NOTES

Designing Recombinant Pseudomonas Strains To Enhance Biodesulfurization

MARIA ESTHER GALLARDO,1 ABEL FERRÁNDEZ,1 VÍCTOR DE LORENZO,2 JOSÉ L. GARCÍA,1 AND EDUARDO DÍAZ1*

Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28006 Madrid,1 and Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco, 28049 Madrid,2 Spain

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The dsz biodesulfurization cluster from Rhodococcus erythropolis IGTS8 has been engineered under the control of heterologous broad-host-range regulatory signals to alleviate the mechanism of sulfur repression, and it was stably inserted into the chromosomes of different Pseudomonas strains. The recombinant bacteria were able to desulfurize dibenzothiophene more efficiently than the native host. Furthermore, these new biocatalysts combine relevant industrial and environmental traits, such as production of biosurfactants, with the enhanced biodesulfurization phenotype.

Combustion of fossil fuels leads to the release of toxic sulfur dioxide into the environment, contributing significantly to air pollution and being the principal cause of acid rain (10, 17). As a result of concern over sulfur emissions, environmental regulations require the use of fossil fuels with low sulfur content, but worldwide reserves of such a low-sulfur crude are being depleted. Most of the sulfur in petroleum is in the form of heterocyclic organic compounds, which are recalcitrant to the current chemical method for sulfur removal (hydrodesulfurization) (17). Thus, a cost-effective alternative to this chemical process is needed. Since biocatalytic processes are cheap, operate under mild conditions, and are endowed with high selectivity, there has been considerable effort to develop bioprocesses for fossil fuel desulfurization (10).

Dibenzothiophene (DBT) is generally considered as the model compound for sulfur heterocycles present in hydrodesulfurization-treated fuel (10). Although a significant number of organisms have been found to remove sulfur from DBT via a hydrocarbon degradative pathway, such a means of sulfur removal involves the destruction of carbon-carbon bonds and thus results in an unacceptable reduction of fuel value (10, 17). However, a small number of microorganisms, mainly Rhodococcus, Bacillus, Corynebacterium, and Arthrobacter species (9, 11, 15), have been shown to remove sulfur from DBT via a sulfur-specific pathway, selectively cleaving sulfur from DBT without ring destruction and therefore maintaining the fuel content. Most probably, all these strains possess the desulfurization pathway that has been already characterized for Rhodococcus erythropolis IGTS8 (formerly R. rhodochrous IGTS8 [12], ATCC 53968). Three catabolic genes, dszA, -B, and -C (formerly named sox), responsible for DBT desulfurization are clustered on a 120-kb linear plasmid of strain IGTS8 (5). These genes have been cloned and sequenced, and their products have been characterized (5, 19). As shown in Fig. 1, enzyme DszC catalyzes two consecutive monooxygenation reactions that convert DBT to DBT-sulfone (14). Subsequently, DszA, a second flavomonooxygenase, and DszB, a desulfinate that catalyzes the rate-limiting step in the pathway, transform DBT-sulfone into 2-hydroxybiphenyl (HBP) and sulfur (Fig. 1) (6). Both terminal oxygenases, DszC and DszA, require an NAD(P)H-flavin mononucleotide oxidoreductase as a source of reducing equivalents (6, 22). Although HBP is not further metabolized and accumulates in the medium, the inorganic sulfur released can be used by the bacterium as the sole source of sulfur. In accordance with the physiological role of Dsz activity, the expression of the dsz gene cluster is strongly repressed by sulfate and sulfur-containing amino acids, and the promoter and associated regulatory regions have been characterized (15).

Up to now, only gram-positive microorganisms, mainly R. erythropolis IGTS8, have been used to develop a commercial biodesulfurization process (6). In this work, we describe a genetically engineered dsz cluster that can be stably maintained and efficiently expressed in different Pseudomonas strains.

Since previous reports showed that R. erythropolis IGTS8 dsz gene products were stable and active in Escherichia coli (5, 19), we used this bacterium as the host for construction of a recombinant dsz cassette whose expression was under control of a heterologous promoter and which thus lacked the native sulfur repression mechanism. A 3.8-kb DreI-SnaBI DNA fragment from plasmid pSAD225-32 (5) spanning the dszABC cluster without the promoter and associated regulatory regions (15) was subcloned under the control of the E. coli lac promoter into the HincII-digested pUC18 (20) cloning vector, resulting in plasmid pESOX1 (Fig. 2). To check the ability of the recombinant cells to desulfurize DBT and use this substrate as the sole source of sulfur for growth, a sulfur bioavailability test (19) was performed. E. coli JM109 (20) transformants harboring plasmids pESOX1 or pUC18 were incubated with shaking at 30°C in liquid basal salts medium (BSM) (5) supplemented with thiamine (1 μg ml⁻¹), ampicillin (100 μg ml⁻¹), and 0.2% glycerol and DBT as the sole carbon and
sulfur sources, respectively. Whereas cells containing plasmid pESOX1 were able to grow, control cells harboring plasmid pUC18 did not grow even after extended incubation (10 days). To confirm that plasmid pESOX1 conferred a desulfurization phenotype on E. coli JM109 cells, with the concomitant formation of HBP, culture broth samples were mixed extensively with ethanol (1:1 [vol/vol]) to solubilize any water-insoluble intermediates. Supernatants of the extracted preparations were analyzed by reversed-phase high-performance liquid chromatography (HPLC) by using a Lichrosphere 5 RP-8 column and an isocratic flow of a 50% acetonitrile–H2O mobile phase pumped at a flow rate of 1 ml/min. Two peaks with retention times of 20.1 and 7.4 min, corresponding to those of DBT and HBP standards, respectively, were monitored at 248 nm (data not shown). All these results indicated that the EcoR1-HindIII dsz cassette was functional in E. coli and that it could be used to engineer the expression of the catabolic dsz-ABC genes under the control of heterologous transcriptional regulatory elements.

To check whether the DszABC proteins were also active in other gram-negative bacteria, the dsz cassette was subcloned into the broad-host-range plasmid pVL731 (3) under the control of a hybrid promoter, Ptac, that has been shown to be functional in a wide range of bacteria (7). The resulting plasmid, pESOX3 (Fig. 2), conferred on isopropyl-β-D-thiogalactopyranoside-induced E. coli JM109 cells a Dsz phenotype similar to that reported above for cells harboring plasmid pESOX1. Since plasmid pESOX3 contains the RP4-mediated mobilization functions, it can be transferred to different recipient cells by triparental filter matings (4) by using E. coli JM109 (pESOX3) as the donor and E. coli HB101(pRK600) as the helper. Control strains can be obtained in a similar way by using E. coli JM109(pVL731) as the donor. Pseudomonas strains were used as recipients since they are among the best-studied and most abundant microorganisms found in crude oil (13), and a wide variety of genetic tools are now available for their rational manipulation (4). Furthermore, several properties of biotechnological interest for design of biocatalysts targeted to industrial processes such as biodesulfurization are present in Pseudomonas species. For example, while the solvent tolerance of Rhodococcus is the lowest reported (log P values from 6.0 to 7.0), that of the genus Pseudomonas (log P values from 3.1 to 3.4) is the highest known (8), and several Pseudomonas strains highly resistant to heavy metals present in fossil fuels have been reported (2). Pseudomonas putida KT2442 (4) cells harboring plasmid pESOX3 or pVL731 were selected at 30°C on Luria-Bertani (20) plates containing tetracycline (20 μg ml⁻¹) and rifampin (50 μg ml⁻¹). The sulfur bioavailability assay indicated that P. putida KT2442 (pESOX3) was able to use DBT as the sole sulfur source. Moreover, HPLC analysis of whole cells showed the accumulation of HBP (data not shown), suggesting that the strain was not able to further metabolize this compound. In accordance with these results, no growth was detected when the recombinant Pseudomonas strain was incubated in BSM liquid medium supplemented with tetracycline (20 μg ml⁻¹), 2 mM SO₄²⁻ as the sulfur source, and 0.2% DBT as the sole carbon source. As expected, P. putida KT2442(pVL731) control cultures were unable to use DBT as the sole sulfur source. These data, taken together, indicated that the dsz cluster is also functional in gram-negative bacteria other than E. coli.

In the development of engineered strains with potential industrial or environmental applications, a high degree of predictability in their performance and behavior is desirable. To achieve this goal, the stable chromosomal insertion of the gene(s) conferring the new trait is required. Therefore, to demonstrate whether this Ptac-driven dsz cassette was also functional in a single copy when stably inserted into the chromosome of the host cell, we constructed plasmid pESOX4 (Fig. 2). This plasmid is a PBSL118 (1) derivative that carries, within a mini-Tn5 transposon, the Ptac::dsz fusion together with a gene that confers resistance to kanamycin. This construction allows the stable integration of the heterologous DNA segment into the chromosomes of a variety of gram-negative bacteria. To integrate the dsz cluster into the chromosome of P. putida KT2442, we performed triparental filter matings by using E. coli CC118 and pESOX4 as the donor and E. coli HB101(pRK600) as the helper. P. putida KT2442::miniTn5Km(Ptac::dsz) transconjugants were selected at 30°C on M63 minimal medium (16) agar plates supplemented with 0.2% citrate and kanamycin (50 μg ml⁻¹), and they were analyzed by performing a sulfur bioavailability test. All transconjugants checked were able to use DBT as the sole sulfur source, and one, hereafter designated P. putida EGSOX, was selected for further studies (see below).

To improve the biodesulfurization process, it would be interesting to design a recombinant biocatalyst that combines the Dsz phenotype with another trait of potential interest, such as the production of biosurfactants. To accomplish this goal, we have used a soil strain, P. aeruginosa PG201 (18), that cannot use DBT as the sole carbon and/or sulfur source and produces rhamnolipid biosurfactants, which are of increas-
ing industrial relevance because of their applications in emulsification, wetting, phase separation, and viscosity reduction (18). Plasmid pESOX4 was used to insert the Ptac::dsz cassette into the chromosome of P. aeruginosa PG201; however, as P. aeruginosa PG201 is kanamycin resistant, the selection of recombinant cells after triparental filter mating was carried out on BSM liquid medium supplemented with 0.2% citrate and DBT. The selection was performed in liquid medium because the agar contains trace amounts of sulfur that allow growth of Dsz⁺ strains. After 5 days of incubation at 30°C, growth was observed and several P. aeruginosa PG201::miniTn5Km(Ptac::dsz) transconjugants were isolated on M63 minimal medium plates supplemented with 0.2% citrate. The desulfurization phenotype of one of these transconjugants, hereafter designated P. aeruginosa EGSOX, was confirmed by a sulfur bioavailability test. Like the parental strain, P. aeruginosa EGSOX formed halos on blue-agar plates (21), confirming the production of extracellular rhamnolipid biosurfactants (data not shown).

The absence of plasmids in P. putida EGSOX and P. aeruginosa EGSOX cells, and the results of a Southern blot experiment (Fig. 3), demonstrated the chromosomal location of the Ptac::dsz cassette in both recombinant strains. All these data indicated, therefore, that the dsz cluster was functional when

FIG. 2. Scheme of construction of the pESOX series of plasmids. Plasmid pSAD225-32 has been described previously (5). DNA from R. erythropolis IGTS8 is shown as hatched blocks. Thin arrows indicate the direction of transcription of the genes. Thick arrows represent the promoters. Vector-derived sequences are shown as broken lines. The black blocks indicate the Shine-Dalgarno (SD) region of the dsz cluster. Plac, native promoter of the dsz cluster. The replication (rep and oriR6K) and RP4-mediated mobilization functions (mob and oriTRP4) are also indicated. The letters I and O represent the 19-bp I and O terminal ends of Tn5. tnp*, gene devoid of NotI sites encoding Tn5 transposase. Antibiotic resistances are indicated as follows: Amp, ampicillin; Km, kanamycin; Tet, tetracycline. Only relevant restriction sites are shown.
stably inserted in a single copy into the chromosomes of different *Pseudomonas* strains.

Figure 4 shows the time course of DBT desulfurization by the two recombinant strains, *P. putida* EGSOX and *P. aeruginosa* EGSOX, and the wild-type strain *R. erythropolis* IGTS8.

At 48 h of incubation, cultures of strain IGTS8 still showed the presence of DBT; however, this compound was exhausted by the two engineered *Pseudomonas* strains. *P. aeruginosa* EGSOX showed the fastest metabolism of DBT: while this strain had transformed 95% of the DBT at 24 h of incubation, only 18% of the DBT was transformed by *R. erythropolis* IGTS8. Interestingly, although *P. putida* EGSOX did not show any significant increase in optical density at 600 nm after 24 h of incubation (Fig. 4), it was able to consume 40% of the DBT, a behavior that resembles a resting-cell process. Remarkably, DBT depletions were concomitant with HBP accumulation in all three strains, and the amount of this compound did not decrease during stationary-phase cultivation up to 5 days (data not shown), indicating that, as already reported for strain IGTS8 and other Dsz gram-positive bacteria (9), HBP is a dead-end metabolite that cannot be further catabolized or used as a carbon source. These data demonstrated that the IGTS8-derived Dsz cassette was efficiently expressed, allowing the elimination of sulfur with no loss of the carbon atoms of DBT, both in *P. putida* EGSOX and *P. aeruginosa* EGSOX. Moreover, in comparison with the wild-type *R. erythropolis* IGTS8 strain, the two recombinant biocatalysts described here showed an enhanced biodesulfurization ability.

In summary, we have reported here that the Dsz cluster from the gram-positive bacterium *R. erythropolis* IGTS8 can be engineered as a DNA cassette under the control of heterologous regulatory signals and used in a single copy to expand the ability of some *Pseudomonas* strains to efficiently desulfurize DBT. The alleviation of the native sulfur repression of the Dsz cluster by heterologous regulation and the chromosomal location of Dsz, conferring stability and containment, are relevant features of the recombinant strains described here. Moreover, we have constructed a *Pseudomonas* strain that combines two traits of industrial interest, that is, a desulfurization phenotype and the ability to produce a biosurfactant that should increase the aqueous concentrations of hydrophobic compounds, resulting in higher mass transfer rates in two-liquid-phase bioreactors (18). Nevertheless, since rhamnolipid production by *P. aeruginosa* PG201 occurs with limiting concentrations of nitrogen and iron during late exponential and stationary phases of growth (18), for checking the influence of these surfactants on DBT desulfurization further research will be required. The design of well-suited recombinant biocatalysts endowed with a desulfurization phenotype offers, therefore, new alternatives for the development of commercially viable desulfurization processes.

M.E.G. and A.F. made equal contributions to this work.

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