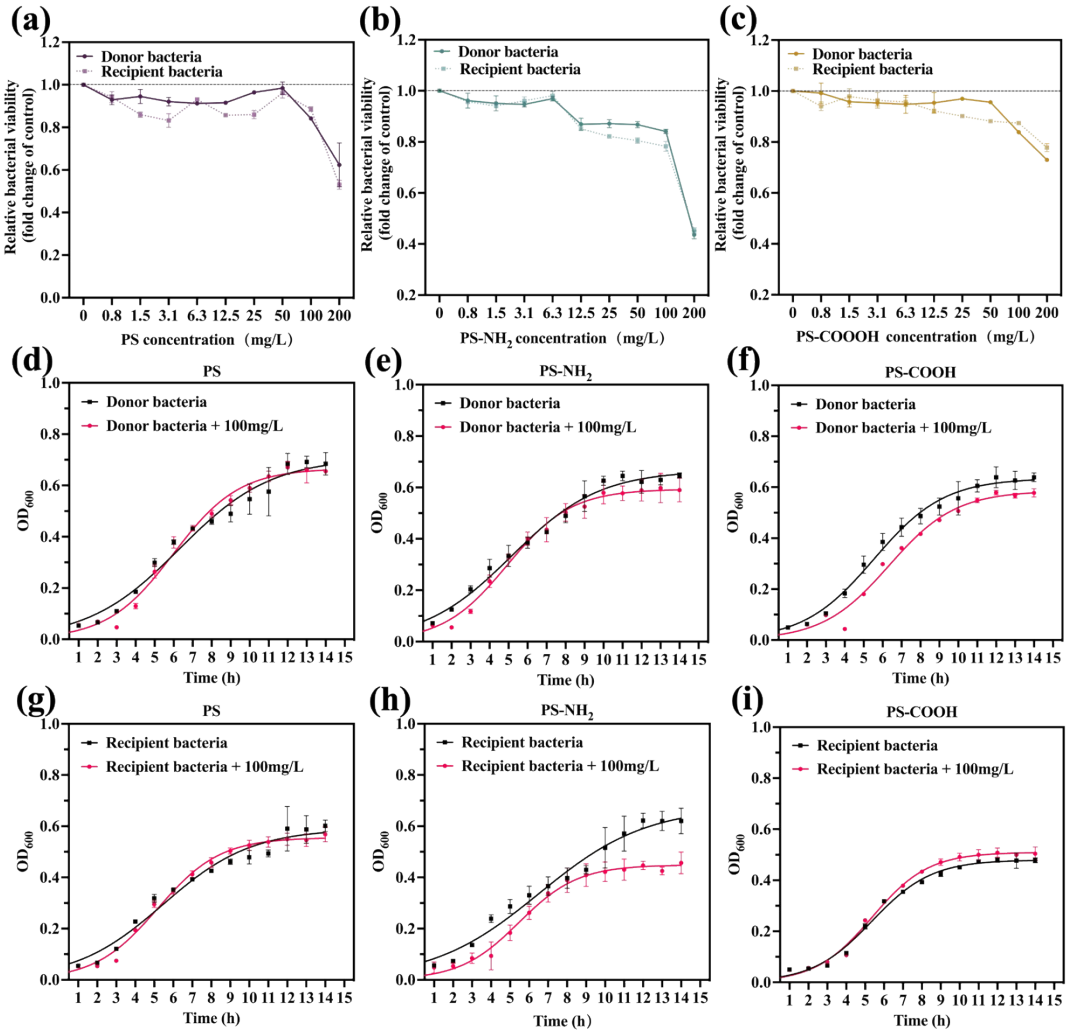

Fig. S2. The viability of the donor and recipient strains exposed to a wide range of concentrations of PS (a), PS-NH_2 (b), and PS-COOH (c). (d - i) Growth curves of untreated donor and recipient bacteria (control) versus donor and recipient bacteria exposed to 100mg/L PS (d, g), PS-NH_2 (e, h), PS-COOH (f, i) at 14h.

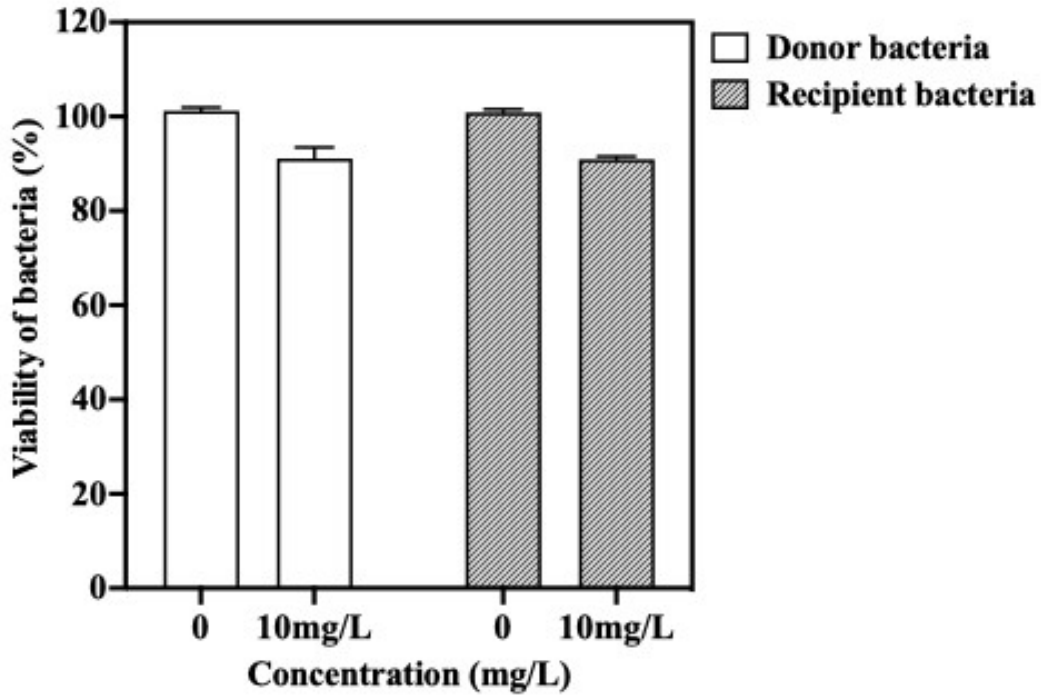

Fig. S3. Viability of donor bacteria and recipient bacteria after incubating with 10mg/L ROS scavenger (N-acetyl-L-cysteine).

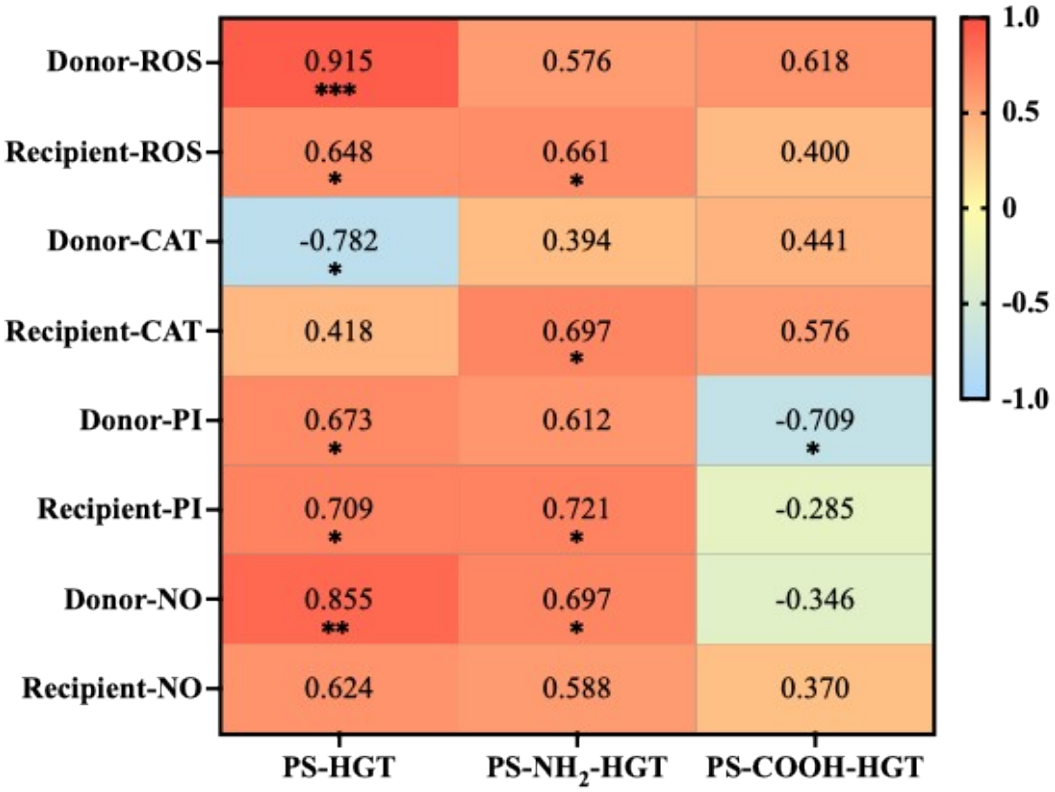

Fig. S4. Spearman correlation coefficient analysis for the relationships among the conjugative transfer frequency (HGT), ROS, NO, CAT and membrane permeability (PI) level in donor bacteria and recipient bacteria. Statistical significant differences between and within groups were tested with two-way analysis of variance (ANOVA), followed by Dunnett's test, (* represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$).

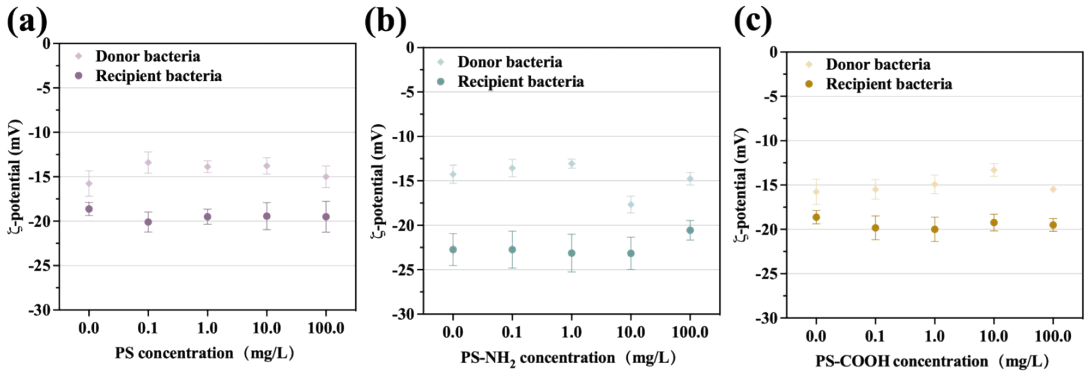

Fig. S5. The zeta potential of donor bacteria and recipient bacteria incubated with different concentrations of PS (a), PS-NH_2 (b), and PS-COOH (c).

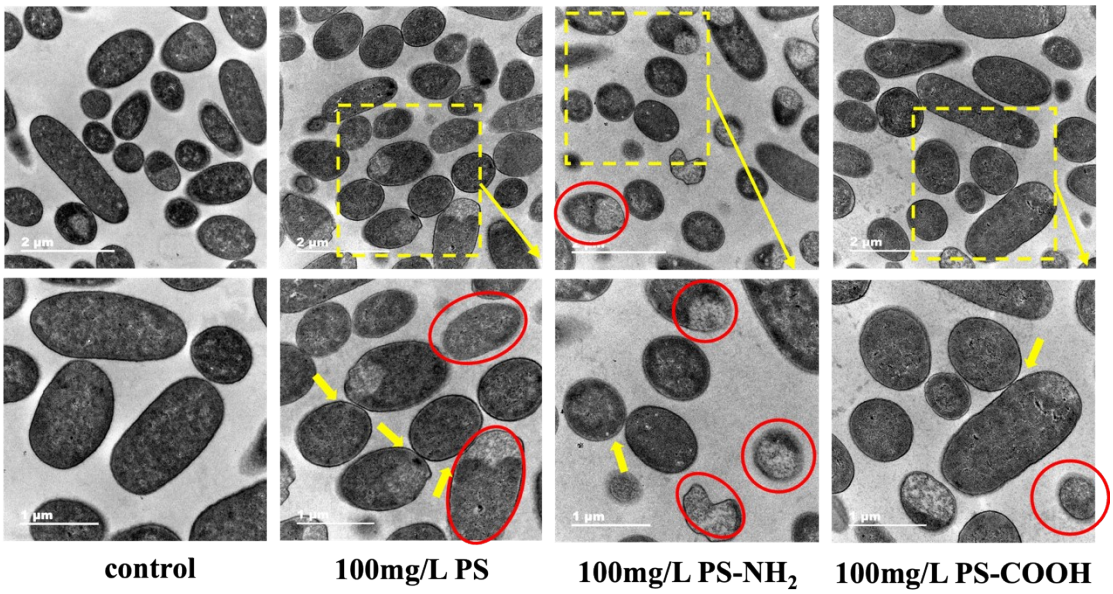

Fig. S6. Representative TEM image of the conjugation transfer microcosm containing both donor and recipient bacteria exposed to 100 mg/L of PSNPs. Yellow arrows indicate typical cell-cell contacts. Red circles indicate cells appearing blurred membrane boundaries or membrane damage.

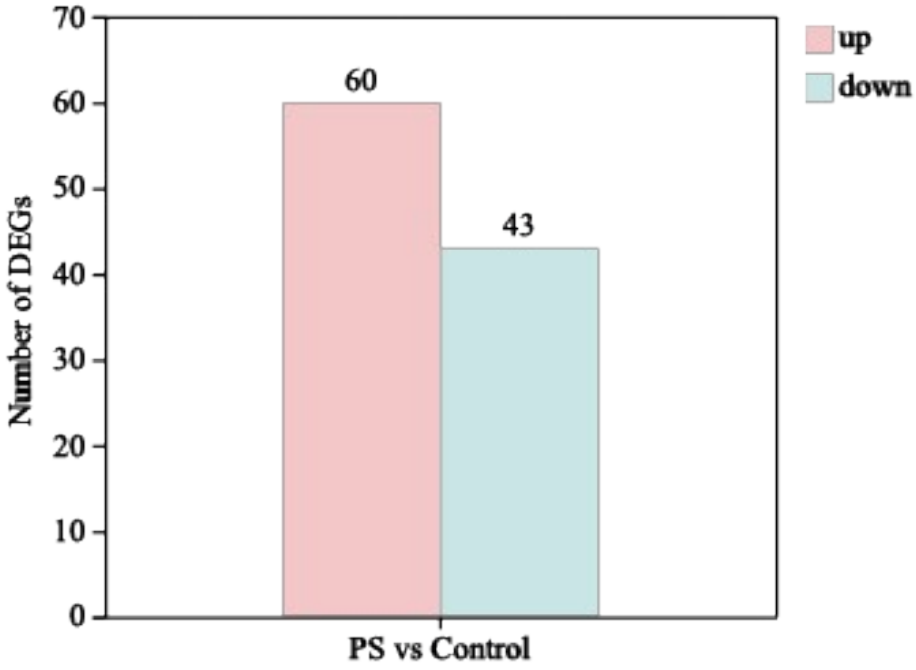

Fig. S7. Up-regulated and down-regulated genes of the DEGs.

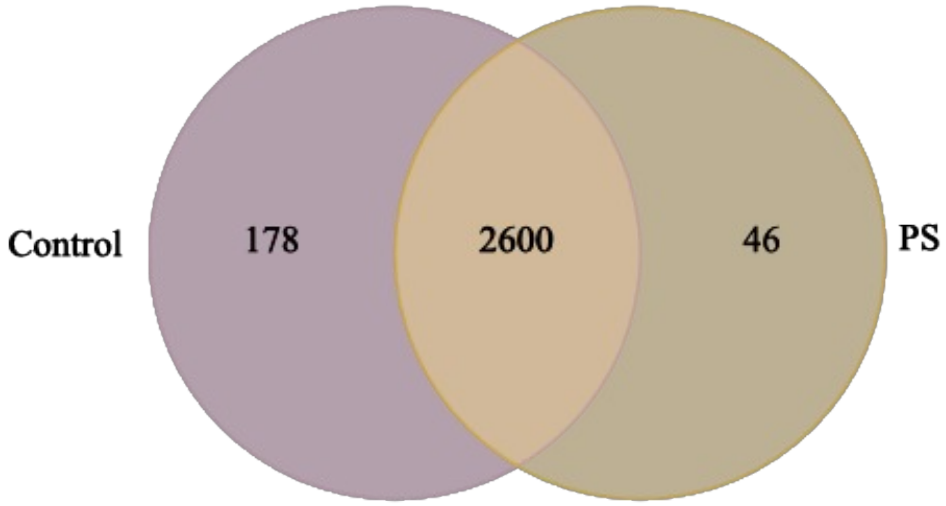

Fig. S8. Venn diagram of the genes that were unique to the control and PS group.

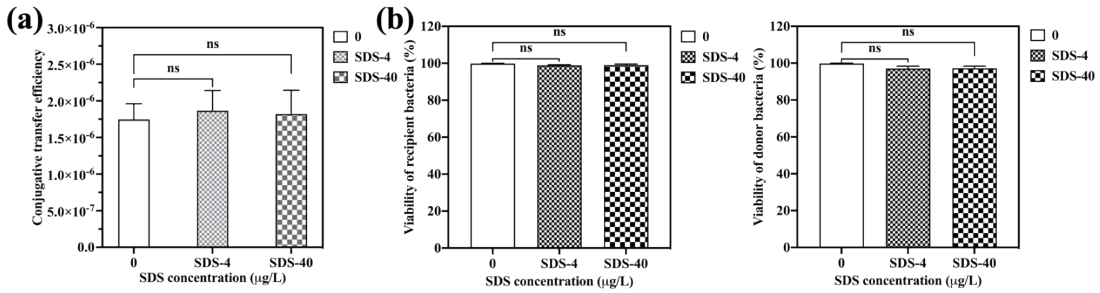

Fig. S9. Influence of SDS on gene transfer efficiency (a) and bacteria viability (b). No significance was found between the control group (pure PBS) and the SDS treated group. (“ns” means not significant).

Table S1. Primer sequences used in this study.

Gene	primer	Sequence (5'-3')	Length of product (bp)
16S rRNA	*16S rRNA-F*	CCTACGGGAGGCAGCAG	194
	16S rRNA-R	ATTACCGCGGCTGCTGG	
trbBp	*trbBp-F*	CGCGGTCGCCATCTTCACG	131
	trbBp-R	TGCCCGAGCCAGTACCGCCAATG	
traF	*traF-F*	GGCAACCTCGTCGCCTTTA	118
	traF-R	GCAAGTCGGCGTGTTTTCG	
rpoS	*rpoS-F*	AATCCACCAGGTTGCGTATGTTGAG	111
	rpoS-R	CTGGCGTTGCTGGACCTTATCG	
lexA	*lexA-F*	GAAGAAGGGTTGCCGCTGGTAG	125
	lexA-R	CGCAGCAGGAAATCAGCATTCG	
recA	*recA-F*	CCACGGAGTCAACGACGATAACG	76
	recA-R	GGCGAGCAGGCACTGGAAATC	
ompA	*ompA-F*	AGTGTAGATGTCCAGGTCGTCAGTG	116
	ompA-R	GGTTAGGTCGTATGCCGTACAAAGG	
ompC	*ompC -F*	AAGCAGCGGTGTTCTGAGCATC	96
	ompC-R	GCGTCGGCGGTTCTATCACTTATG	
trfAp	*trfAp-F*	GAAGCCCATCGCCGTCGCCTGTAG	183
	trfAp-R	GCCGACGATGACGAACTGGTGTGG	

Table S2. Table of detailed gene expression differences.

Gene Type	Gene_id	Gene name	FC	Log2FC	Pvalue	Gene Type	Gene_id	Gene name	FC	Log2FC	Pvalue
Membrance Related	**b3508**	*yhiD*	2.209	1.14371	0.00425	Oxidative stress Related	**b3867**	*hemN*	1.166	0.22199	0.16722
	b4149	*blc*	1.105	0.14457	0.40894		**b3781**	*trxA*	1.126	0.17061	0.41059
	b4376	*osmY*	1.552	0.63445	0.00480		**b0812**	*dps*	1.447	0.53345	0.01703
	b1739	*osmE*	1.523	0.60712	0.02517		**b4063**	*soxR*	1.157	0.21095	0.32711
	b1615	*uidC*	1.454	0.53997	0.46110		**b4062**	*soxS*	2.717	1.44221	9.97E-05
	b3584	*yiaT*	1.55	0.63246	0.36516		**b1646**	*sodC*	1.216	0.28181	0.0865
	b2617	*bamE*	1.177	0.23552	0.28533		**b1656**	*sodB*	1.236	0.30523	0.27073
	b3035	*tolC*	1.267	0.34162	0.05972		**b1732**	*katE*	1.114	0.15532	0.41821
	b0241	*phoE*	1.871	0.90365	0.15674		**b0606**	*ahpF*	1.238	0.30764	0.08812
	b1319	*ompG*	1.837	0.87728	0.19305		**b0605**	*ahpC*	1.112	0.15307	0.44257
	b3875	*ompL*	1.733	0.79308	0.14728	Flagellar Related	**b1075**	*flgD*	1.577	0.65746	0.23662
	b0957	*ompA*	0.922	-0.11717	0.47501		**b1074**	*flgC*	2.421	1.27545	0.32486
	b2215	*ompC*	1.145	0.19483	0.29624		**b1945**	*fliM*	1.193	0.25414	0.59796
SOS Related	**b1848**	*yebG*	1.302	0.38028	0.15804		**b1880**	*flhB*	1.779	0.83093	0.25019
	b0607	*uspG*	1.348	0.43095	0.02147		**b1892**	*flhD*	1.344	0.42626	0.26338
	b1376	*uspF*	1.295	0.37319	0.06283		**b4319**	*fimG*	10.86	3.44097	0.0104

	b1333	*uspE*	1.071	0.09833	0.56712	**Efflux pump Related**	**b0464**	*acrR*	3.963	1.98667	0.00026
	b3923	*uspD*	1.485	0.5706	0.04246		**b0463**	*acrA*	1.516	0.60056	0.00256
	b0958	*sulA*	1.34	0.42179	0.03879		**b4082**	*mdtN*	3.379	1.75678	0.01315
	b2616	*recN*	1.253	0.32532	0.05925		**b1600**	*mdtJ*	1.138	0.18657	0.81347
	b3822	*recQ*	0.921	-0.1186	0.50514		**b3513**	*mdtE*	1.683	0.75145	0.00338
	b2699	*recA*	1.233	0.30192	0.07455						
	b4043	*lexA*	1.262	0.33538	0.05901						
NO Related	**b0463**	*acrA*	1.516	0.60056	0.00256						
	b4070	*nrfA*	1.457	0.54253	0.16300						
	b4063	*soxR*	1.157	0.21095	0.32711						
	b4178	*nsrR*	1.195	0.25695	0.33187						
	b1656	*sodB*	1.236	0.30523	0.27073						
	b3366	*nirD*	5.966	2.57671	0.05099						
	b3367	*nirC*	26.583	4.73241	0.01120						
	b2552	*hmp*	2.267	1.18077	0.01262						
	b0015	*dnaJ*	0.842	-0.24756	0.16868						
	b0014	*dnaK*	0.966	-0.24756	0.78208						
	b0873	*hcp*	1.295	0.37345	0.47136						