APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2002, p. 691–698 Vol. 68, No. 2
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.2.691–698.2002

Chemostat Approach for the Directed Evolution of Biodesulfurization Gain-of-Function Mutants

Joseph J. Arensdorf,* A. Katrina Loomis, Philip M. DiGrazia, Daniel J. Monticello, and Philip T. Pienkos

Enchira Biotechnology Corporation, The Woodlands, Texas 77381

Received 7 June 2001/Accepted 14 November 2001

Chemostat enrichment is a classical microbiological method that is well suited for use in directed-evolution strategies. We used a two-phase sulfur-limited chemostat to select for gain-of-function mutants with mutations in the biodesulfurization (Dsz) system of *Rhodococcus erythropolis* IGTS8, enriching for growth in the presence of organosulfur compounds that could not support growth of the wild-type strain. Mutations arose that allowed growth with octyl sulfide and 5-methylbenzothiophene as sole sulfur sources. An isolate from the evolved chemostat population was genetically characterized and found to contain mutations in two genes, *dszA* and *dszC*. A transversion (G to T) in *dszC* codon 261 resulted in a V261F mutation that was determined to be responsible for the 5-methylbenzothiophene gain-of-function phenotype. By using a modified RACHITT (random chimeragenesis on transient templates) method, mutant DszC proteins containing all possible amino acids at that position were generated, and this mutant set was assayed for the ability to metabolize 5-methylbenzothiophene, alkyl thiophenes, and dibenzothiophene. No mutant with further improvements in these catalytic activities was identified, but several clones lost all activity, confirming the importance of codon 261 for enzyme activity.

The term directed evolution comprises a collection of methods for isolating genetic variants with desired characteristics from a large diverse population by using screens or selections (1). The genetic diversity can refer to a collection of random mutants generated by a variety of methods, including chemical mutagenesis, misincorporation mutagenesis, and cassette mutagenesis (27), or it can refer to a collection of homologous genes which encode proteins with similar but not identical properties. Both forms of genetic diversity have been exploited by the recent development of several methods of in vitro recombination. These methods generate large libraries of chimeric genes by recombining gene fragments obtained from collections of mutants or homologous genes. Two of the first in vitro recombination methods described were staggered-extension PCR (20, 34) and sexual PCR (30, 31). More recently Coco et al. (4) have described a novel method of in vitro recombination known as random chimeragenesis on transient templates (RACHITT).

Powerful tools are necessary to sort through the large libraries generated by in vitro recombination to isolate the phenotype of interest. Typically, high-throughput screening methodologies utilizing robotic systems to screen large numbers of samples in a microtiter plate format have been developed (3, 25). One classical method for the selection and isolation of desired mutants that is often overlooked is chemostat selection (8), which can allow for the manifestation of rare mutants within a large population. A chemostat selection can be designed to enrich for a desired phenotype without knowledge of the genotype or underlying molecular mechanisms, and thus it can be used in directed-evolution strategies with samples ranging from well-characterized cloned genes to uncharacterized microorganisms.

In any population, mutations occur spontaneously at a low but measurable frequency. In a chemostat, continuous cell division ensures that new cells are continuously generated at a frequency of 10^{10} to 10^{14} cells per day, and thus, even at low spontaneous mutation rates, reasonably large numbers of random mutants can be generated. Any random mutant which is better adapted than the wild type to grow under the selective pressures established within the chemostat will eventually establish itself as the dominant population. The number of mutants in the population can be increased by inoculation into the chemostat of libraries of genetic variants generated by such methods as random mutagenesis or in vitro recombination.

Chemostats have been designed to select for mutants that produce enzymes with increased activity rates (10, 32, 33) or with altered substrate specificity (29). The latter type have been termed gain-of-function mutants. It is a challenge to the researcher to design the chemostat environment such that a mutant with the desired phenotype will have a selective advantage for growing to dominance in the culture. It can be especially difficult to design an environment to select for improvements in the metabolism of minor nutrients such as sulfur, for which the alteration of enzyme rate or specificity do not much influence growth rate.

An example of a sulfur metabolic pathway in need of improvement is the Dsz pathway, which could be of great value in the development of a biocatalytic process for the desulfurization of petroleum streams. Increasingly stringent requirements for sulfur levels in fossil fuels are being mandated by environmental agencies worldwide, and biodesulfurization (BDS) represents a novel process to help refiners achieve goals for low sulfur levels (23). The Dsz pathway employs two mono-

* Corresponding author. Mailing address: Enchira Biotechnology Corporation, 4200 Research Forest Dr., The Woodlands, TX 77381. Phone: (281) 419-7000. Fax: (281) 364-6112. E-mail: jarensdorf@enchira.com.

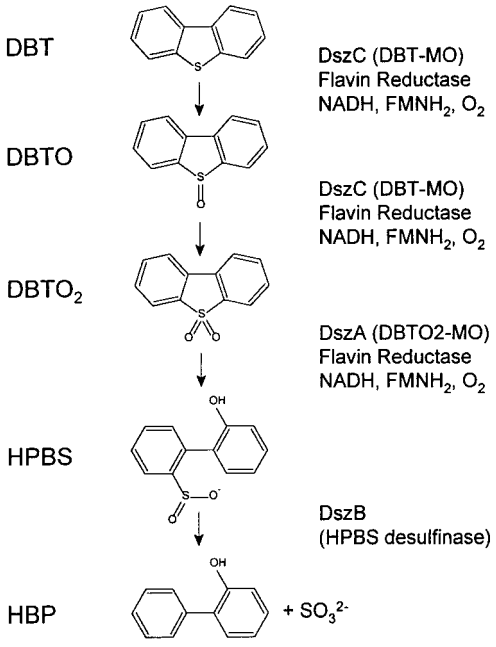

FIG. 1. Proposed Dsz pathway for BDS of DBT by *R. erythropolis* IGTS8. DBTO, DBT sulfoxide; $DBTO_2$, DBT sulfone; HPBS, 2-(2′-hydroxyphenyl)-benzene sulfinate; HBP, 2-hydroxybiphenyl; DBT-MO, DBT monooxygenase; DBTO2-MO, $DBTO_2$ monooxygenase; FMN, flavin mononucleotide.

oxygenases (DszC and DszA) and a desulfinase (DszB) to transform organosulfur molecules such as dibenzothiophene (DBT), yielding a partially oxidized hydrocarbon and sulfite. The Dsz pathway was first described for *Rhodococcus erythropolis* IGTS8 (J. J. Kilbane and B. A. Bielaga, presented at the First International Symposium on the Biological Processing of Coal, Palo Alto, Calif., 1990). The genetics (7, 21) and biochemistry (12) of the IGTS8 BDS pathway are summarized in Fig. 1, with DBT as substrate.

DBT has historically been a good model compound for diesel BDS because alkylated DBTs are one of the main classes of sulfur compounds present in this oil stream (19). Accordingly, to date, most BDS studies have focused on DBT and alkylated derivatives (9, 17, 18), but the need to achieve extremely low levels of sulfur in diesel and in other oil streams such as gasoline has demanded a broader substrate range than the Dsz system can provide. Certain petroleum streams contain, in addition to alkylated DBTs, a variety of alkyl and aryl sulfides, thiophenes, and benzothiophenes (6, 19). There has been limited characterization of the ability of IGTS8-derived strains to biotransform sulfur compounds from these other major classes. In this study, we used model compound assays to explore the limits of BDS for several non-DBT sulfur classes. We then chose several model compounds that were not substrates for the Dsz system, and we used the chemostat as a directed-evolution tool to generate Dsz gain-of-function mutants that could transform these selected model compounds.

MATERIALS AND METHODS

Chemicals. DBT, benzothiophene, 2-methylbenzothiophene (2-MBT), 2,5-di(*tert*-butyl)thiophene, 3-phenylthiophene, hexyl sulfide, octyl sulfide, decyl sulfide, and dodecyl sulfide were purchased from Sigma-Aldrich. 2,3,5-Trimethylthiophene (TMT) and 3-*n*-octylthiophene were purchased from TCI America. 5-Methylbenzothiophene (5-MBT), 3-methylbenzothiophene (3-MBT), and 3,5-dimethylbenzothiophene were purchased from Alfa Aesar. Dodecane was purchased from Fisher Scientific. Sulfones of selected model compounds were synthesized as follows. The model compound was dissolved in dodecane and stirred overnight with a 20-fold molar excess of peroxyacetic acid. After treatment with platinum on carbon to eliminate unreacted peroxyacetic acid, the sample was evaporated to dryness under a nitrogen stream and the residue was resuspended in dodecane.

Bacteria and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. *R. erythropolis* IGTS8 is a natural soil isolate obtained from

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
R. erythropolis IGTS8	BDS type strain	16
R. erythropolis I-19	Derivative of *R. erythropolis* IGTS8 with multiple copies of *dszACD*	9
R. erythropolis BKO53	DszB derivative of *R. erythropolis* I-19	9; B. R. Folsom, presented at the First International Conference on Petroleum Biotechnology, Mexico City, Mexico, 21–23 February 2000
R. erythropolis JB55	Δ*dszABC* derivative of IGTS8; *dszD* still present	4
R. erythropolis C4-104	Chemostat mutant with octyl sulfide gain of function; contains mutant plasmid pJA101	This study
R. erythropolis C4-224	Chemostat mutant with 5-MBT gain of function; contains mutant plasmid pJA102	This study
E. coli DH10B	Electrocompetent cells used to amplify plasmid libraries before transformation into *Rhodococcus*	Gibco BRL
Plasmids		
pEBC388	*dszABC*, chloramphenicol resistance	21
pEBC1100	*dszABCD*, kanamycin resistance	4
pJA101	pEBC388 derivative isolated from chemostat mutant *R. erythropolis* C4-104; contains *dszA1* allele	This study
pJA102	pEBC388 derivative isolated from chemostat mutant *R. erythropolis* C4-224; contains *dszA2* and *dszC1* alleles	This study
pJA103	pEBC1100 derivative with *dszC* allele replaced by *dszC1* allele from pJA102	This study
Codon 261 mutant plasmids	Series of plasmids derived from pEBC1100, each with one of all possible codons at position 261 of DszC (see Results)	This study

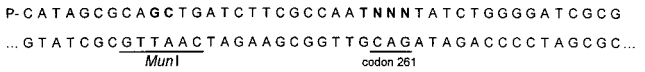

FIG. 2. A synthetic oligonucleotide (upper strand) was 5′ phosphorylated to allow ligation repair from upstream sequence. The oligonucleotide was randomized at codon 261, and the *Mun*I site was removed with two silent mutations.

an enrichment culture with DBT as a sole sulfur source (Kilbane and Bielaga, presented at the First International Symposium on the Biological Processing of Coal). All of the *R. erythropolis* strains used in this study are derivatives of IGTS8.

Culture conditions. (i) Continuous culture. A two-phase sulfur-limited continuous culture of *R. erythropolis* JB55(pEBC388) was maintained with glucose (20 g/liter) and minimal medium (4 g of KH_2PO_4 per liter, 0.4 g of $MgCl_2$ per liter, and 10 ml of Huntners mineral base per liter) (5) in a 2-liter stirred tank reactor (Applikon Dependable Instruments). Sulfur limitation was imposed in the first stage of the chemostat by supplementation of the minimal medium with $MgSO_4$ to 20 μM. The pH was controlled at 7.0 with NH_4OH, and dissolved oxygen was controlled by the agitation rate. Chloramphenicol was introduced in the aqueous feed at 12.5 μg/ml to ensure plasmid stability. The oil phase, dodecane, was introduced on a separate feed and maintained at a constant 10:1 water/oil ratio in a total culture volume of 1.1 liters. Model compounds that were not substrates for the Dsz system were dissolved in the influent oil phase. Initially, an influent sulfate concentration of 20 μM was introduced in the aqueous feed to maintain a small population of approximately 1.5 g (dry weight) of cells per liter. The hydraulic retention time of the chemostat was 46 h. The chemostat dilution rate was 0.022 h^{-1}. Changes in cell density or BDS capability were monitored by monitoring dissolved oxygen, glucose consumption, and effluent model compound concentration.

(ii) Emulsion plate culture. For screening of bacterial growth on organosulfur compounds, emulsion agar containing 1% (vol/vol) dodecane was prepared as described previously (2). The sulfur concentration in the oil was 25 mM.

Assays. BDS activity was determined using two-phase shake flask assays. Assays with BKO53 used frozen cell paste that was thawed in 156 mM phosphate buffer (pH 7.5) with 2% glucose. Assays with other strains were carried out with cells that had been grown in batch culture in BSM1 medium (21). DBT assays were performed as described previously (21) with DBT delivered in hexadecane. DBT specific activity was based on accumulation of 2-(2′-hydroxyphenyl)-benzene sulfinate for BKO53 and on accumulation of hydroxybiphenyl for strains with an active DszB. For assays with other model compounds, the compound was added as a dodecane solution, the assay mixture was incubated at 30°C with shaking, and the oil was recovered by centrifugation for analysis. Total sulfur in the oil phase was determined by combustive UV fluorescence, and substrate and product peaks were analyzed by gas chromatography with sulfur chemiluminescence detection (GC-SCD). In some cases, structures of intermediates were confirmed by gas chromatography-mass spectrometry (GC-MS). A cell-free control was incubated with each model compound to determine the level of volatilization.

Instrumental analysis. Total sulfur in the oil phase was determined by combustive UV fluorescence with the Antek 9000VS Pyro-fluorescent sulfur analyzer (Antek Instruments, Inc., Houston, Tex.). Analysis of sulfur compounds by GC-SCD was performed as described previously (9). GC-MS was performed as described previously (9).

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination of Sanger et al. (28) using the DYEnamic ET Terminator cycle sequencing system (Amersham Pharmacia Biotech, Inc.) and an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

Isolation of *dszC1* allele. pEBC1100 (4) is a plasmid containing an artificial *dszABCD* operon in an *Escherichia coli-Rhodococcus* shuttle vector. The *dszC* gene in pEBC1100 is flanked by *Xba*I and *Sfi*I sites. The mutant *dszC1* allele was PCR amplified from plasmid JA102 using the following primers: forward, GTA GTTCTAGATTCGAAACCGATAGGAACATCCGCA (the *Xba*I site is underlined); reverse, TCGAAGGCCGTCGAGGCCACTACATCAGGAGGTGA AGCCGG (the SfiI site is underlined). The PCR product was restricted with XbaI and SfiI and ligated into pEBC1100 from which the wild-type dszC gene was removed with the same enzymes. The ligation mixture was transformed by electroporation into E. coli DH10B to amplify the plasmids. A pool of plasmids was isolated and transformed by electroporation into R. erythropolis JB55, and selections were done on kanamycin agar plates. The final construct was confirmed by DNA sequencing and designated pJA103.

Codon 261 mutagenesis. To generate a library of derivative strains randomly mutated at codon 261, a modification of the RACHITT method (4) was used. The *dszC* gene was made into a single-stranded full-length template as described above, and then a synthetic oligonucleotide (donor fragment) was annealed to the template across codon 261. The same forward primer and anchor oligonucleotides as described by Coco et al. (4) were used. The synthetic donor (Fig. 2) was designed to be degenerate at codon 261. Several silent mutations were also introduced into the synthetic oligonucleotide. The nucleotide immediately upstream of codon 261, i.e., the third position of codon 260, was changed from C to T in an effort to create a larger mismatch region, open up the first position of codon 261 to more mismatching, and increase the degeneracy of the RACHITT product. A *Mun*I site upstream of codon 261 was eliminated by the introduction of two silent mutations. Chimeric products therefore did not have the *Mun*I site. This allowed for elimination of nonchimeric template read-through sequences by restriction of the primary PCR product with *Mun*I followed by secondary PCR.

The annealed complexes were repaired by extension and ligation to generate full-length genes, and then the template strands were destroyed. The remaining top strands, containing the randomization at codon 261, were amplified by PCR, inserted into pEBC1100 as described above, and then transformed by electroporation into *E. coli* DH10B. A pool of plasmids was isolated and transformed by electroporation into *R. erythropolis* JB55, and selections were done on kanamycin agar plates.

Nucleotide sequence accession number. The nucleotide sequence of the *dsz* genes has been assigned GenBank accession number U08850.

RESULTS

Model compound assays. Table 2 summarizes the results of resting-cell assays with *R. erythropolis* BKO53 to test the substrate range of the Dsz BDS system. DBT was compared with representatives of three non-DBT sulfur compound classes found in a variety of petroleum streams, including gasoline and straight-run diesel: benzothiophenes, thiophenes, and dialkyl sulfides. In addition to total sulfur analysis, GC-SCD was performed on each sample to confirm losses of parent sulfur compounds. In some cases, the SCD indicated the accumula-

TABLE 2. Summary of organosulfur model compound biotransformations by *R. erythropolis* BKO53[a]

Compound	Total sulfur in oil (μM)		% Remaining
	Stock	24 h	
DBT	940	0	0
Benzothiophenes			
Benzothiophene	1,200	920	77
2-MBT	670	230	34
3-MBT	970	250	26
5-MBT	850	850	100
3,5-Dimethylbenzothiophene	810	0	0
Thiophenes			
2,3,5-Trimethylthiophene	760	530	100[b]
2,5-Di(*tert*-butyl)thiophene	250	280	112
3-*n*-Octylthiophene	830	940	113
3-Phenylthiophene	780	760	97
Dialkyl sulfides			
Hexyl sulfide	8,400	1,080	13
Octyl sulfide	760	740	97[c]
Decyl sulfide	600	690	115
Dodecyl sulfide	940	940	100

[a] Assay results are corrected for the presence of the internal standard (dodecyl sulfide).
[b] For 2,3,5-trimethylthiophene, a similar loss was observed for the cell-free control, indicating volatilization.
[c] GC-SCD indicated the appearance of a product peak that was confirmed by GC-MS to be octyl sulfone.

tion of partially oxidized products, which are likely to be sulfoxides or sulfones.

BKO53 was able to transform 100% of the DBT (the standard model compound for BDS) and remove it from the oil phase. Although Oldfield et al. (24) have shown that benzothiophene is not a substrate for the IGTS8 Dsz system, we observed that BKO53 was able to transform and partially clear from the oil phase benzothiophene, 2-MBT, and 3-MBT, although it could not transform 5-MBT. Furthermore, strains that carry the entire Dsz pathway (e.g., IGTS8) can use benzothiophene, 2-MBT, and 3-MBT but not 5-MBT as a sulfur source (data not shown). For benzothiophene, a no-cell control was also tested to confirm that substrate loss was not due to volatilization (data not shown). Although the attenuation of parent substrate peaks on GC-SCD chromatograms and the reduction in total sulfur indicate that these compounds are substrates for DszC, the incomplete clearing of these compounds from the oil phase suggests that they are poor substrates relative to DBT. For each of these compounds, no product peaks were observed on GC-SCD chromatograms, suggesting that the sulfone products of DszC are substrates for DszA. The sulfones of both 5-MBT and 2-MBT were synthesized and assayed as described above, resulting in their complete removal from the oil phase, which is further evidence that they are substrates for DszA. No-cell controls with both 5-MBT sulfone and 2-MBT sulfone indicated that their disappearance in these assays was not due to abiotic losses (data not shown). In summary, for the benzothiophene model compounds tested, DszA had a wider substrate specificity than DszC, and DszC is the major bottleneck for BDS of this class of compounds.

BKO53 did not transform any of the thiophene model compounds tested. This indicates that none of these compounds are a substrate for DszC. TMT sulfone was synthesized and assayed as described above. Abiotic losses in no-cell controls were high (80%) but not complete, while the sulfone was completely removed from the oil phase by BKO53. This suggests that TMT sulfone is a substrate for DszA. These data suggest that DszA may have a wider substrate specificity than DszC toward this class of compounds.

Di-aryl sulfides and aryl-alkyl sulfides have been shown to be substrates for DszC, although in some cases oxidation stops at the level of sulfoxide, leading to the accumulation of chiral sulfoxides with a high enantiomeric excess (H. L. Holland, A. Kerridge, and P. T. Pienkos, presented at the Gordon Conference on Green Chemistry, 11 to 16 July 1999). In this study we observed that symmetric dialkyl sulfides with chain length lower than C_8 appear to be substrates for DszC. Octyl sulfide is itself a poor substrate for DszC and is only partially transformed to the sulfone in a 24-h assay. For symmetric dialkyl sulfides, the substrate range for DszA appears to be narrower than that for DszC. Octyl sulfone accumulated in the 24-h assay and was not further transformed. Octyl sulfone was the only product observed from octyl sulfide and was positively identified by GC-MS. By contrast, hexyl sulfide was largely cleared from the oil, suggesting that hexyl sulfone is a substrate for DszA. Hexyl sulfide also served as a sole sulfur source for growth of strain IGTS8, offering further evidence that the sulfone is a substrate for DszA.

Chemostat selection of gain-of-function mutants. In order to select for mutants with increased substrate range for the Dsz system, 5-MBT, *n*-octylthiophene, and octyl sulfide were selected for use in the sulfur-limited chemostat. After 63 days of operation, *n*-octylthiophene was replaced by TMT because TMT was judged to be more representative of alkyl thiophenes in petroleum products. The chemostat was initially operated with limiting sulfate to maintain a stable population. The organosulfur compounds, supplied in the dodecane oil phase, represented a sulfur source unavailable to the wild-type population. It was hypothesized that mutations in *dszC* could arise to allow growth with 5-MBT or TMT or that mutations in *dszA* could arise to allow growth with octyl sulfide. Such a gain-of function mutant would then have access to additional sulfur and would come to dominate the chemostat population. The long hydraulic retention time of 46 h was chosen to allow the emergence of a mutant with even poor activity towards a model compound.

The chemostat was inoculated with *R. erythropolis* JB55 (pEBC388) and operated for 240 days without apparent contamination. This construct was chosen because it contained a single copy of the *dszABC* operon on a plasmid with *dszD* present on the chromosome. The products of this operon allowed growth on organosulfur compounds as a sole sulfur source. Initially, model compounds were supplied at 1.0 mM in the oil phase feed, and at this concentration, each individual model compound could support a cell density of 3.5 g (dry weight) of cells per liter (based on empirical studies of sulfur-limited cultures of strain IGTS8). The concentration of these compounds was increased to 4 mM on day 29. Octyl sulfone was detected in the effluent oil initially but dropped to trace amounts after several weeks, indicating a shift in DszA. The influent octyl sulfide concentration was increased in steps to 8 mM on day 77, and the cell density in the chemostat increased in response as evidenced by increased glucose consumption (traditional methods of measuring cell density, such as absorbance or dry weight measurement, are difficult in two-phase cultures of *R. erythropolis* due to cell attachment to oil droplets). Total sulfur analysis of influent and effluent samples indicated a loss of approximately 2 mM S in the chemostat, and GC-SCD analysis confirmed that the loss was due to the degradation of octyl sulfide. These data indicated that a population that could grow on octyl sulfide as a sole sulfur source had become established, and so sulfate was removed from the influent medium as a maintenance sulfur source on day 87. The chemostat population remained stable after removal of sulfate, indicating that a transition to utilization of the sulfur compounds in the oil phase had indeed occurred.

With evidence that octyl sulfide was now being metabolized by the chemostat population, we wished to change the selective pressure, and octyl sulfide was removed from the feed on day 143. 3-MBT (which can support growth) was supplied in the influent oil at 2 mM to maintain a baseline population. The concentration of 3-MBT in the chemostat feed was reduced in steps (to 1, 0.3, and 0.15 mM) over a period of 45 days. By day 170, the concentration of total sulfur removed in the chemostat began to exceed the concentration of 3-MBT in the influent oil (0.3 mM). This indicated that the chemostat population had begun to metabolize a new compound. Analysis of the effluent oil indicated that 5-MBT was being utilized. However, when

TABLE 3. Summary of model compound assays[a]

Rhodococcus strain	Genotype	μmol removed			DBT sp act[e] (μmol g [dry wt] of cells^{-1} min^{-1})
		5-MBT[b]	Benzothiophene[c]	Octyl sulfide[d]	
JB55	Δ*dsz*	−0.7	−0.3	1.2	0.06
JB55(pEBC388)	*dszA*$^+$*B*$^+$*C*$^+$	0	−1.3	0.3	0.65
JB55(pEBC1100)	*dszA*$^+$*B*$^+$*C*$^+$*D*$^+$	1.2	NT[f]	NT	1.14
C4-104	*dszA1B*$^+$*C*$^+$	−0.1	NT	8.9[g]	0.54
C4-224	*dszA2B*$^+$*C1*	3.9	3.9	9.2	0.45
JB55(pJA103)	*dszA*$^+$*B*$^+$*C1D*$^+$	7.5	6.9	3.5	1.02

[a] Assays ended at 24 h unless otherwise stated.
[b] The initial amount of 5-MBT was 8.6 μmol.
[c] The initial amount of benzothiophene was 8.6 μmol.
[d] The initial amount of octyl sulfide was 58 μmol.
[e] The initial amount of DBT was 115 μmol; the assay ended at 30 min.
[f] NT, not tested.
[g] The assay ended at 16 h.

3-MBT was completely removed from the chemostat on day 188, the cell density declined even at a dilution rate of 0.022 h^{-1}. Even though the culture would wash out at this growth rate, it could be restored in batch mode with 5-MBT as the sole sulfur source. This batch growth was accompanied by significant reduction of 5-MBT in the oil phase, indicating that a 5-MBT-utilizing population had emerged.

At no point in the 8-month period of chemostat operation was there any evidence that a shift towards alkyl thiophene utilization had occurred.

Screening of chemostat isolates. Chemostat effluent samples were regularly plated on rich medium plates and DBT emulsion plates. During the life of the chemostat, the diversity of colony morphologies observed on these plates increased. However, all isolates had the distinct orange pigmentation of *Rhodococcus*, suggesting they were derivatives of the parental strain used to inoculate the chemostat. On day 104 (54 generations), the chemostat was clearly established on octyl sulfide, and a sample from the chemostat was streaked onto a DBT plate. Five randomly picked isolates were selected, grown in liquid culture in minimal medium, and tested in two-phase resting-cell assays for 16 h with octyl sulfide in dodecane at 58 μmol (6:1 water/oil ratio). Control strains with the wild-type Dsz system removed <2.3 μmol of octyl sulfide. Two of the five chemostat isolates removed 7.7 and 8.9 of μmol octyl sulfide, respectively. This represented specific activities toward octyl sulfide of 0.13 and 0.15 μmol g (dry weight) of cells^{-1} min^{-1}, respectively. The remaining three chemostat isolates removed no octyl sulfide, suggesting that the wild-type strain had not yet washed out, perhaps because the rate of octyl sulfide transformation was sufficient to support cross feeding. One of the octyl sulfide-transforming isolates was selected for further characterization and was designated *R. erythropolis* C4-104. The specific activity of C4-104 toward DBT was 0.54 μmol g (dry weight) of cells^{-1} min^{-1}, compared with a specific activity of the parental strain of 0.65 μmol g (dry weight) of cells^{-1} min^{-1}.

On day 224 (117 generations), the chemostat was clearly established on 5-MBT, and an effluent sample was plated onto 5-MBT emulsion agar. Growth was slow, but after several weeks a subpopulation of large orange colonies was established on these plates. Four of these colonies were randomly picked and grown in liquid culture. Two-phase resting-cell assays were performed with 5-MBT in dodecane at 3.45 mM (6:1 water/oil ratio). Three of the four isolates removed 2.76 mM 5-MBT in a 72-h assay. The fourth isolate was similar to the control strains carrying the wild-type Dsz system that removed only 0.3 mM 5-MBT in the same assay. One of the positive isolates was selected for further characterization and was designated *R. erythropolis* C4-224.

The ability of C4-224 to transform both octyl sulfide and DBT was retained (Table 3). This indicated that the octyl sulfide gain of function was not lost during the 5-MBT phase of the enrichment and that the wild-type function (DBT oxidation) was not lost by strains containing these mutations.

Characterization of gain-of-function mutants. (i) Sequences of *dszA* and *dszC*. The pEBC388 derivative plasmid of the octyl sulfide-utilizing strain C4-104 was isolated and designated pJA101. The *dszA* and *dszC* genes of pJA101 were sequenced, and the sequences were compared to those of the wild type (7, 26). A three-nucleotide mutation was found in *dszA*: GCA→CGC. This mutated *dszA* allele was designated *dszA1*. This mutation spanned DszA codons 344 (silent mutation) and 345 (Q345A). As expected, the *dszC* sequence of pJA101 was unchanged from the wild-type.

The pEBC388 derivative plasmid in strain C4-224 was isolated and designated pJA102. The *dszA* and *dszC* genes of pJA102 were sequenced from both directions. The *dszA* gene of pJA102 contained the same three-nucleotide mutation at codons 344 and 345 as observed in pJA101. An additional G-to-T transversion was observed (G60V), and this allele was designated *dszA2*. A G-to-T transversion was detected in the *dszC* gene of pJA102, and this allele was designated *dszC1*.

(ii) Localization of 5-MBT gain-of-function phenotype to DszC V261F. We were more interested in the 5-MBT gain-of-function phenotype because benzothiophenes are more of a problem in petroleum streams than long-chain alkyl sulfides. Because DszC is the bottleneck in transformation of 5-MBT, we surmised that the DszC V261F mutation was responsible for the 5-MBT gain-of-function phenotype. To test this, the mutant *dszC1* allele was inserted into a vector, pEBC1100 (4), that had been constructed with the wild-type *dszABD* genes. The *dszC* gene of pEBC1100 was removed and replaced with the PCR-amplified mutant allele. The resulting construct, pJA103, was transformed into *R. erythropolis* JB55. *R. erythropolis* JB55(pJA103) was able to grow with 5-MBT as the

sole sulfur source. Table 3 summarizes the results of resting-cell assays performed with *R. erythropolis* JB55(pJA103) and control strains. Comparing strain C4-224 to strain JB55 (pJA103), strain JB55(pJA103) retained the ability to transform 5-MBT but transformed octyl sulfide poorly, if at all (evidence that the octyl sulfide gain of function was due to the mutations in *dsz*A). Strain JB55(pJA103) removed more than twice the amount of 5-MBT as did C4-224, but the ratios of 5-MBT activity to DBT activity in the two strains were nearly the same. This is likely due to increased levels of flavin reductase in pEBC1100 and its derivatives. Flavin reductase is encoded by *dszD*. Although *dszD* is located on the chromosomes of both strains, pJA103 contains an extra copy. In addition to the 5-MBT gain of function, strains with the V261F DszC mutation had improved activity against benzothiophene.

In vitro mutagenesis of *dszC* codon 261. Because the V261F mutation in DszC resulted in a broadening of the substrate range to include 5-MBT, we decided to make further changes at codon 261 to explore the potential for further improvements in substrate range to expand the utility of DszC in BDS. By using a modified RACHITT method (4), a library containing clones randomized with respect to the sequence at codon 261 was constructed. Clones were picked at random for sequencing to identify at least one clone with each of the possible amino acids substitutions. A total of 321 clones were sequenced. The library was quite randomized, as 53 distinct codon 261 mutations were represented, including at least one of all possible amino acid substitutions.

Assays with codon 261 mutant set. A set of 19 mutants, each with a different amino acid substitution at codon 261, was tested in resting-cell assays for transformation of DBT, 5-MBT, and benzothiophene. Most of the substitution mutants that were tested represented those sequences of most frequent codon usage for each respective amino acid in *Rhodococcus*. As a control, a strain with the wild-type sequence (valine) at codon 261 was also tested. The phenylalanine mutant was the only mutant that was able to transform 5-MBT and was the only mutant that transformed benzothiophene more efficiently than the wild-type (valine) strain. Interestingly, the activity on DBT of many of the mutants was reduced or, in some cases, eliminated (Table 4). No mutant had a DBT specific activity greater than that of the wild-type strain, and although the V261F mutant had gained the ability to transform 5-MBT, it had relatively low DBT activity compared to the wild type.

TABLE 4. Summary of DBT activities of codon 261 substitution mutants

Amino acid at DszC codon 261	Transformation of DBT[a]
Nonpolar	
Ala	+
Val	+++
Lcu	+++
Ile	+++
Met	+++
Phe	++
Trp	−
Pro	−
Polar uncharged	
Asn	−
Gln	−
Gly	−
Ser	−
Thr	+++
Cys	++
Tyr	+
Charged	
Lys	−
Arg	−
His	−
Asp	−
Glu	−

[a] Assayed as described in Materials and Methods. Results indicate percent activity of the wild type: +++, >75%; ++, 25 to 75%; +, 2 to 25%; −, <2%.

DISCUSSION

The substrate range of the wild-type *R. erythropolis* Dsz system was observed to be broader than previously reported and to extend beyond the alkylated DBT class of sulfur compounds. It has been reported that strain IGTS8 could not grow on benzothiophene or 5-MBT (15, 22) as a sole sulfur source and that resting cells of IGTS8 were unable to metabolize benzothiophene (11). Our study confirmed the lack of measurable activity toward benzothiophene by IGTS8 derivative strain JB55(pEBC388), which has low DBT activity. However, strain BKO53, which has DBT activity an order of magnitude higher than that of strain IGTS8 , did metabolize benzothiophene, although poorly. Kobayashi et al. (18) have reported that *R. erythropolis* KA2-5-1, which has a Dsz system practically identical to that of IGTS8, could grow on 3-MBT and could transform a variety of methyl and ethyl benzothiophenes. However, enzyme assays with the DszC of strain KA2-5-1 indicated only nominal activity toward benzothiophene and 5-MBT. In this study, 5-MBT was not discernibly transformed by resting cells of BKO53. It was interesting that 3,5-dimethylbenzothiophene was a better substrate than benzothiophene, 3-MBT, or 5-MBT. This suggests that the deficiency in DszC with regard to benzothiophenes is one of substrate binding.

There have been no previous reports of the ability of the Dsz system to transform alkyl sulfides. Growth of IGTS8 on dimethyl sulfoxide has been reported (15), but we have observed that Dsz$^-$ strains can transform dimethyl sulfoxide (unpublished observations). Our study has shown that dialkyl sulfides are substrates for the complete Dsz system, although increased alkylation resulted in less efficient transformation. The accumulation of sulfones in octyl sulfide transformations indicates that DszA has a narrower substrate range than DszC for dialkyl sulfides.

The sulfur-limited chemostat was effective for the directed evolution of gain-of-function mutants with an expanded substrate range for the enzymes involved in BDS. Furthermore, the same chemostat was effective for the sequential accumulation of different evolved phenotypic targets in a single strain. This was accomplished by changing the selective pressure after the appearance of the first mutation to select a second mutation. The resultant population had accumulated both octyl sulfide and 5-MBT gain-of-function phenotypes, and both of these traits were present in a single isolate. The unique three-nucleotide mutation in *dszA* of C4-104 was conserved in C4-

224, indicating that the latter strain was a descendant of the former. It was anticipated that the octyl sulfide gain of function would require a DszA mutation, and this was borne out with the mutations detected in C4-104 and C4-224. The nature of the DszA mutations has not been characterized, and it remains to be determined how each contributes to the gain-of-function phenotypes. The DszC mutation was isolated and shown to be responsible for the 5-MBT gain of function.

Only the clone with the V261F mutation was able to transform 5-MBT, but the loss of DBT activity as a result of several other substitutions at codon 261 shows the importance of this codon for DBT monooxygenase activity. The transformation by DBT monooxygenase of substituted thiophenes remains intractable. No mutant that utilized alkylated thiophenes appeared in the chemostat, and none of the codon 261 mutants demonstrated activity with alkylated thiophenes. Additional broadening of the substrate range of DszC will likely require mutations at different positions. A structural model of DszC has not been reported, and it is not known which residues contribute to the active site. It is interesting that the ability to transform 5-MBT was gained by replacement of the small hydrophobic valine residue with the larger hydrophobic phenylalanine residue. We propose that a smaller binding pocket allows a better fit for the 5-MBT structure. If this is true, one might expect a corresponding loss in activity for larger structures, and in fact the ability to transform 5-MBT was gained at the expense of DBT activity in the V261F mutant. DBT activity was generally lost when polar residues were substituted and retained when nonpolar residues were substituted at codon 261. All reported natural homologs of DszC contain either valine or leucine at codon 261 (7, 13, 14). There have been reports of bacterial isolates that can metabolize benzothiophene and thiophene but not DBT (15, 22), and it has been suggested that no single enzyme system can oxidize all of these classes of sulfur heterocycles. The V261F DszC mutant represents an enzyme that can transform both DBT and benzothiophene.

Recently, homologous *dszC* genes from two different sources were employed in an in vitro recombination study using RACHITT (4). Although clones that had higher activity and altered substrate specificity were obtained, it is worth noting that the V261F mutant selected in the present study could not have been generated in the RACHITT experiment because the two parental genes were homozygous at the codon 261 locus. This demonstrates that random mutagenesis, while perhaps not as powerful as in vitro recombination, remains a useful technique for the generation of genetic diversity. The chemostat has a role to play in screening large libraries for desirable phenotypes. The success of this approach in selecting for desired gain-of-function mutants confirms that, if care is taken to design the appropriate selective pressure, the chemostat can be an important tool in the directed-evolution toolbox.

The broad substrate range of the Dsz system is one of the driving forces for the development of BDS as a commercial process, but it also presents opportunities for the biocatalytic production of specialty chemicals. These include sulfinates (the product of both DszC and DszA from DBTs), a starting material for novel surfactants (23). In addition, a number of prochiral sulfides have been transformed by DszC to yield chiral sulfoxides with the opposite stereospecificity of the better-studied fungal biocatalysts (Holland et al., presented at the Gordon Conference on Green Chemistry). The ability to broaden the substrate range of the Dsz system through directed evolution increases the likelihood that low sulfur target levels in petroleum streams can be achieved with BDS, and it also expands opportunities to generate specialty chemicals.

ACKNOWLEDGMENTS

This work was supported by a grant from the Department of Energy.
We thank Wayne Coco and Hans Hektor for helpful discussions and Maureen Downey and Chanda Tavoloni for technical assistance.

REFERENCES

1. **Arnold, F. H., and A. A. Volkov.** 1999. Directed evolution of biocatalysts. Curr. Opin. Chem. Biol. **3:**54–59.
2. **Bredholt, H., K. Josefsen, A. Vatland, P. Bruheim, and K. Eimhjellen.** 1998. Emulsification of crude oil by an alkane-oxidizing *Rhodococcus* species isolated from seawater. Can J. Microbiol. **44:**330–340.
3. **Burbaum, J. J., and N. H. Sigal.** 1997. New technologies for high-throughput screening. Curr. Opin. Chem. Biol. **1:**72–78.
4. **Coco, W. M., W. E. Levinson, M. J. Crist, H. J. Hektor, A. Darzins, P. T. Pienkos, C. H. Squires, and D. J. Monticello.** 2001. DNA shuffling method for generating highly recombined genes and evolved enzymes. Nat. Biotechnol. **19:**354–359.
5. **Cohen-Bazire, G., W. Sistrom, and R. Stanier.** 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J. Cell. Comp. Physiol. **49:**25–68.
6. **Czogalla, C. D., and F. Boberg.** 1983. Sulfur compounds in fossil fuels. Sulfur Rep. **3:**121–167.
7. **Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young.** 1994. Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. J. Bacteriol. **176:**6707–6716.
8. **Dykhuizen, D. E., and D. L. Hartl.** 1983. Selection in chemostats. Microbiol. Rev. **47:**150–168.
9. **Folsom, B. R., D. R. Schieche, P. M. DiGrazia, J. Werner, and S. Palmer.** 1999. Microbial desulfurization of alkylated dibenzothiophenes from a hydrodesulfurized middle distillate by *Rhodococcus erythropolis* I-19. Appl. Environ. Microbiol. **65:**4967–4972.
10. **Gasent-Ramirez, J. M., and T. Benitez.** 1997. Lysine-overproducing mutants of *Saccharomyces cerevisiae* baker's yeast isolated in continuous culture. Appl. Environ. Microbiol. **63:**4800–4806.
11. **Gilbert, S. C., J. Morton, S. Buchanan, C. Oldfield, and A. McRoberts.** 1998. Isolation of a unique benzothiophene-desulphurizing bacterium, Gordona sp. strain 213E (NCIMB 40816), and characterization of the desulphurization pathway. Microbiology **144:**2545–2553.
12. **Gray, K. A., O. S. Pogrebinsky, G. T. Mrachko, L. Xi, D. J. Monticello, and C. H. Squires.** 1996. Molecular mechanisms of biocatalytic desulfurization of fossil fuels. Nat. Biotechnol. **14:**1705–1709.
13. **Ishii, Y., J. Konishi, H. Okada, K. Hirasawa, T. Onaka, and M. Suzuki.** 2000. Operon structure and functional analysis of the genes encoding thermophilic desulfurizing enzymes of *Paenibacillus* sp. A11-2. Biochem. Biophys. Res. Commun. **270:**81–88.
14. **Kaneko, T., Y. Nakamura, S. Sato, E. Asamizu, T. Kato, S. Sasamoto, A. Watanabe, K. Idesawa, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, Y. Mochizuki, S. Nakayama, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada, and S. Tabata.** 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* (supplement). DNA Res. **7:**381–406.
15. **Kayser, K. J., B. A. Bielaga-Jones, K. Jackowski, O. Odusan, and J. J. Kilbane.** 1993. Utilization of organosulphur compounds by axenic and mixed cultures of *Rhodococcus rhodochrous* IGTS8. J. Gen. Microbiol. **139:**3123–3129.
16. **Kilbane, J. J.** 1989. Desulfurization of coal: the microbial solution. Trends Biotechnol. **7:**97–101.
17. **Kobayashi, M., K. Horiuchi, O. Yoshikawa, K. Hirasawa, Y. Ishii, K. Fujino, H. Sugiyama, and K. Maruhashi.** 2001. Kinetic analysis of microbial desulfurization of model and light gas oils containing multiple alkyl dibenzothiophenes. Biosci. Biotechnol. Biochem. **65:**298–304.
18. **Kobayashi, M., T. Onaka, Y. Ishii, J. Konishi, M. Takaki, H. Okada, Y. Ohta, K. Koizumi, and M. Suzuki.** 2000. Desulfurization of alkylated forms of both dibenzothiophene and benzothiophene by a single bacterial strain. FEMS Microbiol. Lett. **187:**123–126.
19. **Kropp, K. G., and P. M. Fedorak.** 1998. A review of the occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. Can. J. Microbiol. **44:**605–622.
20. **Levichkin, I. V., A. A. Shul'ga, F. T. Kurbanov, and M. P. Kirpichnikov.** 1995. A new method of designing hybrid genes–the homolog recombination method. Mol. Biol. (Mosk) **29:**983–991. (In Russian).

21. **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.
22. **Matsui, T., T. Onaka, Y. Tanaka, T. Tezuka, M. Suzuki, and R. Kurane.** 2000. Alkylated benzothiophene desulfurization by *Rhodococcus* sp. strain T09. Biosci. Biotechnol. Biochem. **64:**596–599.
23. **Monticello, D. J.** 2000. Biodesulfurization and the upgrading of petroleum distillates. Curr. Opin. Biotechnol. **11:**540–546.
24. **Oldfield, C., N. T. Wood, S. C. Gilbert, F. D. Murray, and F. R. Faure.** 1998. Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus *Rhodococcus*, and related taxa. Antonie Leeuwenhoek **74:**119–132.
25. **Olsen, M., B. Iverson, and G. Georgiou.** 2000. High-throughput screening of enzyme libraries. Curr. Opin. Biotechnol. **11:**331–337.
26. **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.
27. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schneider, K. H., G. Jakel, R. Hoffmann, and F. Giffhorn.** 1995. Enzyme evolution in *Rhodobacter sphaeroides*: selection of a mutant expressing a new galactitol dehydrogenase and biochemical characterization of the enzyme. Microbiology **141:**1865–1873.
30. **Stemmer, W. P.** 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc. Natl. Acad. Sci. USA **91:**10747–10751.
31. **Stemmer, W. P.** 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature **370:**389–391.
32. **Tidswell, E. C., A. N. Thompson, and J. G. Morris.** 1991. Selection in chemostat culture of a mutant strain of *Clostridium tyrobutyricum* improved in its reduction of ketones. Appl. Microbiol. Biotechnol. **35:**317–322.
33. **Weikert, C., U. Sauer, and J. E. Bailey.** 1997. Use of a glycerol-limited, long-term chemostat for isolation of *Escherichia coli* mutants with improved physiological properties. Microbiology **143:**1567–1574.
34. **Zhao, H., L. Giver, Z. Shao, J. A. Affholter, and F. H. Arnold.** 1998. Molecular evolution by staggered extension process (StEP) in vitro recombination. Nat. Biotechnol. **16:**258–261.