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Use of Alkaline Phosphatase Fusions To Study Protein Secretion in *Bacillus subtilis*

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We have constructed a vector designed to facilitate the study of protein secretion in *Bacillus subtilis*. This vector is based on a translational fusion between the expression elements and signal sequence of *Bacillus amyloliquefaciens* alkaline protease and the mature coding sequence for *Escherichia coli* alkaline phosphatase (*phoA*). We show that export of alkaline phosphatase from *B. subtilis* depends on a functional signal sequence and that alkaline phosphatase activity depends upon secretion. The vector design facilitates the insertion of heterologous coding sequences between the signal and *phoA* to generate three-part translational fusions. Such *phoA* fusions are easily analyzed by monitoring alkaline phosphatase activity on agar plates or in culture supernatants or by immunological detection. Exploitation of this methodology, which has proven to be extremely useful in the study of protein secretion in *E. coli*, has a variety of applications for studying protein secretion in *B. subtilis*.

Fusion proteins which possess an easily assayed activity have proven to be valuable tools for the study of protein secretion in *Escherichia coli*. For example, translational fusions to *lacZ*, the *E. coli* gene coding for β-galactosidase, have been used extensively in the identification of genes involved in protein secretion and the analysis of signal sequence structure and function (2, 4–6, 29). Hoffman and Wright adapted the utility of fusion proteins for the analysis of protein secretion in *E. coli* by developing a reporter system based on translational fusions to *phoA*, the *E. coli* gene coding for alkaline phosphatase (AP) (13). AP proved to be particularly suited as a reporter in *E. coli* secretion studies because (i) AP has activity only when secreted, (ii) AP retains activity even with an amino-terminal extension, and (iii) AP is easily detected and assayed even at very low levels. Manoil and Beckwith extended the use of AP as a secretion reporter in *E. coli* by using a transposon derivative of Tn*5* which contains *phoA* without the promoter and signal sequence (22). The use of *phoA* fusions has proven to be a powerful tool for the study of protein secretion compatibility, intrinsic membrane topology, protein export signals, and the identification of genes for cell envelope and extracellularly secreted proteins (for a review, see reference 23).

Most studies of protein secretion in bacteria have focused on *E. coli*, but the gram-positive *Bacillus* species merit investigation since they secrete certain proteins into growth media at high levels (1, 26, 32). Although this secretion capability has long been important for commercial enzyme production, little is known about the mechanism of protein export in *Bacillus* species. One reason that protein secretion in *Bacillus* species has remained largely unexplored is the lack of convenient genetic tools. In this article we present an extension of the *phoA* fusion technology to the study of protein secretion in *Bacillus subtilis*. We describe how the various assays for secretion that are based on *phoA* fusions, which have been so valuable in studies of *E. coli* protein export, can be used to analyze secretion from *B. subtilis*. A plasmid was constructed which contains the *Bacillus amyloliquefaciens* alkaline protease (*apr*) signal sequence fused to *E. coli phoA* in a fashion which allows the insertion of heterologous protein coding sequences between the signal and *phoA*. We show that this signal sequence is able to direct the secretion of AP from *B. subtilis* and that AP activity is dependent upon secretion. To demonstrate one application of this system, three different heterologous coding regions were inserted between the *Bacillus* signal and *phoA*, and assays of AP activity were used to analyze the secretion compatibility of these fusion proteins in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* NM522 (12) and XL1- Blue (9) were used in this study. Cultures were grown in LB media (21) with 50 μg of ampicillin (Sigma) per ml at 37°C with shaking and on LB plates with 50 μg of ampicillin per ml at 37°C for 16 h. Expression studies were done in *B. subtilis* BE1010 (*trpC2 metB10 lys-3 ΔaprE66 Δnpr-82*), a low protease derivative of strain BR151 (37) which we constructed. In strain BE1010, deletions in *aprE* (codons 77 to 223) and *npr* (codons 74 to 250) have rendered the chromosomal genes coding for subtilisin and neutral protease nonfunctional, greatly reducing the level of extracellular proteases produced from this strain.

Plasmids. Plasmids pHI1 from A. Wright (16), pJF2 from P. Bassford (11), and pHK412 from L. Enquist and A. Robbins were used. V. Nagarajan provided plasmids pBE20, pBE25, pBE26, pBE64, and pBE85, which were constructed by Nagarajan as follows. Plasmid pBE20 (31) is an *E. coli-B. subtilis* shuttle vector created by fusing pTZ18R (Pharmacia) and pC194 (14) at the *Hin*dIII sites. Plasmid pBE25 was made by cloning the *apr* gene (35, 36) from *B. amyloliquefaciens* between the *Sma*I and *Bam*HI sites of pBE20. Plasmid pBE26 was derived from pBE25 by site-directed mutagenesis and contains an *Eco*RV site between codons 32 and 33, two codons downstream from the signal processing site (34). An additional *Eco*RV site was created 3′ to the *apr* transcriptional terminator because of the nonspecific binding

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of the mutagenic oligonucleotide. Plasmid pBE64, a derivative of pBE26, contains a defective signal sequence (*apr* 13R14PΔ13-24) because of the replacement of *apr* codons 13 to 24 with AGGCCT encoding Arg-Pro. The protein A gene (*spa*) from pRIT5 (Pharmacia) was engineered by site-directed mutagenesis so that codons 23 to 263 of *spa* were flanked by *Eco*RV sites. Plasmid pBE85 contains the *apr* signal sequence fused to this *Eco*RV *spa* fragment.

To construct a convenient fragment containing the *phoA* mature coding sequence, a 2.7-kb *Hin*dIII-*Xho*I fragment from the plasmid pHI1 containing the *E. coli* K-12 *phoA* gene was subcloned between the *Hin*dIII and *Sal*I sites of pTZ18R to make pBE230. An *Eco*RV site was then inserted between the signal sequence and the mature portion of *phoA* to generate pBE231. To make pBE240, in which the *apr* signal sequence is fused to *phoA* (see Fig. 1), the *Eco*RV fragment from pBE26 which contains the *apr* pro and mature sequences was replaced by a 1.5-kb *Eco*RV-*Bst*EII fragment containing *phoA* (BstEII end filled by DNA polymerase I Klenow fragment) from pBE231. The *apr* signal-*phoA* mature fusion junction of pBE240 was confirmed by DNA sequencing (27). The *apr*-derived fragment in pBE240 is 1,200 bp. To generate pBE244, in which the defective signal sequence *apr* 13R14PΔ13-24 is fused to *phoA*, the *Eco*RV-*Pst*I fragment from pBE64 (*apr* Pro mature) was replaced by the *phoA*-containing *Eco*RV-*Pst*I fragment from pBE240.

Three different tripartite fusions of *apr* signal sequence-insert-*phoA*, where the insert is a gene fragment encoding either a secreted or an intracellular protein, were constructed as follows.

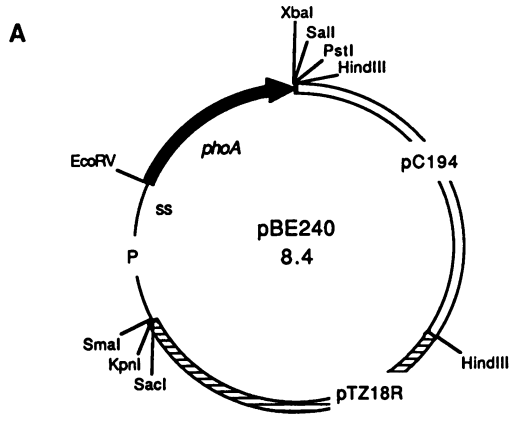
For fusion *apr-spa-phoA*, a 723-bp *Eco*RV fragment encoding *spa* codons 23 to 263 from pBE85 was subcloned in frame into the *Eco*RV site of pBE240 between the *apr* signal sequence and the *phoA* mature coding sequence to generate pBE270.

For fusion *apr-malE-phoA*, a 741-bp *Bgl*II-*Hin*fI fragment encoding *malE* codons 146 to 392 (ends filled by DNA polymerase I Klenow fragment) from pJF2 was subcloned in frame into the *Eco*RV site of pBE240 to generate pBE266.

For fusion *apr-lacZ-phoA*, a 624-bp *Hpa*I fragment encoding *lacZ* codons 148 to 355 from pHK412 was subcloned in frame into the *Eco*RV site of pBE240 to generate pBE267.

All constructions except pBE267 were initially propagated in *E. coli* and then transferred to *B. subtilis*. Plasmid pBE267 was recovered by transforming *B. subtilis* directly and screening by colony immunoassay (25, 28) for colonies which produce cross-reactive material with antiseras directed against AP and β-galactosidase. The structure of pBE267 thus obtained was confirmed by restriction analysis. Attempts to transform *E. coli* with this plasmid were unsuccessful.

AP activity assays. AP activity was detected as blue color when BE1010 transformants were plated on GYEXP indicator medium which is G-minimal medium (15 mM NH_4SO_4, 1 mM $MgSO_4$, 100 mM MOPS [morpholinepropanesulfonic acid; pH 7.0], 0.5 mM $FeCl_3$, 1 mM NaH_2PO_4, 1.5% agar) containing 0.2% glucose, 25 μg of L-tryptophan per ml, 25 μg of L-methionine per ml, 25 μg of L-lysine per ml, 1 μg of thiamine per ml, 0.1% yeast extract, 5 μg of chloramphenicol (Sigma) per ml, and 50 μg of XP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt; Sigma) per ml (24). Plates were incubated at 37°C for 16 h. AP activity in AM3 (Difco) liquid cultures was quantitated by measuring the conversion of *p*-nitrophenol phosphate (pNPP; Sigma) to *p*-nitrophenol (pNP) by samples of culture supernatants (8, 33). Typically, cultures were grown at 37°C with shaking, and cells were



B

					EcoRV				
GCC	*CAG*	*GCG*	GCA	GGG	GAT	ATC	CGG	ACA	CCA
CGG	*GTC*	*CGC*	CGT	CCC	CTA	TAG	GCC	TGT	GGT
A	*Q*	*A*	A	G	D	I	R	T	P
			↑				-->		
			processing site				*phoA*		

FIG. 1. (A) Map of pBE240. This secretion reporter vector is described in the text. The *phoA* gene and portions of the vector derived from plasmids pC194 and pTZ18R are indicated, as are the *apr* promoter (P) and signal sequence (ss). Some unique restriction sites are shown. (B) Sequence of the fusion junction between the *apr* signal sequence (in italics) and *phoA* gene (in boldface). The two codons preceding the *Eco*RV site come from the *apr* pro region. The predicted processing site is indicated (35).

removed by centrifugation. Fifty microliters of the supernatant was diluted to 0.5 ml in Tris-HCl (pH 8.0), mixed with 0.5 ml of 0.5% pNPP, and incubated at 37°C for 10 min. The reaction was stopped by the addition of 0.5 ml of 2 N NaOH, and the A_{410} was read. One unit is defined as that amount of enzyme required to convert 1 μmol of pNPP to pNP in 1 min at 37°C (33).

Labeling and immunoprecipitation. Mid-exponential-phase BE1010 cultures grown in S7 media with 5 μg of chloramphenicol per ml and 50 μg each of L-tryptophan, L-methionine, and L-lysine per ml were starved for methionine for 5 min and then labeled with 100 μCi of [^{35}S]methionine per ml as described previously (25). Acid-precipitable (5% trichloroacetic acid) material from an aliquot of culture was isolated and immunoprecipitated with anti-AP antisera as described previously (25). Immune complexes were recovered by binding to *Staphylococcus aureus* cells as described previously (25). Washed cells were boiled for 3 min in Laemmli buffer, and supernatants were fractionated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels (17) and visualized by fluorography.

RESULTS

Secretion of *E. coli* AP from *B. subtilis*. The secretion reporter vector which we constructed is pBE240 (Fig. 1). In this *E. coli-B. subtilis* shuttle vector, the *E. coli* AP

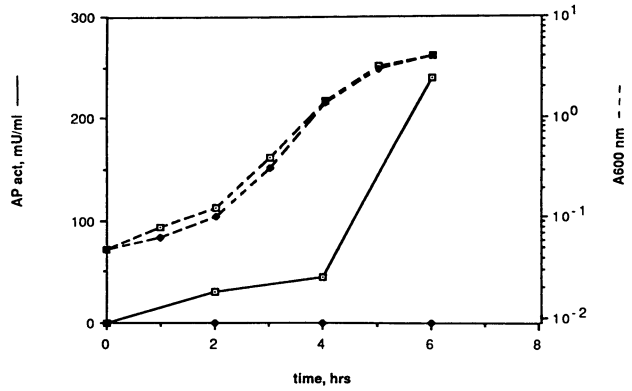


FIG. 2. Growth (– – –) and supernatant AP activity (——) as a function of time for BE1010/pBE240 (□) and BE1010/pBE244 (◆) cultures grown in AM3 medium with 5 μg of chloramphenicol per ml. Assays were performed as described in Materials and Methods.

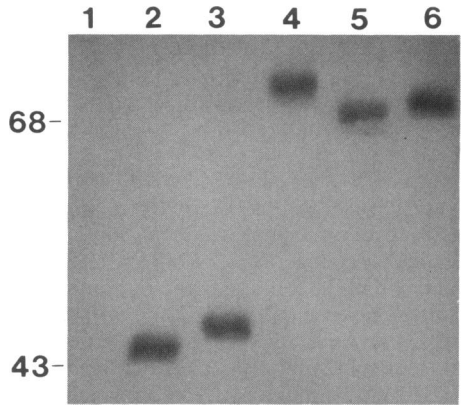


FIG. 3. Labeling and immunoprecipitation of AP and AP fusions from *B. subtilis*. BE1010 cultures harboring pBE20 (lane 1), pBE240 (lane 2), pBE244 (lane 3), pBE266 (lane 4), pBE267 (lane 5), and pBE270 (lane 6) were grown in S7 medium and labeled for 1 min with [^{35}S]methionine. Trichloroacetic acid-precipitable material from a 0.5-ml aliquot was immunoprecipitated with anti-AP serum as described in Materials and Methods. Markers in kilodaltons are shown on the left.

mature coding sequence (*phoA*) is fused in frame to the *B. amyloliquefaciens apr* signal sequence under control of the *apr* expression elements. A unique *Eco*RV restriction site located at the junction of *apr* and *phoA* sequences permits heterologous protein coding sequences to be inserted between the *apr* signal and *phoA*. The polypeptides produced from such translational fusions retain the region of alkaline protease signal processing, including two residues distal to the cleavage site (34). The vector contains several unique restriction sites at each end of the insert (from the pTZ18R polylinker) which facilitate the exchange of promoter-signal or mature regions. The plasmid pBE244 was made by moving the *phoA* mature coding sequence into a vector which carries a defective *apr* signal sequence.

To determine whether pBE240 directs secretion of active AP from *B. subtilis*, strain BE1010 was transformed to chloramphenicol resistance by pBE240 or pBE244 and culture supernatants were assayed for AP activity during growth (Fig. 2). The results show that pBE240 directs the secretion of AP from *B. subtilis*. The time course of appearance of activity is in accord with the known growth-phase dependence of the *apr* promoter (32). In contrast, BE1010/pBE244 gives no detectable AP activity, demonstrating that extracellular activity depends upon secretion of AP. The same conclusion is reached by monitoring AP activity on XP indicator plates where BE1010/pBE240 colonies are blue and BE1010/pBE244 colonies are white.

The secretion of AP from *B. subtilis* was also demonstrated by Western blot (immunoblot) analysis (data not shown). A single protein, with an apparent molecular mass of 47 kDa, which comigrates with purified AP, was detected by anti-AP antibody in BE1010/pBE240 culture supernatant but not in BE1010/pBE244 culture supernatant.

To determine whether the absence of extracellular AP in BE1010/pBE244 cultures is due to a secretion defect and not to lack of synthesis, cultures were pulse labeled with [^{35}S]methionine, immunoprecipitated with anti-AP antibody, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3, lanes 1 to 3). During a 1-min pulse, similar levels of AP were seen from both BE1010/pBE240 and BE1010/pBE244 cultures. The pBE240 band (lane 2) has the mobility expected for mature AP (47 kDa), while the slower migration of the pBE244 band (lane 3) presumably results from the presence of the unprocessed defective signal sequence.

We conclude that *E. coli* AP is secreted from *B. subtilis* when the *phoA* gene is fused to the *B. amyloliquefaciens apr* signal sequence and promoter and that AP activity is a measure of secretion.

Use of *phoA* fusions to monitor secretion of foreign proteins from *B. subtilis*. One application of this work is the use of AP as a reporter for secretion of heterologous polypeptides from *B. subtilis*. To demonstrate this application, we inserted coding sequences for three different polypeptides in frame between the *apr* signal sequence and *phoA* at the *Eco*RV site of pBE240 (Fig. 1; Table 1). Protein A (59 kDa) is an immunoglobulin G binding protein found on the surface of *S. aureus* cells (20) and has been shown to be secreted from *B. subtilis* (10, 37). A fragment of the protein A gene (*spa*) was inserted at the *Eco*RV site of pBE240 to generate pBE270. Maltose-binding protein (MBP) is a 40 kDa protein normally secreted into the periplasm of *E. coli* (3) but untested for secretion in *B. subtilis*. A fragment of the *malE* gene which codes for MBP was inserted at the *Eco*RV site of pBE240 to generate pBE266. We also inserted a 624-bp fragment (codons 148 to 355) of the *E. coli* β-galactosidase coding sequence (*lacZ*) at the *Eco*RV site of pBE240 to generate pBE267. The portion of β-galactosidase encoded by this fragment is unable to pass through the cytoplasmic membrane of *E. coli* (18) but has not been tested for secretion in *B. subtilis*.

BE1010 transformed to chloramphenicol resistance by

TABLE 1. Extracellular AP activities of fusion strains

Plasmid	Signal sequence	Fusion	Size (kDa)[a]	AP activity (mU/ml)[b]
pBE240	wt[c]	*phoA*	50	280
pBE244	Deletion	*phoA*	49	<1
pBE270	wt	*spa-phoA*	74	80
pBE266	wt	*malE-phoA*	75	100
pBE267	wt	*lacZ-phoA*	71	<1

[a] Predicted size of protein encoded by the translational fusion including the signal sequence.
[b] Supernatant assayed at 6 h after inoculation.
[c] wt, Wild type.

pBE270 or pBE266 is blue on XP indicator plates, whereas transformants of pBE267 are white. Assays of culture supernatants show significant levels of AP activity from BE1010/pBE270 and BE1010/pBE266 but none from BE1010/pBE267 (Table 1). Fusion proteins of the expected sizes are detected from supernatants of BE1010/pBE270 and BE1010/pBE266 by Western blot (data not shown). The lack of AP activity from BE1010/pBE267 is not due to lack of synthesis, since comparable amounts of the three fusions are labeled in 1 min (Fig. 3, lanes 4 to 6). The protein A and MBP fusions are secreted from *B. subtilis*, but the β-galactosidase fusion is not.

DISCUSSION

We have shown that *E. coli* AP can be used as a reporter for secretion in *B. subtilis*. When the *E. coli phoA* mature coding sequence is fused in frame to a *Bacillus* alkaline protease signal sequence, active AP is secreted into the culture supernatant (see also reference 30). The appearance of AP activity in the supernatant requires a functional signal sequence, since a fusion with a defective signal sequence results in synthesis of intracellular AP but no extracellular activity is found. Therefore, in *B. subtilis* AP activity depends on secretion as it does in *E. coli* and some other bacterial species (23).

A variety of convenient assays based on the *E. coli* system have now been shown to work well with *B. subtilis*. Simple assays for AP activity, either on indicator plates or in culture supernatants, can be used to detect secretion. Synthesis and cellular localization of AP can be followed immunologically. A colony immunoassay can be used in conjunction with XP indicator plates to screen large numbers of colonies for synthesis and secretion of AP (25, 28). For more quantitative or kinetic measurements, isotopically labeled AP is measured after immune precipitation.

B. subtilis is known to synthesize several APs (7, 15). However, under the conditions described here, there is no background from endogenous APs in any of the assays used. Therefore it will be possible to use the AP reporter for many applications without constructing phosphatase-deficient hosts. However, a *B. subtilis* AP mutation has recently been described (7). *B. subtilis* strains unable to synthesize one or more APs may be required for some uses of the AP reporter.

The *B. subtilis-E. coli* shuttle vector pBE240 described in this work is useful for constructing translational fusions to AP. To determine if a polypeptide functions as an export signal, the coding sequence can be substituted for the *apr* signal sequence in pBE240. Alternatively, a polypeptide sequence can be tested for secretion compatibility by inserting it in frame between the *apr* signal and *phoA* at the unique *Eco*RV site (Fig. 1). In this case, secretion of the fusion polypeptide can be monitored without requiring a direct assay for the heterologous protein. Examples of this application are the three fusions of AP to portions of *S. aureus* protein A, *E. coli* β-galactosidase, and *E. coli* MBP. Since protein A was previously shown to be secreted from *B. subtilis* (10, 34), it was expected that the fusion polypeptide would be secreted, as was observed. The two *E. coli* protein fragments used had not previously been tested for secretion from *Bacillus* species. MBP is normally secreted into the *E. coli* periplasm (for a review, see reference 4), while a large body of evidence has shown that β-galactosidase cannot be exported into the periplasm (4, 18; for a review, see reference 6). The particular fragment of β-galactosidase that we used has been shown to contain sequences that are incompatible with secretion in *E. coli* (18). With all of our fusions, we avoid a net positive charge in the first five residues following the signal sequence since this has been shown in *E. coli* to interfere with export (19). Immune precipitation of isotopically labeled proteins showed that all three fusion polypeptides are synthesized in *B. subtilis* (Fig. 3). Both the protein A-AP and MBP-AP translational fusions produced extracellular AP activity, while the β-galactosidase-AP fusion did not, although a fusion protein of the expected size was synthesized. This result shows that protein A and MBP sequences are compatible with secretion in *B. subtilis* but the β-galactosidase fragment is not.

The plasmid pBE240 produces active AP in *E. coli*, demonstrating that the *apr* signal functions in *E. coli*. In *E. coli*, expression of fusions in which an export signal is attached to β-galactosidase may be lethal because of abortive entry of the fusion polypeptide into the export pathway (4). The plasmid pBE267, in which a fragment of the *lacZ* gene is inserted between the *apr* signal and *phoA* of pBE240, could not be recovered in *E. coli*, suggesting that expression of this fusion is lethal. However, *B. subtilis* BE1010 carrying pBE267 grows normally. In *E. coli*, the lethality of fusions of β-galactosidase to export signals depends on the amount of fusion synthesized. In *B. subtilis*, two different fusions of the entire β-galactosidase sequence to large amino-terminal portions of the *B. subtilis* exoenzyme levansucrase were lethal only when expression was greatly elevated (38). It is possible that higher levels of the fusion protein encoded by pBE267 would inhibit growth of *B. subtilis*. Alternatively, this fusion may not enter the export pathway in *B. subtilis*.

Although extracellular AP activity is evidence of secretion of the fusion polypeptide, the amount of AP activity found is more difficult to interpret. The activity levels from the protein A and MBP fusions are intermediate between BE1010/pBE240 (AP alone) and BE1010/pBE244 (defective signal) (Table 1). Therefore, we compared secretion of the protein A fusion with secretion of protein A alone by fusing only the 723-bp *Eco*RV *spa* fragment to the *apr* signal sequence to give pBE245. Supernatants from BE1010/pBE270 (protein A fusion) and BE1010/pBE245 cultures showed similar levels of protein A when assayed by enzyme-linked immunosorbent assay (data not shown), suggesting that the amount of fusion as measured by AP activity accurately reflects the extracellular accumulation of protein A.

Our results do not reveal the basis of the reduced accumulation of AP activity from the tripartite fusions. Perhaps the direct juxtaposition of the *apr* signal to AP is exported more efficiently than any of the other fusions tested. Alternatively, the tripartite fusions might be translocated as well as AP alone but might be slower to assume a protease-resistant conformation, or they might be more likely to bind to the cell wall, or they might dimerize with reduced efficiency. Although our experiments do not distinguish among these possibilities, they do show that extracellular AP activity is evidence for secretion of the heterologous sequence.

Most knowledge about protein export in prokaryotes rests on genetic and biochemical experiments in *E. coli*. The use of AP as a reporter for secretion has contributed significantly to those investigations. Our results show that *E. coli* AP can likewise be used conveniently to study protein targeting in *B. subtilis*. The methods described in this study can be applied to such areas as analysis of export signals, isolation of genes involved in secretion, and analysis of membrane protein topology. These methods should find extensive use in the study of protein export in *B. subtilis*.

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REFERENCES

1. **Arbige, M. V., and W. H. Pitcher.** 1989. Industrial enzymology: a look towards the future. Tibtech **7**:330–335.
2. **Bankaitis, V. A., J. P. Ryan, B. A. Rasmussen, and P. J. Bassford.** 1985. The use of genetic techniques to analyze protein export in *Escherichia coli*. Curr. Top. Membr. Transp. **24**:105–150.
3. **Bassford, P. J.** 1990. Export of the periplasmic maltose-binding protein of *Escherichia coli*. J. Bioenerg. Biomembr. **22**:401–439.
4. **Bassford, P. J., T. J. Silhavy, and J. R. Beckwith.** 1979. Use of gene fusion to study secretion of maltose-binding protein into *Escherichia coli* periplasm. J. Bacteriol. **139**:19–31.
5. **Beckwith, J., and T. J. Silhavy.** 1983. Genetic analysis of protein export in *Escherichia coli*. Methods Enzymol. **97**:3–11.
6. **Benson, S. A., M. N. Hall, and T. J. Silhavy.** 1985. Genetic analysis of protein export in *Escherichia coli* K12. Annu. Rev. Biochem. **54**:101–134.
7. **Bookstein, C., C. W. Edwards, N. V. Kapp, and F. M. Hulett.** 1990. The *Bacillus subtilis* 168 alkaline phosphatase III gene: impact of a *phoA*III mutation on total alkaline phosphatase synthesis. J. Bacteriol. **172**:3730–3737.
8. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96**:307–316.
9. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques **5**:376–379.
10. **Fahnestock, S., and K. E. Fisher.** 1986. Expression of the *Staphylococcal* protein A gene in *Bacillus subtilis* by gene fusions utilizing the promoter from *Bacillus amyloliquefaciens* α-amylase gene. J. Bacteriol. **165**:796–804.
11. **Fikes, J. D., and P. J. Bassford.** 1987. Export of unprocessed precursor maltose-binding protein to the periplasm of *Escherichia coli* cells. J. Bacteriol. **169**:2352–2359.
12. **Gough, J. A., and N. E. Murray.** 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. **166**:1–19.
13. **Hoffman, C. S., and A. Wright.** 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82**:5107–5111.
14. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150**:815–825.
15. **Hulett, F. M., C. Bookstein, and K. Jensen.** 1990. Evidence for two structural genes for alkaline phosphatase in *Bacillus subtilis*. J. Bacteriol. **172**:735–740.
16. **Inouye, H., S. Michaelis, A. Wright, and J. Beckwith.** 1981. Cloning and restriction mapping of the alkaline phosphatase structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vitro. J. Bacteriol. **146**:668–675.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lee, C., P. Li, H. Inouye, E. R. Brickman, and J. Beckwith.** 1988. Genetic studies on the inability of β-galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. J. Bacteriol. **171**:4609–4616.
19. **Li, P., J. Beckwith, and H. Inouye.** 1988. Alteration of the amino terminus of the mature sequence of a periplasmic protein can severely affect protein export in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85**:7685–7689.
20. **Lofdahl, S., B. Guss, M. Uhlen, L. Philipson, and M. Lindberg.** 1983. Gene for *Staphylococcal* protein A. Proc. Natl. Acad. Sci. USA **80**:697–701.
21. **Maniatis, T., E. F. Fritsch, and K. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
23. **Manoil, C., J. J. Mekalanos, and J. Beckwith.** 1990. Alkaline phosphatase fusions: sensors of subcellular location. J. Bacteriol. **172**:515–518.
24. **Michaelis, S., H. Inouye, D. Oliver, and J. Beckwith.** 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154**:366–374.
25. **Nagarajan, V.** 1990. System for secretion of heterologous proteins in *Bacillus subtilis*. Methods Enzymol. **185**:214–223.
26. **Priest, F. G.** 1984. Extracellular enzymes. American Society for Microbiology, Washington, D.C.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
28. **Saunders, C. W., B. J. Schmidt, R. L. Mallonee, and M. S. Guyer.** 1987. Secretion of human serum albumin from *Bacillus subtilis*. J. Bacteriol. **169**:2917–2925.
29. **Silhavy, T. J., and J. R. Beckwith.** 1985. Uses of *lac* fusions for the study of biological problems. Microbiol. Rev. **49**:398–418.
30. **Sloma, A., D. Pawlyk, and J. Pero.** 1988. Development of an expression and secretion system in *Bacillus subtilis* utilizing *sacQ*, p. 23–26. *In* A. T. Ganeson and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 2. Academic Press, Inc., New York.
31. **Tang, L., R. Lenstra, T. V. Borchert, and V. Nagarajan.** 1990. Isolation and characterization of levansucrase-encoding gene from *Bacillus amyloliquefaciens*. Gene **96**:89–93.
32. **Thompson, L. D., and N. Vasantha.** 1986. Production and secretion of a heterologous protein by *Bacillus subtilis* using protease gene-based secretion vectors, p. 129–140. *In* A. T. Ganesan and J. A. Hoch (ed.), Bacillus molecular genetics and biotechnology applications. Academic Press, Inc., New York.
33. **Torriani, A.** 1960. Influence of inorganic phosphate in the formation of phosphatases by *Escherichia coli*. Biochim. Biophys. Acta **38**:460–469.
34. **Vasantha, N., and L. D. Thompson.** 1986. Secretion of a heterologous protein from *Bacillus subtilis* with the aid of protease signal sequences. J. Bacteriol. **165**:837–842.
35. **Vasantha, N., L. D. Thompson, C. Rhodes, C. Banner, J. Nagle, and D. Fipula.** 1984. Genes for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a large open reading frame between the regions coding for signal sequence and mature protein. J. Bacteriol. **159**:811–819.
36. **Wells, J. A., E. Ferrara, D. J. Henner, D. A. Estell, and E. Y. Chen.** 1983. Cloning, sequencing and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucleic Acids Res. **11**:7911–7924.
37. **Young, F. E., C. Smith, and B. E. Reilly.** 1969. Chromosomal location of genes regulating resistance to bacteriophage in *Bacillus subtilis*. J. Bacteriol. **98**:1087–1097.
38. **Zagorec, M., and M. Steinmetz.** 1990. Expression of levansucrase-β-galactosidase hybrids inhibits secretion and is lethal in *Bacillus subtilis*. J. Gen. Microbiol. **136**:1137–1143.