microbial biotechnology

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

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Summary

Bacterial biofilms provide high cell density and a superior adaptation and protection from stress conditions compared to planktonic cultures, making them a very promising approach for bioremediation. Several *Rhodococcus* strains can desulfurize dibenzothiophene (DBT), a major sulphur pollutant in fuels, reducing air pollution from fuel combustion. Despite multiple efforts to increase *Rhodococcus* biodesulfurization activity, there is still an urgent need to develop better biocatalysts. Here, we implemented a new approach that consisted in promoting *Rhodococcus erythropolis* biofilm formation through the heterologous expression of a diguanylate cyclase that led to the synthesis of the biofilm trigger molecule cyclic di-GMP (c-di-GMP). *R. erythropolis* biofilm cells displayed a significantly increased DBT desulfurization activity when compared to their planktonic counterparts. The improved biocatalyst formed a biofilm both under batch and continuous flow conditions which turns it into a promising candidate for the development of an efficient bioreactor for the removal of sulphur heterocycles present in fossil fuels.

Introduction

Bacterial biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix (Costerton *et al.*, 1999). Biofilms constitute a protected type of growth and thus, bacteria living in the form of biofilms are characterized by a superior resilience and robustness compared to planktonic cells that allows survival in hostile environments (Costerton *et al.*, 1999; Edel *et al.*, 2019). Such decreased susceptibility to environmental stressors together with the long-term activity shown by biofilm cells, the high microbial cell density, the ability to immobilize compounds and the easiness of separation of the end-product from the catalytic biomass, make biofilm systems a very practical and promising tool in bioremediation strategies (Singh *et al.*, 2006; Rosche *et al.*, 2009; Edel *et al.*, 2019). Fundamental to these properties is the role of the self-produced polymeric matrix that encases the cells of the biofilm and is mainly composed of polysaccharides, proteins and extracellular DNA (eDNA) (Flemming *et al.*, 2016). Although the diversity in matrix composition and regulatory mechanisms controlling its synthesis is high, many diverse and phylogenetically distant bacterial species share common features that promote biofilm development such as the production of some exopolysaccharides (poly-β(1-6)-N-acetyl-glucosamine (PIA/PNAG), cellulose) (Cywes-Bentley *et al.*, 2013; Römling and Galperin, 2015), proteins with amyloid or amyloid-like conformation (Taglialegna *et al.*, 2016a) and synthesis of the secondary messenger cyclic di-GMP (c-di-GMP) (Römling *et al.*, 2013).

Rhodococcus genus is a heterogeneous group of Gram positive bacteria that present unique enzymatic

capabilities of great biotechnological importance (Zampolli *et al.*, 2019). Amongst these bacteria, several strains of *Rhodococcus erythropolis* are considered as model organisms for biodesulfurization (BDS), an environmentally friendly method that aims at the removal of sulphur from refractory organic compounds of fossil fuels, at mild operating conditions, without lowering the calorific value of the fuel (Kilbane, 2016; Mohebali and Ball, 2016). This method is a complementary alternative to hydrodesulfurization (HDS), the most common technology used by refineries, that suffers from significant technical and economic limitations (Kilbane, 2006; Soleimani *et al.*, 2007). Removal of sulphur from crude oil products has great environmental impact, because fuel combustion results in sulphur dioxide emission into the environment, causing air pollution and acid rain. The *R. erythropolis* IGTS8 strain was the first bacterium in which the 4S pathway was reported (Gallagher *et al.*, 1993), by which dibenzothiophene (DBT), a major sulphur-containing aromatic compound in fuels that is recalcitrant to HDS, is converted to 2-hydroxybiphenyl (2HBP) and sulphite. The 4S pathway occurs via serial reactions in which two monooxygenases, DszC and DszA, and a desulfinase, DszB, are involved. The genes encoding these biodesulfurization enzymes are clustered in the *dszABC* operon present in a megaplasmid (Piddington *et al.*, 1995). Also, and since the monooxygenation reactions catalysed by DszA and DszC require electrons, a fourth enzyme, the flavin reductase DszD, encoded by the chromosomal *dszD* gene, is needed for DBT desulfurization (Gray *et al.*, 1996). Desulfurization activity of naturally occurring bacterial cultures is very low due to different reasons, such as the lack of a *dszD* gene in the native *dsz* operon, the sulphate-mediated repression of the biodesulfurization operon (Li *et al.*, 1996), the poorly known transcriptional regulation of *dsz* genes (Kilbane, 2006), the inhibition, activity levels and restricted substrate range of Dsz enzymes (Kilbane, 2006) and the limited solvent tolerance of the host cell (Tao *et al.*, 2011; Kilbane, 2016). Thus, multiple efforts mostly based on genetic engineering of the *dsz* operon have been made to achieve higher desulfurization rates. However, the desulfurization levels reached by genetic manipulation are still inadequate to fulfil the industrial requirements and seem to be restricted by still unknown host factors that also limit the success of several process engineering approaches (Martínez *et al.*, 2017). Therefore, it has long been proposed that not only manipulation of the desulfurization genes, but also alterations to other host functions are needed in order to develop desulfurization biocatalysts with higher specific activity (Kilbane, 2006; Martínez *et al.*, 2017; Wang *et al.*, 2017).

Taking into account the features of biofilm systems described above that make them especially suitable for the treatment of recalcitrant compounds and also, the fact that biofilm-dwelling microorganisms are widely accepted as being responsible for the majority of pollutant degradation in natural environments (Lear, 2016), in this work, we aimed at enhancing R. *erythropolis* biodesulfurization capacity by promoting its ability to form a biofilm. A selected strategy based on the overexpression of optimized *dsz* cassettes together with a heterologous diguanylate cyclase, named AdrA (Simm *et al.*, 2004), that drives the synthesis of elevated levels of the biofilm trigger molecule, c-di-GMP, highly promoted biofilm development under batch and continuous flow conditions and significantly improved specific desulfurization activity of R. *erythropolis* cells.

Results and discussion

Selection of an optimal strategy to induce biofilm formation in Rhodococcus erythropolis IGTS8

The *bap* gene of the bacterial pathogen *Staphylococcus aureus* encodes the biofilm associated protein (Bap), a cell wall-associated protein of 2276 amino acids. This surface protein self-assembles, under conditions of low calcium concentrations and acidic pH, into aggregates with an amyloid-like conformation and promotes biofilm formation. One of the main features of this protein is its modular organization, being the B region (Bap_B) responsible for the formation of the amyloid-like fibres and thus sufficient to promote biofilm development (Taglialegna *et al.*, 2016b). Based on a previous study where it was demonstrated that the exogenous addition of recombinant Bap_B (rBap_B) at a concentration of 2 μM is enough to drive biofilm formation in *S. aureus* (Taglialegna *et al.*, 2016b), we purified rBap_B and then cultured *R. erythropolis* IGTS8 in LB medium supplemented with 0.25% glucose and 2 μM rBap_B for 24 h at 30°C. Under laboratory conditions, wild-type *R. erythropolis* showed a basal capability of forming a biofilm that could be observed as a labile ring of cells adhered to the glass wall (Fig. 1A). This observation is in agreement with the characteristic hydrophobicity of *Rhodococcus* cells provided by fatty acids of the cell wall, whose composition changes in response to the surface to which *Rhodococcus* adheres, resulting in different biofilm architectures and biomass (de Carvalho and Fonseca, 2007; Rodrigues and Carvalho, 2015). Results showed that addition of rBap_B did not lead to an increase in *Rhodococcus* biofilm formation with respect to the non-treated culture (Fig. 1A), probably because *Rhodococcus* growth does not result in medium acidification, a requisite for rBap_B mediated biofilm formation. We thus adjusted the pH of LB medium to 4.5 and used it to culture *R. erythropolis* in the presence of rBap_B. However, at this low pH *Rhodococcus* growth was arrested and therefore, this strategy for inducing biofilm formation was discarded.
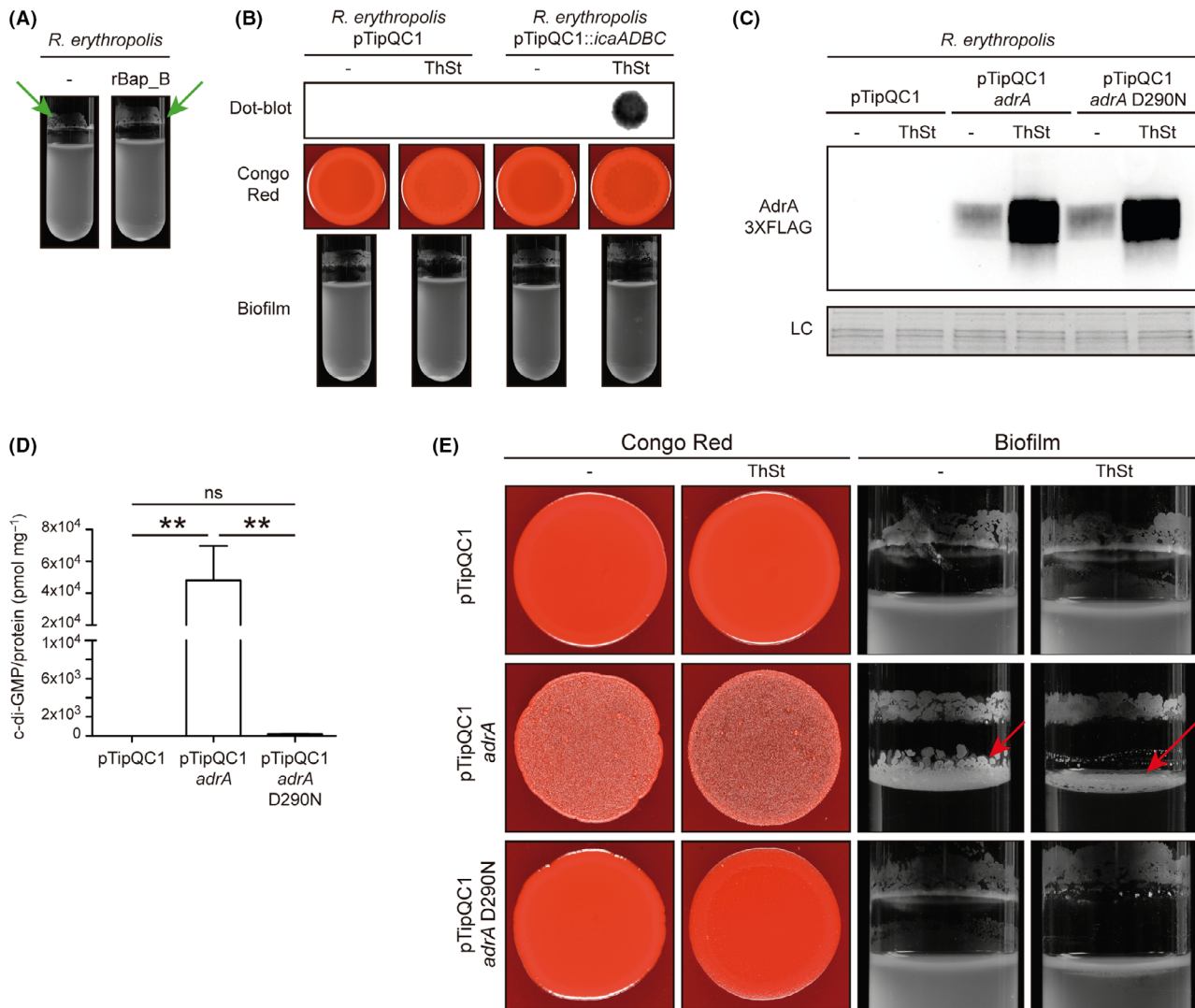
Fig. 1. Evaluation of different strategies to promote *R. erythropolis* IGTS8 biofilm formation.A. Biofilm phenotypes of the wild-type strain in the absence or presence of 2 μM rBap_B after incubation in LB broth supplemented with 0.25 % glucose for 24 h at 30°C. Green arrows point to the basal biofilm that *R. erythropolis* forms under these conditions and that can be observed as a labile ring of cells adhered to the glass wall.B. Analysis of PIA/PNAG exopolysaccharide production by dot-blot, colony morphology on Congo red agar and biofilm phenotype in LB medium of *R. erythropolis* containing the pTipQC1 empty plasmid or pTipQC1::*icaADBC* in the absence or presence of 1 μg ml^{-1} thiostrepton (ThSt).C. Western blot showing AdrA levels in *R. erythropolis* containing the pTipQC1 empty plasmid, pTipQC1::*adrA* or pTipQC1::*adrA* D290N coding for an inactive AdrA protein, in the absence or presence of 1 μg ml^{-1} thiostrepton (ThSt). Anti-3xFLAG antibodies were used to detect the AdrA 3xFLAG-tagged protein. A stain-free gel portion is shown as a loading control (LC).D. *R. erythropolis* strains described in C were grown in LB containing 1 μg ml^{-1} thiostrepton. c-di-GMP was extracted, quantified by mass spectrometry and correlated to protein concentration. Data represent the mean ± standard deviation calculated from three independent experiments. Statistical significance was determined with one-way ANOVA followed by Bonferroni multiple comparison test. **P value < 0.01. ns; no significant difference.E. Colony morphology on Congo red agar and biofilm phenotype in LB medium of the strains described in C. Red arrows point to the biofilm formed at the air-liquid interface by *R. erythropolis* pTipQC1::*adrA* both in the absence and presence of thiostrepton. Note that cultures become transparent since most bacteria are encased inside the biofilm formed at the interface.

PIA/PNAG, a homopolymer of N-acetylglucosamine, has been described as a conserved surface polysaccharide synthesized by several Gram positive and Gram negative bacteria to build the biofilm matrix (Cywes-Bentley *et al.*, 2013). In order to test a second strategy to promote biofilm formation in *R. erythropolis*, we amplified the *icaADBC* operon of *S. aureus*, which encodes the

Ica proteins responsible for PIA/PNAG synthesis in this species (Cramton *et al.*, 1999), and cloned it into the pTipQC1 vector for its expression in *Rhodococcus* under the control of a thiostrepton inducible promoter (Nakashima and Tamura, 2004). *R. erythropolis* cells carrying either the empty pTipQC1 or the pTipQC1::*icaADBC* plasmid were incubated in LB broth, at 30°C for 24 h

under shaking conditions in the absence or presence of thiostrepton and heterologous PIA/PNAG production was monitored using a dot-blot assay and an anti-PIA/PNAG antiserum. Results showed that *R. erythropolis* pTipQC1::*icaADBC* incubated in the presence of 1 μg ml^{-1} thiostrepton produced detectable levels of PIA/PNAG (Fig. 1B). PIA/PNAG mediated biofilm formation has been associated to a rough colony morphology phenotype on solid media containing the Congo red dye (Freeman *et al.*, 1989). Also, the production of this exopolysaccharide in different species and the subsequent biofilm formation can be visualized, after incubation in a glass tube under shaking conditions, as a ring of cells strongly adhered to the glass wall at the air–liquid interface (Valle *et al.*, 2003; Echeverz *et al.*, 2017). However, when these two phenotypes were investigated, *R. erythropolis* cells carrying the *icaADBC* operon showed a smooth morphotype on Congo red agar, indicative of non-biofilm producers, and were also unable to build a visible PIA/PNAG mediated biofilm in a glass tube, even under the presence of the thiostrepton inducer (Fig. 1B). These results were unexpected, since we have previously shown that heterologous expression of the *pgaABCD* operon (responsible for PIA/PNAG synthesis in *Escherichia coli*) in *Salmonella* makes *Salmonella* capable of building a PIA/PNAG mediated biofilm (Echeverz *et al.*, 2017). In the present case, and although heterologous *icaADBC* expression in *R. erythropolis* IGTS8 drives PIA/PNAG synthesis, the resulting polysaccharide levels may be insufficient for inducing a significant biofilm development.

A third strategy for promoting biofilm formation focused on producing high levels of the intracellular dinucleotide c-di-GMP with the aim of inducing the synthesis of *Rhodococcus* own exopolysaccharides that may be part of the biofilm matrix (Pérez-Mendoza and Sanjuán, 2016). C-di-GMP is synthesized by enzymes called diguanylate cyclases harbouring a GGDEF domain and is degraded by specific phosphodiesterases that contain an EAL or HD-GYP domain (Schirmer and Jenal, 2009). Thus, to promote c-di-GMP synthesis, we overexpressed in *R. erythropolis* the *adrA* gene from *Salmonella enterica* serovar Enteritidis, encoding a very active diguanylate cyclase and thus, able to synthesize c-di-GMP (Simm *et al.*, 2004). In order to monitor *adrA* correct expression and translation in *Rhodococcus*, the *adrA* gene was amplified from a genetically modified *Salmonella* strain that encodes a labelled AdrA protein with a 3xFLAG translational fusion (Solano *et al.*, 2009) and was then cloned in the pTipQC1 plasmid. A Western blot assay confirmed the correct production of AdrA in *R. erythropolis* transformed with the pTipQC1::*adrA* plasmid (Fig. 1C) that correlated with very high c-di-GMP levels in the *Rhodococcus* cytoplasm (Fig. 1D). Notably, basal levels of AdrA were produced even in the absence of thiostrepton induction (Fig. 1C). As a result of AdrA production, *R. erythropolis* pTipQC1::*adrA* cells, incubated both in the absence and presence of the inducer, showed a dry and rough colony phenotype on Congo red agar plates and formed a biofilm at the air-broth interface that encased most of the bacterial cells present in the culture (Fig. 1E). Of note, the elevated AdrA synthesis and subsequent great c-di-GMP levels reached upon thiostrepton induction caused a marked negative effect in the *Rhodococcus* growth rate, that was much less pronounced in the absence of induction (Fig. S1). Biofilm phenotypes were dependent on c-di-GMP synthesis, since transformation of *R. erythropolis* with pTipQC1::*adrA* D290N, producing an altered AdrA protein with a degenerate GGNEF motif (Fig. 1C) and therefore, unable to synthesize c-di-GMP (Fig. 1D), did not result in a biofilm associated phenotypic change (Fig. 1 E).

According to the results presented above, moderate AdrA overexpression and subsequent c-di-GMP production was chosen as an optimum strategy to induce biofilm development in *R. erythropolis* cells.

Engineering a dsz-adrA cassette that leads to moderate c-di-GMP production and biofilm formation in R. erythropolis

Based on a previous work where a synthetic *dsz* cassette for improved conversion of DBT into 2HBP was assembled and expressed in *Pseudomonas putida* (Martínez *et al.*, 2016), we constructed a pTipQC1 derived plasmid named pTipQC1::*dsz*-P$_{lac}$D-*adrA* (Fig. 2 A) to promote biofilm formation and *dsz* genes overexpression in *Rhodococcus* cells. This plasmid contained the synthetic *dszB1*, *dszA1* and *dszC1* genes arranged as an operon under the control of the *tipA* promoter, thus avoiding repression of transcription of the *dsz* native promoter in the presence of inorganic sulphate (Li *et al.*, 1996). Taking into account that increased expression of *dszD* has been reported to have a toxic effect (Galan *et al.*, 2000) and that, as stated above, AdrA production has to be tightly regulated to avoid a negative impact on growth, the cassette contains *dszD1* followed by the *adrA* gene, arranged as an operon, under control of the weak *Plac* promoter and separated from the *dszB1A1C1* operon by a strong transcriptional terminator of the lambda phage (Fig. 2A) (Martínez *et al.*, 2016). A second plasmid, pTipQC1::*dsz*-P$_{lac}$D, without the *adrA* gene was also constructed as a control to be used in biofilm and BDS analyses (Fig. 2A).

Both plasmids were then introduced in *R. erythropolis* IGTS8 and AdrA production was evaluated by Western blot (Fig. 2B). As expected, *R. erythropolis* pTipQC1::

(A)

pTipQC1::*dsz*-P*lac*D

pTipQC1::*dsz*-P*lac*D-*adrA*

(B)

(C)

(D)
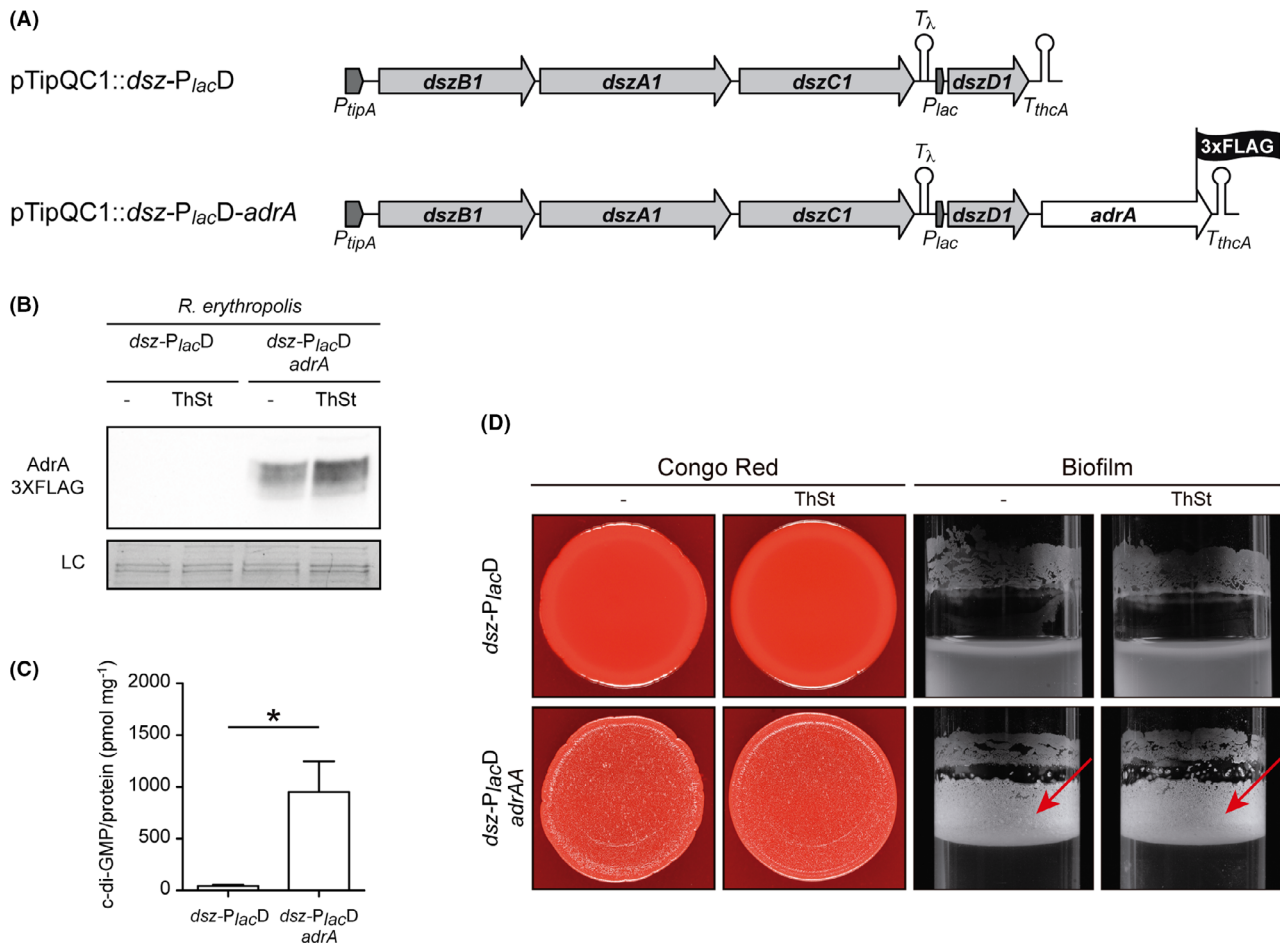
Fig. 2. Construction and analysis of the biofilm formation capacity of *R. erythropolis* expressing *dsz* genes and *adrA*.A. Schematic representation drawn to scale of the *dsz*-P*lac*D cassette alone or in combination with *adrA* that was cloned in the pTipQC1 plasmid.B. Western blot showing AdrA levels in *R. erythropolis* carrying plasmids shown in A and incubated in the absence or presence of 1 μg ml^{-1} thiostrepton (ThSt). Anti-3xFLAG antibodies were used to detect the AdrA 3xFLAG-tagged protein. A stain-free gel portion is shown as a loading control (LC).C. *R. erythropolis* strains carrying plasmids shown in A were grown in LB containing 1 μg ml^{-1} thiostrepton. c-di-GMP was extracted, quantified by mass spectrometry and correlated to protein concentration. Data represent the mean ± standard deviation calculated from three independent experiments. Data were analysed by using Student's *t* test.D. Colony morphology on Congo red agar and biofilm phenotype in LB medium. Red arrows point to the biofilm formed at the air-liquid interface by *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* both in the absence and presence of thiostrepton. Note that cultures become almost transparent since most bacteria are encased inside the biofilm formed at the interface.

dsz-P*lac*D-*adrA* cells produced moderate levels of AdrA, independently of thiostrepton induction. Western blot results were consistent with c-di-GMP quantities in the *Rhodococcus* cytoplasm (Fig. 2C) that were fifty times lower than when the *adrA* gene was expressed from the *tipA* promoter in the pTipQC1::*adrA* plasmid (Fig. 1D). We finally evaluated biofilm related phenotypes and confirmed that such moderate levels of AdrA production did not have a negative impact on growth (Fig. S1) and were enough for *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* cells to show a dry and rough colony phenotype on Congo red agar plates and to form a biofilm at the air-broth interface (Fig. 2D). Hence, this strain was considered for subsequent analysis of DBT desulfurization improvement in relation to the control strain, *R. erythropolis* pTipQC1::*dsz*-P*lac*D, that shows only basal natural

levels of c-di-GMP (Fig. 2C), and therefore stays in a planktonic physical state (Fig. 2D).

R. erythropolis *biofilm cells show a significantly improved capacity to convert DBT into 2HBP when compared to their planktonic counterparts*

In order to test whether biofilm formation through AdrA overexpression in *R. erythropolis* enhances its biodesulfurization capacity, resting cell assays in aqueous phase were performed. To do so, cells (0.3 g dry cell weight per litre) were suspended in 50 mM HEPES buffer, 25 μM DBT was added and BDS was conducted at 30°C. Triplicate samples were prepared for each strain so that at 0, 15 and 60 min of incubation, one sample of each strain was collected and DBT and 2HBP

concentrations were determined. Figure 3 shows the time courses for DBT utilization and 2HBP production (Fig. 3A) and also the conversion efficiency of each strain, indicated as the ratio of 2HBP produced to the initial concentration of DBT (Fig. 3B). After 1 h of incubation, the conversion efficiency increased from a basal point of 20.8% shown by *R. erythropolis* harbouring the empty pTipQC1 plasmid to 35.8% when control *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D planktonic cells were used. Remarkably, an almost complete conversion of DBT into 2HBP was achieved in the case of *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA* biofilm cells (98.2%). Hence, these results demonstrated that biofilm formation through c-di-GMP production is a very productive alternative to the conventional strategy based on the sole overexpression of *dsz* genes. In fact, the specific desulfurization activity attained with the biofilm approach proposed in this study is 84.2 μmol$_{2HBP}$/g DCW/h. This is the highest

value described in the literature with either *Rhodococcus* or other bacterial species when performing microbial resting cell cultures in aqueous phase (Maghsoudi *et al.*, 2001; Martin *et al.*, 2005; del Olmo *et al.*, 2005; Rashtchi *et al.*, 2006; Davoodi-Dehaghani *et al.*, 2010; Alves and Paixão, 2014; Martínez *et al.*, 2016; Ismail *et al.*, 2016; Dejaloud *et al.*, 2017). Additional research to confirm increased bioconversion efficiency may be focused on carrying out resting cells assays in a two-phase system where desulfurization activity is higher than in aqueous media (Davoodi-Dehaghani *et al.*, 2010).

Here, we increased the concentration of c-di-GMP through the overexpression of AdrA, a heterologous diguanylate cyclase. In this regard, *R. erythropolis* c-di-GMP signalling system is composed of fourteen proteins containing a GGDEF domain, four proteins containing both GGDEF and EAL domains and one protein containing an EAL domain (https://www.ncbi.nlm.nih.gov/
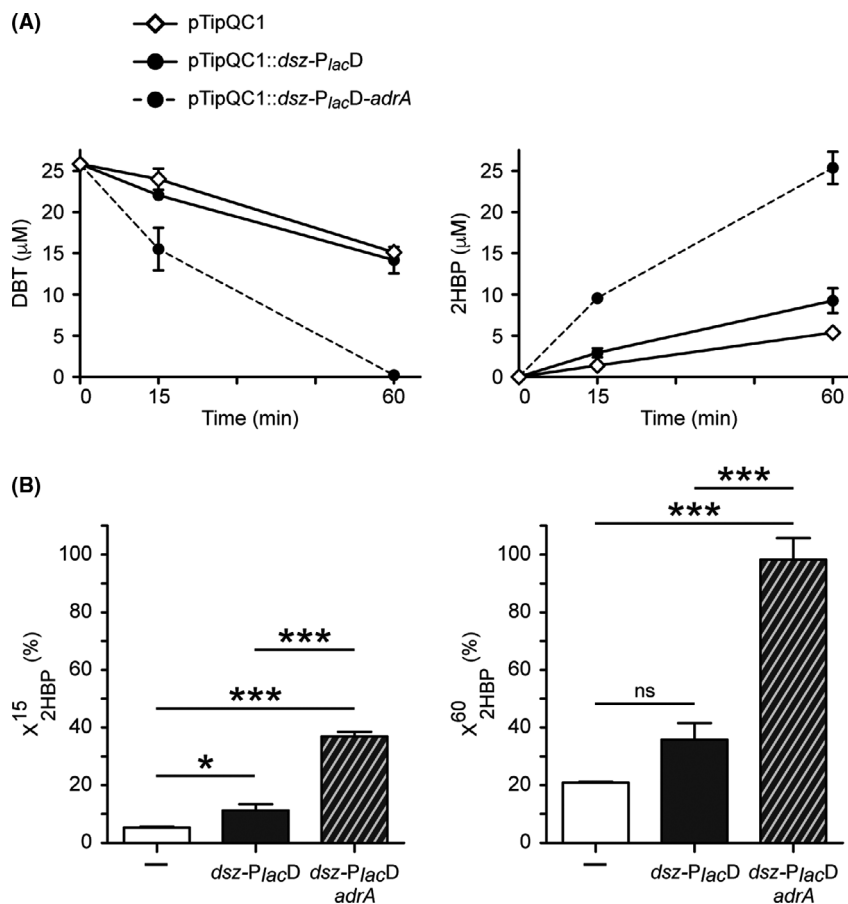
Fig. 3. Conversion of DBT into 2HBP by *R. erythropolis* strains expressing the *dsz*-P$_{lac}$D cassette alone (planktonic) or in combination with *adrA* (biofilm). Resting cells assays were carried out using 25 μM DBT as initial substrate.A. DBT utilization (left) and 2HBP production (right) by resting cells at 15 and 60 min of desulfurization.B. Conversion efficiency of DBT into 2HBP is denoted as the percentage of 2HBP production after 15 min (left) and 60 min (right) according to equation $X_{2HBP}^{time\,(min)} = (C_{2HBP}^{time\,(min)}/C_{DBT}^{0}) \times 100$. Data of *R. erythropolis* containing the pTipQC1 empty plasmid (-), *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA* (*dsz*-P$_{lac}$D-*adrA*) and the control strain *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D (*dsz*-P$_{lac}$D) were subjected to one-way analysis of variance (ANOVA) using Bonferroni post-test. *P value < 0.05; ***P value < 0.001. ns; no significant difference. Data represent the mean $\pm$ standard deviation calculated from three independent experiments.

Complete_Genomes/c-di-GMP.html). Although the putative diguanylate cyclase or phosphodiesterase activity of these proteins has never been tested, further research might be dedicated to increase c-di-GMP levels by means of either overexpressing *Rhodococcus* GGDEF domain containing proteins or mutating EAL domain containing proteins through recently described genome editing systems (DeLorenzo *et al.*, 2018; Liang *et al.*, 2020). Also, future improvements of the recombinant strain might consider the insertion of the synthetic *dsz* cassette into the chromosome of the target host cell in order to avoid recombination events between the synthetic *dsz* genes present in the pTipQC1::*dsz*-P*lac*D-*adrA* plasmid and the native genes.

With regard to the extracellular matrix composition of the biofilm formed by *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA*, dispersion experiments with proteinase K (which degrades proteinaceous constituents of the matrix), sodium metaperiodate (which oxidizes polysaccharide bonds) and DNase (which digests matrix eDNA) showed that the biofilm was only sensitive to sodium metaperiodate, indicating that the major component of the biofilm matrix is of polysaccharide nature (Fig. S2). Importantly, the identification of the exopolysaccharide that is being overproduced in *Rhodococcus* under elevated c-di-GMP conditions and also the genes involved in its synthesis may inspire the construction of an engineered *R. erythropolis* strain in which such exopolysaccharide would be expressed at will in order to tightly modulate *Rhodococcus* biofilm formation. In this sense, we treated *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* biofilm cells with Dispersin B, an enzyme produced by *Aggregatibacter actinomycetemcomitans* that specifically hydrolyses the glycosidic linkages in PIA/PNAG (Kaplan *et al.*, 2004), and also with cellulase, that disrupts cellulose based biofilms (Solano *et al.*, 2002). However, none of the treatments digested the extracellular matrix of the *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* biofilm (Fig. S2), indicating that an exopolysaccharide of different nature is being produced by this strain to readily form a biofilm. The marine bacterium *R. erythropolis* PR4 is known to produce a fatty acid-containing extracellular polysaccharide (PR4 FACEPS) and an acidic extracellular polysaccharide named mucoidan, whose chemical composition and structure have been characterized (Urai *et al.*, 2007a,b). The genetic determinants responsible for the synthesis of these exopolysaccharides and whether *R. erythropolis* IGTS8 produces them in response to c-di-GMP remains to be determined.

To our knowledge, this is one of the few studies performed to date that have shown c-di-GMP production as an optimal approach to enhance biofilm formation and subsequent bacterial biotransformation activities (Wu *et al.*, 2015; Benedetti *et al.*, 2016; Guo *et al.*, 2017; Hu

et al., 2019). Overall, our results evidence that increasing the levels of c-di-GMP might be a promising general strategy to enhance biodegradation kinetics of bacteria. Thus, this approach might have a significant impact on the biotechnological potential of *Rhodococcus* genus in ecological and bioremediation applications since it could be applied to boost many biodegradation capabilities of not only *R. erythropolis* but also other species.

R. erythropolis *cells overproducing c-di-GMP form a biofilm on polystyrene and silicone surfaces under flow conditions inside microfermenters*

Microbial cells immobilization in a reactor offers advantages such as superior operational stability, continuous processing and simpler separation from the reaction media for reuse (Soleimani *et al.*, 2007; Rosche *et al.*, 2009). Previous attempts to immobilize *R. erythropolis* cells include gel entrapment in resin polymers (Naito *et al.*, 2001) and decoration of bacteria with magnetic nanoparticles (Ansari *et al.*, 2009). However, these artificial immobilization approaches may show several limitations, such as the extra elaborate preparation steps that have a negative impact on costs, lower bacterial viability and restricted mass transfer through the immobilization matrix. Conversely, biofilms produce their own immobilization matrix and are naturally adapted to be viable within it (Rosche *et al.*, 2009). The above results indicated that c-di-GMP is able to induce *R. erythropolis* biofilm development in batch cultures. Next, we wondered whether c-di-GMP could also induce *R. erythropolis* biofilm development under continuous flow conditions that might be used to develop an engineered biofilm process for fuel desulfurization. For this, we assessed biofilm formation with the use of microfermenters, under continuous flow conditions and with a flow rate high enough to prevent planktonic growth (Ghigo, 2001). The microfermenters contain submerged, removable Pyrex slides, that serve as substratum for bacterial attachment. To test other types of surfaces apart from glass, polystyrene and silicone rubber disc coupons with a diameter of 12.7 mm were fixed on the glass slides (Fig. 4A). Microfermenters were inoculated with either the biofilm catalytic strain *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* or the control non-biofilm forming strain *R. erythropolis* pTipQC1::*dsz*-P*lac*D. After 48 h of incubation at 30°C, Pyrex slides were removed and biofilm formation on glass, polystyrene and silicone was visually inspected. Results showed that the *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* biocatalyst formed a very robust biofilm both on polystyrene and silicone surfaces whilst the control strain barely attached to these surfaces. None of the two strains was able to form a biofilm on the glass spatula (Fig. 4B). In order to analyse biofilm structure, a

(A)

(B)

(C)
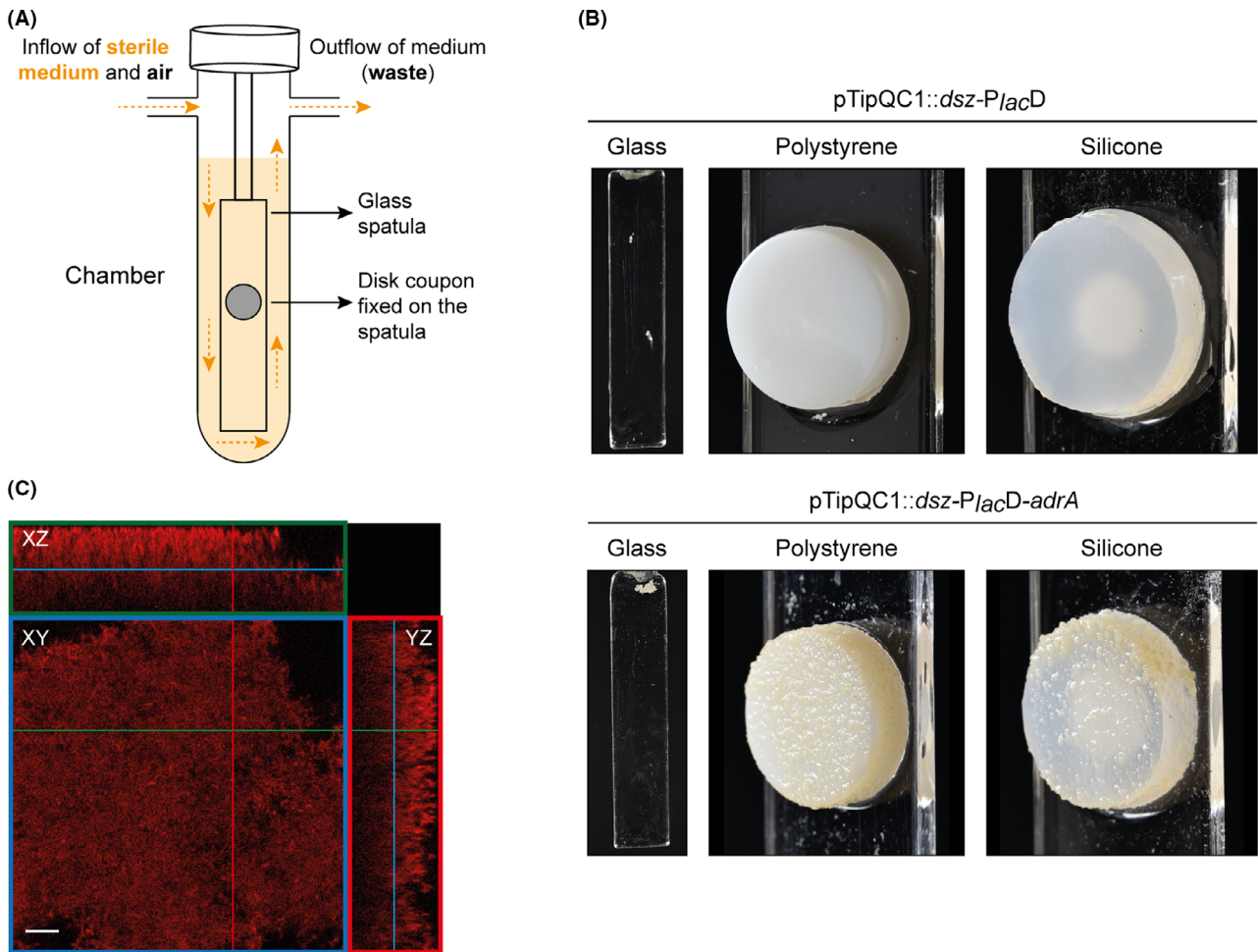
Fig. 4. Biofilm formation on different surfaces by *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA* grown under flow conditions inside microfermenters.A. Schematic diagram of a microfermenter setup containing a glass spatula inside with a fixed 12.7 mm diameter disc coupon (depicted as a grey circle).B. Images of the biofilm formed on the glass spatula (the entire spatula is shown), polystyrene and silicone rubber disc coupons by *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA* and the control strain *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D after 48 h of incubation at 30°C under continuous flow conditions.C. CLSM image of the top-down view (XY plane; large panel, at 14 μm above the coupon surface) and orthogonal views (YZ and XZ planes; side panels) of the biofilm formed by *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA*-*mCherry* on a polystyrene disc coupon after 48 h of incubation at 30°C under continuous flow conditions. Scale bar 10 μm. The experiment was performed in triplicate and a representative image is shown.

derivative of *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA* expressing the fluorescent mCherry protein was constructed. *R. erythropolis* pTipQC1::*dsz*-P$_{lac}$D-*adrA*-*mCherry* was incubated inside microfermenters and then, the biofilm formed on polystyrene coupons was inspected by confocal laser scanning microscopy (CLSM). Images confirmed the presence of a characteristic biofilm architecture that was many cells thick and showed a uniform substratum coverage (Fig. 4C). Altogether, these results suggested that the engineered *R. erythropolis* biofilm forming strain constructed in this study could be used as an effective biocatalyst in the development of an efficient bioreactor for an improved BDS process.

Conclusion

We found a role of c-di-GMP in enhancing *R. erythropolis* BDS capability. A novel biocatalyst with a significantly improved capacity to desulfurize DBT and the ability to form a strong biofilm under batch and continuous flow conditions was developed by combining an optimized *dsz* cassette and the *adrA* gene, that encodes a very active diguanylate cyclase that drives c-di-GMP synthesis in the cell. The demonstration of an interplay between c-di-GMP and the biotechnological potential of *R. erythropolis* may have profound implications in bioremediation applications of not only *R. erythropolis* but also other environmentally relevant bacterial species.

Table 1. Bacterial strains used in this study.

Strains	Relevant characteristics	MIC[a]	Reference/source
Escherichia coli			
XL1Blue	*endA1 gyrA96*(*nalR*) *thi-1 recA1 relA1 lac glnV44* F'[::Tn10 *proAB+lacIq* Δ(*lacZ*)M15] *hsdR17*(*rK- mK+*).	0797	Stratagene
BL21 (DE3)	Strain for heterologous expression and protein production.	0076	Novagen
Rhodococcus erythropolis			
IGTS8	Wild-type strain able to transform DBT into 2HBP via the 4S pathway.	5638	(Kilbane and Jackowski, 1992; Gallagher *et al.*, 1993)
Salmonella enterica subsp. *enterica serovar* Enteritidis			
3934 *adrA*-3xFLAG	Wild-type clinical isolate expressing a 3xFLAG-tagged AdrA protein from the chromosome	2429	(Solano *et al.*, 2009)
3934 *adrA* D290N-3xFLAG	Wild-type clinical isolate expressing a 3xFLAG-tagged mutated AdrA protein from the chromosome. The mutated AdrA D290N version is unable to synthesize c-di-GMP.	4688	Laboratory of Microbial Pathogenesis (unpublished data)
Staphylococcus aureus			
15981	Clinical isolate. Biofilm positive strain.	0532	(Valle *et al.*, 2003)

a. Number of each strain in the culture collection of the Laboratory of Microbial Pathogenesis, Navarrabiomed-Universidad Pública de Navarra.

Experimental procedures

Bacterial strains, plasmids, oligonucleotides and culture conditions

Bacterial strains, plasmids and oligonucleotides used in this work are listed in Tables 1 and 2, respectively. *Escherichia coli* strains were routinely grown in LB broth (Condalab) at 37°C. *R. erythropolis* strains were incubated in LB broth, or Basal Salt Medium (BSM) (del Olmo *et al.*, 2005) at 30°C. Media, when required, were supplemented with appropriate antibiotics at the following concentrations: ampicillin (Amp, 100 μg ml^{-1}), chloramphenicol (Cm, 34 μg ml^{-1}) and kanamycin (Km, 30 μg ml^{-1}). Bacteriological agar was used as gelling agent (VWR). A stock solution of 20 mg ml^{-1} thiostrepton (Sigma-Aldrich) in glacial acetic acid was prepared and added to cultures at 1 μg ml^{-1} to induce gene expression under the *tipA* promoter (P$_{tipA}$).

DNA manipulations

Routine DNA manipulations were performed using standard procedures unless otherwise indicated. Oligonucleotides were synthesized by StabVida. Plasmids were purified using a Macherey Nagel plasmid purification kit. FastDigest restriction enzymes, Phusion DNA polymerase and Rapid DNA ligation kit (Thermo Scientific, Waltham, MA, USA) were used according to the instructions of the manufacturer. All constructed plasmids were confirmed by Sanger sequencing.

Plasmids were transformed in *E. coli* and *R. erythropolis* by electroporation (0.1 cm cuvette; 200 Ω, 25 μF, 1.25 kV; Bio-Rad Gene Pulser X-Cell electroporator). Rhodococcal electrocompetent cells were generated as follows. *R. erythropolis* cells were incubated in LB medium for 48 h at 30°C, diluted 1:200 in 100 ml of fresh LB broth and grown at 30°C, 200 r.p.m, to an optical density

(OD$_{595nm}$) of 0.7–0.8. Cells were cooled down on ice for 30 min, and then, washed twice, first with 50 ml and second with 2 ml of ice-cold 10% glycerol. Cells were finally resuspended in 250 μl of ice-cold 10% glycerol and stored in aliquots at −80°C.

Construction of plasmids containing different genetic determinants involved in biofilm formation

To produce PIA/PNAG in *R. erythropolis*, the *icaADBC* operon of *Staphylococcus aureus* was amplified using oligonucleotides 1 and 2 and genomic DNA of the *S. aureus* 15981 strain as template (Valle *et al.*, 2003). Plasmid pTipQC1 (Nakashima and Tamura, 2004) was digested with NcoI and BamHI enzymes and the insert was directionally cloned into pTipQC1 using the In-Fusion HD Cloning Kit (Takara), leading to pTipQC1::*icaADBC*.

The gene encoding for the diguanylate cyclase AdrA was amplified from the genome of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) 3934 *adrA*-3xFLAG strain (Solano *et al.*, 2009), using primers 13 and 17. The PCR product was cloned into the pJET 1.2 vector (ThermoFisher Scientific), and then subcloned into the pTipQC1 plasmid digested with NcoI and XhoI enzymes leading to plasmid pTipQC1::*adrA*. An *adrA* mutated version, coding for a 3xFLAG tagged AdrA carrying a D290N substitution, was cloned into pTipQC1 following the same strategy as the one detailed above with the use of genomic DNA from the strain *S.* Enteritidis 3934 *adrA* D290N-3xFLAG (Table 1).

Construction of the dsz-adrA plasmid for enhanced biodesulfurization and biofilm formation

The synthetic *dszB1A1C1-D1* cassette was amplified from plasmid pIZdszB1A1C1-D1 (Martínez *et al.*, 2016) using primers 40 and 41. The PCR product was cloned

Table 2. Plasmids and oligonucleotides used in this study.

Plasmid	Relevant characteristics	Reference/source
pET46-Ek/LIC::*rbap_B*	Plasmid for rBap_B heterologous expression and production	(Taglialegna *et al.*, 2016b)
pRC3	Plasmid for Dispersin B heterologous expression and production	(Ramasubbu *et al.*, 2005)
pJET1.2/blunt	Cloning vector	Thermo Scientific
pIZdszB1A1C1-D1	pIZ1016 derivative plasmid expressing a synthetic *dszB1A1C1-D1* cassette	(Martínez *et al.*, 2016)
pTipQC1	Shuttle vector *Escherichia coli- Rhodococcus erythropolis*	(Nakashima and Tamura, 2004)
pTipQC1::*icaADBC*	pTipQC1 containing the *icaADBC* operon of *S. aureus* 15981 under the control of the inducible *tipA* promoter	This study
pTipQC1::*adrA*	pTipQC1 containing the *adrA* gene of *S.* Enteritidis 3934 tagged with the 3xFLAG epitope under the control of the inducible *tipA* promoter	This study
pTipQC1::*adrA* D290N	pTipQC1 containing a mutated *adrA* gene of *S.* Enteritidis 3934, encoding for AdrA D290N and tagged with the 3xFLAG epitope under the control of the inducible *tipA* promoter	This study
pTipQC1::*dsz*-P$_{lac}$D	pTipQC1 containing optimized *dszB1A1C1* genes organized as a transcriptional unit under the control of the inducible *tipA* promoter and also, *dszD1* under the *lac* promoter	This study
pTipQC1::*dsz*-P$_{lac}$D-*adrA*	pTipQC1 containing optimized *dszB1A1C1* genes organized as a transcriptional unit under the control of the inducible *tipA* promoter and also, *dszD1* and the *adrA* gene tagged with the 3xFLAG epitope under the *lac* promoter	This study
pTipQC1::*dsz*-P$_{lac}$D-*adrA*-*mCherry*	pTipQC1 containing optimized *dszB1A1C1* genes organized as a transcriptional unit under the control of the inducible *tipA* promoter and also, *dszD1*, the *adrA* gene tagged with the 3xFLAG epitope and the *mCherry* gene under the *lac* promoter	This study

Oligonucleotide	Sequence[a]
1	AGGAGATATACCATGGGTTGCAATTTTTTAACTTTTTGCTTTTTTATCCTG
2	AGAGATCTAAGCTTGGATCCCCACTCCCATTGGCATTTACGA
13	**CCATGG**GCATGTTCCCAAAAATAATGAATG
17	**CTCGAG**TTACTATTTATCGTCGTCATC
40	**GAATTC**AAGCTTCTCGAGACGCGTTT
41	**GGATCC**ACTAGTTACGTAATCGATTT
42	**GGATCC**TTTGTTTAACTTTAAGAAGGAGATATACC
44	**GGATCC**TTTATCGTCGTCATCTTTGTAG
496	GATGACGACGATAAATAGTAACTCGAGAGGAGGTGAATAATGGTGAGCAAGGGCGAGGA
497	GG**GGATCC**TTACTTGTACAGCTCGTCCA

a. Restriction enzymes sites and overlapping sequences for In-Fusion HD cloning are indicated in bold and underlined format, respectively.

into the pJET 1.2 vector and then subcloned into the pTipQC1 plasmid digested with EcoRI and BamHI enzymes, leading to plasmid pTipQC1::*dsz*-P$_{lac}$D. *adrA*-3xFLAG together with the phage T7 gene 10 ribosome binding site (LG10-RBS) was amplified from plasmid pTipQC1::*adrA* using primers 42 and 44. The PCR product was cloned into the pJET 1.2 vector and then subcloned into pTipQC1::*dsz*-P$_{lac}$D digested with BamHI, leading to plasmid pTipQC1::*dsz*-P$_{lac}$D-*adrA*.

Biofilm assays

The B region of the biofilm associated protein (Bap) from *Staphylococcus aureus* was produced and purified as described elsewhere (Taglialegna *et al.*, 2016b). This region polymerizes to form amyloid-like fibres under specific environmental conditions (acidic pH and low concentrations of calcium) to build the biofilm matrix. To induce bacterial aggregation and biofilm formation

through the B region of Bap, a previously described protocol was used (Taglialegna *et al.*, 2016b). Briefly, an overnight culture of *R. erythropolis* IGTS8 was diluted 1:100 in fresh LB medium containing glucose (0.25% w/v) and rBap_B 2 μM was added. The culture was incubated for 24 h at 30°C, 200 rpm and then, biofilm formation was visually inspected.

To analyse PIA/PNAG and c-di-GMP mediated biofilms, overnight cultures of *R. erythropolis* IGTS8 carrying pTipQC1, pTipQC1::*icaADBC*, pTipQC1::*adrA*, pTipQC1::*adrA* D290N, pTipQC1::*dsz*-P$_{lac}$D or pTipQC1::*dsz*-P$_{lac}$D-*adrA* and grown in LB Cm were diluted 1:100 in 4 ml of fresh LB Cm medium and incubated for 12 h at 30°C, 200 r.p.m. Thiostrepton was then added, cultures were further incubated for 12 h at 30°C, 200 r.p.m., and biofilm formation was visually examined.

Biofilm formation under flow conditions was analysed using microfermenters (Pasteur Institute's laboratory of Fermentation) with a continuous 40 ml h^{-1} flow of LB

Cm and constant aeration with sterile compressed air (0.3 bar) (Ghigo, 2001). Polystyrene and silicone rubber disc coupons (Biosurface Technologies Corporation) were fixed to the internal microfermenter glass slides (spatulas) and served as biofilm substrata. Microfermenter starter cultures of *R. erythropolis* pTipQC1::*dsz*-P*lac*D and *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* strains were grown in LB Cm for 24 h at 30°C and 200 r.p.m., and then diluted 1:100 in fresh LB Cm and incubated again for 24 h at 30°C, 200 r.p.m. Each microfermenter was subsequently inoculated with 250 µl of the corresponding strain. Biofilm development was recorded after 48 h of incubation at 30°C.

Construction of a reporter strain expressing mCherry and biofilm CLSM imaging

To construct a *R. erythropolis* biofilm forming strain expressing the gene encoding the red fluorescent protein mCherry, *adrA*-3xFLAG together with the phage T7 gene 10 ribosome binding site (LG10-RBS) was amplified from plasmid pTipQC1::*adrA* using primers 42 and 44. The gene encoding mCherry was amplified from pAD$_1$-cCherry (Balestrino *et al.*, 2010) using primers 496 and 497. The two aforementioned PCR fragments were fused through overlapping PCR using primers 42 and 497, cloned into the pJET 1.2 vector and then subcloned into pTipQC1::*dsz*-P*lac*D digested with BamHI, leading to plasmid pTipQC1::*dsz*-P*lac*D-*adrA*-*mCherry*.

Plasmid pTipQC1::*dsz*-P*lac*D-*adrA*-*mCherry* was transformed into *R. erythropolis* IGTS8. The resulting strain was grown under flow conditions using the microfermenter system described above and a polystyrene disc coupon as substrate. After biofilm development, the coupon was rinsed with PBS and placed upside down on a 35 mm glass bottom dish (1.5 coverslip, 14 mm glass diameter, uncoated; MatTek). Confocal laser scanning microscopy (CLSM) images were acquired at 0.27 µm z intervals using a Zeiss LSM 800 confocal system. mCherry was detected using a diode laser for excitation at 561 nm and emission from 565 to 700 nm was collected. Images were obtained using a 63×/1.4 objective. The biofilm images were processed using Zeiss Zen software (Zeiss).

Phenotype on Congo red agar plates

Colony morphology on Congo red agar plates to monitor extracellular matrix production was evaluated using a modified medium from Freeman *et al.* (1989) (30 g l^{-1} tryptic soy broth (Condalab), 15 g l^{-1} agar, 0.8 g l^{-1} Congo red (Sigma) and 20 g l^{-1} sucrose). Strains were grown on LB Agar Cm for 72 h at 30°C, cells were harvested and resuspended in sterile water to an optical density (OD$_{595nm}$) of 0.5–0.6 and then, 20 µl drops were spotted on Congo red plates supplemented with Cm, with and without the thiostrepton inducer. Plates were incubated for 48 h at 30°C.

PIA/PNAG and AdrA detection by immunoblotting

R. erythropolis pTipQC1 and *R. erythropolis* pTipQC1:: *icaADBC* were grown in LB Cm for 24 h at 30°C, and then diluted 1:100 in fresh LB Cm and incubated for 12 h at 30°C, 200 r.p.m. Thiostrepton was added and cultures were further incubated for 12 h at 30°C, 200 r.p.m. 2 ml of each culture were collected and PIA/PNAG was extracted and detected as described (Cramton *et al.*, 1999; Valle *et al.*, 2003). Briefly, cultures were centrifuged at 18 000 × *g* for 5 min. Pellets were resuspended in 50 µl of 0.5 M EDTA (pH 8.0) and suspensions were incubated for 5 min at 100°C and centrifuged at 18 000 × *g* for 5 min. Each supernatant (40 µl) was incubated with 10 µl of proteinase K (20 mg ml^{-1}) (Sigma) for 30 min at 37°C. After the addition of 10 µl of Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.4]) containing 0.01% bromophenol blue, 5 µl were spotted on a nitrocellulose membrane using a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was blocked overnight with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween 20, and incubated for 2 h with specific anti-PNAG antibodies diluted 1:10 000 (Maira-Litrán *et al.*, 2005). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immuno-globulin G antibodies (Jackson ImmunoResearch Laboratories) diluted 1:10 000 and developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). All extracts were analysed on the same membrane.

Correct expression and translation of *adrA* in *R. erythropolis* pTipQC1::*adrA*, *R. erythropolis* pTipQC1::*adrA* D290N and *R. erythropolis* pTipQC1::*dsz*-P*lac*D-*adrA* was monitored by Western blot as follows. Cultures were grown in LB Cm for 24 h at 30°C, and then diluted 1:100 in fresh LB Cm and incubated for 12 h at 30°C, 200 r.p.m. Thiostrepton was added and cultures were incubated for 12 h at 30°C, 200 r.p.m. 4 ml of each culture were collected, centrifuged at 20 800 × *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 × *g* and 4°C and supernatants were collected. For normalization purposes, protein quantification was carried out using a Bradford protein assay kit (Bio-Rad). Equal amounts of protein per sample were loaded on a 12% stain-free acrylamide

gel (Criterion Bio-Rad). Proteins were transferred onto Hybond-ECL nitrocellulose membranes (GE Healthcare) by electroblotting and then, membranes were blocked overnight in PBS containing 0.1% Tween 20 and 5% skimmed milk under shaking conditions and incubated with monoclonal anti-FLAG antibodies labelled with horseradish peroxidase (Sigma) diluted 1:1000 in blocking solution for 2 h at room temperature. Bands were developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

c-di-GMP extraction and measurement

Cultures were grown in LB Cm for 24 h at 30°C, and then diluted 1:100 in fresh LB Cm and incubated for 12 h at 30°C, 200 r.p.m. Thiostrepton was added and cultures were incubated for 12 h at 30°C, 200 r.p.m. 4 ml of each culture were collected and c-di-GMP extraction was carried out following a protocol described in (Bähre and Kaever, 2017). c-di-GMP measurements were performed by HPLC-coupled tandem mass spectrometry (LC-MS/MS). A duplicate of each culture was used to determine protein content for normalization purposes. Duplicates were centrifuged at $20\,800 \times g$ for 5 min and pellets were washed twice and resuspended in 1 ml HEPES buffer 50 mM pH 8.0. 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 $20\,800 \times g$ and 4°C and supernatants were collected. A Bradford assay kit (Bio-Rad) was used for protein quantification. Intracellular levels of c-di-GMP from biological triplicates are shown as mean values of picomoles of c-di-GMP per milligram of protein.

Desulfurization assays

Cultures were grown in LB Cm for 24 h at 30°C, and then diluted 1:100 in fresh LB Cm and incubated for 24 h at 30°C, 200 r.p.m. Cultures were collected and washed twice with BSM. Bacterial pellets were resuspended in 50 ml BSM Cm in a 250 ml Erlenmeyer flask at an OD_{595nm} of 0.1. Since cell cultures expressing AdrA showed an aggregated phenotype, normalization was further adjusted based on protein quantification. Cultures were grown for 24 h at 30°C, 200 r.p.m., and then thiostrepton was added to induce gene expression under the P_{tipA} promoter. Cultures were further incubated for 24 h at 30°C and cells were collected by centrifugation and washed twice with 50 mM HEPES buffer pH 8.0. Bacterial pellets were resuspended (30 ml) in 50 mM HEPES buffer pH 8.0 at an OD_{595nm} of 1. Normalization was further adjusted based on protein quantification. Then, each culture was split in three samples

of 10 ml each in 100 ml Erlenmeyer flasks. These resting cells preparations were used for the BDS assays, which were performed after adding 25 μM DBT (25 mM stock solution in acetonitrile), at 200 rpm and 30°C. At 0, 15 and 60 min of incubation, each ten ml sample was mixed with an equal volume of acetonitrile and centrifuged at $14\,000 \times g$ for 10 min to be analysed by high-performance liquid chromatography (HPLC). All BDS experiments were conducted in triplicate.

Desulfurization activity was analysed by calculating the 2HBP specific production rate defined as the quantity of 2HBP produced per hour per gram of dry cell weight [μmol$_{2HBP}$/g DCW/h] and conversion efficiency [$X_{2HBP}^{time\,(min)} = (C_{2HBP}^{time\,(min)}/C_{DBT}^{0}) \times 100$] defined as the ratio of the 2HBP produced to the initial concentration of DBT (del Olmo *et al.*, 2005).

HPLC was used as described (Martínez *et al.*, 2016) to analyse the concentration of DBT and 2HBP employing a C18 column (Teknokroma C18 150 × 4.6 mm, 5 μm particles). The mobile phase was a mix of acetonitrile/water (55:45) at 1 ml min^{-1} flow rate. Peaks were monitored at 234 nm for DBT and 206 nm for 2HBP. Calibrations were performed using highly purified standards of each compound.

Statistics

Statistical analyses were performed with the GRAPHPAD PRISM 5.01 program. For single comparisons, data were analysed using an unpaired, two-tailed Student's *t* test. For multiple comparisons, a one-way analysis of variance (ANOVA) with Bonferroni or Dunnett's posttest was used. In all tests, P values of less than 0.05 were considered statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Conflict of interests

The authors declare no conflict of interest regarding the results of this research.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. 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 harbouring plasmid pTipQC1, pTipQC1::*adrA*, pTipQC1::*adrA* D290N or pTipQC1::*dsz*-P$_{lac}$D-*adrA* were grown in LB Cm for 24 h at 30°C, and then diluted 1:100 in fresh LB Cm and incubated for 12 h at 30°C, 200 r.p.m. Thiostrepton was added and cultures were incubated for 12 h at 30°C, 200 r.p.m. The control non-induced cultures were also incubated for 12 h at 30°C, 200 r.p.m. 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.

Fig. 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 h at 30°C and 200 r.p.m. The biofilm attached to the well walls was treated for 2 h 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.