Rapid Paper

Acidic-phospholipid Deficiency Represses the Flagellar Master Operon through a Novel Regulatory Region in *Escherichia coli*

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The *Escherichia coli* pgsA3 mutation, which causes acidic-phospholipid deficiency, was found to repress the flagellar master operon, as assessed by the β-galactosidase activities of *flhD-lacZ* fusions. This explained the impairment of flagellar formation and motility by the mutation. A series of deletion analysis indicated that a 40-bp region, at the 5′ end of the *flhD* locus examined, was responsible for the repression of a downstream transcription initiation that was catabolite-repression sensitive. This novel regulatory region was 200 bp upstream of the first possible translation initiation site.

To understand the regulatory and functional aspects of membrane phospholipids, molecular genetic approaches to manipulate their biosynthesis are proving especially useful. The *Escherichia coli* pgsA3 allele encoding a defective phosphatidylglycerophosphate (PGP) synthase†2 causes severe acidic-phospholipid deficiency and thus has been used in various areas of biochemistry to examine the possible biological roles of phosphatidylglycerol and cardiolipin (for a review, see ref. 3). We observed that pgsA3 mutants were nonmotile and severely impaired in flagellar formation, whereas a null *cls* mutant lacking cardiolipin synthase6 was normal in these respects.9 Tomura and coworkers also reported similar observations.9

In flagellar formation and chemotaxis in *E. coli*, more than forty genes are involved, being organized into a regulatory hierarchy: expression of the master operon, *flhD-flhC* (formerly *flbB-flaI*), is essential for the function of all other genes (for a review, see ref. 7). Various growth conditions adverse to *E. coli* cells,8 defects in heat shock proteins,9 and mutations defective in phosphatidyethanolamine synthesis (*ppsA* and *psd* mutations)10 have been reported to cause flagellar deficiency by repressing the master operon, although the molecular mechanisms underlying these phenomena have been totally unknown. Catabolite repression is the only regulation in flagellar formation, the mechanism of which is fairly well understood in biochemical terms.7 The primary structure of the master operon has been reported11 but no particular sequences related to these regulations, except for a possible cAMP receptor protein (CRP)-cAMP binding site, have been identified.

Here we describe observations that acidic-phospholipid deficiency caused by the pgsA3 mutation severely represses the flagellar master operon and this repression requires a sequence upstream from the *flhD* coding region.

Materials and Methods

Bacterial strains and culture conditions. *E. coli* K-12 strains W3110, JL5513 (*ppgA2 lacZ::Tn5*), and YL5513 (pgsA3 JL5513) were described previously.9 A chromosomal *flhD-lacZ* operon fusion strain YK4332 [(*flhD::Mu d(Amp' lac)*)]12 and a *ΔacrA* mutant XL1-Blue13 were also described. The *ppgA2* and *pgsA3* alleles were introduced by P1 phage transduction15 by using *aroD::Tn5* and *wcrC279::Tn10* as linked selectable markers, respectively, into strain YK4332 to construct strains FLD1 and FLD2. The *ppgA2* was used to suppress the lethal nature of *pgsA3*.16 Cells were grown in LB medium17 or in a tryptone broth18 with appropriate antibiotics.

Plasmids. Plasmid pKY11 was constructed from pBR322 to contain a chloramphenicol resistance gene and a genomic fragment covering the functional *pgsA* gene (nucleotides 1 to 681 of Fig. 1 of ref. 2). Plasmids pFM1 and pFM7 were constructed by inserting DNA fragments of 353 and 236 bp, respectively, prepared by PCR amplification of the upstream regions of *flhD* of strain W3110, into a *lacZ* operon fusion vector pRS55117 (for structures of the products and PCR primers, see Fig. 1). To generate deletions, pFM1 was digested with EcoRI, differentially shortened with exonuclease III, treated with S1 nuclease and the Klenow fragment, and an EcoRI linker was ligated to the ends. They were digested with EcoRI and SalI. Fragments of approximately 6.5 kb, thus obtained, were ligated with the 6.2-kb EcoRI-SalI fragment of pRS551 to form a series of deletion plasmids, pFM2 through pFM6 (see Fig. 1). The 353-bp PCR product was also digested with *SalI* and EcoRI and ligated with the pRS551 that was previously treated with *BamHI* and the Klenow fragment and then with EcoRI. This product was religated to form pFM11. The primary structures of all newly constructed plasmids were analyzed by sequencing the junction regions.

Methods. Recombinant DNA techniques and DNA sequencing were conducted essentially as described previously21 except that the operon fusion plasmids were directly analyzed to identify the structures of the junction regions by using a fluorescent M13 universal primer that complemented the sequence corresponding to codons 8 to 15 of *lacZ* (Pharmacia). PGP synthase was assayed in sonically ruptured cells as described.19 β-Galactosidase activity was measured by the method of Tesfa-Selase and Drabell.18 Phospholipid composition and contents were measured as previously described.30

Results

The pgsA3 mutation represses the flagellar master operon

The β-galactosidase activities in chromosomal *flhD-lacZ* operon fusions were measured (Table). A pgsA3 derivative, FLD2, was deficient in PGP synthase, resulting in acidic-phospholipid deficiency, and the expression of the flagellar master operon was severely inhibited. Introduction of a plasmid harboring a minimum length of the functional *pgsA* gene (pKY11) fully restored both the β-galactosidase

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Abbreviations: CRP, cAMP receptor protein; PGP, phosphatidylglycerophosphate.
activity and the contents of two acidic phospholipids. Although pgsA and flhD are very close to each other on the E. coli chromosome, the restoration by the plasmid indicated that the defective transcription was caused by acidic-phospholipid deficiency. Since the flagellar master operon was disrupted in these strains by phage Mu and thus the e^r subunit, the fiIA gene product, was defective, the r^10 RNA polymerase was most probably responsible for the transcriptional expression of the flhD described in the Table.

An upstream region of the flhD gene is required for the repression by pgsA3

To identify the region in the flhD locus that is responsible for the repression by pgsA3, we constructed a series of plasmids in which flhD upstream regions of various lengths were fused to lacZ (for structures, see Fig. 1). During the plasmid construction, we repeatedly and carefully sequenced the regions and found that our sequence data differed in three points from that previously reported by Bartlett and coworkers as indicated in Fig. 1. The C of position 38 and the A of position 242 were missing and the C of position 210 was G in the previous work. Although the reasons for these discrepancies remain to be clarified, we believe that our sequence is correct at least for strain W3110. The previous work analyzed a DNA fragment that was derived from strain CSS20.

The \( \beta \)-galactosidase activities of strains JL5513 and JL5513 with Glc both harboring a series of deletion plasmids were measured for cells cultured in the absence and presence of 100 \( \mu \)M d-glucose (Fig. 2). In this experiment,
we used a tryptone broth at 35°C, instead of LB medium used for the temperature-sensitive fusion strains at 30°C (Table), since the catabolite repression by α-glucose was observed more clearly in this medium in which $pgsA3$ mutants were difficult to grow at 30°C. It should be noted, however, that the repression by $pgsA3$ was dependent on the culture temperature; the β-galactosidase activities of $JL5513/pFM1$ and $YL5513/pFM1$ in LB medium at 30°C were 160 ± 8 and 43 ± 3 nanomoles per min per ml culture at 100 Klett units, respectively, but those at 35°C were 171 ± 7 and 72 ± 3, respectively. Figure 2 shows that the $pgsA3$ repression requires a region, nucleotides 20 to 60, whereas catabolite repression was observed to almost the same extent in the entire region examined. The results suggest that the two types of repression are independent and the region responsible for catabolite repression resides downstream from the above-examined region.

In the new version of the nucleotide sequence is present a hitherto unrecognized σ70-type promoter sequence in this region: sequences A and B of Fig. 1 corresponded to the −35 and −10 regions, respectively, giving rise to a homology score of 51.5. However, this sequence alone was found not to be functional as a transcriptional promoter; strain XL1-Blue harboring a lacZ operon fusion plasmid, pFM11, did not have an appreciable β-galactosidase activity (specific activity was 14 above the value 3 of the vector pRS551). Therefore, the 40-bp sequence responsible for the $pgsA3$ repression that was farthest upstream in the fragment examined appears to be, or at least to be a part of, a regulatory sequence conferring a positive effect on the expression of the flagellar master operon.

**Discussion**

This study has shown that the transcriptional expression of the flagellar master operon is severely repressed by the $pgsA3$ mutation, well explaining the defects in flagellar formation and motility previously observed with $pgsA3$ mutants. We have also shown that this repression is dependent on an upstream 40-bp sequence of the $flhD$ locus that does not function as a promoter. Instead, the sequence appears to have a novel regulatory role in the expression of the operon by enhancing the downstream, catabolite-repression sensitive transcription initiation. Bartlett and coworkers indicated that nucleotides 228 to 249 (Fig. 1) is partially homologous to the consensus CRP-cAMP binding sequence. Whether this particular sequence represents the entire regulatory region or only a part of it remains to be seen. The difference in the extents of repression by $pgsA3$ between with the single copy operon fusions (about 7-fold; Table) and with fusion plasmids (about 4-fold) under the same growth conditions suggests that a more upstream region beyond that contained in plasmid pFM1 may be necessary for the full repression by $pgsA3$. Alternatively, a trans-acting factor responsible for this regulation might have been titrated out with the multicopy fusion plasmids.

The biological significance of the repression of the flagellar synthesis by acidic-phospholipid deficiency is not clear. The observed temperature dependency of the repression may give a clue to this question: the phase transition temperature of phosphatidyglycerol is some 30°C lower than those of phosphatidylethanolamine and cardiolipin of the same fatty acid composition and the membrane fluidity of a $pgsA3$ mutant at lower temperatures might therefore be too low to allow proper function. Since the biosynthesis of a flagellum is highly costly, it should be advantageous to regulate its synthesis according to need.73 The flagellar master operon thus appears to possess characteristic features responsible for such regulations. Abnormal phospholipid compositions, causing variously defective membrane functions, may generate an important signal to the flagellar master operon. Work to understand the molecular mechanism underlying these phenomena is in progress in our laboratory.

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