Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5107–5111, August 1985
Genetics

Fusions of secreted proteins to alkaline phosphatase: An approach for studying protein secretion

(hybrid proteins/protein transport/signal sequence)

CHARLES S. HOFFMAN AND ANDREW WRIGHT

Department of Molecular Biology and Microbiology, Tufts University, Health Sciences Campus, 136 Harrison Avenue, Boston, MA 02111

Communicated by Jonathan Beckwith, April 22, 1985

ABSTRACT **We have constructed a series of plasmids containing a modified form of the *phoA* gene of *Escherichia coli* K-12 that have general utility for studies of protein secretion. In these plasmids, the promoter and signal sequence-encoding region of the *phoA* gene have been deleted; thus, expression of the gene, giving rise to active alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], is absolutely dependent upon fusion in the correct reading frame to DNA containing a promoter, a translational start site, and a complete signal sequence-encoding region. Alkaline phosphatase, which is normally located in the periplasm of *E. coli*, is efficiently secreted to the periplasm when fused either to a signal sequence from another periplasmic protein, β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6), or to signal sequences from the outer membrane proteins LamB and OmpF. These heterologous signal sequences are processed during secretion. In the absence of a complete signal sequence, phosphatase becomes localized in the cytoplasm and is inactive. Phosphatase fusion proteins lacking up to 13 amino-terminal amino acids beyond the signal sequence show the same specific activity as that of the wild-type enzyme. However, a significant decrease in activity is seen when 39 or more amino-terminal amino acids are deleted. Addition of approximately 150 amino acids from the enzyme β-lactamase to the amino terminus of alkaline phosphatase has little effect on the specific activity of the enzyme. The ability to change the amino terminus of phosphatase without altering its activity makes the enzyme particularly useful for construction of protein fusions. The fact that phosphatase is designed for transport across the cytoplasmic membrane makes it an ideal tool for study of protein secretion.**

Protein synthesis in *Escherichia coli* occurs in the cytoplasm, yet many protein species are localized exclusively in the cytoplasmic membrane, the periplasm, or the outer membrane. Proteins destined for the latter two compartments are always synthesized with an amino-terminal signal sequence that is removed in the course of secretion, while cytoplasmic membrane proteins, in general, are able to become localized in the membrane without the benefit of a signal sequence (1). Much of the current understanding of protein secretion in prokaryotic cells has been obtained by the study of fusion proteins containing a segment of a secreted protein attached to β-galactosidase. β-Galactosidase, the product of the *lacZ* gene of *E. coli*, is normally found in the cytoplasm and possesses an activity that can be readily detected and assayed. Fusion proteins containing β-galactosidase that become localized in membranes show greatly reduced activity. Mutants have been isolated from strains containing such fusions, which show increased β-galactosidase activity, leading to the identification of genes involved in protein secretion and allowing detailed analysis of the important elements of signal sequence structure (2–7).

There are several advantages to using protein fusions with enzymatic activity for analysis of protein secretion. Such fusions make it possible to isolate transport mutations that affect the localization of the secreted proteins without eliminating the activity of the fused enzyme. In mutant searches where fusion proteins are not utilized, transport mutants would be greatly outnumbered by mutants with defects in transcription or translation of the protein in question. Fusion proteins also can be used to demonstrate the presence of transport information in segments of proteins that, by themselves, have no assayable activity. Finally, the fusion of an enzyme to a truncated protein can lead to stabilization of that protein fragment.

There are severe limitations to transport studies involving β-galactosidase fusions because β-galactosidase is normally found in the cytoplasm and is not necessarily capable of crossing a membrane. In fact, fusion proteins containing β-galactosidase at the carboxyl terminus are never found in the periplasm, suggesting that the amino-terminal portion of a secreted protein is not always able to direct proper localization of a fusion protein.

To further exploit fusion proteins for analysis of protein secretion, we have developed a fusion vector that encodes a protein with secretion characteristics different from those of β-galactosidase. This vector contains a modified form of the *phoA* gene, which encodes alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1; PhoA], an easily assayable enzyme normally located in the periplasm of *E. coli* K-12. Like β-galactosidase, PhoA can be detected, even at low levels, in bacterial colonies by use of a chromogenic substrate [in this case 5-bromo-4-chloro-3-indolyl phosphate (*XP*)]. Since it is a periplasmic protein, PhoA can cross the cytoplasmic membrane efficiently. In fact, its activity is absolutely dependent upon secretion from the cytoplasm. We have altered the *phoA* gene, removing its promoter and signal sequence-encoding region. In this paper we show that the signal sequences from β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6), a periplasmic protein, and from OmpF and LamB, two outer membrane proteins, can direct efficient secretion of this altered phosphatase to the periplasm. Furthermore, we show that the amino-terminal amino acids of PhoA are not required for enzymatic activity, which makes this protein ideal for the construction of fusions to be used in transport studies.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Strains AW1046 (Δ*phoA*) and AW1061 (Δ*phoA phoB phoR*) are derivatives of *E. coli* K-12 strain MC1000 (8). Plasmids used were as follows: pHI1 (9) from H. Inouye; pKT218, pKT279, pKT280, and pKT287

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

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

(10) from K. Talmadge; pORF1 (11) from T. Silhavy; and pSM5 from S. Michaelis. Plasmid pSM5 is identical to plasmid pHC2 described elsewhere (12).

Cells were grown in L broth (13) as rich medium or in WTB (14) or LST (15) minimal medium as indicated. Ampicillin and tetracycline (Sigma) were used at 50 μg/ml and 20 μg/ml, respectively.

Enzymes and Chemicals. Restriction endonucleases, T4 DNA ligase, exonuclease BAL-31, polynucleotide kinase, DNA polymerase I large fragment, and *Pst* I linkers were obtained from New England Biolabs and used according to the manufacturer's instructions. *XP* and *p*-nitrophenyl phosphate were obtained from Sigma. IgGSorb was obtained from The Enzyme Center, Inc. Preparations of rabbit antisera directed against periodate-treated PhoA were provided by Susan Michaelis and Yolanta Fishman.

Assay of PhoA Activity. PhoA activity was measured by the production of *p*-nitrophenol from *p*-nitrophenyl phosphate as described previously (6), except that cultures were grown in L broth because activity was no longer regulated by phosphate concentration.

Protein Labeling, Fractionation, and Immunoprecipitation. Cells growing in either WTB medium containing 1% glucose and 2.5% methionine assay medium (Difco) or LST medium containing glucose (10 mg/ml), leucine (50 μg/ml), proline (50 μg/ml), and vitamin B1 (1 μg/ml) were labeled for 2 min during logarithmic-phase growth by addition of [^{35}S]methionine (10 μCi/ml; 1 Ci = 37 GBq). Cultures were chilled rapidly, and cold osmotic shock was used to release the periplasmic proteins (16). Samples were boiled in the presence of 1% $NaDodSO_4$ and immunoprecipitated (5) with anti-PhoA antiserum.

DNA Sequence Analysis of *phoA* Fusions. Plasmid DNA was isolated from cells (17) and purified on CsCl gradients prior to DNA sequence analysis by the method of Maxam and Gilbert (18). All of the plasmids contained a *Hin*fI site within the *phoA* gene less than 100 base pairs from the *Pst* I fusion joint. Plasmid DNAs were digested with *Hin*fI, labeled either at the 5′ or 3′ positions by using polynucleotide kinase or the Klenow fragment of DNA polymerase I, and subsequently digested with *Hin*dIII. The *Hin*fI–*Hin*dIII fragments containing the fusion joint were separated from other labeled fragments by electrophoresis on a 5% polyacrylamide gel and were used for sequence analysis.

RESULTS

Plasmid Construction. We have made use of plasmid pHI1, which carries the *phoA* gene of *E. coli* K-12, to construct a series of vector plasmids suitable for fusion of secreted proteins to PhoA. Our approach was to replace the *phoA* promoter and the region coding for the signal sequence with the corresponding sequences from the *bla* gene (coding for β-lactamase). This was done in such a way as to introduce a *Pst* I restriction site at the resulting point of fusion (Fig. 1). Plasmid pHI1 was digested with *Hpa* I, which cuts the plasmid approximately 400 base pairs upstream from the *phoA* promoter. Exonuclease BAL-31 was used to remove various amounts of the amino terminus-encoding region of *phoA*. After addition of a *Pst* I linker, the *phoA*-containing DNA was inserted by ligation into three vectors constructed by Talmadge *et al.* (10), each of which possesses a unique *Pst* I site immediately downstream from the *bla* signal sequence-encoding region. (The three *Pst* I sites vary in their placement with respect to the β-lactamase reading frame.) The ligated DNA was used to transform *E. coli* strain AW1061 to tetracycline resistance. PhoA-producing transformants, identified by their blue color on *XP* indicator medium, were found to contain vector plasmids with *phoA* inserts of various sizes. Further studies were carried out on plasmids with some

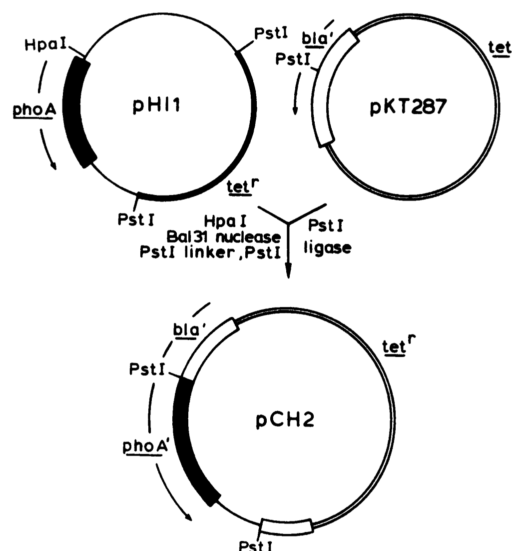

FIG. 1. Construction of plasmid pCH2. The figure shows construction of a fusion between the genes for β-lactamase and PhoA in which the promoter and signal sequence-encoding region are derived from the *bla* gene. The *phoA* gene segment lacks its promoter, signal sequence-encoding region, and the first 13 codons of its coding sequence. A *Pst* I linker sequence is present at the fusion junction.

or all of the *phoA* signal sequence-encoding region deleted, representing all three vectors.

Sequence of Fusion Sites. The fusion sites of a series of selected plasmids were sequenced by the procedure of Maxam and Gilbert (18). The DNA sequences of the fusion regions are presented in Table 1, beginning with the coding sequences for the mature β-lactamase protein and extending through the *Pst* I linker into the *phoA* coding region. All of the fusions shown in Table 1 had lost the *phoA* promoter and most or all of the region coding for the PhoA signal sequence.

Table 1. Structure of β-lactamase-PhoA fusion proteins determined by DNA sequence analysis

Plasmid	Vector	Amino acid and nucleotide sequences of fusion junctions
pCH2	pKT287	14 His Pro Glu Thr Ala Ala Ala *Gln* CAC CCA GAA ACG GCT GCA GCT CAG
pCH4	pKT287	−3 His Pro Glu Thr Ala Ala Ala *Thr* CAC CCA GAA ACG GCT GCA GCG ACA
pCH9	pKT280	40 His Pro Leu Gln Arg *Asp* CAC CCG CTG CAG CGC GAT
pCH39	pKT279	6 His Arg Cys Ser *Pro* CAC CGC TGC AGC CCT
pCH40	pKT280	14 His Pro Leu Gln Pro *Gln* CAC CCG CTG CAG CCT CAG
pCH58	pKT218	14 Met Ser Ile Gln Ala Ala Ala *Gln* ATG AGT ATT CAA GCT GCA GCT CAG

DNA sequence of the *bla-phoA* amino terminus-encoding region and the corresponding protein sequence of mature β-lactamase-PhoA fusion products. The first five DNA sequences begin with the *bla* codons immediately following the leader peptidase cleavage site and continue to the first intact codons of the *phoA* gene (numbered and indicated in italics). Since pCH58 encodes an incomplete signal sequence and is therefore unprocessed, the displayed sequence begins at the translational start site. *Pst* I linker DNA is underlined.

Fusions representing all three reading frames were obtained by using the three vector plasmids pKT279, pKT280, and pKT287. These fusions ranged from that in pCH4, which contained the last three codons of the *phoA* signal sequence-encoding region and the complete mature PhoA-encoding region, to that in pCH9, which lacked the entire signal sequence-encoding region plus 39 codons beyond. The fusion protein specified by pCH9 is considerably less active enzymatically than are other larger fusion proteins described here as seen by lowered intensity of blue color in colonies and by liquid assay (Table 2). The *phoA* gene in plasmid pCH8 lacks ≈55 codons at the 5′ end of the region encoding the mature protein as characterized by restriction analysis. This plasmid specifies an enzyme with even less activity than that specified by pCH9.

A Large *bla-phoA* Fusion. The *bla* gene in plasmid pBR322 possesses a *Pst* I site located approximately two-thirds of the way into the gene. The *Pst* I *phoA* fragment from plasmid pCH2 was inserted at this site, giving rise to plasmid pCH38. This plasmid specifies production of a large fusion protein of the size predicted from the DNA sequence (see below).

Fusions to Other Signal Sequences. To test the general usefulness of fusions to PhoA, we linked *phoA* to the *ompF* and *lamB* genes that code for outer membrane proteins in *E. coli*. The complete signal sequence-encoding region of the *ompF* gene is present on a 174-base-pair *Pst* I fragment. Insertion of this fragment into the *Pst* I site that lies within the *bla-phoA* coding region of plasmid pCH2, creating plasmid pCH57, gave rise to an in-frame protein fusion having essentially no contribution from the mature OmpF protein. Transcription of this fusion gene is initiated from the *bla* promoter present in pCH2, since the *ompF* fragment contains no promoter (Ron Taylor, personal communication). We constructed *lamB-phoA* fusions by introducing new *Pst* I sites at various positions within the *lamB* structural gene (to be reported elsewhere) and using these to insert *Pst* I fragments containing the truncated *phoA* gene. One such fusion, giving rise to active PhoA, encoded on plasmid pCH45, contained the signal sequence-encoding region and approximately the first 60 codons of *lamB* encoding the mature LamB protein linked to the *phoA* fragment present in plasmid pCH2.

Table 2. Effect of alteration of the amino terminus of PhoA on enzymatic activity

Plasmid	Genetic origin: Promoter	Genetic origin: Signal sequence DNA	*phoA* fusion junction*	PhoA activity, units
pCH2	*bla*	*bla*	7/14	1338
pCH4	*bla*	*bla*	7/−3	1293
pCH39	*bla*	*bla*	4/6	1234
pCH40	*bla*	*bla*	5/14	1321
pCH57	*bla*	*ompF*	2/14	1444
pCH9	*bla*	*bla*	5/40	80
pCH8	*bla*	*bla*	5/55†	14
pCH38	*bla*	*bla*	160/14	576
pCH45	*malK*′‡	*lamB*	60†/14	39
pCH58	*bla*	—	7§/14	<5
—	—	—	−/−	<5

PhoA enzymatic activity (6) in strain AW1061 (Δ*phoA phoB phoR*) carrying plasmids containing the indicated *phoA* gene fusions.
*The first number indicates the number of heterologous amino acids at the amino terminus of each mature fusion protein. The second number indicates the PhoA amino acid to which the heterologous sequences are fused.
†Determined by restriction analysis.
‡A weak promoter internal to *malK*, originally referred to as pB3 (19).
§This fusion encodes an incomplete and therefore unprocessed signal sequence. The number represents the total number of heterologous residues prior to the *phoA* coding region.

Structure and Localization of Fusion Proteins. Cells containing *phoA* fusion plasmids were labeled with [35S]methionine and subjected to cold osmotic shock to release periplasmic proteins. The PhoA-specific polypeptides were precipitated from the shock fluid by using anti-PhoA antibody and were analyzed on $NaDodSO_4$/polyacrylamide gels (20). The proteins varied in size, as predicted by the sequences shown in Table 1 (Fig. 2). The proteins encoded by pCH2 and pCH4 had lower mobilities than predicted by their DNA sequences. This does not appear to be due to lack of signal sequence processing because we were able to identify the larger precursor forms of both proteins in the nonperiplasmic fraction of the labeled cells (data not shown). The aberrant mobility of the mature proteins may be due to the amino acids contributed by the common vector, pKT287 (Table 1). Furthermore, since protein sequencing was not done, we cannot be absolutely sure that processing occurred at the wild-type site in all cases. Occasionally we have observed a minor band running just ahead of the major *phoA* product, such as that seen in lane 6 of Fig. 2. We do not know whether this is due to incorrect processing of the fusion protein or to its degradation.

Multiple bands are seen from immunoprecipitates of cells carrying pCH38 (Fig. 2, lane 5). The upper band is that of the fusion protein, while the lower bands appear to result from specific proteolysis of the fusion protein. This is not uncommon for PhoA fusion proteins possessing a large number of amino acids prior to the PhoA region (unpublished results).

Almost all of the 35S-labeled PhoA was released from cells carrying each of the fusion plasmids described in the previous section, by cold osmotic shock under conditions that left cytoplasmic proteins cell-associated. Release of the hybrid proteins encoded by plasmids pCH9 and pCH57 is shown in Fig. 3. For both of those fusions, approximately 94% of the protein was released by cold osmotic shock. The proteins encoded by the other plasmids, apart from that encoded by pCH58, behaved in an identical way. Thus, it appears that the fusion proteins are efficiently secreted under the conditions used for growth and labeling of cells.

The presence of the pCH45-encoded LamB-PhoA fusion protein in the periplasm indicates that the first 60 amino acids of LamB are not sufficient to anchor PhoA to either the

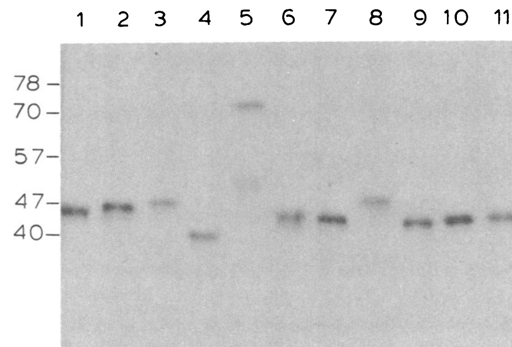

FIG. 2. $NaDodSO_4$/polyacrylamide gel analysis of PhoA-specific polypeptides encoded by fusion plasmids. Cells of strains AW1046 (Δ*phoA*) or AW1061 (Δ*phoA phoB phoR*) carrying various plasmids were grown in WTB medium supplemented with 2.5% methionine assay medium, labeled with [35S]methionine (10 μCi/ml), and subjected to cold osmotic shock. The proteins were immunoprecipitated from the shock fluid. Each lane represents equivalent amounts of radioactivity, not equivalent cell volumes. Lanes: 1 and 11, AW-1046/pHI1; 2, AW1061/pCH2; 3, AW1061/pCH4; 4, AW1061/pCH9; 5, AW1061/pCH38; 6, AW1061/pCH39; 7, AW1061/pCH40; 8, AW1061/pCH45; 9, AW1061/pCH57; 10, AW1061/pCH58. Sizes are shown in kDa.

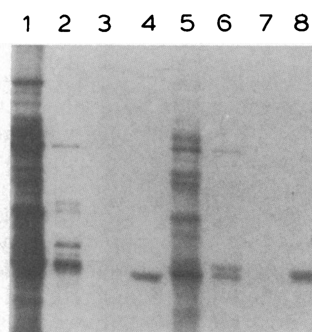

FIG. 3. Localization of fusion proteins encoded by pCH9 and pCH57. Cells were grown in LST and labeled for 2 min with 10 μCi of [^{35}S]methionine per ml. Cold osmotic shock and immunoprecipitation were performed as in Fig. 2. Unprecipitated fractions were examined in order to visualize the degree of cross-contamination between fractions. The figure shows analysis of the labeled fractions on an $NaDodSO_4$/polyacrylamide gel. Lanes: 1–4, AW1061/pCH9; 5–8, AW1061/pCH57; 1 and 5, unprecipitated, nonperiplasmic fractions; 2 and 6, unprecipitated, periplasmic fractions; 3 and 7, immunoprecipitated, nonperiplasmic fractions; 4 and 8, immunoprecipitated, periplasmic fractions. Cell equivalents were examined for the two fractions in both unprecipitated and immunoprecipitated fractions, though 5 times more of the immunoprecipitated material was used over unprecipitated.

cyoplasmic or the outer membrane. However, we have observed membrane-associated PhoA by liquid assay and immunoprecipitation in fusions containing larger segments of both OmpF and LamB (unpublished data).

PhoA Activity of Protein Fusions. The results in Table 2 show that strains carrying plasmids pCH2, pCH4, pCH39, pCH40, and pCH57 produced similar levels of PhoA activity. Transcription of the fusion genes in all of these plasmids is assumed to occur from the *bla* promoter. The first four plasmids encode fusion proteins with the β-lactamase signal sequence, while plasmid pCH57 specifies a fusion protein with the OmpF signal sequence, thus the β-lactamase signal sequence and the OmpF signal sequence appear to behave similarly in the formation of an active PhoA molecule.

Although transcription of the hybrid *bla-phoA* genes in pCH9 and pCH38 is also initiated at the *bla* promoter, the plasmids give rise to significantly less PhoA activity than do the other plasmids. The PhoA portion of the pCH9 fusion protein lacks the first 39 amino acids of the mature protein, 26 more than those deleted in pCH2 and pCH40. Plasmid pCH8 carries a *phoA* deletion removing approximately 15 more codons than are deleted in pCH9, as characterized by restriction analysis. Cells containing this plasmid produce 1/6th as much activity as do those containing pCH9. These deletions help to define a limit to the manipulation of the PhoA amino terminus with regard to effect on enzymatic activity. Similarly, the presence of a large portion of the β-lactamase protein present in the pCH38 fusion reduces activity, but to a much lesser extent than in the case of the large deletions into *phoA*.

Cells carrying pCH45, encoding a LamB-PhoA fusion, also possess a relatively low level of PhoA activity. Transcription in this case is assumed to initiate at a relatively weak promoter within the *malK* gene, which presumably accounts for this result (19). Therefore, we do not know whether the specific activity of the encoded protein differs from those of fusions encoded by plasmids such as pCH2.

Further Flexibility of the PhoA Amino Terminus. As indicated above, deletion of portions of the PhoA amino terminus has little or no effect on enzymatic activity, while addition of >150 amino acids of β-lactamase has only a minor (2–3 fold) effect. The fact that a considerable portion of the amino terminus of PhoA can be removed and/or replaced makes it ideal for constructions of protein fusions.

PhoA of *E. coli* K-12 consists of two identical noncovalently linked protein subunits complexed with zinc. We found that the two subunits of the fusion protein present in cells containing plasmid pCH39 are covalently linked through a disulfide bond. The dimer is observed on polyacrylamide gels when samples are boiled in the absence of β-mercaptoethanol (data not shown), while only the monomer is present after boiling in the presence of the reducing agent. The *Pst* I linker in the pKT279 vector encodes a cysteine residue that must be involved, therefore, in the new disulfide linkage because it is the only cysteine residue unique to the fusion protein of pCH39. This bond has no effect on enzymatic activity.

Does Phosphate Secretion Require a Signal Sequence? Thus far, we have assumed that secretion of the *phoA* product depends upon its fusion to an intact signal sequence. In order to test this hypothesis, we ligated the *phoA* coding sequence from pCH2 into the vector pKT218, forming plasmid pCH58. Plasmid pKT218 encodes only the first four amino acids of the β-lactamase signal sequence; the four codons in *bla* are adjacent to the *Pst* I site. Transformants possessing the new fusion plasmid gave rise to white colonies on *XP* medium and were shown by liquid assay to produce no active PhoA (Table 2). However, [^{35}S]methionine labeling followed by precipitation with anti-PhoA antibody showed the production of fusion protein in an amount similar to that specified by plasmids such as pCH2. Because the PhoA was inactive, it seemed likely that it was located in the cytoplasm, as in the case for signal sequence mutants (ref. 6; see *Discussion*). Unexpectedly, a significant, but variable fraction of the *phoA* gene product specified by pCH58 was released from the cells by cold osmotic shock. The results shown in Fig. 4 indicate that 56% of the PhoA is present in the periplasmic fraction. Despite the significant release of the pCH58 product by osmotic shock, we consider it likely that the protein is localized solely in the cytoplasm. The observed release (56%) is considerably less than that seen for all fusions containing signal sequences and is typified by the pCH9 product shown in Fig. 4. It has been observed that a significant amount of the cytoplasmic protein, EF-Tu, is released from cells by osmotic shock under conditions where most other cytoplasmic proteins are not (21). Release of the pCH58-specified PhoA may occur by a similar, and as yet undefined, mechanism. The amino termini of the proteins encoded by pCH2 (the mature form) and pCH58 are shown in Table 1. It seems unlikely that

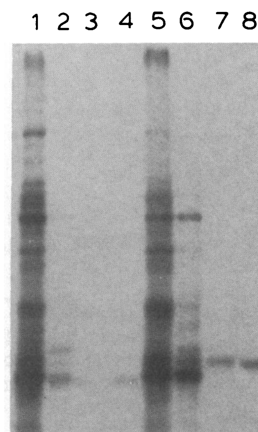

FIG. 4. Fractionation of PhoA-specific polypeptides in AW1061 carrying plasmids pCH9 and pCH58. The protocol was the same as in Fig. 2 except that cells were grown in LST medium. Lanes: 1–4, AW1061/pCH9; 5–8, AW1061/pCH58. The order of periplasmic and nonperiplasmic, unprecipitated and immunoprecipitated fractions is as in Fig. 3.

the small difference in these proteins could affect specific activity, especially in the light of other constructions that have little or no effect on activity.

The difference in the activity of the fusion proteins encoded by plasmids pCH2 and pCH58 displays the requirement for an intact signal sequence for transport of these alkaline phosphatase fragments.

DISCUSSION

The properties of the protein fusions described in this work indicate that the *phoA* gene is ideal for construction of gene fusions for analysis of protein secretion. We have shown that the PhoA signal sequence can be replaced by signal sequences from both periplasmic and outer membrane proteins and that these sequences will allow efficient secretion of the enzyme into the periplasm. We also have shown that the presence of an intact signal sequence-encoding region at the amino terminus-encoding region of the *phoA* gene is essential for enzymatic activity. This is in agreement with the work of Michaelis *et al.* (6) in which mutations in the signal sequence-encoding region gave rise to a cytoplasmic precursor protein species that lacked activity. This loss of activity is not due to the retention of the signal sequence because the precursor has been shown to be active when translated *in vitro* (22). It has been proposed that the reducing environment of the cytoplasm prevents disulfide bond formation necessary for proper folding of the enzyme. Alteration of the amino terminus of PhoA does not appear to affect its activity to any great extent, which provides for considerable flexibility in the construction of gene fusions. A β-lactamase-PhoA fusion protein containing two-thirds of the β-lactamase polypeptide was efficiently secreted and was enzymatically active. Removal of as many as 13 amino acids from the amino terminus of the mature PhoA protein had no effect on enzyme activity. A protein lacking the 39 amino-terminal residues was also enzymatically active, but in this case the specific activity was considerably reduced. Deletion of approximately the first 55 amino acids of mature PhoA reduced activity by 99% and yet gave sufficient activity for its detection among colonies carrying plasmids lacking *phoA*. Even the formation of a new disulfide linkage between the two subunits of the enzyme at the amino terminus had no effect on activity. Clearly, the amino-terminal region of the protein is not essential for enzyme function under the assay conditions used here. However, this region of the protein might play a role in the *in vivo* function of the enzyme. The wild-type *phoA* gene product exists in two forms differing by a single amino-terminal amino acid, giving rise to three dimeric isozymes, all of which are active. Whether they are functionally different is unclear.

Fusions of transported proteins to β-galactosidase are frequently lethal to the cell when produced in large amounts (3, 23, 24) because of the inability of β-galactosidase to cross the inner membrane. PhoA provides an alternative fusion vector for use in monitoring production of transported proteins. Furthermore, since PhoA requires a signal sequence for activity, it is unlikely that a translational restart in the protein would give rise to an active product. In the case of *lacZ* fusions, translational restarts can give rise to enzyme and artificially raise the measured levels of protein synthesis. Fusions to PhoA also should prove useful for selection of regulatory mutations in genes encoding secreted proteins, since a method is available for selection of cells with increased levels of PhoA activity (25). Screening colonies for an altered intensity of blue color on plates containing *XP* also can be used to identify mutants with altered levels of gene expression.

Studies similar to the use in this laboratory of *phoA* fusions to examine protein transport to the outer membrane have been done with β-galactosidase (26, 27). Since phosphatase differs from β-galactosidase in its secretion characteristics, these new fusions should provide information that will complement that obtained in previous fusion experiments. The efficient secretion of PhoA by other signal sequences shows this to be an excellent tool for protein secretion studies and the measurement of expression of transported proteins.

This work was initiated in Dr. Jon Beckwith's laboratory. We thank Jon Beckwith, Hiroshi Inouye, and Susan Michaelis for continued advice and encouragement. We thank D. Michael Gill and A. L. Sonenshein for critically reading the manuscript. This work was supported by grants from the National Institutes of Health.

1. Michaelis, S. & Beckwith, J. (1982) *Annu. Rev. Microbiol.* **36**, 435–465.
2. Emr, S. D., Schwartz, M. & Silhavy, T. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5802–5806.
3. Bassford, P. J. & Beckwith, J. (1979) *Nature (London)* **277**, 538–541.
4. Emr, S. D. & Silhavy, T. J. (1980) *J. Mol. Biol.* **141**, 63–90.
5. Oliver, D. B. & Beckwith, J. (1981) *Cell* **25**, 765–772.
6. Michaelis, S., Inouye, H., Oliver, D. & Beckwith, J. (1983) *J. Bacteriol.* **154**, 366–374.
7. Kumamoto, C. A. & Beckwith, J. (1983) *J. Bacteriol.* **154**, 253–260.
8. Casadaban, M. J. & Cohen, S. N. (1980) *J. Mol. Biol.* **138**, 179–207.
9. Inouye, H., Michaelis, S., Wright, A. & Beckwith, J. (1981) *J. Bacteriol.* **146**, 668–675.
10. Talmadge, K., Stahl, S. & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3369–3373.
11. Weinstock, G. M., ap Rhys, C., Berman, M. L., Hampar, B., Jackson, D., Silhavy, T. J., Weisemann, J. & Zweig, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4432–4436.
12. Clement, J. M. & Hofnung, M. (1981) *Cell* **27**, 507–514.
13. Miller, J. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
14. Bennett, R. L. & Malamy, M. H. (1970) *Biochem. Biophys. Res. Commun.* **40**, 496–503.
15. Hershey, A. (1955) *J. Gen. Physiol.* **36**, 777–789.
16. Neu, H. C. & Heppel, L. A. (1965) *J. Biol. Chem.* **240**, 3685–3692.
17. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
18. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560.
19. Braun-Breton, C. & Hofnung, M. (1978) *Mol. Gen. Genet.* **159**, 143–149.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Jacobson, G. R., Takacs, B. J. & Rosenbusch, J. P. (1976) *Biochemistry* **15**, 2297–2303.
22. Inouye, H. & Beckwith, J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1440–1444.
23. Bassford, P. J., Silhavy, T. J. & Beckwith, J. (1979) *J. Bacteriol.* **139**, 19–31.
24. Michaelis, S., Gaurente, L. & Beckwith, J. (1983) *J. Bacteriol.* **154**, 356–365.
25. Torriani, A. & Rothman, F. (1961) *J. Bacteriol.* **81**, 835–836.
26. Benson, S. A. & Silhavy, T. J. (1983) *Cell* **32**, 1325–1335.
27. Benson, S. A., Bremer, E. & Silhavy, T. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3830–3834.