The Repressor Protein, Bm3R1, Mediates an Adaptive Response to Toxic Fatty Acids in Bacillus megaterium*

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Bm3R1 is a helix-turn-helix transcriptional repressor from Bacillus megaterium whose binding to DNA is inhibited by fatty acids and a wide range of compounds that modulate lipid metabolism. The inactivation of Bm3R1/DNA binding activity results in the activation of transcription of the operon encoding a fatty acid hydroxylase, cytochrome P450 102. The metabolic role of this operon is unknown. It is possible that it is involved in the synthesis of modified fatty acids as part of normal cellular metabolism or may represent a protective mechanism by which B. megaterium detoxifies harmful foreign lipids. In this report we demonstrate that polyunsaturated fatty acids (PUFA) activate the transcription of the CYP102 operon. These PUFA are the most potent activators of the CYP102 operon observed to date, and we show that their effects are due to binding directly to Bm3R1. In addition, cultures that have been treated with the CYP102 inducer, nafenopin, are protected against PUFA toxicity. Resistance to PUFA toxicity is also seen in a Bm3R1-deficient strain that constitutively expresses CYP102. The resistant phenotype of this Bm3R1 mutant strain is reversed by specific chemical inactivation of CYP102. These data demonstrate that Bm3R1 can act as a direct sensor of toxic fatty acids and, in addition, provide the first direct evidence of fatty acids binding to a prokaryotic transcription factor.

Unsaturated fatty acids are essential structural components of the cell membrane. They are also sophisticated signaling molecules that can mediate a myriad of processes involved in cellular communication, differentiation, and cell death (1–5). It is for these reasons that all organisms require tight regulation of the lipid composition of the cell. Perturbations in the levels of different types of lipid may be fatal due to disruption of membrane structure and metabolic or regulatory processes (2). The mammalian liver responds to perturbations in lipid homeostasis by the induction of cytochrome P450 fatty acid hydroxylases and the enzymes for peroxisomal β-oxidation (6, 7). Perturbations in lipid homeostasis may take the form of high fat diet, diabetes, or treatment with fatty acid mimetics such as peroxisome proliferators or non-steroidal anti-inflammatory drugs (8). The regulation of lipid metabolism under such conditions has been shown to be a direct genomic response where a transcription factor responds directly to free fatty acids and acts as a molecular switch for the regulated transcription of genes encoding fatty acid-metabolizing enzymes (9–13); however, the role of mammalian fatty acid hydroxylases in the clearance of fatty acids is not well defined.

The cytochrome P450 fatty acid hydroxylases are regulated by peroxisome proliferators in many other types of organisms including plants and Bacillus megaterium (14, 15). It could therefore be hypothesized that inducible fatty acid hydroxylation represents an ancient regulatory metabolic response to lipid overload.

The simplest of these organisms, B. megaterium, is a soil-living Gram-positive bacterium that utilizes branched fatty acids rather than straight chain fatty acids as its main membrane phospholipid (16). B. megaterium only synthesizes small amounts of unsaturated fatty acids as a transient response to cold (17), and exogenously applied unsaturated fatty acids are toxic (18). This raises the intriguing question, how does B. megaterium cope with these toxic yet highly abundant carbon sources, i.e. plant-derived unsaturated fatty acids?

It has been known for some time that B. megaterium has the capacity to hydroxylate and epoxygenate unsaturated fatty acids (19). The enzyme responsible for these activities is a soluble cytochrome P450, designated CYP1021 or cytochrome P450BM3 (20). Intriguingly, the repressor that controls the transcription of the CYP102 operon in response to a wide range of xenobiotic compounds is a helix-turn-helix DNA-binding protein known as Bm3R1 (21). This protein was originally characterized as mediating the induction of CYP102 by barbiturates. It has been shown that barbiturates abolish the binding of Bm3R1 to its operator DNA sequence and that this allows transcription to proceed through the Bm3R1 and CYP102 coding sequences. We have since shown that DNA binding by Bm3R1 is inhibited, and cytochrome CYP102 is induced, by a wide range of compounds that are known to perturb lipid metabolism in mammals (14, 18). These compounds include hypolipidemic drugs and non-steroidal anti-inflammatory drugs that all appear to act as fatty acid mimetics. We have also shown that the chlorophyll metabolite, phytanic acid, induces CYP102 and is metabolized to a less potent inducing form by CYP102 (22). These findings raised the possibility that one function of CYP102 was to detoxify foreign lipids. In support of this concept was the observation that the most potent inhibitors of Bm3R1 DNA binding in vitro are polyunsaturated fatty acids (18). In these studies induction of CYP102 was not observed due to the toxicity of these compounds at the high concentrations used. In this report we provide the first evidence that unsaturated fatty acids are activators of transcrip-

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The abbreviations used are: CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; PUFA, polyunsaturated fatty acids; PBS, phosphate-buffered saline; 12-AO, 12-anthracene oleic acid; 17-ODA, 17-octadecynoic acid.
tion of the CYP102 operon over a very narrow range of concentrations and that these unsaturated fatty acids bind directly to Bm3R1. The narrow range of concentrations that are required for induction by unsaturated fatty acids is due to toxicity at concentrations immediately above the binding constants for Bm3R1. We have observed that treating cultures with appropriate concentrations of unsaturated fatty acids produces a very transient induction of the levels of CYP102 and that pretreatment of cultures with the peroxisome proliferator, nafenopin, allows growth in normally toxic concentrations of unsaturated fatty acids. This work demonstrates that the CYP102 operon encodes a finely tuned sensor for unsaturated fatty acids and a fatty acid hydroxylase that can mediate the detoxification of these compounds. This is the first demonstration of this cytoprotective response to toxic fatty acids.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Fatty acids were purchased from Sigma and Cayman Research Laboratories. Nafenopin was a generous gift from Zeneca Central Toxicology Laboratories, Macclesfield, UK, and from Dr. Brian Lake at Bibra, Carshalton, Surrey, UK. Antibodies and oligonucleotides were prepared in dimethyl sulfoxide (Me2SO) and added as required. In aeration to an optical density of 0.2 at 600 nm. Fatty acids and drugs obtained using Ultrafit for Macintosh.

**Purification of Recombinant Bm3R1**—Histidine tagged-Bm3R1 was expressed from the pET15b plasmid in *Escherichia coli* strain BL21(DE3) as described previously (18), with the following modifications. Induction of a 10-liter culture with 1 mM isopropyl-1-thio-β-D-galactopyranoside was performed at 30 °C for 4.5 h. The cells were then harvested by centrifugation and resuspended in 600 ml of PBS containing 0.1 mg/ml lysozyme. The suspension was incubated on ice for 15 min and then the spheroplasts were harvested by centrifugation. The spheroplasts were then thawed in cold water, and β-mercaptoethanol was added to 5 mM. The cells were lysed by sonication, and the cell debris was removed by centrifugation in a microcentrifuge at 4 °C for 10 min. The protein concentration of the supernatant was assayed using the Bio-Rad protein assay. 40 μl of the lysate was added to 160 μl of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 20 mM NaCl, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS) and incubated at 37 °C on a microtiter plate. Optical density was monitored at 600 nm at hourly intervals.

**Protein Immunoblotting**—Lysates were prepared as described for the measurement of β-galactosidase activity, and proteins within these lysates (30 μg) were resolved using SDS-polyacrylamide gel electrophoresis. A 7.5% acrylamide gel was used to resolve CYP102. Following separation by SDS-polyacrylamide gel electrophoresis, the proteins were transferred to nitrocellulose. CYP102 was identified using a polyclonal rabbit anti-α-synthase (1/2000), raised to the reductase domain of CYP102 (English et al. (18)), followed by mouse anti-rabbit serum (1/2000) conjugated with horseradish peroxidase (Sigma). Following detection with ECL (Amersham Pharmacia Biotech), the bands were visualized by autoradiography.

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSAs were carried out as described previously (18). A double-stranded oligonucleotide, encompassing the high affinity binding site of Bm3R1 designated OII (5′-CCGAAATTCCTGCTATTCCG-3′) (21), was incubated with purified recombinant Bm3R1. His-tagged Bm3R1 has a dissociation constant of 1.5 mM for this DNA sequence (24). All assays were carried out in 30 μl final volume, 60 mM KCl, 12 mM Hepes, 1 mM EDTA, 1 mM dithiothreitol, and glycerol (10% v/v) (EMSA buffer) containing 1 μg of carrier DNA poly(dI-dC) on ice for 15 min. 10 fmol of radioactive oligonucleotide was then added, and the sample was incubated for a further 15 min on ice. Drugs, diluted in EMSA buffer, were added to the incubations prior to the addition of the oligonucleotide. The fatty acids used in this study do not disrupt the binding of mammalian nuclear proteins to DNA. Following incubation, 4 μl was loaded onto a 4% non-denaturing polyacrylamide gel, electrophoresed at 16 mA constant current, dried, and autoradiographed. The relative radioactivity present in the free and protein bound oligonucleotide fractions was determined using a Bio-Rad PhosphorImager. *K*ᵣ estimates were obtained using Ultrasoft for Macintosh.

**Filter Binding Assay**—Purified recombinant Bm3R1 was combined with 300 mM 12-anthracene oleic acid (Molecular Probes) in 1 ml of 25 mM Tris-HCl, pH 7.5. Fluorescence was measured using a excitation wavelength of 383 nm and emission wavelength of 460 nm using a Perkin-Elmer LS-5 fluorescence spectrophotometer. The background fluorescence resulting from the protein alone and the 12-α-oleic acid alone were combined and deducted from each experimental value. *Kᵣ* values were obtained using Ultrasoft for Macintosh.

**Growth of Bacterial Cultures**—Growth of bacterial cultures was as described previously (18). *B. megaterium* were grown at 37 °C with aeration to an optical density of 0.2 at 600 nm. Fatty acids and drugs were prepared in dimethyl sulfoxide (Me2SO) and added as required. In the case of controls, Me2SO alone was added. The final Me2SO concentration did not exceed 0.5% (v/v) in any incubation.

**Reporter Constructs**—Genomic fragments corresponding to the CYP102 regulatory sequences were isolated by polymerase chain reaction. Fragment C143 corresponds to positions 62 to 1573 and fragment A45 corresponds to positions 62 to 949 of the CYP102 operon. GenBank™ accession numbers J04832 (23). These were subcloned into the EcoRI site of the *Bacillus subtilis* E. coli shuttle vector pSB355. The luciferase cDNA of pSB355 was excised by digestion with KpnI and SacI, and a KpnI/SacI fragment containing the cDNA encoding β-galactosidase from pV85-gal was ligated downstream of the CYP102 sequences. These constructs were used to transform a lacZ mutant strain of *B. megaterium*, PV586. The resulting strains were designated

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PV586/C143 and PV586/A45. Measurement of β-Galactosidase Activity—Cells were harvested from 1 ml of culture by centrifugation and resuspended in 200 μl of PBS containing 0.2 mg/ml lysozyme. This suspension was incubated on ice for 30 min and then 37 °C for 5 min. The resulting spheroplasts were harvested by centrifugation (10 000 × g for 3 min). The cell debris was removed by centrifugation in a microcentrifuge at 4 °C for 10 min. The protein concentration of the supernatant was assayed using the Bio-Rad protein assay. 40 μl of the lysate was added to 160 μl of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 20 mM NaCl, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS) and incubated at 37 °C on a microtiter plate. Optical density was monitored at 600 nm at hourly intervals.

**RESULTS**

**Unsaturated Fatty Acids Bind Bm3R1 in Vitro**—Initial studies were directed toward establishing the role of fatty acids in regulating the CYP102 operon. In previous work we demonstrated that polyunsaturated fatty acids (PUFA) are the most potent of the compounds tested in dissociating the Bm3R1-DNA complex in *vitro* (18). The finding that these compounds disrupt the binding of purified recombinant Bm3R1 to DNA provides strong evidence that the fatty acids bind to the repressor; however, this evidence is indirect. In order to demonstrate a direct interaction between unsaturated fatty acids and Bm3R1, we carried out binding experiments using a fluorescent fatty acid probe, 12-anthracene oleic acid (12-α-OA). This fatty acid has only a low fluorescence in aqueous solution; however, 12-α-OA becomes highly fluorescent when bound to the hydro-
phobic lipid binding sites of proteins. This interaction has been directly visualized by the crystallization of 12-AO in the lipid binding pocket of the adipocyte lipid-binding protein (25, 26).

Binding studies were carried out using purified recombinant His-tagged Bm3R1. In DNA binding experiments, 12-AO inhibited the formation of the Bm3R1-DNA complex with a $K_i$ of 1.05 $\mu M$, as previously observed for other unsaturated fatty acids.

**Fig. 1.** The fluorescent probe, 12-anthracene oleic acid, displaces Bm3R1 from DNA and binds directly to Bm3R1 in solution. A, EMSA were performed as described under “Experimental Procedures” using 500 ng of recombinant Bm3R1 and 10 fmol of the radiolabeled oligonucleotide pair corresponding to the CYP102 operator sequence. Increasing concentrations of 12-anthracene oleic acid were added to these assays as indicated. The signals corresponding to the free (FREE) and protein-bound (BOUND) oligonucleotide are noted. B, increasing amounts of recombinant Bm3R1 protein were added to 300 nM 12-AO, and the resulting fluorescence was determined as described under “Experimental Procedures” (filled squares). PhosphorImage analysis of the above EMSA is also plotted (open squares). The values are from a single representative of three independent experiments. C, increasing amounts of recombinant Bm3R1 protein were added to 3 $\mu M$ 12-AO, and the resulting fluorescence was determined as described under “Experimental Procedures.”

**Fig. 2.** A, inhibition of Bm3R1/DNA binding by linoleic acid. EMSAs were performed as described under “Experimental Procedures” using 500 ng of recombinant Bm3R1 and 10 fmol of the radiolabeled oligonucleotide pair corresponding to the CYP102 operator sequence. Increasing concentrations of linoleic acid were added to these assays as indicated. The signals corresponding to the free (FREE) and protein-bound (BOUND) oligonucleotide are noted. B, saturation analysis of Bm3R1 binding to $^{14}$C-labeled linoleic acid. Bm3R1 (240 nM) was incubated in solution with increasing concentrations of $^{14}$C-labeled linoleic acid. The bound linoleic acid was determined by filtration analysis as described under “Experimental Procedures” (open squares). The values represent means from triplicate filters. The experiment was repeated twice with similar results. PhosphorImage analysis of the above EMSA is also plotted (filled squares). C, displacement of $^{14}$C-labeled linoleic acid from Bm3R1 by nafenopin. Bm3R1 (2.4 $\mu M$) was incubated in solution with 0.8 $\mu M$ $^{14}$C-labeled linoleic acid. Shown is the relative amounts of $^{14}$C-labeled linoleic acid bound to Bm3R1 in the presence of increasing concentrations of nafenopin. The values are expressed as a percentage of the radioactivity bound in the absence of competitor and are means from triplicate filters.
Fig. 3. Structure of CYP102 reporter constructs and their activation by peroxisome proliferators. The CYP102 operon consists of two coding sequences. The coding sequence for the repressor Bm3R1 lies immediately 5' of the coding sequence for the fatty acid hydroxylase CYP102. The operator sequence that binds Bm3R1 lies between the transcriptional start site and the coding sequence for Bm3R1 (27). The genomic DNA fragments corresponding to the CYP102 regulatory sequences were cloned upstream of the Bm3R1 binding site (Operator III), and the entire coding sequence for Bm3R1. The A45 construct contains the same regulatory sequences but contains a truncated Bm3R1 coding sequence. The C143 construct shows tighter regulation by peroxisome proliferators than the A45 construct, but the A45 construct displays higher constitutive activity. The requirement for the entire coding sequence of Bm3R1 was also observed for the effective regulation of CYP102 transcription by barbiturates (27).

In addition to these experiments we also studied fatty acid binding to recombinant Bm3R1 using a rapid filtration binding assay with 14C-labeled linoleic acid. Linoleic acid disrupts the Bm3R1-DNA complex with a Kd of 645 nM. These values are comparable with those obtained using the adipocyte lipid-binding protein which has a Kd for 12- AO of 2 μM (25, 26). A non-lipid binding protein, trypsinogen, displayed no detectable activation of fluorescence in similar experiments (data not shown). Incubation of increasing concentrations of recombinant Bm3R1 with 3 μM 12-AO (above Kd) showed linear binding to 3 μM protein and a sharp saturation of binding at higher concentrations (Fig. 1C). This experiment demonstrated that the protein preparation is largely active and binds one molecule of 12-AO per monomer unit of Bm3R1.

We then investigated the relationship between fatty acid concentration and CYP102 transcriptional activation. The regulation of the reporter was studied over a range of concentrations of several different unsaturated fatty acids. The dieneic fatty acid, linoleic acid, was induced over a very narrow range of concentrations between 2 and 7 μM (Fig. 4C), whereas the trienoic, γ-linolenic and linolenic acids were effective inducers between 2 and 20 μM (Fig. 4C). At concentrations above 20 μM significant toxicity was observed with all three unsaturated fatty acids.
unsaturated fatty acids bind with high affinity to Bm3R1 and activate the CYP102 gene. In order to establish the consequences of this induction we examined the relationship between induction and the ability to inhibit Bm3R1 binding to DNA and fatty acid toxicity (Fig. 5). Two closely related fatty acids were chosen for these experiments, linoleic acid and ricinoleic acid. Ricinoleic acid was chosen as it only differs from linoleic acid by the loss of a double bond and the addition of a single hydroxyl group at carbon 12 (Fig. 5A). The concentrations of these fatty acids required to abolish Bm3R1 binding to DNA (Fig. 5, A and B) correlated closely with those required to induce transcription in vivo, with linoleic acid \( (K_i = 648 \text{ nM}) \) being about 30-fold more potent than ricinoleic acid \( (K_i = 21 \mu M) \). These data further demonstrate that subtle differences in fatty acid structure can have profound effects on Bm3R1 binding affinity. At higher concentrations there was a rapid loss of CYP102 induction. This was accompanied by a profound inhibition of cell growth, with the transcriptional activation being abolished at 8 \( \mu M \) linoleic acid (Fig. 5C), and above 300 \( \mu M \) in the case of ricinoleic acid (data not shown). The higher levels of induction of CYP102 transcription by ricinoleic acid would appear to be due to the large amounts of inducer present in the medium at the effective concentration. The reduced potency of signaling by the hydroxylated fatty acid is similar to that previously observed with phytic acid and hydroxyphytanic acid (22).

Fatty Acyl-CoA Esters Bind Bm3R1 in Vitro, but Appear Not to Be the Endogenous Regulators of CYP102—The metabolism of fatty acids can involve esterification with coenzyme A. It is therefore possible that in vivo it is the fatty acyl-CoA ester that mediates the activation of CYP102 transcription. This is thought to be the case for the transcription factor, FadR. FadR mediates the regulation of genes involved in lipid metabolism in E. coli. FadR can act as both a repressor and transcriptional activator in response to millimolar concentrations of saturated and unsaturated fatty acids (29). Free fatty acids are approximately 1000-fold less potent at inhibiting the FadR binding to operator DNA sites when compared with their CoA esters, with no apparent preference for saturated or unsaturated fatty acids (30, 31). These observations have led to the conclusion that fatty acyl-CoA esters are the regulators of FadR activity in vivo. In order to investigate the possible role of fatty acyl-CoA esters in the regulation of CYP102 transcription, we included the various fatty acyl-CoA esters in the EMSA assays using Bm3R1 (Fig. 6). The fatty acyl-CoA esters also inhibited Bm3R1/DNA binding activity and did so at lower concentrations than the free fatty acids. In fact all of the fatty acyl-CoA esters were effective over a similar range of concentrations (40–1000 \( \mu M \)), including the saturated, stearoyl-CoA ester.

These findings contrast to the activity of saturated fatty acid, stearic acid, which only activates CYP102 transcription and inhibits Bm3R1/DNA binding activity at concentrations greater than 200 \( \mu M \) (18). The 100-fold difference in potency between stearic acid and linoleic acid in the activation of CYP102 transcription in vivo therefore appears to correlate with the ability of Bm3R1 to bind free fatty acids rather than their CoA esters (Fig. 5B). This would suggest that fatty acyl-CoA esters do not accumulate to effective concentrations in B. megaterium upon treatment of cultures with the low concentrations of free fatty acids used in this study.

**Regulation of CYP102 by Bm3R1 Generates Resistance to Toxic Fatty Acids**—In order to establish the consequences of CYP102 induction, we studied the effects of increased CYP102 activity on fatty acid toxicity. The growth inhibition observed in the earlier experiments was due to the toxic effects of these fatty acids. This is more readily observed at high concentrations where toxicity leads to cell lysis. For example, treatment with 20 \( \mu M \) linoleic acid resulted in a decrease in culture density of 75% in 6 h (Fig. 7A). Plating out these cultures confirmed an identical drop in the number of viable cells, and inclusion of 25 \( \mu M \) linoleic acid produced a greater than 99% loss of viability within 1 h (data not shown). At 15 \( \mu M \) linoleic acid growth was initially inhibited; however, by 5 h the cultures re-entered logarithmic growth (Fig. 7B). This recovery
could be explained by the metabolism and detoxification of the unsaturated fatty acids. The induction of CYP102 and subsequent oxidation of the fatty acids would represent a plausible explanation for this effect. This would imply that CYP102 provides an adaptive response mechanism for the detoxification of unsaturated fatty acids. In order to test this hypothesis, cultures of \textit{B. megaterium} were pretreated with the peroxisome proliferator, nafenopin, for 1 h prior to the addition of linoleic acid. This treatment resulted in a marked induction of CYP102 (Fig. 7C, inset) and an almost complete protection from the toxic effects of the linoleic acid (Fig. 7C).

A strain of \textit{B. megaterium}, G39E, has been isolated with a mutation in the Bm3R1 repressor which can no longer bind to DNA and as a consequence constitutively expresses CYP102 (27). The hypothesis that CYP102 is responsible for the detoxification of the fatty acids would predict that this strain would be more resistant to fatty acid toxicity. This was indeed the case, and the G39E strain was resistant to 20 μM linoleic acid (Fig. 8A). The above experiments indicated that Bm3R1 can mediate resistance to toxic fatty acids. To test further the involvement of CYP102 in the detoxification pathway, we inhibited CYP102 activity using 17-octadecynoic acid (17-ODA). This acetylenic fatty acid is known to specifically and irreversibly inactivate cytochrome P450 fatty acid \( \beta \)-\( \gamma \) hydroxylases, including CYP102 (32, 33). Treatment of cultures of the mutant strain with concentrations of 17-ODA that are known to inhibit CYP102 did not affect growth; however, when these cultures were also treated with 20 μM linoleic acid, a marked growth inhibition was observed (Fig. 8B). Therefore, the resistant phenotype of the G39E strain is dependent on the activity of CYP102.

\section*{DISCUSSION}

Many fatty acids, particularly unsaturated fatty acids, are extremely toxic to cells; however, in most cases the mechanism could be explained by the metabolism and detoxification of the unsaturated fatty acids. The induction of CYP102 and subsequent oxidation of the fatty acids would represent a plausible explanation for this effect. This would imply that CYP102 provides an adaptive response mechanism for the detoxification of unsaturated fatty acids. In order to test this hypothesis, cultures of \textit{B. megaterium} were pretreated with the peroxisome proliferator, nafenopin, for 1 h prior to the addition of linoleic acid. This treatment resulted in a marked induction of CYP102 (Fig. 7C, inset) and an almost complete protection from the toxic effects of the linoleic acid (Fig. 7C).

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Fatty Acid Toxicity in B. megaterium

The genetic and biochemical analysis of the B. subtilis system is in progress.

The exquisite sensitivity to unsaturated fatty acids displayed by B. megaterium and B. subtilis contrasts with the binding of a prokaryotic transcription factor to free fatty acids. A previous study noted the toxic effects of unsaturated fatty acids on B. megaterium (18) but did not observe induction of CYP102 at the high concentrations used. Other investigators who have studied these toxic effects on B. subtilis have noted that the histidine protein kinase essential for sporulation, KinA, was inhibited by unsaturated fatty acids in vitro (34). However, in vivo studies of the effects of unsaturated fatty acids on the sporulation were also hampered by fatty acid toxicity. Inhibition of KinA by fatty acids exhibited an identical rank order of potency to that observed for toxicity in B. megaterium, i.e. oleic acid > linoleic > linolenic > stearic acid. Indeed the concentrations of each fatty acid required to inhibit KinA in vitro are virtually identical to the concentrations that we have found to be toxic to B. megaterium. We have recently found that B. subtilis is sensitive to linoleic acid toxicity over the same range of concentrations as observed for B. megaterium. The genetic and biochemical analysis of the B. subtilis system is in progress.

3 C. N. A. Palmer, E. Axen, and C. R. Wolf, unpublished data.
tolerance to high concentrations of unsaturated fatty acids found in both *E. coli* and *Saccharomyces cerevisiae*. Both of these latter organisms can utilize unsaturated fatty acids as their sole carbon source at concentrations over 3 orders of magnitude higher than those tolerated by *B. megaterium* (35–37). Both *E. coli* and *S. cerevisiae* display transcriptional responses to fatty acids which increase lipid metabolism. *E. coli* and *Hemophilus influenzae* express a transcription factor called FadR that acts as a fatty acid sensor (29, 38). The binding of FadR to DNA is disrupted by fatty acids in an analogous manner to Bm3R1. In contrast to Bm3R1, however, the *E. coli* FadR protein is relatively nonspecific in its regulation by fatty acids and has only a slight preference for polyunsaturated fatty acids. It is thought that the FadR proteins are regulated in vivo by the acyl-CoA esters of the lipids as there is a 1000-fold difference in the apparent affinity, as judged by gel shift assays, between the free fatty acids and the respective acyl-CoA esters. The situation in shift assays, between the free fatty acids and the respective analogues, is 1000-fold different in the apparent affinity, as judged by gel and responses to fatty acids which increase lipid metabolism. The specificity of regulation is totally concordant with the affinity for the repressor of the free fatty acids observed in vitro. However, further genetic analysis of *B. megaterium* is required to completely rule out the involvement of acyl-CoA esters in the regulation of CYP102.

The role of CYP102 as a fatty acid detoxification enzyme is supported by the finding that either induction or constitutive overexpression of CYP102 results in a high level of fatty acid resistance, and chemical inactivation of CYP102 results in a sensitization to these compounds. CYP102 is one of the most catalytically active cytochrome P450s known, with a turnover number of several thousand catalytic cycles per min, which is about 100 times the average catalytic rate of mammalian cytochrome P450. This extremely high activity is consistent with the attenuation of CYP102 induction after 2 h due to removal of the active fatty acid.

This metabolic pathway therefore generates a regulatory loop where exposure to fatty acids removes the repressor from the CYP102 operator resulting in the induction of CYP102. On induction of the CYP102 the fatty acid is metabolized and no longer binds the Bm3R1 repressor which results in the transcription of CYP102 being switched off. Also, upon activation of the CYP102 operon, Bm3R1 levels are increased which provides an additional “feedback” component for the reimplosion of CYP102 repression (22).

One of the most intriguing aspects of this work is the close parallel between the response of *B. megaterium* and mammalian cells to fatty acids. Many of the chemical agents that induce CYP102 also induce fatty acid hydroxylases in mammals (6, 41). The mammalian system also appears to provide an adaptive response to alterations in fatty acid homeostasis which may protect against their toxic effects by hydroxylation and increased rates of peroxisomal β-oxidation (7, 42). In mammalian cells this response is mediated by the direct binding of fatty acids to a family of nuclear receptors known as peroxisome proliferator activated receptors (9, 11–13). On this basis, these studies in *B. megaterium* may be of direct relevance to the understanding of cellular responses to fatty acids in mammalian systems.