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Cloning of and Complementation Tests with Alkaline Phosphatase Regulatory Genes (*phoS* and *phoT*) of *Escherichia coli*

MITSUKO AMEMURA,[1] HIDEO SHINAGAWA,[1] KOZO MAKINO,[1] NOZOMU OTSUJI,[2]† AND ATSUO NAKATA[1]*

The Research Institute for Microbial Diseases, Osaka University, Suita, Osaka,[1] and Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka,[2] Japan

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The regulatory genes of alkaline phosphatase, *phoS* and *phoT*, of *Escherichia coli* were cloned on pBR322, initially as an 11.8-kilobase *Eco*RI fragment. A restriction map of the hybrid plasmid was established. Deletion plasmids of various sizes were constructed in vitro, and the presence of *phoS* and *phoT* genes on the cloned DNA fragments was tested by introducing the plasmids into *phoS64* and *phoT9* strains for complementation tests. One set complemented only *phoS64* but not *phoT9*; the other set complemented only *phoT9* but not *phoS64*. We conclude that *phoS64* and *phoT9* mutations belong to different complementation groups and probably to different cistrons. The hybrid plasmid with the 11.8-kilobase chromosomal fragment also complemented the *phoT35* mutation. A smaller derivative of the hybrid plasmid was constructed in vitro which complemented *phoT35* but did not complement *phoS64*, *phoT9*, or *pst-2*. Our results agree with the suggestion that *phoT35* lies in a different complementation group from *phoS*, *phoT*, or *pst-2* (Zuckier and Torriani, J. Bacteriol. **145**:1249–1256, 1981). Therefore, we propose to designate *phoT35* as *phoU*. The effect of amplification of *phoS* or *phoT* on alkaline phosphatase production was examined. It was found that multiple copies of the *phoS* gene borne on pBR322 repressed enzyme production even in low-phosphate medium, whether it was introduced into wild-type strains (partially repressed) or *phoR* (*phoR68* or *phoR17*) strains (fully repressed), whereas the introduction of multicopy plasmids bearing the *phoT* gene did not affect the inducibility of the enzyme.

The synthesis of alkaline phosphatase (AP) is repressed by inorganic phosphate. The expression of its structural gene, *phoA*, is under complex genetic control. Three positive regulatory genes, *phoB* (4, 15), *phoR* (8, 9, 15), and *phoM* (30), and three negative regulatory genes, *phoR* (7–9, 15), *phoS*, and *phoT* (7, 10, 31) have been identified. It has been shown that the *phoS* and *phoT* genes are also responsible for highly specific phosphate transport (31).

Recently, Levitz et al. (17) reported that the *phoS* and *phoT* genes did not complement each other in *cis-trans* tests and concluded that they belong to the same cistron. However, these investigators failed to give a satisfactory explanation for the fact that *phoT* mutants produce the phosphate-binding protein which has been shown to be the *phoS* gene product (10–12, 21, 32). Furthermore, Zuckier and Torriani (33) suggested, on the basis of their genetic studies, that *phoS* and *phoT* belong to different cistrons. No physiological difference was demonstrated.

Our interest in the control of AP synthesis and the related complex regulatory circuit motivated us to clone the relevant genes, which would provide powerful tools and materials for elucidation of the molecular mechanisms. In this paper, we report the cloning and characterization of *phoS* and *phoT* genes, and the results of complementation tests between them utilizing the cloned genes.

† After 2 years of heroic struggle against cancer, Nozomu Otsuji passed away on 2 July 1982, at the age of 50. Despite weakened health and debilitating therapy, his passion for science never weakened. He stimulated and participated in the work on the *pho* regulon, to which he made great contributions, until his last days. He was loved and admired by everybody who knew him.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work, other than the BC strains described below, are listed in Table 1.

To compare the synthesis of AP, a set of strains isogenic except for the relevant mutation sites was

TABLE 1. Bacterial strains

Strain	Genotype[a]	Source or reference
ANC24	F⁻ *leu trp his argG rpsL ilv met thi*	Hfr cross: K10 × CSH57
ANCC2	F⁻ *leu phoR68 trp his argG rpsL ilv met thi*	Hfr cross: C2 × CSH57
ANCC3	F⁻ *leu phoR69 trp his argG rpsL ilv met thi*	Hfr cross: C3 × CSH57
ANCC4	F⁻ *leu purE trp his argG rpsL phoT35 met thi*	P1 transduction: C4 × CSH57
ANCC5	F⁻ *leu phoR17 trp his argG rpsL ilv met thi*	Hfr cross: C5 × CSH57
ANCC75	F⁻ *leu purE trp his argG rpsL phoS64 met thi*	P1 transduction: C75 × CSH57
ANCC90	F⁻ *leu purE trp his argG rpsL phoT9 met thi*	P1 transduction: C90 × CSH57
C2	HfrC(PO2A) *phoR68 relA1 pit-10 spoT1 tonA22* T2ʳ	(25)
C3	HfrC(PO2A) *phoR69 relA1 pit-10 spoT1 tonA22* T2ʳ	CGSC 6318 (15)
C4	HfrC(PO2A) *phoT35 relA1 pit-10 spoT1 tonA22* T2ʳ	F. G. Rothman (10)
C5	HfrC(PO2A) *phoR17 relA1 pit-10 spoT1 tonA22* T2ʳ	F. G. Rothman (6)
C75	HfrC(PO2A) *phoS64 spoT1 relA1 tonA22* T2ʳ	(10)
C90	HfrC(PO2A) *phoT9 spoT1 relA1 tonA22* T2ʳ	(10)
CSH57	F⁻ *ara leu lacY purE gal trp his argG malA rpsL xyl mtl ilv metA*(or *B*) *thi*	Cold Spring Harbor Laboratory (24)
GS5	F⁻ *proC24 purE41 pyrF30 his-53 thyA25 pit-1 pst-2 metB1 nalA12 rpsL97 tsx-63*?	CGSC 5507 (29)
K10	HfrC(PO2A) *pit-1 spoT1 relA1 tonA22* T2ʳ	(25)
KLF48/ KL159	F148/ *thi-1 his-4 aroD5 proA2 recA1 xyl-5* or *xyl-7 nalA12 tsx-1*? or *tsx-29* λ⁻ *supE44*	CGSC 4302
KH693	F⁻ *thr-1 leu-6 trp-1 his-1 argH-1 metE dnaA46 tna bglB bglR mtl-2 malA1 thi ara-13 gal6 lacY1 rpsL9 tonA2 supE44*	T. Miki (14)
KY7388	*gal-1 gal-2 lac glmS* λ *glmS-1* (λ i^{21} *glmS*) λ *tna*(λ i^{21} *tna*)	T. Miki (22)

[a] For the strains constructed in our laboratory, only genotypes confirmed after isolation are described.

constructed. The *phoR* strains, C2, C3, and C5, were crossed by conjugation with strain CSH57. Colonies constitutive for AP synthesis were purified from the Ade⁺ and Strʳ recombinants (ANCC2, ANCC3, or ANCC5). The *phoS64*, *phoT9*, or *phoT35* mutation was transferred into CSH57 by P1 phage transduction (24). Cells of strain CSH57 were infected with P1 *vir* grown in strain C75, C90, or C4, and Ilv⁺, AP-constitutive transductants were purified (ANCC75, ANCC90, and ANCC4, respectively).

BC strains were isolated as follows: strain BE269 (F⁻ *ilv trp tna lac-2* Strʳ [1]) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Nakarai Chemical Co.) as described by Nakata et al. (26). AP-constitutive mutants were purified. They were then crossed, as recipients, with NS31-11 (HfrH *leu lacI proC thi* [26]) as a donor. When no recombinant colonies in which AP synthesis was repressed appeared among the Trp⁺ Strʳ recombinants, the mutants were classified as R2 groups by the old nomenclature (7).

Media. T-broth contained 10 g of tryptone (Difco Laboratories) and 5 g of NaCl per liter. TG medium, a minimal salt solution buffered with Tris (pH 7.2) and containing 0.2% glucose, supplemented with either 6.4×10^{-4} M (excess phosphate) or 6.4×10^{-5} M (limited phosphate) KH_2PO_4, as described by Nakata et al. (26), was used. It was also supplemented, as needed, with amino acids or nucleosides at concentrations previously described (6). Plates contained 1.5% agar. Tetracycline (10 μg/ml) or ampicillin (40 μg/ml) was added to the medium for direct selection of transformants or to ensure the presence of plasmids in liquid cultures used for enzyme assays.

We observed that growth inhibition by ampicillin was very weak in the TG medium we used. Therefore, we used the Tcʳ gene on the plasmids to select against the plasmid-cured cells which would produce AP constitutively.

Assay of AP activity. The AP phenotype was determined by spraying colonies with a mixture of α-naphthylphosphate (Sigma Chemical Co.) and tetrazotized *o*-dianisidine (Fast Blue B Salt; Sigma). With this spray, colonies synthesizing AP constitutively on T-broth plates or on TG plates with excess phosphate were stained dark brown (26, 28).

The assay of enzyme activity in liquid culture was carried out as follows. One drop of toluene was added to about 1 ml of culture, which was vigorously blended in a Vortex mixer for 1 min and then incubated at 37°C with shaking for 60 min, until the toluene evaporated completely. A 0.1-ml sample of the toluene-treated culture was added to 2.4 ml of 1 M Tris-hydrochloride (pH 8.0) containing 10^{-3} M *p*-nitrophenylphosphate (Nakarai Chemical Co.). After incubation at 37°C, the reaction was stopped with 0.5 ml of 0.5 M Na_2HPO_4, and the optical absorbance at 410 nm was measured in cuvettes with a 1-cm lightpath, within 30 min after the addition of Na_2HPO_4. The optical density at 410 nm due to the disrupted cells was negligible at this dilution. The enzyme activity was expressed as micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein (measured with the Folin reaction [19]).

Test of *pst*. For the test of the *pst* genotype, strain GS5 (*pit-1 pst-2*) was used. The cells to be tested were inoculated on a plate of TGly medium (TG medium in

which 0.2% glucose was replaced by 0.6% glycerol) supplemented with K_2HPO_4 (5×10^{-5} M) and DL-glycerol-3-phosphate (0.4 mg/ml [29, 33]). Strain GS5 requires phosphoglycerol as a phosphate source, because it cannot transport inorganic phosphate due to its *pit pst* genotype. This strain becomes independent of added organic phosphate when either the *pst*$^+$ or the *pit*$^+$ gene is introduced (29). Transformants of GS5 resulting from the introduction of hybrid plasmids carrying the *phoS-phoT* region, which could grow on plates without added phosphoglycerol, were scored as *pst*$^+$, since the *pit* gene was mapped at a different locus on the chromosome (2, 29).

Test of *bgl*. For the test of the *bgl* genotype, eosin methylene blue-salicin (1%) plates were used. Colonies of strain KH693 (*bglB bglR*) were white, and colonies of the *bglB*$^+$ *bglR* strain were dark red on this plate (22, 27). When strain KH693 was transformed by a hybrid plasmid and the transformant formed dark red colonies on the plate, the plasmid was considered to carry the *bglB*$^+$ gene.

Purification of λ *glmS* phage particles. Bacteriophage lambda was induced by the addition of mitomycin C (0.5 μg/ml) to a 200-ml culture of strain KY7388. Phage particles were collected by high-speed centrifugation (25,000 rpm in a no. 30 rotor for 90 min) and suspended in 10 mM Tris–0.1 mM Na_2 EDTA (pH 8.0). They were then collected by CsCl blocl gradient centrifugation followed by CsCl equilibrium centrifugation (6). Two bands of phage particles were obtained after the equilibrium centrifugation, of which the lower, containing the λ *glmS* phage, was withdrawn.

Extraction of bacterial, phage, and plasmid DNA. Whole cell DNA was prepared from *Escherichia coli* K-12 strain KLF48/KL159 (no. 4302; Coli Genetic Stock Center, Yale University, New Haven, Conn.). Exponentially growing cells (absorbance at 600 nm, 0.6) were harvested and treated with solution I (lysozyme, 2 mg/ml; 10 mM EDTA; 25 mM Tris-hydrochloride [pH 8.0]) for 30 min at 0°C and lysed with 1% sodium dodecyl sulfate at 0°C for 60 min. After the cell debris was removed by centrifugation at 10,000 rpm in an SS34 rotor for 15 min, the supernatant was treated with solution II (phenol-chloroform-isoamyl alcohol 25:24:1 [vol/vol/vol]). The aqueous phase was withdrawn, and phenol was extracted with chloroform-isoamyl alcohol 24:1 (vol/vol). DNA was precipitated twice with 2 volumes of ethanol and further purified by CsCl equilibrium centrifugation at 38,000 rpm in a no. 40 rotor for 40 h.

Phage DNA was extracted from λ *glmS*-1 phage particles with formamide (6).

Plasmid DNA was prepared according to the method of Birnboim and Doly (3). The crude plasmid DNA solutions were further purified by cesium chloride-ethidium bromide equilibrium centrifugation. Ethidium bromide was removed from the DNA solution with isopropanol saturated with aqueous 5 M NaCl–10 mM Tris–1 mM Na_3-EDTA (pH 8.5) (6).

Cloning of *E. coli* genes. Purified chromosomal and pBR322 DNA were digested separately with restriction endonuclease *Eco*RI. The vector DNA was treated with *E. coli* AP to remove 5′-phosphate to prevent self-ligation (13). Recombinant plasmids were constructed in vitro by ligation of the *E. coli* DNA fragments and the pBR322 vector by using bacteriophage T4 ligase. T4 ligase was kindly supplied by S. Harashima, Osaka University. Ligation was performed at 8°C overnight in ligation buffer containing 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP. The DNA concentrations were about 30 μg/ml for vector DNA and 100 μg/ml for passenger DNA in a total volume of 50 to 100 μl. One unit of T4 ligase per μg of DNA to be ligated was used.

Transformation was performed according to the method of Lederberg and Cohen (16) by using an appropriate recipient with a mutation in the gene to be cloned. To check the efficiency of these processes, we picked eight transformant colonies, and the plasmids were prepared by the rapid method (3). If more than 20% of the transformants contained hybrid plasmids, the transformants were transferred to duplicate plates (TG with antibiotic), one for a master plate and the other for the AP phenotype test. When more than 80% of the transformants possessed independent hybrid plasmids, the mixture of plasmids prepared from such transformants was stored as a gene bank.

Restriction map. The restriction maps of recombinant plasmids were constructed by digesting the DNA with combinations of restriction endonuclease and subsequent electrophoresis on agarose or polyacrylamide gels. The procedures employed have been described by Davis et al. (6). Most of the restriction enzymes were purchased from Takara Shuzo Co. Ltd.

RESULTS

Cloning of a chromosomal fragment complementing the *phoS* and *phoT* mutations. The chromosomal fragment complementing the *phoS64* and *phoT9* mutations was isolated from two different sources, λ *glmS*-1 phage and *E. coli* cells. The transducing λ *glmS*-1 phage contains the *E. coli* chromosomal fragment covering the region from *tna* to *glmS*, including *bgl* (14, 23). The *phoS* gene is located between *bgl* and *glmS* (14). We thought it likely that the *phoT* gene is located on this fragment, since the *phoT* gene is closely linked to the *phoS* gene (10). The DNA isolated from λ *glmS* particles was digested with *Eco*RI and ligated to the *Eco*RI site on plasmid pBR325. This DNA was used to transform either *phoS* or *phoT* mutant cells. Several ampicillin-resistant transformants that are repressed for AP synthesis, that is, they show complementation of the *phoS* or *phoT* mutation, were isolated. Plasmids were extracted from several colonies, and they were able to transform and to complement either the *phoS* or *phoT* mutation. The cloned *Eco*RI fragments of these plasmids were compared by digestion with restriction enzymes *Eco*RI, *Hin*dIII, *Mlu*I and *Pst*I, and all the fragments showed the same restriction pattern (pSN400, Fig. 1).

DNA was also prepared from *E. coli* strain KLF48/KL159, digested with *Eco*RI, and ligated into plasmid pBR322, as described in Materials and Methods. Transformation experiments were performed with a *phoR* mutant (strain ANCC2) as a recipient. Transformants resistant to tetra-

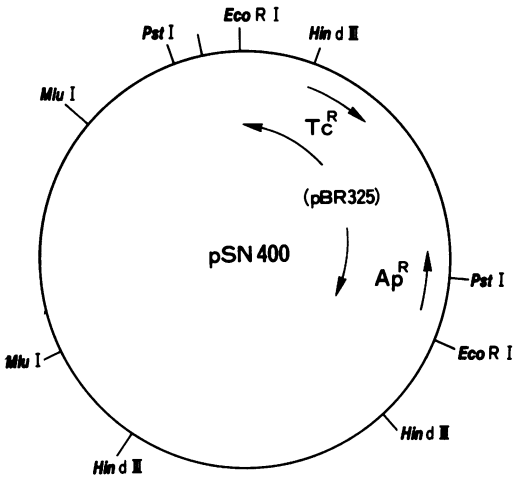

FIG. 1. Restriction map of pSN400. An *Eco*RI fragment of λ *glmS*-1 phage DNA was cloned on plasmid pBR325. The plasmid complemented *phoS*, *phoT*, *pst*-2, and *bglB* mutations.

cycline were selected on a T-broth plate containing tetracycline, and AP⁻ transformants (white or slightly stained colonies by chromogenic substrate) were purified. The plasmid DNA was extracted from these transformants, and their sizes and the pattern of DNA fragments generated by digestion with restriction endonucleases were compared. They were classified into four groups according to the restriction patterns. The *Eco*RI fragment cloned in one of them (pSN401) was found to show the same restriction map for *Hin*dIII, *Mlu*I, and *Pst*I as the DNA fragment isolated from transducing phage λ *glmS* (Fig. 2). Strains ANCC75 (*phoS64*) and ANCC90 (*phoT9*) were transformed by this plasmid (pSN401). It was found that this plasmid could complement both of these mutations. Although pSN401 was originally isolated as a plasmid complementing the *phoR* mutation, strains C2 (*phoR68*) and C5 (*phoR17*), harboring pSN401, were unable to synthesize AP not only on TG plates with excess phosphate but also on plates with limited phosphate. This phenomenon also occurred with pSN400.

The identity of the *Eco*RI fragment on the hybrid plasmid with fragment derived from λ *glmS* was also supported by the genetic markers borne on the plasmids. Strains KH693 (*bglB bglR*) and GS5 (*pit-1 pst-2*) were transformed with either pSN400 or pSN401. All the tetracycline-resistant transformant colonies from the former strain were dark red on eosin methylene blue-salicin plates (BglB⁺ phenotype), and those from the latter strain could grow on TGly (5×10^{-5} M K_2HPO_4) plates (Pst⁺ phenotype). We have not examined whether pSN400 and pSN401 carry the *bglR* allele.

The similarity of the two hybrid plasmids (pSN400 and pSN401) derived from different sources and screened in different genetic backgrounds is summarized in Table 2. Both of them showed the same phenotypes in each genetic background and thus are likely to carry the same alleles.

Restriction map of the plasmids and complementation tests on plates. A restriction map of the cloned chromosomal fragment (*Eco*RI fragment) was constructed (Fig. 2). There were four *Hpa*I, two *Hin*dIII, two *Mlu*I, and two *Pst*I cleavage sites. A variety of deletion plasmids were constructed by partial or complete digestion with an appropriate enzyme followed by self-ligation. Their properties were examined by complementation tests. We also examined the sizes and restriction patterns of the deletion plasmids, and they were confirmed to be the ones shown in Fig. 2. A plasmid from which the *Hin*dIII$_1$-*Hin*dIII$_2$ fragment was deleted (pSN507) complemented both *phoS* and *phoT* mutations, but not *bglB*. The following deletion plasmids were constructed from pSN507. A plasmid lacking the *Hpa*I$_1$-*Hpa*I$_2$ fragment (pSN517) complemented *phoT9* but not *phoS64*. Plasmid pSN537, which lacks both *Hpa*I$_1$-*Hpa*I$_2$ and *Hpa*I$_3$-*Hpa*I$_4$ fragments, complemented only *phoT9*. Plasmids lacking the fragment between *Hpa*I$_2$ and *Hpa*I$_4$ (pSN557), and between *Hpa*I$_1$ and *Hpa*I$_4$ (pSN567) did not complement either *phoS64* or *phoT9*.

In plasmid pSN577, the *Mlu*I$_1$-*Mlu*I$_2$ segment of pSN517 was excised and religated in the

TABLE 2. AP activity of various strains transformed by hybrid plasmids derived from λ *glmS*-1 DNA (pSN400) and from *E. coli* chromosomal DNA (pSN401)

Plasmid	Pst GS5 (*pit-1 pst-2*)	BglB KH693 (*bglB bglR*)	AP activity[a]				
			ANC24 (wild type)	ANCC2 (*phoR68*)	ANCC75 (*phoS64*)	ANCC90 (*phoT9*)	GS5 (*pit-1 pst-2*)
pSN400	+	+	0.005 ± 0.001	0.004 ± 0.001	0.008 ± 0.001	0.004 ± 0.0004	0.005 ± 0.001
pSN401	+	+	0.005 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.003 ± 0.001	0.006 ± 0.001
pBR322	−	−	0.009 ± 0.001	0.41 ± 0.18	3.86 ± 0.19	3.86 ± 0.52	1.86 ± 0.072

[a] In the medium supplemented with excess phosphate, except GS5, for which the medium was supplemented with DL-glycerol-3-phosphate (0.04%) (29, 33). Activity was measured as micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein ± standard deviation.

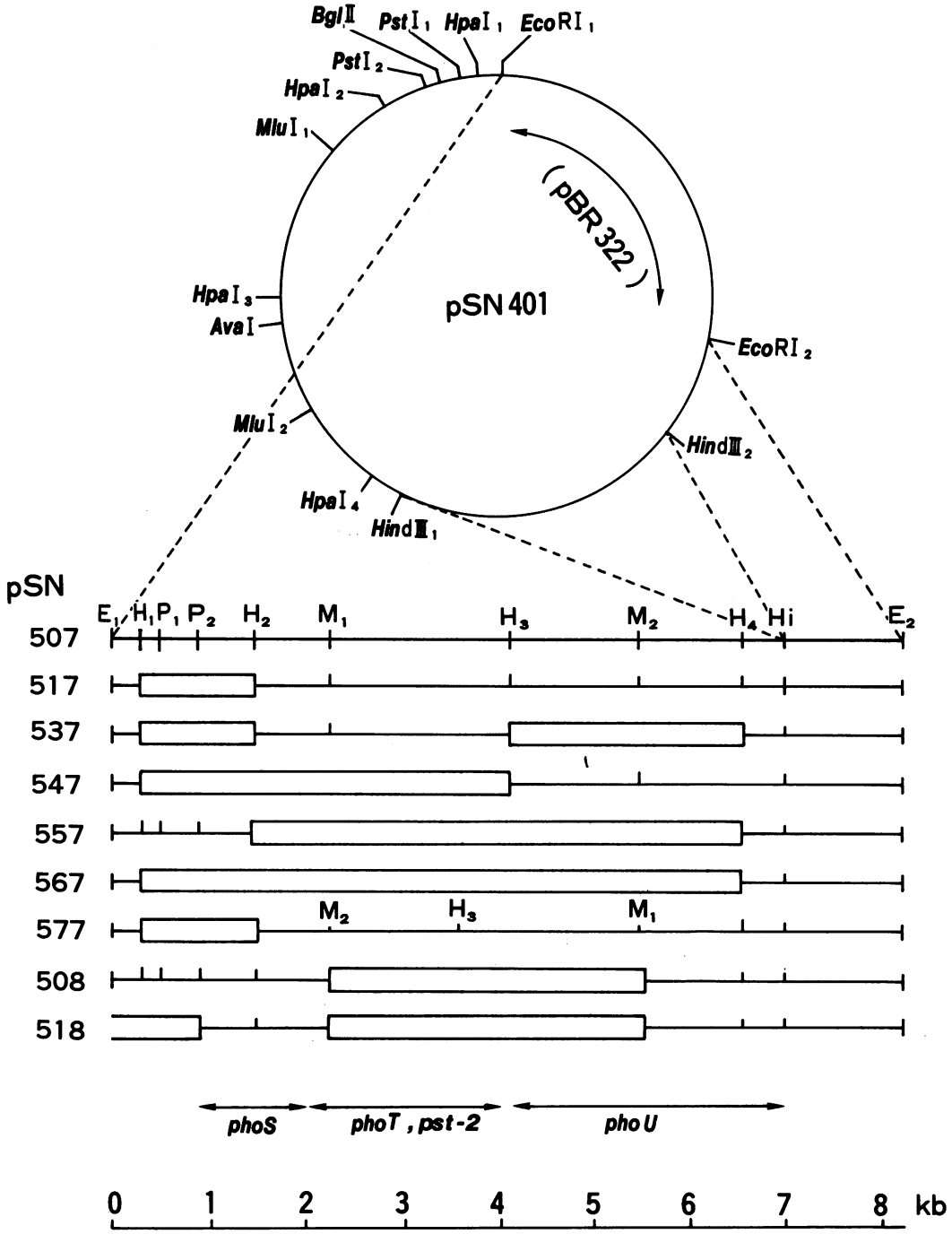

FIG. 2. Restriction map of pSN401 containing an *Eco*RI fragment covering the *phoS phoT* region of the *E. coli* chromosome and of deletion plasmids derived from it. Plasmid pSN507 was formed by deletion of a *Hin*dIII fragment, measuring 3.6 kb, located between *Hpa*I$_4$ and *Eco*RI$_2$ sites on pSN401. Other deletion plasmids were constructed from pSN507. Cleavage sites: E, *Eco*RI; H, *Hpa*I; Hi, *Hin*dIII; M, *Mlu*I; P, *Pst*I.

opposite orientation. This plasmid did not complement the *phoT9* mutation. Thus, it is clear that the *phoT* gene lies within the 2.6-kilobase (kb) *Hpa*I$_2$-*Hpa*I$_3$ segment covering site *Mlu*I$_1$.

Plasmid pSN508, lacking the *Mlu*I$_1$-*Mlu*I$_2$ fragment, complemented *phoS64* but not *phoT9*. When the *Pst*I fragment of pSN508 (from the *Pst*I$_2$ site to the *Pst*I site on the Ap^r gene in

TABLE 3. Classification of AP constitutive mutants by complementation tests on plates[a]

Strain	pSN518 (*phoS*$^+$)	pSN537 (*phoT*$^+$)	pSN547 (*phoU*$^+$)
BC68	+	−	−
BC69	−	+	−
BC72(*phoT* [1])	−	+	−
BC73	+	−	−
BC74	−	+	−
BC76	−	−	+
BC77	−	+	−
BC78	+	−	−
BC79	+	−	−

[a] Ten tetracycline-resistant transformed colonies were transferred to TG plates with either excess or limited phosphate. These plates were also supplemented with the required amino acids and tetracycline (20 μg/ml). +, White colonies (complementation took place); −, colored colonies (complementation did not take place) after spraying with chromogenic substrate.

pBR322) was removed, the deletion plasmid (pSN518) still complemented the *phoS64* mutation. It is obvious that the *phoS* gene is located on the 1.3-kb fragment between the *PstI*$_2$ and *MluI*$_1$ cleavage sites and covers site *HpaI*$_2$. Therefore, we conclude that the *phoS* and *phoT* genes can be independently complemented by the wild-type alleles, that is, the wild-type allele is transdominant over each mutant.

Plasmid pSN547, which lacks the *HpaI*$_1$-*HpaI*$_3$ fragment (Fig. 2) was unable to complement either *phoS64* or *phoT9*. However, when it was introduced into another *phoT* mutant (strain C4; *phoT35*), the synthesis of AP was repressed, which meant that complementation took place. The plasmids pSN517, pSN537, and pSN547 were transferred into strain ANCC4 (*phoT35*). AP synthesis was repressed in transformants carrying either pSN517 or pSN547 but not in those carrying pSN537. Plasmid pSN577, in which the *MluI* fragment of pSN517 was religated in the opposite orientation, did not complement the *phoT35* mutation. These results indicate that a wild-type allele which complements *phoT35*, but not *phoT9*, lies on the DNA fragment between the *HpaI*$_3$ and *Hin*dIII cleavage sites on pSN507.

We isolated nine AP-constitutive mutants which belong to the R2 group by the old classification (7). Plasmids pSN518 (*phoS*$^+$), pSN537 (*phoT*$^+$), and pSN547 (*phoT35*$^+$) were transferred into these strains and tested for AP synthesis on plates. As shown in Table 3, all of these mutants could be assigned to either the *phoS* or *phoT* group, except one which was complemented only by pSN547 (BC76).

These results indicate that the mutations *phoT9* and *phoT35* belong to different complementation groups, which was initially suggested by Zuckier and Torriani (33). Therefore, we propose to designate the *phoT35* mutation as *phoU* instead of *phoT*

Complementation tests were also performed with the *pst-2* mutant, which was isolated as a mutation deficient for highly specific phosphate transport and was found to be partially repressible for AP synthesis (29, 31, 32). The plasmids mentioned above were transferred into the *pit pst* mutant (strain GS5) and tested for Pst phenotype. It was found that the plasmids which complemented *phoT9* also complemented *pst-2*, and none of those that failed to complement *phoT9* complemented *pst-2*. Thus, *phoT9* and *pst-2* are as yet inseparable by complementation tests, and they belong to a different complementation group from *phoS* or *phoU*.

Effect of hybrid plasmids on AP synthesis. The complementation tests were also performed by measuring AP activities of cells grown in either excess-phosphate or limited-phosphate medium. Although pSN401 was originally isolated as a plasmid complementing the *phoR* mutation, its derivative pSN507 (*phoS*$^+$ *phoT*$^+$ *pst*$^+$ *phoU*$^+$), which carries a deletion of the *Hin*dIII fragment, provokes a negative AP phenotype even in low-

TABLE 4. AP levels of strains carrying pSN507 and its derivatives in various genetic backgrounds

Plasmid	Medium[a]	AP level[b]			
		ANC24 (wild type)	ANCC2 (*phoR68*)	ANCC57 (*phoS64*)	ANCC90 (*phoT9*)
pSN507	HP	0.005 ± 0.002	0.006 ± 0.001	0.006 ± 0.001	0.005 ± 0.001
	LP	0.032 ± 0.01	0.007 ± 0.001	0.094 ± 0.037	0.127 ± 0.04
pSN518	HP	0.007 ± 0.002	0.004 ± 0.001	0.015 ± 0.006	ND[c]
	LP	0.18 ± 0.11	0.007 ± 0.002	1.73 ± 0.02	ND
pSN537	HP	0.012 ± 0.001	0.57 ± 0.036	2.52 ± 0.35	0.016 ± 0.002
	LP	3.63 ± 0.21	0.323 ± 0.011	3.63 ± 0.31	3.03 ± 0.21
pSN567	HP	0.008 ± 0.002	0.65 ± 0.02	1.80 ± 0.20	2.68 ± 0.31
	LP	5.42 ± 0.32	0.20 ± 0.02	1.65 ± 0.7	4.18 ± 0.28

[a] TG medium supplemented with excess phosphate (HP) or limited phosphate (LP).
[b] Micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein ± standard deviation.
[c] ND, Not determined.

TABLE 5. AP levels of strains carrying the *phoS* gene on a low-copy-number plasmid

Plasmid	Medium[a]	AP level[b]					
		ANC24 (wild type)	ANCC2 (*phoR68*)	ANCC3 (*phoR69*)	ANCC75 (*phoS64*)	ANCC90 (*phoT9*)	ANCC4 (*phoT35*)
pSN5083	HP	0.008 ± 0.001	0.84 ± 0.08	1.52 ± 0.03	0.015 ± 0.001	2.29 ± 0.14	2.22 ± 0.19
	LP	6.99 ± 0.5	0.22 ± 0.01	1.82 ± 0.05	3.18 ± 0.44	4.74 ± 0.21	3.10 ± 0.45
pMF3	HP	0.008 ± 0.001	1.39 ± 0.01	2.94 ± 0.08	2.02 ± 0.35	1.28 ± 0.24	1.28 ± 0.01
	LP	5.57 ± 0.1	0.45 ± 0.07	1.65 ± 0.17	6.19 ± 0.92	4.62 ± 0.65	3.55 ± 0.07

[a] TG medium supplemented with excess phosphate (HP) or limited phosphate (LP).
[b] Micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein ± standard deviation.

phosphate medium (Table 4). This effect is due to *phoS*, since a *phoS*$^+$ (*phoT*$^-$) plasmid (pSN518) prevents full induction of AP synthesis in wild-type *E. coli* and totally prevents synthesis in the *phoR68* mutant.

When pSN518 was introduced into ANCC90 (*phoT9*), all the transformant colonies showed the AP constitutive phenotype on T-broth plates. In TG medium, the transformants grew at a growth rate one-third that of the strain carrying only pSN567, and they frequently segregated AP-repressible colonies. The level of AP activity in ANCC90 carrying pSN518 varied considerably from experiment to experiment, which we consider to reflect the degree of the segregation (data not shown). We interpret this phenomenon as follows. Overproduction of the *phoS* gene product in *phoT* cells has an adverse effect on cell growth, and rare PhoT$^+$ revertants resulting from mutation or recombination between the *phoT* gene on the chromosome and a portion of the *phoT* gene borne on the plasmid eventually comprise a considerable portion of the cell population after growth in TG medium, in which PhoT$^+$ cells grow more rapidly than PhoT$^-$ cells.

To test this assumption, we introduced the *phoS* gene into a low-copy-number plasmid. Plasmid DNA of pSN508 was digested with *Eco*RI and inserted in the *Eco*RI site of the low-copy-number plasmid pMF3, which possesses the F replicon (20). ANCC75 was transformed with this DNA. The cells were screened for transformants resistant to ampicillin but sensitive to tetracycline and showing repressed synthesis of AP. Plasmid DNA was isolated and was confirmed to be the result of insertion of the *Eco*RI fragment of pSN508, containing the *phoS* gene, into pMF3 (pSN5083). This plasmid was introduced into strains with various genetic backgrounds, and the levels of AP were measured in liquid medium (Table 5). The results of AP synthesis under repressed or derepressed conditions were as expected: no inhibition of AP synthesis by pSN5083 in *phoR* mutants and full derepression in a wild-type strain carrying the plasmid. When pSN5083 was introduced into ANCC90, no growth inhibition of the transformed cells was observed, and the transformants did not segregate AP-repressible colonies after they were grown in TG medium, in contrast to the case of the multicopy plasmid, pSN518. Complementation of *phoS64* but not *phoT9* or *phoT35*, by the plasmid was observed, as expected.

The deletion plasmid pSN547 repressed AP synthesis in the *phoT35* mutant but not in *phoT9* and *phoS64* mutants (Table 6). It did not prevent full derepression in the wild-type strain or the *phoR68* mutant. AP synthesis in the *phoT35* mutant was not repressed by pSN537 (*phoT*$^+$) (data not shown) or by pSN5083 (*phoS*$^+$) (Table 5).

All of these results are consistent with those obtained in the plate tests with the chromogenic substrate.

DISCUSSION

We have cloned an *E. coli* chromosomal fragment which complements the *phoS* and *phoT* mutations. This fragment was also found to contain the *bglB* and *pst-2* genes. A series of deletion plasmids were constructed in vitro, including one which complemented only the *phoS64* mutation but not *phoT9*, and one which complemented only *phoT9* but not *phoS64*.

TABLE 6. AP levels of strains carrying pAN547

Medium[a]	AP level[b]				
	ANC24 (wild type)	ANCC2 (*phoR68*)	ANCC75 (*phoS64*)	ANCC90 (*phoT9*)	ANCC4 (*phoT35*)
HP	0.007 ± 0.002	0.46 ± 0.03	2.50 ± 0.18	2.28 ± 0.09	0.01 ± 0.001
LP	5.57 ± 0.18	0.22 ± 0.02	1.61 ± 0.22	3.56 ± 0.11	2.39 ± 0.10

[a] TG medium supplemented with excess phosphate (HP) or limited phosphate (LP).
[b] Micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein ± standard deviation.

Therefore, we conclude that the *phoS* and *phoT* mutations are located in different genes.

Our results are contradictory to the conclusion drawn by Levitz et al. that *phoS* and *phoT* are not separate cistrons (17). Their conclusion was based on their result that F'111 *phoS* and F'111 *phoT* did not complement *phoT* and *phoS* recipients, respectively. Although these workers used *recA* strains for recipients, they could not rule out some genetic rearrangements, such as deletion or insertion involving transposable genetic elements, since these processes have been shown to be *recA* independent. Their conclusion was also based on several assumptions, some of which were not substantiated by experimental evidence. We have also experienced difficulties in complementation tests with large F' factors such as F'111 due to instability of the plasmid state. We tried to test complementation between *phoS* and *phoT* alleles by isolating F' factors as described by Low (18). The strain Hfr P13(PO104) *ilv*$^+$ *phoS phoT*$^+$ (or *phoS*$^+$ *phoT*) *tna*$^+$ *cys* was crossed with F$^-$ *ilv*$^-$ *phoS*$^+$ *phoT*$^-$ (or *phoS phoT*$^+$) *tna cys*$^+$ and *recA*, and colonies Pho$^-$ (white after spraying with a chromogenic AP substrate) were observed among Ilv$^+$ Cys$^+$ recombinants. Some of them were found to be also Tna$^+$. However, we could not confirm the fertility of the recombinants, apparently because of their instability.

Cox et al. (5) reported that, based on complementation tests with partial diploids, there are three complementation groups in addition to the *phoS* group, represented by the alleles *pstA2*, *phoT32*, and *pstB401*, and all four genes are part of an operon. The gene order was reported to be *pstA-(pstB phoT)-phoS . . . uncDC*, with the *pstA* gene being promoter proximal. Our present work indicates that the gene order in this region is *bglB . . . phoU-(pst-2 phoT)-phoS*. The *pst-2* mutation was refered to as *pstA2* by Cox et al. (5). We have not tested the *pstB401* mutation. We believe that the promoter of the *phoS* gene is not located near *pstA* but instead is on the *PstI*$_2$-*MluI*$_1$ fragment which contains the *phoS* gene (Fig. 2). Plasmid DNA of pSN518 (Fig. 2) was digested with *Eco*RI and *Mlu*I, and the resulting single-stranded DNA ends were filled in by using the four deoxyribonucleoside triphosphates and T4 DNA polymerase. The blunt ends of the DNA were ligated with T4 DNA ligase. This plasmid (pSN5182) complemented a *phoS* mutation, as judged by the repression of AP synthesis and the production of the phosphate-binding protein (Morita et al., unpublished data). This suggests that a promoter and signals for initiation of translation must be located within the 1.3-kb *PstI*$_2$-*MluI*$_1$ segment containing the *phoS* gene. Furthermore, it is likely that the *pstA* (*pst-2*) region and the *phoU* (*phoT35*) gene comprise independent operons, since deletion of the region containing *pstA* or *phoU* did not prevent the remaining gene from complementing the appropriate mutation (Fig. 2 and Table 7).

The restriction map of the cloned fragment and complementation tests revealed that the *phoS* gene is located on a 1.3-kb *PstI*$_2$-*MluI*$_1$ segment and the *phoT* gene on a 2.6-kb *HpaI*$_2$-*HpaI*$_3$ segment. Since the *phoT* gene overlapped the *MluI*$_1$ cleavage site, the length of the *phoS* gene is presumed to be less than 1.3 kb. The maximal length of *phoS* identified in the present studies, is only slightly more than the calculated size of the gene coding for the *phoS* precursor protein of molecular weight 39,000 (estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Morita et al., manuscript in preparation).

The *phoT35* mutation is constitutive for AP synthesis and produces *phoS* protein (10, 33) and therefore was classified as *phoT*. Zuckier and Torriani (33) reported that *phoS* and *pst-2* mutants are deficient in the phosphate-specific transport system, whereas the *phoT35* mutant is not. Because of its distinct phenotype and separate position on the chromosome, they have suggested that the *phoT35* represents a gene different either from *phoT* or *pst-2*. We could not separate the *phoT* gene from the *pst-2* gene physically, since all deletion plasmids which complemented *phoT* also complemented the *pst-2* mutation. We have shown in this paper that the DNA fragment which complements *phoT35* is separable from those that complement *phoT9* or *pst-2*. Therefore, from the work of Zuckier and Torriani and our present results, we proposed to designate *phoT35* as a new gene, *phoU*.

Cells containing multicopy *phoS* plasmids

TABLE 7. AP levels of GS5(*pit-1 pst-2*) carrying various hybrid plasmids

Medium[a]	AP level[b]			
	pSN507 (*phoS*$^+$ *phoT*$^+$ *phoU*$^+$)	pSN537 (*phoT*$^+$)	pSN547 (*phoU*$^+$)	pSN567
HP	0.006 ± 0.001	0.147 ± 0.011	1.03 ± 0.14	2.44 ± 0.02
LP	0.013 ± 0.001	0.165 ± 0.013	1.34 ± 0.08	2.68 ± 0.15

[a] TG medium supplemented with DL-glycerol-3-phosphate (0.04%) and with either excess (HP) or limited (LP) phosphate.

[b] Micromoles of *p*-nitrophenol liberated per minute per milligram of cellular protein ± standard deviation.

showed decreased levels of AP even under derepressed conditions. The strong inhibition by *phoS* plasmids was more evident when the host was a *phoR* strain. When *phoS*$^+$ plasmids were introduced into a *phoT* strain, the generation time was increased three to four times relative to the *phoT*$^+$ strain carrying the same plasmids. The prolonged generation time was not observed when the plasmids contained both *phoS* and *phoT* genes. Our preliminary analysis of the cellular proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggested that a large amount of *phoS* protein was produced in the *phoS* mutant carrying the *phoS*$^+$ plasmid. Excess phosphate-binding protein produced by the multicopy *phoS* gene seems to cause adverse physiological effects. The *phoT* gene might play a regulatory role in *phoS* gene expression, or the *phoT* gene product might work cooperatively with *phoS* protein.

We cloned the *phoS* gene while we were trying to clone the *phoR* gene on pBR322. During the course of the attempts, we have cloned four different chromosomal DNAs on pBR322 which repress AP synthesis in the *phoR68* mutant grown on TG plates with excess phosphate. One of them was found to contain *phoS* gene. The remaining three showed different restriction cleavage patterns, and we have not yet characterized them. They might carry one of the genes similar to *phoS* or *phoA* which are regulated by phosphate and by the regulatory genes, such as *phoB* and *phoR*. Such genes might compete with *phoA* for a positive regulatory factor, such as *phoB* gene product. Their gene products might compete for processing machinery with *phoA* if they are genes coding for secretory proteins. The effect of these genes on AP synthesis in the *phoR68* mutant is likely to be due to the increased gene dosage or amount of the gene product as the result of their presence on the high-copy-number plasmid pBR322.

Since all the *E. coli* strains used in the present work were Rec$^+$, the complementation experiments might reflect genetic recombination rather than true complementation. We think this is unlikely. The *phoR68* mutant was complemented by the *phoS* plasmid (pSN518). This strain recovered AP constitutivity after it was cured of the plasmid. The plasmid prepared from the transformant was introduced into the *phoS* and *phoT* mutants; it still complemented the *phoS* mutation but not the *phoT* mutation.

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