Supplementary Information for

Elevated c-di-GMP levels promote biofilm formation and biodesulfurization capacity of *Rhodococcus erythropolis*

Pedro Dorado-Morales[1], Igor Martínez[2], Virginia Rivero-Buceta[3], Eduardo Díaz[3], Heike Bähre[4], Iñigo Lasa[1] and Cristina Solano[1]*

[1] Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra (UPNA)-Complejo Hospitalario de Navarra (CHN), IdiSNA, Irunlarrea 3, Pamplona 31008, Navarra, Spain

[2] Department of Systems Biology, Centro Nacional de Biotecnología, Agencia Estatal Consejo Superior de Investigaciones Científicas, Darwin 3, 28049 Madrid, Spain

[3] Department of Microbial and Plant Biotechnology, Centro de Investigaciones Biológicas Margarita Salas, Agencia Estatal Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

[4] Research Core Unit Metabolomics, Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover, Germany

This file includes:

Supplementary experimental procedures

Fitness evaluation

R. erythropolis harboring plasmid pTipQC1, pTipQC1::*adrA*, pTipQC1::*adrA* D290N or pTipQC1::*dsz*-P_{lac}D-*adrA* were grown in LB Cm for 24 hours at 30 ˚C, and then diluted 1:100 in 5 ml of fresh LB Cm and incubated for 12 hours at 30 ˚C, 200 rpm. Thiostrepton was added and cultures were incubated for 12 hours at 30 ˚C, 200 rpm. Cultures were collected, centrifuged at 20,800 x g for 5 min and pellets were washed twice and resuspended in 1 ml HEPES buffer 50 mM pH 8.0 containing PMSF 1 mM. Bacteria were lysed using 100 µm acid-washed glass beads (Sigma) and a FastPrep cell disrupter (MP Biomedicals; speed 6, 45 s, twice). Samples were centrifuged for 10 min at 20,800 x g and 4 ˚C, supernatants were collected and protein quantification was carried out using a Bradford protein assay kit (Bio-Rad).

Activity of dispersing enzymes on the biofilm formed by R. erythropolis pTipQC1::dsz-P_{lac}D-adrA

Dispersin B enzyme was purified from a recombinant *Escherichia coli* BL21 (DE3) strain carrying plasmid pRC3 as previously described (Ramasubbu *et al.*, 2005). After purification, fractions containing Dispersin B were dialyzed against ultrapure water at 4 °C using a Slide-A-Lyzer Dialysis Cassette (ThermoFisher Scientific) with a molecular mass cut-off of 10 kDa and subsequently lyophilized. Lyophilized Dispersin B was finally resuspended in Storage Buffer (50 mM sodium phosphate pH 5.8; 100 mM NaCl; 50% glycerol) at a concentration of 2 mg ml^{-1} and stored at –20 ºC. Protein concentration was determined with a Bradford protein assay kit (Bio-Rad).

The standard microtiter dish biofilm formation assay was performed as previously described with minor modifications (O'Toole *et al.*, 1999). Briefly, overnight cultures of *R. erythropolis* pTipQC1::*dsz*-P_{lac}D grown in LB Cm were diluted 1:100 in 1.5 ml of LB Cm and incubated for 72 hours at 30 ˚C and 200 rpm in a 24 flat-well microtiter plate (Costar). Liquid media was then removed, wells were gently washed twice with PBS and the biofilm formed as an adhered ring to the well wall was treated for 2 hours at 37 ˚C with: (i) 100 µg ml^{-1} proteinase K (Sigma) in 20 mM Tris pH 7.5, 100 mM NaCl (Kaplan *et al.*, 2004); (ii) 100 U ml^{-1} DNAse I (Sigma) in PBS (Sugimoto *et al.*, 2018); (iii) 40 µg ml^{-1}

Dispersin B in PBS (Kaplan *et al.*, 2004); (iv) 5 mg ml^{-1} cellulase (from *Trichoderma viride*, Sigma) in 50 mM citrate buffer pH 4.6 (Trivedi *et al.*, 2016); (v) 10 mM sodium metaperiodate (Scharlau) in 50 mM sodium acetate buffer pH 4.5 (Kaplan *et al.*, 2004). Control wells were filled with each reaction buffer. After treatment, wells were gently washed twice with PBS and biofilms were stained with 2 ml of 0.25% crystal violet (VWR) for 5 min at room temperature. The excess crystal violet was removed and wells were washed twice with PBS and air dried. Crystal violet-stained cells were quantified by solubilizing the dye with 2 ml of ethanol-acetone (80:20, vol/vol) and determining the optical density at 595 nm (OD_{595nm}). Each treatment was evaluated in triplicate.

Supplementary Figures

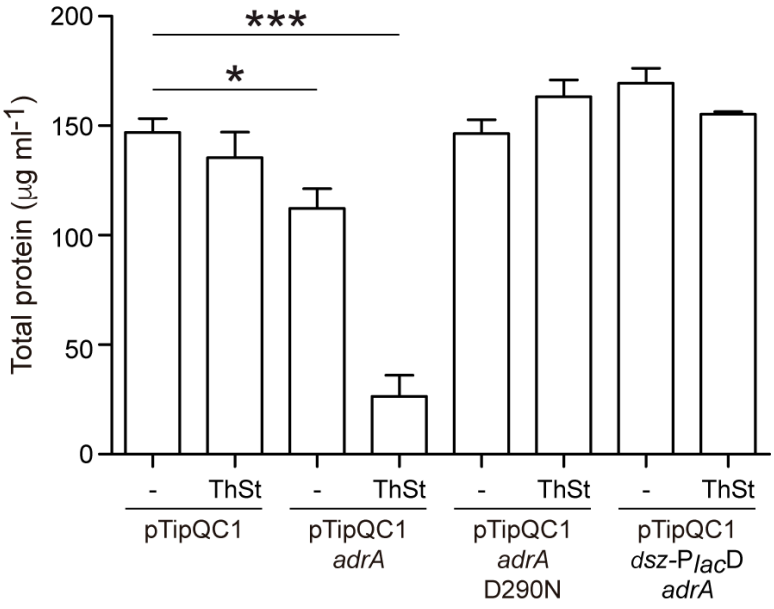

Figure S1. AdrA overexpression and subsequent very high c-di-GMP levels in *R. erythropolis* pTipQC1::*adrA* upon thiostrepton induction have a very significant negative impact on *Rhodococcus* growth. *R. erythropolis* strains harboring plasmid pTipQC1, pTipQC1::*adrA*, pTipQC1::*adrA* D290N or pTipQC1::*dsz*-P_{lac}D-*adrA* were grown in LB Cm for 24 hours at 30 ˚C, and then diluted 1:100 in fresh LB Cm and incubated for 12 hours at 30 ˚C, 200 rpm. Thiostrepton was added and cultures were incubated for 12 hours at 30 ˚C, 200 rpm. The control non-induced cultures were also incubated for 12 hours at 30 ˚C, 200 rpm. Cells were collected, lysed and the total protein content was calculated (μg ml^{-1}). Data were compared to the total protein content of the non-induced *R. erythropolis* pTipQC1 culture by one-way analysis of variance (ANOVA) with Dunnett's post-test. **P* value < 0.05; ****P* value < 0.001. Data represent the mean ± standard deviation calculated from two independent experiments performed in triplicate.

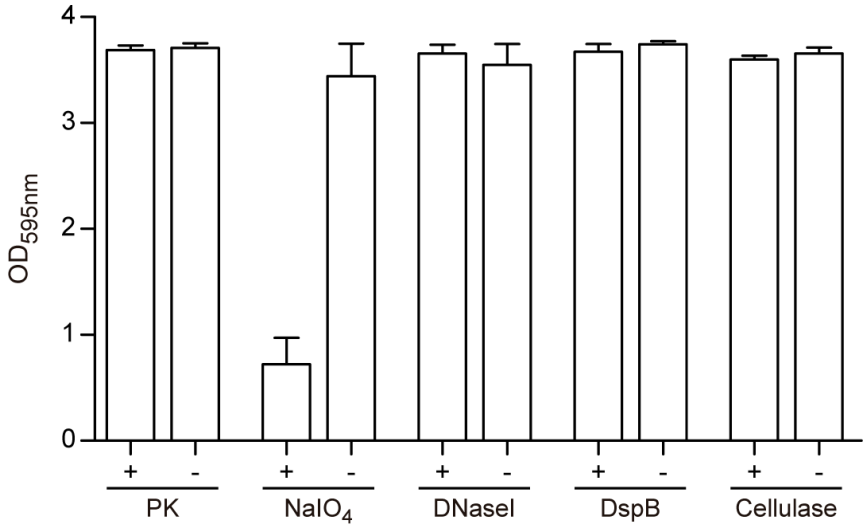

Figure S2. Analysis of the nature of the biofilm extracellular matrix produced by *R. erythropolis* pTipQC1::*dsz*-P_{lac}D-*adrA* through biofilm treatment with dispersing agents. *R. erythropolis* pTipQC1::*dsz*-P_{lac}D-*adrA* was grown in a 24 flat-well microtiter plate for 72 hours at 30 ˚C and 200 rpm. The biofilm attached to the well walls was treated for 2 hours at 37 ˚C with 100 µg ml^{-1} proteinase K (PK) in 20 mM Tris pH 7.5, 100 mM NaCl; 10 mM sodium metaperiodate ($NaIO_4$) in 50 mM sodium acetate buffer pH 4.5; 100 U ml^{-1} DNAse I in PBS; 40 µg ml^{-1} Dispersin B (DspB) in PBS and 5 mg ml^{-1} cellulase in 50 mM citrate buffer pH 4.6. Control wells (-) only contained the corresponding reaction buffer. After treatment, wells were washed and 0.25% crystal violet was added to stain non-dispersed biofilms. The amount of crystal violet-stained cells was quantified by solubilizing the dye in ethanol/acetone and determining the absorbance at 595 nm. Data represent the mean ± standard deviation calculated from three independent experiments. The biofilm formed by *R. erythropolis* pTipQC1::*dsz*-P_{lac}D-*adrA* was only dispersed by sodium metaperiodate, indicating that an exopolysaccharide (neither PIA/PNAG nor cellulose) is the main component of the biofilm extracellular matrix.

Supplementary References

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