

Cloning and Characterization of *sigA* and *sigB* Genes from *Rhodococcus opacus* Strain B4: Involvement of *sigB* in Organic Solvent Tolerance

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We cloned and sequenced the *sigA* and *sigB* genes from organic solvent-tolerant *Rhodococcus opacus* strain B4. The predicted SigA and SigB proteins showed high similarity with the mycobacterial principal sigma factor, σ^A proteins, and the mycobacterial alternative sigma factor, σ^B proteins, respectively. The *sigA* gene was transcribed almost constitutively during growth and after environmental stress, while transcription of the *sigB* gene was induced during transition from exponential growth phase to stationary phase and after environmental stress including heat shock and ethanol stress. The *sigB* mutant of *R. opacus* strain B4 showed decreased growth at higher temperature and in the presence of ethanol. In addition, the *sigB* mutant increased susceptibility to benzene, toluene, and *n*-hexane, although it was as tolerant to other organic solvents as the parental strain. Our results indicate that SigB is involved in cell tolerance to specific organic solvents in *R. opacus* strain B4. It was found that SigB is required for expression of benzene dioxygenase operon. However, genetic analysis revealed that organic solvent degradation is not involved in organic solvent-tolerance even under the *sigB* deficient background.

Key words: solvent tolerance, *Rhodococcus opacus*, *sigA*, *sigB*, RpoD family

1. Introduction

It was 1989 when Inoue and Horikoshi first reported the presence of a bacterium which can thrive in high concentrations of toluene¹³. Since then, several organic solvent-tolerant bacteria have been isolated³⁵. They include *Pseudomonas putida* strains, *Bacillus* spp., *Flavobacterium* strain DS-711, *Arthrobacter* ST-1, and *Rhodococcus* sp. strain 33 (reference 4, and see references cited in reference 35). We isolated *Rhodococcus opacus* strain B4 from gasoline-contaminated soil as a benzene-tolerant bacterium²⁴. This strain not only tolerates a wide variety of aliphatic and aromatic hydrocarbons but also utilizes them as a sole source of carbon and energy. It was demonstrated that this strain is able to survive and exhibit biocatalytic activity in anhydrous organic solvents for at least 5 days^{33,39}. Therefore, *R. opacus* strain B4 is expected to be a good candidate of a host strain for bioproduction of value added hydrophobic chemicals.

The mechanisms underlying bacterial tolerance to organic solvents have been investigated intensively with Gram-negative bacteria *P. putida* and *Escherichia coli*³⁵. They include modifications in cell envelope to increase cell membrane rigidity and decrease permeability^{9,10,15}, solvent-inactivating enzymes⁵, active efflux of solvents by solvent efflux pumps^{16,19,31}, and release of membrane vesicles with solvent

molecules adhering to cells¹. However, little is known about solvent-tolerant mechanisms in Gram-positive bacteria. Although Iwabuchi *et al.* reported involvement of extracellular polysaccharide in organic solvent-tolerance in *Rhodococcus rhodochromus*¹⁴, it is unclear even whether Gram-positive bacteria possess solvent-tolerant mechanisms similar to those of Gram-negative bacteria. Sardesai and Bhosle paid attention to sigma B in *Bacillus subtilis* as a factor involved in solvent tolerance because this secondary sigma factor is induced by environmental stress including ethanol shock and involved in bacterial survival after exposure to ethanol³⁵. They also pointed out that SigB had been shown to regulate expression of *bmrUR* operon encoding proteins that may contribute to resistance to multidrug compounds²⁸. However, this speculation has not yet been verified experimentally.

Rhodococcus, together with *Mycobacterium*, *Corynebacterium*, *Nocardia*, and *Gordonia*, belongs to the mycolata, a broad and diverse group of mycolic-acid-containing actinomycetes. In *Mycobacterium tuberculosis*, *Corynebacterium glutamicum*, and *Brevibacterium flavum*, two σ^{70} family proteins, σ^A and σ^B , have been identified and characterized^{2,12,26}. The mycobacterial σ^A protein is a principal and essential sigma factor, while mycobacterial σ^B is an alternative sigma factor and plays roles similar to those of *B. subtilis* sigma B. Transcription of the σ^B gene is in-

duced upon entry into the stationary phase and by environmental stress^{7,11}), and involved in cell responses to environmental stress including ethanol stress⁸). In this paper, we cloned and characterized genes encoding σ^A and σ^B from *R. opacus* strain B4. We also constructed the *sigB* mutant of *R. opacus* strain B4 and examined it for its responses to organic solvents to investigate the role of SigB in solvent tolerance in *R. opacus* strain B4.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Rhodococcus opacus strain B4²⁴) and its derivatives were used in this study. *R. opacus* strains were grown at 28°C with shaking in MSB medium²⁴) with 0.5% glucose and tryptic soy broth (TSB; Difco Laboratory, Detroit, MI, USA). For solid media, 2% agar was added. Whenever necessary, media were supplemented with 5 mg/l of gentamycin, 25 mg/l of chloramphenicol, and 20% sucrose. *E. coli* MV1184³⁸), which was used for plasmid construction and DNA manipulation, was grown at 37°C with shaking in 2x YT medium³²) supplemented with appropriate antibiotics.

2.2. DNA manipulation and sequencing

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations, agarose gel electrophoresis, and Southern hybridization³²). Polymerase chain reactions (PCRs) were carried out

using KOD plus DNA polymerase (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Oligonucleotides used for PCR are listed in Table 1. Before cloning, PCR products were electrophoresed and purified from the 1% agarose gel using the GeneCleanII kit (Bio101, Carlsbad, CA, USA). *R. opacus* was transformed by electroporation as described previously²⁵). The nucleotide sequence was determined by the dideoxynucleotide sequencing method³⁴). Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information databases).

2.3. Cloning and sequencing of the *sigA* and *sigB* genes from *R. opacus* strain B4

The genome sequence of *Rhodococcus* sp. strain RHA1, which is closely related to *R. opacus* strain B4, has been completely sequenced²¹). We designed PCR primer sets B4sigAf/B4sigAr and B4sigBf/B4sigBr (Table 1) based on nucleotide sequences of the *sigA* and *sigB* genes from *Rhodococcus* sp. strain RHA1 (accession number NC 008268, locus tags RHA1 ro06823 [*sigA*] and RHA1 ro06813 [*sigB*]). The *sigA* and *sigB* genes were amplified from the *R. opacus* strain B4 genome by PCR using these primer sets and the resulting PCR products were cloned into the *Sma*I site of pBluescript II KS+ (Stratagene, TX, USA). Then, the *R. opacus* strain B4 genome was analyzed by Southern hybridization with the cloned *R. opacus* strain B4 *sigA* and *sigB* sequences as probes. The *sigA* probe

Table 1. Oligonucleotides used for PCR and qRT-PCR

Primer	Sequence (5' to 3')
Primers for PCR	
B4sigAf	GTGGCAGCCACCGATACACGTCAGA
B4sigAr	GCAGGTGCTGCGCGACTACCTGGAC
B4sigBf	ATGACAAGCCCCAGCACCCTACTC
B4sigBr	CGAGCGGCTGCGCTCCTACGCCAGC
sigB1f	GGAAGCTTACCGTGGGTGCTCTCCGACCTCGA
sigB1r	GGAGATCTGACTTGCCTCCTCATCGTGGTGTG
sigB2f	GGAGATCTTCTCTCCCTCCGCATCAGCGCCGGT
sigB2r	GGTCTAGATTCGACTGTGCGCTGCTGTTCCGGT
sigB3f	AACTGATCGAACTTCTCGCTGCCGC
sigB3r	GCCGAGGGTGCCTGGTACCCGATT
Primers for qRT-PCR	
(For measurement of <i>sigA</i> mRNA)	
sigA-RT-F	GACATGGACGACATCGAC
sigA-RT-R	CTTGTCCTTCTCGGACG
(For measurement of <i>sigB</i> mRNA)	
sigB-RT-F	CACCACTACTCGCATTCG
sigB-RT-R	ACAGTCCACCTCGTCTG
(For measurement of <i>bnzA1</i> mRNA)	
bnzA1-RT-F	TGTACGAACAGGAACTGGAG
bnzA1-RT-R	ATCCTCGCCCATGTAGTTC
(For measurement of <i>gyrB</i> mRNA)	
gyrB-RT-F	GTCTACAGCTTCGAGACG
gyrB-RT-R	TGACGACCTTTCGGTG

detected 1.1-kb and 0.6-kb *Bam*HI genomic fragments, while the *sigB* probe detected a 4.7-kb genomic fragment. These *Bam*HI and *Not*I fragments were cloned into pBlue-script II KS+. The PCR products and cloned *Bam*HI and *Not*I fragments were sequenced to determine nucleotide sequences of the *sigA* and *sigB* genes in *R. opacus* strain B4.

2.4. Plasmid construction

PCR using primer sets sigB1f/sigB1r and sigB2f/sigB2r (Table 1) was conducted to amplify 1.3-kb regions upstream and downstream of *sigB* from the *R. opacus* strain B4 genome, respectively. Amplified upstream and downstream regions were digested with *Hind*III-*Bgl*II and *Bgl*II-*Xba*I, respectively, and ligated with the backbone of *Hind*III-*Xba*I-digested pG19II²²) to obtain pG19-dsigB. Plasmid pG19-Pkm-bnz¹⁷) was used to exchange a promoter region of the *bnz* operon in *R. opacus* strain B4 with a promoter of kanamycin resistant (Km^r) gene from pUC4K³⁷). The multi-cloning site from pBluescript II KS+ (Stratagene, Cedar Creek, TX, USA) was introduced into *Rhodococcus-E. coli* shuttle vector pKNR01.1²⁵) to obtain pKNR10.1. The *sigB* gene was amplified from *R. opacus* strain B4 genome by PCR using a primer set sigB3f/sigB3r and the PCR product was cloned into pKNR10.1 to construct pKNR-sigB.

2.5. Unmarked genomic gene modification

Plasmid pG19II²²) was used for the unmarked gene modification in *R. opacus* strain B4. pG19II was originally constructed for the insertion-deletion mutation of chromosomal genes in *Pseudomonas aeruginosa*. This plasmid contains gentamycin resistant (Gm^r) gene as a selectable marker, *sacB* (conferring sucrose sensitive phenotype) as a counter-selectable marker, the multi-cloning sites, an *E. coli* replication origin, and an origin of transfer. To delete a genomic gene, a derivative of pG19II was constructed by cloning upstream and downstream regions of the target gene into pG19II. Strain B4 was transformed with the pG19II derivative and Gm^r transformant cells were obtained. The Gm^r transformants were grown in TSB without gentamycin to allow the second homologous chromosomal recombination. After cultivation for 6 h, culture broth was spread on TSB agar plates containing 20% sucrose and the plates were incubated at 28°C to select sucrose resistant colonies. Theoretically, half of the sucrose resistant colonies were unmarked deletion mutants and the other half has a parental genotype. Unmarked gene modification in derivatives from *R. opacus* strain B4 was confirmed by PCR analysis.

2.6. Organic solvent-sensitive growth assays

R. opacus strains were cultivated with shaking in TSB. Overnight cultures were diluted with TSB and adjusted to optical density at 600 nm (OD₆₀₀)=0.1. To assay sensitivity to organic solvents, 5 µl of 10-fold serial dilutions of the diluted cultures were spotted onto TSB agar plates. The plates were incubated at 28°C in a desiccator with a beaker

containing each of liquid organic solvents. Colony growth was inspected after 72 h of incubation.

2.7. Quantitative reverse transcription PCR (qRT-PCR)

R. opacus strains were cultivated in TSB in the absence and presence of toluene in the vapor phase. Cells were harvested by centrifugation at 4°C for 10 min. Total RNA was extracted from *R. opacus* cells using an RNeasy kit (QIAGEN, Inc., Valencia, CA) with a RNAprotect bacteria reagent (QIAGEN) for the stabilization of RNA. RNA samples were treated with an RNase-free DNase set (QIAGEN) to eliminate contaminating DNA. Complete removal of DNA was confirmed by PCR. A One Step SYBR PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan) was used for cDNA generation and qRT-PCR. qRT-PCR was carried out using a LightCycler system (Roche Diagnostics) with an reverse transcription at 42°C for 15 s followed by cycles of denaturation at 95°C for 5 s, primer annealing at 60°C for 10 s, and extension at 72°C for 6 s. Primer sets used in qRT-PCR were listed in Table 1. The *gyrB* mRNA was also measured as the standard using primer set *gyrB*-RT-F/*gyrB*-RT-R and used for normalization.

2.8. Nucleotide sequence accession numbers

The DDBJ accession numbers of the *sigA* and *sigB* genes of B-4 are AB477528, and AB477529, respectively.

3. Results and Discussion

3.1. Cloning and sequence analysis of *sigA* and *sigB* from *R. opacus* strain B4

The *sigA* and *sigB* genes from *R. opacus* strain B4 were cloned and sequenced as described in "Materials and Methods" section. Sequence analysis revealed that the *sigA* gene of 1371 bp and the *sigB* gene of 969 bp could code for proteins of 457 and 323 amino acid residues having calculated molecular weights of 50.0 and 36.0 kDa, respectively. Blastp analysis indicated that *R. opacus* strain B4 SigA shows a high similarity to mycobacterial principal sigma factors (σ^{70} family group 1), σ^A proteins. In the σ^{70} family proteins, regions 1.2, 2, 3, and 4 are conserved²⁰), and in these regions, *R. opacus* strain B4 SigA shares 88–97% identical amino acid residues with *Mycobacterium tuberculosis* H37Rv MysA (NP 212719), *Mycobacterium smegmatis* strain MC2 155 SigA (YP 887090), and *Corynebacterium glutamicum* ATCC 13032 SigA (NP 601117). Especially in the highly conserved regions (RpoD box in region 2.3, 14-mer sequence in region 2.4, and 20-mer sequence in region 4.2²⁰), *R. opacus* strain B4 SigA shows a perfect match with *M. tuberculosis* H37Rv MysA. In contrast, there is much lower similarity among these sigma factors in region 1.1 which is poorly conserved in the principal sigma factors although *R. opacus* strain B4 SigA is almost identical to *Rhodococcus* sp. strain RHA1 SigA in this region. The conserved regions (regions 1.2, 2, 3, and 4) were also found in *R. opacus* strain B4 SigB. However, this

protein lacks region1.1. Moreover, *R. opacus* strain B4 SigB has higher similarity (79–88% identity) to mycobacterial σ^B proteins (*M. tuberculosis* H37Rv MysB [NP 217226], *C. glutamicum* ATCC 13032 SigB [NP 601125], and *M. smegmatis* strain MC2 155 SigB [YP 887083]) than to σ^A proteins. These results suggest that *R. opacus* strain B4 SigB belongs to the mycobacterial σ^B .

3.2. Transcriptional analysis of *sigA* and *sigB*

Mycobacterial σ^A proteins are principal and essential sigma factors and responsible for most RNA synthesis in exponentially growing cells^{6,7,11,20,27}. The σ^A genes are transcribed almost constitutively during growth and after stress conditions. Mycobacterial σ^B proteins are dispensable alternative sigma factors and thought to play roles similar to those of *E. coli* RpoS^{8,11,18,23}. Transcription of the σ^B genes is induced during transition from exponential growth phase to stationary phase and after several stress conditions such as acid stress, ethanol shock, heat shock, and H₂O₂ exposure^{8,11,23,27}. To confirm that *R. opacus* strain B4 SigA and SigB belong to mycobacterial σ^A and σ^B , respectively, transcriptional analysis was conducted for *sigA* and *sigB* by qRT-PCR.

Fig. 1 shows time course data of *sigA* and *sigB* transcription when *R. opacus* strain B4 was cultivated in TSB with shaking at 28°C. The level of transcription of *sigA* was almost constant during different growth phase, suggesting that *sigA* is constitutively transcribed. Transcription pattern of *sigB* is similar to those of other mycobacterial σ^B genes and the level of *sigB* transcription increased during the transition from exponential to stationary growth phase. We then investigated effects of environmental stress on transcription of *sigA* and *sigB*. *R. opacus* strain B4 cells at the late exponential growth phase were subjected to heat shock

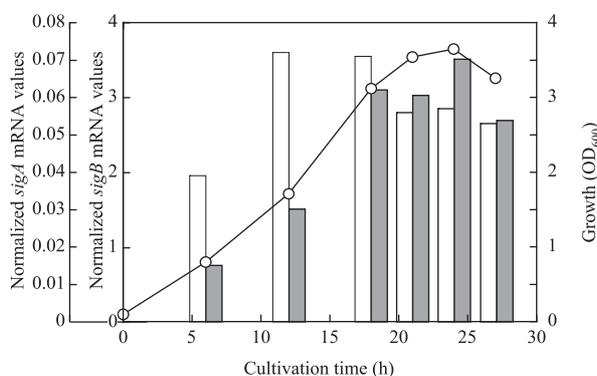


Fig. 1. Time course of transcription levels of *sigA* and *sigB* during growth in MSB medium with glucose.

R. opacus strain B4 was cultivated in MSB medium with glucose. Bacterial cells were sampled at 6, 12, 18, 21, 24, and 28 hours of incubation and total RNA was isolated from cells. Transcription levels of *sigA* and *sigB* were measured by qRT-PCR. Normalized mRNA values were obtained by dividing the mRNA values by that of the *gyrB* mRNA value for the same template. Open circles, bacterial growth; open bars, normalized *sigA* mRNA values; grey bars, normalized *sigB* mRNA values.

stress (33°C) and ethanol stress (3% ethanol) and transcription levels of *sigA* and *sigB* in cells were measured. As controls, transcription levels of *sigA* and *sigB* were measured in cells which were not exposed to the environmental stress. Heat shock and ethanol stress induced the transcription of the *sigB* mRNA (Fig. 2). Compared with controls, 1.7-fold and 4.8-fold induction of the *sigB* mRNA was observed after exposure to heat shock and ethanol stress, respectively. In contrast, transcription of *sigA* was not affected by heat shock and ethanol stress. There was no significant difference in transcriptional level of the *sigA* gene between control cells and stressed cells. Thus, it was shown that transcriptional responses of *R. opacus* strain B4 *sigA* and *sigB* to growth rate and environmental stress are similar to those of mycobacterial σ^A and σ^B genes, respectively.

3.3. Construction of unmarked *sigB* deletion mutant of *R. opacus* strain B4

We further characterized *R. opacus* strain B4 SigB. To investigate the role of SigB in *R. opacus* strain B4 cells, an unmarked *sigB* deletion mutant of *R. opacus* strain B4, designated *R. opacus* strain dsigB, was constructed using pG19-dsigB as described in “Materials and Methods” section. Deletion mutation of the genomic *sigB* gene was confirmed by PCR analysis (data not shown). Strain dsigB showed normal growth when it was cultivated under normal conditions (in MSB medium with glucose at 28°C with shaking) (Fig. 3A). To investigate the influence of *sigB* deletion on responses to environmental stress, we examined strain dsigB for its growth under different environmental stress. Strain

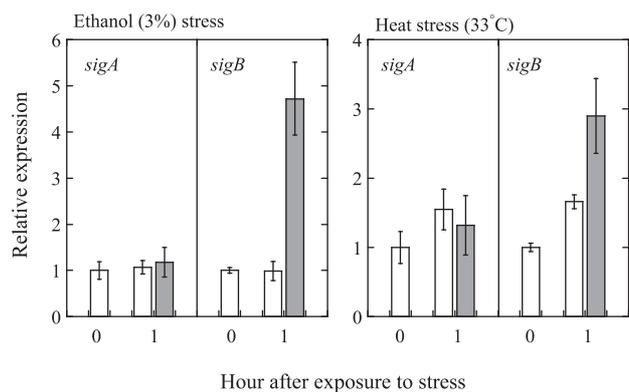


Fig. 2. Effects of ethanol stress and heat shock on transcription of *sigA* and *sigB* in *R. opacus* strain B4.

R. opacus strain B4 was grown in MSB medium with glucose. When OD₆₀₀ of cultures reached at 1.0, ethanol was added to cultures at a concentration of 3% (ethanol stress) or cultivation temperature was shifted from 28°C to 33°C (heat shock). After one hour of incubation under stressed conditions, total RNA was isolated and transcription of *sigA* and *sigB* was measured by qRT-PCR. Relative expression of *sigA* and *sigB* was obtained by dividing normalized mRNA values after one hour of incubation under stressed conditions by normalized mRNA values just before exposure to stress. Values represent the mean and standard deviation of at least three observations. Open bars, relative expression in unstressed cells; grey bars, relative expression in stressed cells.

dsigB showed growth similar to that of the parental strain under acidic conditions (pH=5.0 and 6.0) (Fig. 3B). In contrast, the sigB mutation affected growth at higher temperature (33°C) and in the presence of ethanol. As shown in Fig. 3C and D, strain dsigB showed significantly decreased growth compared to the parental strain when they were grown in MSB with glucose under higher temperature (33°C) or in the presence of 3–4% ethanol. Simultaneous exposure of cells to higher temperature and ethanol stress severely affected growth of strain dsigB. Strain dsigB failed to grow at 33°C in the presence of 3% ethanol, while the parental strain showed decreased but significant growth (Fig. 3E). These results together with transcriptional responses of sigB to environmental stress indicate that SigB is involved in the regulation of the gene expression in response to environmental stress.

3.4. The sigB gene is involved in tolerance to specific organic solvents

In this study, it was demonstrated that SigB plays a role in a response to ethanol stress in *R. opacus* strain B4. Therefore, we then conducted organic solvent-sensitive growth assays to assess involvement of sigB in tolerance to organic solvents in *R. opacus* strain B4. Organic solvents tested were aliphatic hydrocarbons (*n*-hexane, *n*-octane, and *n*-decane) and aromatic compounds (benzene, toluene, *o*-, *m*-, *p*-xylenes, ethylbenzene, *o*-, *m*-, *p*-cresols, dimeth-

ylphthalate, and diethylphthalate). The sigB mutant showed increased susceptibility to benzene, toluene, and *n*-hexane, but not to other organic solvents (Fig. 4). Introduction of pKNR-sigB containing the functional sigB gene complemented the mutant phenotype of strain dsigB, confirming that increased susceptibility of strain dsigB to benzene, toluene, and *n*-hexane was due to the sigB mutation (Fig. 4). It is the first demonstration that SigB is involved in bacterial tolerance to hydrocarbons.

It was found that SigB is involved in tolerance to specific organic solvents (*i.e.* benzene, toluene, and *n*-hexane) in *R. opacus* strain B4. How can this specificity be explained? We paid attention to inactivating (degrading) activities of organic solvents for this explanation because both benzene and toluene are metabolized via the common pathway, benzene dioxygenase pathway²⁴). In the previous study, we demonstrated that organic solvent-tolerance was not affected by inactivation of benzene dioxygenase (*bnz*) operon in *R. opacus* strain B4²⁴). However, we speculated that inactivation (degradation) of organic solvents are required for organic solvent-tolerance in the sigB-deficient background, where expression of some other functions required for organic solvent-tolerance might be also defective, and that SigB regulates expression of *bnz* operon. To assess this possibility, we measured transcription level of *bnzA1* encoding the benzene dioxygenase large subunit in cells grown on TSB agar plates supplemented with toluene in the vapor

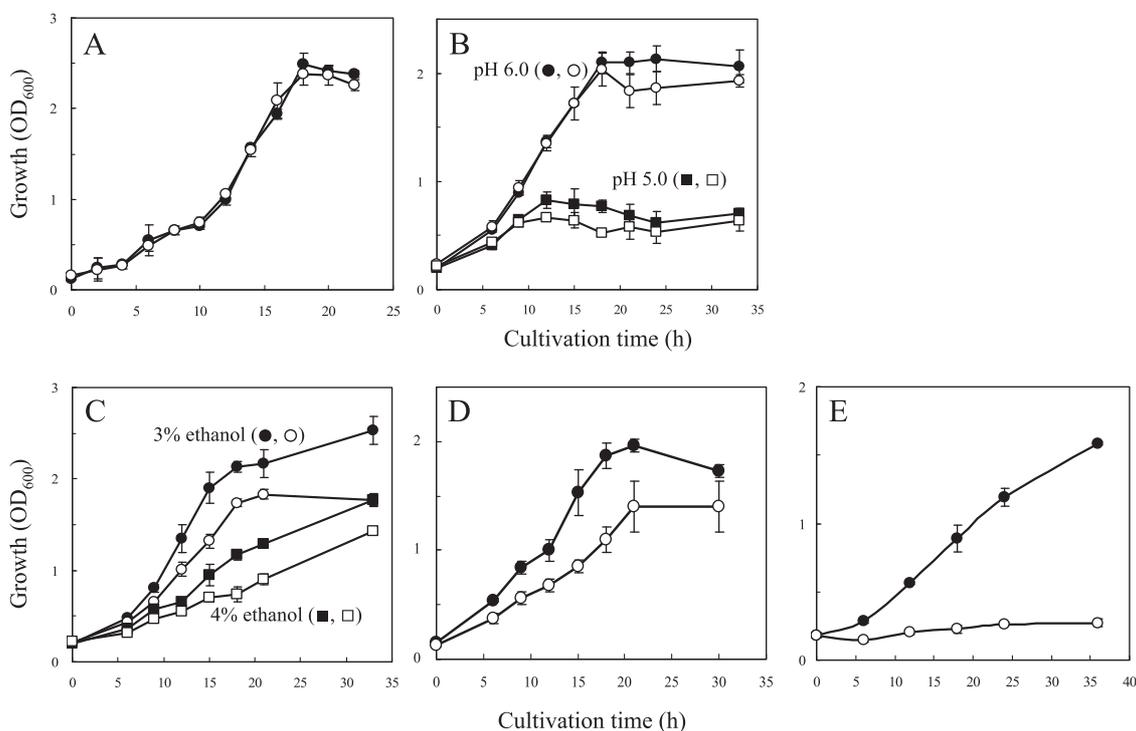


Fig. 3. Growth of the sigB mutant (strain dsigB) and parental strains of *R. opacus* strain B4 under various environmental stresses.

Each experiment was performed using triplicate samples. The values represent the mean and the standard deviation for each point. MSB supplemented with glucose as a carbon source was used for growth medium. (A) Growth at 28°C and initial pH 7.0 (control), (B) growth at 28°C and initial pH 5.0 and 6.0 (low pH stress), (C) growth at 28°C and initial pH 7.0 in the presence of 3 and 4% ethanol (ethanol stress), (D) growth at 33°C and initial pH 7.0 (heat stress), (E) growth at 33°C and initial pH 7.0 in the presence of 3% ethanol (ethanol+heat stresses). Closed symbols, the parental strain; open symbols, the sigB mutant strain.

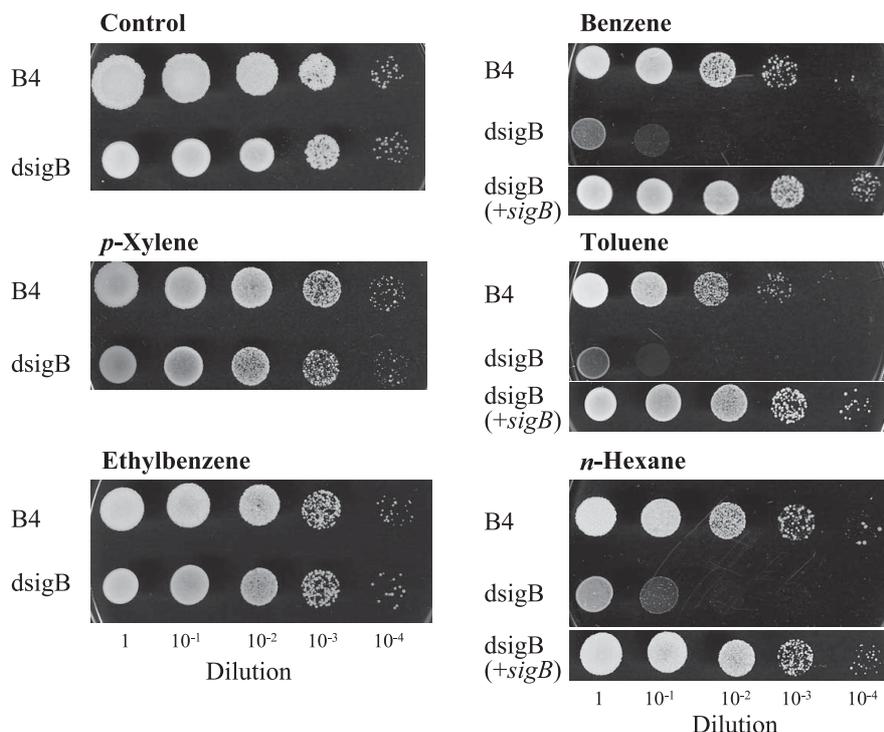


Fig. 4. Organic solvent-sensitive growth assays of *R. opacus* strain B4 and its derivatives.

Overnight cultures were diluted and adjusted to $OD_{600}=0.1$. Five μ l of 10-fold serial dilutions of the diluted cultures were spotted onto TSB agar plates. The plates were incubated at 28°C in a desiccator with a beaker containing each of liquid organic solvents. Colony growth was inspected after 72 h of incubation. B4, the parental strain; dsigB, the *sigB* mutant; dsigB (+*sigB*), strain dsigB harboring pKNR-*sigB*. Although data are not presented here, the *sigB* mutant showed growth similar to that of the parental strain when exposed to other organic solvents tested.

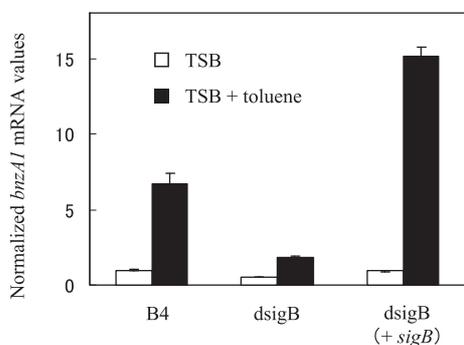


Fig. 5. Transcription levels of *bnzA1* in *R. opacus* strain B4 and its derivatives.

The parental strain, the *sigB* mutant, and the *sigB* mutant harboring pKNR-*sigB* were cultivated on TSB agar plates with and without toluene in the vapor phase. After cultivation of 2 days, cells were harvested. Total RNA was isolated from harvested cells and subjected to qRT-PCR to measure *bnzA1* mRNA. Opened bars, cells grown on TSB agar without toluene in the vapor phase; closed bars, cells grown on TSB agar with toluene in the vapor phase.

phase. When grown on TSB plates, toluene induced transcription of the *bnzA1* gene in the parental strain (Fig. 5). However, transcription of *bnzA1* was not induced in the *sigB* mutant even in the presence of toluene. Introduction of pKNR-*sigB* restored inducible transcription of *bnzA1* in the *sigB* mutant. These results clearly demonstrate that SigB is required for inducible transcription of *bnz* operon.

To assess whether susceptibility to toluene in strain dsigB is caused by failure of the degradation pathway, *i.e.* *bnz* operon, we inserted Km^r gene promoter from pUC4K in the upstream region of *bnz* operon for forced expression of *bnz* operon in strain dsigB. The resulting strain was designated dsigB(Pk-*bnz*). Although expression of *bnz* operon in dsigB(Pk-*bnz*) was confirmed by qRT-PCR (data not shown), it did not restore the ability of *sigB* mutant to tolerate toluene (Fig. 6). Rather, strain dsigB(Pk-*bnz*) seemed more sensitive to toluene than strain dsigB. It may be because a metabolite(s) of toluene exerts a toxic effect on strain dsigB(Pk-*bnz*) cells. Considering these results and our previous result that solvent-tolerance is not affected by deletion of *bnz* operon²⁴), it is unlikely that susceptibility of strain dsigB is due to its failure of expression of degradation pathways.

P. putida DOT-T1E³⁰) is a Gram negative bacterium highly tolerant to organic solvents, and the mechanisms of solvent tolerance have been most extensively studied in this strain. In *P. putida* DOT-T1E, three efflux pumps belonging to the Resistance-Nodulation-Cell-Division (RND) family of bacterial transporters (TtgABC, TtgEFG, and TtgGHI) are responsible for its solvent tolerance as well as drug resistance²⁹). It was found that these efflux pumps exhibit significant differences in substrate specificity^{3,29,36}). Although there has been no report demonstrating involvement of efflux pumps in organic solvent tolerance of Gram positive bacteria, sequence analysis predicts the presence of many multidrug

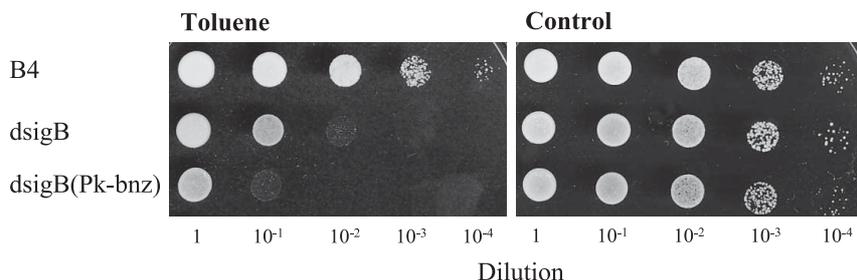


Fig. 6. Toluene-sensitive growth assays of *R. opacus* strains B4, dsigB, and dsigB(Pk-bnz). Susceptibility of bacterial cells to toluene was assayed as described in Fig. 4.

resistant transporters in their genomes. For example, *Rhodococcus* strain RHA1, which is closely related to *R. opacus* strain B4, is predicted to possess more than 40 putative major facilitator superfamily multidrug resistance protein genes and more than 10 putative multidrug ABC transporter genes in its genome (NC 008268). Based on these data, we speculate that *R. opacus* strain B4 also possesses many genes encoding efflux pump in its genome and that some of them are responsible for organic solvent tolerance. And we hypothesize that one of efflux pump is specific for toluene, benzene, and *n*-hexane, which is required for tolerance to these organic solvents, and that its expression is regulated by SigB. We are now searching candidates for efflux pump genes involved in solvent-tolerance in *R. opacus* strain B4 by utilizing the genome sequence data of *Rhodococcus* sp. strain RHA1.

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