Effects of *pho* Regulatory Mutations and *phoA* Gene Amplification on Alkaline Phosphatase Synthesis and Release by *lky* Mutants of *Escherichia coli* K12

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lky mutants of *Escherichia coli* K12 spontaneously released alkaline phosphatase (APase) into the extracellular medium to give up to 300 units ml⁻¹. APase is a phosphate repressible periplasmic enzyme encoded by the gene *phoA*. With a view to establishing a method of easy purification, we have analysed APase synthesis and release patterns of isogenic *lky* strains containing either a constitutive *pho* regulatory mutation, or a hybrid plasmid carrying the structural gene *phoA*⁺ and *pho* regulatory genes, or a transducing $\phi 80$ *phoA*⁺ phage. In the presence of the *phoS2333* mutation, F⁻ *lky* strains lysogenized with $\phi 80$ *phoBin phoA*⁺ phage and grown in high phosphate medium were able to release eight times more APase activity (2300 units ml⁻¹) than haploid strain 2336 (*phoS*⁺ *lky*) grown in low phosphate medium. Neither protein synthesis, the cell export machinery nor leakage mechanisms were limiting for APase release. Sufficient APase was released into the medium to facilitate its purification.

INTRODUCTION

In *Escherichia coli*, the enzyme alkaline phosphatase (EC 3.1.3.1; APase), which hydrolyses organic phosphate esters and releases inorganic orthophosphate (P_i), is localized in the periplasmic space, an extracytoplasmic cell compartment bounded by the inner (cytoplasmic) and outer membranes of the bacterial cell envelope (Lugtenberg, 1981). APase synthesis is induced by growth with limiting concentrations of P_i (Torriani, 1967). The expression of its structural gene, *phoA*, as well as that of other genes belonging to the phosphate (*pho*) regulon and coding for periplasmic (P2: *sn*-glycerol 3-phosphate binding protein; P4: phosphate binding protein) or outer membrane (PhoE) proteins is subject to a highly complex genetic control (Argast & Boos, 1980). It has been proposed that *phoA* gene expression is directly and positively regulated by the *phoB* gene product, and that *phoB* gene expression, in turn, is positively regulated by the *phoM* and *phoR* gene products, and negatively by the *phoR*, *phoS*, *phoT* (*pstA*), *pstB* and *phoU* gene products, directly or indirectly; it has also been shown that *phoB*, as well as *phoA*, is derepressed by phosphate limitation (for reviews see Tommassen & Lugtenberg, 1982; Shinagawa *et al.*, 1983; Ludtke *et al.*, 1984; Nakata *et al.*, 1984).

Translation of cell envelope proteins is initiated in the cytoplasm on membrane-bound ribosomes and transfer of these exported proteins across the inner membrane involves co-translational and/or post-translational mechanisms (for reviews see Silhavy *et al.*, 1983; Brickman *et al.*, 1984). APase translocation into the periplasmic space is carried out by a co-translational process (Inouye *et al.*, 1982).

Many Gram-positive bacteria produce exoenzymes but Gram-negative bacteria, like *E. coli*, generally do not release proteins into the culture medium (Glenn, 1976). We have described the

Abbreviations: APase, alkaline phosphatase; XP, 5-bromo-4-chloro-3-indolyl phosphate.

isolation and characterization of *lky* and *exc* mutants which are spontaneously able to release periplasmic proteins like APase and β -lactamase into the culture medium (Lazzaroni & Portalier, 1979, 1981; Fognini-Lefebvre & Portalier, 1984), and we have determined the influence of culture conditions on APase synthesis and excretion by *lky* mutants (Atlan & Portalier, 1984). In this study, we analyse the effects of *pho* regulatory mutations and *phoA* gene amplification on the properties of *lky* strains.

METHODS

Strains. All strains used in this study were derived from *Escherichia coli* K12 (Table 1). *phoS65*, *phoS27*, *phoU* (formerly called *phoT35*), *phoR69* mutations and *zib501::Tn10* insertion were generously given by B. Bachmann (*E. coli* Genetic Stock Center, New Haven, Connecticut 06515, USA). *Tn10* and *Tn5* insertions in *proC* and *gal* genes respectively, were obtained from M. Gottesman's laboratory (NIH, Bethesda, Maryland 20205, USA). $\phi 80$ *phoA*⁺ and $\phi 80$ *phoBin phoA*⁺ hybrid bacteriophages were a gift of J. Beckwith (Harvard Medical School, Boston, Massachusetts 02115, USA).

Table 1. *Bacterial strains and plasmids*

Strain	Sex	Relevant genotype or phenotype	Source
PA601	F ⁻	Thr ⁻ <i>leuC tonA proA lacY purE galT His⁻ thyA</i> <i>rpsL malA Xyl⁻ Mtl⁻ argH</i>	M. Schwartz
207	Hfr	Thi ⁻ <i>metB lacI lky-207</i>	Lazzaroni & Portalier (1979)
706	F ⁻	Thr ⁻ <i>leuC proA lky-207 His⁻ rpsL phoS2333</i> <i>argH</i>	Periplasmic-leaky mutant of strain 2333 (Atlan & Portalier, 1984)
791	F ⁻	<i>proC His⁻ rpsL argH</i>	Derivative of strain PA601; this work
2122	F ⁻	<i>proC lky-207 His⁻ rpsL phoS2333 argH</i>	Derivative of strain 706; this work
2333	F ⁻	Thr ⁻ <i>Leu⁻ proA rpsL phoS2333 argH</i>	APase constitutive mutant of strain PA601 (Atlan & Portalier, 1984)
2335	F ⁻	<i>proC recA rpsL argH</i>	<i>recA</i> derivative of strain 791; this work
2336	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL argH</i>	Periplasmic-leaky mutant of strain 791; this work
2338	F ⁻	<i>phoA8 His⁻ rpsL argH</i>	APase deficient mutant of strain 791; this work
2340	F ⁻	<i>proC::Tn10 phoR69 His⁻ rpsL argH</i>	APase constitutive mutant of strain 791; this work
2355	F ⁻	<i>proC::Tn10 phoR69 gal::Tn5 lky-207 His⁻</i> <i>rpsL argH</i>	Periplasmic-leaky mutant of strain 2340; this work
2356	F ⁻	<i>proC::Tn10 phoR69 gal::Tn5 lky-207 recA</i> <i>rpsL argH</i>	<i>recA</i> derivative of strain 2355; this work
2362	F ⁺	<i>proC recA rpsL argH/ColE1 phoA⁺ proC⁺ phoB⁺</i>	Transformed strain 2335 with hybrid plasmid pJC719; this work
2364	F ⁺	<i>proC recA rpsL argH/ColE1 phoA⁺ proC⁺ phoB⁺</i> <i>phoR⁺</i>	Transformed strain 2335 with hybrid plasmid pJC721; this work
2370	F ⁺	<i>proC::Tn10 phoR69 gal::Tn5 lky-207 recA rpsL</i> <i>argH/ColE1 phoA⁺ proC⁺ phoB⁺ phoR⁺</i>	Transformed strain 2356 with hybrid plasmid pJC721; this work
2385	F ⁻	<i>proC gal::Tn5 lky-207 recA rpsL argH</i>	<i>recA</i> derivative of strain 2336; this work
2386	F ⁻	<i>proC His⁻ rpsL argH $\phi 80$ phoA⁺</i>	Lysogen of strain 791; this work
2387	F ⁻	<i>proC His⁻ rpsL argH $\phi 80$ phoBin phoA⁺</i>	Lysogen of strain 791; this work
2388	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL argH $\phi 80$</i> <i>phoA⁺</i>	Lysogen of strain 2336; this work
2389	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL argH $\phi 80$</i> <i>phoBin phoA⁺</i>	Lysogen of strain 2336; this work
2390	F ⁻	<i>phoA8 His⁻ rpsL argH $\phi 80$ phoA⁺</i>	Lysogen of strain 2338; this work
2391	F ⁻	<i>phoA8 His⁻ rpsL argH $\phi 80$ phoBin phoA⁺</i>	Lysogen of strain 2338; this work
2392	F ⁻	<i>proC::Tn10 phoR69 His⁻ rpsL argH $\phi 80$ phoA⁺</i>	Lysogen of strain 2340; this work
2393	F ⁻	<i>proC::Tn10 phoR69 His⁻ rpsL argH $\phi 80$</i> <i>phoBin phoA⁺</i>	Lysogen of strain 2340; this work
2394	F ⁻	<i>proC::Tn10 phoR69 gal::Tn5 lky-207 His⁻</i> <i>rpsL argH $\phi 80$ phoA⁺</i>	Lysogen of strain 2355; this work
2395	F ⁻	<i>proC::Tn10 phoR69 gal::Tn5 lky-207 His⁻</i> <i>rpsL argH $\phi 80$ phoBin phoA⁺</i>	Lysogen of strain 2355; this work
2396	F ⁻	<i>proC lky-207 His⁻ rpsL phoS2333 argH</i> <i>$\phi 80$ phoA⁺</i>	Lysogen of strain 2122; this work

Table 1 (continued)

Strain	Sex	Relevant genotype or phenotype	Source
2397	F ⁻	<i>proC lky-207 His⁻ rpsL phoS2333 argH ϕ80 phoBin phoA⁺</i>	Lysogen of strain 2122; this work
2399	F ⁺	<i>proC gal::Tn5 lky-207 recA rpsL argH/ColE1 phoA⁺ proC⁺</i>	Strain 2385 transformed with hybrid plasmid pJC719; this work
2401	F ⁺	<i>proC gal::Tn5 lky-207 recA rpsL argH/ColE1 phoA⁺ proC⁺ phoB⁺ phoR⁺</i>	Strain 2385 transformed with hybrid plasmid pJC721; this work
2407	F ⁻	<i>proC His⁻ rpsL zib501::Tn10 phoS65 argH</i>	APase constitutive mutant of strain 791; this work
2408	F ⁻	<i>proC His⁻ rpsL zib501::Tn10 phoS27 argH</i>	APase constitutive mutant of strain 791; this work
2409	F ⁻	<i>proC His⁻ rpsL zib501::Tn10 phoU argH</i>	APase constitutive mutant of strain 791; this work
2410	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL zib501::Tn10 phoS65 argH</i>	Periplasmic-leaky mutant of strain 2407; this work
2411	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL zib501::Tn10 phoS27 argH</i>	Periplasmic-leaky mutant of strain 2408; this work
2421	F ⁻	<i>proC gal::Tn5 lky-207 His⁻ rpsL zib501::Tn10 phoU argH</i>	Periplasmic-leaky mutant of strain 2409; this work
2438	F ⁻	<i>proC::Tn10 lky-207 phoR69 His⁻ rpsL phoS2333 argH ϕ80 phoBin phoA⁺</i>	Derivative of strain 2397; this work
2442	F ⁻	<i>proC lky-207 His⁻ rpsL phoS2333 argH ϕ80</i>	Lysogen of strain 2122; this work
<i>Plasmid</i>			
pJC719	-	ColE1 <i>phoA⁺ proC⁺ phoB⁺</i>	Hybrid ColE1 plasmid (Lazzaroni & Portalier, 1982)
pJC721	-	ColE1 <i>phoA⁺ proC⁺ phoB⁺ phoR⁺</i>	Hybrid ColE1 plasmid (Lazzaroni & Portalier, 1982)

Media, growth conditions and biomass. Modified rich Luria Broth (LB 8-3) was used as high phosphate medium, and minimal TRIS medium, supplemented with (per litre): glucose (4 g), proteose-peptone (2 g), thiamin hydrochloride (1 mg) and appropriate amino acids (40 mg), was used as low phosphate medium (Atlan & Portalier, 1984). Isopropyl- β -D-thiogalactopyranoside (IPTG, Sigma) was added to the culture medium at a final concentration of 1 mM to induce β -galactosidase synthesis. Solid media contained 1.5% (w/v) agar, and, if required, tetracycline hydrochloride (10 mg l⁻¹, Sigma), kanamycin sulphate (20 mg l⁻¹, Sigma), or 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 mg l⁻¹, Sigma).

Routinely, 5 ml cultures were inoculated from 8 h subcultures in the same medium, and allowed to grow for 16 h at 37 °C.

Biomass was estimated by measuring the OD₆₀₀ of cell suspensions with a Jean & Constant spectrophotometer (Prolabo, France): 100 OD₆₀₀ units correspond to 3 × 10⁸ cells ml⁻¹ or 0.20 mg of bacterial dry weight ml⁻¹.

Enzyme extracts and assays. Cellular and extracellular extracts were obtained as described previously (Atlan & Portalier, 1984). APase and β -galactosidase activities were assayed according to the procedures of Torriani (1967) and Miller (1972) respectively. One unit (U) of APase or β -galactosidase activity was defined as the amount of enzyme that hydrolyses 1 nmol substrate min⁻¹. An enzyme activity of 100% corresponds to the total intracellular plus extracellular activities. Total specific production corresponds to the increase of total (intracellular and extracellular) enzyme activity (mg bacterial dry weight increase)⁻¹.

Electrophoretic analysis. Before electrophoresis, protein extracts were concentrated by ethanol precipitation at -20 °C for 4 h (1 vol. extract:2 vols ethanol). After centrifugation (40000 g, 30 min, -20 °C), protein pellets were resuspended in electrophoresis cracking buffer (Laemmli, 1970). Samples were boiled for 3 min under reducing conditions in the presence of 0.1 M- β -mercaptoethanol.

Sodium dodecylsulphate-polyacrylamide slab gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (1970), except that TEMED (*N,N,N',N'*-tetramethylethylenediamine) and ammonium persulphate concentrations were respectively 0.033% and 0.035%. Polyacrylamide gradients (7-15%) were used routinely. Samples were electrophoresed for 16 h at 100 V; gels were stained with 2% Coomassie blue as described by Lazzaroni & Portalier (1981) and scanned with a Phi5 densitometer (Vernon, Paris, France).

Genetic techniques. Tn10 or Tn5 transposons or *phoA8* mutation were transferred by transduction with phage P1kc (Miller, 1972). Clones carrying transposons Tn10 or Tn5 were selected by their tetracycline or kanamycin resistance, respectively. *phoA* mutated clones were identified *in situ* by a qualitative test (Lazzaroni & Portalier, 1979) among Pro⁺ transductants selected on proline deficient TRIS medium (*phoA* and *proC* genes are 99% co-transducible).

$\phi 80$ lysogens and lysates were obtained as described by Miller (1972); $\phi 80$ *phoA*⁺ prophage excision was induced by UV light; lysates were used to infect an APase deficient strain (2338) on low phosphate solid medium containing XP (Inouye *et al.*, 1981); $\phi 80$ *phoA*⁺ lysogenized clones appeared blue-coloured.

Hybrid ColE1 plasmids were transferred into F⁻ *proC recA* recipient strains (*lky*⁺ or *lky*) by F-mediated conjugation (Miller, 1972). Pro⁺ ColE1 resistant conjugants were selected, and the presence of the plasmid was confirmed, by a second transfer into an appropriate recipient strain.

RESULTS AND DISCUSSION

Influence of pho regulatory mutations on APase synthesis and release

During growth in low phosphate medium, *pho*⁺ periplasmic-leaky strain 2336, carrying mutation *lky*-207, had the same derepression pattern as parental strain 791 (*pho*⁺ *lky*⁺) and produced equivalent amounts of APase, although it released up to 50% of its production into the medium (cellular lysis was estimated by measuring β -galactosidase activity in the medium) (Table 2). We have constructed isogenic derivatives of parental strains 791 and 2336 that carry a *phoU*, *phoS*, *phoR* or *phoBin* mutant allele and synthesize APase constitutively, even in the presence of a high phosphate concentration (about 10⁻³ M). The *phoBin* mutation is a promoter-up mutation in the *phoA* gene promoter which makes the *phoA* gene expression independent of the positive effect mediated by the *phoB* gene product (Wanner *et al.*, 1979). Strain 2333 was selected as an arsenate-resistant derivative of parental strain PA601, and had an APase constitutive pattern (Atlan & Portalier, 1984); as plasmid pSN518 carrying gene *phoS*⁺ (Amemura *et al.*, 1982) suppressed this mutation, we named it *phoS2333* (J. C. Lazzaroni & R. Portalier, unpublished results).

After growth in low phosphate medium (Table 2), *phoR* strains 2340 (*lky*⁺) and 2355 (*lky*) did not synthesize more APase than parental strains 791 and 2336, respectively. However, other constitutive strains carrying a *lky*⁺ or *lky* allele showed a marked increase in APase activities. *phoS2333* clones, which synthesized three times more enzyme than parental strains, were characterized by the highest specific production of APase. *phoS65* leaky strain 2410 had the highest proportion of APase leakage (60% of total APase production).

After growth in high phosphate medium (Table 2), all *lky* strains carrying a constitutive *pho* mutation synthesized and released APase. However, the lowest APase production, measured with *phoBin* strain 2389 (111 U ml⁻¹), was 14 times less than the highest, measured in *phoS2333* strain 2122 (1483 U ml⁻¹). The specific production of APase of constitutive derivatives grown in high phosphate medium was always lower than that observed in low phosphate medium; moreover, in the former medium, *lky* mutants released a greater proportion of APase and cell lysis, reflected by the extracellular level of β -galactosidase activity, was lower. We have checked that the presence of Tn10 or Tn5 transposons in *lky* strains did not alter APase leakage level and cellular stability (data not shown).

These results showed that *lky* mutants, except strain 2122, produced as much APase as their non-excretory isogenic parents; the marked increase in the specific activity of APase observed with strain 2122 grown in low phosphate medium could indicate a new regulatory mechanism for APase synthesis induced in the *phoS2333* mutant by enzyme excretion in order to maintain a constant periplasmic enzyme content. Moreover, total APase derepression was not achieved in a *pho*⁺ wild-type strain; even in low phosphate medium, some *pho*⁺ regulatory gene products still exerted a repressive effect on APase synthesis or export.

Protein content of extracellular fluids from APase constitutive lky mutants

The protein composition of culture supernatants after growth in high phosphate medium was analysed by SDS-PAGE (Fig. 1). As we have shown previously (Atlan & Portalier, 1984), four major periplasmic proteins were released into the medium by APase constitutive *lky* mutants. One protein had a molecular weight of 58 kDal, and three were phosphate-regulated polypeptides: APase monomer (P1; 45 kDal); *sn*-glycerol-3-phosphate binding protein (P2; 41.5 kDal) and phosphate binding protein (P4; 31.5 kDal). These four periplasmic proteins, as well as other minor unidentified polypeptides, were released by *lky* mutants 2411 (*phoS27*), 2355

Table 2. *Effect of pho regulatory mutations on APase synthesis and release*

The data are mean values from four experiments.

Strain	Relevant genotype	High phosphate medium					Low phosphate medium				
		APase					APase				
		Total specific production* (U mg ⁻¹)	Total activity† (U ml ⁻¹)	Intracellular activity (U ml ⁻¹)	Extracellular activity (U ml ⁻¹)	Extracellular β -galactosidase activity (%)	Total specific production* (U mg ⁻¹)	Total activity† (U ml ⁻¹)	Intracellular activity (U ml ⁻¹)	Extracellular activity (U ml ⁻¹)	Extracellular β -galactosidase activity (%)
791	<i>pho⁺ lky⁺</i>	0	0	0	0	0.1	1235	647	633	14	1.6
2336	<i>pho⁺ lky</i>	0	0	0	0	2.0	1230	669	337	332	9.9
2333	<i>phoS2333</i>	1612	1531	1518	13	0.8	2411	1254	1240	14	1.2
2122	<i>phoS2333 lky</i>	1483	1705	508	1197	70.2	3823	2003	966	1037	13.5
2409	<i>phoU</i>	304	331	331	0	1.0	1990	1066	1045	21	5.6
2421	<i>phoU lky</i>	1051†	568	36	532	93.7	1810	796	398	398	9.1
2407	<i>phoS65</i>	522	561	561	0	0.5	2260	1167	1143	24	2.4
2410	<i>phoS65 lky</i>	527	380	16	364	95.7	2055	1002	413	589	5.6
2408	<i>phoS27</i>	604	650	650	0	0.4	2165	1039	1018	21	2.0
2411	<i>phoS27 lky</i>	545	480	46	434	90.5	2186	993	444	549	6.4
2340	<i>phoR69</i>	372	380	373	7	1.7	1287	680	653	27	1.9
2355	<i>phoR69 lky</i>	287	332	40	292	88.0	1205	646	316	330	—
2387	791 ϕ 80	133	146	140	6	4.1	1798	936	912	24	2.4
	<i>phoBin</i>										
	<i>phoA⁺</i>										
2389	2336 ϕ 80	111	122	26	96	78.7	1835	998	520	478	11.0
	<i>phoBin</i>										
	<i>phoA⁺</i>										

* See Methods.

† Equal to intracellular plus extracellular activities.

‡ This high value mainly reflected the very low growth of strain 2421 in high phosphate medium.

Fig. 1. SDS-polyacrylamide gradient (7–15%) gel electrophoretic analysis of commercial APase (Sigma P9761) (a) and extracellular proteins released by APase constitutive *lky* strains: 2122 (b), 2411 (c), 2355 (d) and 2421 (e). Samples containing 350 U APase activity were loaded in each well. Bacteria were grown in high phosphate medium and were collected in late stationary phase.

(*phoR69*), 2421 (*phoU*) and 2122 (*phoS2333*); P1 monomer accounted for 15%, 8%, 15% and 26% of the total amount of excreted proteins, respectively. Moreover, with the latter mutant, APase was the major released protein.

Effect of phoA gene amplification on APase synthesis and release

phoA⁺-ColE1 hybrid plasmids pJC719 (*phoA*⁺ *proC*⁺ *phoB*⁺) and pJC721 (*phoA*⁺ *proC*⁺ *phoB*⁺ *phoR*⁺) (Lazzaroni & Portalier, 1982) were introduced by F-mediated conjugation into *recA lky*⁺ (2335) or *lky*[−] (2385) recipient strains. Total APase activities of plasmid-bearing *lky*⁺ transconjugants 2362 and 2364 grown in derepression conditions were eight times higher than those of haploid strain 2335, whereas *phoR*⁺ or *phoR lky* transconjugants only synthesized twice the amount of APase produced by haploid strains 2385 or 2356 (Table 3). We do not know if the presence of the mutation *lky*-207 in the genetic background of strain PA601 limited the replication of ColE1-derived plasmids or the cell's capacity to synthesize or export APase.

In high phosphate medium, APase was constitutively synthesized by plasmid-bearing derivatives of repressible strain 2335, although strain 2364 containing plasmid pJC721 was the only one to produce high enzyme levels. We were unable to transfer *phoA*⁺-ColE1 plasmids into the *phoS2333 recA lky*⁺ or *lky* strains: in order to get more information on the ability of periplasmic-leaky mutants to synthesize and release high amounts of APase in high phosphate medium, we used an independent genetic approach, as described below.

*APase synthesis and release by ϕ 80 *phoA*⁺ or ϕ 80 *phoBin phoA*⁺ lysogenized cells*

lky⁺ and *lky* strains carrying wild-type or mutant *pho* regulatory alleles were lysogenized with hybrid bacteriophage ϕ 80 *phoA*⁺ or ϕ 80 *phoBin phoA*⁺. APase synthesis and release properties of lysogenized strains are shown in Table 4.

Expression of the *phoA*⁺ gene carried by the ϕ 80 hybrid phages, and present as a single copy in strains 2390 and 2391, was phosphate-repressible, as it was for the *phoA*⁺ chromosomal gene in wild-type strain 791. On the other hand, during growth in low phosphate medium, strains 2386 and 2388, which carry two copies of the *phoA*⁺ gene, synthesized twice as much APase as their haploid parents 791 and 2336. Gene dosage experiments with *phoR* or *phoS* constitutive strains

Table 3. *APase synthesis and release in strains harbouring plasmids carrying the *phoA*⁺ region*
The data are mean values from three experiments.

<i>recA</i> strain	Relevant genotype	High phosphate medium					Low phosphate medium				
		APase					APase				
		Total specific production* (U mg ⁻¹)	Total activity† (U ml ⁻¹)	Intracellular activity (U ml ⁻¹)	Extracellular activity U ml ⁻¹	Extracellular β-galactosidase activity (%)	Total specific production* (U mg ⁻¹)	Total activity† (U ml ⁻¹)	Intracellular activity (U ml ⁻¹)	Extracellular activity U ml ⁻¹	Extracellular β-galactosidase activity (%)
2335	<i>pho</i> ⁺ <i>lky</i> ⁺	0	0	0	0	0.8	598	267	261	6	2.2
2362	2335/pJC719†	36	34	34	0	0.9	3342	2539	2428	111	4.4
2364	2335/pJC721‡	670	684	672	12	1.8	4792	2375	2299	76	3.2
2385	<i>pho</i> ⁺ <i>lky</i> ⁻	0	0	0	0	2.2	773	557	145	412	74.0
2399	2385/pJC719	24	25	0	25	1.0	1239	966	440	526	54.5
2401	2385/pJC721	27	31	5	26	1.2	1431	1116	597	519	46.5
2356	<i>phoR69 lky</i> ⁻	411	328	20	308	93.9	1061	658	297	361	54.9
2370	2356/pJC721	187	221	105	116	52.5	2239	1164	337	827	71.0

* See Methods.

† Equal to intracellular plus extracellular activities.

‡ Plasmid pJC719 carried *proC*⁺, *phoA*⁺ and *phoB*⁺ genes, whereas pJC721 carried *proC*⁺, *phoA*⁺, *phoB*⁺ and *phoR*⁺ genes.

2340	<i>phoR69</i>	373	380	373	7	1.8	0.2	1288	680	653	27	4.0	1.9
2355	<i>phoR69 lky</i>	287	333	41	292	87.7	—	1205	646	316	330	51.1	—
2392	2340 ϕ 80	636	693	677	16	2.3	0.9	1932	1074	1037	37	3.4	3.3
	<i>phoA</i> ⁺												
2394	2355 ϕ 80	534	555	45	510	91.9	—	2551	1143	492	651	57.0	—
	<i>phoA</i> ⁺												
2393	2340 ϕ 80	684	698	677	21	3.0	1.1	1728	940	903	37	3.9	2.9
	<i>phoBin</i>												
	<i>phoA</i> ⁺												
2395	2355 ϕ 80	503	533	50	483	90.6	—	2020	921	402	519	56.4	—
	<i>phoBin</i>												
	<i>phoA</i> ⁺												
2333	<i>phoS2333</i>	1612	1531	1518	13	0.8	0.1	2411	1254	1240	14	1.1	0.1
2122	<i>phoS2333 lky</i>	1483	1705	508	1197	70.2	2.9	3823	2003	966	1037	51.8	13.5
2442	2122 ϕ 80	1455	1499	448	1051	70.1	3.2	—	—	—	—	—	—
2396	2122 ϕ 80	3350	2948	740	2208	74.9	6.6	7009	2972	1469	1503	50.6	15.8
	<i>phoA</i> ⁺												
2397	2122 ϕ 80	2586	2974	682	2292	77.1	7.9	5647	2530	1188	1342	53.0	16.6
	<i>phoBin</i>												
	<i>phoA</i> ⁺												
2438	2122 <i>phoR69</i>	3012	3494	1418	2076	59.4	3.7	—	—	—	—	—	—
	ϕ 80 <i>phoBin</i>												
	<i>phoA</i> ⁺												

* See Methods.

† Equal to intracellular plus extracellular activities.

Table 5. *Relative contribution of periplasmic proteins to the extracellular medium from lky phoS mutants grown in high phosphate medium*

Cells were grown in high phosphate medium. The data are mean values from two experiments.

Strain	Relevant genotype	Relative contribution of periplasmic proteins			
		58 kDal	P1 (APase monomer)	P2	P4
2122	<i>phoS2333 lky</i>	20	26	7	18
2442	2122 ϕ 80	20	25	4	23
2396	2122 ϕ 80 <i>phoA</i>	14.5	40	1.5	16.5
2397	2122 ϕ 80 <i>phoBin phoA</i>	16.5	43	1.5	18

grown in low or high phosphate medium similarly showed that partial diploid strains synthesized about twice as much APase as haploid strains. These results also demonstrated that *lky* mutants 2396 and 2397 were able to export and process high APase levels (3000 U ml^{-1}), and to release up to 2300 U ml^{-1} after growth in rich, high phosphate medium, i.e. twice the amount of enzyme released by haploid strain 2122.

Molecular mechanisms involved in the APase leakage process were not saturated when cells were grown in high phosphate medium, and APase did not accumulate in the periplasm of *lky* mutants; however, this was not the case for *lky* strains 2396 and 2397 during growth in low phosphate medium.

The *phoBin* mutated promoter enabled APase synthesis to occur in repressed conditions, but was less efficient than the wild-type promoter for initiating *phoA* gene transcription in derepressed conditions.

For further studies on extracellular fluids of *lky* mutants we used rich, high phosphate medium (Atlan & Portalier, 1984) which allowed fast growth, low cellular lysis and high APase release.

Protein content of extracellular fluids from lky phoS2333 mutants lysogenized with ϕ 80 phoA⁺ or ϕ 80 phoBin phoA⁺ phages

Quantitative relationships between major periplasmic proteins released by *lky phoS2333* lysogens are shown in Table 5. The increase in total extracellular APase activities observed with lysogenized strains (Table 4) was correlated with an increase in the relative APase content in the extracellular medium. The enzyme accounted for up to 43% of total released proteins (strain 2397), a proportion which facilitates its purification.

Conclusions

Several conclusions can be drawn from our study of the properties of the leaky strains we have constructed. First, in low phosphate medium, all haploid constitutive *lky* strains released 50% of their total APase activity whatever the nature of the constitutive mutation was, and even when specific activities of APase were very high. These results suggest that the mechanisms involved in the APase leakage operate independently of the *pho* regulon.

Second, availability of APase constitutive strains allowed us to study the release process during growth in rich medium, in the presence of excess phosphate. Under these conditions, specific activities were lowered but the proportion of extracellular APase increased significantly from 50% to 70–95%. These results suggest that a rich-medium-inducible mechanism directly or indirectly stabilizes the inner membrane (hence less β -galactosidase leakage) but destabilizes the outer membrane, so increasing leakage of periplasmic constituents. Studies are now underway to elucidate the nature of this regulation.

APase overproduction had already been obtained by several authors (Berg, 1981; Inouye *et al.*, 1981; Lazzaroni & Portalier, 1982) but their studies were done in conditions of phosphate starvation. We have now taken advantage of the high quantities of APase released by constitutive mutants in high phosphate medium to develop a new and simple method for APase purification (D. Atlan & R. Portalier, unpublished results).

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