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Genetic Analysis of the *dsz* Promoter and Associated Regulatory Regions of *Rhodococcus erythropolis* IGTS8

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The *dsz* gene cluster of *Rhodococcus erythropolis* IGTS8 comprises three genes, *dszA*, *dszB*, and *dszC*, whose products are involved in the conversion of dibenzothiophene (DBT) to 2-hydroxybiphenyl and sulfite. This organism can use DBT as the sole sulfur source but not as a carbon source. Dsz activity is repressed by methionine, cysteine, Casamino Acids, and sulfate but not by DBT or dimethyl sulfoxide. We cloned 385 bp of the DNA immediately 5′ to *dszA* in front of the reporter gene *lacZ* of *Escherichia coli*. We showed that this region contains a *Rhodococcus* promoter and at least three *dsz* regulatory regions. After hydrazine mutagenesis of this DNA, colonies that were able to express β-galactosidase in the presence of Casamino Acids were isolated. Sequencing of these mutants revealed two possible regulatory regions. One is at −263 to −244, and the other is at −93 to −38, where −1 is the base preceding the A of the initiation codon ATG of *dszA*. An S1 nuclease protection assay showed that the start of the *dsz* promoter is the G at −46 and that transcription is repressed by sulfate and cysteine but not by dimethyl sulfoxide. The promoter encompasses a region of potential diad symmetry that may contain an operator. Immediately upstream of the promoter is a protein-binding domain between −146 and −121. Deletion of this region did not affect repression, but promoter activity appeared to be reduced by threefold. Thus, it could be an activator binding site or an enhancer region.

The release of sulfur oxides after combustion of fossil fuels is an important environmental problem, as it contributes to air pollution and is the principal cause of acid rain (23). Virtually all of the sulfur in liquid petroleum fuels is organic and is difficult and expensive to remove. The current method of choice for sulfur removal is hydrodesulfurization (HDS). This is treatment of the fuel with hydrogen under high temperature and pressure in the presence of a metal catalyst (9, 32). However, with the depletion of low-sulfur-containing petroleum reserves and increasingly stringent regulations, adequate desulfurization by HDS alone is becoming progressively more difficult to achieve. A number of different organosulfur compounds, including dibenzothiophene (DBT) and its alkylated derivatives, are refractory to HDS and are the predominant sulfur species in HDS-treated fuel (31). Thus, their removal by microbiological methods could have significant environmental benefits.

A number of organisms have been shown to be able to metabolize DBT to CO_2 and biomass with the destruction of carbon-carbon bonds (19), but this reduces the fuel value. Several strains of *Rhodococcus erythropolis* have been shown to remove sulfur from DBT without ring destruction; these include SY1 (27, 28), IGTS8 (17), D-1 (15), and N1-36, N1-43, and Q1a-22 (36). These strains are probably identical or very closely related and probably have the same pathway of desulfurization (17, 30). Another strain, tentatively identified as an *Arthrobacter* species, is also able to convert DBT to 2-hydroxybiphenyl (HBP) (20). The three genes responsible for the desulfurization of DBT, *dszA*, *-B*, and *-C* (*dsz* for desulfurization), are clustered on a 120-kb linear plasmid; strains cured of this plasmid, UV1 and CPE648, are Dsz^- (3–5, 29). The Dsz phenotype is repressed by sulfate (24, 29, 37) and the sulfur-containing amino acids methionine (Met) and cysteine (Cys) (13, 24) but not by DBT, DBT sulfone, or dimethyl sulfoxide (DMSO); the Dsz phenotype is obtained by growing IGTS8 with any of the latter three compounds as the sole sulfur source. An understanding of the mechanism of sulfur regulation in IGTS8 may permit the design of strains with higher desulfurizing activities.

In order to further investigate the regulation of Dsz metabolism, we cloned a fragment of the 5′ untranslated region of the *dsz* gene cluster in front of the reporter gene *lacZ* in an *Escherichia coli-Rhodococcus* shuttle vector, pEBC317 (Fig. 1). Here we show that this fragment contains the *dsz* promoter and identify the transcription start site. The results of mutagenesis experiments revealed two potential regulatory regions within this fragment.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains and plasmids used are listed in Table 1. IGTS8, previously designated an unknown *Rhodococcus* species, has recently been shown to be a strain of *R. erythropolis* (10).

Growth of *R. erythropolis* strains. Two different defined media, BSM1 and BSM2, were used for the growth of *R. erythropolis* strains.

(i) BSM1. BSM1 contained the following (per liter): phosphate buffer, 100 mmol (pH 7.2); glucose, 20 g; NH_4Cl, 2 g; $MgCl_2 \cdot 6H_2O$, 644 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; nitriloacetic acid, 0.1 g; $FeCl_2 \cdot 4H_2O$, 2.6 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.1 mg; $CuCl_2 \cdot 2H_2O$, 0.15 mg; $Co(NO_3)_2 \cdot 6H_2O$, 0.125 mg; $ZnCl_2$, 2.6 mg; $CaCl_2 \cdot 2H_2O$, 33 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.09 mg; and EDTA, 1.25 mg.

(ii) BSM2. BSM2, a medium that gives higher levels of Dsz-specific activity than does BSM1, contained the following (per liter): KH_2PO_4, 4 g; NH_4NO_3, 3 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; succinate, 1 g; glycerol, 10 g; ethanol, 8 g; $MnCl_2 \cdot 4H_2O$, 20 mg; $FeCl_2 \cdot 4H_2O$, 50 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.02 mg; $CuCl_2$, 0.02 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; $ZnCl_2$, 0.08 mg; $FeCl_3 \cdot 6H_2O$, 0.4 mg; $CaCl_2$, 20 mg; $MoCl_5$, 0.1 mg; and $2NiCO_3 \cdot 3Ni(OH)_2 \cdot 4H_2O$, 5 μg (adjusted to pH 7.3 with NaOH).

The sulfur source for both media was either 1.5 or 15 mM DMSO, 1 mM cysteine, 1.5 mM methionine, 1 to 50 mM SO_4^{2-}, 200 μM DBT or DBT sulfone, or 1% Casamino Acids (CAA).

To obtain cells for assays, a *Rhodococcus* seed culture was grown for 24 h in nutrient broth (8 g per liter of Difco nutrient broth), diluted 20-fold in BSM2, and grown for a further 36 to 48 h.

Cell extracts. Cells grown in the appropriate medium were concentrated to an optical density at 600 nm of 45 by centrifugation and resuspension in 10 mM phosphate buffer (pH 7.0). Cells were disrupted in a French press, and debris was

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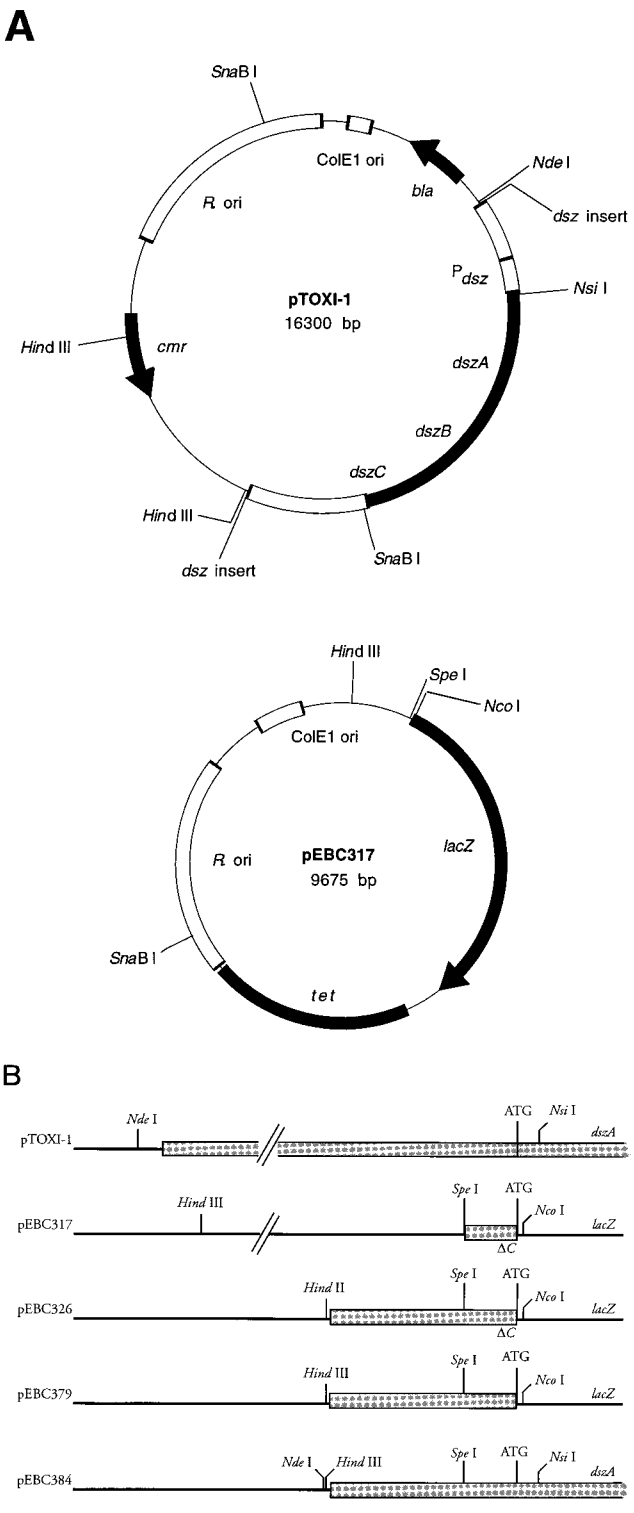

FIG. 1. (A) Maps of the *E. coli-Rhodococcus* shuttle vectors pTOXI-1 and pEBC317. pTOXI-1 is a derivative of pUC18 with a 6.9-kb *Rhodococcus* DNA fragment containing the *Rhodococcus* origin (*R.* ori) of replication and the *cmr* gene from pRF29 (6) and a 6.7-kb insert of *Rhodococcus dsz* DNA (29). pEBC317 is also a derivative of pUC18 with the *Rhodococcus* origin of replication from pRR6, the *tet* gene from pRK290 (ATCC 37168), and the *lacZ* gene. The 26 bases from *Spe*I to the initiation codon of pEBC317 are the same as the 27 bases prior to the initiation codon of *dszA* of pTOXI-1 except for the deletion of a C at −10 in pEBC317 (Fig. 3). The arrows indicate the direction of transcription. (B) Sites used for cloning 5′ untranslated *dsz* DNA. Shaded bars represent *Rhodococcus* DNA (not drawn to scale).

removed by centrifugation at 3,500 × *g* for 10 min. The resulting supernatant contained 1 to 2 mg of protein per ml.

Assays. Dsz activity was assayed by the ability of whole cells to convert DBT to HBP in a 1-h assay at 30°C and pH 7.0. The HBP produced was determined by high-pressure liquid chromatography (HPLC) (30). The specific activity was expressed in micromoles of HBP produced min^{-1} g (dry weight) of $cells^{-1}$. One gram (dry weight) of cells is equivalent to 1 liter of cells at an optical density at 600 nm of 3.5.

β-Galactosidase was measured by the method of Miller (22), except that activity was measured in cell extracts and was expressed per milligram of protein rather than per optical density at 600 nm.

Construction of *dsz* promoter-*lacZ* fusions. The 385 bp of *Rhodococcus* DNA immediately 5′ to *dszA* was cloned immediately 5′ to the *E. coli lacZ* gene. This was done by inserting a PCR product of *dsz* DNA from −385 to −34, where −1 is the base preceding the A of the initiation codon, with *Hin*dIII and *Spe*I (Fig. 2A) into pEBC317 (Fig. 1); pEBC317 has the remaining *dsz* DNA to the initiation codon of *lacZ*. The resulting plasmid, pEBC326, could express β-galactosidase only if the *dsz* DNA contained a promoter. A ribosome binding site is the sequence from about −20 to +13 (33); the *lacZ* binding site in pEBC326 is a fusion of *dszA* from −20 to −1 and then *lacZ* to +13. The control Dsz plasmid, pEBC384, was made by partially deleting the 1,275 bp of 5′ untranslated *Rhodococcus dsz* DNA in pTOXI-1 to 385 bp by PCR. All PCR products were sequenced to ensure that no mutations had been introduced.

During cloning, two changes were made in the *dsz* DNA. One was the insertion of a *Spe*I site at −33 to aid in cloning by changing GCCA at −32 to −29 to CTAG, and the other was the deletion of the C at −10 of pEBC317 and pEBC326 that resulted in a change in the Shine-Dalgarno region from AGGACG to AGGAG (Fig. 3). Although it might be expected that the latter change would improve ribosome binding and *dsz* gene expression, neither change had a significant effect on Dsz activity or its repression by CAA when these changes were introduced into pTOXI-1 (data not shown).

Hydrazine mutagenesis. The 385-bp *dsz* promoter-containing DNA fragment (Fig. 3) was cloned from pEBC326 into M13mp18N by using *Hin*dIII and *Nco*I for mutagenesis. Single-stranded DNA was treated with 13 M hydrazine for 50 min by the method of Walton et al. (35). The mutated fragments were subcloned into pEBC317 with *Hin*dIII and *Spe*I and transformed into CPE648. In the presence of sulfate or CAA, the *dsz* promoter was repressed; therefore, the colonies on BSM1–X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–CAA were pale yellow. However, any mutations that relieved the repression were expected to be blue. Such colonies were found but could have arisen from chromosomal or plasmid mutations. The latter could be in the *Rhodococcus* DNA or could have arisen from plasmid rearrangement. To differentiate between these possibilities, plasmid DNA from blue colonies was used to retransform CPE648. Plasmid mutations should give rise to further blue colonies. To verify that the plasmid-borne mutations were within the 385-bp fragment, this DNA was subcloned into pEBC317 and examined for its ability to give rise to blue colonies. This 385-bp DNA was then sequenced. The mutations selected are likely to either increase promoter strength or affect regulation.

Deletion analysis. PCR was used to generate deletion mutations in the 385-bp fragment. The 5′ deletion set (Fig. 2B) was cloned into pEBC317 with *Hin*dIII and *Nco*I for LacZ expression and into pEBC384 with *Nde*I and *Spe*I for Dsz expression. The 3′ set (Fig. 2C) was cloned into pEBC317 and pEBC384 with *Hin*dIII and *Spe*I.

S1 nuclease mapping. Total RNA was isolated from IGTS8 cells grown in BSM2 with 1.5 mM DMSO as the sulfur source by using a QIAGEN (Chatsworth, Calif.) total RNA isolation kit. S1 nuclease mapping was carried out with an Ambion (Austin, Tex.) S1 assay kit. The ^{32}P-end-labeled single-stranded probe, complementary to nucleotides −272 to +86, was synthesized by asymmetric PCR as described by Ambion. It was purified after agarose gel electrophoresis with a Qiagen QIAquick extraction kit. The PCR primers used were 5′-GTGGTGCTTGGCTATTGACC-3′ (forward) and 5′-CGACAGAAAGTC ATTCGACGC-3′ (reverse). The reverse primer was also used as the primer for the sequencing ladder. The single-stranded probe, complementary to nucleotides 91 to 459, used to determine the effects of sulfur sources on transcription was made with the following PCR primers: 5′-TCGAATGACTTTCTGTCGGG-3′ (forward) and 5′-ATGCTGATTAATGCCGAAGTTGC-3′ (reverse).

DNA-binding proteins. Ten nanograms of DNA labeled with ^{32}P (6×10^4 cpm ng^{-1}) was incubated with 10 μg of a crude extract of IGTS8 for 10 min at room temperature in a total volume of 20 μl. The binding buffer contained 50 mM Tris HCl (pH 7.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μg of poly(dI-dC), and 15% glycerol (2). This mixture was then analyzed on a 6% polyacrylamide gel. The cold competing DNA used was the same 385-bp fragment, pUC19 double-stranded DNA, or a 412-bp PCR fragment of *R. fascians* containing the *cmr* promoter (7).

Protein gels. Protein separations were done with Novex (San Diego, Calif.) precast 10% gels with Tris-glycine-sodium dodecyl sulfate (SDS) (Laemmli) running buffer.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
R. erythropolis IGTS8	*dsz*$^+$	16
R. erythropolis CPE648	*dsz* mutant that has lost a 120-kb linear plasmid containing the *dsz* gene cluster (5)	29
E. coli DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara-leu*) *7697 galU galK* λ$^-$ *rpsL endA1 nupG*	Gibco BRL
Plasmids		
pRR6	An *E. coli-Rhodococcus* shuttle vector conferring resistance to chloramphenicol	29
pTOXI-1	6.7-kb *Taq*I fragment from IGTS8 containing the *dsz* gene cluster and 1,275 bp of 5′ untranslated *Rhodococcus* DNA in pRR6	29
pEBC317	Derived from pBluescript II SK+ with deletion of the *bla* gene and insertion of a promoterless *E. coli lacZ* gene with the *dszA* ribosome binding site the *tet* gene from pRK290 (ATCC 37168), and the *Rhodococcus* origin of replication from pRR6 (Fig. 1)	This study
pEBC326	385 bp of 5′ untranslated *dsz* DNA from pTOXI-1 (Fig. 3) inserted before *lacZ* in pEBC317 with a *Spe*I site at −33 and the deletion of C at −10	This study
pEBC379	Same as pEBC326 except that it has a C at −10	This study
pEBC384	pTOXI-1 with the *Nde*I site followed by *Hin*dIII and with the 1,275 bp of 5′ untranslated DNA replaced with the 385 bp from pEBC379	This study
pEBC388	pEBC384 with the deletion of the G at −57	This study
pUC19N	pUC19 with an *Nco*I site inserted between *Eco*RI and *Bam*HI of the polylinker	This study
M13mp18N	M13mp18 with an *Nco*I site inserted between *Eco*RI and *Bam*HI of the polylinker	This study

RESULTS

Dsz repression. *R. erythropolis* IGTS8 was grown in BSM1 with 15 mM DMSO and increasing amounts of sulfur in the form of Cys, Met, or sulfate; DMSO has not been observed to repress Dsz activity. After 48 h of growth, Dsz activity was measured. As shown in Fig. 4, activity declined with increasing amounts of Cys, Met, or sulfate. Complete repression of the Dsz phenotype was obtained with 375 μM Met, Cys, or sulfate. At concentrations of 250 μM and lower, Met appeared to repress more than either Cys or sulfate did. On the basis of the detection limit of HPLC for HBP, repression was at least 50-fold (the ratio of the maximum activity to the minimum detectable).

Dsz proteins from the soluble fraction of lysates of IGTS8 cells grown with DMSO or DBT as the sole sulfur source can clearly be seen on a Coomassie blue-stained SDS-polyacrylamide electrophoresis gel (Fig. 5). The identities of these proteins were established by N-terminal sequencing of the purified proteins and by Western blotting (immunoblotting) with antibodies to these proteins (11). The most prominent of these protein bands were DszA and DszC, while the amount of the DszB protein appeared to be much smaller, particularly as it may comigrate with another *Rhodococcus* protein present in the extract of the Dsz$^-$ strain CPE648 (Fig. 5, lane 9). When IGTS8 cells were grown in the presence of Met, Cys, or sulfate, none of the Dsz proteins could be detected either by Coomassie blue staining or by Western blotting (data not shown). These proteins also cannot be detected in a lysate of the Dsz$^-$ strain CPE648 grown with DMSO (Fig. 5, lane 9).

LacZ expression from the *dsz* promoter. The *lacZ* gene from *E. coli* was used as a reporter for *dsz* promoter activity in a series of plasmids based on the *E. coli-Rhodococcus* shuttle vector pEBC317. Denome et al. (3) have identified a possible transcriptional terminator 335 bp before the start of *dszA*. If this is correct, then a *dsz* promoter should be located between this terminator and the start of *dszA*. Therefore, a 385-bp fragment immediately 5′ to *dszA* was cloned immediately 5′ to *lacZ* in pEBC317 and in pTOXI-1 in place of the 1,275 bp of 5′ untranslated *Rhodococcus dsz* DNA in this clone (see Materials and Methods). These plasmids were numbered pEBC326 and pEBC384, respectively.

Cells of strain CPE648 containing plasmid pTOXI-1, pEBC326, or pEBC384 were grown in BSM2 under repressing and nonrepressing conditions and assayed for β-galactosidase or Dsz activity. It was shown that the fragment had promoter activities for both β-galactosidase and Dsz and that the Dsz activities were the same for both pTOXI-1 and pEBC384 (Table 2), indicating that it had full *dsz* promoter activity. Furthermore, repression of β-galactosidase activity was up to 80-fold, which was similar to that of Dsz activity (>50-fold). This indicates that the 385-bp fragment contains both the *dsz* promoter and its regulatory regions.

Mutagenesis of the *dsz* promoter fragment. Hydrazine was used in order to obtain mutations within the 385-bp promoter-containing fragment in pEBC326 that reduced repression by CAA (Fig. 3). Colonies of CPE648 carrying pEBC326 are blue on BSM1–X-Gal plates but light yellow on the same plates with the addition of 1% CAA. After mutagenesis with hydrazine (see Materials and Methods), blue colonies that appeared with a frequency of about 0.2% on BSM1–X-Gal–CAA plates were selected. The plasmid DNAs from these strains were analyzed to verify that the blue phenotype was due to mutations in the 385-bp fragment (see Materials and Methods), which were then sequenced. Four different mutants, R1 to R4, were obtained (Table 3). The first had a double mutation at −263 and −244; the second had a mutation at −93; the third had two mutations, one at −93 and one at −38; and the fourth had a mutation at −57. Two to four isolates of each were obtained, but these were probably sibs. β-Galactosidase activities were determined, and it was found that all of the mutants examined had both increased activity compared with that of the wild type (WT) when grown under nonrepressing conditions and decreased repression, from more than 50-fold for the WT to as little as 4-fold for two of the mutants (Table 3). R4, with the deletion of the G at −57, had the highest activity in both DMSO and CAA. The β-galactosidase activity of this mutant under repressing conditions was as high as that of pEBC326 with the WT promoter under nonrepressing conditions.

Effects of mutants on Dsz activity. The effects of each of the four mutants on Dsz activity in pEBC384 were determined. When grown with DMSO, there was no increase in specific activity compared with that of the WT (Table 3). However,

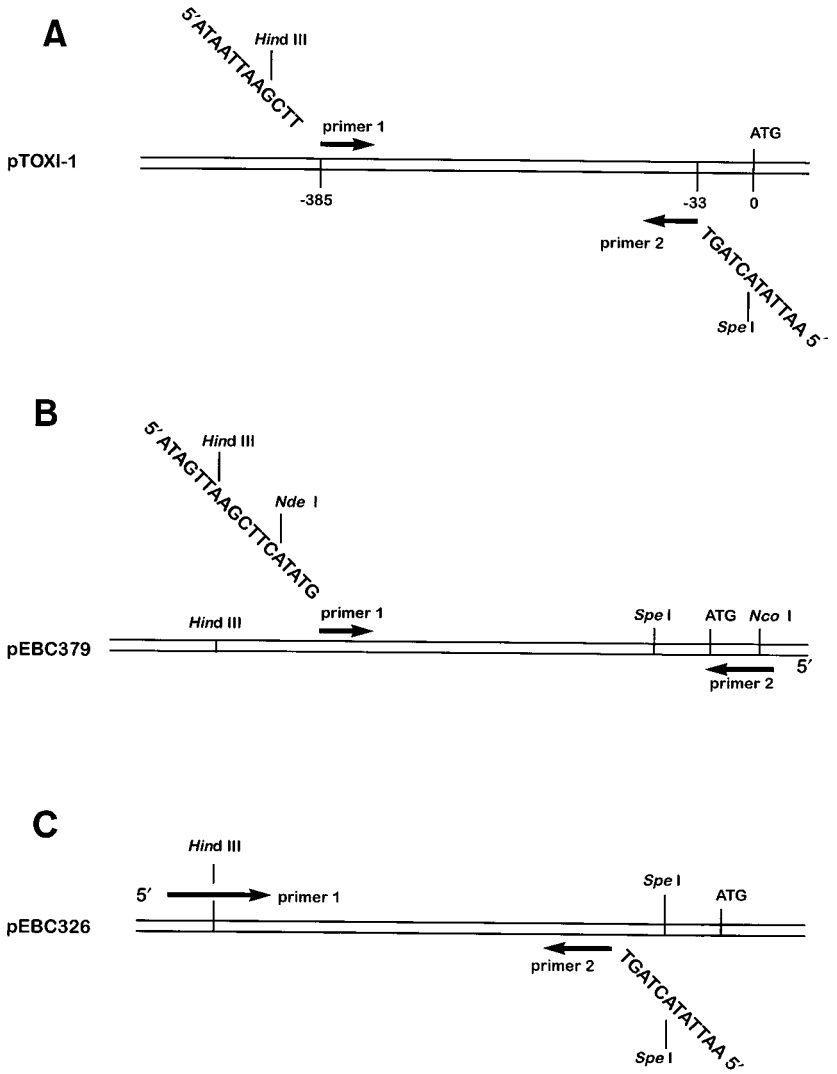

FIG. 2. Primers used for cloning promoter and promoter deletion fragments. (A) Cloning the 385-bp fragment of 5′ untranslated DNA from pTOXI-1 into pEBC317. (B) Construction of the 5′ promoter deletions. The PCR fragments were cloned into pUC19N with *Hin*dIII and *Nco*I for sequencing and then into pEBC317 for LacZ expression by using the same restriction sites. For Dsz expression, the *Nde*I and *Spe*I sites were used for cloning into pEBC384. (C) Construction of the 3′ promoter deletions. The PCR fragments were cloned into pEBC317 and pEBC384 for LacZ and Dsz expression, respectively. *Hin*dIII-*Nco*I fragments from the pEBC317 constructs were subcloned into pUC19N for sequencing.

under repressing conditions, Dsz activity was detected for three of the four mutants but not for R1 or WT pEBC384. R4, which had the highest level of LacZ activity, had the highest level of Dsz activity in the presence of CAA.

Deletion analysis of the *dsz* promoter fragment. Deletions of the promoter-regulatory fragment were made in order to localize the promoter and in an attempt to delete the regulatory regions while maintaining full promoter activity. Two sets of mutants with deletions of increasing size were made by PCR. One set started at the *Hin*dIII site at the 5′ side of the promoter, and the second set ended at the unique *Spe*I site of pEBC326 at −33 at the 3′ side (Fig. 2B and C). Both sets were subcloned into pEBC317 and pEBC384 for Dsz and LacZ activity measurements, respectively (see Materials and Methods). With the increasing size of the 5′ deletion, there appeared to be a progressive decrease in promoter activity for LacZ, with almost complete loss of activity with the 305-bp deletion (Fig. 6A). This was not reflected in Dsz activity, which was the same for all deletions except the largest deletion, which had no activity. For the 3′ set, the smallest deletion (10 bp) had only slightly reduced LacZ and Dsz activities. All the remaining deletions had very low levels of LacZ activity and no detectable Dsz activity (Fig. 6B). The deletions defined a region from −120 to −44 within which the promoter was located. A part of the promoter must be located in each of the regions from −120 to −80 and −60 to −44, as deletion of either of these segments eliminated promoter activity. None of the deletions described retained promoter activity and eliminated repression; each deletion that had promoter activity was fully repressed by CAA.

Mapping the mRNA start and regulation of transcription. The 5′ end of the *dsz* mRNA was determined by S1 nuclease protection using an end-labeled single-stranded probe complementary to nucleotides −272 to +86 (Fig. 3). Only one protected band was detected, allowing the start to be unambiguously assigned to a G at −46 (Fig. 7A). This is in agreement with the deletion data shown above.

The effects of growth with different sulfur sources on Dsz

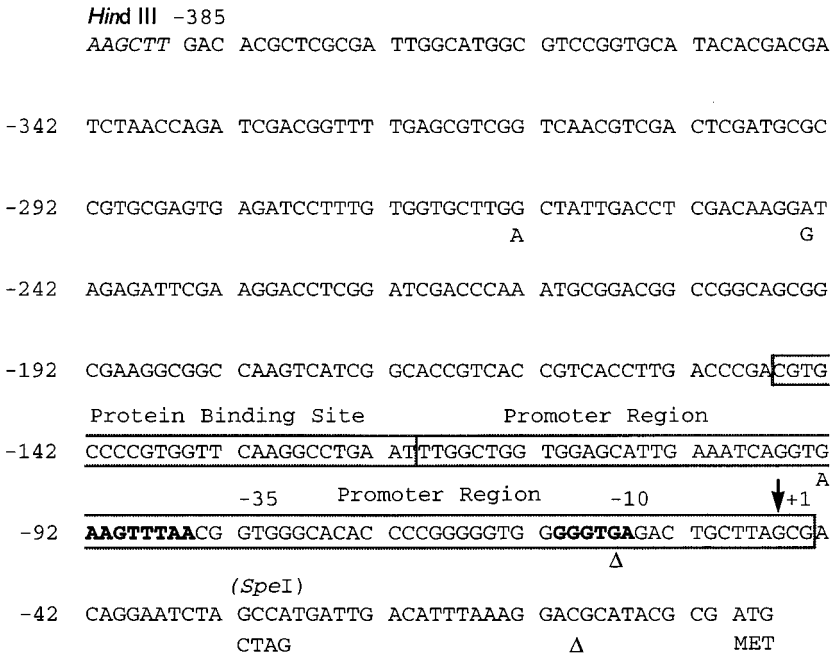

FIG. 3. Sequence of the 385-bp *dsz* promoter-containing fragment that starts immediately after the *Hin*dIII site. Nucleotides below the line indicate the positions of mutations, including the deletion of a C at −10. Boxed sequences are the protein-binding domain and the promoter region deduced from deletion analysis. The arrow indicates the G residue at +1 in the 5′ end of the mRNA, and the nucleotides in bold are the putative −10 and −35 regions of the promoter. The *Hin*dIII site at the 5′ end and the *Spe*I site near the 3′ end were added for cloning purposes. Sequence numbering is for the native fragment, where −1 is the base preceding the A of the ATG initiation codon of *dszA*.

transcription was determined by probing mRNA from cells grown with DMSO alone or in combination with SO_4^{2-} or Cys with a probe within *dszA* from 91 to 459. The results (Fig. 7B) show that with the WT promoter (pEBC384), mRNA was detected only from cells grown with DMSO as the sulfur source. With the mutant promoter R4 (pEBC388), mRNA was detected under all growth conditions. The intensity of the protected band appeared to be highest from cells grown with

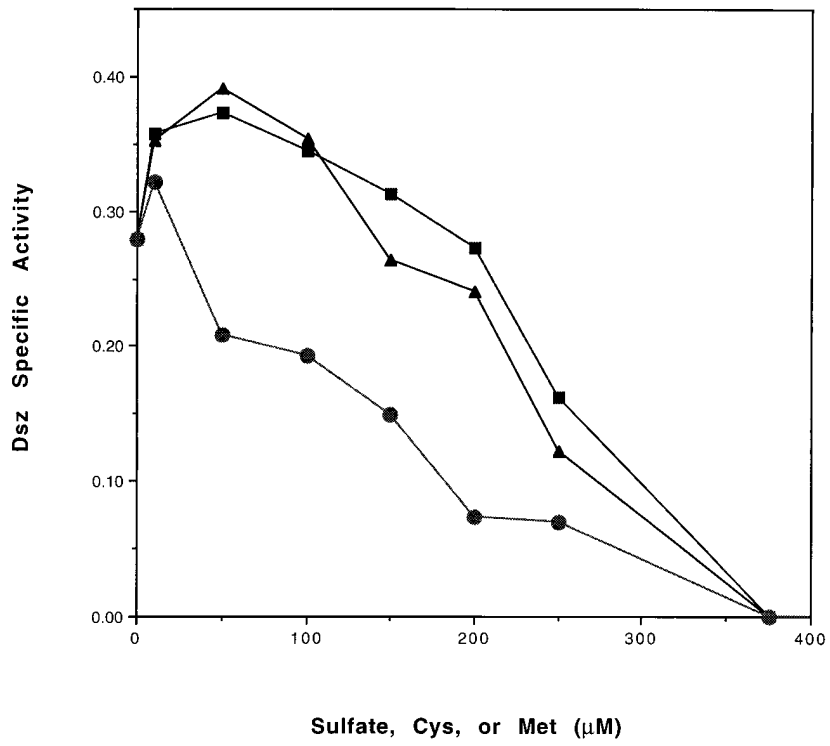

FIG. 4. Effects of concentrations of sulfur sources Met, Cys, and sulfate on Dsz activity of IGTS8. Activity was measured after 48 h of growth in BSM1 supplemented with 15 mM DMSO and increasing concentrations of sulfate (▲), Cys (■), or Met (●).

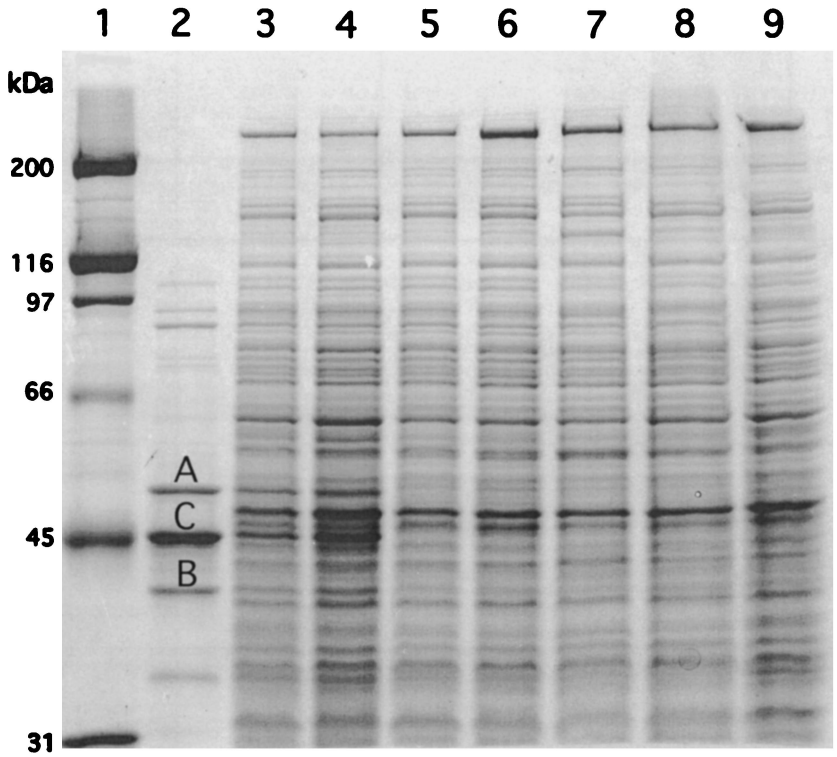

FIG. 5. Effects of sulfate on the expression of Dsz proteins. Proteins from IGTS8 or CPE648 were prepared as described in Materials and Methods, separated on an SDS–10% polyacrylamide gel, and stained with Coomassie blue. Each lane had a total of 100 μg of protein. Lanes: 1, molecular mass standards (Bio-Rad broad range); 2, mixture of purified DszA, -B, and -C proteins; 3 to 8, IGTS8 cells grown with 15 mM DMSO and the following additional sulfur sources: none (lane 3), 200 μM DBT (lane 4), 1.5 mM sulfate (lane 5), 1.0 mM Cys (lane 6), 1.5 mM Met (lane 7), and 1% CAA (lane 8); 9, CPE648 cells grown with 15 mM DMSO.

only DMSO. With extended exposure of the X-ray film (16 rather than 2.5 h), faint bands of S1-protected mRNA could be detected with the WT promoter in cells grown with SO_4^{2-} or Cys (data not shown).

DNA-binding protein. The results discussed above show that the *dsz* promoter is repressed by sulfate; this makes it likely that there is a repressor protein that can bind in the vicinity of the promoter. Therefore, the 385-bp *dsz* promoter-containing fragment was labeled with ^{32}P and incubated with protein extracts of IGTS8 cells grown in the presence of either sulfate, CAA, or DMSO as the sole sulfur source. DNA-binding protein was detected by a gel shift assay (Fig. 8). This binding was specific for the *dsz* promoter fragment, as it could be competed by the addition of unlabeled *dsz* promoter DNA but not by the addition of a similar-size fragment of unlabeled pUC19 DNA or *R. fascians cmr* promoter DNA (7). The sulfur source for IGTS8 growth did not appear to influence the amount of binding protein.

The deletion fragments generated for promoter activity were used to determine the location of the protein-binding domain. *Hin*dIII-*Spe*I fragments from each mutant were used as cold competing DNAs in the gel shift assay. It was found that only promoter fragments that had the region −144 to −121 (Fig. 6) were able to compete for binding with the full-size ^{32}P-labeled DNA. This region is immediately upstream of the promoter.

TABLE 2. Effects of sulfur sources on β-galactosidase and Dsz activities with the 385-bp *dsz* promoter-containing fragment[a]

Sulfur source (concn)	pEBC326 (*lacZ*)		pTOXI-1 (*dsz*)		pEBC384 (*dsz*)	
	β-Galactosidase[b]	Repression (fold)[c]	Dsz[b]	Repression (fold)[c]	Dsz[b]	Repression (fold)[c]
DMSO (1.5 mM)	3,700	1	0.34	1	0.32	1
SO_4^{2-} (1 mM)	301	12	0.00	>50	0.00	>50
SO_4^{2-} (50 mM)	103	36	NT[d]		NT	
Cys (1 mM)	50	74	NT		NT	
Met (1 mM)	46	80	NT		NT	
CAA (1%)	63	59	NT		NT	

[a] pEBC326 and pEBC384 with the 385-bp *dsz* promoter-containing fragment and pTOXI-1 transformed into CPE648 cells were grown in BSM2 with the indicated sulfur sources.
[b] β-Galactosidase and Dsz activities were measured as described in Materials and Methods.
[c] The level of β-galactosidase or Dsz activity in 1.5 mM DMSO was set at 1.
[d] NT, not tested.

TABLE 3. Effects of mutations in the 385-bp promoter-containing DNA fragment on LacZ and Dsz activities

dsz regulatory DNA	Mutation(s)	LacZ activity[a]			Dsz sp act[b]		
		DMSO	1% CAA	Repression[c]	DMSO	1% CAA	Repression[c]
WT (pTOXI-1)	None				0.34	0.00	>50
WT (pEBC326 and pEBC384)	None	1,820	48	38	0.32	0.00	>50
R1	G to A at −263 and A to G at −244	4,280	410	10	0.37	0.00	>50
R2	G to A at −93	3,820	870	4	0.30	0.025	12
R3	G to A at −93 and A to G at −38	2,320	890	4	0.30	0.022	14
R4	ΔG at −57	8,110	1,770	5	0.27	0.071	4

[a] LacZ activities in pEBC326 with different regulatory mutations were measured in cells grown in either 1.5 mM DMSO or 1% CAA.
[b] Dsz activities in pTOXI-1 and pEBC384 with different regulatory mutations were measured in cells grown as described above for LacZ activity measurements.
[c] Repression is the ratio of the activity in DMSO to that in 1% CAA.

DISCUSSION

Expression of the *dsz* gene cluster is required for desulfurization of DBT, a major source of sulfur in fossil fuels, by *R. erythropolis* IGTS8. The pathway from DBT to HBP has previously been elucidated (4, 8, 16, 29, 30), and the three genes and their products have been identified (3, 29, 30). This gene cluster has several of the attributes of an operon (29), although expression of the middle gene of the cluster, *dszB*, appears to be expressed at a level lower than that of the gene on either side (Fig. 5). Furthermore, a frameshift mutation at the *Nsi*I site in *dszA*, which should result in a TGA stop codon after 15 amino acids, did not appear to have a polar effect (29). There is no indication that the gene cluster is inducible, but it was strongly repressed by sulfate and sulfur-containing amino

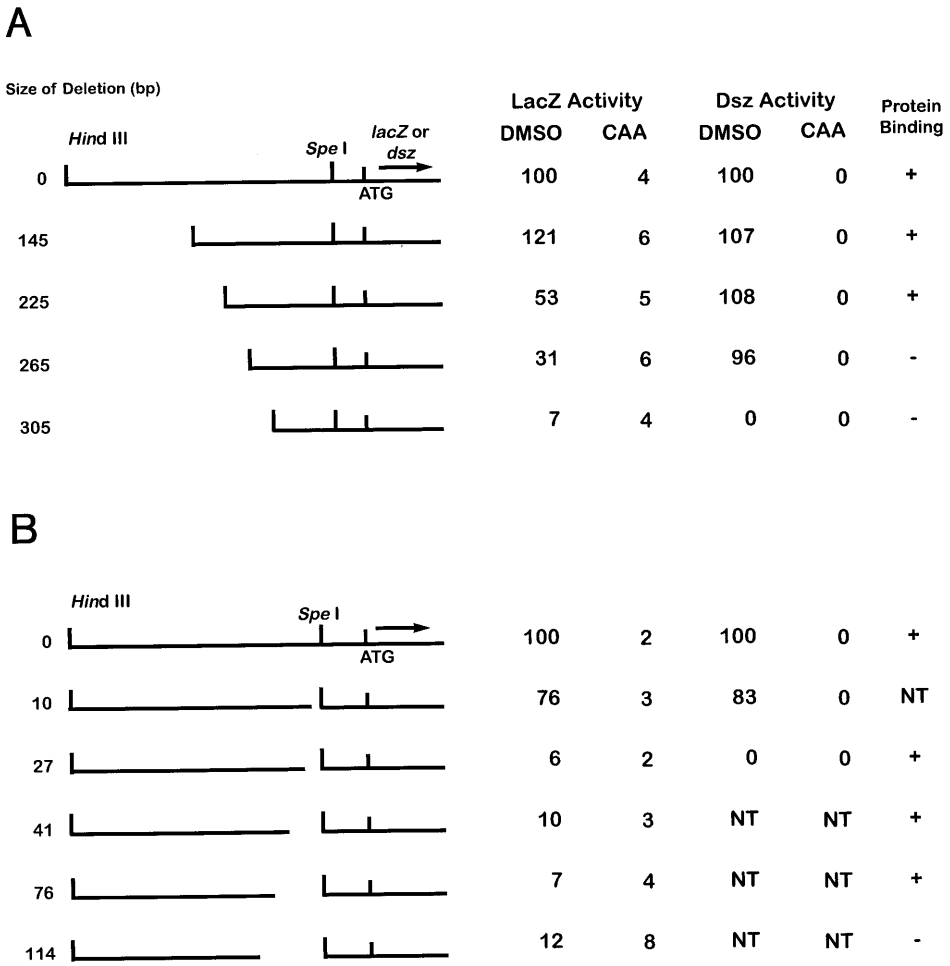

FIG. 6. Effects of deletions within the 385-bp *dsz* promoter fragment on LacZ and Dsz activity. (A) Deletions were made from the 5′ end, starting at the *Hin*dIII site. (B) Deletions were made on the 3′ side, ending at the *Spe*I site. Activities were normalized to 100 for the 385-bp promoter fragment in pEBC326 (*lacZ*) or pEBC384 (*dsz*) in CPE648 cells grown with DMSO as the sole sulfur source. + and −, presence and absence of protein binding, respectively; NT, not tested.

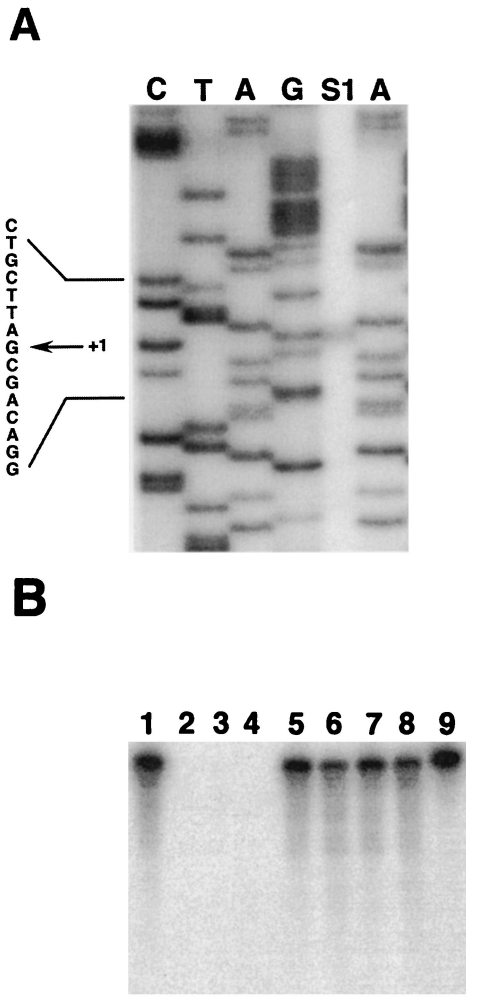

FIG. 7. (A) S1 nuclease mapping of the 5′ start of the *dsz* mRNA. This was carried out as described in Materials and Methods. The dideoxy ladder, lanes C, T, A, and G, and the extra A lane were generated with the same primer used to make the single-stranded probe. Lane S1 is the S1-protected mRNA from IGTS8 cells grown in the presence of DMSO. (B) Effects of sulfur source on transcription. mRNA was extracted from CPE648 cells containing either pEBC384 with the WT *dsz* promoter (lanes 1 through 4) or pEBC388 with the *dsz* promoter with the G deletion at −57 (lanes 5 through 8). Cells were grown with 1.5 mM DMSO and the following additional sulfur sources: DMSO only (lanes 1 and 5), 50 mM SO_4^{2-} (lanes 2 and 6), 1.0 mM Cys (lanes 3 and 7), and 1% CAA (lanes 4 and 8). Lane 9 contained only the probe. The mRNA was protected from S1 nuclease digestion with a single-stranded probe from bases 91 to 459 (see Materials and Methods).

acids (Fig. 4, 5, and 8B). Various organosulfur compounds have been tested for the ability to induce Dsz activity, but none of these induced activity in the presence of sulfate or increased activity in the absence of sulfate (18). Elucidation of the details of the regulatory circuits will help in the development of a commercially viable desulfurization process using this organism.

We have shown that a promoter for the *dsz* gene cluster is located in a 385-bp region immediately upstream of *dszA*. On the basis of deletion analysis, the promoter was localized to the region from −121 to −44. The S1 nuclease protection assay confirmed this and showed that the 5′ end of the *dsz* mRNA was the G at −46 (Fig. 7A). Only one S1 nuclease-protected band was detected, indicating that there is only one *dsz* promoter before the start of *dszA*. No meaningful homology could be detected by comparison with the −35 and −10 regions of *E. coli* promoters (14) or to the promoter for the chloramphenicol resistance gene, *cmr*, of *R. fascians* (7). A possible −35 promoter region with the sequence AAGTTTAA and a −10 region of GGGTGA are similar to those of *Bacillus subtilis* promoters that use sigma factor σ^B (25). Three of these promoters have a consensus sequence of (A/G)GG(A/T)TT(A/G)A and GGGTAT for the −35 and −10 regions, respectively (1, 34). The sequence at the transcription initiation site of these promoters, TAG, is also the same as that of the *dsz* promoter, with two starting at the middle A and one starting at the G as does the *dsz* promoter. The main difference between these promoters is that the *Bacillus* promoters have a 14-bp spacer region between the −10 and −35 regions, whereas the spacer region for the *dsz* promoter is 23 bp. σ^B is a nonessential sigma factor that is maximally expressed in early stationary phase (1).

Sulfate and sulfur-containing amino acids repressed Dsz activity (Fig. 4). There is no evidence of feedback inhibition of the enzymes themselves, as the addition of sulfate to cells grown with DBT did not inhibit Dsz activity (37). Furthermore, we have found that sulfate or CAA added to IGTS8 cells growing with DMSO as the sole sulfur source did not affect Dsz activity for more than 6 h (data not shown). However, transcription from the *dsz* promoter was strongly repressed by SO_4^{2-}, Cys, and CAA but not by DMSO (Fig. 7B), indicating that there could be a Dsz repressor. Repression by these sulfur-containing compounds may be achieved by direct interaction with this protein, or they may induce its expression. Each deletion that retained promoter activity was fully repressible, indicating that if there is a Dsz repressor protein, it binds to a site that overlaps the promoter. Overlaps between operators and promoters are not uncommon, e.g., LexA binds to at least nine operators, of which two overlap −10 regions and one overlaps a −35 region (for a review, see reference 14).

Five mutations which alone or in combination resulted in a partially derepressed Dsz phenotype were found in two regions, one at −263 to −244 and the second at −93 to −38. The mutation at −38 was isolated as a double mutation with another at −93 and may not be relevant, as a deletion from −33 to −43 had the WT phenotype. The other two mutations in the second region, at −57 and −93, are within the promoter; therefore, they are likely to be mutations that increase pro-

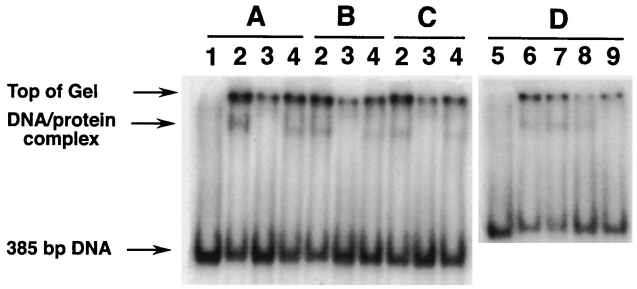

FIG. 8. Gel mobility shift assay indicates that a protein from IGTS8 binds to the 385-bp promoter-containing fragment (P_{dsz}). ^{32}P-labeled DNA was incubated with extracts of IGTS8 cells grown in BSM2 with 1% CAA (A), BSM2 with 1.5 mM SO_4^{2-} (B), BSM2 with 1.5 mM DMSO (C), and RM (10 g of glucose, 8 g of Difco nutrient broth, 0.5 g of Difco yeast extract per liter) (D). The competing unlabeled DNAs were P_{dsz}, pUC19, and *R. fascians cmr* promoter (P_{cmr}) DNAs. All lanes contained ^{32}P-labeled P_{dsz} DNA incubated with IGTS8 protein extracts and competing DNAs at a 15-fold excess, except as noted. Lanes: 1 and 5, ^{32}P-labeled DNA alone; 2 and 6, no competing DNA; 3, competing P_{dsz} DNA; 4, competing pUC19 DNA; 7, competing P_{cmr} DNA; 8, competing P_{cmr} DNA (100-fold excess); 9, competing P_{dsz} DNA.

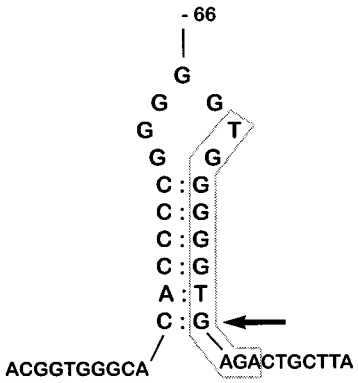

FIG. 9. Potential hairpin structure located in the *dsz* promoter. The hairpin is between −75 and −57 and has a free energy value of −15.4 kcal (1 cal = 4.184 J). Deletion of the G at −57 (indicated by the arrow), as in mutant R4, would reduce this to −13.2 kcal.

moter strength but could also affect repressor binding. Site-directed mutagenesis will probably be required to construct mutants with full promoter activity and no repression. The promoter encompasses a region of potential dyad symmetry (Fig. 9), including the mutation at −57, that could be part of an operator. An almost identical inverted repeat occurs within *dszB* from 1562 to 1578 and could be part of another operator.

lacZ was found to be very useful as a reporter gene to select promoter and regulatory *dsz* mutations. However, there was only a qualitative correlation between the LacZ and Dsz activities of mutants. Thus, for LacZ the mutants had both increased apparent promoter strength and simultaneously decreased repression. For Dsz, activity was increased only under repressing conditions and only for three of four mutants. For these three mutants, repression was decreased from >50-fold to between 4- and 14-fold. Coomassie blue gels (data not shown) indicated that the production of Dsz proteins was higher than might be expected from the activity measurements. It seems likely that Dsz activity is controlled not only by *dsz* gene expression but by other as-yet-unidentified factors.

A gel shift assay indicated that a protein bound to the DNA just upstream of the *dsz* promoter (Fig. 8). The binding appeared to be specific, as it could not be inhibited by pUC19 DNA or by DNA containing an *R. fascians* chloramphenicol promoter. The gel shift caused by the protein binding was relatively large and indicated that binding caused bending of the DNA. Such bending is frequent in A-rich regions (12, 38), but this was not true for the *dsz* protein-binding region (Fig. 3). This protein does not seem to be a *dsz* repressor, as deletion of the binding site did not appear to have any effect on repression, but LacZ activity was reduced by threefold. Thus, it is possible that it contains an enhancer region similar to the one before the promoter for gene 10 of phage T7 (26) or an activator similar to MetR. Mutations of two *metR* binding sites upstream from *glyA*, a gene positively regulated by MetR, reduced expression by only twofold (21). It is possible that DNA bending causes interaction between the DNA-binding protein and the −10 region of the promoter and that this is required for maximum LacZ activity. Homocysteine is a coregulator of MetR and can either increase or decrease its effect, depending on the gene that is regulated. As yet, the only evidence we have for coregulators of Dsz expression is the effects of Met and Cys on *dsz* expression. It would be interesting to isolate the binding protein and clone the gene coding for it. The gel shift assay also indicated that a protein-DNA complex which did not significantly migrate into the gel was formed. It appeared to be partially inhibited by an excess of unlabeled *dsz* DNA but not by pUC19 DNA (Fig. 8) and therefore warrants further investigation.

Thus, the 385 bp of 5′ untranslated *dsz* DNA contains a number of interesting elements; it appears to contain a promoter and at least three regions that affect Dsz activity. The first, at −263 to −244, reduced repression, but deletions did not affect repression or gene expression. The second region, between −144 and −121, was able to bind a protein that could be an activator; deletion of this region reduced gene expression but not repression. The third region, between −57 and −98, may be a repressor binding site that overlaps the promoter −10 and −35 regions. It is possible that combinations of these mutations could further decrease repression.

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