# Supplementary Information

## Bismuth drug as antibiotic adjuvant to inhibit biofilm formation via a dual mechanism

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### Supplementary methods

#### Construction of overexpression strain

The *cyoC* and *cydA* overexpression plasmid was constructed based on the vector pMLS7, which was designed by Lefebre et al. and purchased from addgene (#32056).[1] The DNA fragment of *cyoC* and *cydA* used the genome of *B. cepacia* 6349 as template and amplified by PCR. The plasmid was first digested by two restriction enzymes, Xbal (Thermo ScientificTM) and HindIII (Thermo Scientific™) at 37°C for 1 h and purified by MiniBEST DNA Fragment Purification Kit Ver.4.0 (Takara, 9761).

#### Biofilm inhibition and killing assay

The detailed protocols of these two assays were reported previously.[2] In brief, the overnight *B. cepacia* bacterial suspension was inoculated into 96-well MBEC biofilm inoculator (19113, Innovotech Inc.) for 8 h to allow bacteria to attach to the surface of the inoculator. The supernatant was replaced with fresh brain heart infusion (BHI) medium with or without drugs for another 36 h incubation at 37°C. As for the biofilm killing assay, bacterial suspension was inoculated into a 96-well MBEC biofilm inoculator (19113, Innovotech Inc.) for 8 h first and replaced with fresh medium for another 16 h. The inoculator was gently washed by PBS for three times and then fresh BHI was added at indicated concentration of combination drugs for 24 h incubation. Next, biofilms were washed by PBS for three time, followed by dispersed by sonication for 45 min. The bacterial content was enumerated by agar plating. All these tests were performed in triplicate.

**Transcriptomic analysis**

*B. cepacia* 6349 was grown to the mid-log phase ($OD_{600}$ = 0.5) and treated with CBS (50 μg/mL) for 1, 2, and 4 h, respectively. The cells were then washed three times with 1× PBS to remove residual CBS. Bacterial pellets were resuspended in 10 mg/mL lysozyme, incubated at 37°C for 1 h, and harvested by centrifugation at 12,000 rpm for 15 min. RNA extraction for *B. cepacia* was performed following a modified protocol from Li et al.[2] Ribosomal RNA (rRNA) was depleted from the extracted total RNA, which was then reverse-transcribed into cDNA. Prior to PCR amplification, the cDNA underwent end repair, dA-tailing, and purification. Subsequently, all samples were amplified, cleaned up, and quantified. Library preparation was performed on the Illumina HiSeq/NovaSeq platform, and image analysis was conducted using NovaSeq Control Software + OLB + GAPipeline-1.6 (Illumina). The reference genome GCF_006094315.1 (NCBI) was used in this study; reads were mapped and aligned to the reference genome for annotation using Bowtie2 (v2.2.6). After file conversion, gene expression levels were estimated using HTSeq (v0.6.1p1). Differential expression analysis was carried out using the DESeq2 Bioconductor package, with the Benjamini-Hochberg method employed to control the false discovery rate (FDR)[3]. Adjusted p-values (Padj) were used to verify statistical significance, with a cutoff of <0.05. Differentially expressed genes (DEGs) were further subjected to GO and KEGG enrichment analyses, novel transcript prediction, SNV analysis, gene structure analysis, SD sequence prediction, Rho-independent terminator prediction, and sRNA analysis. These analyses were performed using various software tools, including GOSeq (v1.34.1), Samtools (v0.1.19, mpileup command), Rockhopper (v2.0.3), RBSfinder (v1.0), TransTermHP (v2.09), and RNAfold (v2.3.2).

**Confocal laser scanning microscopy image**

The protocol was modified from the method reported by Wang et al.[3] In brief, the overnight bacterial culture was inoculated in a cell culture imaging dish (Thermofisher, 150680) for 8 h for bacteria attachment, followed by replacing the used BHI medium with the fresh medium with or without drugs for another 36 h incubation. The mature biofilm was stained by 2 μM DMAO (Beyotime, C2030S) at 37°C for 15 min. 1X PBS was used to wash out the extra dye. Then fresh BHI medium was added into each well for directly imaging by confocal laser scanning microscopy (Leica TCS SP8 CLSM). Fluorescein was excited at 488 nm.

**The cAMP and c-di-GMP ELISA assays**

The protocols for detecting cAMP and c-di-GMP levels were similar. The c-di-GMP level was detected using a c-di-GMP ELISA kit (GenScript, L00461). The total protein concentration of the prepared bacterial lysate was first determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) and adjusted to a uniform concentration with ddH2O. The cAMP level in the bacterial lysate was then measured using a cAMP ELISA kit (GenScript, L00460). A standard curve was generated using cAMP standards ranging from 0 to 243 pmol/mL (0, 1, 3, 9, 27, 81, and 243 pmol/mL). For the assay, 100 μL of anti-cAMP mAb was added to each well and incubated at 25°C for 1 h, followed by washing five times with 1× washing solution (diluted from the 20× washing buffer provided in the kit). The residual liquid in the ELISA plate was removed by blotting on filter paper several times until no liquid remained. Next, 100 μL of buffer A, along with different concentrations of cAMP standards or bacterial samples, was added to the non-specific binding (NSB) wells and the remaining wells, respectively. This was followed by the addition of 50 μL of buffer B and cAMP-HRP conjugate, and the plate was incubated at 4°C for 2 h. After incubation, the liquid was discarded, and the plate was washed four times with 1× washing solution. The wells were ensured to be empty before adding 100 μL of TMB substrate to each well for a 20 min incubation at room temperature (RT). Finally, 50 μL of stop

solution was added to each well, and the absorbance was measured at 450 nm using a microplate reader (SpectraMax M3, Molecular Devices).

**Measurement of intracellular ATP level**

The *crp*-overexpressing strain was grown to mid-log phase ($OD_{600}$ = 0.5) and treated with different concentrations of CBS (0, 12.5, 25, and 50 μg/mL) for 4 h, followed by washing three times with 1× PBS to remove residual CBS. Lysozyme (10 mg/mL) was added, and the samples were incubated at 37°C for 1 h. The total protein concentration of the samples was determined by BCA assay and adjusted to the same level by diluting with ATP lysis buffer. The intracellular ATP level was then measured using an ATP assay kit (Beyotime, SS0026). The ATP working reagent was prepared by diluting it with ATP working buffer at a ratio of 1:9, and an ATP standard (0.5 mM) was used as a positive control. Subsequently, 100 μL of ATP working solution was mixed with 20 μL of bacterial sample in each well of a 96-well plate. Luminescence was measured within 5 min using a microplate reader. The entire procedure was performed on ice.

**NO detection**

*B. cepaica* 6349 and two variant strains (with 100 μg/mL TMP) were grown to mid-log phase ($OD_{600}$= 0.5) and treated with different concentrations of CBS (0, 20, 40 μg/mL) for 4 h. Next, bacterial suspension was collected and centrifuged by centrifugation (Eppendorf) at 12,000 rpm for 5 min, followed by washing with 1X PBS (Sigma-Aldrich) three times. Lysozyme was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the working concentration for *B. cepacia* is 10 mg/mL. The washed bacterial cells were resuspended in lysozyme solution at 37°C for 1h for lysis, followed by centrifugation for 5 min. The supernatant was used for NO detection and the debris was discarded. The RNS (referring to NO) was detected by cellular ROS/RNS assay kit (ab139473). The NO detection reagent was first added into the bacterial samples at the ratio of 2.5:1000 and incubated at room temperature for 2 h with gentle shaking. The NO inducer was added into the sample at the last 30 min of incubation and 1x washing buffer was used to remove extra NO detecting probe by washing three times. Every 1 mL sample suspension was washed by 5 mL washing buffer. After the last washing, the bacteria were resuspended by 1x washing buffer and appropriate volume of the suspension was used to detect the fluorescence ($\lambda_{ex}/\lambda_{em}$ of 650/670 nm) by microplate reader.

**Time-kill kinetics assay**

*B. cepacia* 6349 was grown to mid-log phase ($OD_{600}$= 0.5) and treated with different concentration of CBS, tetracycline, tobramycin and the combinations of CBS and antibiotics for different time points (0, 2, 4, 6 and 24 h). In each time point, 10 μL bacterial suspension of each group was taken into the 96-well plate and serially diluted by 1x PBS. Bacterial load was counted by plate enumeration on BHI agar plate.

**The construction of *B. cepacia* 6349 *pcrp*-pEX18TC**

The plasmid vector pEX18TC was kept in our lab and was first transformed into *E. coli* S17 as donor strain. Next, *B. cepacia* 6349 *pcrp (crp*-overexpressed strain) and *E. coli* S17 were cultured together by conjugation and *B. cepacia* 6349 *pcrp*-pEX18TC were screened on agar plates containing multiple antibiotics (100 μg/mL trimethoprim and 50 μg/mL kanamycin).

**β-galactosidase activity detection**

The β-galactosidase activity was assessed with β-galactosidase assay kit (Beyotime, RG0036). The sample preparation was the same as other assays. In brief, *B. cepacia* 6349 *pcrp*-pEX18TC was treated with 2% L-arabinose (m/v), 1 mM cAMP, 50 μg/mL CBS or 1 mM cAMP+50 μg/mL CBS for 4 h, followed by washing with PBS and collection via centrifugation. The whole protein concentration of each sample was adjusted by PBS to the same level as determined by BCA assay. Each working well consisted of 25 μL of bacterial sample, 25 μL of lysis buffer and 50 μL of working reagent, incubated at 37℃ for 30 min. After 30 min incubation, 150 μL of stop solution was added into each well and the plate was read at $OD_{420}$.

**Supplementary figures and tables**

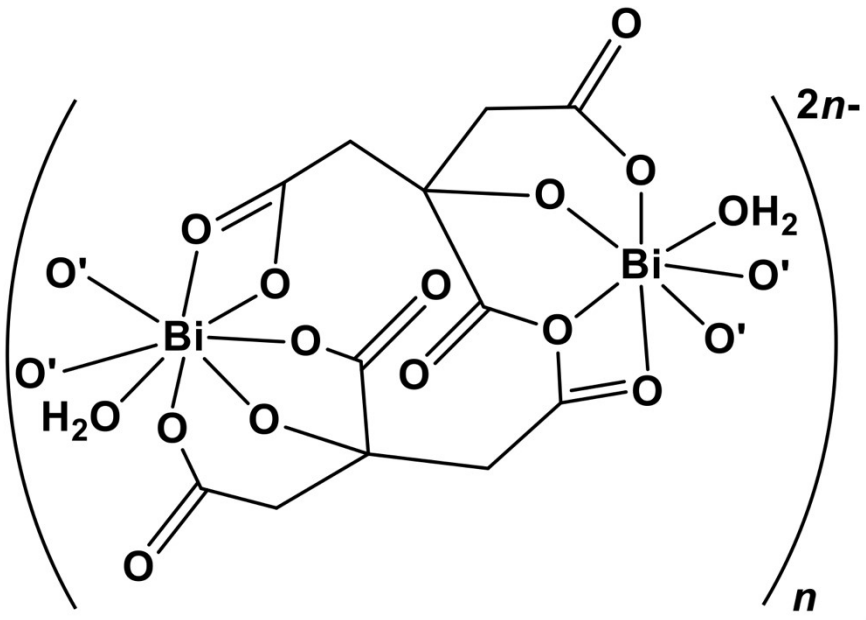


**Figure S1.** The chemical structure of colloidal bismuth subcitrate (CBS). Note that the basic dimeric unit $[Bi(citrate)_2Bi]^{2-}$ further assembles to a polymeric structure. O' represents an oxygen from another citrate.

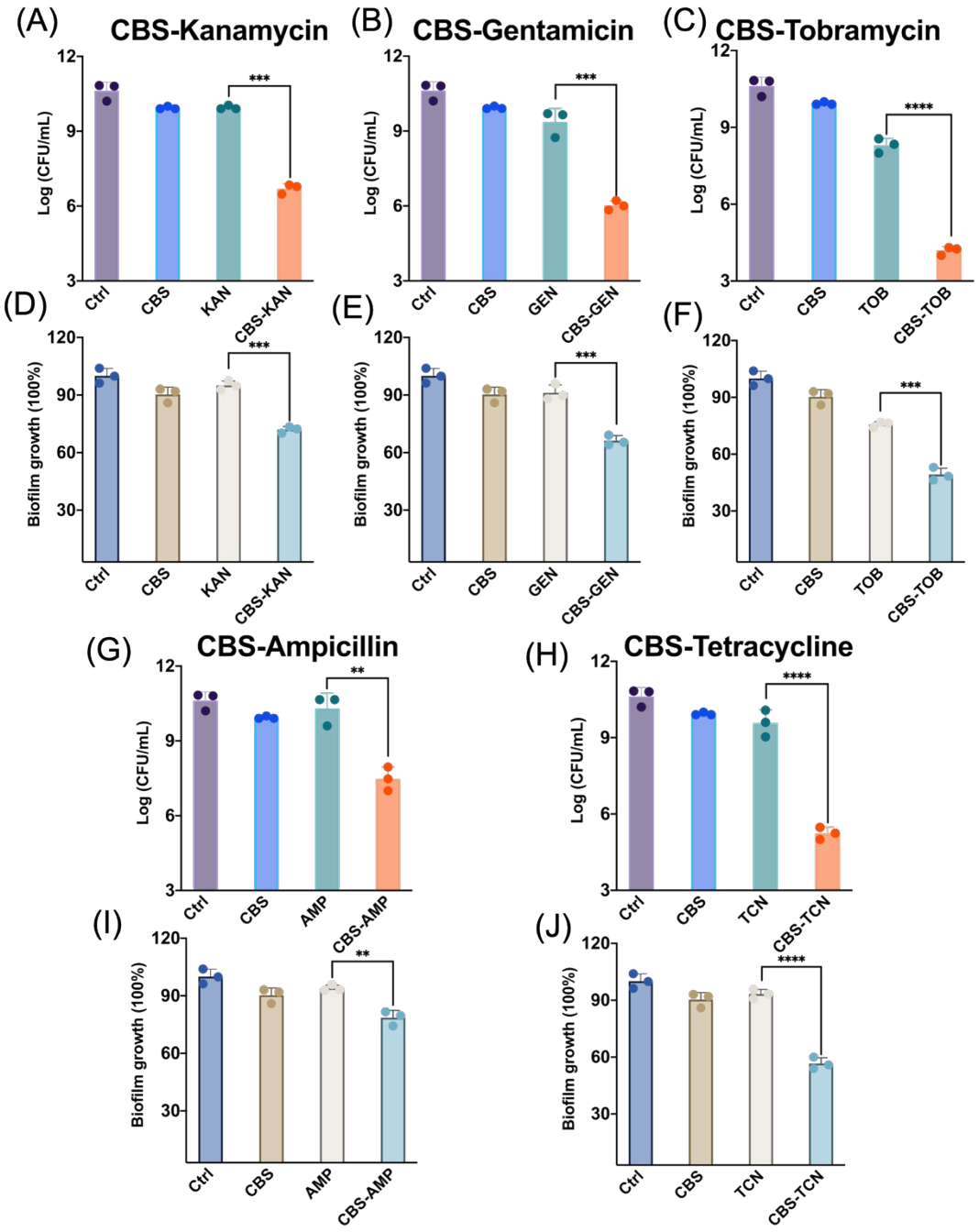


**Figure S2.** Anti-biofilm activities of bismuth-antibiotic combinations. Biofilm inhibitory assays for the combinations of CBS with kanamycin (A), gentamycin (B), tobramycin (C), ampicillin (G) and tetracycline (H) by sonication. Biofilm inhibitory assays for the combinations of CBS with kanamycin (D), gentamycin (E), tobramycin (F), ampicillin (I) and tetracycline (J) by staining with crystal violet.

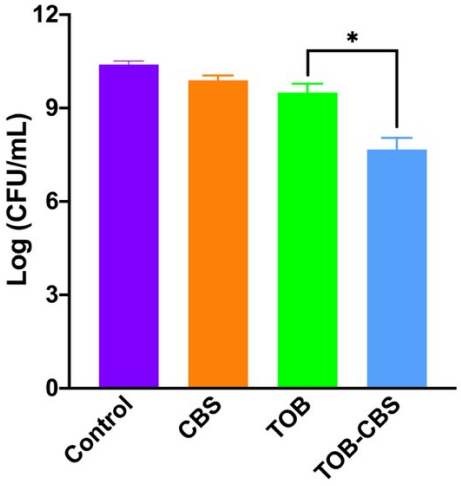


**Figure S3.** CBS-TOB combination significantly reduces biofilm-associated bacterial load in *B. cepacia*. The established *B. cepacia* biofilm were treated with CBS, tobramycin (TOB) or their combination (CBS-TOB) for 24 h. Biofilm were then dispersed by sonification, and the remaining viable bacterial load was quantified by colony counting. The concentrations used were 12.5 μg/m L for CBS and 30 μg/mL for TOB.

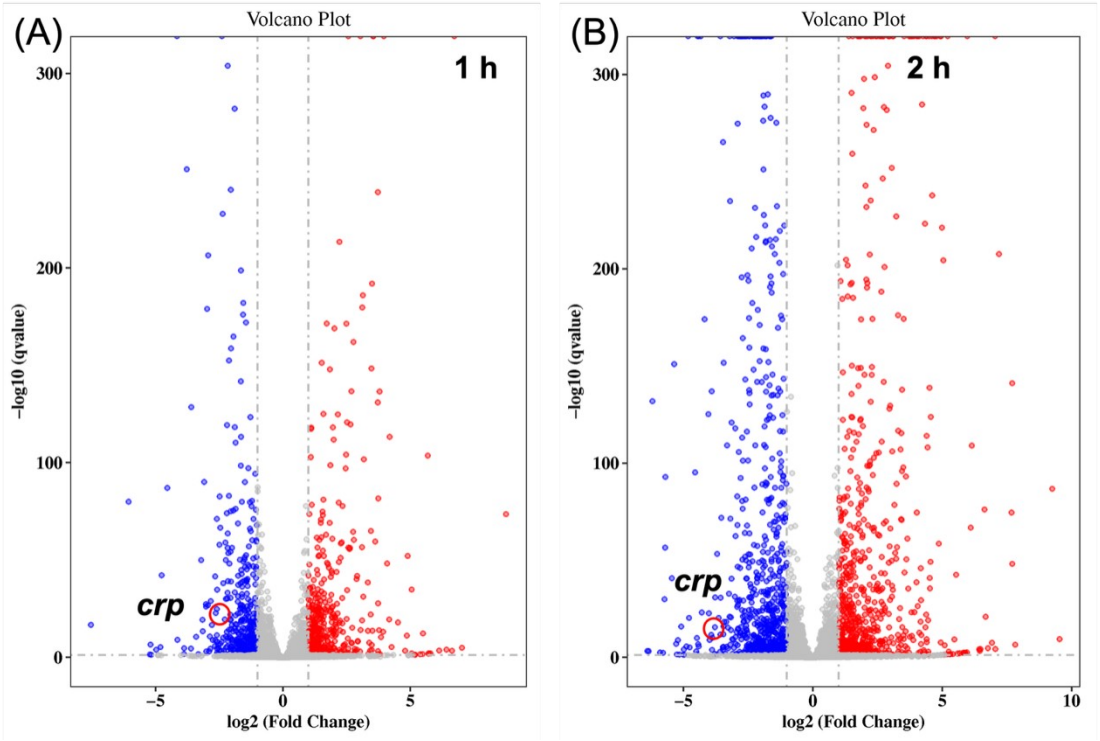


**Figure S4.** Short-term bismuth exposures induce downregulation of *crp* expression. Volcano plots illustrate differentially expressed genes (DEGs) in *B. cepacian* after treatment with 50 μg/mL CBS for 1 h (A) and 2 h (B).

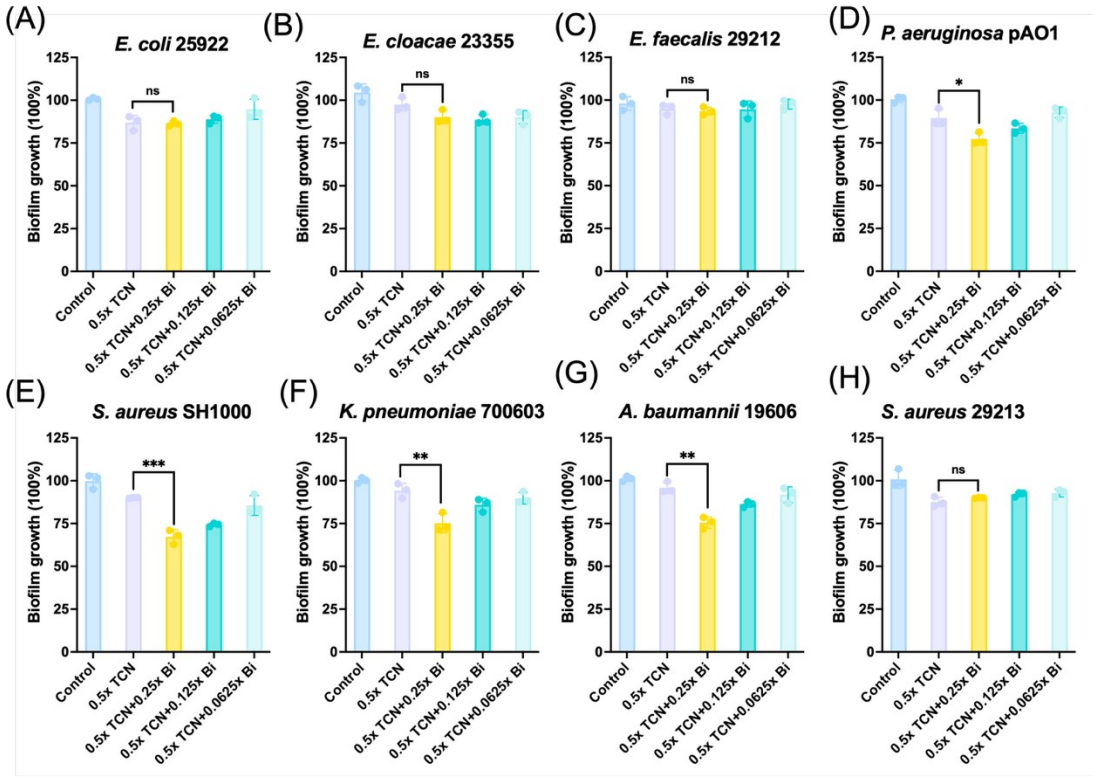


**Figure S5.** CBS-TCN combinations exhibit potent anti-biofilm activity against ESKAPE pathogens at low concentrations. Biofilm inhibitory assays were conducted with CBS combined with tetracycline (TCN) against representative ESKAPE pathogens: *E. coli* 25922 (A), *E. cloacae* 23355 (B), *E. faecalis* 29212 (C), *P. aeruginosa* pao1 (D), *S. aureus* SH1000 (E), *K. pneumoniae* 700603 (F), *A. baumannii* 19606 (G) and *S. aureus* 29213 (H). Biofilm were quantified by crystal violet staining. CBS were tested at 0.25x, 0.125x and 0.0625x of the reference concentration (corresponding to 128, 64 and 32 µg/mL, respectively), while TCN were used at 0.5x MICs for each strain.

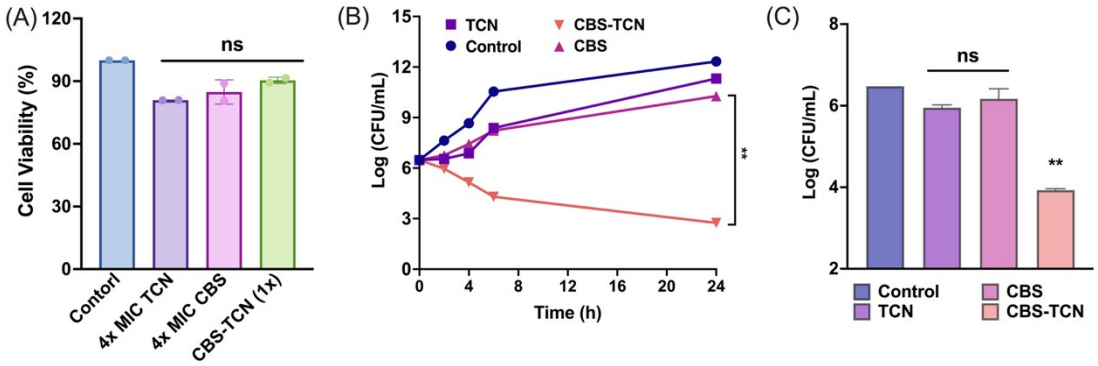


**Figure S6.** CBS-TCN combination exhibits strong antibacterial activity against *B. cepacia*. (A) Cytotoxicity assay of CBS, TCN and their combination CBS-TCN toward mammalian cells. The concentration used were 32 µg/mL (TCN) and 25 CBS µg/mL (CBS) and 128 µg/mL in the combination. (B) Time-kill curves of CBS (12.5 µg/mL) and TCN (16 µg/mL) and the CBS-TCN combination against *B. cepacia* 6349 over 24 h. (C) Bacterial load of *B. cepacia* 6349 attached by human A549 epithelial cells in the presence of CBS (12.5 µg/mL) and TCN (16 µg/mL), or their combination.

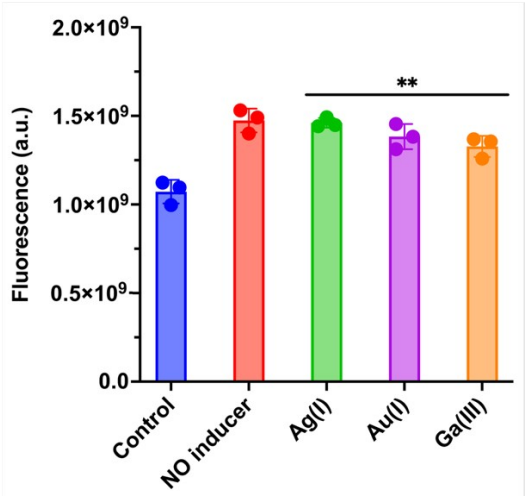


**Figure S7.** Various metal complexes induce nitric acid (NO) accumulation in *B. cepacia*. $AgNO_3$, $Ga(NO_3)$ and Au-6TG were used in the experiments. *B. cepacia* 6349 was treated with each metal complexes at 32 μg/mL for 4 h. The MIC values of these metal complexes against *B. cepacia* are all greater than 32 μg/mL.

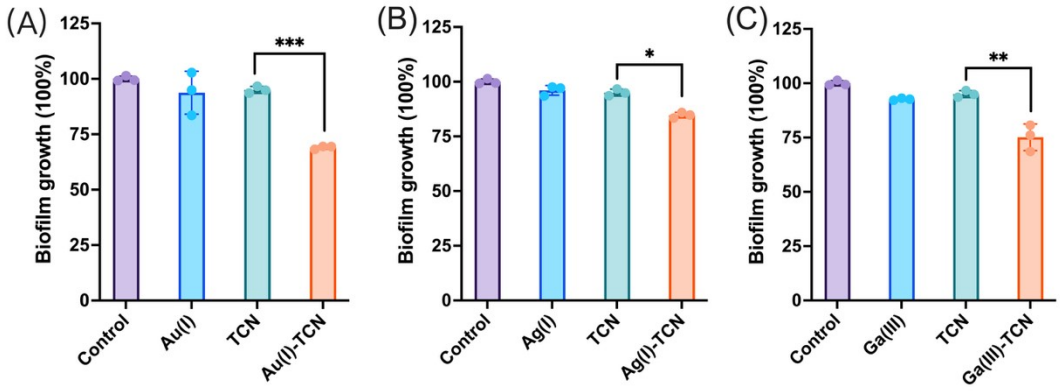


**Figure S8.** Metallodrug-TCN combinations display strong anti-biofilm efficacy against *B. cepacia*. Biofilm inhibition by combinations of TCN with Au(I) (A), Ag(I) (B), and Ga(III) (C). The concentration of the metal complexes and TCN were 32 μg/mL and 16 μg/mL respectively.

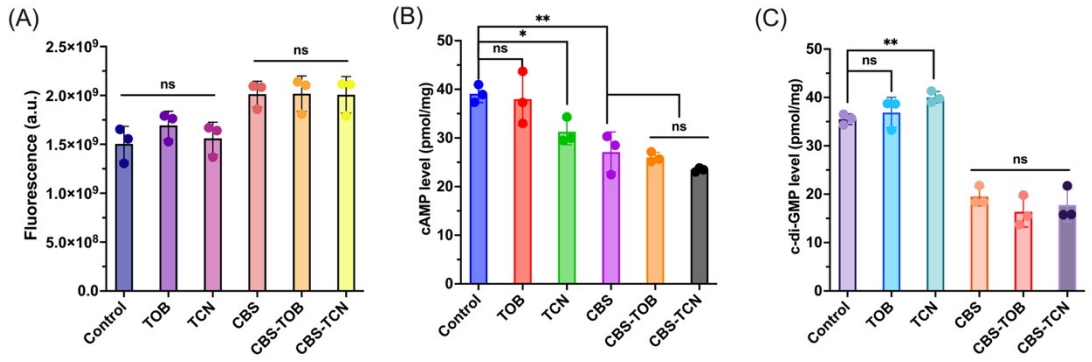


**Figure S9.** The effects of CBS, antibiotics, and their combination on intracellular NO, cAMP, and c-di-GMP levels in wild-type *B. cepacia*. (A) NO accumulation, (B) cAMP levels, and (C) c-di-GMP levels in *B. cepecia* WT strain following treatment with sub-inhibitory concentrations of tobramycin (TOB), tetracycline (TCN), CBS or their combinations. The concentrations used were 15 μg/mL (TOB), 16 μg/mL (TCN) and 12.5 μg/mL (CBS)for both single agents and combinations.

**Table. S1** The Bliss independent model scores of bismuth-antibiotic combinations and antibiotic susceptibility against *B. cepacia* 6349.

| Antibiotics | Bliss model score (in the combination with CBS) | MIC value (μg/mL) |
|---|---|---|
| Ampicillin | 0.524 ± **0.013** | >70 |
| Amoxicillin | 0.109 ± **0.044** | >93.4 |
| Kanamycin | 0.556 ± **0.053** | >124 |
| Gentamicin | 0.580 ± **0.064** | 30.5 |
| Tobramycin | 0.835 ± **0.028** | 60 |
| Tetracycline | 0.613 ± **0.034** | 32 |
| Eravacycline | 0.420 ± **0.097** | 4.5 |
| Doxycycline | 0.132 ± **0.032** | >3.5 |
| Trimethoprim | -0.557± **0.011** | 0.3 |
| Chloramphenicol | -0.221± **0.032** | 16 |
| Meropenem | -0.231± **0.043** | 3 |
| Rifabutin | 0.054 ± **0.040** | 54 |
| Rifamycin | -0.119± **0.056** | 22.3 |
| Streptomycin | 0.196 ± **0.067** | >150 |
| Clarithromycin | 0.128 ± **0.091** | >191 |
| Clindamycin | 0.255 ± **0.054** | >108 |
| Colistin | 0.112 ± **0.063** | >9 |
| Levofloxacin | 0.289 ± **0.037** | 0.7 |
| Ciprofloxacin | 0.116 ± **0.019** | 1 |

**Table. S2** MIC values of CBS and TCN against ESKAPES pathogens.

| Strains | MIC of TCN (μg/mL) | MIC of CBS (μg/mL) |
|---|---|---|
| *E. coli* 25922 | 2 | >256 |
| *E. cloacae* 23355 | 4 | >256 |
| *E. faeclis 29212* | 32 | >256 |
| *S. aureus* 29213 | 1 | >256 |
| *P. aeruginosa* pao1 | 64 | >256 |
| *A. baumannii* 19606 | 32 | >256 |
| *K. pneumoniae* 700603 | 64 | >256 |
| *S. aureus* SH1000 | 0.25 | >256 |

**Table. S3** Bacterial strains and plasmids used and constructed in this study.

| Strain | Application |
|---|---|
| *B. cepacia* 6349 | Susceptibility test |
| *E. coli* DH5α | Plasmid transformation/conjugation |
| *E. coli* S17 | |
| *B. cepacia* 6349-p*cyoC* | Target validation/overexpression strain |
| *B. cepacia* 6349-p*cydA* | |
| *B. cepacia* 6349-p*crp*-pEX18TC | |
| *E. coli* 25922 | Anti-biofilm test |
| *E. cloacae* 23355 | |
| *E. faeclis 29212* | |
| *S. aureus* 29213 | |
| *P. aeruginosa* pao1 | |
| *A. baumannii* 19606 | |
| *K. pneumoniae* 700603 | |
| *S. aureus* SH1000 | |

| **Plasmid** | **Application** |
|---|---|
| pEX18TC | Target validation |
| pMLBAD | Gene overexpression |
| pMLBAD-*cyoC* | |
| pMLBAD-*cydA* | |

**Table. S4** Primers used in this study.

| Gene | Forward | Reverse |
|---|---|---|
| *rpoS* | GAACGCACGACATACTTGCC | GCGTGAAAACCTCGCTGAAG |
| *gryB* | CCACTGAGGCTTCGACTGAG | CGTGATGGGTACGTTCGACA |
| *crp* | GCCGGTTCACTCACGATAGG | GAACTGCCGCCAGTATTGC |

**References**


1. M. D. Lefebre and M. A. Valvano, Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in Burkholderia cepacia complex isolates, *Appl Environ Microbiol*, 2002, **68**, 5956-5964.
2. J. Li, H. Wang, P. Gao, R. Wang, C. L. Chan, R. Yi-Tsun Kao, H. Li and H. Sun, Bismuth drug eradicates multi-drug resistant Burkholderia cepacia complex via aerobic respiration, *Chem Sci*, 2025, **16**, 12372–12384.
3. C. Wang, Y. Xia, R. Wang, J. Li, C. L. Chan, R. Y. Kao, P. H. Toy, P. L. Ho, H. Li and H. Sun, Metallo-sideromycin as a dual functional complex for combating antimicrobial resistance, *Nat Commun*, 2023, **14**, 5311.