JOURNAL OF BACTERIOLOGY, July 1987, p. 2913–2916
0021-9193/87/072913-04$02.00/0

Vol. 169, No. 7

Cloning and Nucleotide Sequence of *phoP*, the Regulatory Gene for Alkaline Phosphatase and Phosphodiesterase in *Bacillus subtilis*

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Received 10 December 1986/Accepted 23 March 1987

Two DNA fragments which complement the alkaline phosphatase-negative mutation *phoP* of *Bacillus subtilis* were cloned from a *B. subtilis* chromosome with the prophage vector φCM (a derivative of φ105). One of the fragments contained the regulatory gene *phoR* in addition to *phoP*. Nucleotide sequence analysis of the *phoP* region revealed that the *phoP* gene product consists of 241-amino-acid residues and that the sequence of these amino acids is extensively homologous with the sequence of the *phoB* gene product. This protein is the positive regulator for the phosphate regulon in *Escherichia coli*. It therefore appears that *phoP* is a regulatory gene for alkaline phosphatase synthesis in *B. subtilis*.

During vegetative growth under low-phosphate conditions, *Bacillus subtilis* produces an alkaline phosphatase (APase). APase-less mutants have been isolated by several investigators and mapped at the same *phoP* locus (9, 12, 16, 18), but it remained uncertain whether *phoP* is the structural gene for APase or the regulatory gene for its production (7–9, 12, 25).

In *Escherichia coli* a number of genes, including *phoA*, *phoS*, *phoE*, and *ugpAB*, are expressed when the cell is starved for P_i. The expressions are controlled by the *phoB* gene product, which is the positive regulatory factor for the phosphate regulon (23). The amino acid sequences of APase (*phoA*) and the positive regulator (*phoB*) were determined on the basis of DNA sequences (1, 13). Thus the comparison of their base sequences with that of *B. subtilis phoP* should help us to understand the function of *phoP*. In this paper we report the cloning of *phoP* by the improved prophage transformation system (11, 21) and the determination of the DNA sequence of the gene. The function of the *phoP* gene product is discussed on the basis of the DNA sequence.

MATERIALS AND METHODS

***B. subtilis* strains and bacteriophages.** Strain UOT-0278 (*hisA1 metB51*) was used as the *phoP*$^+$ donor. The *phoP* and *phoR* mutations of strains SP25 (*argA15 phoP17*), V12 (*argA15 phoR212*), and BC14 (*trpB3 phoR114*) (16) were transferred by transformation to strain UOT-0531 (*trpC2 metB51 leuA8 nonB1*), giving rise to strains UOT-1073 (*trpC2 leuA8 argA15 nonB1 phoP17*), UOT-1077 (*trpC2 leuA8 nonB1 phoR212*), and UOT-1079 (*trpC2 leuA8 nonB1 phoR114*), respectively. UOT-1073 lysogenized and bacteriophage φ105 (19) or φ105 *ind-1* (an induction-negative mutant of phage φ105 [5]) was used as the cloning host. A single-copy phage vector φCM was described previously (21).

Media. LPXP plates were used to detect the APase phenotype of the colonies under low-P_i conditions. They contained 1% peptone, 1% NaCl, 0.1 M Tris (pH 7.5), 0.5% glucose, 5 μg of chloramphenicol per ml, 50 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml, and 1% agar. For high-P_i conditions, LPXP plates were used with 2 mM P_i added (HPXP plates). For liquid culture, the LP and HP broths described by Yamane et al. (25) were used, with the concentration of phosphate in the HP broth changed to 5 mM. Other media used for competent cell transformation and transduction and for preparation of chromosomal and phage DNA were described previously (21).

Assay of APase. *B. subtilis* cells were grown in LP or HP broth at 37°C for 24 h. After centrifugation, the cells were suspended in an equal volume of 50 mM Tris (pH 7.5) and treated with a few drops of toluene. A 50-μl portion of the toluenized cell suspension was mixed with 500 μl of 5 mM *p*-nitrophenyl phosphate in 1 M Tris (pH 9.0) and incubated at 37°C for 5 to 30 min. At intervals, the reaction was stopped by addition of 500 μl of 2 N NaOH to the reaction mixture. A_{410} was measured after the cells were centrifuged. APase activity is expressed as 1,000 × A_{410} per milliliter of 24-h culture per minute.

DNA sequencing. The nucleotide sequence was determined by the dideoxy chain termination method (20); the procedure described by Messing (15) was used.

RESULTS

Cloning of *phoP*. *B. subtilis* UOT-0278 (*phoP*$^+$) chromosomal DNA (0.3 μg) and 0.1 μg of φCM phage DNA were digested separately with *Bgl*II, and then ligated together and used to transform *B. subtilis* UOT-1073 (φ105 *ind-1*) competent cells. Of 1.6 × 10^4 chloramphenicol-resistant (Cm^r) transformants obtained on LPXP plates, 5 clones formed blue colonies (APase positive). To transfer the cloned DNA to the inducible prophage, total DNAs were prepared independently from the clones and used to transform *B. subtilis*

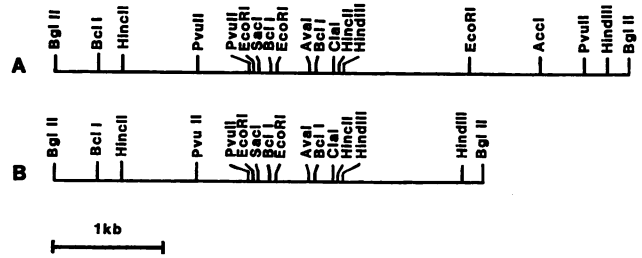

FIG. 1. Restriction maps of the inserted DNA fragments of φCMHO3 (A) and φCMHO1 (B).

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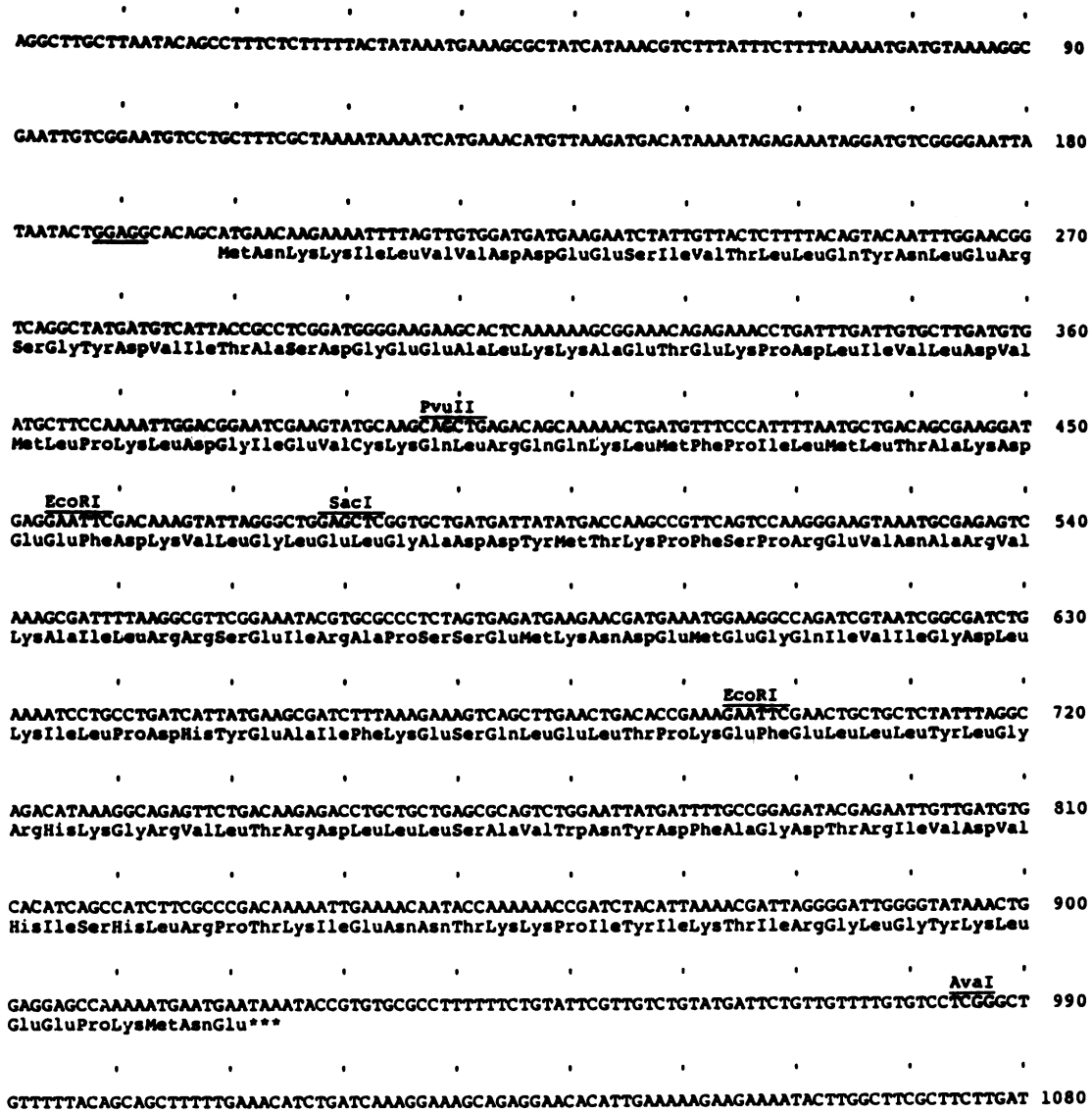

FIG. 2. Nucleotide sequence of the *phoP* region. The deduced amino acid sequence of the *phoP* gene product is given below the DNA sequence. A putative ribosome-binding site is underlined. The numbers on the right indicate the last nucleotide on the line.

UOT-1073 (ϕ105). In two of the five clones, all the Cm^r transformants formed blue colonies on LPXP. From each transformant, phage DNA was prepared for cleavage analysis. Phages ϕCMHO1 and ϕCMHO3 contained 3.9- and 5.2-kilobase *Bgl*II fragments, respectively. Although the two fragments were different in size, they both had $phoP^+$ transforming activity (data not shown). To explain this, restriction analysis of the two inserts was carried out. The left part (*Bgl*II to *Hin*dIII) and the right end (*Hin*dIII to *Bgl*II) of the two fragments were identical (Fig. 1); however, the portion of ϕCMHO3 containing the *Eco*RI, *Acc*I, and *Pvu*II sites was deleted from ϕCMHO1.

Complementation test. *phoR*, the regulatory gene for APase, has been mapped close to *phoP* (16, 18). Miki et al. (15) classified the APase-negative mutations into two clusters, PI and PII. PI belongs to the *phoP* region. PII and RI, the cluster of the APase-constitutive mutations, seem to be within the *phoR* region. To check the ability of the cloned fragments to complement these mutations, the APase mutants were transduced with ϕCM, ϕCMHO1, and ϕCMHO3,

TABLE 1. APase activities of *B. subtilis* cells harboring various phages

Strain	Genotype	Phage	APase activity[a]	
			LP	HP
UOT-0531	Wild type	ϕCM	628	13
UOT-1073	*phoP* (PI)	ϕCM	5	21
		ϕCMHO1	490	15
		ϕCMHO3	302	12
UOT-1077	*phoR* (PII)	ϕCM	11	17
		ϕCMHO1	10	21
		ϕCMHO3	260	29
UOT-1079	*phoR* (RI)	ϕCM	1,880	280
		ϕCMHO1	2,640	240
		ϕCMHO3	930	12

[a] APase activities of the cells grown in LP or HP broth were assayed as described in Materials and Methods. Activity is expressed as $1,000 \times A_{410}$ per milliliter of 24-h culture per minute.

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          10        20        30        40        50
PhoB  MARRILVVEDEAPIREMVCFVLEQNGFQPVEAEDYDSAVNQLNEPWPDLI
      *  **** ** ;*  ;; ; **  *;; ; * * ; *;   ;   ***
PhoP  MNKKILVVDDEESIVTLLQYNLERSGYDVITASDGEEALKKAETEKPDLI
          10        20        30        40        50

          60        70        80        90       100
PhoB  LLDWMLPGGSGIQFIKHLKRESMTRDIPVVMLTARGEEEDRVRGLETGAD
      ;** ***   **;  * *; ; ;    *;;****; ** *;* *** ***
PhoP  VLDVMLPKLDGIEVCKQLRQQKLMF  PILMLTAKDEEFDKVLGLELGAD
          60        70         80        90

         110       120       130              140
PhoB  DYITKPFSPKELVARIKAVMRR  ISPMAVEEVI EMQG      LSLDP
      **;******;*; **;**;;**  * ; ; *    **;*      * ; *
PhoP  DYMTKPFSPREVNARVKAILRRSEIRAPSSEMKNDEMEGQIVIGDLKILP
             110       120       130       140

       150       160       170       180       190
PhoB  TSHRVMAGEEPLEMGPTEFKLLHFFMTHPERVYSREQLLNHVWGTNVYVE
        ;  *  **;;* ** ** ;   *  ** ;*; **  **  *   ;
PhoP  DHYEAIFKESQLELTPKEFELLLYLGRHKGRVLTRDLLLSAVWNYDFAGD
     150       160       170       180       190

         200       210       220
PhoB  DRTVDVHIRRLRKA LEPGGHDRMVQ TVRGTGYRFSTRF
       * ***** ;** ; ;*  ;;  ;   *;** **;
PhoP  TRIVDVHISHLRPTKIENNTKKPIYIKTIRGLGYKLEEPKMNE
     200       210       220       230       240
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FIG. 3. Comparison of amino acid sequences of the *E. coli phoB* and *B. subtilis phoP* proteins. Identical residues (*) and conservative residues (;) are marked. Gaps have been inserted to align the two sequences for the greatest homology.

and then the APase activities were assayed. The results given in Table 1 show that ϕCMHO3 complements both *phoP* (PI) and *phoR* (PII and RI) mutations in *trans*, whereas ϕCMHO1 complements only *phoP* (PI). The deleted 1.3-kilobase region on ϕCMHO1 is thought to correspond to a part of the *phoR* gene.

DNA sequence of the *phoP* region. Deletion analysis with restriction enzymes has shown that the 1.9-kilobase fragment from the left *Hin*cII site to the *Ava*I site is enough for *phoP* complementation and that the *Sac*I site is within the gene. The nucleotide sequence was determined, and the open reading frame was searched around the *Sac*I site. An open reading frame of 241 amino acid residues (M_r 27,716) was found (Fig. 2). A putative ribosome-binding site GGAGG (position 188) lies upstream from the initiation codon ATG (position 199) (14). The start site of the transcription has not yet been determined, but the sequences TTGTCG (position 94) and TAAAAT (position 117) show homology with the consensus sequence for the major RNA polymerase holoenzyme $E\sigma^{43}$ (TTGACA for −35 and TATAAT for −10) (6, 17). The sequence CTATCA TAAACGTCTTTA (position 47) seems to be homologous to the phosphate box, which is a common sequence for the genes involved in the phosphate regulon of *E. coli* (13). There is no stem-loop structure strong enough to terminate transcription in the region sequenced.

Sequence comparison between PhoP and PhoB proteins. The deduced amino acid sequence was compared with that of PhoB, the positive regulator for the phosphate regulon of *E. coli*. Throughout the sequence, extensive homology was found between the two proteins. Figure 3 shows their amino acid sequences. The homology was 40% for identical amino acids and 20% for conservative replacements. The hydropathy profiles at a span setting of 9 were analyzed, and the results are shown in Fig. 4. These profiles are also highly comparable, indicating that the secondary structures of the two proteins are also similar. From these results it appears that in *B. subtilis*, the function of the *phoP* gene product is to regulate APase, as PhoB does in *E. coli*.

DISCUSSION

DNA fragments which complement the APase-negative mutation, *phoP*, were cloned by the improved prophage transformation system (11, 21). This system has turned out to be useful for the cloning of genes, even those such as *phoP*, which are not selected directly. The prophage vector ϕCM was used to perform complementation tests against *phoP* (PI) and *phoR* (PII and RI) mutants. There was spontaneous deletion of 1.3 kilobases within the insert of ϕCMHO1. This deletion caused the simultaneous loss of ability to complement both PII and RI mutations, indicating that the *phoR* gene is the positive and negative regulatory gene.

Although the *phoP* mutation brings about a simultaneous defect in the production of both APase and phosphodiesterase (12, 25; T. Miki, Ph.D. thesis, University of Tokyo, Tokyo, Japan, 1966), the function of the *phoP* gene has remained uncertain. Glenn showed that the loss of activities was due to decreased production of proteins, with no changes in the enzymatic properties of the proteins, suggesting a regulatory function for the *phoP* gene product (7).

Nucleotide sequence analysis revealed that PhoP protein functions as the regulator, since the amino acid sequence of the *phoP* gene product was highly homologous to that of the *phoB* gene product, which is the positive regulator for phosphate regulon in *E. coli* (23). The level of identical plus conservative residues was 60%. This result, together with the observation that the positive and negative regulatory genes, *phoR* in *B. subtilis* and *phoR* in *E. coli*, are located close to the positive regulatory genes, *phoP* and *phoB*, respectively, suggests that the regulatory mechanisms for APase synthesis in *B. subtilis* and *E. coli* are very similar. It

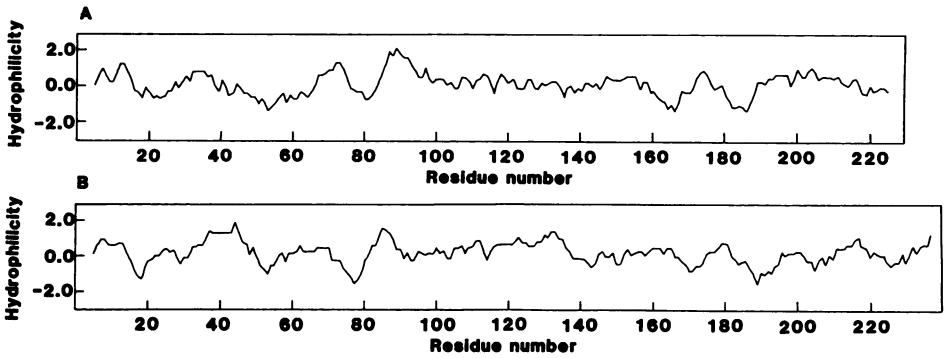

FIG. 4. Hydropathy profiles of PhoB (A) and PhoP (B) proteins.

is therefore likely that *phoP* is the common regulatory gene for the synthesis of APase and phosphodiesterase (probably two kinds of phosphodiesterases are produced in *B. subtilis* [12, 25]).

PhoB belongs to a family of regulatory proteins including OmpR for the outer membrane proteins of *E. coli* (24), Dye for the envelope proteins of *E. coli* (3), NtrC for nitrogen fixation in *Klebsiella pneumoniae* (2), CheY and CheB for chemotaxis in *Salmonella typhimurium* (22), and Spo0A and Spo0F for sporulation in *B. subtilis* (4, 10, 26). These are thought to act as transcriptional factors. PhoP also is thought to activate the transcription of the APase gene(s) in *B. subtilis*.

ACKNOWLEDGMENTS

We thank T. Miki for the gift of bacterial strains, A. Nakata for supplying information on functions of *E. coli pho* genes, and F. Kawamura for helpful discussion.

This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

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