ENGINEERING SYNTHETIC BACTERIAL CONSORTIA FOR ENHANCED DESULFURIZATION AND REVALORIZATION OF OIL SULFUR COMPOUNDS

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ABSTRACT

The 4S pathway is the most studied bioprocess for the removal of the recalcitrant sulfur of aromatic heterocycles present in fuels. It consists of three sequential functional units, encoded by the *dszABCD* genes, through which the model compound dibenzothiophene (DBT) is transformed into the sulfur-free 2-hydroxybiphenyl (2HBP) molecule. In this work, a set of synthetic *dsz* cassettes were implanted in *Pseudomonas putida* KT2440, a model bacterial “chassis” for metabolic engineering studies. The complete *dszBIAIC1-D1* cassette behaved as an attractive alternative — to the previously constructed recombinant *dsz* cassettes — for the conversion of DBT into 2HBP. Refactoring the 4S pathway by the use of synthetic *dsz* modules encoding individual 4S pathway reactions revealed unanticipated traits, e.g., the 4S intermediate 2HBP-sulfinate (HBPS) behaves as an inhibitor of the Dsz monooxygenases, and once secreted from the cells it cannot be further taken up. That issue should be addressed for the rational design of more efficient biocatalysts for DBT bioconversions. In this sense, the construction of synthetic bacterial consortia to compartmentalize the 4S pathway into different cell factories for individual optimization was shown to enhance the conversion of DBT into 2HBP, overcome the inhibition of the Dsz enzymes by the 4S intermediates, and enable efficient production of unattainable high added value intermediates, e.g., HBPS, that are difficult to obtain using the current monocultures.

1. Introduction

Crude oils contain undesirable contaminant molecules, such as thiophenic aromatics compounds, which have a negative impact on oil processing and pose serious environmental threats (Soleimani et al., 2007). A wide spectrum of desulfurization technologies have been developed to remove sulfur mainly from finished refinery products (Stanislaus et al., 2010). Hydrodesulfurization (HDS) treatment has proved to be the common technology of choice to reduce the level of sulfur in crude oil products. Significant environmental, technical and economic limitations have been reported in applying the HDS process (Babich and Moulijn, 2003).

During the past 30 years, research to develop alternative desulfurization technologies resulted in a biotechnological strategy to eliminate sulfur from thiophenic compounds (biodesulfurization (BDS)) via serial reactions known as the 4S pathway (Gray et al., 2003; Gupta et al., 2005; Kilbane, 2006; Monticello, 2000; Nuhu, 2013; Xu et al., 2009). This pathway was firstly reported in the gram-positive bacterium *Rhodococcus erythropolis* IGTS8 (Gallagher et al., 1993), but the 4S pathway has been also found in other bacteria (Duarte et al., 2001; Kilbane, 2006; Mohebbi and Ball, 2008).

The 4S pathway provides a nondestructive oxidative process used by the cells to obtain the sulfur required for growth, which involves the transformation of dibenzothiophene (DBT), the model compound for sulfur heterocycles present in oil and refractory to HDS, into 2-hydroxybiphenyl (2HBP) and sulfite (Fig. 1A) (Gallagher et al., 1993). Sulfite is further assimilated via its oxidation into sulfate by a sulfite oxidoreductase (Aggarwal et al., 2012). In the 4S pathway, DBT undergoes three successive oxidation steps leading to the formation of DBT-sulfoxide (DBTO), DBT-

sulfone (DBTO₂) and 2HBP-sulfinate (HBPS) mediated by two monooxygenases (DszC and DszA), followed by a hydrolytic step for sulfur removal as sulfite, and formation of 2HBP, which is mediated by a desulfinase (DszB). The three oxidation steps require reducing equivalents (FMNH₂) supplied by a flavin-reductase (DszD) (Fig. 1A) (Gray et al., 1996). The genetics of the 4S pathway has been elucidated in *R. erythropolis* IGTS8 being coded by three megaplasmid-born genes (*dszABC*) and one chromosomal-born gene (*dszD*) (Fig 1B) (Gray et al., 1996).

Attempts to remove sulfur from crude oil based on the 4S pathway have identified two major technical bottlenecks, which need to be overcome to develop a feasible bioprocess for sulfur removal in the oil industry. These major bottlenecks are bioprocess-related hurdles to develop a continuous bioprocess, and biocatalyst-related limitations to develop robust biocatalysts with a high catalytic desulfurization rate. Since low amounts of sulfur are required for bacterial growth, and the native regulation of *dsz* gene expression is sulfur-dependent (Li et al., 1996), the *dsz* genes are expressed at low level in almost all naturally occurring bacteria harboring the 4S pathway, which leads to low rates of DBT desulfurization from an industrial point of view (Kilbane, 2006). Moreover, decay of the BDS activity due to the accumulation of the sulfur-free end product 2HBP in bacterial biocatalysts has been reported (Abin-Fuentes et al., 2013). It has been shown that bacteria harboring identical *dsz* genes provide different BDS rates due to the influence of the host metabolism (Kilbane, 2006). To enhance the catalytic rate of DBT desulfurization, the genetic determinants of the 4S pathway have been the target for enormous genetic boosting strategies, including: i) expression under control of strong promoters that alleviate sulfur-dependent transcriptional regulation ; ii) removal of *dszAB* gene overlap, iii) cloning of multiple copies of *dsz* genes; iv) cloning of more efficient versions of *dszD*; v) genetic rearrangement of *dsz* genes to have better

expression and optimized catalytic activities; vi) enhancing the level of expression of *dszB*, that encodes the rate-limiting step of the 4S pathway, by mutating its '5 untranslated region; vii) adding a synthetic gene coding for a polypeptide of sulfur-containing amino acids to increase sulfur consumption; viii) cloning *dsz* genes in different robust hosts; and ix) gene shuffling as well as other directed evolution methods on the Dsz enzymes (Kilbane, 2006; Li et al., 2008; Ma et al., 2006; Shavandi et al., 2009; Wang et al., 2011). Despite all these successful trials to produce genetically improved biocatalysts, so far, none of the deployed gene manipulation techniques has resulted in a BDS rate meeting the industrial requirement for the development of a commercial BDS process (Boniek et al., 2015; Kilbane, 2006). Moreover, no real efforts have been taken to solve HBPS and 2HBP-related feedback inhibition of the BDS process (Abin-Fuentes et al., 2013). Since the 4S pathway consists of three serial and irreversible functional modules (Fig. 1A) that can be successfully expressed in several heterologous hosts unable to desulfurize DBT (Diaz and Garcia, 2010), it constitutes an ideal target for improvement through synthetic biology and metabolic engineering approaches.

In the present study, we developed a collection of synthetic *dsz* cassettes and “à la carte” bacterial consortia to overcome bottlenecks of the 4S pathway and enhance DBT bioconversions into unattainable chemicals.

2. Materials and methods

2.1. Chemicals

Chemicals used in this work, including DBT and DBTO₂, were from Sigma. 2HBP was purchased from Fluka. HBPS was synthesized as described in previous studies (Hanson and Kemp, 1981; Oldfield et al., 1997).

2.2. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are listed in Table 1. Bacterial strains were grown in lysogeny broth (LB) medium (Sambrook and Russell, 2001) at 30°C (*P. putida* and *Rhodococcus* strains) or 37°C (*E. coli* strains). When required, the strains were grown at 30°C in Basal Salt Medium (BSM) composed of the following: NaH₂PO₄·H₂O 4 g L⁻¹; K₂HPO₄·3H₂O 4 g L⁻¹; NH₄Cl 2 g L⁻¹; MgCl₂·6H₂O 0.0245 g L⁻¹; CaCl₂·2H₂O 0.001 g L⁻¹; FeCl₃·6H₂O 0.001 g L⁻¹; MgSO₄·7H₂O 0.444 g L⁻¹; glycerol 2.45 g L⁻¹; 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and glutamic acid 20 g L⁻¹ (Martin et al., 2004). When appropriate, antibiotics were added at the following concentrations: ampicillin (100 µg mL⁻¹), kanamycin (50 µg mL⁻¹), gentamicin (10 µg mL⁻¹), and tetracycline (10 µg mL⁻¹).

2.3. Molecular biology techniques

Standard molecular biology techniques were performed as previously described (Sambrook and Russell, 2001). Plasmid DNA was prepared with a High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIOgene). Oligonucleotides were supplied by Sigma. All cloned inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled

dideoxynucleotide terminators (Sanger et al., 1977) and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). Transformation of bacterial cells was carried out by electroporation (Gene Pulser; Bio-Rad) (Sambrook and Russell, 2001). The protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as the standard. Nucleotide sequence analyses were done at the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov>).

2.4. Construction of *dsz* cassettes

A complete *dszB1A1C1-D1* cassette was synthesized by ATG:biosynthetics GmbH (Merzhausen, Germany) and supplied as a 4.6 kb EcoRI/XbaI DNA fragment (the nucleotide sequence of this cassette was submitted to GenBank with Accession number KR270527) cloned into the EcoRI/XbaI double digested pUC-derived pGH vector, or into the broad-host range plasmid pIZ1016 (Table 1), giving rise to plasmids pGHdszB1A1C1-D1 (Table 1) and pIZdszB1A1C1-D1 (Table 1, Fig. 1C), respectively. The *dszB1A1-D1*, *dszC1-D1* and *dszB1* cassettes were constructed by StuI-, XhoI- and ClaI-digestion and further re-ligation of the pIZdszB1A1C1-D1 plasmid, respectively, giving rise to plasmids pIZdszB1A1-D1, pIZdszC1-D1 and pIZdszB1 (Table 1, Fig. 1C). The *dszA1C1-D1* cassette was constructed by MluI-digestion and further re-ligation of pGHdszB1A1C1-D1 plasmid, and the resulting 3.5-kb EcoRI/XbaI *dszA1C1-D1* cassette was subcloned into the EcoRI/XbaI double digested pIZ1016 vector to generate plasmid pIZdszA1C1-D1 (Table 1, Fig. 1C). The *dszA1-D1* cassette was constructed by StuI-digestion and further re-ligation of pIZdszA1C1-D1 giving rise to plasmid pIZdszA1-D1 (Table 1, Fig. 1C). All synthetic *dsz* cassettes are flanked by unique restriction enzymes that allow its expression under different regulatory systems.

In the pIZ1016-derived plasmids, the *dsz* cassettes are expressed under control of the IPTG-dependent P_{tac} , $P_{lac}/lacI^{\Delta}$ regulatory system, and these expression modules can be transferred as NotI DNA cassettes (Fig. 1C).

2.5. *Biocatalysts production and resting cells assays*

P. putida cells were cultured 12 h at 30 °C with shaking in LB medium containing the appropriate antibiotic, i.e., gentamicin for cells harboring pIZ1016-derived plasmids, and tetracycline for cells harboring the pESOX3 plasmid. These cultures were used to inoculate 50 mL of BSM medium containing the appropriate antibiotics in a 250 mL Erlenmeyer flask at an initial A_{600} of 0.1. To induce the expression of the *dsz* genes under the P_{tac} promoter, 1 mM IPTG was added to the medium. Cultures were incubated 24 h at 30 °C. *R. erythropolis* IGTS8 cells were cultured as previously described (del Olmo et al., 2005). Cells were harvested by centrifugation, washed twice with 0.9% NaCl, and the cell pellet was resuspended (20 mL) in 50 mM HEPES buffer pH 8.0 at an A_{600} of 2.0 in a 100 mL Erlenmeyer flask. This resting cells preparation was used for the BDS assays, which were carried out in an orbital shaker at 30 °C and 200 rpm after adding 4S compounds (DBT, DBTO₂ or HBPS). Samples were collected periodically and, unless otherwise specified, extracted with an equal volume of acetonitrile for HPLC analysis of substrate consumed and product(s) generated after centrifugation at 14000 x g for 10 min. To check the extracellular location of some 4S-pathway intermediates, resting cells were centrifuged at 14000 x g for 10 min and the supernatant was extracted with acetonitrile and analyzed by HPLC. Three/four different biological samples were used to calculate substrate consumption and intermediates/product formation in all experiments reported in this work.

2.6. DszB assays in cell extracts

For the DszB assays using crude cell extracts, *P. putida* KT2440 (pIZdszB1) cells were grown as detailed above (section 2.5), washed twice with 0.9% NaCl and resuspended in 50 mM HEPES buffer pH 8.0 at an A_{600} of 20, and disrupted by passage through a French press operated at a pressure of 20000 psi. Cell debris were removed by centrifugation at 16000 g for 20 min at 4°C, and the resulting supernatant was used as crude cell extract. DszB assays were carried out after adding HBPS to the cell extract in a 100 mL Erlenmeyer flask that was incubated in an orbital shaker at 30 °C and 200 rpm. Samples were collected periodically and extracted with an equal volume of acetonitrile for analysis of HBPS consumed and 2HBP generated.

2.7. Flavin reductase assay

Flavin reductase activity was measured in crude cell extracts of *P. putida* KT2440 (pIZdszB1A1C1-D1) and *P. putida* KTH2 (pESOX3) strains were obtained as detailed above (section 2.6). Flavin reductase activity was detected by a spectrophotometric assay measuring the oxidation of NADH at 340 nm ($\epsilon = 6.22 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (Galan et al., 2000a). The reaction consists of 200 μM NADH, 10 μM FMN in 25 mM sodium phosphate buffer pH 7.4 containing 100 mM NaCl and 0.5 mM DTT (Gray et al., 1996), in a final volume of 0.7 mL. The assay was initiated by adding 0.03 mL of the enzyme extract and was run at 22°C during a controlled period of time.

2.8. Analytical methods

Absorbance at 600 nm (A_{600}) was measured using a Shimadzu UV-Vis spectrophotometer (model UV mini 1240). HPLC was employed to analyze the

concentration of the 4S compounds (DBT, DBTO₂, HBPS and 2HBP) employing a C18 column (Teknokroma C18 150 x 4.6 mm, 5 µm particles); the mobile phase was a mixture acetonitrile/water (55:45) at 1 mLmin⁻¹ flow rate. Peaks were monitored at different wave lengths (234 nm for DBT and DBTO₂; 206 nm for 2HBP). HBPS was monitored at 206 nm after acidification of the sample with HCl (75 µL/mL). Calibrations were performed using highly purified standards of each compound.

3. Results and discussion

3.1. *The dszBIAIC1-D1 cassette enhances conversion of DBT into 2HBP*

Based on the sequence of the *R. erythropolis* IGTS8 *dsz* genes (Gray et al., 1996; Oldfield et al., 1997; Piddington et al., 1995; Tao et al., 2011), we have synthesized modified genes, called *dszA1*, *dszB1*, *dszC1* and *dszD1*, to engineer a synthetic *dszBIAIC1-D1* cassette (Fig. 1C). This was achieved with the following: i) the native *dszABC* operon was rearranged to avoid overlapping genes and to increase the expression of the *dszB* gene that encodes the rate-limiting step of the 4S pathway; ii) the native gene expression signals that are under sulfur-dependent control were substituted by optimized transcriptional (based on the *lacI^q/P_{lac}* and *lacI^q/P_{lac}* regulatory couples) and translational (consensus ribosome-binding site, optimized codon usage) signals for *Pseudomonas* strains that are not subject of sulfate-dependent inhibition; iii) the Y63F amino acid substitution — previously reported to enhance the activity and stability of the DszB desulfinate (Ohshiro et al., 2007) — was engineered in the synthetic *dszB1* gene; iv) a synthetic *dszD1* gene — encoding the flavin reductase needed for the two monooxygenase reactions of the 4S pathway and that is not linked to the *dszABC* genes in strain IGTS8 (Fig. 1B) — was included in the *dsz* cassette. To avoid the potential toxic effect of an increased expression of the *dszD* gene (Galan et al., 2000a), it was arranged as an independent operon under control of the weak *P_{lac}* promoter and separated from the *dszBIAIC1* operon by a strong transcriptional terminator of the lambda phage (Fig. 1C). The modularity of the *dsz* synthetic cassette was facilitated by removing some restriction enzyme sites present in the native *dsz* genes and by adding other sites that allow the easy removal/insertion of the synthetic genes in different vectors and under control of different regulatory elements (Fig. 1C). The *dszBIAIC1-*

D1 gene cassette was cloned and expressed in a broad-host range vector, plasmid pIZ1016, able to replicate in a wide variety of gram-negative bacteria (Moreno-Ruiz et al., 2003), giving rise to plasmid pIZdszB1A1C1-D1 (Fig. 1C; Table 1).

The functionality of the *dszB1A1C1-D1* cassette was tested in *P. putida* KT2440, a bacterium that has been proposed as an optimal bacterial "chassis" endowed with metabolic, physiological and stress-endurance traits of biotechnological interest (Nikel et al., 2014). In a resting cell process, *P. putida* KT2440 (pIZdszB1A1C1-D1) converted all DBT to nearly stoichiometric amounts of 2HBP after 180 min incubation (Fig. 2), hence demonstrating that the *dszB1A1C1-D1* cassette was fully and efficiently functional. When we compared these results with those achieved by using *P. putida* KTH2 (pESOX3), a recombinant *P. putida* strain expressing the native *dszABC* genes (Galan et al., 2000a), we observed an almost 3-fold increase in the production of 2HBP (Fig. 2B), suggesting that the *dszB1A1C1-D1* synthetic module behaves as an attractive and efficient alternative to previous recombinant *dsz* cassettes.

The rate of consumption of DBT was lower when using the *P. putida* KT2440 (pIZdszB1A1C1-D1) biocatalyst than when using the *P. putida* KTH2 (pESOX3) strain (Fig. 2A), suggesting that a higher rate of DBT consumption leads to a lower efficiency of 2HBP formation, as previously pointed out (Reichmuth et al., 2000). Since DBT removal is due to the DszC activity (Fig. 1A), and this monooxygenase depends on the FMNH₂ cellular levels, the flavin reductase activity levels of the host cell become critical for an efficient initial oxidation of DBT. In this sense, whereas *P. putida* KT2440 (pIZdszB1A1C1-D1) cells express a *dszD* gene encoding the cognate Dsz flavin reductase under control of the weak *P_{lac}* promoter, the *P. putida* KTH2 (pESOX3) cells express the *hpaC* gene encoding a heterologous flavin reductase under control of the stronger *P_{tac}* promoter (Galan et al., 2000b). When we monitored the

flavin reductase activity of both biocatalysts, we observed that it was $1.49 \cdot 10^{-2} \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ in *P. putida* KTH2 (pESOX3) and $5.08 \cdot 10^{-3} \mu\text{mol min}^{-1} \text{mg protein}^{-1}$ in *P. putida* KT2440 (pIZdszB1A1C1-D1). Thus, these results confirm a significantly higher flavin reductase activity in the *P. putida* KTH2 (pESOX3) strain which, in turn, is in agreement with the higher DBT removal rate observed with this biocatalyst (Fig. 2A). Although the molecular basis of this finding is still unknown, the fast oxidation of DBT may lead to the transient accumulation of intermediates that act as Dsz enzyme inhibitors, causing a lower 2HBP formation (Abin-Fuentes et al., 2013). All of these results suggest that the tight control of the expression of the *dszD* flavin reductase gene in the *dszB1A1C1-D1* cassette is critical to designing an efficient process for complete bioconversion of DBT into 2HBP.

3.2. Design of a collection of functional dsz cassettes for bioconversions of 4S-pathway compounds: identifying unanticipated bottlenecks

A major bottleneck of the current 4S-pathway expressing biocatalysts is the observed reduction of DBT oxidation when 2HBP accumulates (Abin-Fuentes et al., 2013), hence suggesting that the BDS process could be improved by engineering an artificial 4S pathway, where the enzymatic steps were compartmentalized in different host biocatalysts for individual optimization, and to avoid exposure of the Dsz enzymes to the inhibitory effects of the 4S pathway intermediates. To accomplish this unprecedented approach in BDS, three synthetic DNA cassettes containing single *dsz* genes (*dszB1*), or combinations of two (*dszA1-D1*, *dszC1-D1*) or three (*dszA1C1-D1*, *dszB1A1-D1*) *dsz* genes, were constructed (Fig. 1C). These *dsz* cassettes were introduced into *P. putida* KT2440 and their functionality was studied in resting cell assays.

The *P. putida* KT2440 cells expressing the *dszC1-D1* cassette completely converted DBT into DBTO₂ in about 30 min (Fig. 3A). Remarkably, the rate of DBT removal was higher with a biocatalyst expressing the *dszC1-D1* cassette (Fig. 3A) than with a biocatalyst expressing the complete *dszB1A1C1-D1* module (Fig. 2A), pointing out the inhibitory effect of 4S-pathway compounds on the activity of DszC monooxygenase (Abin-Fuentes et al., 2013). To study further this inhibitory effect *in vivo*, we monitored the conversion of DBT into HBPS by using the *dszA1C1-D1* cassette (Fig. 1C). As shown in figure 4A, a high initial removal rate of DBT was observed, but the reaction slowed down after 15 min and 30% of the substrate (DBT) remained in the medium at the end of the resting cell process. These results, therefore, confirmed *in vivo* the inhibitory effects of HBPS on the DszC activity observed *in vitro* (Abin-Fuentes et al., 2013).

The *dszA1-D1* cassette (Fig. 1C) completely converted DBTO₂ into HBPS in about 120 min (Fig. 3B). A faster removal of DBTO₂ was observed by using the *dszB1A1-D1* cassette (Fig. 1C) that is also able to convert HBPS into 2HBP (Fig. 4B). Thus, these results reveal a previously unknown inhibitory effect of HBPS on the activity of the DszA monooxygenase.

Interestingly, when 25 μ M HBPS was added to *P. putida* KT2440 (pIZdszB1) cells, no production of 2HBP was observed even after 180 min of incubation of the resting cells, and all HBPS was recovered in the extracellular medium (Fig. 5A), suggesting that the *dszB1* module (Fig. 1C) was not functional in a resting cell assay. To confirm that this behavior was due to a lack of uptake of HBPS inside the biocatalyst rather than to an inactive DszB1 enzyme, cell free crude extracts of *P. putida* KT2440 (pIZdszB1) were used, and an efficient conversion of HBPS into 2HBP was observed (Fig. 5B). Thus, this result confirms that the *dszB1* cassette is functional but only when

used as a cell-free biocatalyst, suggesting that *P. putida* whole cells are unable to uptake HBPS. To explore whether HBPS could be transported inside other bacterial biocatalysts, we checked the behavior of *R. erythropolis* IGTS8 resting cells in the presence of HBPS. Again, all HBPS was recovered in the extracellular medium (data not shown), suggesting that this 4S-pathway intermediate is unable to enter Rhodococcal cells.

Analysis of the extracellular medium of *P. putida* KT2440 (pIZdszB1A1-D1) resting cells incubated with DBTO₂ for 180 min (Fig. 4B) revealed that both 2HBP (about 8 μ M) and HBPS (about 15 μ M, data not shown) were present outside the cells. This observation is in agreement with the fact that HBPS is easily secreted to the medium and, as indicated above, unable to enter again into the cells for its further biotransformation to 2HBP. In summary, these results highlight the importance of HBPS secretion as a previously unrecognized bottleneck that may reduce the conversion of DBT into 2HBP when using whole cell biocatalysts. Thus, it seems critical to coordinate the production of HBPS with its transformation to 2HBP, since the over-accumulation of HBPS in the cytoplasm may conduct to its premature release to the medium.

3.3. Enhancing conversion of DBT into 2HBP by engineering synthetic bacterial consortia

As suggested above, both formation and consumption of HBPS must be precisely programmed within a single biocatalyst. Moreover, we have demonstrated that the rate of DBT and DBTO₂ removal can be significantly enhanced, with respect to that obtained with the complete *dszB1A1C1-D1* synthetic module, by using the *dszC1-D1* (Fig. 3A) and *dszB1A1-D1* (Fig. 4B) cassettes, respectively. Hence, to increase the

bioconversion of DBT into 2HBP we designed an artificial 4S pathway based on the construction of synthetic bacterial consortia. This approach of sequential desulfurization using segregated steps of the 4S pathway should also avoid the inhibitory effect that HBPS and 2HBP exert on DszC in monocultures (Abin-Fuentes et al., 2013).

A first bacterial consortium was made of *P. putida* KT2440 (pIZdszC1-D1) cells, which convert DBT into DBTO₂ (Fig. 3A), and *P. putida* KT2440 (pIZdszB1A1-D1) cells, which convert DBTO₂ into 2HBP (Fig. 4B). We have observed that a high rate of DBT consumption leads to a lower production of 2HBP due to the formation of HBPS that inhibits the DszC and DszA monooxygenases (Fig. 4); therefore to generate the most efficient consortium we tested different ratios of both biocatalysts for optimizing the rate of DBTO₂ formation and consumption. As shown in Fig. 6A, when the consortium was arranged as a *P. putida* KT2440 (pIZdszC1-D1): *P. putida* KT2440 (pIZdszB1A1-D1) cell ratio of 2:1, the efficiency of 2HBP formation (about 15%) was even lower than that obtained when using the *P. putida* KT2440 (pIZdszB1A1C1-D1) biocatalyst (about 20%). This result is in agreement with our previous observation that a fast oxidation of DBT to DBTO₂ would turn into a high amount of HBPS that will become toxic and block the whole Dsz pathway. On the contrary, when we shifted the cell ratio in the consortium by increasing the amount of the *P. putida* KT2440 (pIZdszB1A1-D1) biocatalyst with respect to that of the *P. putida* KT2440 (pIZdszC1-D1) biocatalyst, we observed a higher 2HBP formation efficiency than with a similar amount of the *P. putida* KT2440 (pIZdszB1A1C1-D1) biocatalyst, reaching values of 2HBP formation up to 50% at a cell ratio of 1:4 (Fig. 6A). Thus, these results reveal that generating an artificial 4S pathway by combining the *dszC1-D1* and *dszB1A1-D1* cassettes in a balanced synthetic bacterial consortium is a new and promising strategy to increase bioconversion of DBT into 2HBP.

We have shown above that the *dszB1A1-D1* cassette is able to efficiently oxidize DBTO₂ but it only synthesizes 35% 2HBP due to the fact that a significant amount of HBPS is secreted to the culture medium and then it cannot enter the cell anymore (Fig. 4B). To overcome this bottleneck for improving further the efficiency of the 4S artificial pathway, it would be desirable to recover and channel the secreted HBPS for 2HBP formation. Since the *dszB1* cassette that converts HBPS into 2HBP does not require reducing power and, therefore, it is fully active in cell-free extracts (Fig. 5B), we tested the combination of cell-free extracts obtained from the *dszB1* module with the optimized *P. putida* KT2440 (pIZdszC1-D1): *P. putida* KT2440 (pIZdszB1A1-D1) (1:4) bacterial consortium and also with *P. putida* KT2440 carrying the complete cassette (pIZdszB1A1C1-D1). As shown in Fig. 6B, when DszB1 extracts were added to the target bacterial consortium an almost full conversion of DBT into 2HBP was achieved. However, when DszB1 extract was added to the complete cassette, it was not possible to achieve a complete conversion (Fig. 6B) since about 30 μ M DBT was still remaining in the medium probably due to the inhibitory effect of HBPS and 2HBP on the DszC activity. Taken together all these results, we can conclude that the rational combination of appropriated *dsz* cassettes in synthetic bacterial consortia becomes a suitable alternative to the use of naturally existing or recombinant *dsz* pathways (Diaz and Garcia, 2010; Galan et al., 2000a; Gray et al., 2003; Kilbane, 2006; Li et al., 2008; Ma et al., 2006; Xu et al., 2009) for developing a process toward the bioproduction of 2HBP from DBT.

The specific desulfurization activity reached with the microbial consortia presented in this work is 23 μ mol_{2HBP}/g DCW/h. This value is the highest of those described previously in the literature, i.e., 21.6 (Davoodi et al., 2010), 11.9 (Martín et al., 2005), 8.9 (del Olmo et al., 2005), 3.6 (Alves and Paixao, 2014) and 2.1 (Rashtchi et

al., 2006) $\mu\text{mol}_{\text{HBPS}}/\text{g DCW/h}$, when using microbial resting cell cultures in aqueous phase. Further research to increase bioconversion efficiencies may be focused on the problem of desulfurizing HBPS in medium broth. Several strategies can be addressed and may imply either: i) the use of immobilized cell-free extracts or purified DszB enzyme into, for instance, magnetic beads would allow its recovery for a continuous BDS process; ii) the development of bioreactors with membranes coated with DszB; iii) the development of a bacterial chassis permeable to HBPS which could allow the uptake of HBPS into the cells, e.g. engineering a membrane channel similar to those described for hydrocarbons or phenols uptake (Parales et al., 2008); iv) engineering an active extracellular DszB enzyme anchored to the bacterial surface of the biocatalyst.

3.4. Developing bacterial synthetic consortia for the production of HBPS

Segregating the steps of the 4S pathway can be used to terminate the BDS at the level of some intermediates, which are unattainable by monocultures and can be used as precursors for other petrochemicals (e.g., DBTO₂ and HBPS). Since HBPS is not commercially available and it is of biotechnological interest for the chemical synthesis of biosurfactants (Monticello, 2000; Xu et al., 2009), we have used our synthetic cassettes to develop an efficient cellular system for the conversion of DBT into HBPS. As shown in Fig. 4A, a biocatalyst expressing the *dszAIC1-D1* cassette is able to convert DBT into HBPS but this conversion is far from being complete due to the inhibitory effects of HBPS on the DszC/DszA monooxygenases. Therefore, we proposed that the combination of the *dszC1-D1* cassette, that efficiently converts DBT into DBTO₂ (Fig. 3A), with the *dszAI-D1* cassette, which converts DBTO₂ into HBPS (Fig. 3B), by generating a synthetic bacterial consortium made of the *P. putida*

(pIZdszC1-D1) and *P. putida* (pIZdszA1-D1) biocatalysts, could be a rational strategy to enhance HBPS production. Hence, we tested different ratios of both biocatalysts in resting cell experiments to settle the most efficient bacterial consortium. As shown in Fig. 7, when we increased the amount of the *P. putida* KT2440 (pIZdszA1-D1) biocatalyst with respect to that of the *P. putida* KT2440 (pIZdszC1-D1) biocatalyst, the conversion of DBT to HBPS was enhanced, and we already achieved full conversion at a *dszC1-D1:dszA1-D1* ratio of 1:1. These results demonstrate that the compartmentalization of the monooxygenase-dependent steps of the 4S pathway for DBT desulfurization in two different cell biocatalysts and their further combination to generate an artificial bacterial consortium constitutes a successful strategy toward the efficient conversion of DBT into the high added value HBPS compound.

4. Conclusions

In this work, a new functional *dszBIAIC1-D1* synthetic cassette has been designed, demonstrating that it behaves as an attractive and efficient alternative to the previously constructed recombinant *dsz* cassettes (Diaz and Garcia, 2010; Galan et al., 2000a; Gray et al., 2003; Kilbane, 2006; Li et al., 2008; Ma et al., 2006; Xu et al., 2009) for the bioconversion of DBT into 2HBP. We have shown that a higher rate of DBT consumption leads to a lower efficiency of 2HBP formation, which agrees with the fact that some 4S intermediates, such as HBPS, behave as inhibitors of the DszC and DszA enzymes. Thus, a tight control of the expression of the *dszD* flavin reductase gene becomes critical to control the activity of the two Dsz monooxygenases and, hence, to design an efficient BDS. Moreover, our results highlight the importance of reducing the HBPS release to the extracellular medium due to the impossibility of its further uptake by the bacterial cells. This previously unrecognized bottleneck will reduce the conversion of DBT into 2HBP when using whole cell biocatalysts. All these previously unanticipated limitations should be taken into consideration when designing efficient DBT bioconversion processes.

To overcome some of these limitations, we have engineered for the first time the 4S enzymatic steps as independent synthetic cassettes that are functional both alone and by combination. Our work revealed that generating an artificial 4S pathway by combining the *dszC1-D1* and *dszBIA1-D1* cassettes in an optimized synthetic bacterial consortium, and the *dszB1* cassette as a cell-free extract, is a new and promising alternative to the use of naturally existing or recombinant *dsz* pathways for enhanced conversion of DBT into 2HBP. The compartmentalization of the monooxygenase-dependent steps of the 4S pathway for DBT desulfurization in two different cell

biocatalysts and their further combination to generate an artificial bacterial consortium constitutes a successful strategy toward the efficient conversion of DBT into the high added value HBPS compound. The *in vitro* evolution or isolation of new Dsz enzymes (Coco et al., 2001; Kamali et al., 2010) not susceptible to the inhibitions caused by the 4S intermediates are future challenges that should be taken into consideration for enhancing DBT bioconversions.

As far as we know, this work represents the first example of using synthetic consortia (Bacchus and Fussenegger, 2013; Du et al., 2013; Kalyuzhnaya et al., 2015; Ortiz-Marquez et al., 2014; Zargar et al., 2015) to compartmentalize the 4S pathway into different hosts for individual optimization, and for demonstrating that through division of labor, synthetic consortia can overcome process limitations difficult to achieve when using monocultures. Since the *dsz* cassettes reported here have been engineered in broad-host range plasmids, they can be tested and optimized in a wide range of potential gram-negative biocatalysts of predictable metabolic background and that can survive operating conditions in bioreactors, which makes them appealing as valuable genetic tools for DBT bioconversions in microbial cell factories (Sauer and Mattanovich, 2012).

This new strategy will facilitate further improvements of the BDS process to achieve the target desulfurization efficiencies (1-3 mmol₂HBP/g DCW/h) that have been reported to be needed for industrial applications (Kilbane, 2006). The compartmentalization or segregation of functional units of the 4S pathway should also facilitate and support the development of faster fuel desulfurization hybrid technologies which could involve combination of biological and physicochemical methods, e.g., the *dszC1D1* harboring biocatalysts could be used to efficiently convert DBT into the more polar DBTO₂ which could be easily separated from the fuel and further desulfurized by

physicochemical methods; the DBTO₂ delivered by oxidative desulfurization technologies (Campos-Martin et al., 2004) could be efficiently desulfurized by using the *dszBIA1-D1* harboring biocatalysts.

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TABLES

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant genotype-phenotype	Reference/Source
<i>E. coli</i> DH10B	F ⁺ , <i>mcrA</i> Δ(<i>mrr hsdRMS-mcrBC</i>) φ80d <i>lac</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> λ <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Invitrogen
<i>P. putida</i> KT2440	Wild-type strain	(Franklin et al., 1981)
<i>P. putida</i> KTH2	Km ^r , <i>P. putida</i> KT2442 with a chromosomal insertion of the <i>E. coli</i> <i>hpaC</i> gene	(Prieto et al., 1996)
<i>R. erythropolis</i> IGTS8	Wild-type strain	(Kilbane and Jackowski, 1992)
Plasmid	Description	Reference/Source
pESOX3	Tc ^r , broad-host range plasmid that expresses the native <i>dszABC</i> operon from <i>R. erythropolis</i> IGTS8 under control of the <i>lacI^f/Ptac</i> regulatory system	(Gallardo et al., 1997)
pGHdszB1A1C1-D1	Ap ^r , pUC derivative carrying the synthetic <i>dszB1A1C1-D1</i> cassette	This work
pIZ1016	Gm ^r , <i>ori</i> pBBR1 Mob ⁺ , <i>lacZα</i> , <i>Ptac/lacI^f</i> , broad- host-range cloning and expression vector	(Moreno-Ruiz et al., 2003)
pIZdszB1A1C1-D1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszB1A1C1-D1</i> cassette	This work
pIZdszB1A1-D1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszB1A1-D1</i> cassette	This work
pIZdszA1C1-D1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszA1C1-D1</i> cassette	This work
pIZdszC1-D1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszC1-D1</i> cassette	This work
pIZdszA1-D1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszA1-D1</i> cassette	This work
pIZdszB1	Gm ^r pIZ1016 derivative expressing the synthetic <i>dszB1</i> gene	This work

FIGURE LEGENDS

Fig.1. The 4S pathway for DBT desulfurization, its genetic determinants, and design of *dsz* cassettes. (A) Scheme of the 4S pathway for DBT desulfurization. The three functional modules are based on the activity of the DszC monooxygenase, DszA monooxygenase and DszB desulfinase, and they are shown by blue, green and yellow boxes, respectively. Reducing power (FMNH₂) needed for the activity of the DszC and DszA monooxygenases is provided by the DszD flavin reductase (orange box). The metabolites are as follows: dibenzothiophene (DBT), dibenzothiophene sulfoxide (DBTO), dibenzothiophene sulfone (DBTO₂), 2-(2-hydroxybiphenyl) sulfinic acid (HBPS), 2-hydroxybiphenyl (2HBP). (B) Organization of the *dsz* genes encoding the 4S pathway in a megaplasmid (*dszABC*) and in the chromosome (*dszD*) of *R. erythropolis* IGTS8. The color code of the *dsz* genes correspond to that of the enzymatic reactions in panel A. *P_{dsz}*, represents the promoter of the *dszABC* operon. (C) Scheme of the pIZ1016-derivative plasmids harbouring the different *dsz* cassettes. The arrows indicate the direction of transcription of the genes. The colour code of the synthetic *dsz* genes correspond to that of the enzymatic reactions in panel A. Only relevant restriction sites are shown (E, EcoRI; H, HindIII; N, NotI; S, SacI; Sn, SnaBI; Sp, SpeI; X, XbaI). The transcriptional regulatory elements, i.e., *lacI^q/Ptac* and *Plac* are shown by black and grey arrows, respectively. Tλ, indicates a transcriptional terminator of the lambda phage. The genes encoding gentamicin resistance (Gm^r), and the plasmid replication (*rep*) and mobilization (*mob*) functions are shown by white arrows.

Fig. 2. The *dszB1A1C1-D1* cassette is functional in *P. putida*. DBT consumption (A) and 2HBP formation (B) by *P. putida* KT2440 (pIZdszB1A1C1-D1) (filled symbols) and *P. putida* KTH2 (pESOX3) (empty symbols) in resting cells assays containing 25 μ M of DBT as initial sulfur substrate.

Fig. 3. Checking the functionality of the *dszC1-D1* and *dszA1-D1* cassettes. Evolution of the 4S route compounds in resting cells of *P. putida* KT2440 containing plasmids pIZdszC1-D1 (A) or pIZdszA1-D1 (B). Resting cell assays were performed by using 25 μ M of DBT (A) or 25 μ M DBTO₂ (B) as initial sulfur substrates. C_j indicates the concentration of the monitored compound.

Fig. 4. Checking the functionality of the *dszA1C1-D1* and *dszB1A1-D1* cassettes. Evolution of the 4S route compounds in resting cells of *P. putida* KT2440 containing plasmids pIZdszA1C1-D1 (A) or pIZdszB1A1-D1 (B). Resting cell assays were performed by using 25 μ M of DBT (A) or 25 μ M DBTO₂ (B) as initial sulfur substrates. C_j indicates the concentration of the monitored compound.

Fig. 5. Checking the functionality of the *dszB1* cassette. Bioconversion of HBPS into 2HBP in resting cells (A) and cell extracts (B) of the *P. putida* KT2440 (pIZdszB1) biocatalyst. Assays were performed using 25 μ M of HBPS as initial sulfur substrate. C_j indicates the concentration of the monitored compound.

Fig. 6. Conversion of DBT into 2HBP by synthetic bacterial consortia. (A) Experiments were carried out in resting cells using different ratios of biocatalysts *P. putida* KT2440 containing plasmid pIZdszC1-D1 (C1-D1) and *P. putida* KT2440

containing plasmid pIZdszB1A1-D1 (B1A1-D1) (grey bars), achieving a final A_{600} of 2.0 and with an initial concentration of DBT of 100 μ M. Similar resting cells experiments were carried out with the single biocatalyst *P. putida* KT2440 containing plasmid pIZdszB1A1C1-D1 (B1A1C1-D1) (white bar). (B) Addition of crude cell extracts from *P. putida* KT2440 containing plasmid pIZdszB1 (0.8 mg_{protein}/mL) to the *P. putida* KT2440 (pIZdszC1-D1): *P. putida* KT2440 (pIZdszB1A1-D1) (1:4) bacterial consortium (grey sparse bar) and to the *P. putida* KT2440 (pIZdszB1A1C1-D1) (white sparse bar). The conversion efficiency is indicated as the percentage of 2HBP production after 360 min of the reaction assay according to equation $X_{2HBP}^{360} = \frac{C_{2HBP}^{360}}{C_{DBT}^0} \times 100$

Fig. 7. Conversion of DBT into HBPS by synthetic bacterial consortia. Experiments were carried out in resting cells using different ratios of biocatalysts *P. putida* KT2440 containing plasmid pIZdszC1-D1(C1-D1) and *P. putida* KT2440 containing plasmid pIZdszA1-D1 (A1-D1) (black bars), at a final A_{600} of 2.0 and with an initial concentration of DBT of 100 μ M. Similar resting cells experiments were carried out with the single biocatalyst *P. putida* KT2440 containing plasmid pIZdszA1C1-D1 (A1C1-D1) (white bar). The conversion efficiency is indicated as the percentage of HBPS production after 360 min of the reaction assay according to equation $X_{HBPS}^{360} = \frac{C_{HBPS}^{360}}{C_{DBT}^0} \times 100$

FIGURES

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

Fig. 7