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

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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, dehydratation, 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 FabF 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...
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 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 Joulin-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 database, 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 1. Gel Shift Assay Showing the Binding of FapR to the fabHA-F Promoter Region (A) fabHA-F promoter region sequence. Upstream of the translation initiation codon are underlined; and the 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. Opposite 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 (PfabHAFΔ17). The fragments were labeled with [γ-32P]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 μg for lanes 2 to 7, respectively, and 2 and 5 μg for lanes 8 and 9, 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 μg, respectively, and 0.5, 2.5, and 5 μg 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.
Global Control of Membrane Lipid Biosynthesis

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

We disrupted the fabR 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 fabR 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 fabR 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 fabR parent strain cultures (Figure 2). This dramatic reduction in viability during cold-shock could be relieved by expression of a wild-type copy of the fabR gene into fabR-deficient cells (Figure 2). The severe cold-sensitive phenotype of fabR null mutants is probably due to alterations in the membrane fatty acid composition that are described below.

In the fabR null mutant, expression of fabHAF was significantly higher than in a fabR+ isogenic strain (Figure 3A). Moreover, when the fabR gene was provided in trans into fabR 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 fabR 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 fabR 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 fabR, 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 (yhfO 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-CoAACP 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 fabR, fabI, and yhfO 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...
Inactivation of the FabH dehydrase of B. subtilis derepresses the expression 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 fabR mutant 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 PsyP promoter, was
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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 fap 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 fap 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cerulenin*</th>
<th>Triclosan*</th>
<th>Function*</th>
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<tbody>
<tr>
<td>fabHB</td>
<td>10.5</td>
<td>9.1</td>
<td>Type III β-ketoacyl-ACP synthase</td>
</tr>
<tr>
<td>fabF</td>
<td>4.5</td>
<td>4.8</td>
<td>Type II β-ketoacyl-ACP synthase</td>
</tr>
<tr>
<td>fabHA</td>
<td>4.1</td>
<td>6.6</td>
<td>Type III β-ketoacyl-ACP synthase</td>
</tr>
<tr>
<td>fabI</td>
<td>3.3</td>
<td>2.6</td>
<td>Enoyl-ACP reductase</td>
</tr>
<tr>
<td>yfHC</td>
<td>2.9</td>
<td>2.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>plsX</td>
<td>2.9</td>
<td>3.1</td>
<td>Related to transacylation</td>
</tr>
<tr>
<td>fabD</td>
<td>2.7</td>
<td>3.4</td>
<td>Malonyl CoA-ACP transacylase</td>
</tr>
<tr>
<td>fabG</td>
<td>2.5</td>
<td>3.4</td>
<td>β-hydroxyacyl-ACP reductase</td>
</tr>
<tr>
<td>yhdO</td>
<td>2.5</td>
<td>3.9</td>
<td>Similar to acyltransferases</td>
</tr>
<tr>
<td>fapR</td>
<td>2.4</td>
<td>3.0</td>
<td>New transcriptional regulator</td>
</tr>
</tbody>
</table>

A culture of the B. subtilis wild-type strain JH642 was grown in LB medium at 37°C to an OD600 of 0.3. Two samples were treated with either cerulenin (3.3 μg/ml) or triclosan (0.4 μg/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.

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

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

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

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Percentage (w/w) of Fatty Acid Type</th>
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<tbody>
<tr>
<td></td>
<td>GS268</td>
</tr>
<tr>
<td>Iso-C16:0</td>
<td>5.3</td>
</tr>
<tr>
<td>n-C16:0</td>
<td>1.1</td>
</tr>
<tr>
<td>Iso-C17:0</td>
<td>10.6</td>
</tr>
<tr>
<td>Anteiso-C17:0</td>
<td>27.9</td>
</tr>
<tr>
<td>n-C18:0</td>
<td>0.7</td>
</tr>
<tr>
<td>Iso-C18:0</td>
<td>8.7</td>
</tr>
<tr>
<td>n-C18:1</td>
<td>9.3</td>
</tr>
<tr>
<td>Iso-C18:2</td>
<td>13.1</td>
</tr>
<tr>
<td>Anteiso-C18:2</td>
<td>13.7</td>
</tr>
<tr>
<td>n-C19:0</td>
<td>0.9</td>
</tr>
<tr>
<td>Iso-C19:0</td>
<td>1.4</td>
</tr>
<tr>
<td>n-C20:0</td>
<td>7.6</td>
</tr>
</tbody>
</table>

| Ratio iso/anteiso | 0.93 | 1.25 |
| Ratio LCFA/SCFA   | 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-C16:0, 12-methyltetradecanoic; n-C16:0, n-tetradecanoic; Iso-C17:0, 13-methylpentadecanoic; Anteiso-C17:0, 12-methyltetradecanoic; n-C17:0, n-pentadecanoic; Iso-C18:0, 14-methylpentadecanoic; n-C18:0, n-hexadecanoic; Iso-C18:1, 15-methylhexadecanoic; Anteiso-C18:0, 14-methylhexadecanoic; n-C18:2, n-heptadecanoic; Iso-C19:0, 16-methylheptadecanoic; n-C18:3, n-octadecanoic.

ND, not detected.

*Ratio of iso-fatty acids to anteiso-fatty acids.

Summarized in Table 2, the ratio of iso-fatty acids to anteiso-fatty acids is significantly higher in the fapR- strain compared to the wild-type, indicating a decrease in the production of anteiso-fatty acids in the fapR- strain.

To investigate the role of FapR in the regulation of fatty acid composition, the expression of fatty acid biosynthetic genes was analyzed. 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...
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 on 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 μg/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/cgi/content/full/4/5/663/DC1, lacZ transcriptional fusions to
the promoter region of genes yhdO, fabHA, fabI, and fapF were
constructed using plasmids pDG1729 or pJM116. The fusions were
integrated by a double event of recombination in the nonessential
amyE locus. fabHB-lacZ fusion was constructed and integrated isoo-
topically using plasmid pMULTIN4. fapF strain was obtained replacing
364 bp of the 5’-extreme of the gene with the cat cassette from plasmid pJM105B. Complementation of fapF strains was achieved
by transformation of the strains with the low copy number plasmid
pHPKS containing the fapF gene and its promoter region (pHPKS-
fapR).

Primer Extension
Strain JH642 was grown in LB medium until midexponential phase.
RNA was isolated using RNaseasy Mini-columns (Qiagen). Primer ex-
tension was performed essentially as described (Aguilar et al., 1999)
using 15 μg 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 OD600 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 centrifugia-
tion (40,000 × g for 25 min), the crude extract was recovered and loaded onto a 20 ml-DEAE agarose column (HiPrep 16/10 Amer-
sham-Pharmacia). Elution was performed by using buffer A con-
taining increasing concentrations of sodium chloride. Fractions be-
tween 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). After elution was achieved, the fraction containing increas-
ing 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 fabHA promoter
region were bound. After extensive washing with buffer B (buffer A
plus Nonidet P40 0.05% (v/v) and MgCl2 10 mM) containing poli-
didC 2 μM, the remaining protein(s) were eluted with buffer A
containing NaCl 500 mM. The proteins were subjected to SDS-PAGE
electrophoresis and electroblotted to PVDF, and N-terminal sequence of the majority
one determined by automatic sequencing.

Full-length FapR was expressed as N-terminal his 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 (Brad-
ford, 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 μl reactions containing
buffer B. The reactions were kept for 15 min at room temperature
and then loaded onto a 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 appro-
priate 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 In-
struments, Inc.).

Western Blot Analyses and β-Galactosidase Assays
Western blot analyses were performed as previously described
(Schujman et al., 2001). YbdO 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 (ΔA420 per min per ml of culture
× 1000/A600) 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 chro-
matographer-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 web-
site (http://www.ncbi.nlm.nih.gov/). Contig sequences for each spe-
cies 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).

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