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Citation: Schujman, Gustavo E et al. "FapR, a Bacterial Transcription Factor Involved in Global Regulation of Membrane Lipid Biosynthesis." *Developmental Cell* 4.5 (2003): 663–672. Copyright © 2003 Cell Press

As Published: [http://dx.doi.org/10.1016/S1534-5807\(03\)00123-0](http://dx.doi.org/10.1016/S1534-5807(03)00123-0)

Persistent URL: <http://hdl.handle.net/1721.1/83849>

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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FapR, a Bacterial Transcription Factor Involved in Global Regulation of Membrane Lipid Biosynthesis

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Summary

Bacterial cells exert exquisite control over the biosynthesis of their membrane lipids, but the mechanisms are obscure. We describe the identification and purification from *Bacillus subtilis* of a transcription factor, FapR, that controls the expression of many genes involved in fatty acid and phospholipid metabolism (the *fap* regulon). Expression of this *fap* regulon is influenced by antibiotics that specifically inhibit the fatty acid biosynthetic pathway. We show that FapR negatively regulates *fap* expression and that the effects of antibiotics on *fap* expression are mediated by FapR. We further show that decreasing the cellular levels of malonyl-CoA, an essential molecule for fatty acid elongation, inhibits expression of the *fap* regulon and that this effect is FapR dependent. Our results indicate that control of FapR by the cellular pools of malonyl-CoA provides a mechanism for sensing the status of fatty acid biosynthesis and to adjust the expression of the *fap* regulon accordingly.

Introduction

One of the most daunting challenges in biology is elucidating the mechanisms by which cells sense and respond to changes in the biosynthesis of essential building blocks that support a majority of cellular activities. Among the critical metabolic changes that occur during various conditions in all living cells are fluctuations in the biosynthesis of fatty acids. In all organisms, fatty acids and their derivatives are essential components of membranes, important sources of metabolic energy, and important effector molecules that regulate metabolism. Due to the essential roles that fatty acids have within the cell, the complex processes that govern the synthesis of these compounds are regulated in such a manner as to allow biological membranes to maintain stable compositions that are characteristic for different

organisms, tissues, and intracellular organelles (DiRusso and Nystrom, 1998; Dobrosotskaya et al., 2002; Rock and Jackowski, 2002). For instance, bacteria and most (if not all) poikilothermic organisms have to remodel the membrane lipid composition to survive at low temperatures (Aguilar et al., 2001; Cybulski et al., 2002; Sakamoto and Murata, 2002; de Mendoza and Cronan, 1983).

All organisms produce fatty acids via a repeated cycle of reactions involving the condensation, reduction, dehydration, and reduction of carbon-carbon bonds (Campbell and Cronan, 2001; Rock and Cronan, 1996). In mammals and other higher eukaryotes, these reactions are catalyzed on a type I synthase (FAS I), a large multifunctional protein in which the growing fatty acid chain is covalently attached to the protein (Campbell and Cronan, 2001; Rock and Cronan, 1996). In contrast, bacteria, plant chloroplasts, and *Plasmodium falciparum* contain a type II system (FAS II) in which each reaction is catalyzed by a discrete protein and reaction intermediates are carried through the cytosol as a thioester of the small acyl carrier protein (ACP) (Rock and Jackowski, 2002; Campbell and Cronan, 2001). The chain elongation step in fatty acid biosynthesis consists of the condensation of acyl groups, which are derived from acyl-ACP or acyl-coenzyme A (acyl-CoA), with malonyl-ACP by the β -ketoacyl-ACP synthases (often referred as condensing enzymes) (Cronan and Rock, 1996). These enzymes are divided in two groups. The first (FabH) class of condensing enzymes is responsible for the initiation of fatty acid elongation and utilizes acyl-CoA primers (Rock and Jackowski, 2002). *Escherichia coli* produces straight chain and unsaturated fatty acids, and *E. coli* FabH selectively uses acetyl-CoA to initiate the pathway (Rock and Jackowski, 2002). In contrast, *Bacillus subtilis* produces mainly branched chain fatty acids and contains two FabH isozymes (named FabHA and FabHB) that differ from the *E. coli* enzyme in that they are selective for branched chain acyl-CoAs (Choi et al., 2000). The second (FabF-FabB) class of condensing enzymes is responsible for the subsequent rounds of fatty acid elongation in the pathway (Campbell and Cronan, 2001). These enzymes condense malonyl-ACP with acyl-ACP to extend the acyl chain by two carbons. While *E. coli* expresses both types of these condensing enzymes, in *B. subtilis* the FabF protein is the sole condensing enzyme able to carry out the subsequent elongation reactions in fatty acid synthesis (Schujman et al., 2001; de Mendoza et al., 2002).

We recently reported that in *B. subtilis* the expression of a gene cluster, the *fabHAF* operon, coding for the FabHA and FabF condensing enzymes, is upregulated in response to inhibition of fatty acid synthesis (Schujman et al., 2001). We proposed that *B. subtilis* has the ability to sense a decrease in the activity of the pathway and to respond by adjusting the synthesis of the FabHA and FabF condensing enzymes (Schujman et al., 2001). Nevertheless, the mechanism by which the transcription of the *fabHAF* operon is regulated by the activity of fatty acid synthesis remains unsolved. It is also not known

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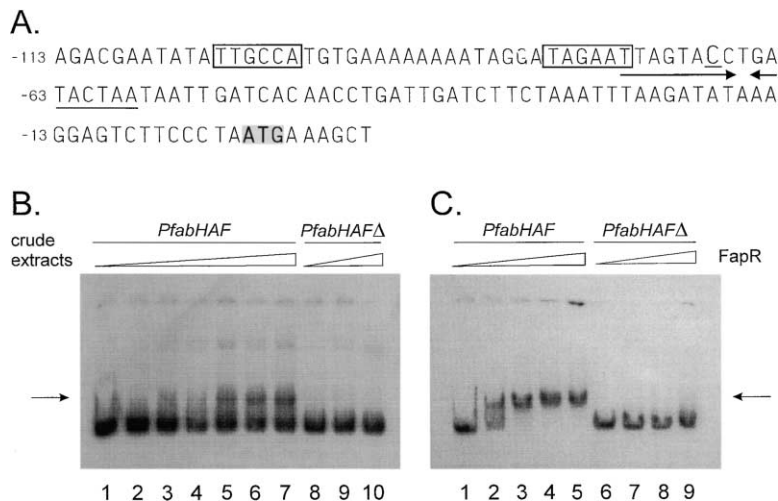


Figure 1. Gel Shift Assay Showing the Binding of FapR to the *fabHA-F* Promoter Region
(A) *fabHA-F* promoter region sequence. σ^A -RNA polymerase binding boxes are framed; C residue taken to be the start site of transcription by primer extension (see Experimental Procedures) is underlined; and the translation start codon is shaded. Opposed arrows indicate the 17 bp inverted repeats. Bases are numbered relative to the first one of the translation initiation codon.

(B) The gel shift assays experiments were performed with a 300 bp *PfabHAF* fragment or with the same DNA fragment containing a partial deletion of the 17 bp inverted repeats identified in the promoter region of *PfabHAF* (*PfabHAFΔ*). The fragments were labeled with [α - 32 P]dATP (see Experimental Procedures). Crude extracts of *B. subtilis* wild-type strain JH642 were obtained from cultures in exponential phase of growth and incubated with the labeled probes. The amounts of extract

used in each lane were 0.5, 1, 2, 3, 4, and 5 μ g for lanes 2 to 7, respectively, and 2 and 5 μ g for lanes 9 and 10, respectively. The migration of the free probes without protein addition is shown in lanes 1 and 8.

(C) FapR-His6 was overexpressed in *E. coli* and purified as described in Experimental Procedures. The labeled DNA fragments described in (B) were incubated with the purified protein and the mobility assayed. The amount of protein used in lanes 2 to 5 were 0.5, 1, 2.5, and 5 μ g, respectively, and 0.5, 2.5, and 5 μ g in lanes 7 to 9, respectively. The migration of the free probes is shown in lanes 1 and 6. The arrows indicate the migration of the probe interacting with the protein.

how other genes involved in lipid biosynthesis in *B. subtilis* are regulated.

To identify *B. subtilis* genes regulated by the activity of the fatty acid biosynthetic pathway, we performed DNA microarray analysis comparing RNA of wild-type cells treated with specific inhibitors of fatty acid synthesis to RNA from untreated control cells. Our results indicate that ten genes, contained in five different operons, coding for proteins involved in fatty acid and phospholipid metabolism are induced in response to inhibition of fatty acid synthesis.

We isolated a protein from crude extracts of *B. subtilis* cells that is able to bind a fragment of DNA containing the regulatory region upstream from one of the operons responsive to fatty acid deprivation. This protein, FapR (fatty acid and phospholipid biosynthesis regulator), is a negative regulator of itself and of genes involved in fatty acid and phospholipid biosynthesis. In vitro, it binds directly to a regulatory site found upstream of all of these genes. In toto, our results provide evidence for a novel mechanism for global control of membrane biogenesis in which the FapR regulator couples the status of fatty acid biosynthesis in the cells with the expression of genes involved in lipid metabolism.

Results

Purification of a Protein that Binds to the Promoter Region of Genes Required for Membrane Lipid Biosynthesis

We wished to identify the transcription factor(s) contributing to regulation of the fatty acid biosynthetic genes in *B. subtilis*. The *fabHAF* operon encodes one of the FabH isoenzymes required for initiation of fatty acid elongation and the FabF protein that is essential to catalyze the remaining elongation steps of the pathway (Schujman et al., 2001). The transcription of this operon

is induced in response to inhibition of fatty acid biosynthesis (Schujman et al., 2001). The promoter region of *fabHAF* (*PfabHAF*) contains a 17 bp inverted repeat (Figure 1A), and we therefore attempted to determine if this dyad symmetric element was the binding site of a putative regulatory protein. To this end, we performed band shift assays with crude extracts obtained from the wild-type strain JH642. After confirming that the crude extract obtained was able to shift the mobility of a 300 bp DNA fragment carrying the *PfabHAF* region, but not the mobility of a version of *PfabHAF* containing a deletion of the palindromic sequence extending from position -73 to -56 (Figure 1B), we used this gel shift assay to monitor the purification of the putative regulator. To purify the protein associated with binding, we used a modification of the procedure described by Jourlin-Castelli et al. (2000) to purify DNA binding proteins. In brief, DNA carrying the *PfabHAF* was bound to streptavidin-coated magnetic spheres, and partially purified proteins fractions, which retained the ability to shift the *PfabHAF* region, were applied to these magnetic spheres (see Experimental Procedures). After thorough washing, one or more proteins able to bind the *PfabHAF* were eluted with 500 mM NaCl. The active fractions proved to contain a major protein that had a mobility in SDS-PAGE corresponding to a mass of about 26.5 kDa (data not shown). This protein was electroblotted to polyvinylidene difluoride membrane and subjected to microsequencing. A partial N-terminal amino acid sequence was obtained as MXXNKXXRQ. Searching the entire *B. subtilis* genome (Kunst et al., 1997), we found that this N-terminal sequence was only contained in the putative product of the *ylpC* gene. This hypothetical protein has a predicted mass of 21,255 Da and is similar to other bacterial proteins of unknown function. In addition, *ylpC* is the first gene in a cluster containing *plsX*, *fabD*, *fabG*, and *acp*, each of which codes for a protein involved in fatty acid or phospholipid synthesis.

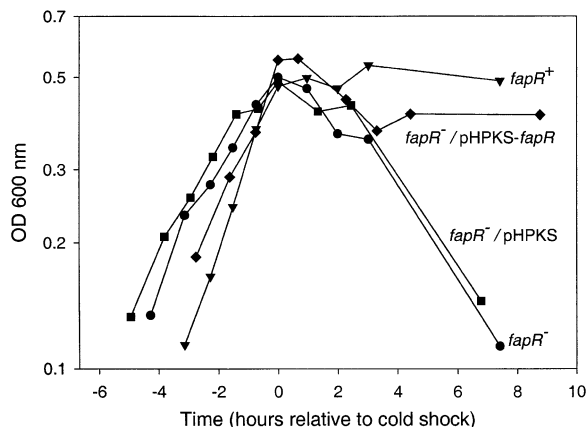


Figure 2. *fapR* Cells Are Cold Sensitive

Cells were grown in Spizizen minimal medium at 37°C. At an OD₆₀₀ of 0.50–0.55, cultures were shifted to 15°C. Growth was monitored by measuring the absorbance at 600 nm of the cultures. Symbols: circles, GS268 (*fapR*⁻); squares, GS283 (*fapR*⁻/pPHKS); rhombuses, GS284 (*fapR*⁻/pPHKS-*fapR*); triangles, JH642 (*fapR*⁺).

To determine if YlpC binds to *PfabHAF*, we first constructed a His-tag version of YlpC and used it to purify YlpC-His6 from *E. coli* by affinity chromatography. The binding of purified YlpC-His6 to *PfabHAF* was tested by mobility shift assays (Figure 1C). The YlpC-His6 protein was able to shift the mobility of the DNA fragment carrying the *PfabHAF* region but not the mobility of *PfabHAF* lacking the palindromic sequence extending from position -73 to -56 (Figure 1C). These data directly demonstrate that YlpC-His6 binds to the *PfabHAF* promoter and strongly suggest that the dyad symmetric element is essential for binding activity. Thus, based on the localization of *ylpC* in a lipid biosynthetic gene cluster and the ability of its gene product to bind to *PfabHAF*, we have named this gene *fapR* for fatty acid and phospholipid regulator.

Deletion of *fapR* Leads to Defects in Cell Growth and to Constitutive Expression of the *fabHAF* Gene Cluster

We disrupted the *fapR* gene with a chloramphenicol resistance cassette, which provides read-through transcription to maintain the expression of the essential downstream genes, required for fatty acid synthesis. In defined glucose minimal medium, the *fapR* mutant was viable at 37°C, although the generation time (~154 min) was significantly longer than that of wild-type strain (~86 min). However, the shift of exponentially growing cultures of *fapR* cells from 37°C to 15°C resulted in a profound reduction in culture optical density as well as in colony forming units when compared with *fapR*⁺ parental strain cultures (Figure 2). This dramatic reduction in viability during cold-shock could be relieved by expression of a wild-type copy of the *fapR* gene into *fapR*-deficient cells (Figure 2). The severe cold-sensitive phenotype of *fapR* null mutants is probably due to alterations in the membrane fatty acid composition that are described below.

In the *fapR* null mutant, expression of *fabHAF* was

significantly higher than in a *fapR*⁺ isogenic strain (Figure 3A). Moreover, when the *fapR* gene was provided in *trans* into *fapR* null mutants, expression of the *PfabHAF* lowered to levels similar to the wild-type strain (data not shown). These experiments, together with those showing that the FapR-His6 protein was able to shift the mobility of a DNA fragment carrying the *PfabHAF* region, suggest that FapR acts negatively on *fabHAF* transcription and that its effect is exerted by binding directly to *PfabHAF*.

It is worth noting that upregulation of *fabHAF* transcription in the *fapR* deficient strain is similar to the selective response of this operon, in a wild-type strain, to inhibitors of fatty acid synthesis such as cerulenin or triclosan (Schujman et al., 2001). Moreover, in the *fapR* strain, no additional derepression of *fabHAF* was observed after the addition of either cerulenin or triclosan (Figure 3A), implying strongly that FapR responds directly or indirectly to fatty acid deprivation.

FapR Is a Global Negative Regulator of Membrane Lipid Synthesis

To see if other genes involved in lipid synthesis, in addition to *fabHA* and *fabF*, are upregulated in response to inhibition of fatty acid synthesis, we compared the whole transcriptome of cells treated with either cerulenin or triclosan. To this end, the wild-type reference strain (JH642) was grown until early exponential phase and then divided into three samples. One sample was treated with cerulenin, the second one was treated with triclosan, and the third sample remained untreated. The cultures were grown for 40 min and the genomic expression profiles of the samples were analyzed using DNA microarrays containing 4074 of the 4106 protein coding genes of *B. subtilis* (see Experimental Procedures). As expected from our previous results, the amount of RNA from the *fabHAF* operon and the *fabHB* gene, which code for the three *B. subtilis* condensing enzymes (Figure 4A), increased upon block of fatty acid synthesis (Table 1). Notably, RNA from *fapR*, identified here as a negative regulator of the *fabHAF* operon, was also induced by fatty acid deprivation. In addition to the above-mentioned genes, another seven genes coding for proteins with similarity to enzymes involved in fatty acid and phospholipid synthesis were significantly induced in the presence of cerulenin and triclosan (Table 1). The products of two of them (*yhdO* and *plsX*) have a putative function in the transacylation of long chain acyl-ACPs to glycerol-1-phosphate (Figure 4A). The product of the *yhfC* gene is of unknown function but it is immediately divergent to *fabHB*. The other upregulated genes are *fabD*, coding for malonyl-CoA:ACP transacylase (Morbidoni et al., 1996), and *fabI* and *fabG*, which code for 3-ketoacyl-ACP-reductase (Heath et al., 2000) and enoyl-ACP reductase (de Mendoza et al., 2002), respectively (Figure 4A).

We confirmed the data obtained by the transcriptome analysis, determining that the β-galactosidase activities of strains bearing *lacZ* fusions to the promoter regions of the *fapR*, *fabI*, and *yhdO* genes were significantly elevated after the addition of cerulenin or triclosan to the culture medium (data not shown). These data demonstrate that the inhibition of fatty acid biosynthesis

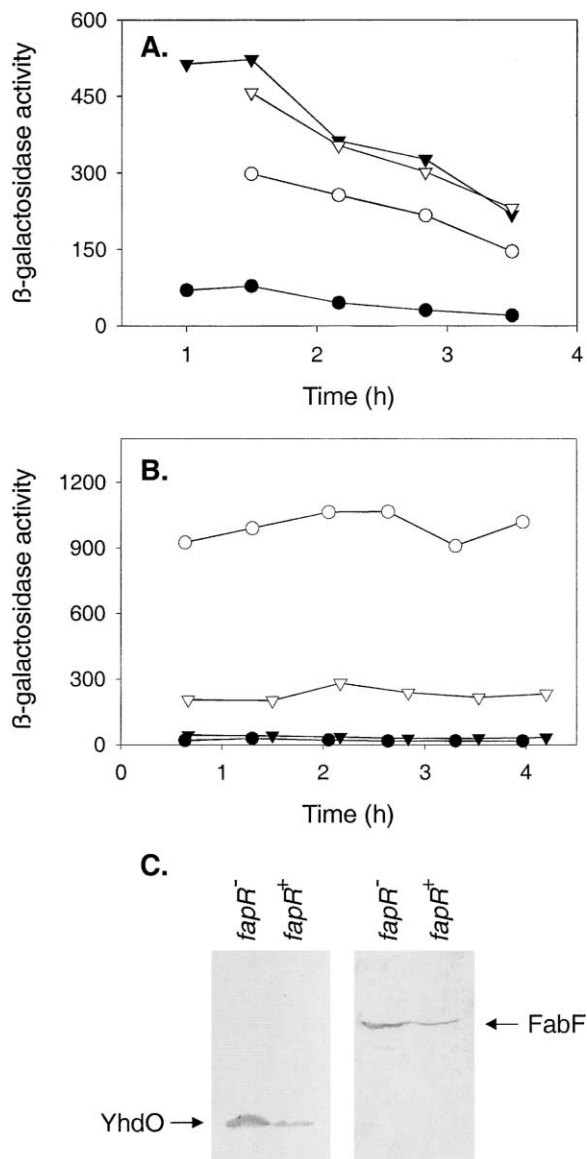


Figure 3. Effect of a *fapR* Mutation on Expression of Fatty Acid Biosynthetic Genes

(A) *B. subtilis* strains GS273 (*fapR*⁺) (circles) and GS274 (*fapR*⁻) (triangles) harboring a *PfabHAF-lacZ* fusion located in the *amyE* locus were grown in LB medium at 37°C. At an OD₅₂₅ of 0.3, half of each culture was treated with cerulenin 3.3 μg/ml (open symbols), while the other half remained untreated (closed symbols). At the indicated times, samples of each culture were removed to assay β -galactosidase-specific activity. A single experiment representative of four repeats is shown.

(B) *B. subtilis* strains GS277 (*fapR*⁺) (closed triangles) and GS278 (*fapR*⁻) (open triangles) harboring a *PfapR-lacZ* fusion and strains GS282 (*fapR*⁺) (closed circles) and GS285 (*fapR*⁻) (open circles) containing a *PfabHB-lacZ* transcriptional fusion were grown in LB medium at 37°C. At the indicated times, samples were collected and assayed for β -galactosidase activity. A single experiment representative of four repeats is shown.

(C) Strains JH642 (*fapR*⁺) and GS268 (*fapR*⁻) were grown in LB medium at 37°C. Cells were collected in exponential phase of growth, and YhdO and FabF contents were determined by immunoblotting as described in Experimental Procedures.

triggers increased transcription of at least ten genes encoding for key enzymes of the fatty acid and phospholipid biosynthetic pathway.

As FapR acts negatively on transcription of *fabHAF*, we determined whether the *fapR* null mutation was able to derepress other genes controlled by fatty acid starvation. Figure 3B displays the results of these experiments, in which the expression of *fabHB-lacZ* and *fapR-lacZ* reporter was measured. These experiments demonstrate that the inactivation of *fapR* derepresses the expression of *fabHB-lacZ* and *fapR-lacZ* (Figure 3B). No additional derepression of these transcriptional fusions was obtained after addition of cerulenin (data not shown). Also, the levels of FabF and YhdO proteins in *fapR*⁺ and *fapR*⁻ cells were determined by immunodetection and quantification. Consistent with the operon fusion analysis, these experiments demonstrate that inactivation of *fapR* resulted in approximately 5-fold increase of the FabF and YhdO proteins (Figure 3C). The effect of a *fapR* deletion on the activity of the fatty acid synthase of *B. subtilis* was determined in vitro. We found that extracts from *fapR* cells possessed a fatty acid synthase activity that was about 5-fold higher than those of wild-type cells (data not shown).

To determine whether the negative effects of FapR on *yhdO* and *fapR* operon expression might be a result of a direct binding to these gene promoters, gel shift assays were performed using radiolabeled fragments with sequences of the putative *yhdO* and *fapR* promoter regions and purified FapR-His6. The results of these experiments demonstrated that FapR-His6 indeed binds to the *yhdO* and *fapR* promoters (see Supplemental Figure S1 at <http://www.developmentalcell.com/cgi/content/full/4/5/663/DC1>). Analysis of the sequences upstream of the lipid biosynthetic genes that are upregulated by inhibitors of fatty acid synthesis revealed sequences similar to the FapR binding site determined for *PfabHAF* (Figure 4B). We conclude that FapR is a global negative regulator of genes involved in both fatty acid and phospholipid lipid synthesis (the *fap* regulon).

Malonyl-CoA Regulates FapR Activity

A key issue in the regulation of the *fap* regulon by FapR is to understand how the status of fatty acid synthesis controls FapR activity. The observation that FAS inhibition produces the same transcriptional response as a null mutation in *fapR* suggests that FapR is controlled by a ligand that is either an intermediate or an end product of the lipid biosynthetic pathway. Taking into account that inactivation of the *E. coli* condensing enzymes triggers a dramatic increase in the accumulation of the FAS substrate malonyl CoA (Furukawa et al., 1993; Heath and Rock, 1995), we postulate that fluctuations in the levels of this metabolite could be coupled to FapR activity. This model predicts that inhibition of the synthesis of malonyl-CoA (Figure 4A, shaded box), which drastically reduces de novo fatty acid synthesis in *B. subtilis* (Perez et al., 1998), should reduce the expression of the *fap* regulon both in the absence and in the presence of FAS inhibitors. To test this, the *B. subtilis accBC* operon (boldfaced in Figure 4A), coding for two subunits of the acetyl-CoA carboxylase (ACC) (Marini et al., 1995), under the control of the xylose-inducible *PxyIA* promoter, was

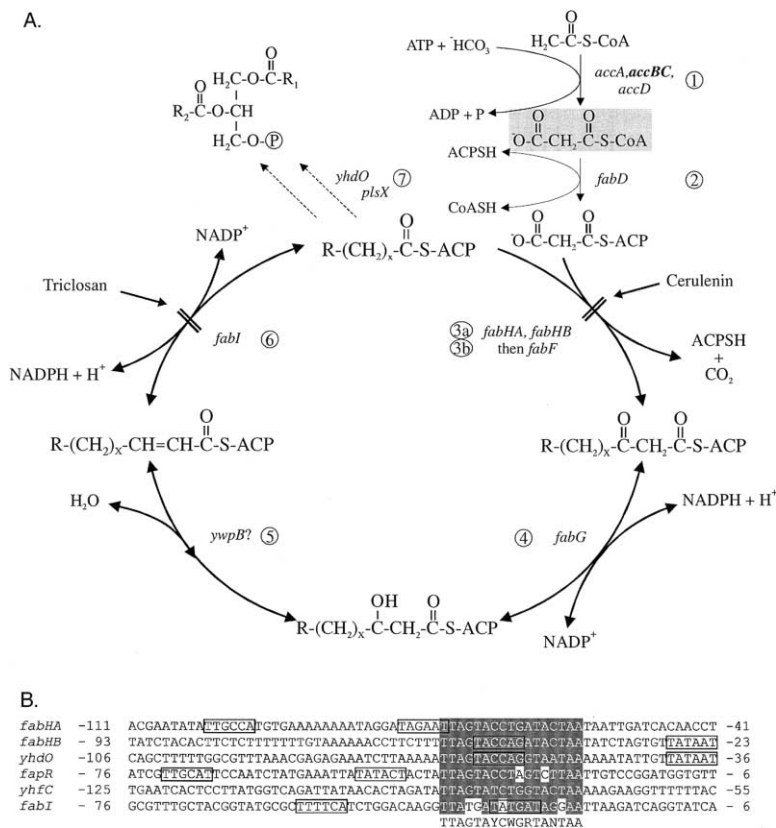


Figure 4. Promoter Regions of Genes Regulated by FapR

(A) Pathway of lipid synthesis in *Bacillus subtilis*. Cycles of fatty acid elongation are initiated by the condensation of straight and branched chain acyl-CoA primers with malonyl-ACP catalyzed by the FabHA and FabHB condensing enzymes (3a). The next step is the reduction of β -ketoesters, catalyzed by β -ketoacyl reductase (4). The β -hydroxyacyl-ACP is then dehydrated to the *trans*-2 unsaturated acyl-ACP by β -hydroxyacyl-ACP dehydrase (5), which is reduced by enoyl reductase (6) to generate an acyl-ACP two carbons longer than the original acyl-ACP. The last step of the cycle is inhibited by triclosan. Subsequent rounds of elongation are initiated by the elongation condensing enzyme FabF (3b). This step is blocked by cerulenin. The acyl-ACP ends products of fatty acid synthesis are transacylated to glycerol-phosphate to generate phosphatidic acid, which is an intermediate in the synthesis of phospholipids (7). These enzymes were not characterized yet in *B. subtilis*. Malonyl-CoA is generated from acetyl-CoA by acetyl-CoA carboxylase (ACC) (1) and then is transferred to ACP by malonyl-CoA transacylase (2). The genes coding for the enzymes involved in each step are indicated. R denotes the terminal group of branched chain fatty acids derived from ketoacids from valine, leucine, or isoleucine. The *accBC* genes coding for two subunits of ACC are in boldface. Shaded box, malonyl-CoA.

(B) Alignment of the promoter regions of the genes and operons regulated by FapR. Predicted σ^A -RNA polymerase binding boxes (Jarmer et al., 2001) are indicated with rectangles. The conserved inverted repeat sequence is shaded and a consensus sequence is indicated at the bottom line. Bases are numbered related to the first base of the translation initiation codon.

integrated isotopically in a strain bearing a *fabHAF-lacZ* fusion. In this strain, the basal transcription of *fabHAF* was almost completely eliminated when cells were grown in the absence of xylose (Figure 5A). As expected, the upregulation of *fabHAF* in the presence of cerulenin was greatly decreased when the levels of malonyl-CoA were depleted in xylose-deprived cells (Figure 5A). Similar results were obtained when we monitored transcription of another member of the *fab* regulon, the *fabHB*

gene, during inhibition of malonyl-CoA synthesis (Figure 5B). However, in a *fapR* background derepression of the *fabHB* gene was not significantly affected when *accBC* expression was blocked (Figure 5B), confirming that FapR is responsible for the potent transcriptional inhibition of the *fab* genes when the levels of malonyl-CoA are reduced. These data strongly support the hypothesis that FapR responds directly or indirectly to the intracellular levels of the malonyl-CoA pool.

Table 1. Newly Identified Lipid Biosynthetic Genes Induced by Inhibition of Fatty Acid Synthesis

Gene	Cerulenin ^a	Triclosan ^a	Function ^b
<i>fabHB</i>	10.5	9.1	Type III β -ketoacyl-ACP synthase
<i>fabF</i>	4.5	4.8	Type II β -ketoacyl-ACP synthase
<i>fabHA</i>	4.1	6.6	Type III β -ketoacyl-ACP synthase
<i>fabI</i>	3.5	2.6	Enoyl-ACP reductase
<i>yhfC</i>	2.9	2.3	Unknown
<i>plsX</i>	2.9	3.1	Related to transacylation
<i>fabD</i>	2.7	3.4	Malonyl CoA-ACP transacylase
<i>fabG</i>	2.5	3.4	β -hydroxyacyl-ACP reductase
<i>yhdO</i>	2.5	3.9	Similar to acyltransferases
<i>fapR</i> (<i>ylpC</i>)	2.4	3.0	New transcriptional regulator

A culture of the *B. subtilis* wild-type strain JH642 was grown in LB medium at 37°C to an OD₅₂₅ of 0.3. Two samples were treated with either cerulenin (3.3 μ g/ml) or triclosan (0.4 μ g/ml), while a third sample remained untreated. Cultures were incubated for 45 min and RNA extracted and analyzed by microarrays as described in Experimental Procedures.

^a Ratio of expression of genes in antibiotic-treated cells compared to untreated cells. The average ratios of four independent experiments are shown.

^b The biosynthetic steps catalyzed by the products of the induced genes are shown in Figure 4A.

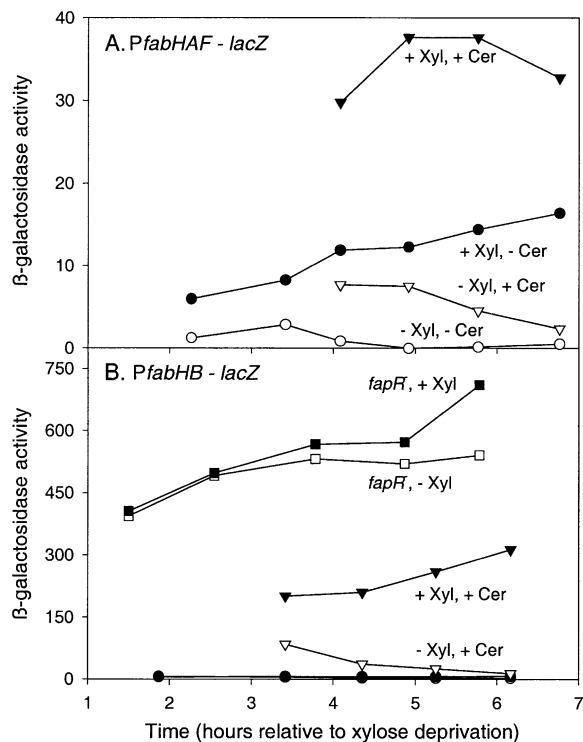


Figure 5. Effect of Malonyl-CoA Synthesis on Expression of Fatty Acid Biosynthetic Genes

(A) *B. subtilis* strain GS54 (*PxyIA-accBC*) harboring a *PfabHAF-lacZ* fusion located in the *amyE* locus was grown in Spizizen minimal medium supplemented with xylose 0.1% (w/v) at 37°C. At an OD₆₂₅ of 0.2, the culture was filtered, washed, and resuspended in the same medium without xylose. Xylose was added to half of the culture at a final concentration of 0.1% (w/v) (closed symbols), while the other half was not supplemented (open symbols). The cultures were incubated at 37°C until an OD₆₂₅ of 0.3 was reached and half of each culture was treated with cerulenin 3.3 μg/ml (triangles), while the other half remained untreated (circles). At the indicated times, samples of each culture were removed to assay β-galactosidase-specific activity. A single experiment representative of three repeats is shown.

(B) The same protocol described in (A) was used with strain GS289 (*PxyIA-accBC*), which contains an isotopic *PfabHB-lacZ* fusion, and with the *fapR*⁻ isogenic strain GS290 (*PxyIA-accBC*, *PfabHB-lacZ*, *fapR*⁻). Closed symbols, xylose-supplemented cultures; open symbols, xylose-deprived cultures. Circles, strain GS289; triangles, strain GS289 treated with cerulenin; squares, strain GS290. A single experiment representative of four repeats is shown, and the relative standard deviation for each data point was less than 15%.

Deletion of *fapR* Leads to Overproduction of Long Chain Fatty Acids

The *B. subtilis* membrane is characterized by a fatty acid profile dominated by a large extent (>90%) by even- and odd-numbered terminally methyl-branched fatty acids (de Mendoza et al., 2002; Cybulski et al., 2002). The effect of *fapR* deletion on the production of the major fatty acids of *B. subtilis* was determined. In a *fapR*⁺ strain, the ratio of long chain fatty acids (LCFA; chain length of C16, C17, and C18) to short chain fatty acids (SCFA; chain length of C14 and C15) was 0.58 (Table 2). However, a *fapR*-deficient strain produces significantly more LCFA, increasing the ratio LCFA/SCFA

Table 2. Fatty Acid Compositions of the Phospholipids of Strains JH642 (*fapR*⁺) and GS268 (*fapR*⁻)

Fatty Acid	Percentage (w/w) of Fatty Acid Type	
	GS268	JH642
Iso-C _{14:0}	5.3	3.1
n-C _{14:0}	1.1	1.1
Iso-C _{15:0}	10.6	25.8
Anteiso-C _{15:0}	27.9	30.4
n-C _{15:0}	0.7	2.9
Iso-C _{16:0}	8.7	4.2
n-C _{16:0}	9.3	9.2
Iso-C _{17:0}	13.1	13.9
Anteiso-C _{17:0}	13.7	7.1
n-C _{17:0}	0.9	1.3
Iso-C _{18:0}	1.4	ND
n-C _{18:0}	7.6	1.1
Ratio iso/anteiso ^a	0.93	1.25
Ratio LCFA/SCFA ^b	1.20	0.58

Cells were grown in Spizizen minimal medium with glucose as carbon source at 37°C to an OD₆₀₀ of 0.5. Total lipids were extracted and transesterified to yield fatty acids methyl esters. The fatty acids methyl esters were subjected to gas chromatography-mass spectrometry analysis. The methyl esters were Iso-C_{14:0}, 12-methyltridecanoic; n-C_{14:0}, n-tetradecanoic; Iso-C_{15:0}, 13-methyltetradecanoic; Anteiso-C_{15:0}, 12-methyltetradecanoic; n-C_{15:0}, n-pentadecanoic; Iso-C_{16:0}, 14-methylpentadecanoic; n-C_{16:0}, n-hexadecanoic; Iso-C_{17:0}, 15-methylhexadecanoic; Anteiso-C_{17:0}, 14-methylhexadecanoic; n-C_{17:0}, n-heptadecanoic; Iso-C_{18:0}, 16-methylheptadecanoic; n-C_{18:0}, n-octadecanoic.

ND, not detected.

^a Ratio of iso-fatty acids to anteiso-fatty acids.

^b Ratio of long chain fatty acids (LCFA) (C16, C17, and C18) to short chain fatty acids (SCFA) (C14, C15).

to 1.20. Unlike the wild-type strain that synthesizes low levels (<1%) of n-octadecanoic acid (stearic acid), the levels of this fatty acid were about 7% in the *fapR* mutant. Notably, the *fapR* strain synthesized about 2% of methyl stearic acid that was undetectable in the wild-type parental strain.

These compositional data support the conclusion that the abnormal high levels of cellular LCFA are, at least in part, responsible of the cold-sensitive phenotype of *fapR* strains. It is well established that to survive cold-shock, bacteria need to maintain membranes in a fluid or liquid-crystalline state (Sakamoto and Murata, 2002; de Mendoza and Cronan, 1983). However, phospholipids containing LCFA have significantly higher lipid-phase transition than do phospholipids containing SCFA (Cronan and Gelmann, 1975). This means that, upon cold-shock, membranes of *fapR* strains may become too rigid for growth and adaptation at low growth temperatures.

FapR Is Conserved in Other Gram-Positive Species

FapR is highly conserved in many gram-positive organisms sequenced to date. The gene was found in all the species of the *Bacillus*, *Listeria*, and *Staphylococcus* genera and also in *Clostridium difficile* and other related genera (Figure 6), but was not detected in gram-negative bacteria or other gram-positive genera. It is noteworthy

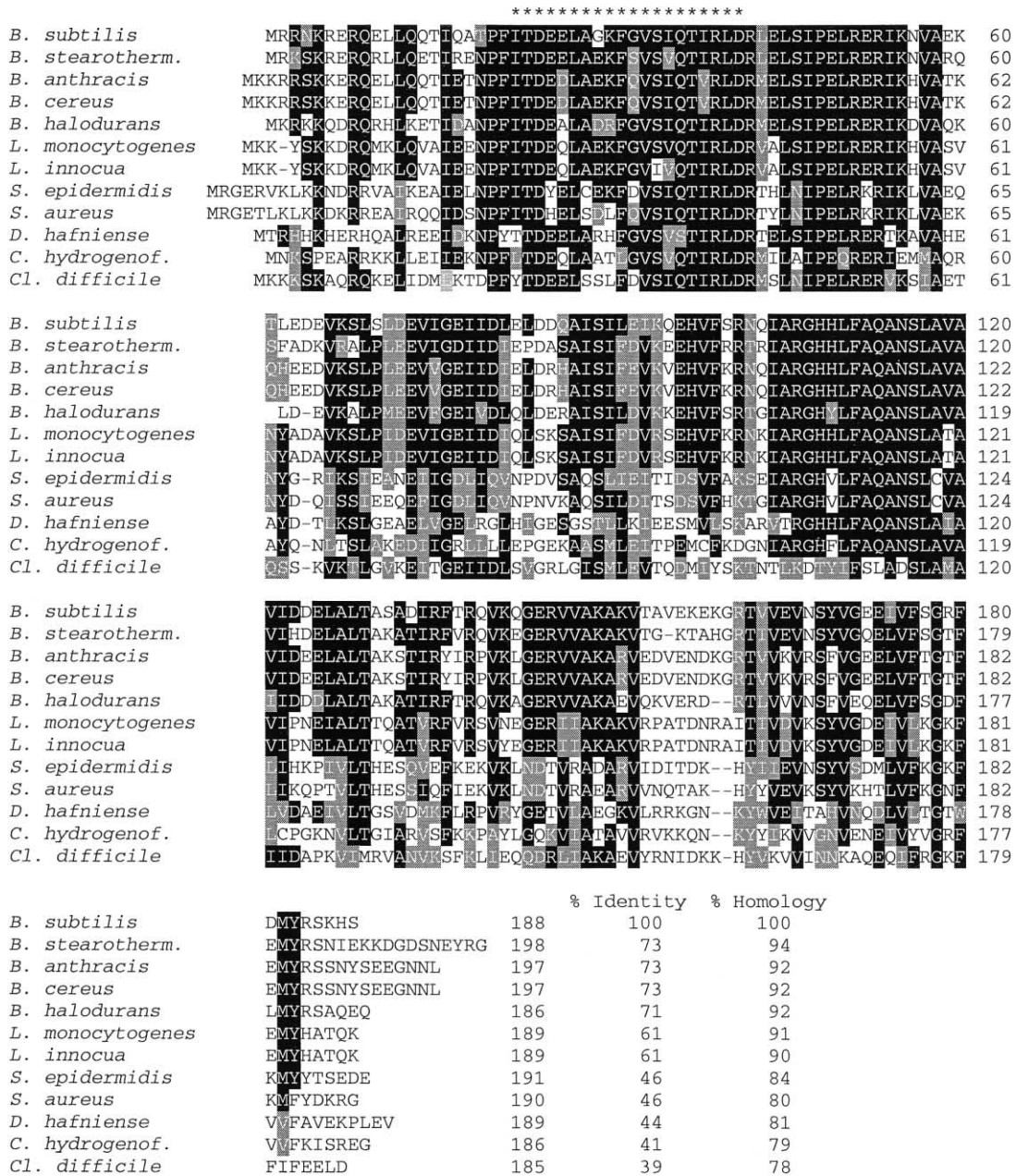


Figure 6. FapR Is Highly Conserved in Many Gram-Positive Bacteria

Alignment of FapR sequences from many organisms of the *Bacillus/Clostridium* group. Identity and homology percentages of each sequence with respect to *B. subtilis* are shown. Asterisks indicate the localization of a putative helix-turn-helix motif. Abbreviations: *B.*, *Bacillus*; *L.*, *Listeria*; *S.*, *Staphylococcus*; *D.*, *Desulfotobacterium*; *C.*, *Carboxidothermus*; *Cl.*, *Clostridium*; *hydrogenof.*, *hydrogenoformans*; *stearotherm.*, *stearothermophilus*.

that many of the organisms containing FapR are human pathogens, including *Bacillus anthracis*, *Bacillus cereus*, and *Listeria monocytogenes*. In all the organisms analyzed, the *fapR* gene was found associated to the gene *plsX*, like in *B. subtilis*, suggesting that the same operon structure is conserved in those organisms. The consensus binding sequence of FapR is also highly conserved in the putative promoter regions of the *fapR* gene in those species. All FapR primary structures share an HTH

motif beginning in the position 22–25 as predicted by software analysis (Figure 6). These observations indicate that the regulation mechanism observed in *B. subtilis* is probably conserved in many other organisms.

Discussion

We discovered a regulatory pathway, which is intriguing with respect to cell physiology and its particular mode

of action. We have identified a protein, FapR, that controls the homeostasis of the lipid bilayer through the transcriptional regulation of several crucial genes encoding enzymes of fatty acid and phospholipid synthesis in *B. subtilis*. FapR negatively regulates the expression of lipid metabolic genes by binding to a consensus sequence contained in the promoter region of the biosynthetic *fap* regulon.

Because the metabolic building blocks of membrane lipid synthesis are constantly exchanged, the use of a single protein, FapR, might suffice as a device for sensing and controlling the entire repertoire of fatty acid-derived membrane lipids. We propose that fluctuations in membrane lipid production, and possibly other conditions affecting lipid synthesis, alter the balance of a key intermediate in the pathway and thus regulate expression of fatty acid and phospholipid synthesis. Thus, we suggest that an essential function of FapR is to monitor cell lipid synthesis and to adjust membrane composition accordingly.

FapR and Transcriptional Regulation

FapR is a transcription factor controlling the expression of almost an entire set of genes involved in the type II fatty acid biosynthetic pathway. This transcription factor also controls two key genes involved in the transfer of acyl-ACPs, the end products of fatty acid biosynthesis, to glycerol-3-phosphate. This is the first step in phospholipid formation and represents the transition from soluble intermediates to membrane bound enzymes and products. Thus, FapR is a global regulator ideally suited to adjust the composition of membranes, which depends largely of the activity of key enzymes that control the synthesis and incorporation of fatty acid among the various lipids.

Physiology

The correct ratio of high melting point to low melting point fatty acids is crucially important for maintaining the optimal fluidity of the membrane (Sakamoto and Murata, 2002; de Mendoza and Cronan, 1983). Suboptimal levels of membrane lipid fluidity lead to a severe impairment of membrane systems that are essential for normal cell function. Evidently, cells use homeostatic mechanisms that maintain the concentrations of different membrane lipids at particular levels to optimize its fluidity. We discovered here that FapR is required to optimize membrane composition since *fapR* mutants produce significantly more high melting point LCFA than wild-type strains. It is likely that this unbalanced synthesis of LCFA in *fapR* strains contributes to suboptimal growth at physiological temperatures and a severe cold-sensitive phenotype. Highlighting the central importance of the FapR pathway for lipid homeostasis in *B. subtilis*, this transcriptional regulator has clearly identifiable homologs in other gram-positive bacteria. We therefore assume that FapR is the first identified member of a family of transcriptional regulators, which is of paramount importance to control the chemical and physical properties of the cell membranes in many organisms.

Elucidation of the mechanistic details that control

FapR binding to DNA may have important and widespread implications for the still largely unresolved question of how type II FAS is regulated and coordinated in response to growth-limiting levels of new acyl chains produced within the cells. Recent exciting work has demonstrated that fatty acid biosynthesis is an emerging target for the development of novel antibacterial chemotherapeutics (Campbell and Cronan, 2001). Thus, unraveling the mechanisms that sense the amount of fatty acids within the cell and transduce this physiological information into an adaptive transcriptional response could have important implications for drugs targeting this signal-transducing pathway.

The FapR Pathway as a Monitor of Lipid Synthesis

The activity of FapR in transcriptional regulation of fatty acid and phospholipid synthesis seems to be controlled by the status of fatty acid synthesis. Expression of the *fap* regulon is derepressed in the absence of FapR but also can be derepressed by the addition of either cerulenin or triclosan, inhibitors of key enzymes of the fatty acid biosynthetic pathway. Since these fatty acid inhibitors do not upregulate the expression of lipid biosynthetic genes in *fapR*-deficient cells, the simplest model for the function of FapR in regulation of lipid synthesis is that FapR repression is relieved during a decrease in the activity of the pathway. Moreover, *fapR* is an auto-regulated gene since it is a member of the *fap* regulon. Thus, it is tempting to speculate that the activity of FapR is controlled by a ligand that is an integral component of endogenous de novo fatty acid biosynthesis. The intracellular concentration of malonyl-CoA is dramatically increased when the FabF condensing enzyme is inhibited by cerulenin (Furukawa et al., 1993; Heath and Rock, 1995), and it is assumed that a similar increase in this metabolite takes place during inhibition of enoyl-ACP reductase with triclosan (Schujman et al., 2001). Our results now show that a decrease in the cellular levels of malonyl-CoA greatly decreases the expression of the *fap* regulon and that this effect is FapR dependent. In the light of these findings, we propose that the intracellular pool of malonyl-CoA regulates FapR-mediated repression of target promoters. The levels of malonyl-CoA are then in turn regulated in concert with fatty acid biosynthesis through feedback inhibition by the acyl-ACP end products (Davis and Cronan, 2001). Thus, if the rate of fatty acid synthesis falls below the normal levels, a transient increase in the intracellular concentration of malonyl-CoA would relieve, or partly relieve, FapR-mediated repression of lipid biosynthetic genes. In this regard, we draw attention to recent work attributing the effect of reduced food intake and body weight in mice to a putative sensor in hypothalamic neurons of malonyl-CoA levels (Loftus et al., 2000).

Experimental Procedures

Media

Cells were grown in Luria-Bertani (LB) medium or Spizizen minimal medium supplemented with glucose 0.5%, mineral traces, and required amino acids (40 μ g/ml) (Nicholson and Setlow, 1990). Appropriate antibiotics were added to the media, as needed.

Bacterial Strains and Plasmids

The *B. subtilis* strains used in this study were derived from JH642 and are listed in Supplemental Table S1 at <http://www.developmentalcell.com>.

com/cgi/content/full/4/5/663/DC1. *lacZ* transcriptional fusions to the promoter region of genes *yhdO*, *fabHA*, *fabI*, and *fapR* were constructed using plasmids pDG1728 or pJM116. The fusions were integrated by a double event of recombination in the nonessential *amyE* locus. *fabHB-lacZ* fusion was constructed and integrated isotopically using plasmid pMUTIN4. *fapR* strain was obtained replacing 364 bp of the 5' extreme of the gene with the *cat* cassette from plasmid pJM105B. Complementation of *fapR* strains was achieved by transformation of the strains with the low copy number plasmid pHPKS containing the *fapR* gene and its promoter region (pHPKS-*fapR*).

Primer Extension

Strain JH642 was grown in LB medium until midexponential phase. RNA was isolated using RNeasy Mini-columns (Qiagen). Primer extension was performed essentially as described (Aguilar et al., 1999) using 15 μ g RNA/reaction and a primer with 5' end at position 23 relative to the A of the *fabHA* AUG start codon.

FapR Isolation

B. subtilis strain JH642 (wild-type) was grown at 37°C in LB medium with strong aeration. When the culture reached an OD₅₂₅ of 0.8, the cells were harvested by centrifugation and washed with buffer A (20 mM Tris-HCl [pH 8], 0.5 mM EDTA, 1 mM DTT, 5% (v/v) glycerol) containing 50 mM NaCl and 1 mM phenylmethylsulfonylfluoride. The pellet was resuspended in the same buffer and the cells were disrupted by two passages through a French press. After centrifugation (40,000 \times g for 25 min), the crude extract was recovered and loaded onto a 20 ml-DEAE agarose column (HiPrep 16/10 Amersham-Pharmacia). Elution was performed by using buffer A containing increasing concentrations of sodium chloride. Fractions between 250 and 400 mM of NaCl were joined, diluted with buffer A, and concentrated using a CentriPrep (cutoff 3000 Da, Millipore) and loaded onto a 1 ml MonoQ FPLC column (HR 5/5, Amersham-Pharmacia). Elution was with buffer A containing increasing concentrations of sodium chloride. Fractions between 285 and 315 mM of NaCl were joined and diluted 6-fold with buffer A. Nonidet P40 and MgCl₂ were added to final concentrations of 0.05% (v/v) and 10 mM, respectively. The proteins were incubated at room temperature for 20 min with magnetic spheres coated with streptavidin (Promega) to which biotinylated DNA fragments containing the *fabHAF* promoter region were bound. After extensive washing with buffer B (buffer A plus Nonidet P40 0.05% (v/v) and MgCl₂ 10 mM) containing polydIdC 2 μ g/ml, the remaining protein(s) were eluted with buffer A containing NaCl 500 mM. The proteins were subjected to SDS-PAGE and electroblotted to PVDF, and N-terminal sequence of the majority one determined by automatic sequencing.

Full-length FapR was expressed as N-terminal his₆ fusion protein in *E. coli* from plasmid pGES229 and affinity purified on Ni²⁺-NTA resin according to the manufacturer's protocol (Qiagen).

Protein concentrations were determined by Bradford assay (Bradford, 1976) using BSA as a standard.

Gel Shift Assays

Labeled DNA fragments were produced by PCR using [α -³²P]ATP and incubated with protein samples in 20 μ l reactions containing buffer B. The reactions were kept for 15 min at room temperature and then loaded onto a running, nondenaturing 6% polyacrylamide gel prepared in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA [pH 8.0]). Electrophoresis was performed at 100 V, and the dried gel was subjected to autoradiography.

Microarray Analyses

RNA was extracted, labeled, and analyzed with *B. subtilis* DNA microarrays as described (Britton et al., 2002). Briefly, at the appropriate times, culture samples were fixed with -20°C methanol and RNA extracted with RNeasy mini kit (Qiagen). Labeled cDNA was generated from RNA samples by direct incorporation of Cy3- or Cy5-labeled dUTP into cDNA. Differentially labeled samples from two different conditions were mixed and hybridized to the DNA microarrays, and each experiment was done four times. Images were processed and analyzed with GenePix 3.0 software (Axon Instruments, Inc.).

Western Blot Analyses and β -Galactosidase Assays

Western blot analyses were performed as previously described (Schujman et al., 2001). YhdO and FabF antibodies were used at a dilution of 1:1000. Proteins were detected using AP-conjugated anti-rabbit secondary antibodies (BioRad).

β -galactosidase specific activity (ΔA_{420} per min per ml of culture \times 1000/A₅₂₅) was determined as described (Miller, 1972) after pelleting cell debris.

Fatty Acids Analysis and Fatty Acid Synthase Assays

Lipids were extracted and fatty acids converted to their methyl esters with sodium methoxide (Aguilar et al., 1998). The methyl esters were separated in a Perkin-Elmer Turbo mass gas chromatographer-mass spectrometer, equipped with a PEG column, and analyzed with Perkin-Elmer TurboMass software. Each fatty acid was identified by comparing its mass spectrum with those obtained from methyl esters of fatty acid standards (Sigma).

Fatty acid synthase was measured in vitro as described (Schujman et al., 1998).

Sequence Analysis

Preliminary genome sequence data was searched at the NCBI website (<http://www.ncbi.nlm.nih.gov>). Contig sequences for each species were obtained at NCBI, TIGR, and Institute Pasteur databases. Protein sequences were compared by using CLUSTAL algorithm (Thompson et al., 1994). Helix-turn-helix domains were searched with the program HTH (Dodd and Egan, 1990).

Acknowledgments

We thank R.A. Britton for his valuable advice with DNA microarray analysis and S. Altabe and M. Hourcade for helpful advice in the fatty acid analysis. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica (FONCYT) to D. de M. G.E.S. is a fellow from CONICET and D. de M. is a Career Investigator from CONICET and an International Research Scholar from Howard Hughes Medical Institute. This work was also supported in part by Public Health Services Grant GM50895 to A.D.G. and an International Fellowship from the American Society for Microbiology to G.E.S.

Received: September 30, 2002

Revised: February 19, 2003

Accepted: February 24, 2003

Published: May 5, 2003

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