

The LipB protein is a negative regulator of *dam* gene expression in *Escherichia coli*.

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Abstract

Transcription initiation of the major promoter (P2) of the *Escherichia coli dam* gene increases with growth rate. The presence of three partially palindromic motifs, (TTCAGT(N₂₀)TGAG), designated G(growth)-boxes, within the -52 to +31 region of the promoter, may be related to growth rate control. Deletion of two of these repeats, downstream of the transcription initiation point, result in constitutive high activity of the promoter. The unlinked *cde-4::miniTn10* insertion also results in several fold higher activity of the *dam* P2 promoter, suggesting that this mutation resulted in the loss of a putative *dam* P2 repressor. The *cde-4* mutation was mapped to the *lipB* (lipoic acid) gene, which we show encodes a 24 kDa protein initiating at a TTG codon. LipB is a highly conserved protein in animal and plant species, other bacteria, Archaea, and yeast. Plasmids expressing the native or hexahistidine-tagged LipB complement the phenotype of the *cde-4* mutant strain. The level of LipB *in vivo* was higher in exponentially growing cells than those in the stationary phase. Three G-box motifs were also found in the *lipB* region. Models for the regulation of expression of the two genes are discussed.

1. Introduction

The *dam* gene of *Escherichia coli* encodes a DNA methyltransferase which transfers methyl groups from S-adenosyl-L-methionine to adenine residues in the sequence 5'-GATC-3' in double stranded DNA [1]. Since methylation lags behind the replisomal proteins, newly synthesized DNA is in a hemimethylated configuration for a short time [2]. Hemimethylated DNA is an important feature in a variety of cellular processes [1] including *dam*-directed mismatch repair which is active on this type of DNA but not fully methylated DNA to remove replication errors [3]. Hemimethylated origins (*ori*) of certain plasmids and *E. coli* minichromosomes cannot be used to initiate chromosome replication *in vivo* until they become fully methylated [4]. Although efficient initiation of *E. coli* chromosome replication occurs at a fully methylated *oriC* region of the chromosome, the *oriC* sequence remains hemimethylated for about 30% of the cell cycle to prevent premature initiation [2]. Hemimethylated DNA is the basis to ensure that initiation from multiple *oriC* regions occurs simultaneously [5]. Transcription initiation from certain promoters is more active in a hemimethylated versus methylated state, thereby linking their activity to the cell cycle [6].

Alteration in the amount of hemimethylated DNA, by changing the level of Dam methyltransferase in the cell from its normal level of about 130 molecules per cell, can have significant effects [7]. The spontaneous mutation rate is increased [8;9], the level of gene expression can be altered [10] and initiation of chromosome replication at multiple origins becomes asynchronous [5].

The *dam* gene is located in a transcriptional unit that includes *aroK*, *aroB*, *urf74.3*, *rpe*, *gph* and *trpS* [11-13]. This gene cluster includes at least five transcription initiation sites (P1 through P5) as well as one strong transcription terminator [12]. Transcription of the *dam* gene *in vivo* is growth rate-dependent as measured by assaying β -galactosidase activity in extracts of cells containing a single-copy chromosomal *dam::lacZYA* fusion [14;15]. Of the five promoter regions, only the major *dam* promoter (P2) shows growth rate-dependent transcription initiation as determined by *lacZ* fusion and primer extension measurements [14;15].

In order to probe the molecular mechanism of growth rate control at the transcriptional level, a mutation (*cde-4*), which uncouples growth rate-dependence, has been isolated [14]. The mini*Tn10*-induced *cde-4* mutation was mapped by Southern blotting in or near the *lipB* (lipoic acid) gene and is unlinked to *dam* [15]. Lipoic acid biosynthesis requires the product of the *lipA* gene, which converts octanoic acid to lipoic acid, and *lipA* mutants are auxotrophic [16]. Although *lipB* mutants have been reported to have a lipoic acid requirement [17], they are not defective in lipoic acid biosynthesis but have reduced levels of lipoic acid ligase activity [18]. The residual activity is due to a second lipoic acid ligase encoded by the *lpIA* gene [19].

In the present communication, we describe the mapping of the *cde-4* mutation to the *lipB* gene, the purification of the LipB protein and preliminary data suggesting that expression of the gene is regulated.

2. Materials and methods

2.1 Bacterial strains and plasmids

All strains used were *Escherichia coli* K-12 and are listed in Table 1. The 50-bp region encompassing the minimal *dam* gene P2-50 promoter (-49 to +1) was amplified by PCR using primers containing additional *Bam*HI and *Eco*RI sites. The PCR amplified fragment was fused to *lacZ* in pRS415 [20] and the plasmid was sequenced. Nucleotide sequencing was performed essentially as described by Sanger et al., [21] using plasmid DNA prepared from strain MM294. The promoter-*lacZ* fusion was integrated in single copy at the phage lambda attachment site in the *E. coli* chromosome [22]. The promoter:: *lacZ* fusion was transferred to strain LJ24 [23] by phage P1 transduction and the resulting transductant designated UF800. Strain LJ391 (*dam* P1-5) and strain LJ555 (*dam* P2-80) were described previously [14;15].

All plasmids used are listed in Table 1. A hexahistidine tagged *lipB* derivative of pET15b, pMQ404, was constructed by PCR amplification of the *lipB* gene from pKR112 using the 5' primer MM192 (5'-GGGTTTAACCATATGTATCAGGATAAAATTC-3') tagged with an *Nde*I (underlined) site and a 3' primer MM189 (5'CAAGGATCCGAATTAAGCGGTAATATATTCG-3') tagged with a *Bam*HI (underlined) site. Vent polymerase (New England Biolabs) was used for all PCR reactions in the buffer supplied by the manufacturer. The amplified fragment was purified using a QiaQuick (Qiagen) column, digested with *Nde*I and *Bam*HI and inserted into the corresponding sites of pET15b (Novagen), to yield pMQ404. The plasmids pMQ405 and pMQ409 were constructed using the

same strategy but different primers. The coding sequence for pMQ405 was PCR-amplified from pCTV625 using 5' primer MM178 (5'-GGGTTTAACCATATGAAAACCAAACCTTAACG-3') and 3' primer MM189. The coding sequence for pMQ409 was PCR-amplified from pKR112 using 5' primer MM188 (5'-GGGTTTAACCATATGCCGGGTGATATTCCGG-3') and 3' primer MM189. The pMQ408 plasmid was constructed by following strategy. Plasmid pKR112, which contains the *lipB* gene was digested with *Ppu10I*, the ends filled in with DNA polymerase I Klenow fragment (New England Biolabs) and digested with *BamHI* to obtain the *lipB* coding sequence. This fragment was sub-cloned into pET15b that had been digested with *NdeI*, the ends filled in DNA polymerase I Klenow fragment and digested with *BamHI*. Plasmid pMQ410, which carries the *lipB* gene without a hexahistidine tag, was constructed by ligating a 650-bp *NdeI-BamHI* fragment from pMQ404 into expression vector pET3a (Novagen) digested with the same enzymes. Plasmid pMQ411, which has a disrupted *lipB* gene, was constructed by inserting a 1.1-kb blunt ended *BamHI* fragment containing the kanamycin-resistance into the blunt-ended *Ppu10I* site of pMQ410. Fill-in reactions to create blunt ends utilized DNA polymerase I Klenow fragment.

2.2 Growth conditions, media, β -galactosidase assay and lac fusion techniques

Cells were grown in AB minimal medium [24] supplemented with either 0.2% glucose and 0.5% casamino acids or 0.8 % acetate and 50 $\mu\text{g/ml}$ leucine as described previously [14]. β -Galactosidase activity was assayed in either crude extracts obtained by sonication or in toluene treated cells and the specific activity expressed as Miller units [25].

2.3 *Purification of His-tagged LipB and antisera preparation*

The pMQ404 plasmid was transfected into *E. coli* strain GM5861 and proteins were expressed as described by Studier et al., [26]. LipB protein was purified to apparent homogeneity using nickel-chelation affinity chromatography essentially as described by the manufacturer (Novagen). Briefly, bacteria were grown in 100 ml LB medium to a density of approximately 5×10^8 cells/ml. IPTG (isopropyl-1-thio- β -D-galactopyranoside) was added to a final concentration of 1 mM, and incubation was continued for a further 3 h at 37°C. Cells were collected by centrifugation, resuspended in 5 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) and lysed using a French pressure cell. After centrifugation in a Beckman Ti70 rotor for 30 min. at 39,000 rpm at 4 C, the supernatant was applied to a nickel-agarose column (Novagen). The column was washed with 40 mM imidazole in nickel-chelation affinity chromatography buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 1 mM β -mercaptoethanol). Hexahistidine-tagged LipB protein was eluted with 500 mM imidazole in the same buffer, and the purified protein stored at -70°C. Thrombin cleavage of hexahistidine-tagged LipB and purification of LipB was carried out according to the manufacturer's (Novagen) instructions. Rabbit polyclonal antiserum to hexahistidine-tagged LipB was prepared by BAbCO (Richmond, CA).

2.4 *Sequence Analysis*

Wisconsin Package Version 9.0, Genetics Computer Group (Madison, WI) software programs were used to analyze amino acid sequences. Sequence comparison of the polypeptides was

conducted with BESTFIT and PILEUP programs. The BLAST program at the National Center for Biotechnology Information was used to search for homology with the GenBank/EMBL sequence data [27].

2.5 *Gel electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis (PAGE) in 10% acrylamide gels was carried out using a Hoeffer SE 250 slab gel apparatus as described by Laemmli [17]. The proteins were stained with Coomassie Brilliant Blue (Sigma). The following proteins (obtained from BioRad) were used as molecular weight markers: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysosyme (14.5 kDa). For western immunoblot analysis, the separated proteins in acrylamide gels were transferred to Immobilon-P membranes (Millipore) using a semi-dry electroblotter (Owl Scientific). LipB was detected using chemiluminescence according to the manufacturer's (Tropix, Inc.) instructions.

3. Results

3.1 *The growth rate determinants of the dam operon P2 promoter*

The determinants for growth rate regulation of the *dam* P2 promoter are located in an 83 bp region (-52 to +31) encompassing the promoter and its transcriptional start point [14]. In order to localize these determinants further, a DNA fragment (P2-50) containing the minimal P2

promoter region (-49 to +1) was fused to *lacZ*, integrated into the phage λ attachment site in single copy, and assayed for activity (Table 2). Although the level of Dam varies by more than 5-fold under different growth conditions [14], we have chosen to use two growth conditions in which the level differs by a factor of 2.5 if growth rate regulation is present [14]. These conditions are growth in minimal medium supplemented with glucose and casamino acids (fast growth) and minimal medium with acetate and required amino acids (slow growth). Cells containing *lacZ* fusions to all the *dam* promoters (P1 through P5) or only *dam* promoter P2 (the sequence from -52 to +31, *dam* P2-83) displayed higher activity when grown in rich relative to poor medium, an indication of growth rate regulation. The *dam* P2 promoter *lacZ* fusion P2-50 (nucleotides -49 to +1) had lost this growth rate regulation. It is probable, therefore, that the determinant for growth rate regulation is located in part between nucleotides +1 and +31. The loss of growth rate regulation was accompanied by an increase in promoter activity suggesting that all or part of a repressor binding site had been deleted in the region of nucleotides +1 and +31 (Table 2). Growth rate regulation of the *dam* P2 promoter may, therefore, be mediated through increased repression at slow growth rates.

3.2 *The cde-4 (constitutive dam expression) mutation maps to the lipB gene*

An *E. coli* mutant (*cde-4*), isolated following miniTn10 mutagenesis, shows elevated *dam* gene expression at low growth rates [14]. The miniTn10 was mapped to the 15.4 min region of the chromosome by genetic methods and Southern hybridization and located in or near the *lipB* gene. In order to identify the exact position of the insertion, we cloned a 5.5 kb *Bgl*III fragment containing the miniTn10 into the low copy number vector pJEL109 [12]. Tetracycline resistant

transformants were selected. DNA sequencing indicated that the insertion was in the *lipB* gene and is predicted to alter the amino acid sequence at alanine 126 of the 213 amino acid protein (Figure 1). A *Tn1000* insertion in the same region (Figure 1) inactivated LipB activity [28]. Unlike the previously described *lipB182::Tn1000dKan* strain, the *cde-4::mTn10* mutant is not auxotrophic for lipoic acid [14].

3.3 Complementation of *cde-4* by the *lipB* gene

On the *E. coli* chromosome, the *lipB* gene is separated from *lipA* by at least one open reading frame with the same direction of transcription as *lipB* (National Center for Biotechnology Information (Online), 2000). The *cde-4::miniTn10* insertion in *lipB* could either affect only this gene, or have a polar effect on the expression of a downstream gene to generate the Cde phenotype. A complementation analysis was used to discriminate between these possibilities. Plasmids pKR94 (bearing *lipB* without a promoter), pKR109 (containing *lipA* expressed from the *tac* promoter) and pKR112 (encoding *lipB* expressed from the *tac* promoter) [28] were introduced into strains LJ391 (wildtype) and LJ507 (*cde-4*). These host strains contain a *dam-lacZ* transcriptional fusion. The data in Table 3 indicate that none of the three plasmids had a significant effect on *dam* gene transcription in the wildtype strain, but the growth rate was decreased upon overexpression of LipA or LipB proteins. This suggests that an increased level of lipoic acid or lipoic acid ligase was detrimental to cell growth. Plasmid pKR112, expressing the *lipB* gene, was able to complement the slow growth phenotype and high *dam* gene expression of the *cde-4* mutant strain, LJ507. Complementation occurred even in the absence of IPTG addition presumably due to the high basal expression level of the *tac* promoter (not shown). No

complementation was observed with pKR94, which is identical to pKR112 but lacks a promoter. β -Galactosidase activity measurements from cells in Table 3 are expressed as units per mg protein rather than the Miller units used in Table 2. This change in the enzyme activity units was necessary because the *cde-4* strain has a somewhat filamentous morphology resulting in a lower protein/OD ratio.

We conclude that the absence of LipB protein uncoupled *dam* gene expression from growth rate, and that this effect was not due to polarity on *lipA* or any other downstream gene.

3.4 Slow growth does not uncouple *dam* gene expression

The *cde-4* mutation causes cells to grow more slowly (Table 3; compare lines 1 and 4) and simultaneously increases the level of *dam* gene expression relative to the wildtype (Tables 2 and 3). The effect of LipB on *dam* expression could be indirect through alteration of growth rate. To explore this possibility, we measured *dam* gene expression in two slow growing strains bearing mutations in either the *cya* (adenylate cyclase) or *pgi* (phosphoglucose isomerase) genes. The results in Table 4 show that the *cya* mutant strain has the same level of *dam* gene expression as the wildtype at equivalent growth rates. Similar data were obtained with the *pgi* mutant strain (data not shown). The *cde-4* mutant, in contrast, shows a higher level of *dam* gene expression at the lower growth rate compared to wildtype (Table 2).

We conclude that it is not slow growth *per se* but some specific aspect of LipB activity which results in uncoupling growth rate control of *dam* gene expression.

3.5 The *lipB* gene

The *lipB* open reading frame contains two putative ATG start codons that would give rise to proteins with a calculated MW of 21,309 and 16,997 Daltons (Figs. 1, 2). The coding sequence with the upstream ATG is displayed in the *E. coli* genome database (National Center for Biotechnology Information (Online), 2000). Amino terminal hexa-histidine tagged versions of these two putative LipB proteins were constructed using the pET system [26]. The plasmid construct pMQ409, which uses the downstream ATG codon, did not produce a protein of the expected size, indicating low stability of the fusion protein. Plasmid pMQ408, utilizing the upstream ATG codon, produced a protein present at low concentration and subsequent experiments showed it to be very unstable (data not shown). Neither of these plasmids complemented the poor growth of a *lipB* mutant strain in minimal medium (Fig. 2).

Since neither of the putative ATG-codon ORFs appeared to produce a stable functional protein, an alternative possibility was that the gene did not have an ATG initiation codon but some other triplet. We decided to test a *lipB* sequence with TTG start codon because it had a reasonable ribosome binding site sequence preceding it (Fig. 1). The resultant construct, pMQ404, has an ATG in place of the original TTG due to the method of construction, as well as an aminoterminal histidine tag. This plasmid produced a stable protein of the predicted size and was able to complement the poor growth of the *cde-4* and *lipB182::Tn1000dKan* (data not shown) mutants in minimal medium. It is probable, therefore, that translation of the *lipB* gene *in vivo* starts with the rare UUG initiation codon.

Complementation of the *cde-4* and *lipB* mutations is observed using the pET18b derivative, pMQ404, carrying the *lipB* gene in the absence of T7 RNA polymerase (Fig. 2). This is due to the low basal level of transcription from the T7 promoter, which is sufficient to complement the normally low level of LipB in the cell (see "The level of LipB in cells" below). We have found that chromosomal *mutS* and *mutL* mutations are complemented under similar conditions by pET vectors bearing these genes in the absence of T7 RNA polymerase [29-31]. The MutS and MutL proteins are present at about 150 molecules per cell [32].

3.6 Purification of histidine-tagged LipB protein

Since plasmid pMQ404, encoding an N-terminal histidine-tagged LipB, was able to complement *cde-4* and *lipB* mutants for growth, the histidine tag cannot appreciably affect the *in vivo* activity of the protein. The purification of LipB, as monitored by SDS-PAGE and Coomassie Brilliant Blue staining, using nickel immobilized affinity chromatography is shown in Figure 3. Induction of transcription produced an intense staining band at the predicted MW of the protein (compare Fig. 3, lanes b and c). The induced protein was partitioned into soluble and insoluble fractions (Fig. 3, lanes d and e). The soluble fraction was further purified over a nickel-agarose column, resulting in 90-95 % pure LipB protein (Fig. 3, lane f). The MW of purified LipB in Figure 3 agrees with the predicted size of 213 amino acids (23,882 Daltons) based on the DNA sequence plus the additional amino acids derived from the histidine-tagged region of pET15b (2094 Daltons).

The purified LipB protein was used to generate polyclonal antibodies in rabbits. The antiserum was used to detect LipB in cells with and without various plasmid constructs (Fig. 4). Plasmid pMQ405, containing the *ybeD-lipB* region directed the synthesis of a protein migrating at 24 kDa, the expected size of the LipB protein utilizing a UUG translation initiation codon (Fig. 4, lane a). A frameshift mutation in the *lipB* coding sequence of this plasmid results in loss of the 24 kDa band (Fig. 4, lane b). The LipB protein produced from a plasmid in which the putative TTG start codon is changed to ATG (pMQ410) migrates with the same MW (Fig. 4, lane c) and no band is detected in the vector only cell extract (Fig. 4, lane d). A 24 kDa protein was detected (Fig. 4, lane e) after thrombin cleavage of the histidine-tagged protein produced by pMQ404 (Fig. 4, lane f). No LipB was detected by immunoblotting in cells with either the pET15b vector alone or pMQ409, which encodes the *lipB* gene starting with the downstream ATG codon (data not shown). When the upstream ATG codon was used as translational start codon (pMQ408), a low level of truncated LipB protein was synthesized (data not shown).

Overexpression of LipB following induction from the various expression plasmids caused markedly decreased growth rates (data not shown) suggesting that excessive amounts of LipB are deleterious to cell growth. We also noticed a difference between the K-12 and B strains of *E. coli* to form stable transformants with pMQ404(pET15b *lipB*). Strain BL21(λ DE3) could not be transformed with pMQ404 but the K-12 strain GM5861 (a λ DE3 lysogen) could. Stable pMQ404 transformants of BL21(λ DE3) could be obtained if the pLysE (lysozyme), but not pLysS, plasmid was present (data not shown). This suggests that even low level transcription of plasmid-borne *lipB* leads to cell death in the B strain.

3.7 The level of LipB in cells

The level of LipB protein in a culture of wildtype *E. coli* cells was found to be near the detection limit of the antibody (Fig. 5A). No LipB protein could be detected in a saturated overnight culture nor when it was concentrated 2.5 and 5 times (Fig. 5A, lanes 1, 2 and 3). LipB was barely detectable in wildtype *E. coli* cells from saturated overnight cultures concentrated 25-fold (Fig. 5A, lane 4), where a faint band appeared to migrate at a position similar to that of the LipB protein synthesized from plasmid pMQ405 (Fig. 5A, lane 6). This band was not detectable in cells with plasmid pMQ407 where the *lipB* gene is interrupted by a kanamycin resistance cassette (Fig. 5A, lane 5). The very low concentration of LipB in either stationary or logarithmic (data not shown) phase cells is consistent with the proposed UUG start codon that is expected to have a low efficiency of translation initiation compared to AUG.

When wildtype cells containing the *ybeD-lipB* encoding plasmid pMQ405 were grown exponentially or in early stationary phase, the presence of LipB was readily detected (Fig. 5B, lanes 1 and 2 respectively). Late stationary phase cells, however, had reduced LipB levels suggesting a diminution during lag phase (Fig. 5B, lanes 3, 4, 5).

There is a higher level of LipB in pMQ405-containing cells compared to those with plasmids pMQ404 and pMQ410. Because these plasmids differ only by the amount of DNA upstream of the *lipB* gene, this suggests that the *ybeD* region upstream of *lipB* in pMQ405 may contain an element that enhances *lipB* expression.

4. Discussion

The *E. coli dam* gene is growth rate regulated at the level of transcription [14;15]. It was suggested that this regulation adjusts the level of Dam methylase to match the synthesis rate of DNA. This would result in the persistence of hemimethylated DNA near the replication fork to ensure that a number of cellular processes, which are coupled to the cell cycle, remain functional over a wide range of growth rates. These processes include DNA mismatch repair and initiation of chromosome replication.

In *E. coli*, growth rate regulation of transcription initiation has been most extensively studied in ribosomal RNA (*rrn*) promoters. It was shown recently that the cellular concentrations of ATP and GTP are important factors for growth rate control of RNA (*rrn* P1) promoters [33]. All but one of the *rrn* P1 promoters initiate transcription with ATP (the remaining one with GTP). High purine nucleotide levels were proposed to stabilize an unusually short-lived complex formed by RNA polymerase at these promoters. Because the level of purine nucleotide increases with growth rate, the activity of the *rrn* P1 promoters does as well.

Although the *dam* P2 and *rrn* P1 promoters behave similarly with respect to growth rate, it is unlikely that the same mechanism operates at *dam* P2. The initiating nucleotide is UTP [12] and initiation at adenines located at +4 and -2 or guanines at +3 and -1 has not been observed.

Furthermore, the distance from the -10 hexamer to the transcription start is 7 nucleotides for the *dam* P2 promoter, compared to 9 for all the *rrn* P1 promoters. The latter was suggested as being at least in part responsible for the short half-life of open complexes formed at *rrn* P1 promoters.

The data in table 2 indicate that a growth rate-dependence determinant of expression of the *dam* P2 promoter is located between +1 and +32. Examination of the nucleotide sequence surrounding the promoter, however, reveals three sequence motifs of TTCAGT(N21)TGAG (the G-box) (Fig. 6). The core nucleotides (in bold) are on the same face of the DNA helix separated by two turns. Deletion analysis of the promoter suggests that G-boxes I and II are sufficient for growth rate regulation of the P2 promoter, albeit somewhat reduced compared to the intact region (Fig 6, Table 2). The minimal promoter (-49 to +1) containing only G-box I was no longer regulated by growth rate (Fig. 6, Table 2). At present, we do not know the significance of this motif but it could represent a recognition sequence for a DNA binding element that might be protein or RNA. If so, it could compete with RNA polymerase for binding at the *dam* P2 promoter. Because growth rate regulation of *dam* P2 is abolished either by deleting sequence motifs II and III or by mutating the *lipB* gene, the LipB protein becomes an obvious candidate for such a DNA binding protein. There is a striking resemblance between the DNA binding region of uracil DNA glycosylase [34] and a sequence in LipB (residues 66-92) suggesting that binding to DNA is a possibility.

Other regions of LipB have sequence homology to LipA (converts octanoic acid to lipoic acid) and LplA (lipoic acid protein ligase) raising the possibility that LipB is itself a lipoic acid protein ligase or functions intimately with another such protein to promote ligase activity. The observation of decreased lipoic acid ligase activity in *lipB* mutants is consistent with this hypothesis, but we have not yet assayed purified LipB protein for such activity. Two such assays have recently become available [35;36]. The dramatic sequence conservation of the protein in plants and animals (see below) is also consistent with a ligase activity.

The level of LipB is higher in exponential growing cells containing plasmid pMQ405 relative to the same cells entering stationary phase (Fig. 5C) suggesting gene expression may be growth-phase related or growth rate-dependent. If the latter, then the levels of Dam and LipB are correlated directly but this is inconsistent with the action of LipB as a repressor at *dam* P2. For this model to be plausible, LipB would have to act at the G-boxes at *dam* P2 as a repressor and as an activator at the G-boxes in and near *lipB*. In this context it is interesting to note that, in addition to the *dam* P2 region, the only location identified on the *E. coli* genome where three G-boxes are found in close proximity is the *lipB* gene. Here, one G-box is in the coding region of *ybeD* and two are in the coding region of *lipB* (data not shown). It is tempting to speculate that the G-box motifs are bound by a hitherto unidentified repressor protein and that the action of LipB on *dam* gene expression is indirect. Lipoylation of this putative repressor by LipB might render it inactive, explaining the derepression of the *dam* P2 promoter at high growth rates when the level of LipB protein is high.

A computer-aided homology search of translated nucleotide and peptide sequence databases was performed with the deduced amino acid sequence of the *lipB* gene [27]. LipB homologs were found in *Haemophilus influenzae* (Hin, 64% identity, 203 amino acids (aa)), *Mycobacterium tuberculosis* (Mtu, 34%, 176 aa), *Mycobacterium leprae* (34%, 181 aa), *Synechocystis* (28%, 191 aa), *Thiobacillus versutus* (Tve, 41%, 116 aa), a member of the Archaea and *Prochlorothrix hollandica* (30%, 111 aa), a cyanobacter. The sequence is also present in the yeasts *Saccharomyces cerevisiae* (Sce, 35%, 152 aa), *Schizosaccharomyces pombe* (40%, 133 aa), and *Kluyveromyces lactis* (31%, 160 aa) and the red alga *Cyanidium caldarium* (29%, 133 aa). Both *Arabidopsis thaliana* (32%, 151 aa) and *Caenorhabditis elegans* have LipB homologs but *Bacillus subtilis* does not. Six motifs of homology are apparent in the peptide sequence (Figure 7A) and these are shown as rectangles in the overall protein sequence (Figure 7B). The sequence of motifs is maintained in the homologs. The sequence conservation suggests an important physiological role for LipB.

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FIGURE LEGENDS

Fig. 1. DNA sequence of the *lipB* gene. The coding sequence of the gene starting with a TTG codon is shown. The sequences in bold preceding the coding sequence indicate possible -10 and ribosome binding sites. The "upstream" and "downstream" ATG codons referred to in the text are located at bolded nucleotides 67-69 and 178-180 respectively.

Fig. 2. The LipB translational start codon. The chromosomal DNA fragments in each plasmid are shown beneath a schematic representation of the *ybeD-lipB* region of the chromosome. The ability to complement strain LJ502 (*cde-4*) for growth in glucose minimal medium is also indicated. The cross in the pMQ406 plasmid insert indicates a frameshift mutation.

Fig. 3. Purification of hexahistidine-tagged LipB. Cells containing pMQ404 (lane b, uninduced) were harvested 3 hr after induction (lane c, induced) lysed, (lane d, supernatant, lane e, pellet) and the extract clarified by centrifugation. The supernatant was applied to a nickel-agarose column, which was then washed with buffer and LipB protein eluted with 500 mM imidazole (lane f). Molecular weight standards are shown in lane a.

Fig. 4. Immunoblotting of LipB in cell extracts. Cell extracts were prepared from strains bearing various plasmids and assayed for the presence of LipB by immunoblotting as described in Materials and Methods. Lane a, pMQ405(*ybeD-lipB*)/GM5861 extract, lane b pMQ406(*ybeD-lipB* frameshift)/GM5861 extract, lane c, pMQ410(pET3a-*lipB*)/GM5861 extract, lane d, pET3a/GM5861 extract, lane e, hexahistidine-tagged LipB pMQ404/GM5861 extract cleaved with thrombin, lane f, hexahistidine-tagged LipB pMQ404/GM5861 extract. Arrows indicate the

position of the uncleaved (left) and thrombin cleaved (right) LipB standards. The upper band represents non-specific cross-reacting material.

Fig 5. Detection of LipB in cell extracts. Cell extracts were prepared from strains with or without various plasmids and assayed for the presence of LipB by immunoblotting as described in Materials and Methods. A. Lane 1, pMQ405(*ybeD -lipB*)/GM5861 extract, lane 2, pMQ407(*ybeD -lipB::Kan*)/GM5861 extract, lane 3, wildtype (CC106) extract, 25-fold concentrated, lane 4, wildtype extract, 5-fold concentrated, lane 5, wildtype extract, 2.5-fold concentrated, lane 6, wildtype extract, not concentrated. The wildtype cells do not contain plasmids. The upper band represents non-specific cross-reacting material. The arrow indicates the position of TTG-LipB. B. Wildtype cells (CC106) bearing pMQ405 (*ybeD -lipB*) sampled at a Klett reading of 70 (lane 1), and 200 (lane 2), after overnight growth (lane 3) and diluted to a Klett reading of 70 (lane 4) and 200 (lane 5).

Fig. 6. The G-boxes in the *dam* P2 promoter. The DNA sequence of the *dam* P2 promoter region was obtained from Løbner-Olesen et al. [12]. The -35 and -10 elements of the promoter are underlined and the italicized transcriptional start (T) is indicated by +1. The location of the three G-boxes is shown above the sequence, and matching bases are indicated in bold. The sequences of two deletion derivatives *dam* P2(-52 to +31) and *dam* P2(-49 to +1) are also shown.

Fig. 7. LipB homologs in various organisms. A. Conserved motifs in the LipB homologs of *Escherichia coli* (Eco), *Haemphilus influenzae* (Hin), *Mycobacterium tuberculosis* (Mtu), *Saccharomyces cerevisiae* (Sce) and *Arabidopsis thaliana* (Ata). White letters on a black

background indicate identical amino acids in the aligned proteins. Shaded letters indicate conserved amino acids. B. Distribution of motifs in the LipB homologs of various organisms. The distribution of each of the six motifs in the protein is indicated. Numbering is from left to right in each case.

Table 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source/Reference
<i>E. coli</i> K-12 strains		
BL21(λ DE3)	<i>ompT gal hsdS_B dcm</i>	[26]
LJ24	<i>supE44 rpsL rfbD1 thi-1 lacY1 tonA21 leuB6 $\Delta(lacIZ)$</i>	[14]
LJ391	As LJ24 but <i>attλ (dam P1-5::lacZ)</i>	[28]
LJ484	As LJ24 but Δ <i>cya</i>	This work
LJ502	As LJ24 but <i>cde-4</i>	[14]
LJ507	As LJ391 but <i>cde-4</i>	This work
CC106	<i>ara thi $\Delta(gpt-lac)5/ F-lacI^q lacZ proAB+$</i>	[37]
GM5861	As CC106 but (λ DE3)	This work
KER184	<i>rpsL lipB182::Tn1000dKan</i>	[19]
MM294	<i>endA1 hsdR17 supE44 thi-1</i>	M. Messelson
UF800	As LJ24 but <i>attλ (dam P2-50::lacZ)</i>	This work
Plasmids		
pCTV625	Contains <i>lip</i> genes on a 5.2 kbp <i>PvuII</i> fragment	[16]

pET3a	Cloning vector	Novagen Inc.
pET15b	Cloning vector	Novagen Inc.
pKR112	<i>lipB</i> under <i>tac</i> promoter	[19]
pMQ404	pET15b plus <i>lipB</i> ORF	This work
pMQ405	pET15b plus <i>ybeD-lipB</i> ORFs	This work
pMQ406	As pMQ405 but with a frameshift mutation in <i>lipB</i> (<i>PpuI01</i> site filled in)	This work
pMQ407	As pMQ405 but with a 1.1 kb kanamycin resistance fragment in the <i>PpuI01</i> site	This work
pMQ408	pET15b with upstream ATG <i>lipB</i> ORF	This work
pMQ409	pET15b with downstream ATG <i>lipB</i> ORF	This work
pMQ410	pET3a with <i>lipB</i> ORF (TTG to ATG)	This work
pMQ411	As pMQ410 but <i>lipB::Kan</i>	This work
pRS415	<i>lacZYA</i> promoter cloning vector	[20]

Table 2. Activity of the *dam* P2 promoter

Promoter fragment ^a	β-Galactosidase activity	Degree of regulation ^c
	Fast/slow ^b	
P1-5 ^d	99/40	2.5
P2-83 (-52 to +31)	133/65	2.0
P2-50 (-49 to +1)	343/381	0.9

^a P1-5 indicates that all five *dam* promoters are present on the fragment. P2-83 and P2-50 indicate that only the *dam* P2 promoter is present on fragments of 83 and 50 bp respectively. Coordinates in parentheses are relative to the transcriptional start point of promoter P2 [12]. For sequences see Figure 6.

^b Fast growth of cell is in minimal medium supplemented with glucose and casamino acids whereas slow growth is in minimal medium supplemented with acetate and required amino acids as described in Materials and Methods. β-Galactosidase activity is expressed in units according to Miller [25].

^c Degree of regulation is defined as the activity at the fast growth rate divided by the activity at the slow growth rate.

^d The β-galactosidase specific activities of LJ391 (*dam* P1-5) were obtained from Rasmussen et al., [14].

Table 3. Complementation of the *cde-4* mutation by *lipB* plasmids.

Host Strain ^a	Plasmid	β -galactosidase activity ^b	Growth rate ^c
LJ391	pKR94	1762	2.0
LJ391	pKR109 (<i>lipA</i>)	1595	1.5
LJ391	pKR112 (<i>lipB</i>)	1792	1.3
LJ507	pKR94	3030	0.5
LJ507	pKR109 (<i>lipA</i>)	2694	0.5
LJ507	pKR112 (<i>lipB</i>)	1592	2.0

^a Strain LJ391 is LJ24 containing a *dam-lacZYA* transcriptional fusion in the λ attachment site of the chromosome. Strain LJ507 is LJ391 containing the *cde-4* mutation (for details see Materials and methods).

^b Specific activity is expressed in units per mg protein in crude extracts. Values have not been corrected for gene dosage. Cells were grown in minimal medium supplemented with glucose, casamino acids, 100 μ g/ml of ampicillin and 1 mM IPTG (for details see Materials and methods).

^c The growth rate of the culture is given as doublings per hour at 37° C.

Table 4. *Dam* gene expression at different growth rates

Strain	Carbon source	Growth rate	β -Galactosidase activity
LJ391	Glucose + casamino acids	2.0	93
LJ391	Glucose	1.0	49
LJ391	Glycerol	0.4	19
LJ484	Glucose + casamino acids	1.0	51
LJ484	Glucose	0.5	27

Strain LJ484 is a *cya* deletion derivative of strain LJ391. β -Galactosidase activity is expressed in units according to Miller [25]. Other details were as given in the legend to Table 3.

tgtccgcatggttctgtaactcgcttctccggttaccgggctcctggtcgggtaactcccc
tctcctcgctgtgat**tatac**ttttccccccacttttactcattctccac**ggag**atgcccgtt

1 ttgtatcaggataaaaattccttggtccgccagctcgggtcttcagccttacgagccaatctcc
M Y Q D K I L V R Q L G L Q P Y E P I S 20
61 caggct**atg**catgaattcaccgatacccgcgatgatagtagtacccttgatgaaatctggctg
Q A M H E F T D T R D D S T L D E I W L 40
121 gtcgagcactatccggtattcacccaagggtcaggcaggaaaagcggagcacatttt**atg**
V E H Y P V F T Q G Q A G K A E H I L M 60
181 ccgggtgatattccggtgatccagagcgatcgcggtgggcagggtgacttatcacgggccg
P G D I P V I Q S D R G G Q V T Y H G P 80
241 gggcaacagggtgatgtatgtgttgcttaacctgaaacgccgtaaactcgggtgtgcgtgaa
G Q Q V M Y V L L N L K R R K L G V R E 100
→ Tn1000
301 ctggtgaccttgcttgagcaaacagtggtgaataccctgggtgaactgggtata|gaagcg
L V T L L E Q T V V N T L A E L G I E A 120
→ mTn10
361 catcctcgggctgacg|cgccagggtgtctatggtggggaaaagaaaatttgctcactgggt
H P R A D A P G V Y V G E K K I C S L G 140
421 ttacgtattcgcgcggttggttcattccacggctctggcattaaacgtcaatatggatctt
L R I R R G C S F H G L A L N V N M D L 160
481 tcaccatttttacgtattaatccttggtgggtatgccggaatggaaatggctaaaatatca
S P F L R I N P C G Y A G M E M A K I S 180
541 caatggaaaccggaagcgacgactaataatattgctccacgtttactggaaaatatttta
Q W K P E A T T N N I A P R L L E N I L 200
601 gcgctactaaacaatccggacttcgaatatattaccgcttaa
A L L N N P D F E Y I T A *

Fig. 2

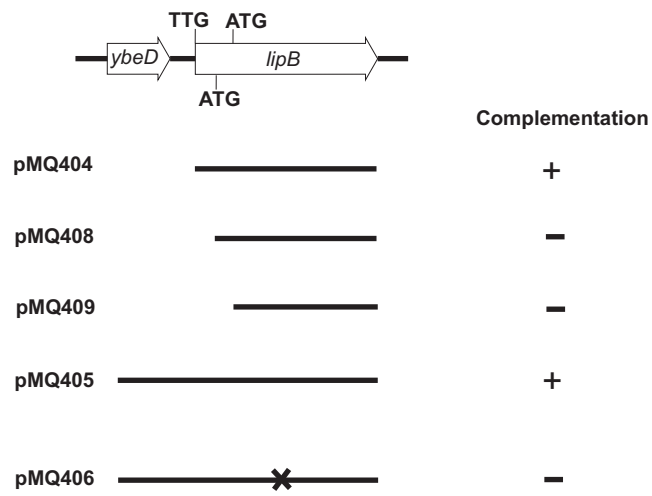


Fig. 3

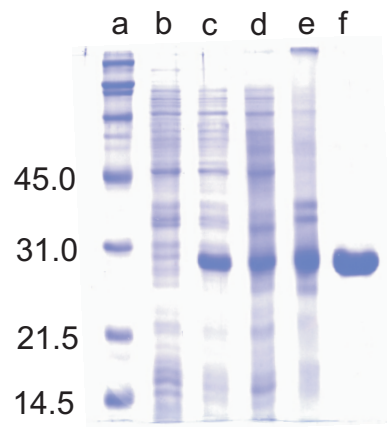


Fig. 4

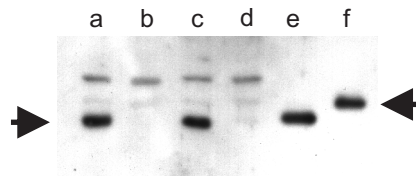
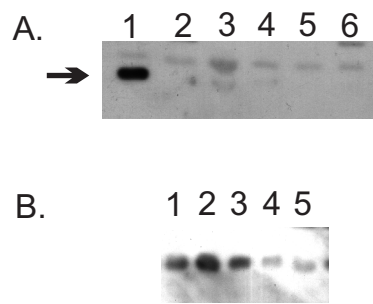


Fig. 5



I

II

III

TTCAGT-----N(20)-----TGAG
|-----|

TTCAGT-----N(20)-----TGAG
|-----|

TTCAGT-----N(20)-----TGAG
|-----|

+1

TGGGTGATTTATTCAGTTGCCAAACCCGCTGGAGTATTGAGATAATTTCAGTCTGACTCTCGCAATATCTTATGAGGTTTCAGTTCATGTCCTGCGGCGCTCTCTGAGCGAAG

GGGTGATTTATTCAGTTGCCAAACCCGCTGGAGTATTGAGATAATTTCAGTCTGACTCTCGCAATATCTTATGAGGTTTCAG

P2-83

GGGTGATTTATTCAGTTGCCAAACCCGCTGGAGTATTGAGATAATTTCAGT

-35

-10

P2-50

A.

		Motif I		Motif II	
Eco	5	KILV R QLGLQFY E	36	DEIWL V EHYPV E T Q QAG K A E	
Hin	4	SLI V RQLGLQDY Q	35	DEIWL V QHYPV E T Q QAG K P E	
Mtu	20	AID V RQLGTVDY R	52	DTLL L LEHPAV Y AGRR T ETH	
Sce	2	SRC I R Q SVCTN F N	111	PIIL T FEFE P TY T G K R I K K T	
Ata	104	TLE V W K L G TVN Y L	126	DTLL S LQ H PE P TY T L G KRR T D H	
		Motif III		Motif IV	
Eco	62	GDIP V I Q SD R GG Q V T Y H GP G Q V MY L LN L K	97	GV R EL V T L LE Q TVV N T L AE L GI	
Hin	61	SEIP V V Q SV R GG Q I T Y H AP G Q V MY V LID I K	97	N V R Q L V T A LE Q TVV K T L AE Y GI	
Mtu	76	DG T PV V DT D R G G K IT W H G PG Q LV G Y P I I GL A	115	Y V R R LE E SL I Q V CAD L GL H AG R	
Sce	135	PR P K F V Q VER G G Q V T Y H GP G Q V I V I I LD L K	189	PA K CL V SC I EQ A T I RT L K N T K M	
Ata	158	IGAEL H Y T Q R GG D IT E H G PH O A I LY P I I SL R	203	G A R N Y V ET L ERS M IE F AS I Y G V	
		Motif V		Motif VI	
Eco	125	AP G V Y V G	132	KK I C S T L GL R I R R G CS F H G L A LN V N M DL S P F LR I N P CG Y AG M EM A K I S Q W	
Hin	126	AP G V Y V D	133	KK I C S T L GL R I R R G CS F H G L A LN I N M DL N P F H Y I N PCG Y AG L EM C Q L AD F	
Mtu	140	RS G V W LP	151	R K V A A I G V R V SR A T T L H G F AL N C D C D L A A F T A I V P CG I S DA A V T S L SA E	
Sce	227	DT G V W VE	237	KK V AS V G I H V RR S IT S H G V A IN V NT D L S Y M NS F EM C GL K NT L T T S I ME Q	
Ata	230	ET G V W V G	240	R K I G A I G V R I SS G IT S H G L A LN I D P DM K Y F E H I V P CG I A D K E V T S L R R E	

B.

