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Cloning and Characterization of the *Escherichia coli* Gene Coding for Alkaline Phosphatase

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The *Escherichia coli* structural gene for alkaline phosphatase, *phoA*, and a promoter-like mutant of *phoA*, called *pho-1003*(Bin) *phoA*$^+$, were cloned by using plasmid vectors. Initially, these genes were cloned on deoxyribonucleic acid fragments of 28.9 kilobases (kb). Subsequently, they were subcloned on fragments of 4.8 and then 2.7 kilobases. A restriction map was developed, and *phoA* was localized to a 1.7-kb region. The promoter end of the gene was inferred by its proximity to another gene cloned on the same deoxyribonucleic acid fragment, *proC*. The stability of the largest plasmid (33.3 kb) was found to be *recA* dependent, although the subcloned plasmids were stable in a *recA*$^+$ strain. Synthesis of alkaline phosphatase directed by the *phoA*$^+$ and *pho-1003*(Bin) *phoA*$^+$ plasmids in a *phoA* deletion strain was assayed under repressing and derepressing levels of phosphate. These data were compared with the copy numbers of the plasmids. It was found that synthesis of alkaline phosphatase was tightly regulated, even under derepressing conditions: a copy number of 17 enabled cells to synthesize only about twofold more enzyme than did cells with 1 chromosomal copy of *phoA*$^+$. Enzyme levels were also compared for cells containing *pho-1003*(Bin) *phoA*$^+$ and *phoA*$^+$.

In *Escherichia coli*, alkaline phosphatase (AP) is coded for by the *phoA* gene, which is located between 8 and 9 min on the *E. coli* map (2). Its synthesis is believed to be regulated by both a negative regulatory element, coded for by *phoR* (11, 15), and positive elements, coded for by *phoR*, *phoB*, and *phoM* (4, 22, 36; B. L. Wanner and P. Latterell, Genetics, in press). *phoB* and *phoR* are separated from *phoA* by *proC* (2). In addition, AP synthesis is repressed by P_i by an unknown mechanism (30). Several other genes, *phoS* and *phoT*, are involved in the transport and accumulation of P_i (34). The regulation of AP biosynthesis is thus complex and is not well understood.

AP is synthesized as a precursor which is processed and secreted into the periplasmic space (13, 17, 27). The amino acid composition of the leader is partially known (23), but nothing is known of the promoter region of *phoA*.

One approach to a better understanding of the regulation of AP biosynthesis and the structure of the promoter-leader region of the *phoA* gene is to clone *phoA* and a promoter mutant on plasmid vectors. This is a first step toward the eventual sequencing of the DNA regions of interest. This paper describes the cloning and restriction enzyme mapping of both *phoA* and *pho-1003*(Bin) *phoA*$^+$, a promoter-like mutant independent of the *phoB* gene product (32). The levels of AP synthesized by plasmids containing the cloned genes were measured and compared with the copy numbers of the respective plasmids.

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MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The strains, bacteriophages, and plasmids used and their relevant genotypes are given in Table 1. Strain Xph90a, which contains deletion E15 within *phoA* (*phoA8*), was obtained from J. Beckwith. PEB100 is a *recA* derivative of Xph90a constructed as follows: Xph90a was treated with trimethoprim to obtain a *thyA* derivative (18) and then used as the recipient in mating with strain KL16-99, a *thyA*$^+$ *recA* strain; then Xph90a colonies were selected which were *thyA*$^+$ and scored for *recA*, using plates containing nitrofurantoin (14). CB1 is a *proC* derivative of Xph90a constructed by using bacteriophage P1 to transduce *lac*$^+$ *proC* from strain χ342 into Xph90a, which is Δ*lac-169* *proC*$^+$. Lac$^+$ colonies were detected on MacConkey agar and screened for Pro$^-$. CB3 was constructed as follows: phage P1 grown on strain C90 (*proC*$^+$ *phoA*$^+$ *phoT9*) was used to transduce CB1; the transductants were selected for *proC*$^+$ and scored for *phoA*$^+$. C90 was used as the donor of *phoA*$^+$, since Xph90a and C90 have the same parent, strain K10 (C. Pratt, personal communication).

DNA isolation. Covalently closed circular DNA

TABLE 1. *Bacterial strains, phages, and plasmids*

Strain, phage, or plasmid	Relevant marker(s)[a]	Source
Strain		
Xph90a	*ΔphoA8 Δlac-169*	J. Beckwith
KL16-99	*recA21*	A. Markovitz
C90	*phoA⁺ phoT⁻*	C. Pratt (12)
χ342	*lac⁺ proC*	R. Curtiss III
PEB100	*ΔphoA8 recA21*	These experiments (see text)
CB1	*ΔphoA8 proC*	These experiments (see text)
CB3	*phoA⁺ proC*	These experiments (see text)
Phage		
λp *phoA⁺*		J. Beckwith (23)
λp *pho-1003*(Bin) *phoA⁺*		J. Beckwith (Wanner and Latterell, in press)
Plasmid[a]		
pBR322	Ap^r Tc^r	H. Boyer (7)
pACYC177	Ap^r Km^r	S. Cohen (9)

[a] Abbreviations used for drug resistance are Ap^r (ampicillin), Tc^r (tetracycline), and Km^r (kanamycin).

was isolated and purified as described by Berg et al. (6).

Media. Antibiotic screening was performed on Penassay plates, using ampicillin at 100 μg/ml and tetracycline at 15 μg/ml.

Plate assays to detect cells producing AP were performed with medium 121 supplemented with 83 μM phosphate (15). The indicator dye was 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine (XP) (8), obtained from Sigma Chemical Co. XP was dissolved in *N,N*-dimethylformamide to give 20 mg/ml then added to minimal medium at a final concentration of 40 μg/ml. Colonies producing AP were blue, and colonies unable to produce AP were white.

Restriction enzymes. All restriction enzymes were obtained from Bethesda Research Laboratories (BRL). Single digests were performed in the buffer recommended by BRL. For double digests, the following buffers were used: for *Hin*dIII-*Bgl*II and *Bgl*II-*Bam*HI, 100 mM Tris (pH 7.4)–10 mM $MgCl_2$–6 mM β-mercaptoethanol; for *Bam*HI-*Xor*II and *Xor*II-*Xho*I, 6 mM Tris (pH 7.4)–12 mM $MgCl_2$–6 mM β-mercaptoethanol–50 μg of bovine serum albumin per ml; for *Xho*I-*Hin*dIII, 20 mM Tris (pH 7.4)–60 mM NaCl–7 mM $MgCl_2$–6 mM β-mercaptoethanol. All digestions were at 37°C for 60 min.

Construction of recombinant plasmids. DNAs were cut with the restriction enzymes described in Results. In some cases the vector was pretreated with calf intestinal AP to remove 5′-terminal phosphates and to prevent recircularization of the plasmid (31). This enzyme was obtained from Sigma.

Ligation was performed with T4-induced DNA ligase (33) from BRL. Reactions were performed at 22°C for 60 min.

Bacterial cells were made competent for transformation by the procedure of Lederberg and Cohen (16).

Agarose gel electrophoresis. Plasmids were analyzed on 1.0% agarose gels, using Tris-acetate buffer (400 mM Tris, 120 mM sodium acetate, 25 mM disodium-EDTA, pH 7.8). Uncut plasmid DNA from colonies scraped from plates was prepared, using a modification of the quick lysis method of Telford et al. (29).

AP assays. Cells were grown for 24 h in medium 121 containing repressing levels of phosphate (10 mM K_2HPO_4) or depressing levels of phosphate (0.1 mM K_2HPO_4). They were then treated with toluene and assayed as described by Kreuzer et al. (15). Units of alkaline phosphatase were calculated by the formula (15): units = (change in optical density at 410 nm/min) × dilution factor × (1/optical density at 540 nm).

Plasmid copy number. All cells were grown in M9 minimal medium (1) supplemented with tryptophan (50 μg/ml) and thiamine (10 μg/ml). The growth medium for CB1(pPB100) and CB1(pPB101) lacked proline, whereas that for CB1(pPB106), CB1(pBR322), and CB1(pACYC177) contained proline (50 μg/ml) and ampicillin (100 μg/ml). DNA was labeled with 5 μCi of [^{3}H]thymidine (45 Ci/mmol) per ml in the presence of deoxyadenosine. Cells were harvested during the log phase of growth, and 2.5 ml was lysed and centrifuged as described by Womble et al. (35). The copy number was determined from two or more experiments, with a variation of ±15%.

RESULTS

Cloning and restriction enzyme mapping of the *phoA* gene. DNAs from λp *phoA⁺* and λp *pho-1003*(Bin) *phoA⁺* were cut with the restriction enzyme *Hin*dIII and mixed with DNA from the vector pBR322 which had been cut with *Hin*dIII and pretreated with calf intestinal AP, as described in Materials and Methods. These DNA fragments were ligated and used to transform strain Xph90a (Table 1). Preliminary experiments showed that plates containing the indicator dye for AP (XP) could not be used for selecting ampicillin-resistant colonies due to a significant decrease in the effective ampicillin concentration. Therefore, primary selection was performed on complex medium containing ampicillin (100 μg/ml), and screening was performed on plates containing XP. In addition, colonies were screened for tetracycline resistance, since *Hin*dIII has one recognition site in pBR322 and this site is in the gene coding for

tetracycline resistance (7). Loss of tetracycline resistance was an indication of the insertion frequency. In this experiment it was found that between 90 and 95% of colonies which were ampicillin resistant were tetracycline sensitive. Approximately 2% of the ampicillin-resistant colonies were also AP positive on the indicator plates.

The plasmid DNAs from several AP-positive colonies were purified by using cesium chloride-ethidium bromide gradients and analyzed by agarose gel electrophoresis. These DNAs, from both the λp *phoA*$^+$ cloning and the λp *pho-1003*(Bin) *phoA*$^+$ cloning, were cleaved with *Hin*dIII. The molecular sizes of the two fragments observed in each case corresponded to linear pBR322 (4.4 kilobases [kb]) and to a λ fragment greater than 20 kb.

The largest *Hin*dIII fragment of λ is approximately 57.1% of the λ genome when λ's cohesive ends were annealed, giving a size of 28.0 kb (10). The size of one of the plasmids originating from λp *phoA*$^+$, called pPB100 (Table 2), was determined by cleaving the DNA with different combinations of restriction enzymes with known recognition sites in λ and with *Xho*I (see below). The following double digests were used: *Hin*dIII-*Bgl*II, *Bgl*II-*Bam*HI, *Bam*HI-*Xor*II, *Xor*II-*Xho*I, and *Xho*I-*Hin*dIII. The inserted fragment was 28.9 kb, and the plasmid was therefore 33.3 kb.

Preliminary restriction mapping of pPB100 indicated the loss of at least two sites present in λ, one *Bam*HI site and one *Bgl*II site, as well as the appearance of a new *Xho*I site (data not shown). The new *Xho*I site and the deleted *Bam*HI and *Bgl*II sites all mapped in the *Hin*dIII-to-*Sma*I segment of λ, indicating that the *phoA* gene should be in this region (Fig. 1). Similar data were determined from the plasmid containing the cloned *pho-1003*(Bin) *phoA*$^+$ gene.

To subclone *phoA*$^+$ on a smaller DNA fragment, with the eventual aim of sequencing the promoter-leader region, pPB100 and pPB200 were cleaved with *Hin*dIII and *Sma*I for subcloning into the vector pACYC177. This plasmid is 3.7 kb, codes for resistance to ampicillin and kanamycin, and contains single sites for both *Hin*dIII and *Sma*I (9). Both of these sites are in the gene coding for kanamycin resistance, and the double digest deletes a segment of DNA of 0.15 kb. After cleavage, DNA from pPB100 or pPB200 was ligated to pACYC177 and used to transform Xph90a with primary selection on Penassay plates containing ampicillin. The *Hin*dIII-*Sma*I fragment was not able to reinsert into pBR322, since there is no recognition site for *Sma*I in pBR322. Colonies were screened on XP-containing plates for production of AP. AP-positive colonies were screened by a quick lysis method for plasmid isolation (see Materials and Methods and reference 29) to detect plasmids having the expected molecular size of about 8 kb. Several plasmids having the correct size were observed from both subclonings. One colony from each was analyzed further. The plasmid containing *phoA*$^+$ was pPB101, and the plasmid containing *pho-1003*(Bin) *phoA*$^+$ was pPB201 (Table 2). The molecular size of the inserted fragment was determined by cleavage of pPB101 or pPB201 with *Hin*dIII and *Sma*I. In contrast to the size of the λ fragment produced by cleavage with *Hin*dIII and *Sma*I, which is 3.8 kb, the cloned fragment was 4.8 kb.

Restriction enzyme mapping of the *Hin*dIII-*Sma*I fragment with single and double digests yielded the information in Fig. 1. Of the original sites on λ, the following were deleted and replaced by chromosomal DNA: *Bgl*II (0.7 kb), *Bam*HI (0.8 kb), *Hpa*I (1.3 kb), and *Sst*II (1.5, 2.6, and 2.7 kb). Although there were two *Eco*RI sites (1.1 and 1.4 kb) and a *Hin*cII site (0.6 kb) near *Hin*dIII on pPB101, these sites in λ are flanked by sites deleted in pPB101. This indicated that the *Eco*RI sites and the *Hin*cII site were on chromosomal DNA. Other evidence, given below, substantiated this. Therefore, DNA from at least 0.7 (*Bgl*II) to 2.7 (*Sst*II) kb on λ had been replaced by chromosomal DNA. The 3.0-kb *Hin*cII site from λ could have been present on pPB101.

The *Hin*dIII-*Xho*I fragment was subcloned into pACYC177. There is one site for each of

TABLE 2. *Plasmids containing the cloned phoA*$^+$ *and pho-1003(Bin) phoA*$^+$

Plasmid	Genotype	Fragment[a]	Fragment size (kb)	Vector
pPB100	*phoA*$^+$	*Hin*dIII	28.9	pBR322
pPB200	*pho-1003*(Bin) *phoA*$^+$	*Hin*dIII	28.9	pBR322
pPB101	*phoA*$^+$	*Hin*dIII/*Sma*I	4.8	pACYC177
pPB201	*pho-1003*(Bin) *phoA*$^+$	*Hin*dIII/*Sma*I	4.8	pACYC177
pPB106	*phoA*$^+$	*Hin*dIII/*Xho*I	2.7	pACYC177
pPB206	*pho-1003*(Bin) *phoA*$^+$	*Hin*dIII/*Xho*I	2.7	pACYC177

[a] The fragment cloned is identified by the restriction enzyme(s) used for its construction.

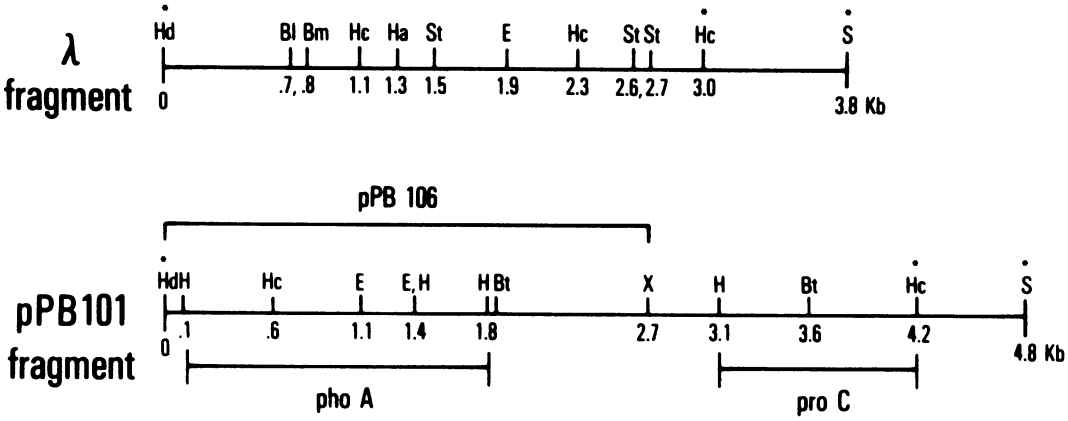

FIG. 1. *Comparison of the restriction map of λ and the cloned DNA fragment from λ containing the phoA⁺ gene. The λ map is from Daniels et al. (10) and Szybalski and Szybalski (28). Abbreviations for the restriction enzymes: E, EcoRI; Bm, BamHI; Bl, BglII; Bt, BstII; H, HaeII; Hc, HincII; Hd, HindIII; Ha, HpaI; S, SmaI; St, SstII; X, XhoI. The asterisks above Hd, Hc, and S indicate that these sites were probably the same on both maps. Sites for HaeII and BstIII at 1.8 kb were indistinguishable by gel analysis.*

these enzymes in pACYC177, both within the gene coding for kanamycin resistance. A double digest by *Xho*I and *Hin*dIII deleted a DNA segment from pACYC177 of 0.3 kb. The vector and pPB101 or pPB201 were cleaved, ligated, and used to transform strain Xph90a. Primary selection was on plates containing ampicillin followed by screening for AP-positive colonies on XP plates. Blue colonies were picked and tested for the presence of a plasmid of 6 kb by quick colony lysis (29). Plasmids subcloned from both pPB101 (*phoA*⁺) and pPB201 [*pho-1000*(Bin) *phoA*⁺] were purified and then analyzed by agarose gel electrophoresis. After cleavage with *Hin*dIII and *Xho*I, the size of the *Hin*dIII-*Xho*I fragment was 2.7 kb (Table 2). Therefore, the new *Xho*I site did not cleave the *phoA* gene and the *Hin*dIII-*Xho*I subcloning cleaved 2.1 kb from the *Hin*dIII-*Sma*I fragment.

To further delineate the *phoA* gene, it was important to find sites which did cleave the gene. The *Eco*RI sites were tested, and at least one was found to be within the *phoA* gene. pPB101 contained two sites for *Eco*RI, both within the cloned DNA. If pPB106 was cleaved with *Eco*RI and inserted into pBR322 at its *Eco*RI site, the joint plasmid was about 10 kb and conferred resistance to ampicillin and tetracycline. Colonies which were resistant to tetracycline were screened on XP plates; none was AP positive. Colonies were also screened by the whole colony lysis technique (29) to determine the molecular sizes of their plasmids. Plasmids of 10 kb were found, but they were phenotypically AP negative. Plasmid DNA from one such colony was isolated and then cleaved with *Eco*RI, yielding one fragment of 4.4 kb from pBR322 and one of 5.8 kb from pPB106. The DNA between 1.1 and 1.4 kb was lost during the subcloning. Therefore, the *Eco*RI site at 1.1 kb was within *phoA*; these data did not give information about the 1.4-kb site.

The *Hin*cII site at 0.6 kb was also found to be within the *phoA* gene. DNA from pPB201 was cleaved with *Hin*cII to give a fragment of 3.6 kb. This was inserted into the single *Hin*cII site on pACYC177, which is in the gene coding for ampicillin resistance. Colonies which were kanamycin resistant and ampicillin sensitive were scored on XP plates. All were AP negative. In addition, colonies were screened for plasmid DNA by the whole colony lysis technique (29), and colonies containing a plasmid of approximately 7 kb were retested on XP plates. Again, all were AP negative. To verify that these plasmids contained the *Hin*cII fragment from pPB201, plasmid DNA was purified from one colony and cleaved with *Hin*cII, yielding the two expected fragments of 3.6 kb for the *Hin*cII fragment and 3.7 kb for pACYC177.

Finally, a *Hae*II digest of pPB106 was performed with ligation of the resulting fragments. Plasmid selection was for ampicillin resistance. One thousand colonies were scored on XP plates for AP, and only one colony which was AP positive was found. Plasmid DNA from this colony was isolated and found to contain the DNA fragment of 1.7 kb extending from the 0.1-kb *Hae*II site to the 1.8-kb *Hae*II site. This DNA segment included the *Hae*II site at 1.4 kb, indicating that the *Hae*II (1.4 kb) site was probably within the *phoA* gene. This supported the idea that both *Eco*RI sites (1.1 and 1.4 kb) were within *phoA*.

Figure 2 presents a summary of the restriction map of pPB101. The map of pPB201 was identical. Included on this map is *proC*, which was located on the *Sma*I-*Xho*I fragment (unpublished data). The *proC* gene was present on plasmids pPB100, pPB200, pPB101, and pPB201.

Stability of the cloned fragments. A large number of white colonies (AP negative) were observed when cells containing pPB100 or pPB200 were streaked on XP plates. When these colonies were tested on ampicillin-containing plates, they were unable to grow. Thus, it appeared that many colonies were losing their plasmids. This was further substantiated by whole colony lysis and agarose gel electrophoresis, since no plasmid DNA was detected from AP-negative colonies (data not shown). In contrast, colonies containing pPB101 or pPB106 did not appear to segregate many AP-negative colonies. For this reason, a more systematic approach was taken to study stability.

Cells containing plasmids were grown to mid-log phase, approximately three generations, in Penassay broth containing 100 μg of ampicillin per ml. They were then diluted 1,000-fold into broth containing or lacking ampicillin and grown for 15 generations. Cells containing pPB100, pPB101, or pPB106 were plated on XP-containing plates both before the initial 3 generations of growth and after 15 generations of growth.

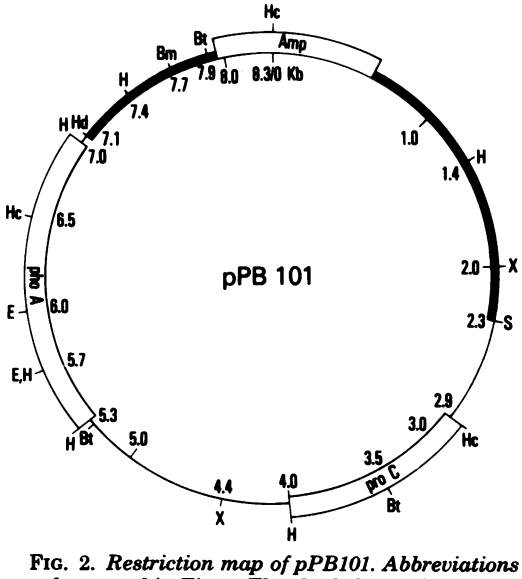

FIG. 2. *Restriction map of pPB101. Abbreviations are those used in Fig. 1. The shaded area from 7.1 to 2.3 kb represents the vector, pACYC177. Restriction enzyme sites on pACYC177 are from Chang and Cohen (9) and these experiments. For restriction enzyme abbreviations, see the legend to Fig. 1.*

They were scored as AP negative or AP positive. Controls containing pBR322 and pACYC177 were plated on nonselective plates and scored on plates containing ampicillin. The results of two or more independent assays for each plasmid tested are shown in Table 3.

Both vectors, pBR322 and pACYC177, were stable. However, results for pPB100 showed that even before growth, 11% of the cells had become AP negative; i.e., 11% of the cells taken from a plate containing 100 μg of ampicillin per ml which were freshly streaked and incubated overnight appeared to have lost their plasmids. After 15 generations of growth in broth containing ampicillin, no more cells had become AP negative. Thus, if the number of AP-positive cells was normalized to 100% before growth in broth, the percentage of AP-positive cells was 100% after growth in broth with the drug. However, the normalized value for Xph90a(pPB100) after 15 generations of growth in broth lacking drug was 85%. One hundred AP-negative colonies were tested and found to be ampicillin sensitive, indicating loss of the plasmid. In contrast, pPB101 and pPB106 were from 97 to 100% stable.

To test whether the stability of pPB100 was *recA* dependent, a *recA* derivative of Xph90a was constructed (see Materials and Methods). The assays (Table 3) showed that pPB100 was stable in this *recA* strain.

Attempts were made to further destabilize pPB100. If pPB100 was streaked from a drug-containing plate to a nonselective plate and then grown 15 generations without the drug, only 33% of the cells retained the plasmid (data not shown). This result reinforced the observations above showing the instability of pPB100 and the importance of maintaining selective pressure at all times.

AP synthesis. Strain CB1 (Δ*phoA8 proC*) and derivatives constructed by transferring all plasmids into CB1 were assayed for biosynthesis of AP under repressing and derepressing phosphate levels. Strain CB3, a *phoA*$^+$ derivative of Xph90a (Table 1), was used as a positive control. Table 4 shows the results of these experiments.

Under derepressing conditions, strain CB3 synthesized 0.5 to 2.5 times less AP than did the plasmid-containing strains. Cells containing pPB200 and pPB201 [*pho-1003*(Bin) *phoA*$^+$] produced about 1.5-fold more enzyme than did pPB100 and pPB101 (*phoA*$^+$), whereas the amounts were similar for pPB106- and pPB206-containing cells.

Under repressing conditions, strain CB3 produced 0.04 U of AP. However, the plasmid-containing strains were not fully repressed. In one

TABLE 3. *Stability of the cloned phoA gene*

Strain	Plasmid	Plasmid stability (%)[a]		
		Before growth	After growth:	
			With ampicillin	Without ampicillin
Xph90a (*recA*$^+$)	pBR322	100	100	100
	pACYC177	100	100	100
	pPB100	89 (100)	89 (100)	76 (85)
	pPB101	97 (100)	97 (100)	94 (97)
	pPB106	100 (100)	100 (100)	99 (100)
PEB100 (*recA*)	pPB100	98 (100)	100 (100)	95 (97)

[a] Percentage of AP-positive colonies in a population. The numbers in parentheses are the data normalized to the stability before growth.

TABLE 4. *Alkaline phosphatase synthesis*[a]

Strain	Plasmid	AP (U)	
		Derepressed	Repressed
CB3	None	6.4	0.04
CB1	None	0.05	0.05
CB1	pPB100	9.1	1.1
CB1	pPB200	13.8	9.7
CB1	pPB101	10.6	0.4
CB1	pPB201	15.7	1.8
CB1	pPB106	12.9	0.3
CB1	pPB206	13.4	1.4

[a] Cells were grown in medium 121 either lacking proline (pPB100, pPB200, pPB101, and pPB201) or supplemented with proline (pPB106 and pPB206). Media for plasmid-containing strains contained ampicillin at a final concentration of 100 μg/ml. Details of the assay are given in the text. The data are the averages of two or more experiments each.

TABLE 5. *Copy numbers of plasmids containing the cloned phoA gene*

Plasmid	Copy no.[a]
pBR322	20
pACYC177	21
pPB100	2
pPB101	15
pPB106	17

[a] The copy number was measured as described in the text.

case, pPB200, the amount of enzyme synthesized was 1.5-fold higher than CB3 when it was derepressed.

Plasmid copy number. To determine whether the level of AP synthesis could be correlated with the number of copies of *phoA*, the copy numbers of plasmids pBR322, pACYC177, pPB100, pPB101, and pPB106 were measured (Table 5). The copy numbers of pBR322 and pACYC177 were both about 20 per chromosome. The copy number of pPB100, derived from pBR322, was only 2, whereas the copy numbers for pPB101 and pPB106 were 15 and 17, respectively. These numbers were all significantly lower than the copy numbers of the parental plasmids.

DISCUSSION

Cloning of *phoA*$^+$ and *pho-1003*(Bin) *phoA*$^+$ from λ phage containing the integrated genes shows that the *phoA* gene integrated between the *Sma*I site at 40.7% on the λ map and the *Hin*dIII site at 48.4% on the λ map. This is in the *b*2 region of λ (10, 28). The resultant DNA fragment which was cloned was 28.9 kb, and subsequent subcloning reduced its size to 2.7 kb.

Restriction mapping of the cloned region was performed (Fig. 1). Results indicate that the *phoA* gene lies between the 0.1- and the 1.8-kb *Hae*II sites and is cleaved by *Hin*cII (0.6 kb), *Eco*RI (1.1 kb), and probably *Hae*II and *Eco*RI (both near 1.4 kb). This places *phoA* on DNA of 1.7 kb. The theoretical size of DNA required for *phoA* can be calculated based on the size of a subunit of AP, which is 43,000 daltons (20); the length of the leader region, which for other secreted proteins, such as the maltose binding protein, is 20 to 30 amino acids (3); and the length of the promoter region, which for a model sequence based on 17 procaryotic promoter sequences is about 45 bases (26). The predicted length of DNA required for the *phoA* gene is about 1.4 kb, using the above information. Thus, *phoA* fits well within the 1.7-kb *Hae*II fragment.

It has been shown that the direction of transcription of the *phoA* gene is clockwise relative to the *E. coli* linkage map (25). This places the promoter region of *phoA* at the 0.1-kb-*Hae*II-site end of the gene (see Fig. 2).

There is a marked difference in stability among the plasmids containing the cloned *phoA* gene (Table 3). pPB100 is less stable than either pPB101 or pPB106, but can be stabilized if put into a *recA* mutant. It is known that *recA* promotes homologous recombination. If the insta-

bility of pPB100 is due to homologous recombination, there is no evidence that this recombination is between pPB100 and the chromosome, since cells become both AP negative and ampicillin sensitive. It is possible that there is recombination either within pPB100 or between two copies of pPB100. It has been reported that recombination in λ can occur either inter- or intramolecularly when λ carries a tandem duplication (5). The resulting bacteriophage contains one or more duplications or a deletion. This process only occurs in a *recA*$^+$ host. If there is a tandem duplication in the cloned λ DNA resulting in a deletion of DNA, this could account for the observed instability of pPB100 and pPB200 in a *recA*$^+$ host. In addition, recombinant λ phages have been isolated (21) that resulted from recombination between λ and pBR322. There were two classes of recombinants, one due to recombination at the phage attachment site (not present in pPB100 or pPB200) and the other due to plasmid integration at other sites on λ. Of the seven nonattachment sites mapped on λ, six are within the region cloned in pPB100 and pPB200. Therefore, it is quite possible that recombination could occur between λ and pBR322 sequences in the constructed plasmids either intra- or intermolecularly, causing loss of the plasmid. Since the average copy number of pPB100 is only two, some cells would be expected to entirely lose their plasmids. Subcloning of the large *Hin*dIII fragment seems to have deleted at least one of the sequences required for recombination, since pPB101 and pPB106 are stable in a *recA*$^+$ host.

Synthesis of AP was assayed in strain CB1 containing each of the chimeric plasmids (Table 4). These strains, under derepressing conditions, produced more enzyme than did strain CB3, which has one chromosomal copy of *phoA*$^+$. This increase is probably due to the increased number of copies of the *phoA*$^+$ gene per cell in plasmid-containing strains. However, the increase is not proportional to the copy number, since pPB101, for example, has a copy number of 15 but makes only 1.7-fold more enzyme than does CB3, whereas pPB100 has a copy number of 2, yet it produces 1.4-fold more enzyme than does CB3. This indicates tight cellular control of synthesis of AP, which could be at the level of transcription, translation, or post-translation (processing of the precursor).

The enzyme levels for the *pho-1003*(Bin) *phoA*$^+$-containing strains are higher than those for the *phoA*$^+$ strains. This is consistent with *bin* being a promoter mutation allowing synthesis of AP independent of the *phoB* gene product. However, the amount of increase ranges from only 0.1 U (pPB206) to 1.5-fold (pPB200 and pPB201) for the *pho-1003*(Bin) *phoA*$^+$-containing strains compared with the *phoA*$^+$-containing strains. If, in fact, *pho-1003*(Bin) *phoA*$^+$ is a promoter mutation, then additional factors regulate the synthesis of AP, in agreement with the data discussed above.

In contrast with the expression of AP, levels of tryptophan synthetase increased up to 16.3-fold that of control cells under partial derepression when the tryptophan operon was cloned in RSF1010 (copy number, 11) (19). Even tryptophan synthetase activities were not proportional to copy number, however.

Repression of AP synthesis is complete for strain CB3, but not for the plasmid-containing strains. This can be explained if the increased number of *phoA*$^+$ copies in the plasmid-containing strains titrate the repressor molecule(s). The higher level of enzyme synthesis directed by pPB200 could be due to read through from a pBR322 promoter. The orientations of the *Hin*dIII fragment in pPB100 and pPB200 were determined by cleavage with *Sal*I, which cleaves only pBR322 once, and *Sma*I, which cleaves only the *Hin*dIII fragment once (data not shown). In pPB100, the *phoA* gene is near the *Eco*RI site of pBR322, but it is near the *Bam*HI site of pBR322 in pPB200. Thus, the *Hin*dIII fragment is in opposite orientations in pPB100 and pPB200. Read through from a pBR322 promoter is possible, since the promoter end of *phoA* is 0.1 kb or less from the *Hin*dIII junction with pBR322 and, if it occurred in pPB200, would not affect transcription of *phoA* in pPB100, which is in the opposite orientation.

In summary, cloning of *phoA*$^+$ and *pho-1003*(Bin) *phoA*$^+$ has given the following information: (i) the *phoA* gene's promoter is inferred to be near the 0.1 kb *Hae*II end of the cloned DNA, (ii) stability of the 33.3-kb plasmid PB100 is *recA* dependent, and (iii) AP synthesis is tightly regulated so that even when there are 15 to 17 copies of the gene, enzyme levels increase only up to 2.5-fold.

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