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Transcriptional Regulation of the *Bacillus subtilis bscR-CYP102A3* Operon by the BscR Repressor and Differential Induction of Cytochrome CYP102A3 Expression by Oleic Acid and Palmitate[1]

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The adjacent *yrhI* and *yrhJ* genes were identified by the *Bacillus subtilis* genome sequencing project. We now report that *yrhJ* (renamed *CYP102A3*) encodes a cytochrome P450 and that *yrhI* (renamed *bscR*) encodes a repressor that negatively regulates the transcription of the *bscR-CYP102A3* operon. The transcriptional initiation site of *bscR* has been mapped by primer extension analysis. An 18-bp perfect palindromic sequence centered 65.5 bp downstream from the transcriptional initiation site of *bscR* has been identified as the binding site for *BscR* by gel mobility shift assays. Base substitutions in the 18-bp inverted repeat resulted in derepression of the *bscR-xylE* transcriptional fusion *in vivo*. *bscR-xylE* fusion studies and Northern blot analysis revealed that oleic acid and palmitate could induce the expression of the *bscR-CYP102A3* operon to a considerable extent. However, only oleic acid was capable of preventing the binding of BscR to its operator DNA *in vitro*, suggesting that the induction of *CYP102A3* expression by oleic acid and palmitate in *B. subtilis* might be mediated through different mechanisms.

Key words: inducer, operator, promoter, repressor, transcriptional regulation.

The cytochromes P450 comprise a very large superfamily of heme-containing monooxygenases that catalyze the monooxygenation of a wide variety of hydrophobic compounds of both endogenous and xenobiotic origin. They are generally divided into two major groups based on the number of protein components that constitute the monooxygenase system (*1*). Class I P450s are of the three-protein component type that occurs in mitochondria and bacteria. Transfer of electrons to the terminal P450 component requires an FAD-containing P450 reductase and a small redox iron-sulfur protein. Class II P450s are of the two-protein component type identified in the endoplasmic reticulum of eukaryotic organisms. Transport of electrons to the P450 component requires an FAD- and FMN-containing reductase. $P450_{BM\text{-}3}$ (CYP102A1) of *Bacillus megaterium* ATCC 14581 is an exception. It is a catalytically self-sufficient monooxygenase containing one equivalent each of heme, FMN, and FAD on a single polypeptide chain (*2*). This soluble enzyme can catalyze the hydroxylation or epoxidation of long-chain fatty acids in the presence of NADPH and O_2. Immediately upstream of the $P450_{BM\text{-}3}$ gene is the *bm3R1* gene, which encodes the Bm3R1 repressor that negatively regulates the expression of the *bm3R1-*$P450_{BM\text{-}3}$ operon at the transcriptional level (*3*). It has recently been found that Bm3R1 mediates an adaptive response that leads to fatty acid detoxification by $P450_{BM\text{-}3}$ in *B. megaterium* (*4*).

The adjacent *yrhI* and *yrhJ* genes, which were identified by the *B. subtilis* genome sequencing project (*5*), show 41 and 58% amino acid sequence identity to the *bm3R1* and $P450_{BM\text{-}3}$ genes of *B. megaterium*, respectively. In this work we show that *yrhJ* (renamed *CYP102A3* by the P450 nomenclature committee) encodes a cytochrome P450 and that *yrhI* (renamed *bscR*) encodes a repressor that negatively regulates the expression of the *bscR-CYP102A3* operon. We have also identified the binding site for the BscR repressor. Its location implies that BscR repressor–DNA interaction probably inhibits transcriptional elongation by a roadblock mechanism. Furthermore, our results suggest that the induction of *B. subtilis CYP102A3* expression by oleic acid and palmitate might be mediated through different mechanisms.

MATERIALS AND METHODS

Growth Conditions—*B. subtilis* 168 (*trpC2*) and *E. coli* JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F′(*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) cells were grown at 37°C in LB medium (*6*). Sodium pentobarbital and sodium palmitate were dissolved in H_2O prior to addition to the culture medium, whereas oleic acid was dissolved in ethanol. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml (*E. coli*); chloramphenicol, 5 μg/ml (*B. subtilis*); tetracycline, 15 μg/ml (*B. subtilis*).

Construction of Plasmids—To facilitate the overexpression and purification of His-tagged BscR, a PCR-amplified

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Abbreviations: bp, base pair(s); kb, kilobase(s); LB, Luria-Bertani; *mbgA*, β-galactosidase of *Bacillus megaterium*; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; XylE, catechol 2,3-dioxygenase.

DNA fragment containing the *bscR* gene was cloned between the *Bam*HI and *Pst*I sites of pQE30 (Qiagen) to generate plasmid pGS482. The various DNA fragments in plasmids pGS562, pGS590, pGS656, and pGS657 were amplified by PCR and cloned individually between the *Eco*RI and *Hin*dIII sites of pLC4 (*7*). The DNA fragment in plasmid pGS563, which contains a 2-bp deletion in the *bscR* coding region, was generated by a two-step PCR method (*8*). The DNA fragment in plasmid pGS645, which contains base subsitutions in the 18-bp inverted repeat, was also generated by the two-step PCR method. For construction of plasmid pGS627, a DNA fragment containing the *bscR* gene was amplified by PCR and cloned between the *Eco*RI and *Hin*dIII sites of pHY300PLK (Takara). The DNA fragment in plasmid pGS688, which contains base subsitutions in the 18-bp inverted repeat, was cloned between the *Eco*RI and *Hin*dIII sites of pLC4.

Overexpression and Purification of the Heme Domain of CYP102A3 and the BscR Protein—E. coli JM109 cells bearing the heme domain-expressing plasmid pGS592 and *bscR*-expressing plasmid pGS482 were grown in LB medium. After the absorbance at 600 nm reached 0.5, the cultures were induced with 0.3 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. Purification of the His-tagged heme domain and His-tagged BscR by Ni-NTA (Ni-nitrilotriacetic acid) affinity column chromatography was performed according to the instructions of the manufacturer (Qiagen).

Primer Extension and Northern Blot Analyses—Total RNA was prepared as previously described (*9*). Primer extension analysis (*10*) and Northern blot analysis (*11*) were carried out as previously described.

Gel Mobility Shift Assays—DNA-binding assays were carried out as previously described (*12*). When double-stranded oligonucleotides were used as the probes, the synthetic oligonucleotides were first annealed in a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 mM NaCl. The annealed double-stranded oligonucleotides were then labeled with [α-^{32}P]dCTP by the Klenow fragment. Sodium pentobarbital and sodium palmitate were dissolved in H_2O prior to addition to the reaction mixture, whereas oleic acid was first dissolved in ethanol. The final concentration of ethanol in each incubation was 10% (v/v).

Other Methods—Transformation of *B. subtilis* cells was achieved by the protoplast method (*13*). Genomic DNA from *B. subtilis* was isolated as previously described (*14*). Established methods were used to determine the spectral characteristics of the heme domain of CYP102A3 (*15*) and for the spectrophotometric measurement of XylE activity (*7*). Protein concentrations were determined by the BCA protein assay method according to the instructions of the manufacturer (Pierce) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Sequence Analysis of CYP102A3—The *yrhJ* gene encodes a putative protein of 1054 amino acids that shows 58% amino acid identity to the cytochrome $P450_{BM-3}$ from *B. megaterium* (GenBank accession number P14779) (*2*). The deduced amino acid sequence FGNGQRACIG (from residues 396 to 405) of the *yrhJ*-encoded protein shows significant homology to the heme-binding consensus motif of [FW]-[SGNH]-x-[GD]-x-[RHPT]-xC-[LIVMFAP]-[GAD], including the essential cysteine residue (boldface) that serves as the heme iron ligand (*16*). The previously proposed FAD-binding regions, FMN-binding regions, and NADPH-binding regions of the *B. megaterium* $P450_{BM-3}$ (*17*) are also well conserved in the deduced amino acid sequence of the *yrhJ*-encoded protein (data not shown).

Purification of the Heme Domain of CYP102A3 and Analysis of Its Spectral Characteristics—To establish that the *yrhJ* gene encodes a protein with the spectral characteristics of cytochrome P450, a DNA fragment corresponding to the putative heme domain (amino acids 1 to 480) of the *yrhJ*-encoded protein was amplified by PCR and cloned into the expression vector pQE30. The His-tagged heme do-

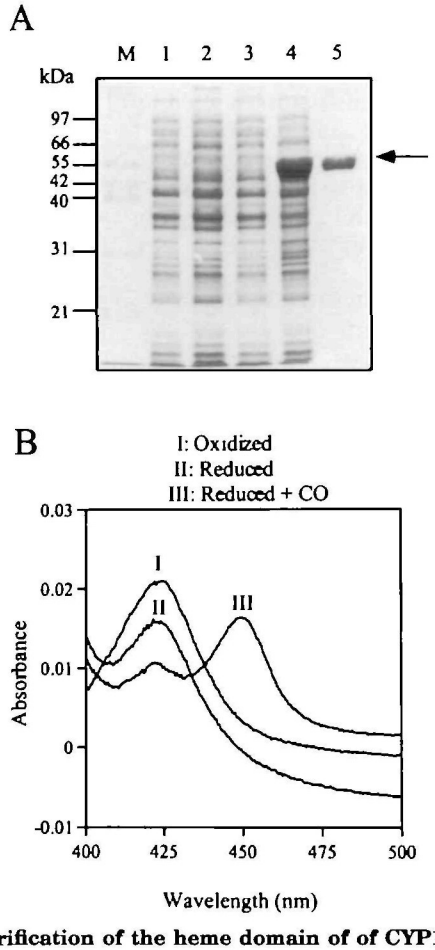

Fig. 1. **Purification of the heme domain of of CYP102A3 and analysis of its spectral characteristics.** (A) SDS-13%PAGE of whole-cell extracts of *E. coli* transformants. Lanes 1 and 2, *E. coli* JM109 cells bearing the control vector pQE30 and grown in the absence and presence of 0.3 mM IPTG, respectively. Lanes 3 and 4, *E. coli* JM109 cells bearing heme domain-expressing plasmid pGS592 and grown in the absence and presence of 0.3 mM IPTG, respectively. Lane 5, purified His-tagged heme domain of CYP102A3. M, molecular mass standards. The position of the heme domain of CYP102A3 is indicated by the arrow at the right. (B) Each sample cuvette contained about 8 μg of the purified His-tagged heme domain of CYP102A3 in 1 ml of 0.1 M phosphate buffer (pH 7.8). I, the absorption spectrum measured with the oxidized form of the protein; II, the spectrum measured with dithionite-reduced protein; III, the spectrum of the protein treated with dithionite followed by bubbling with CO.

main was purified in a single step by Ni-NTA affinity chromatography. The purified heme domain with an apparent molecular mass of approximately 55 kDa as estimated by SDS-PAGE was about 90% pure (Fig. 1A). The purified heme domain exhibited spectral characteristics typical of a cytochrome P450 in oxidized, reduced, and reduced CO-bound forms with Soret peaks of oxidized low spin and dithionate-reduced forms at 424 nm and a reduced CO absorbance maximum at 450 nm (Fig. 1B).

Identification of the Transcriptional Initiation Site and the Promoter of bscR—We first constructed a series of deletion derivatives as shown in Fig. 2A. PCR-generated DNA fragments of various lengths were transcriptionally fused to a promoterless *xylE* reporter gene in the promoter probe vector pLC4 (*7*). Crude protein extracts from *B. subtilis* cells carrying these plasmids were assayed for XylE activities. The results shown in Fig. 2B suggest that plasmid pGS657 still retained the *bscR* promoter sequence, whereas in plasmid pGS590, the *bscR* promoter sequence was either partially or completely deleted. We next isolated RNA from log-phase *B. subtilis* cells carrying plasmid pGS563 (Fig. 2A). An 18-mer synthetic oligonucleotide complementary to the 5′-flanking region of *bscR* (Fig. 3A) was used as the primer in primer extension analysis. As shown in Fig. 3B, only one major extension product was detected. The length of the extension product indicates that the 5′ end of the transcript is located 102 bp upstream from the translational start site of *bscR*. This transcriptional initiation site is at an appropriate distance from a putative promoter sequence (TTTATA for the –35 box and TATAAA for the –10 box) that may be recognized by the *B. subtilis* σ^A (*18*). The location of this promoter is consistent with the results obtained from deletion analyses of the *bscR* regulatory region shown in Fig. 2.

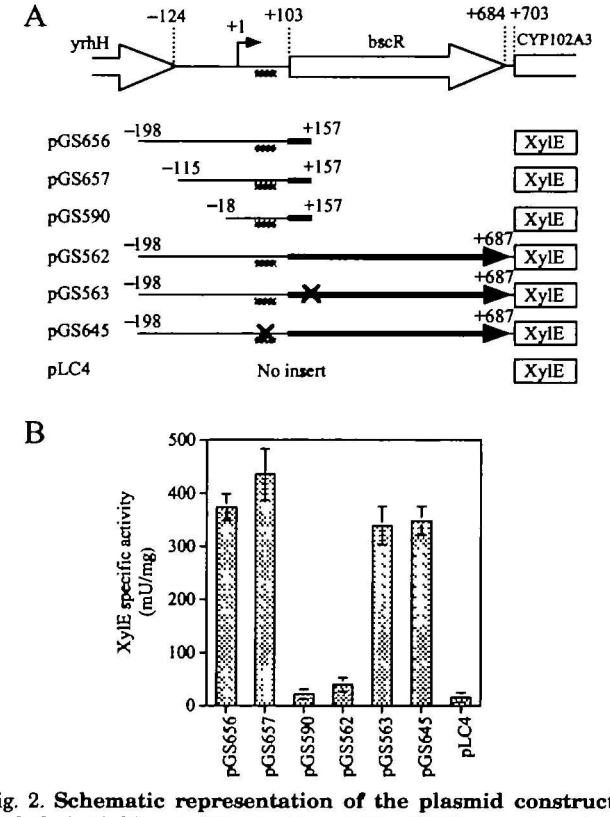

Fig. 2. **Schematic representation of the plasmid constructs and their XylE activities in *B. subtilis*.** (A) The number above each line denotes base position relative to the transcriptional initiation site of *bscR*. The hatched heavy lines below the *bscR* regulatory regions indicate the 18-bp inverted repeat that functions as an operator for the BscR repressor. Base substitutions in the operator DNA or a 2-bp deletion in the *bscR* coding region are represented by crosses. Each pLC4-based plasmid contains a *xylE* reporter gene preceded by one of the deletion derivatives of the *bscR* promoter region and coding region. (B) *B. subtilis* cells carrying the above plasmids were grown at 37°C to an absorbance at 600 nm of 1.0. Each value represents the mean of at least four determinations. Error bars indicate the standard error of the mean.

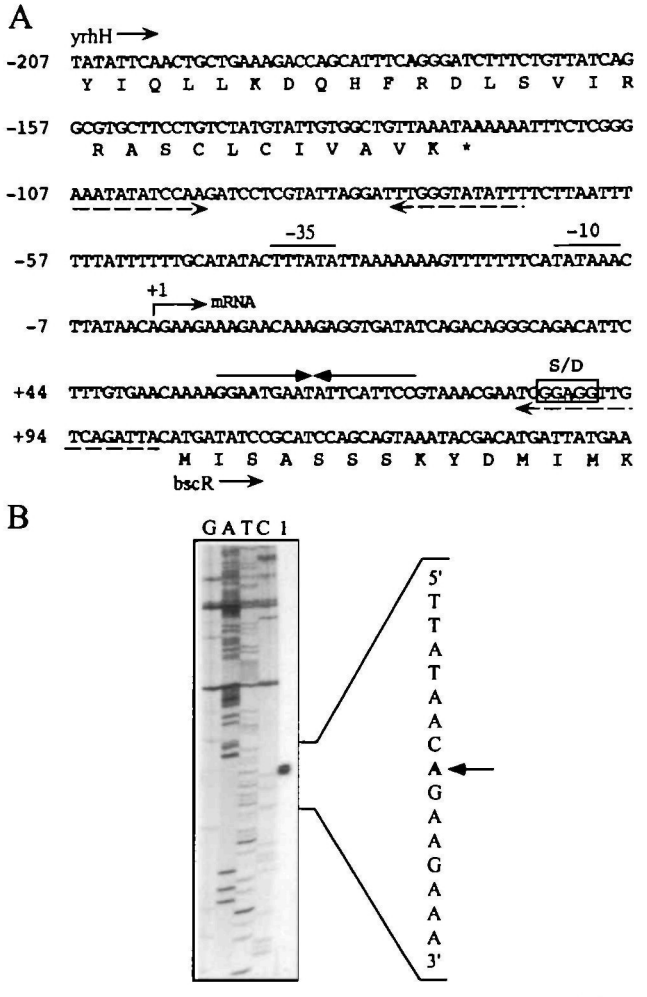

Fig. 3. **Primer extension analysis of the transcriptional initiation site of *bscR*.** (A) Nucleotide sequence of the *bscR* regulatory region. Nucleotide positions are numbered relative to the transcriptional initiation site of *bscR*. The nucleotide sequence complementary to the sequence of the primer used for primer extension analysis is marked by a dashed arrow (from positions +84 to +101). The 18-bp inverted repeat that functions as an operator for BscR is indicated by a pair of solid inverted arrows. An inverted repeat, which presumably acts as a rho-independent transcription terminator for the *yrhH* gene, is indicated by a pair of dashed inverted arrows. (B) Primer extension analysis of the *bscR* transcript was carried out with RNA prepared from *B. subtilis* cells carrying pGS563. Lane 1 shows the primer extension product. Dideoxy sequencing ladders were obtained with the same primer used for primer extension analysis. The sequence shown is complementary to that read from the ladder. The arrow indicates the transcriptional initiation site.

The Regulatory Role of BscR—The *bscR* gene encodes a putative protein of 194 amino acids with 41% identity to the Bm3R1 repressor of *B. megaterium* (GenBank accession number P43506). The N-terminal region of BscR contains a segment of 20 amino acids (from residues 30 to 49) that is most likely to be a helix-turn-helix DNA-binding motif (*19*). A database homology search revealed that BscR resembles a group of proteins belonging to the TetR/AcrR family of transcriptional regulators (data not shown). Figure 2 shows that *B. subtilis* cells carrying plasmid pGS656 exhibited a much higher XylE activity than those carrying plasmid pGS562. When a 2-bp deletion at positions +201 and +202 relative to the transcriptional initiation site of *bscR* was generated in the coding region of *bscR* in plasmid pGS563, this frameshift mutation led to an approximately 8.5-fold increase in XylE activity as compared with that of plasmid pGS562. The observed derepression implies that *bscR* may encode a negative regulator.

Interaction of BscR with the bscR Iregulatory Region In Vitro—A *bscR*-expressing plasmid pGS482 that overproduced His-tagged BscR protein was constructed as described in "MATERIALS AND METHODS," and purified by Ni-NTA affinity column chromatography (data not shown). Gel mobility shift assays were used to show that the purified His-tagged BscR was capable of binding to a 0.18-kb DNA fragment (from positions –18 to +157) containing part of the *bscR* promoter region (Fig. 4A), but not to a control DNA containing the *mbgA* regulatory region (*20*) (Fig. 4B). The *bscR* regulatory region contains an 18-bp perfect palindromic sequence centered 65.5 bp downstream from the transcriptional initiation site of *bscR* (Fig. 3A). When a 0.26-kb DNA fragment (from positions –198 to +54) containing the *bscR* promoter sequence but lacking the 18-bp inverted repeat and its downstream sequence was used as the probe in gel mobility shift assays, no protein-DNA complex was detected (data not shown). This suggests that the 0.26-kb region located immediately upstream of the 18-bp inverted repeat contains no BscR binding site. To determine whether BscR interacts directly with the 18-bp inverted repeat, a double-stranded oligonucleotide containing the 18-bp inverted repeat (Oligo I) was used as the probe in gel mobility shift assays. As shown in Fig. 4C, purified His-tagged BscR was capable of binding to this oligonucleotide but not to a double-stranded oligonucleotide containing substituted bases in the 18-bp inverted repeat (Oligo II) (Fig. 4D). This demonstrates that BscR can interact specifically with the 18-bp inverted repeat.

Effect of Mutations in the 18-bp Inverted Repeat on the Expression of bscR-xylE Transcriptional Fusion In Vivo—We next examined the effect of base substitutions in the 18-bp inverted repeat (shown at the bottom of Fig. 4) on the expression of *bscR-xylE* transcriptional fusion *in vivo*. As shown in Fig. 2, these mutations in plasmid pGS645 caused an approximately 7-fold increase in XylE activity as compared with that of plasmid pGS562. This result, together with the above-mentioned *in vitro* DNA binding assays, strongly suggest that the 18-bp inverted repeat functions as the BscR binding site to negatively regulate the expression of the *bscR-CYP102A3* operon. Since no binding site for the BscR repressor could be detected within the 0.26-kb region located immediately upstream of the 18-bp inverted repeat by gel mobility shift assays or by computer analyses, it is possible that binding of the BscR repressor to its operator may affect the elongation of transcription rather than interfere with the initiation of transcription. Further experiments will be directed toward clarifying this hypothesis.

Effects of Long-Chain Fatty Acids and Pentobarbital on the Expression of bscR-xylE Transcriptional Fusion—Long-chain fatty acids (*21*) and pentobarbital (*22*) have been reported to induce $P450_{BM\text{-}3}$ expression in *B. megaterium*. To determine whether this is the case for *CYP102A3, B. subtilis* cells carrying the *bscR-xylE* transcriptional fusion were

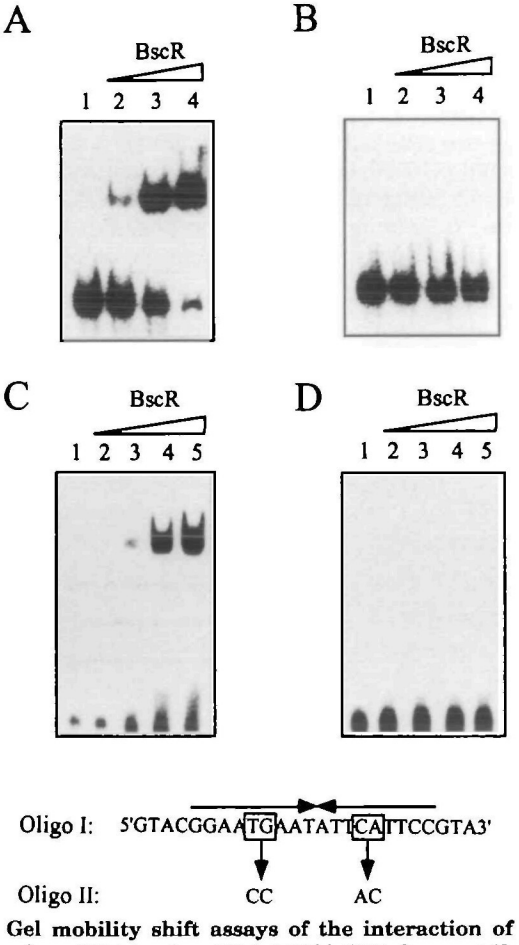

Fig. 4. **Gel mobility shift assays of the interaction of BscR with various DNA probes.** (A) A 0.18-kb DNA fragment (from positions –18 to +157) containing the bscR promoter and the 18-bp inverted repeat was used as the probe. Lane 1, DNA probe alone; lanes 2–4, DNA probe plus increasing amounts of His-tagged BscR (15, 50, and 150 ng, respectively). (B) A 0.18-kb DNA fragment containing the *mbgA* regulatory region was used as the probe. Lane 1, DNA probe alone; lanes 2–4, DNA probe plus increasing amounts of His-tagged BscR (15, 50, and 150 ng, respectively). In panels (A) and (B), about 3 ng of ^{32}P-labeled DNA probe was used in each reaction mixture (20 μl). Double-stranded oligonucleotide I containing the 18-bp inverted repeat was used as the probe. The sequence of its upper strand is shown at the bottom of the figure. Lane 1, DNA probe alone; lanes 2–5, DNA probe plus increasing amounts of His-tagged BscR (5, 15, 50, and 150 ng, respectively). (D) Double-stranded oligonucleotide II containing mutated base pairs in the 18-bp inverted repeat was used as the probe. The mutated bases are shown at the bottom of the figure. Lane 1, DNA probe alone; lanes 2–5, DNA probe plus increasing amounts of His-tagged BscR (5, 15, 50, and 150 ng, respectively). In panels (C) and (D), about 0.3 ng of ^{32}P-labeled DNA probe was used in each reaction mixture (20 μl).

grown in the absence or presence of oleic acid, palmitate, and pentobarbital. The results showed that oleic acid and palmitate could substantially induce *xylE* expression at micromolar concentrations (Fig. 5). Slight induction by pentobarbital was only observed at millimolar concentrations. The previous observation that there was no induction of P450 activity in *B. subtilis* by pentobarbital (*23*) is probably due to the lower sensitivity of the method used to assay P450 activity.

Northern Blot Analysis of CYP102A3 Expression upon Treatment with Palmitate—We further carried out Northern blot analysis to examine the induction of *CYP102A3* expression by palmitate. Total cellular RNA was isolated from *B. subtilis* cells grown in the absence or presence of 200 μM palmitate. A 0.54-kb ^{32}P-labeled single-stranded DNA complementary to the upper strand of the *CYP102A3* coding region was used as the hybridization probe. As shown in Fig. 6, the intensity of a band of about 4 kb, which might represent the cotranscript of the *bscR* and *CYP102A3* genes, was higher in cells treated with palmitate than in untreated cells. This indicates that palmitate can increase *CYP102A3* expression at the RNA level.

In Vitro Effects of Various Inducers on the Interaction of the BscR Protein with a DNA Fragment Containing the bscR Regulatory Region—We next used gel mobility shift assays to examine whether the above-mentioned inducers exert their inductive effects by directly preventing the binding of the BscR repressor to its operator DNA. Figure 7A shows that the formation of the BscR-DNA complex gradually decreased as the oleic acid concentration was increased. In control experiments, ethanol at a final concentration of 10% did not prevent the binding of BscR or Bm3R1 to the target DNA (data not shown). This supports the idea that the mechanism for the oleic acid–mediated induction of *CYP102A3* expression involves interference with the binding of the BscR repressor to its operator DNA by oleic acid. In contrast, palmitate at concentrations ranging from 100 to 1,000 μM did not inhibit the formation of the BscR-DNA complex (Fig. 7B). In a control experiment, we found that purified the His-tagged Bm3R1 protein could also bind to this DNA probe (Fig. 7B), probably due to the sequence similarity between the operator DNAs for Bm3R1 and BscR. However, palmitate at the given range of concentrations interfered with the formation of the Bm3R1-DNA complex (Fig. 7B). These results suggest that the mechanisms for the induction of *CYP102A3* expression by oleic acid and palmitate are distinct. One possiblity is that some metabolic derivative(s) of palmitate rather than palmitate *per se* actually acts as an inducer(s) to dissociate the BscR-DNA complex. In addition, it was also found that 4 mM pentobarbital could inhibit Bm3R1 binding to its target DNA (*24*), but was unable to interfere with the formation of the BscR-DNA complex (Fig. 7C). Based on 41% identity and 54% similarity between the amino acid sequences of BscR and Bm3R1, it would be intriguing to identify the unique structural features or critical residues of Bm3R1 that are important for the interactions with palmitate and pentobarbital. BscR is assumed to lack these unique features or residues.

The *B. subtilis* genome contains another gene called *yfnJ* (renamed *CYP102A2* by the P450 nomenclature committee), which encodes the putative YfnJ protein (GenBank accession number CAB12544) of 1061 amino acids with 61.7% identity to CYP102A3. The CYP102A2 protein contains sequences that show significant homology to the heme-, FAD-, FMN-, and NADPH-binding motifs of CYP102A3 (data not shown). In contrast to *CYP102A3*, the *CYP102A2* gene appears to be monocistronic. Its regulatory

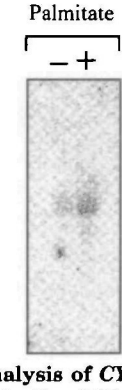

Fig. 6. **Northern blot analysis of *CYP102A3* expression upon treatment with palmitate.** *B. subtilis* cells were grown in LB medium to an absorbance at 600 nm of 0.3, and then treated with (+) or without (–) 200 μM palmitate for 2 h. A 0.54-kb ^{32}P-labeled single-stranded DNA complementary to the upper strand of the *CYP102A3* coding region was used as the hybridization probe.

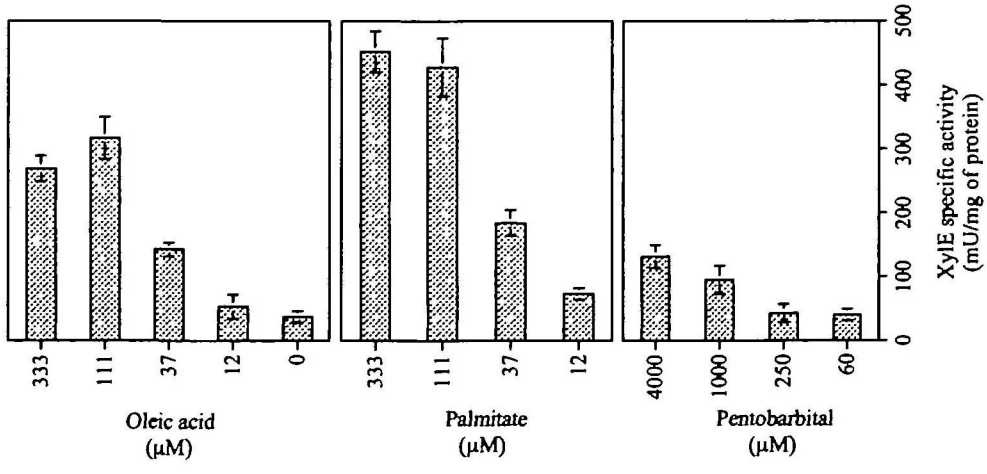

Fig. 5. **Effects of long-chain fatty acids and pentobarbital on the expression of *bscR-xylE* transcriptional fusion in *B. subtilis*.** *B. subtilis* cells carrying the *bscR-xylE* transcriptional fusion in plasmid pGS562 (Fig. 2A) were initially grown at 37°C in LB medium to an absorbance at 600 nm of 0.3. Various amounts of oleic acid (A), palmitate (B), or pentobarbital (C) were then added to the culture medium. Cell growth was continued for 6 h. Each value represents the mean of four determinations. Error bars indicate the standard error of the mean.

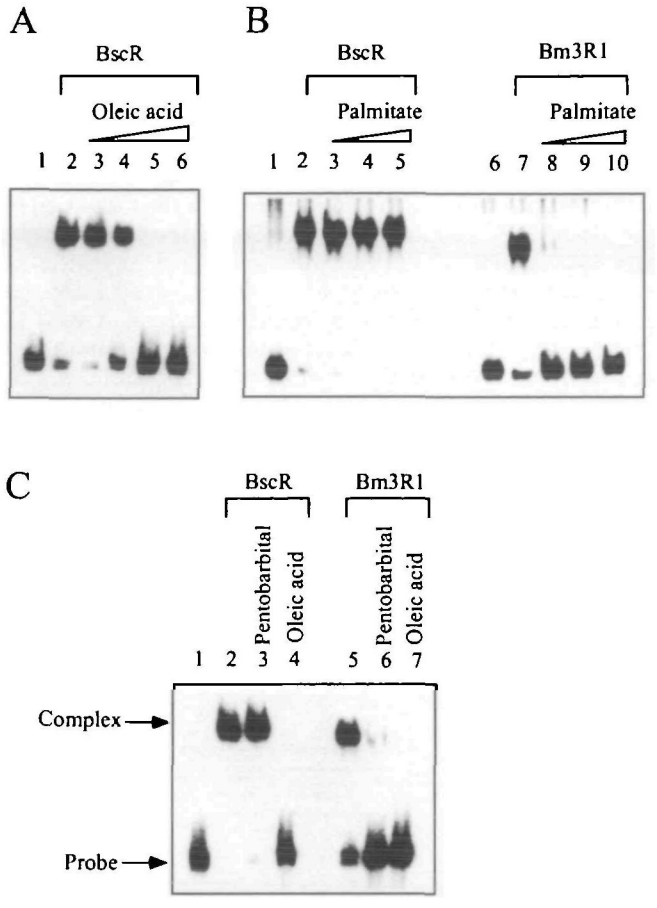

Fig. 7. **Effects of various inducers on the interaction of the BscR protein with a DNA fragment containing the *bscR* regulatory region.** The above-mentioned 0.18-kb DNA fragment containing the *bscR* promoter and the 18-bp inverted repeat was used as the probe in gel mobility shift assays. Purified His-tagged BscR (150 ng) or Bm3R1 (150 ng) was incubated with 3 ng of ^{32}P-labeled probe in the absence or presence of various inducers in a total volume of 20 μl of reaction mixture. (A) Lane 1, DNA probe alone; lane 2, DNA probe plus BscR; lanes 3–6, DNA probe plus BscR in the presence of 3, 10, 30, and 100 μM oleic acid, respectively. (B) Lanes 1 and 6, DNA probe alone; lane 2, DNA probe plus BscR; lanes 3–5, DNA probe plus BscR in the presence of 100, 300, and 1000 μM palmitate, respectively; lane 7, DNA probe plus Bm3R1; lanes 8–10, DNA probe plus Bm3R1 in the presence of 100, 300, and 1000 μM palmitate, respectively. (C) Lane 1, DNA probe alone; lane 2, DNA probe plus BscR; lanes 3 and 4, DNA probe plus BscR in the presence of 4 mM pentobarbital and 30 μM oleic acid, respectively; lane 5, DNA probe plus Bm3R1; lanes 6 and 7, DNA probe plus Bm3R1 in the presence of 4 mM pentobarbital and 30 μM oleic acid, respectively.

region and coding region do not contain any sequence that exhibits significant homology to the 18-bp operator sequence of BscR, suggesting that *CYP102A2* expression is either not or at least not directly regulated by BscR. Further work will be needed to clarify whether or how *CYP102A2* expression is controlled by environmental factors and to distinguish the different physiological roles of CYP102A2 and CYP102A3 in *B. subtilis*.

We thank the reviewers for pointing out the correct names of the cytochromes P450 mentioned in this work.

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