JOURNAL OF BACTERIOLOGY, Nov. 1997, p. 7156–7160 Vol. 179, No. 22
0021-9193/97/$04.00+0

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 DÍAZ[1]*

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

Received 9 June 1997/Accepted 9 September 1997

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 dioxides 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 desulfinase that catalyzes the rate-limiting step in the pathway, transform DBT-sulfone into 2-hydroxybiphenyl (HBP) and sulfite (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 *Dra*I-*Sna*BI 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 *Hin*cII-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

* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, 28006 Madrid, Spain. Phone: 34-1-5611800. Fax: 34-1-5627518. E-mail: CIBDF4F @fresno.csic.es.

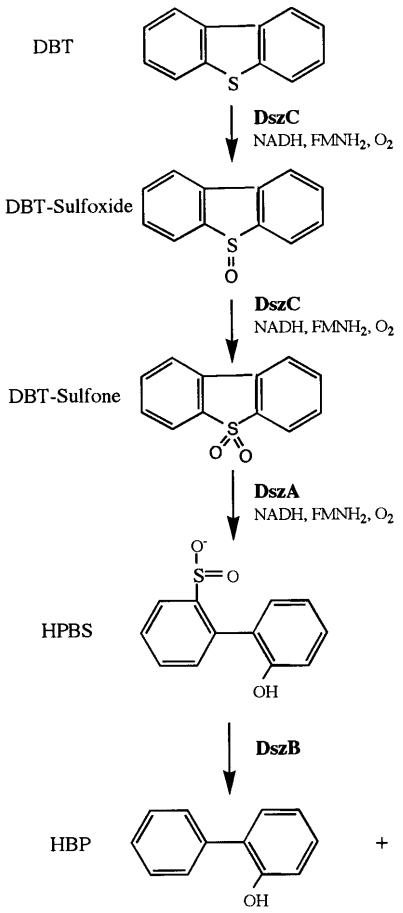

FIG. 1. Proposed pathway for DBT desulfurization by *R. erythropolis* IGTS8. HPBS, 2-(2-hydroxybiphenyl)benzenesulfinate; DszC, DBT monooxygenase; DszA, DBT-sulfone monooxygenase; DszB, HPBS desulfinase.

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–H_2O 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 *Eco*RI-*Hin*dIII *dsz* cassette was functional in *E. coli* and that it could be used to engineer the expression of the catabolic *dszABC* 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 pVLT31 (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(pVLT31) 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 pVLT31 were selected at 30°C on Luria-Bertani (20) agar 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 SO_4^{2-} as the sulfur source, and 0.2% DBT as the sole carbon source. As expected, *P. putida* KT2442(pVLT31) 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 the *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-Tn*5* 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* CC118λpir(pESOX4) as the donor and *E. coli* HB101(pRK600) as the helper. *P. putida* KT2442::miniTn*5*Km(*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-

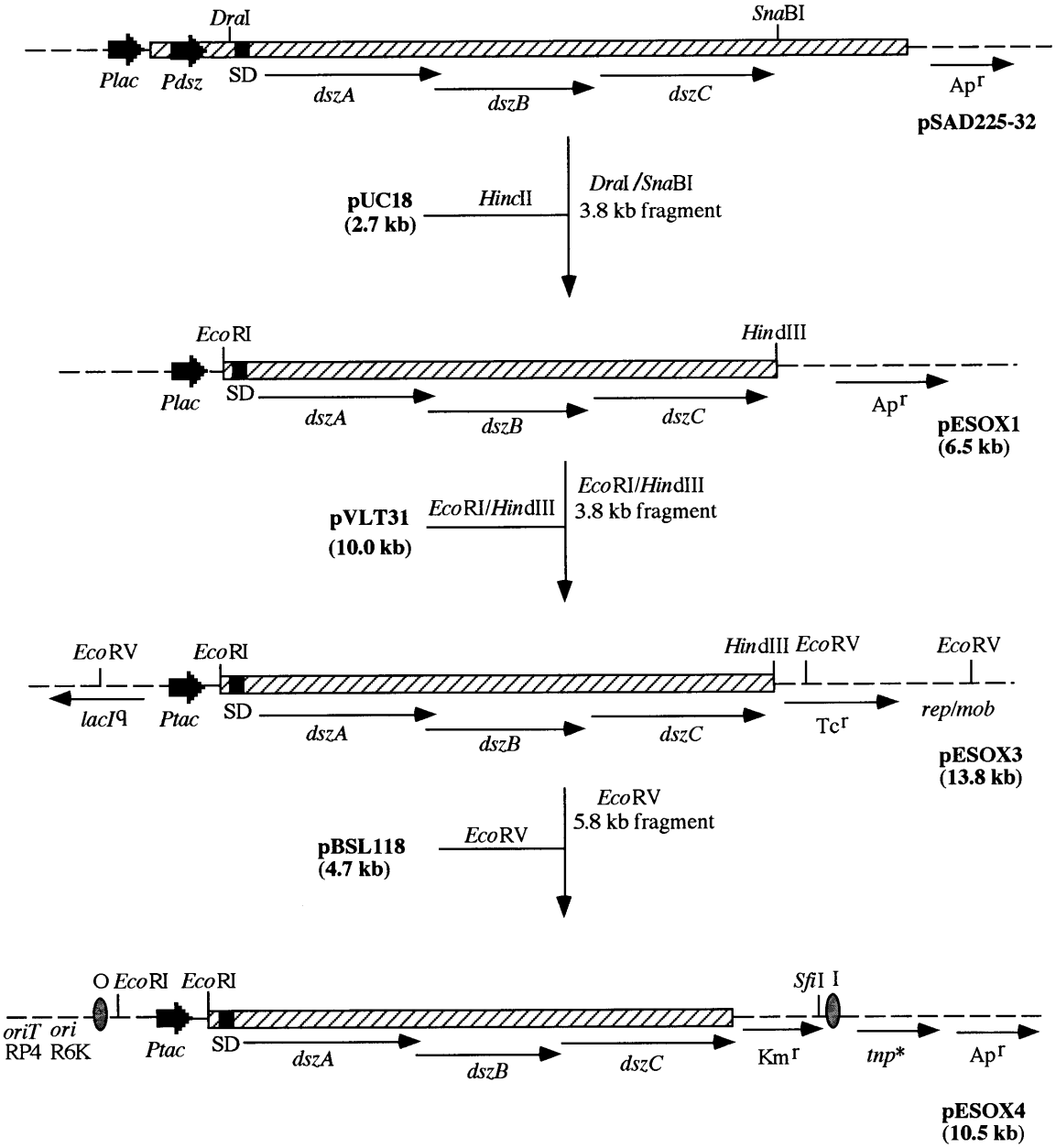

FIG. 2. Scheme of construction of the pESOX series of plasmids. Plasmid pSAD225-32 has been described previously (5). DNA from *R. erythropolis* IGTS8 is shown as hatched blocks. Thin arrows indicate the direction of transcription of the genes. Thick arrows represent the promoters. Vector-derived sequences are shown as broken lines. The black blocks indicate the Shine-Dalgarno (SD) region of the *dsz* cluster. *Pdsz*, native promoter of the *dsz* cluster. The replication (*rep* and *ori*R6K) and RP4-mediated mobilization functions (*mob* and *ori*TRP4) are also indicated. The letters I and O represent the 19-bp I and O terminal ends of Tn*5*. *tnp**, gene devoid of *Not*I sites encoding Tn*5* transposase. Antibiotic resistances are indicated as follows: Ap^r, ampicillin; Km^r, kanamycin; Tc^r, tetracycline. Only relevant restriction sites are shown.

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::miniTn*5*Km(*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

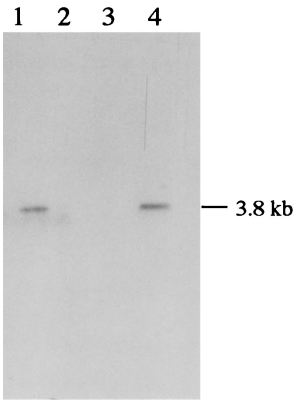

FIG. 3. Southern analysis of chromosomal DNAs from different *Pseudomonas* strains. *Eco*RI/*Hin*dIII double-digested chromosomal DNAs from *P. putida* EGSOX (lane 1), *P. putida* KT2442 (lane 2), *P. aeruginosa* PG201 (lane 3), and *P. aeruginosa* EGSOX (lane 4) were probed with the 3.8-kb *Dra*I/*Sna*BI DNA fragment (genes *dszABC*) from plasmid pSAD225-32 (Fig. 2). The DNA fragment used as a radioactive probe was labeled by the random-primer method (20) with [α-^{32}P]dCTP. Hybridization was performed as described previously (20). The size of the band corresponding to the fragment used as the probe is indicated.

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.

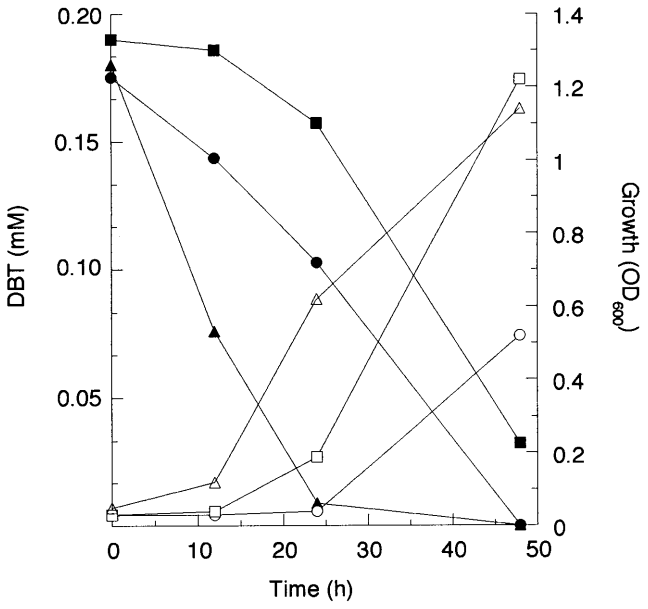

FIG. 4. Cell growth and DBT utilization by *R. erythropolis* IGTS8, *P. putida* EGSOX, and *P. aeruginosa* EGSOX. The strains were grown at 30°C in BSM medium supplemented with 1.5 mM dimethyl sulfoxide (15) and 0.2% glycerol as sulfur and carbon sources, respectively. When the cultures reached an optical density at 600 nm (OD_{600}) of about 2.0, they were diluted to an OD_{600} of about 0.05 into BSM medium supplemented with 0.2% glycerol. Samples (1 ml) were cultivated with shaking in test tubes containing 0.2 mM DBT as the sole sulfur source. After extraction of the samples with 1 volume of ethanol, consumption of DBT was monitored by comparing the peak areas on HPLC with those of standard DBT. Empty symbols indicate growth; closed symbols show DBT concentration. *R. erythropolis* IGTS8, *P. putida* EGSOX, and *P. aeruginosa* EGSOX are represented by squares, circles, and triangles, respectively. Results of one experiment are shown; data were reproducible in three separate experiments.

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 depletion was 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*, providing 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.

We thank S. A. Denome and K. D. Young for sending us plasmid pSAD225-32 and U. Ochsner for providing *P. aeruginosa* PG201.

This work was supported by Repsol S.A. and by CYCYT grant AMB94-1038-CO2-02. M.E.G. and A.F. were the recipients of predoctoral fellowships from Repsol S.A. and the Plan Nacional de Formación de Personal Investigador-MEC, respectively. E.D. was the recipient of a Contrato Temporal de Investigadores-CSIC.

REFERENCES

1. **Alexeyev, M. F., I. N. Shokolenko, and T. P. Croughan.** 1995. New mini-Tn*5* derivatives for insertion mutagenesis and genetic engineering in gram-negative bacteria. Can. J. Microbiol. **41:**1053–1055.
2. **Atlas, R. M.** 1994. Petroleum microbiology. Macmillan Publishing, New York, N.Y.
3. **de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lac*I^q/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
4. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5* and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
5. **Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young.** 1994. Character-

ization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. J. Bacteriol. **176:**6707–6716.
6. **Gray, K. A., O. S. Pogrebinsky, G. T. Mrachko, L. Xi, D. J. Monticello, and C. S. Squires.** 1996. Molecular mechanisms of biocatalytic desulfurization of fossil fuels. Nature Biotechnol. **14:**1705–1709.
7. **Henschke, R. B., and F. R. J. Schmidt.** 1990. Use of wide host range promoters to monitor the fate of recombinant DNA in soil, p. 200–206. *In* J. C. Fry and M. J. Day (ed.), Bacterial genetics in natural environments. Chapman and Hall, London, United Kingdom.
8. **Inoue, A., and K. Horikoshi.** 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J. Ferment. Bioeng. **71:**194–196.
9. **Izumi, Y., T. Ohshiro, H. Ogino, Y. Hine, and M. Shimao.** 1994. Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. Appl. Environ. Microbiol. **60:**223–226.
10. **Kilbane, J. J.** 1989. Desulfurization of coal: the microbial solution. Trends Biotechnol. **7:**97–101.
11. **Kilbane, J. J.** March 1993. U.S. patent 5,198,341.
12. **Kilbane, J. J., and K. Jackowski.** 1992. Biodesulfurization of water-soluble coal-derived material by *Rhodococcus rhodochrous* IGTS8. Biotechnol. Bioeng. **40:**1107–1114.
13. **Leahy, J. G., and R. R. Colwell.** 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54:**305–315.
14. **Lei, B., and S.-C. Tu.** 1996. Gene overexpression, purification, and identification of a desulfurization enzyme from *Rhodococcus* sp. strain IGTS8 as a sulfide/sulfoxide monooxygenase. J. Bacteriol. **178:**5699–5705.
15. **Li, M. Z., C. H. Squires, D. J. Monticello, and J. D. Childs.** 1996. Genetic analysis of the *dsz* promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. J. Bacteriol. **178:**6409–6418.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Monticello, D. J., and W. R. Finnerty.** 1985. Microbial desulfurization of fossil fuels. Annu. Rev. Microbiol. **39:**371–389.
18. **Ochsner, U. A., J. Reiser, A. Fiechter, and B. Witholt.** 1995. Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. Appl. Environ. Microbiol. **61:**3503–3506.
19. **Piddington, C. S., B. R. Kovacevich, and J. Rambosek.** 1995. Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8. Appl. Environ. Microbiol. **61:**468–475.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Siegmund, I., and F. Wagner.** 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnol. Tech. **5:**265–268.
22. **Xi, L., C. H. Squires, D. J. Monticello, and J. D. Childs.** 1997. A flavin reductase stimulates DszA and DszC proteins of *Rhodococcus erythropolis* IGTS8 *in vitro*. Biochem. Biophys. Res. Commun. **230:**73–75.