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Green Fluorescent Protein Functions as a Reporter for Protein Localization in *Escherichia coli*†

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The use of green fluorescent protein (GFP) as a reporter for protein localization in *Escherichia coli* was explored by creating gene fusions between *malE* (encoding maltose-binding protein [MBP]) and a variant of *gfp* optimized for fluorescence in bacteria (GFPuv). These constructs encode hybrid proteins composed of GFP fused to the carboxy-terminal end of MBP. Fluorescence was not detected when the hybrid protein was synthesized with the MBP signal sequence. In contrast, when the MBP signal sequence was deleted, fluorescence was observed. Cell fractionation studies showed that the fluorescent MBP-GFP hybrid protein was localized in the cytoplasm, whereas the nonfluorescent version was localized to the periplasmic space. Smaller MBP-GFP hybrid proteins, however, exhibited abnormal fractionation. Expression of the gene fusions in different *sec* mutants, as well as signal sequence processing assays, confirmed that the periplasmically localized hybrid proteins were exported by the *sec*-dependent pathway. The distinction between fluorescent and nonfluorescent colonies was exploited as a scorable phenotype to isolate *malE* signal sequence mutations. While expression of hybrid proteins comprised of full-length MBP did not result in overproduction lethality characteristic of some exported β-galactosidase hybrid proteins, synthesis of shorter, exported hybrid proteins was toxic to the cells. Purification of MBP-GFP hybrid protein from the different cellular compartments indicated that GFP is improperly folded when localized outside of the cytoplasm. These results suggest that GFP could serve as a useful reporter for genetic analysis of bacterial protein export and of protein folding.

The study of bacterial protein export has been greatly facilitated by the use of reporter genes whose products serve as enzymatic markers for cellular location. It is well established that reporter proteins such as alkaline phosphatase (encoded by *phoA*) and β-lactamase (encoded by *bla*) function optimally only when localized to the periplasmic space. These reporters have been especially valuable in identifying regions of a protein that promote membrane translocation (6, 24) and allow predictions of membrane topology (37, 38).

β-Galactosidase functions in the opposite manner of alkaline phosphatase and β-lactamase in that it is enzymatically active only when retained in the cytoplasm (15, 18, 21, 40). When fused to appropriate export signals, β-galactosidase is localized to cellular locations that render the enzyme inactive. This phenotype has been exploited for the isolation of mutants with restored β-galactosidase activity. Many of these mutants ultimately led to the identification of a number of *sec* genes that encode important components of the *Escherichia coli* protein export machinery (17, 30, 40).

Another phenotype associated with *E. coli* strains that produce exported β-galactosidase fusions is overproduction lethality, resulting from high-level synthesis of the hybrid proteins (3, 13). It has been observed that β-galactosidase is incompatible with components of the export machinery and, as a consequence, the export pathway becomes "jammed" at the translocation step. This jamming event appears to be the result of the conformation assumed by β-galactosidase and not of the presence of specific amino acid sequences that are incompatible with the protein export machinery (32, 47). Upon sufficient overproduction of β-galactosidase hybrid proteins, the export pathway becomes so severely jammed that other exported proteins accumulate in the cytoplasm in their precursor form (3, 13). The overproduction lethality phenotype has also been exploited to characterize the protein export pathway. Specifically, mutations that reverse the lethal effects of hybrid protein overproduction have been used to reveal important features of the signal sequence of exported proteins (2, 4, 11, 12).

Despite the successes of using β-galactosidase fusion proteins for genetic analysis of protein export, biases and limitations to the genetic selections and screens that use this reporter likely exist, potentially limiting the spectrum of export mutants that can be isolated. For example, β-galactosidase fusions have been used in an attempt to isolate mutants defective in the insertion of integral membrane proteins. Although this selection yielded an informative class of mutants defective in disulfide bond formation (1), they were not revealing as to the cellular components important for membrane translocation.

It is predicted that use of an alternative reporter system may lead to the identification of additional components of the translocation machinery and facilitate better characterization of the components currently known to be important for protein export. In particular, a reporter protein that is efficiently translocated across the cytoplasmic membrane and yet is active only in the cytoplasmic compartment would be useful for initiating new screens for export-defective mutants.

To this end, we have investigated the use of green fluorescent protein (GFP) from *Aequorea victoria* as a reporter for protein localization in bacteria. GFP has several features that make it an attractive candidate for protein localization studies in bacteria. For example, the protein is active in *E. coli*, and it has proven to be a useful reporter for a number of investigations in this microorganism, including monitoring gene expression (45), assessing viability (7), and detecting bacteria in the

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environment (33). GFP is active as a chimeric protein and has provided details of bacterial cell division and chromosome partitioning (8, 19, 28, 34, 35, 42, 48, 52, 53, 55, 56). An additional characteristic of GFP that is potentially advantageous for protein localization studies is that GFP has a molecular mass of 27 kDa, which is significantly smaller than β-galactosidase, whose molecular mass is ca. 130,000 kDa. GFP also functions as a monomer, in contrast to the tetrameric configuration required for β-galactosidase activity. The three-dimensional structure of GFP is also known and reveals that GFP attains a relatively uncomplicated "β-can" structure (41, 54) not unlike the structure of bacterial porin proteins (29). This fact suggests that GFP would likely be exported from the cytoplasm if fused to appropriate export signals. Also, GFP emits green light following excitation of an internal fluorophore composed of a Ser-Tyr-Gly sequence positioned near the protein's amino terminus. Excitation of GFP-expressing cells can be performed by exposure to long-wave UV light, making detection of GFP activity simple and obviating the need for specific substrates.

Although GFP is widely used to probe the events that occur within living cells (39), including protein localization in eukaryotic cells (20, 22, 43), the use of GFP for bacterial protein export studies has not previously been reported. Results presented in this study confirm that GFP can function as a reporter for protein localization in *E. coli* and hence provides a new tool for the analysis of bacterial protein export.

MATERIALS AND METHODS

Bacterial strains and plasmids. MC4100 [*araD139* Δ(*argF-lac*)*169 flhD5301 fruA25 relA1 rpsL150 rbsR22*] (46) was used as the host for fluorescence assays and cell fractionations along with the *sec* derivatives MM54 [MC4100, *secA51* (Ts) *leu-59*::Tn*10*] (40), IQ85 [MC100, *secY24*(Ts) *zhe-33*::Tn*10*] (44), and CK2163 (MC4100, *secBL75Q*) (16). DH5α (Gibco-BRL, Gaithersburg, Md.) was used as the host strain for cloning. XL1-Red (*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT*) (Stratagene, Inc., La Jolla, Calif.) was used as a host for mutagenesis of plasmid DNA.

pMGP2 and pMGC2 were constructed by ligating an agarose gel-purified 1.6-kb *Hin*dIII-*Sca*I fragment from pGFPuv (Clontech Laboratories, Palo Alto, Calif.) into *Hin*dIII-*Sca*I-digested pMalp2 and pMalc2 (New England Biolabs, Beverly, Mass.), respectively. Deletion derivatives of these plasmids were made by digesting pMGP2 and pMGC2 with *Bgl*II-*Bam*HI and religating the appropriate gel-purified fragments creating pMGP22 and pMGC22, respectively. Signal sequence mutations isolated by mutagenesis of pMGP2 were subcloned as a 1.0-kb *Bgl*II-*Eco*RV fragment into unmutagenized pMGP2. pJF2 has been described previously (14).

Bacterial growth. Cells were cultured in Luria broth (LB) (46) or E minimal medium (50) at 30°C. Ampicillin was added at a concentration of 100 μg/ml when selecting or maintaining transformants. Transformations were performed as outlined by Inoue et al. (25). Bacterial growth was monitored spectrophometrically at an optical density of 600 nm (OD_{600}). The synthesis of fusion proteins was induced by addition of 5 μM isopropyl-β-D-thiogalactoside (IPTG). Cells were also cultured by distributing a lawn of bacteria in LB top agar (0.7% agar) supplemented with ampicillin onto LB plus ampicillin plates and positioning a sterile 25-mm filter disk that had been immersed in dithiothreitol (1 M) at the center of the plate.

Fluorescence assays. Cells were harvested from the surface of LB plates following incubation overnight at 30°C with sterile saline. The cells were washed once in saline, and the OD_{600} of the cultures was determined. Fluorescence was measured at an excitation wavelength of 365 nm using a Dyna-Quant 200 fluorometer (Amersham Pharmacia Biotech, Piscataway, N.J.) modified with an interference filter (Edmund Scientific, Barrington, N.J.) to detect emission at 509 nm. The fluorescence values of three independently grown cultures were averaged and normalized to the density of the cultures. Fluorescence was expressed as arbitrary units. For comparison between bacterial strains, the fluorescence of MC4100 transformed with pMGC2 was set at a value of 1,000. For detection of fluorescence in colonies, plates were incubated for 3 to 4 days at 30°C. Plates were photographed under UV illumination at 385 nm.

Isolation and identification of export-defective mutants. pMGP2 was transformed into XL1-Red, and several ampicillin-resistant colonies were inoculated into 50 ml of LB, cultured overnight and then subcultured for a second overnight incubation. Plasmid DNA was prepared from the culture and transformed into DH5α. The transformants were screened on plates containing 1,000 to 2,000 colonies using a long-wave UV lamp (395 nm) to identify those that were fluorescent. A reconstruction experiment was performed by mixing IQ85 and MC4100 transformed with pMGP2 at ratios of 1:1,000 and 1:10,000 and plating the mixed populations onto LB Amp plates at various concentrations. After incubation for 4 to 5 days, plates were observed under UV illumination to identify fluorescent colonies. Both fluorescent and nonfluorescent colonies were restreaked at 30 and 42°C to test for temperature sensitivity.

Cell fractionation. Cells were fractionated after growth to an OD of 1.0. Then, 10 ml of cells was pelleted and resuspended in 0.5 ml of periplasting buffer (20% sucrose, 1 mM EDTA, 30,000 U of Ready-Lyse lysozyme [Epicentre Technologies, Madison, Wis.] per ml). Samples were incubated on ice for 5 min. Spheroplasts were then pelleted by centrifugation at 12,000 × *g* for 2 min. The supernatant was reserved as the periplasmic fraction. The pelleted spheroplasts were lysed in 1 ml of water containing 400 U of Omnicleave endonuclease (Epicentre Technologies) per ml. The samples were incubated for 5 min at room temperature followed by brief sonication at 30 to 40% power. Unlysed cells were removed by centrifugation at 12,000 × *g*. The supernatant was removed and centrifuged at 138,000 × *g* for 1 h. The supernatant was reserved as the cytoplasmic fraction, and the pellet containing the membrane fraction was resuspended in 0.5% Sarkosyl, 10 mM Tris-HCl, and 5 mM EDTA. Cells were further fractionated into inner and outer membranes by a modification of the technique described previously (26). Cells were cultured as described above and disrupted by French pressing. The lysate was centrifuged for 20 min at 8,000 × *g* to remove any unbroken cells. The clarified lysate was layered atop a 15 to 70% discontinuous sucrose gradient and centrifuged at 100,000 × *g* for 4 h. A band representing the membrane fraction was isolated, diluted 1:2 with 30 mM Tris-HCl (pH 8.0), and then layered on a 53 to 70% discontinuous sucrose gradient and centrifuged at 247,000 × *g* for 18 h. The fraction representing the inner-membrane proteins was recovered off the top of the 53% sucrose layer, and the outer membrane fraction was collected off the top of the 70% sucrose layer. Each fraction was layered on a second 53 to 70% discontinuous sucrose gradient and centrifuged as described above. The membrane fractions were again diluted, as described above, and centrifuged for 2 h at 100,000 × *g* to pellet the membrane proteins. The pellets were washed once with 1 M KCl, followed by an additional centrifugation step. The pellets and KCl washes were reserved for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cell fractions were analyzed by SDS-PAGE using a standard protocol (23). In brief, proteins were resolved by SDS–12.5% PAGE prior to transfer to nitrocellulose by electroblotting. Immunological detection was performed with rabbit polyclonal antibody specific to either maltose-binding protein (MBP) or GFP (Research Diagnostics, Flanders, N.J.) and alkaline phosphatase-conjugated secondary antibody (Bio-Rad, Inc., Hercules, Calif.). Antibodies to OmpA and ATPase (Research Diagnostics, Flanders, N.J.) were used to monitor fractionation of the outer and inner membranes, respectively.

Cell labeling and immune precipitation. Transformants were cultured in E minimal medium supplemented with amino acids excluding methionine and cysteine to an OD_{600} of 0.3. The cultures were pulse-labeled with 140 μCi of [^{35}S]methionine and [^{35}S]cysteine (Amersham Pharmacia Biotech, Piscataway, N.J.) per ml for 1 min, followed by a 1-min chase using an equal volume of prewarmed E medium containing 0.8% unlabeled methionine and cysteine. Then, 1-ml samples were immediately combined with 50 μl of ice-cold 100% trichloroacetic acid and incubated on ice for 10 min. Precipitated proteins were pelleted for 2 min in a microcentrifuge followed by a wash with cold acetone. The pellet was resuspended in a solution of 20 mM Tris-HCl (pH 7.5), 2% SDS, and 20 mM EDTA and then boiled for 2 min. Insoluble material was removed by centrifugation, and a 30-μl portion was mixed with 650 μl of immunoprecipitation buffer (50 mM Tris-HCl [pH 8.0], 150 mM EDTA, 2% Triton X-100) and GFP rabbit antiserum. After 1 h of incubation at 4°C with gentle mixing, immobilized protein A (Pierce Chemical Co., Rockford, Ill.) was added, and the samples were incubated for an additional hour at 4°C. The immune complexes were isolated by centrifugation and washed twice with immunoprecipitation buffer and twice again with 10 mM Tris-HCl (pH 8.0). The final pellet was resuspended in 50 μl of 10 mM Tris-HCl (pH 8.0) and 50 μl of SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.001% bromophenol blue), boiled for 2 min, and centrifuged for 5 min. Then, 10 μl of supernatant was resolved by SDS–7.5% PAGE. Immune-precipitated proteins were visualized using a Bio-Rad GS-363 phosphorimaging system (Bio-Rad, Inc., Hercules, Calif.).

Hybrid protein purification and characterization. MBP-GFP hybrid protein was purified from MC4100 transformed with pMGP2 and pMGC2. One-liter cultures of each strain were grown in LB supplemented with glucose at 2 g/liter at 30°C for 24 h. Cells were harvested by centrifugation, resuspended in 50 ml of column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol), and lysed by French pressing. The resulting cell lysate was centrifuged for 20 min at 8,000 × *g*. The supernatant was then centrifuged for 2 h at 100,000 × *g* to further clarify the lysate. Then, 3 ml of amylose resin (New England Biolabs), prepared as described by the manufacturer, was incubated with the cleared lysate for 20 min at room temperature. The protein-bound resin was transferred to a 10-ml disposable column and washed with 50 ml of column buffer. The hybrid protein was eluted with 10 ml of a 10 mM maltose solution and concentrated using a Centricon YM-50 concentrator (Amicon, Inc., Beverly, Mass.). After quantifying the protein spectrophotometrically, samples were di-

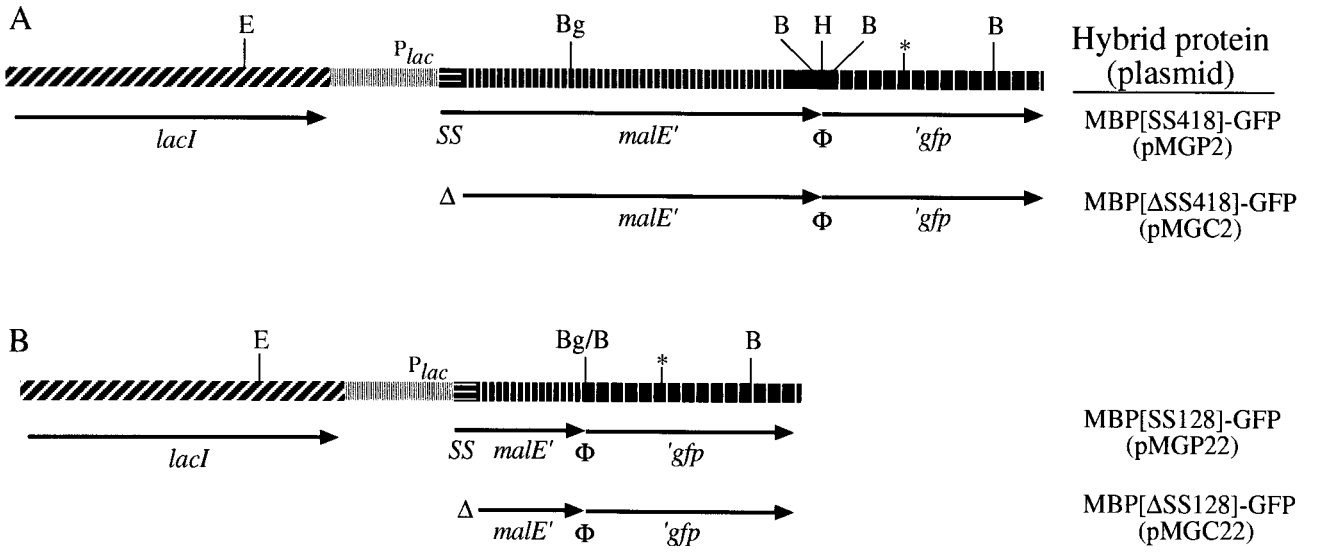

FIG. 1. Constructs encoding MBP-GFP hybrid proteins. (A) Constructs carried by pMGP2 (encoding MBP[SS418]) and pMGC2 (encoding MBP[ΔSS418]). (B) Constructs carried by pMGP22 (encoding MBP[SS128]) and pMGC22 (encoding MBP[ΔSS128]). Features: *lacI* (diagonal stripes), LacI repressor; *malE'* (narrow vertical stripes), MBP; *'gfp*, GFPuv (wide vertical stripes); PO_{lac}, *lac* promoter and operator; SS (horizontal stripes), signal sequence encoding region; and the linker region between *malE* and *gfp* (solid black line). The location of the region of *gfp* encoding the chromophore is indicated by an asterisk. Relevant restriction sites: E, *Eco*RV; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII. Δ, region of *malE* deleted in the constructs. The designation of the hybrid proteins encoded by each construct is shown on the right. The number within the square brackets indicates the number of amino acids of the mature portion of MBP fused to GFP.

luted to 1 mg/ml in column buffer. The purity of the protein samples was assessed by SDS-PAGE and staining with Coomassie dye.

Denaturation-renaturation experiments were performed as described previously (51) by adding 0.5 μl of concentrated HCl to a 100-μl sample containing 0.5 mg of each protein in column buffer. After a 5-min incubation, 1.0 μl of 10 N NaOH was added, and the samples were incubated further for 24 h at 4°C.

Additional samples of the MBP-GFP hybrid protein were dialyzed against 4 liters of column buffer without reducing agent using a microdialysis system (Life Technologies, Bethesda, Md.). β-Mercaptoethanol was added to the dialyzed protein to a final concentration of 10 mM, and the sample was incubated for several days at 4°C. The fluorescence of all samples was monitored using a Fluoreomax-2 spectrofluorometer (Instruments S.A., JOBIN YVON/SPEX Division, Edison, N.J.). Protein samples were also transferred to clear, 0.5-ml thin-wall PCR tubes, and the images were captured using a Kodak DC 260 model digital camera.

RESULTS

Construction and characterization of MBP-GFP hybrid proteins. The GFP variant GFPuv (9) was used to test the utility of GFP as a reporter for protein localization in *E. coli*. GFPuv was chosen because of its increased intensity of fluorescence relative to wild-type GFP and the fact that its codon usage has been optimized for expression in *E. coli*. MBP from *E. coli* was used as the target protein for fusion with GFP because it is a well-characterized exported protein normally localized to the periplasmic space. Gene fusions between *gfp* and *malE* were made as described in Materials and Methods, yielding the constructs shown in Fig. 1A. One construct, carried by pMGP2, encoded a hybrid protein where GFP was tagged onto the carboxy terminus of MBP, designated MBP[SS418]-GFP. Because it was unclear if GFP would function if localized outside of the cytoplasm, a construct was also assembled where *gfp* was fused to a version of *malE* that lacked a signal sequence. This gene fusion, carried by pMGC2, encoded the hybrid protein designated MBP[ΔSS418]-GFP that was retained in the bacterial cytoplasm.

Following transformation of pMGC2 into *E. coli*, it was evident that the MBP[ΔSS418]-GFP protein was active, as revealed by the green fluorescent colonies of the transformants (Fig. 2). No fluorescent colonies were observed with the pMGP2 transformants, however, suggesting that export of MBP-GFP rendered GFP inactive.

Localization of MBP-GFP hybrid proteins. To determine the cellular location of the MBP-GFP hybrid proteins, cells were fractionated into cytoplasmic, periplasmic, and membrane samples. The fractions were resolved by SDS-PAGE and

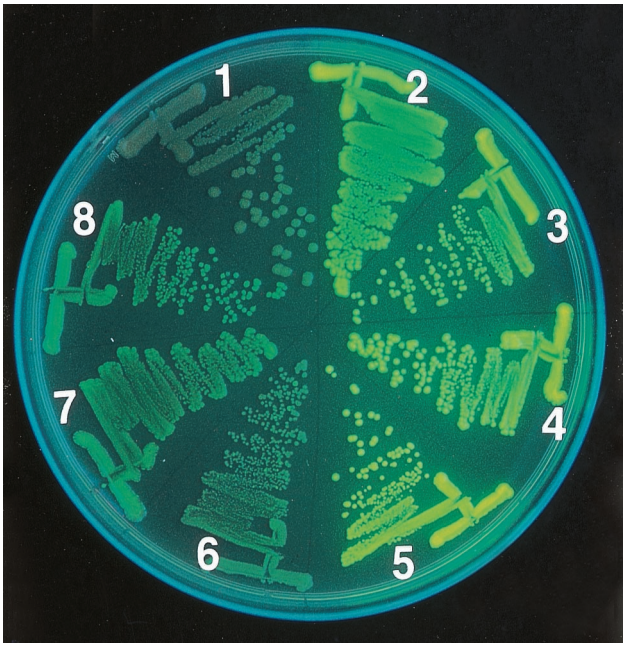

FIG. 2. Fluorescence of transformants expressing MBP-GFP hybrid proteins. MC4100 transformed with the following: 1, pMGP2; 2, pMGC2; 8, MM52 [*secA*(Ts)] transformed with pMGP2; 3, pMGC2; 7, CK2163 (*secB*) transformed with pMGP2; 4, pMGC2; 6, IQ85 [*secY*(Ts)] transformed with pMGP2; and 5, pMGC2.

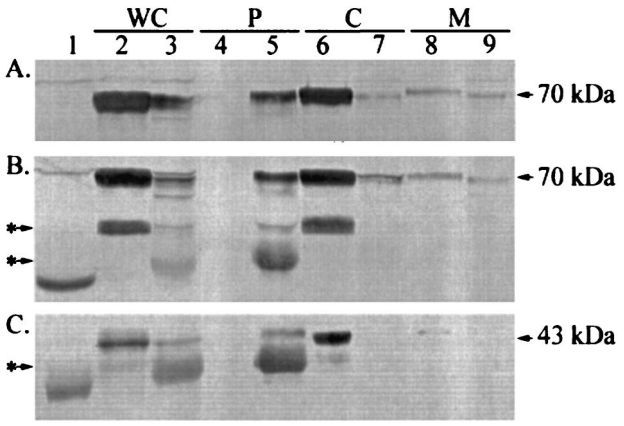

FIG. 3. Immunoblot analysis of MBP-GFP hybrid proteins. Cells transformed with plasmids expressing either MBP-GFP hybrid proteins (A and B) or MBP (C) were fractionated as described in Materials and Methods and analyzed by Western blot analysis. Immunoblots were decorated with anti-GFP antibody (A) or anti-MBP antibody (B and C). For panels A and B, lanes represent transformants of the following: lane 1, pJF2 (expressing wild-type MBP); lanes 2, 4, 6, and 8, pMGC2 (expressing MBP[Δ418]-GFP); lanes 3, 5, 7, and 9, pMGP2 (expressing MBP[SS418]-GFP). (C) MBP expressed from: lane 1, pJF2; lanes 2, 4, 6, and 8, pMalc2; lanes 3, 5, 7, and 9, pMalp2. Lane designations: WC, whole cell; P, periplasmic fraction; C, cytoplasmic fraction; M, membrane fraction. The location of the full-length MBP-GFP protein is noted with arrows next to the nearest corresponding molecular weight marker; the asterisk indicates breakdown products detected with anti-MBP antibody.

immunoblotted with antibodies directed against either GFP or MBP. As shown in Fig. 3, MBP[ΔSS418]-GFP was localized primarily in the cytoplasm, as was the signal sequenceless MBP species encoded by pMalc2 (Materials and Methods). In contrast, MBP synthesized with its signal sequence intact, as well as MBP[SS418]-GFP, was localized to the periplasmic space, indicating that GFP hybrid proteins can be efficiently exported out of the cytoplasmic compartment.

SDS-PAGE analysis of the cell fractions also revealed that MBP[SS418]-GFP was present at reduced levels when localized to the periplasmic space (Fig. 3A and B). Consequently, to achieve the results shown in Fig. 3, threefold-more volume of the exported MBP samples was loaded into each lane of the SDS-PAGE gel. As discussed below, the reduced levels of this protein are likely due, in part, to the incorrect folding of GFP in the periplasmic space, making it prone to proteolytic digestion. No breakdown products that cross-reacted with anti-GFP antibody could be detected in these samples, however. In contrast, when anti-MBP antiserum was used to decorate the MBP-GFP hybrid proteins, breakdown products were observed. Furthermore, MBP encoded by both the pMalc2 and pMalp2 plasmids also proved to be unstable. This was particularly evident with MBP expressed from pMalp2. Apparently, the MBP species encoded by these recombinant plasmids are uniquely prone to proteolysis since wild-type MBP, also encoded by a recombinant plasmid (14), was stable (Fig. 3B and C, lane 1). Taken together, these results suggest that some of the instability of MBP[SS418]-GFP can also be attributed to the MBP moiety.

Activity of MBP-GFP protein in *sec* mutants. To further characterize the MBP-GFP hybrid proteins with respect to protein export, the gene fusions were introduced to *E. coli* strains known to be defective in protein export. If MBP-GFP is exported by the normal *sec*-dependent pathway of protein export, then it was predicted that fluorescence could be detected if the hybrid proteins were expressed in a *sec* mutant. The MBP-GFP-encoding plasmids were transformed into three well-characterized *sec* mutants, previously shown to be important for export of a large number of proteins, including MBP. Figure 2 shows that fluorescence was detected whenever pMGP2 was transformed into a *secA* or *secY* mutant, even at 30°C, the permissive temperature for growth of these mutants (40, 44). Fluorescence was also detected when pMGP2 was transformed into a *secB* mutant. *secB* is a nonessential gene involved in the export of MBP, as well as other *E. coli* proteins (30, 31). As anticipated, transformants of pMGC2, encoding MBP[ΔSS418]-GFP, all remained fluorescent.

Although fluorescence could be visually detected after overnight incubation of the three *sec* mutants, fluorescent intensity increased with prolonged incubation at 30°C. The colony fluorescence shown in Fig. 2 was detected after 4 days of incubation at 30°C. Observably, GFP is a sensitive reporter of the protein export defects of these mutants, even at their permissive growth temperature.

To further quantify the changes in GFP activity observed in the various *E. coli* strains, fluorescent emission was measured at 509 nm, near the peak of emission by GFP (9). Table 1 shows the relative fluorescent intensities of the various transformants. In all cases, strains defective in protein export showed a significant increase in fluorescence (up to 400-fold for *secY*), indicating that export of the MBP-GFP hybrid protein was hindered.

Construction of additional *malE-gfp* fusions. To further characterize exported GFP hybrid proteins, additional *malE-gfp* gene fusions were constructed by deleting the region between the *Bgl*II and the two *Bam*HI sites on both pMGP2 and pMGC2 (Fig. 1B). Similar to the results seen with the full-length MBP-GFP hybrids, transformants expressing these shorter hybrid proteins showed fluorescence only when expressed without the MBP signal sequence (MBP[ΔSS128]-GFP) or when the exported protein (MBP[SS128]-GFP) was expressed in a *secY* mutant (Table 1). However, fractionation of the cells revealed that MBP[SS128]-GFP was not localized to the periplasmic space; rather, it was consistently detected in the membrane fraction. Additional fractionation studies revealed that the protein, with a predicted molecular mass of 40 kDa, fractionated with the outer membrane. In addition, an apparent breakdown product was found associated with the

TABLE 1. GFP fluorescence in *sec* mutants

E. coli strain	Plasmid[a]	Fluorescence[b]	Ratio of fluorescence[c] (*sec* mutant/MC4100)
MC4100 (wild type)	pMGC2	1,000	
	pMGP2	1 ± 1	
	pMGC22	702 ± 48	
	pMGP22	1 ± 8	
IQ85 (*secY*)	pMGC2	2,391 ± 129	2.4
	pMGP2	417 ± 167	417.0
	pMGC22	1,035 ± 384	1.5
	pMGP22	139 ± 7	139.0
CK2163 (*secB*)	pMGC2	1,763 ± 123	1.8
	pMGP2	140 ± 10	140.0
MM54 (*secA*)	pMGC2	1,271 ± 7	1.3
	pMGP2	112 ± 12	112.0

[a] Plasmid constructs are shown in Fig. 1.
[b] Fluorescence is reported in arbitrary units and was determined as described in Materials and Methods.
[c] Ratios indicate the difference in fluorescence between the *sec* mutants and MC4100 and were determined by dividing the fluorescence value of each *sec* mutant transformant with that of MC4100 transformed with the same plasmid.

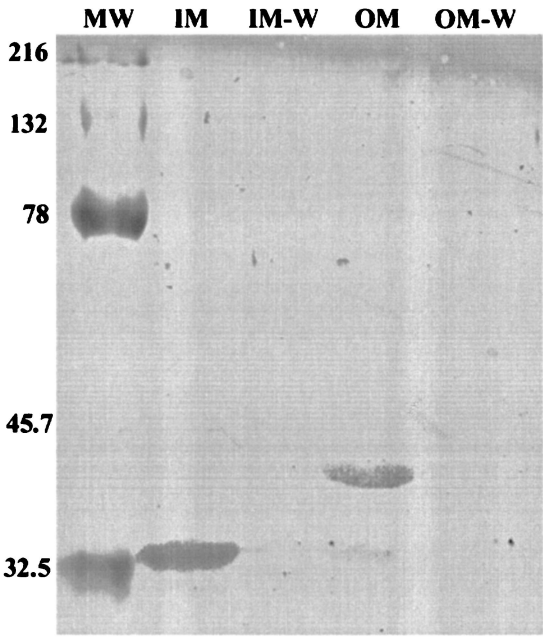

FIG. 4. Cell fractionation of shortened MBP-GFP hybrid protein. Cells expressing MBP[SS128]-GFP were fractionated into inner and outer membranes as described in Materials and Methods. The immunoblot shown was decorated with anti-GFP antibody. MBP[SS128]-GFP migrated at a predicted molecular mass of 40 kDa and fractionated with the outer membrane. An apparent smaller-molecular-weight derivative of this protein was also found in the inner membrane fraction. Molecular weight (MW) standards are as shown. IM, inner membrane fraction; IM-W, KCl wash of inner membrane fraction; OM, outer membrane fraction; OM-W, KCl wash of outer membrane fraction. Although not shown, OmpA and ATPase were used to monitor fractionation of the outer membrane and inner membranes, respectively.

inner membrane fraction (Fig. 4). One possibility considered was that this breakdown product represented GFP that had been clipped free of the MBP sequences. However, this product had a molecular mass of 33 kDa, which is significantly larger than GFP.

We also observed that levels of MBP[SS128]-GFP were significantly reduced in comparison with MBP[ΔSS128]-GFP, suggesting that the shorter hybrid protein was extremely unstable when localized out of the cytoplasm.

Expression of full-length MBP-GFP hybrids does not result in overproduction lethality. Although GFP behaves similarly to β-galactosidase in that neither protein is active when exported out of the cytoplasm, it remained to be determined if GFP hybrid proteins also resulted in an overproduction lethality phenotype characteristic of exported β-galactosidase fusions. To test the effects of induced synthesis of MBP-GFP hybrid proteins, we took advantage of the fact that expression of the *malE-gfp* gene fusions is under control of the *lac* regulatory elements (Fig. 1). We observed that upon induction of MBP[SS128]-GFP synthesis with IPTG, transformants continued to grow at a rate similar to cultures grown without induction (data not shown). Microscopic examination of the cells also revealed no morphological changes and no evidence of cell lysis. Induction of hybrid protein synthesis was confirmed by Western blot analysis (data not shown).

In contrast to these results, induced expression of MBP [SS128]-GFP was toxic to the cells. A reduction in growth rate, as well as cell lysis, was observed when transformants expressing the shorter, exported hybrid protein were grown in the presence of IPTG.

Given the toxicity of this GFP fusion, we constructed additional gene fusions between *gfp* and the complete coding regions of *phoA* (alkaline phosphatase) and *bla* (β-lactamase). These fusions all exhibit the phenotype associated with the full-length *malE* fusions in that they display fluorescence only when localized to the cytoplasmic compartment, and their synthesis is not toxic to the cells (B. J. Feilmeier and G. J. Phillips, unpublished data). Apparently, GFP is not toxic and can be faithfully localized if a sufficient amount of export information is provided at the amino-terminal portion of the protein. In addition, the behavior of these additional hybrid proteins indicates that the folding of MBP in the periplasmic space does not account for the lack of GFP fluorescence.

Isolation of export-defective mutants. Since cells expressing GFP localized to the periplasmic space do not fluoresce, we predicted that fluorescence could be used as a phenotype to isolate export-defective mutants. Given our understanding of the protein export process, it was predicted that mutations that map to *malE-gfp* that block export of the hybrid gene product, including those that alter the signal sequence-encoding region (27), should be isolated.

To test this prediction, pMGP2 was randomly mutagenized by passage through the *E. coli* mutator strain XL1-Red. Plasmid DNA was prepared from this strain that had been grown for several generations and used to transform DH5α. Several thousand transformants were screened by visualization with long-wave UV light, and numerous fluorescent colonies were detected. To determine if these transformants fluoresced as a result of a mutation in the *malE* signal sequence, a 2-kb *Eco*RV-*Bgl*II restriction fragment (Fig. 1) was isolated from a number of the mutant pMGP2 plasmids and used to replace the corresponding segment from unmutagenized pMGP2. The recombinants obtained all yielded fluorescent transformants. As shown in Fig. 5, DNA sequence analysis revealed that the signal sequence was altered in all of the mutants, confirming

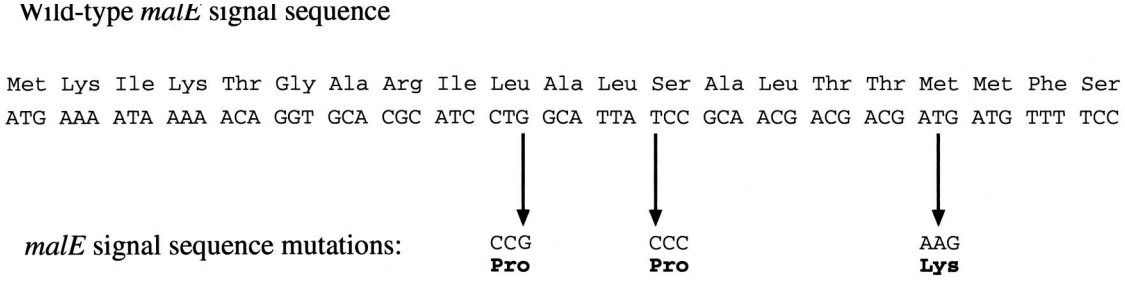

FIG. 5. Signal sequence mutations of MBP. The wild-type *malE* signal sequence encoding region is shown below the amino acid sequence of the amino-terminal region of the precursor MBP protein along with the sequence of three signal sequence mutations.

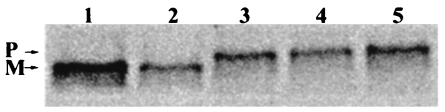

FIG. 6. Signal sequence processing of MBP-GFP hybrid proteins. Shown are pulse-labeled MBP-GFP proteins immune precipitated with anti-GFP antibody. Transformants labeled: lane 1, pMGC2; lane 2, pMGP2; lane 3, pMGP2 (mutant M1); lane 4, pMGP2 (mutant M2.1); lane 5, pMGP2 (mutant M2.3). P, precursor (signal sequence unprocessed) form; M, mature form.

the prediction that GFP can serve as a genetic tool for isolation of export-defective mutants.

Further characterization of the mutant MBP-GFP hybrid proteins was performed by immune precipitation of pulse-labeled polypeptides. Figure 6 shows the results of an experiment in which transformants were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine, and anti-GFP antibody was used to immunoprecipitate both the cytoplasmic and the periplasmic forms of the hybrid proteins. Comparison of the protein encoded by pMGP2 with that encoded by pMGP2 revealed that both proteins have the same molecular weight, indicating that the signal sequence was cleaved off of the periplasmically localized protein. In contrast, the signal sequence mutants remain in the precursor form, i.e., with their signal sequences intact. This experiment offered further evidence that the periplasmic MBP-GFP protein is exported by the *sec*-dependent pathway and requires signal sequence processing for efficient export. The MBP-GFP mutant proteins that retained fluorescent activity, however, were all defective in signal sequence processing.

Identifying export-defective mutants by the approach just described was easily accomplished by detecting a single fluorescent colony among a plate of 1,000 to 2,000 nonfluorescent ones. Since these fluorescent colonies, however, represented signal sequence mutants where export of the hybrid protein was completely blocked (Fig. 6), it was also determined if mutants could be identified that resulted in only a partial block in export. Consequently, a reconstruction experiment was performed by mixing cultures of MC4100 and IQ85 (*secY*), transformed with pMGP2, at various ratios and plating them on LB agar plates. After 3 to 4 days, fluorescent colonies could be distinguished on the plates at a ratio that reflected the proportion of wild-type and *secY* mutant cells in the original populations. Several fluorescent colonies were picked and restreaked at 30 and 42°C. All such colonies displayed a temperature-sensitive phenotype, indicating that they were *secY* mutants. These results indicated that export-defective mutants that only partially block export of the MBP-GFP hybrid protein can also be isolated by screening for colony fluorescence.

Why doesn't GFP function in the periplasmic space? Although it is not immediately obvious why fluorescence of MBP-GFP is not detected when the hybrid protein is localized to the periplasmic space, a number of possible explanations can be considered. For example, the exported protein could be irreversibly inactivated by modification such as proteolytic cleavage. Although full-length MBP-GFP was detected in immune blot analysis, degradation products related to the hybrid protein were also seen (indicated by the asterisks in Fig. 3). In addition, the amount of full-length MBP-GFP protein detected in the periplasmic space was also substantially reduced in comparison to the cytoplasmic version, perhaps explaining the lack of fluorescence.

To better understand why exported GFP was not fluorescent, we purified the MBP[SS418]-GFP hybrid protein to determine if it displayed fluorescence when no longer confined to the periplasmic space. To accomplish this, we took advantage of the affinity of the MBP moiety for amylose to purify MBP [SS418]-GFP from the periplasmic space, as well as MBP [ΔSS418]-GFP from the cytoplasmic compartment. Purified proteins were analyzed by SDS-PAGE and Western blotting to assess the purity of the proteins. While both the cytoplasmic and the periplasmic versions of MBP-GFP yielded a single band of predicted molecular weight, the exported fusion also copurified with a minor fraction of MBP breakdown products (data not shown). As shown in Fig. 7, illumination of the samples by UV showed that MBP[ΔSS418]-GFP isolated from the cytoplasm was highly fluorescent. In contrast, an equivalent amount of MBP[SS418]-GFP isolated from the periplasmic space showed no detectable fluorescence, consistent with the in vivo observations. These results indicated that the lack of fluorescence in vivo cannot be attributed solely to diminished levels of the hybrid protein and that recovery of GFP from the periplasmic environment is not sufficient to immediately restore fluorescence.

To determine if GFP is irreversibly inactivated in the periplasmic space or if the protein is simply misfolded in this environment, we attempted to restore fluorescence to the purified proteins. Samples of purified MBP[SS418]-GFP and MBP[ΔSS418]-GFP were treated with acid under conditions known to denature native GFP, followed by treatment with base to neutralize the sample and promote refolding of the protein (51). As anticipated, treatment of the MBP[ΔSS418]-GFP protein sample with acid completely eliminated fluorescence. However, nearly half of the activity was restored following the addition of base (Fig. 7). Strikingly, GFP fluorescence was also detected in the sample of MBP-GFP purified from the periplasmic space following the acid-base treatment regimen. We attribute the difference in recovery of fluorescence (compare the scales in Fig. 7), in part, to the higher purity of full-length MBP[ΔSS418]-GFP.

Further insight into the properties of MBP-GFP isolated from the periplasmic space was gained by observing that prolonged incubation of the protein sample in column buffer (Materials and Methods) alone also yielded active GFP. A sample of MBP[SS418]-GFP purified from the periplasmic space was extensively dialyzed against column buffer without β-mercaptoethanol to remove the reducing agent. β-Mercaptoethanol was added back to a portion of the purified proteins, and the samples were incubated at 4°C for several days. After prolonged incubation (7 days), samples incubated with β-mercaptoethanol fluoresced upon illumination with UV light. In contrast, samples containing buffer without the reducing agent showed no detectable levels of fluorescence over the same time period (data not shown).

The observation that GFP fluorescence could be detected upon prolonged incubation in buffer containing β-mercaptoethanol suggested that improper disulfide bond formation is contributing to the misfolding of GFP. The MBP[SS418]-GFP protein contains two cysteine residues, both located distal to the fusion joint shown in Fig. 1, that could participate in disulfide bond formation, and as a consequence render GFP inactive. We were, however, unable to detect fluorescence when MBP[SS418]-GFP was expressed in a *dsbA* mutant (1), one known to be defective in periplasmic disulfide bond formation. Likewise, growth of pMGP2 transformants in the presence of dithiothreitol yielded no fluorescent cells.

DISCUSSION

We have explored the use of GFP as a reporter for protein localization in *E. coli*. Because this protein is relatively small

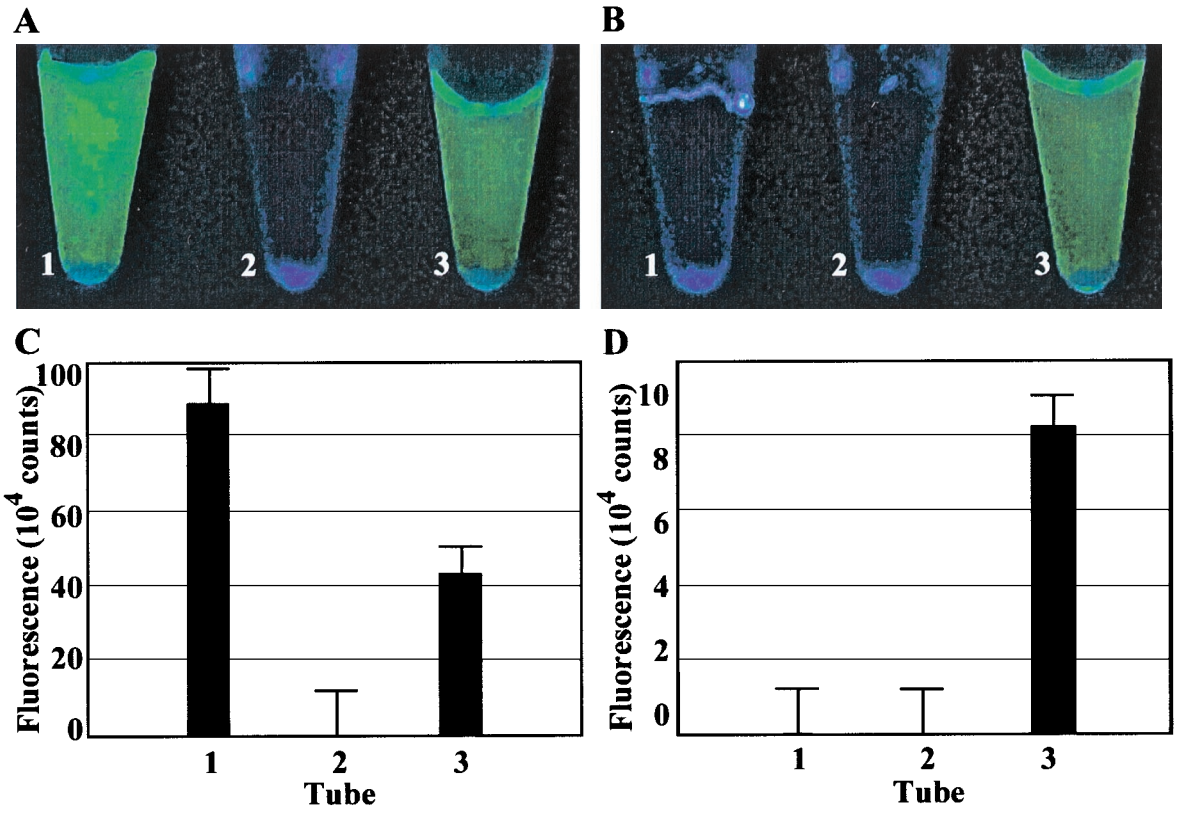

FIG. 7. Characterization of purified MBP-GFP hybrid protein. Hybrid proteins were purified from the periplasmic space or the cytoplasm and subjected to acid-base treatment, as described in Materials and Methods. (A) MBP[ΔSS418]-GFP isolated from the cytoplasm. Tubes: 1, untreated; 2, acid treated; 3, acid-base treated. (B) MBP[SS418]-GFP isolated from the periplasmic space. Tubes: 1, untreated; 2, acid treated; 3, acid-base treated. (C) Relative fluorescence of tubes shown in panel A. (D) Relative fluorescence of tubes shown in panel B.

and attains a conformation that resembles a porin monomer (41), it was predicted to be compatible with the protein export process of bacteria. To test this prediction and to assess the activity of GFP when localized outside of the cytoplasm, fusions between MBP and GFP (the UV-shifted variant, GFPuv) were constructed. Characterization of these fusions revealed that GFP remained active as long as the protein remained localized to the cytoplasm; export of the protein, however, rendered GFP inactive. Cellular fractionation studies revealed that the MBP[SS418]-GFP hybrid was efficiently exported outside of the cytoplasm. However, although fusions that contained the signal sequence plus 128 amino acids of MBP were exported out of the cytoplasm, they were not faithfully localized to the periplasmic space. Export of the hybrid proteins was also shown to be dependent on the *sec* pathway since GFP fluorescence was detected in strains carrying different *sec* alleles. Efficient signal sequence processing of MBP[SS418]-GFP was also confirmed by pulse-chase experiments.

The observation that GFP is active only in the cytoplasm parallels the observation that β-galactosidase fusions are likewise inactive when exported out of the cytoplasm. The distinction between Lac^+ and Lac^- phenotypes has been successfully used to isolate a number of export-defective mutants. In contrast to β-galactosidase, however, overproduction of MBP [SS418]-GFP did not cause a lethal jamming of the protein export machinery. This distinction likely is attributed to the smaller size and relatively simple conformation of GFP compared with β-galactosidase. We did observe, however, that expression of the shorter MBP-GFP hybrid protein (MBP [SS128]-GFP) was toxic when overproduced. It is not likely that this toxicity is the result of jamming of the protein export machinery (3, 12, 32, 47), since the hybrid protein was found to be efficiently localized outside of the cytoplasm (Fig. 4). Furthermore, export jamming is, in general, reduced when shorter β-galactosidase hybrid proteins are expressed (5), while the opposite was observed for GFP. The toxic effects of exported MBP[SS218]-GFP are not unprecedented, however, since expression of certain LamB fusion proteins is lethal to *E. coli* by a mechanism that does not involve export jamming (47). These results indicate that not every GFP fusion construct may be suitable for analysis of protein export.

The distinction in GFP activity in different subcellular compartments manifests itself in a striking phenotype between fluorescent and nonfluorescent colonies. The ease with which fluorescence can be detected and quantified makes GFP a potentially useful reporter with which to genetically track bacterial protein localization. To test the utility of GFP as a screenable marker for the isolation of export-defective mutants, pMGP2 was randomly mutagenized, and transformants were screened for fluorescent colonies. Fluorescent colonies were shown to carry plasmids where the MBP signal sequence had been mutationally altered, hence blocking entry of MBP [SS128]-GFP into the export pathway. The nature of the signal sequence mutations conformed to those previously isolated for *malE* (2, 4). The mutations included those that introduced a positive charge to a region of the signal sequence that requires hydrophobicity (Met18 to Lys) and introduction of a proline that could disrupt a requirement for a specific secondary structure (Ser13 to Pro and Leu10 to Pro). We also noted that the

Leu10 to Pro mutation is identical to the previously isolated 10-1 *malE* signal sequence mutation (2, 4, 10).

The sensitivity of GFP as a reporter for export defective mutants was further tested by performing a reconstruction experiment where mixed cultures of wild-type *E. coli* and a *secY* mutant, both expressing MBP[SS418]-GFP, were cultured together on an agar plate. After 3 to 4 days of growth at 30°C, fluorescent colonies representing the *secY* mutants were clearly visible. Although not tested, the sensitivity of detection of mutants could perhaps be heightened by use of a fluorescence-activated cell sorter to separate fluorescent from nonfluorescent cells, as has been reported for other applications (49). Collectively, these results indicate that GFP can be used as a genetic tool for the isolation of different classes of export-defective mutants. In addition to the use of GFP to yield new insights into the protein export process, it also may serve as a substitute for β-galactosidase as a reporter to determine the topological orientation of integral membrane proteins (15, 18, 36).

The observation that GFP is active only when localized to the cytoplasm also raises the related question of why the protein is inactive in the periplasmic space. By purifying MBP-GFP protein from both the periplasmic and cytoplasmic compartments, we were able to show that the lack of fluorescence in the periplasmic space could not be attributed solely to diminished levels of the protein. When equivalent amounts of MBP-GFP purified from both the periplasmic space and the cytoplasm were compared, only the GFP isolated from the latter compartment fluoresced. Furthermore, GFP fluorescence was not irreversibly inactivated in the periplasmic space, since fluorescence could be restored to the protein by treatment under conditions known to alter protein conformation, including acid-base treatment and prolonged incubation in the presence of a reducing agent. This latter observation suggests that disulfide bond formation plays a role in inactivation of GFP. Indeed, two cysteine residues are located in the GFP moiety (one in GFP, the other encoded by the region linking *malE* with *gfp*) of MBP[SS418]-GFP that could contribute to either intra- or intermolecular disulfide bonds. Altering the reducing environment of the periplasmic space, however, did not restore fluorescence in vivo. Collectively, these results indicate that the periplasmic environment is not suitable for GFP to fold into a conformation required for fluorescent activity. These results further suggest that GFP may also be a useful tool to study protein folding in the periplasmic space.

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ADDENDUM IN PROOF

A sensitive screen has recently been described that used *lacZ* gene fusions to isolate mutants defective in membrane protein localization (H. Tian, D. Boyd, and J. Beckwith, Proc. Natl. Acad. Sci. USA **97:**4730–4735, 2000).

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