Translocation of Jellyfish Green Fluorescent Protein via the Tat System of Escherichia coli and Change of Its Periplasmic Localization in Response to Osmotic Up-shock*

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The bacterial twin arginine translocation (Tat) pathway is capable of exporting cofactor-containing enzymes into the periplasm. To assess the capacity of the Tat pathway to export heterologous proteins and to gain information about the property of the periplasm, we fused the twin arginine signal peptide of the trimethylamine N-oxide reductase to the jellyfish green fluorescent protein (GFP). Unlike the Sec pathway, the Tat system successfully exported correctly folded GFP into the periplasm of Escherichia coli. Interestingly, GFP appeared as a halo in most cells and occasionally showed a polar localization in wild type strains. When subjected to a mild osmotic up-shock, GFP relocalized very quickly at the two poles of the cells. The conversion from the halo structure to a periplasmic gathering at particular locations was also observed with spherical cells of the ΔrodA·pbpA mutant or of the wild type strain treated with lysozyme. Therefore, the periplasm is not a uniform compartment and the polarization of GFP is unlikely to be caused by simple invagination of the cytoplasmic membrane at the poles. Moreover, the polar gathering of GFP is reversible; the reversion was accelerated by glucose and inhibited by azide and carbonyl cyanide m-chlorophenylhydrazione, indicating an active adaptation of the bacteria to the osmolarity in the medium. These results strongly suggest a relocalization of periplasmic substances in response to environmental changes. The polar area might be the preferential zone where bacteria sense the change in the environment.

The periplasmic space lies between the inner and the outer membranes of Gram-negative bacteria. A number of processes that are vital to the growth and viability of the cell occur within this compartment. Proteins residing in the periplasmic space fulfill important functions in the detection, processing, and uptake of essential nutrient substances. These proteins are exported into the periplasm mainly via two pathways: the unfolded proteins via the Sec system (1) and the folded enzymes containing redox cofactor via the Tat (or Mtt) pathway (2–4).

The periplasm might not be a uniformly homogenous compartment; fine structures known as Bayer patches/bridges (5) and periseptal and polar annuli (6–9) have been described. The existence of these structures under physiological conditions is a subject of contention (10, 11). Nevertheless, these structures were proposed to provide sites required for the export of outer membrane components, murein synthesis, secretion of bacteriophages, and cell divisions (12).

On the other hand, polar bacterial organization was observed with a variety of bacterial species and concerns a disparate array of cellular functions (13). In addition to the well known examples of polar organelles such as flagella, pili, and stalk-like appendages at the bacterial surface, accumulating evidence shows that periplasmic, inner membranous, and cytoplasmic proteins may also exhibit polar localization under certain condition. These proteins participate in various cellular processes including maltose sensing and uptake (14), chemotaxis (15), conversion of chorismate to phenylalanine (16, 17), DNA replication, and cell division (18, 19). Despite the convincing data that established the polar localization of these proteins, the relationship between their cellular location and fine periplasmic structure remains unanswered.

The Tat (also called Mtt) system is a recently discovered protein export pathway that is capable of translocating folded proteins with peculiar twin arginine (RR) translocation signal peptides (2, 3). Two classes of genes encoding functional Tat components have been identified and studied in Escherichia coli. The tatC gene encodes an integral membrane protein with six transmembrane segments, and its depletion leads to mislocalization of all the enzymes analyzed (2, 3, 20). On the other hand, the tatA, tatB, and tatE genes code for three proteins. They share sequence homology at their N termini, including one transmembrane segment and an adjacent amphipathic domain, whereas their C termini vary both in sequence and in length (2, 3, 21). The depletion of these genes would affect the translocation of various enzymes differently. In addition to the enzymes containing redox cofactors, the Tat system probably also exports proteins that fold too quickly or too tightly to be handled by the Sec system (2, 3). Green fluorescent protein (GFP) from the jellyfish, Aequorea victoria, has been widely used as a marker for gene expression and localization of gene

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1 The abbreviations used are: Tat, twin arginine translocation; RR, twin arginine; GFP, green fluorescent protein; PCR, polymerase chain reaction; CCCP, cyanide m-chlorophenylhydrazine; MBP, maltose-binding protein.
products. The chromophore is generated by the spontaneous cyclization and oxidation of the sequence Sec^{65}(or Thr^{65})-Tyr^{66}-Gly^{67}. The protein fold consists of an 11-stranded β-barrel 42 Å long and 24 Å in diameter with a central coaxial helix carrying the chromophore (22). GFP is synthesized without an export signal. When it is fused to maltose-binding protein carrying a typical Sec-dependent signal peptide, the hybrid GFP is not fluorescent in a wild type strain, and it is exported into the periplasm in an improperly folded conformation (23). Interestingly, the expression of the hybrid GFP in secA mutants leads to the recovery of fluorescence. Furthermore, deletion of the Sec signal peptide also results in fluorescent colonies. Therefore, the Sec pathway is capable of exporting only the improperly folded GFP.

To assess the capacity of the Tat system to export folded heterologous proteins and to study the implication of the periplasm in various cellular processes, we fused the GFP to the twin arginine signal peptide of the trimethylamine-N-oxide (TMAO) reductase. The folded GFP was exported successfully into the periplasm via the Tat pathway. Interestingly, we observed that GFP gathered at the poles in the periplasm in response to osmotic up-shock. It is a reversible process, implying an adaptation of the bacterium to the medium. Our results suggest that the periplasm is a dynamic heterogeneous space and suggest that bacteria might react to environmental changes by compartmentalization of the periplasm in correlation with the localization of membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media**—The E. coli strains used in this study were: MC1400 (F' lacU169 araD139 rpsL150 thiF1601 deoC7 ptsF25 relA1 lacI57lacY7 lacZ7::Tn10 (a gift from D. Vinella); and TGI (Δlac-pro supE thi hsdD51 F' traD36 proA^+ B^− lacY1 lacZAM15). Arabinose-resistant derivatives of the araD mutants were constructed by selecting purple colonies of these strains spread on eosin methylene blue plates (26) containing 0.2% arabinose. The tat mutants in the arabinose-resistant derivatives were confirmed by PCR and anaerobic growth on minimal medium with TMAO as the energy source. The plasmids pBAD24 and gfmuat2 were described in Refs. 27 and 28, respectively.

The bacteria were routinely grown in Luria-Bertani (LB) medium, on LB plates, or in the minimal M63 or M9 media (26). Anaerobic growth was achieved normally in stopped bottles or tubes filled to the top or on plates in GasPak anaerobic jars (BBL Microbiology Systems). As required, ampicillin (100 μg/ml), rifampicin (150 μg/ml), chloramphenicol (300 μg/ml), TMAO (1 mg/ml), sodium molybdate (2 μm), or sodium selenite (2 μm) were added. Precultures were inoculated from a single colony and used at a 100-fold dilution. To assess the energy dependence of bacterial adaptation to osmotic up-shock, sodium azide and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were used at final concentrations of 2 mM and 10 μM, respectively.

**Construction of RR-GFP Fusion**—The gfp-mut2 gene was amplified by PCR using GFPNHEIUP (5'-agaaagatatactgtagcaaggagc-3') and GFPDOWN2 (5'-tgcatcgatggtcgctgctg-3') as primers. The reaction was performed using the Expand High Fidelity PCR system according to the manufacturer's instruction (Roche Molecular Biochemicals). The amplified fragment was purified, double-digested by NheI and HindIII, and cloned into the corresponding sites of the plasmid pBluescript (laboratory plasmid stock), which is a derivative of pBluescript. The resulting plasmid was transformed into E. coli DH5α, which was grown in the presence of 100 μg/ml ampicillin (or TMAO reductase). The folded GFP was exported successfully into the periplasm via the Tat pathway. In contrast, more than 95% of total fluorescence was assessed by fluorescence spectroscopy and immunoblot analysis using antisera against GFP. As expected, GFP was synthesized only if the cells were grown in the presence of arabinose. After cellular fractionation, the distribution of the GFP was analyzed first by fluorescence spectroscopy. In the wild type strain, more than 65% of total fluorescence was located in the periplasm.

We then confirmed the cellular distribution of the GFP by visualization of GFP on a nondenaturing gel or by immunoblot after electrophoresis. A single fluorescent band was found in the periplasm of the wild type strain whereas it was absent from the periplasm of the tatC mutant (Fig. 1A, lanes 3 and 5).

**RESULTS**

**Construction and Translocation of the RR-GFP Fusion Protein**—To assess the capacity of the Tat pathway and to monitor in vivo protein translocation via this system, we fused the GFP with the RR signal peptide of TMAO reductase. The expression of the fusion protein was under the control of the arabinose-inducible promoter (see "Experimental Procedures"). The synthesis of GFP in a wild type strain and the tatC derivative was assessed by fluorescence spectroscopy and immunoblot analysis using antisera against GFP. As expected, GFP was synthesized only if the cells were grown in the presence of arabinose. After cellular fractionation, the distribution of the GFP was analyzed first by fluorescence spectroscopy. In the wild type strain, more than 65% of total fluorescence was located in the periplasm. In contrast, more than 95% of total fluorescence was found in the cytoplasm of the tatC mutant. We had thus successfully translocated GFP into the periplasm via the Tat pathway.

**Microscopy and Fluorescence Spectroscopy**—An overnight culture was diluted 1:100 in LB + ampicillin + glucose medium and grown at 37 °C until the A_{600} reached 0.6–0.8. Cells were centrifuged, resuspended in LB + ampicillin with 0.2% arabinose, and grown at 30 °C for 45 min. Rifampicin or chloramphenicol was added, and the culture was incubated at 30 °C for the times indicated under "Results." The cells were examined immediately or after the addition of 0.15 M NaCl or other treatment, as described under "Experimental Procedures," by Zeiss PhoMi III fluorescence microscope equipped with a filter set for fluorescein isothiocyanate. Images were captured using a CCD camera (Color Cool View, Photonic Sciences) and Image Pro-Plus software. The fluorescence levels present in crude extracts and different cellular fractions were quantified by using a Spex Fluorolog III fluorimeter equipped with a cooled detector (Jobin Yvon).

The amplified fragment was purified, double-digested by NheI and HindIII, and cloned into the corresponding sites of the plasmid pBluescript (laboratory plasmid stock), which is a derivative of pBAD24 and equipped with a cooled detector (Jobin Yvon).
FIG. 1. Translocation and processing of RR-GFP. The cytoplasmic (lanes 2 and 4) and periplasmic (lanes 3 and 5) fractions prepared from MC4100 (lanes 2 and 3) and B1LK0 (lanes 4 and 5) were separated on 10% nondenaturing polyacrylamide gels (A and B) or SDS-gel (C), and GFP proteins were visualized either after excitation by a transilluminator (UV 365 nm) (A) or immunoblot using antisera against GFP (B and C). GFP indicates native GFP without signal peptide (lane 1) or GFP processed from the fusion precursor (RR-GFP). GFP* and RR-GFP 1, 2, and 3 represent a degraded form of GFP and GFP in complex or under different conformations, respectively. The RR-GFP 1 band in panel B corresponds to the fluorescent band RR-GFP in panel A. Unfortunately, we did not succeed in photographing the fluorescent bands RR-GFP2 and RR-GFP3 in panel A (see “Results”).

FIG. 2. Localization of GFP in wild type strains and in the tatC mutant. After induction of the expression of RR-GFP, protein synthesis in the wild type strains MC4100 (A and B) and TG1 (E and F) and in the tatC mutant (C and D) was blocked by the addition of rifampicin (see “Experimental Procedures”). 45 min later, cells were inspected under a fluorescence microscope immediately (A, C, and E) or after the addition of NaCl at a final concentration of 0.15 M (B, D, and F).

wild type strain (Fig. 1C). The precursor RR-GFP and probably its degraded derivatives (RR-GFP*) were present in the cytoplasm of both the wild type strain and the tatC mutant (Fig. 1C). To confirm the translocation of GFP via the Tat pathway, we also analyzed the cellular distribution of GFP in MCMTA (tatB::Kan) and CU164 (secY39cs). Folded GFP accumulated in the cytoplasm of the tatB::Kan mutant, but was exported in the periplasm of the secY mutant (data not shown). Therefore, we succeeded for the first time to export heterologous cytoplasmic protein GFP into the bacterial periplasm via the Tat pathway.

In Vivo Cellular Location of GFP—It was reported that the GFP-MinD fusion has a halo appearance when it is evenly distributed along the cytoplasmic membrane (19). We reasoned that GFP should also exhibit a halo appearance when it is exported into the periplasm. Therefore, we examined the in vivo cellular localization of GFP by fluorescence microscope. To reduce uneven level of GFP in different individual cells due to the Phad promoter, a saturating concentration of arabinose (0.2%) was added in the culture with cells at logarithm phase. To avoid an overproduction and saturation of GFP, protein synthesis was inhibited by rifampicin or chloramphenicol 45 min after the induction by arabinose. When a wild type strain was directly observed in LB medium, GFP showed a halo distribution in most cells, but it had occasionally a polar localization (Fig. 2, A and E). In contrast, GFP diffused throughout the cells of the tatC mutant (Fig. 2, C and D). Notably, the tatC cells form chains, suggesting a deficiency in a late stage of cell division. Recently it was reported that the tat mutations exhibit pleiotropic defects in the cell envelope (32). Interestingly, no GFP was observed at the septum, which confirmed the cytoplasmic accumulation of GFP in the tatC mutant. Therefore, export of GFP via the Tat pathway resulted in an even distribution of the fluorescence in the periplasm of E. coli.

The occasional polar localization of GFP was a surprise that raised the question of what might lead to such polarization. Since the polar localization was never observed in the tatC mutant, we concluded that GFP must accumulate either in the membrane or in the periplasm at the poles of the wild type strain. Several possibilities might lead to the polar localization of GFP. First, it might be an artifact or a consequence of over-expression of GFP. Second, the Tat translocase might have a polar localization, which would concentrate RR-GFP and GFP at the poles before or immediately after the translocation, respectively. Finally, periplasmic GFP might relocalize at the poles for some reasons. To examine these hypotheses, we studied the kinetics of halo formation and the polar localization of GFP. The fluorescence appeared very slowly and a sufficient level of fluorescence was observed at about 45 min after the induction. Because the Phad promoter has a very fast induction rate (27), the slow appearance of GFP suggests a rather slow process of fluorophore formation under the condition used. Although arabinose was used at saturating concentration, heterogeneity of GFP level in individual cells was observed, as described by Hashemzadeh-Bonehi et al. (33). Therefore, timing of GFP folding and export cannot be accurately measured and hereinafter all phenomena were described for the majority of cells. The GFP halo appeared at 40 min after blockage of its synthesis. One hour later almost all cells showed the GFP halo and about 1% cells showed polarization of GFP. This result clearly indicates that the halo formation occurs before the polarization of GFP, excluding a possible polar localization of the translocase, and that GFP polarization is unlikely to be a consequence of overproduction of the fusion protein. In addition, this result also revealed a long time lag between the inhibition of RR-GFP synthesis and the appearance of the GFP halo. It is fully consistent with the export of GFP through the Tat pathway, which is a slow process (30). We attempted to study the kinetics of halo formation by following individual cells, but this was unsuccessful because the GFP halo appeared very slowly and the fluorescence was quenched after a few excitations.

Polarization of GFP in Response to Osmotic Up-shock—The above results indicate that the GFP halo formation results from
the translocation of GFP into the periplasm via the Tat pathway. We then analyzed the relationship between halo formation and polarization. Surprisingly, resuspending cells in phosphate saline buffer immediately caused the disappearance of the halo and recruitment of GFP at both poles. Further analysis revealed that NaCl or KCl alone could trigger polarization. In addition, the addition of 0.15 M NaCl in the culture could completely convert the halo to the polar spots (Fig. 2B), suggesting that the polarization might be a consequence of cellular response to osmotic shock. Indeed, the addition of 0.15 M potassium or 20% sucrose also triggered the polarization of GFP, although sucrose was less efficient than the sodium and potassium ions. This rapid halo to polar spot conversion was also observed for another wild type strain TG1 (Fig. 2, E and F). Interestingly, fluorescence was often more dominant at one pole than the other. However, GFP remained uniformly distributed in the cytoplasm when the tatC mutant was resuspended in the phosphate saline buffer or was subjected to the osmotic up-shock (Fig. 2, compare panels D and C). Therefore, the polarization of GFP is most likely the consequence of relocalization of the periplasmic GFP to the poles.

Polarization of GFP in Spherical Cells—When cells are subject to an osmotic up-shock, plasmolysis bays are formed as the cytoplasmic membrane separates from the other components of the wall. It was proposed that the plasmolysis bays are restricted at the poles because the bilayer of the cytoplasmic membrane is essentially an incompressible two-dimensional liquid. Therefore, the way to cope with the reduction of cytoplasmic volume while keeping the same surface of the bilayer would be the invagination of the cytoplasmic membrane at the two poles (11). It is thus interesting to know if the polarization of GFP is the simple physics phenomena or represents a gathering of periplasmic proteins at peculiar sites in response to the osmotic up-shock. To check these hypotheses, we assessed the GFP localization in spherical cells.

E. coli owes the rigidity of its rod shape to its peptidoglycan layer, a single macromolecule surrounding the cytoplasmic membrane. Penicillin-binding proteins catalyze the final steps of peptidoglycan synthesis. The depletion of rodA-pbpA genes abolishes the synthesis of the penicillin-binding protein 2 and the mutant strain, GC3904, thus grows as spherical cells (34). Similarly, lysozyme (muramidase) hydrolyzes the peptidoglycan, resulting in the formation of spherical cells. The plasmid pRR-GFP was introduced into GC3094 and the cells were inspected in the same way as described above. We observed a circle appearance of GFP in most cells of the exponentially growing mutant GC3940 in LB medium. Occasionally, GFP also gathered at one or two sites or at one side of the spherical cells (Fig. 3A). Interestingly, the convertible localization of GFP was considerably enhanced when the cells were subjected to an osmotic up-shock (Fig. 3B). It is likely that after EDTA-lysozyme treatment, the cells of the wild type strain became spherical and the GFP appeared as a circle (Fig. 3C). The further addition of 0.15 M NaCl or 20% sucrose resulted in the recruitment of GFP in these cells (Fig. 3, D and F). Intriguingly, GFP gathered at one to three mostly irregular sites in the rodA-pbpA mutant, whereas it was located especially at one or two opposite sites in the lysozyme-treated cells. Such different localization of GFP might be explained by the fact that the rodA-pbpA mutation affects cellular elongation, a process related to a clear definition of the poles, whereas the lysozyme probably hydrolyzes the peptidoglycan at random places. Therefore, these results suggest that the periplasm and cytoplasmic membrane might not be uniform and that the periplasmic proteins might preferentially gather at the poles or the area corresponding to the poles.

Energy-dependent Reconversion of Polar Spots to a Halo Appearance—It is likely that the polarization of GFP is a consequence of cellular response to an osmotic up-shock. In this case, the adaptation of bacteria to a given osmolarity should lead to conversion of polar spots to a halo appearance. Indeed, the polarization of GFP, resulted from the addition of NaCl in the culture of the wild type strain MC4100, disappeared progressively and the polar spots completely converted back to halo 15 min after the osmotic up-shock (Fig. 4, A and B). Importantly, simultaneous addition of 2 mM sodium azide or 10 μM CCCP with 0.15 M NaCl completely inhibited the reconversion of the GFP spot to halo (Fig. 4, C and D). Interestingly, after the induction and the blockage of the GFP synthesis, if the fluorescent cells were centrifuged and then resuspended in M63 medium, GFP in all cells was immediately relocalized from the halo to the two poles and polar localization remained even after 60 min incubation at room temperature (Fig. 4E). In contrast, it converted completely to halo 20 min after resuspension of the cells in M63 in the presence of 0.2% glucose (Fig. 4F). Therefore, the acceleration by glucose together with the blockage by azide and CCCP strongly suggest that the conversion of the GFP appearance is an energy-dependent process. The GFP localization might be thus related to the cellular adaptation to the osmotic change in its medium.

DISCUSSION

The Tat system is a recently identified bacterial protein export pathway with the remarkable ability to transport folded proteins and even enzyme complexes across the cytoplasmic membrane (2, 3). Precursors exported by the Tat system contain a particular signal peptide with a conserved RR motif, which is distinct from the Sec-dependent signal peptides. The twin arginine signal peptide is capable of rerouting proteins that are naturally exported via the Sec pathway to the Tat.
pathway (2). However, export of cytoplasmic protein via the Tat pathway had not been reported. In this paper, we show the successful translocation of a heterologous cytoplasmic protein via the Tat system in E. coli. Although active GFP was observed and obtained from the periplasm, whether the GFP is exported in the active form is not obvious. The generation of chromophore is a post-translational autoxidation process that requires molecular oxygen. It was reported that anaerobically grown E. coli accumulates nonfluorescent GFP at an intermediate stage in protein folding and that the generation of fluorescence occurs after the admission of air both in vivo and in vitro (35). In this study, GFP was synthesized under aerobic conditions; the redox potential in the cytoplasm is high enough to allow the formation of the fluorophore. We observed that fluorescence was developed in the cytoplasm of both the wild type strain and the tat mutant starting from about one-half hour after the synthesis of the GFP protein, whereas the appearance of the periplasmic halo requires an additional half-hour. Therefore, the activation of GFP occurs prior to translocation, and GFP is probably translocated in a folded conformation via the Tat pathway. This conclusion is supported by the recent observation that unfolded GFP fused to a Sec-dependent signal peptide could be exported by the Sec system but could not be folded correctly in the periplasm (23). In this study, we observed two slow migrating, fluorescence-unstable bands in the cytoplasm. They may represent folding intermediates of GFP or GFP associated with some factor required for the export of GFP. We are currently analyzing these isoforms of GFP.

Bacterial chemotaxis involves a phosho-relay system brought about by ligand association with a membrane-bound chemoreceptor. The chemoreceptors sense, alone or indirectly through a periplasmic ligand-binding protein, the gradient of ligands in the medium. Four chemoreceptors have been localized at the poles of E. coli cells by immunoelectron microscopy and indirect immunofluorescence light microscopy (15). Maddock and Shapiro (14) thus proposed that the bacterial cell sequesters different regions of the cell for specialized functions. Interestingly, the maltose-binding protein (MBP), which is associated with the chemoreceptor Tar to sense maltose, also exhibits a polar localization (14). In addition, MBP can diffuse laterally in the periplasm (36), and the induction of its synthesis by adding maltose in the medium triggers polar cap formation in E. coli (37). These observations strongly suggest that bacteria detect the nutrient substances at the poles. Interestingly, we observed in this study a reversible relocation of GFP in the periplasm to the poles in response to an osmotic up-shock. We thus speculate that in addition to the polarization of cytoplasmic membrane-bound receptors, the polar area of the periplasm and the outer membrane might be the preferential zone where bacteria sense the change in the environment. Therefore, challenged to an osmotic up-shock, bacteria would gather periplasmic proteins to the poles to interact with the membrane-bound receptor and trigger the adaptation process. However, the polar gathering should not be specific to the ligand-binding proteins because GFP is a heterologous protein for E. coli. Nevertheless, the polarization might be an active process because it is achieved very quickly and is reversible. We will analyze the influence of other environmental changes on the localization of GFP to determine whether our model of polar gathering for signal detection and transduction could be generalized to other stimuli.

The periseptal annulus is described as a pair of concentric rings, each of which consists of a continuous ring in close apposition with the inner membrane, murein, and outer membrane (6). These organelles mark the sites of future cell division. Following septation and cell separation, each daughter cell inherits one of the two periseptal annuli, which remains a polar annulus at the new pole of the newborn cell. The periseptal and polar annuli divide the periplasm into three types of subcompartments: two polar and two midcell compartments and one compartment at the site of future division. By monitoring fluorescence recovery after photobleaching at the periseptal and polar annuli, Foley et al. (38) found that the recovery of fluorescence was uniformly low over the zones of the periseptal and polar annuli. They proposed that these regions are biochemically sequestered from the remainder of the periplasmic space. However, Anba et al. (7) previously reported that diffusion of the overproduced phosphate-binding protein in the periplasmic space is not interrupted by the periseptal annuli of E. coli under conditions of plasmolysis. In addition, other authors (10, 11) have challenged the biological implication of periseptal and polar annuli, suggesting that these fine structures result from the physical constraints on the membrane imposed by mild plasmolysis and its localized relief during membrane collapse. In this study, we found that the halo of GFP could quickly convert to polar spots, suggesting a free movement of proteins within the periplasm. Therefore, if the periseptal annuli exist under physiological conditions, they should not be permanent periplasmic compartments. The successful translocation of the active GFP into the periplasm should thus represent a powerful tool to study the property of periplasm in various cellular processes.

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FIG. 4. Energy-dependent reconversion of GFP polar spots to halo appearance. The cultivation condition is same as described for Fig. 2. NaCl (0.15 M) was added alone (A and B) or simultaneously with 2 mM sodium azide (C) or 10 μM CCCP (D) in LB medium containing fluorescent halo cells. Cells were examined immediately (A) or 10 min after the addition of NaCl (B–D) under a fluorescence microscope. Alternatively, the fluorescent halo cells were centrifuged and then resuspended in M63 medium (E), supplemented with 0.2% glucose (F), and examined 60 min or 20 min later, respectively.
Addendum—After this paper was accepted for publication, a related paper by Thomas et al. (Mol. Microbiol. 2001, 39, 47–53), showing that GFP is successfully exported by the Tat system, was published in the on-line table of contents. We are grateful to Dr. C. Robinson for sending us a proof copy of their paper prior to its publication.

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