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Null Mutations in a Nudix Gene, *ygdP*, Implicate an Alarmone Response in a Novel Suppression of Hybrid Jamming

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Induction of the toxic LamB-LacZ protein fusion, Hyb42-1, leads to a lethal generalized protein export defect. The *prlF1* suppressor causes hyperactivation of the cytoplasmic Lon protease and relieves the inducer sensitivity of Hyb42-1. Since *prlF1* does not cause a detectable change in the stability or level of the hybrid protein, we conducted a suppressor screen, seeking factors genetically downstream of *lon* with *prlF1*-like phenotypes. Two independent insertions in the *ygdP* open reading frame relieve the toxicity of the fusion protein and share two additional properties with *prlF1*: cold sensitivity and the ability to suppress the temperature sensitivity of a *degP* null mutation. Despite these similarities, *ygdP* does not appear to act in the same genetic pathway as *prlF1* and *lon*, suggesting a fundamental link between the phenotypes. We speculate that the common properties of the suppressors relate to secretion defects. The *ygdP* gene (also known as *nudH*) has been shown to encode a Nudix protein that acts as a dinucleotide oligophosphate (alarmone) hydrolase. Our results suggest that loss of *ygdP* function leads to the induction of an alarmone-mediated response that affects secretion. Using an epitope-tagged *ygdP* construct, we present evidence that this response is sensitive to secretion-related stress and is regulated by differential proteolysis of YgdP in a self-limiting manner.

The general secretory (Sec) pathway is responsible for exporting the majority of proteins destined for extracytoplasmic locations in the gram-negative bacterium *Escherichia coli*. Export via this pathway in *E. coli* takes place largely posttranslationally, after most or all of the polypeptide has been synthesized. Sec substrates are targeted for export through the inner membrane translocation complex SecYEG, due to the presence of signal sequences in the amino-terminal region of the protein and are maintained in unfolded export-competent conformations by the actions of cytoplasmic chaperones. Hydrolysis of ATP by the cytoplasmic protein SecA pushes the protein through the translocator in an unfolded fashion (for recent reviews, see references 14, 29, and 31).

Heterologous cytoplasmic proteins can be directed to the Sec pathway by fusing a signal sequence to the amino terminus of the open reading frame (ORF) (39). The LamB-LacZ hybrid protein Hyb42-1 consists of the signal sequence and 173 residues of the mature portion of the outer membrane maltoporin LamB fused to the cytoplasmic protein LacZ (β-galactosidase) (4). The presence of the LacZ domain confers an uninduced Lac^+ phenotype on lactose MacConkey agar and on Luria-Bertani (LB) agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). LamB production is under the control of the maltose regulon, and expression of the hybrid construct may be induced by the addition of maltose to the medium. Under inducing conditions, strains expressing Hyb42-1 exhibit maltose sensitivity (a Mal^s phenotype). This is due to the fact that the amino-terminal (LamB) region of the hybrid targets the protein for export but the cytoplasmically derived LacZ region folds more quickly than it can be translocated. Thus, the available SecYEG translocators are titrated out by the production of LamB-LacZ molecules that block the translocation pore. Induction of the hybrid ultimately prevents the export of other envelope proteins, resulting in a lethal secretion defect called hybrid jamming. This defect can be detected by a general accumulation of envelope precursor proteins in the cytoplasm (43).

A variety of mechanisms can suppress the maltose sensitivity conferred by the toxic hybrid. In the most trivial case, mutations that disrupt the gene fusion itself relieve jamming by preventing expression of the LacZ moiety. These can easily be distinguished from the parental strain on indicator media due to their Lac^- Mal^r phenotype. A more interesting class of intragenic suppressors identifies the signal sequence. These mutant signal sequences fail to target the hybrid protein for export and are therefore resistant to maltose induction. The cytoplasmic retention of the hybrid protein in these mutants results in increased β-galactosidase activity, conferring a Lac^{++} Mal^r phenotype (15).

Extragenic suppressors of hybrid toxicity have also been isolated. Mutations that cause a decrease in the expression of the hybrid (because of an effect on the maltose regulon) allow compensatory mechanisms to alleviate the toxicity. Alternatively, increasing the activity of envelope stress response pathways allows cells to survive maltose induction (such as by a *cpxA** allele). Suppression of hybrid jamming by the Cpx pathway results in the export of the hybrid protein to the periplasm by an unknown mechanism (42). The elevated Cpx response results in higher levels of the periplasmic protease DegP, which rapidly degrades the hybrid protein upon its entry into the periplasm. In fact, DegP is required for the suppression of hybrid toxicity in *cpxA** strains. In the absence of a functional *degP* gene, these strains remain sensitive to induction of the hybrid, despite the fact that the hybrid-jamming phenotype has

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been relieved (12). While the secretion defect is relieved, the presence of the cysteine-rich LacZ domain in the oxidizing periplasmic environment results in the formation of high-molecular-weight disulfide-bonded aggregates that presumably interfere with normal periplasmic processes (43).

The *prlF1* mutation was identified in a maltose-resistant mutant with a secretion-dependent Lac-down phenotype (Lac^- Mal^r) (22). Spontaneous mutations in the *prlF* gene were independently isolated as cold-sensitive suppressors of the temperature-sensitive growth defect of a *degP* (also known as *htrA*) null strain (2). These alleles, *sohA1* and *sohA2* (for suppressor of *htrA*) are molecularly identical to the *prlF1* allele (N. J. Hand, unpublished data). The *degP* suppression, cold sensitivity, and relief-of-jamming phenotypes conferred by the mutation in question (a 7-bp insertion in the *prlF* ORF that results in a truncated PrlF protein with a frameshifted C terminus) are all gain-of-function phenotypes (21). Null mutations in *prlF* have no detectable phenotype (21).

Selection for cold-resistant suppressors of *prlF1* led to the identification of the cytoplasmic protease Lon as the only known effector of *prlF1* function (44). In addition to suppressing *prlF1* cold sensitivity, null mutations in *lon* revert the (Lac^- Mal^r) *prlF1* phenotype to wild type (Lac^+ Mal^s). Furthermore, it has been shown that the *prlF1* allele causes a posttranslational hyperactivation of Lon (44). However, unlike the activated Cpx strains, the *prlF1* mutation affects neither the steady-state level nor the turnover of the hybrid protein (41). Weak sequence homology between PrlF and a chaperone-like domain of the VAT protein from *Thermoplasma acidophilum* hints at a direct interaction between PrlF and Lon (11). Both Lon and VAT are members of the AAA family of ATP-dependent proteases.

These observations present a conundrum. The suppression of hybrid jamming by *prlF1* indicates that the hybrid protein is no longer interfering with translocator function, while the Lac^- phenotype suggests that the hybrid protein is not in the cytoplasm (which, as in the case of the signal sequence mutants, would result in a Lac^{++} phenotype). Furthermore, the observation that the hybrid protein is toxic in the periplasm, unless it is degraded, means that the *prlF1* mutation cannot simply relocate the hybrid to that compartment. Attempts to directly address the cellular localization of the hybrid protein in the *prlF1* mutant by subcellular fractionation have been confounded by the anomalous behavior of the LamB-LacZ hybrid. Taken together, these observations indicate that the *prlF1* allele links the function of PrlF to two proteases in distinct cellular compartments. The effect of the mutation is to cause an apparent relocalization of the LamB-LacZ hybrid protein to an unknown location, where it is both inactive and nontoxic. The only substantial clue to how this might take place was the involvement of Lon. Since we have shown that proteolysis of the hybrid itself is not responsible for the *prlF1* phenotypes, we speculated that the degradation of an unknown substrate of Lon might instead be responsible for the maltose resistance. We postulated that by looking for *prlF1*-like suppressors of hybrid toxicity in a strain where the *prlF1* phenotype is suppressed by a *lon* null mutation we might identify such a Lon substrate, or at least uncover clues to the mechanism of action of this elusive suppressor. In this paper, we present the surprising results of this screen. Rather than isolating a downstream effector of Lon, we have instead identified a novel suppressor, *ygdP*, which recapitulates the *prlF1* phenotypes via a seemingly independent mechanism. This finding leads us to believe that there may be a fundamental link between cold sensitivity, the relief of hybrid jamming, and the temperature-sensitive defect caused by the *degP* null mutation.

MATERIALS AND METHODS

Media and chemicals. Standard culture media were used for bacterial growth (38). For growth in liquid minimal medium, M63 medium (38) was used and was supplemented with 0.4% sugars, 0.5% (vol/vol) LB broth, 1 mM $MgSO_4$, and 100 μg of thiamine HCl/ml. Where required, minimal medium was supplemented with individual amino acids at a final concentration of 100 μg/ml. For expression from the pTrc99a-derived plasmids and their λInCh derivatives, the medium was supplemented with isopropylthio-β-D-galactoside (IPTG) at 2 mM. Antibiotics were purchased from Sigma (St. Louis, Mo.) and were used at the following concentrations: ampicillin, 125 μg/ml for maintenance of high-copy-number plasmids and 25 μg/ml for selection of single-copy Amp^r λ lysogens; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; tetracycline, 25 μg/ml; amikacin, 3 μg/ml; and spectinomycin, 50 μg/ml.

MalE (MBP) antiserum was from the laboratory collection. X-Gal was purchased from Fisher Scientific (Toronto, Ontario, Canada). *o*-Nitrophenyl-β-D-galactopyranoside, FLAG mouse (M2) monoclonal antibodies, and FLAG (M2) affinity agarose were purchased from Sigma. IPTG was purchased from Ambion (Austin, Tex).

Anti-rabbit immunoglobulin G horseradish peroxidase-linked whole antibodies (from sheep) and anti-mouse immunoglobulin G horseradish peroxidase-linked whole antibodies (from goat) were purchased from Amersham Life Sciences (Piscataway, N.J.). ECL Western blotting reagents were purchased from Amersham Life Sciences.

Reagents for DNA cloning, including restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and were used according to the manufacturer's directions. *Pfu* polymerase, PfuTurbo polymerase, and QuikChange mutagenesis kits were purchased from Stratagene (La Jolla, Calif.) and were used as directed. Deoxyribonucleotides were purchased from Amersham Pharmacia Biotech (Piscataway, N.J.). DNA purification kits were purchased from Qiagen (Valencia, Calif.). Oligodeoxynucleotide primers were obtained from the synthesis/sequencing facility at Princeton University or purchased from IDT (Coralville, Iowa) or Sigma Genosys (The Woodlands, Tex.).

Protran 0.2-μm-pore-size nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, N.H.). Ponceau S was purchased from Sigma. Kodak X-AR film was purchased from the Eastman Kodak Company (New York, N.Y.).

Microbiological techniques. Standard techniques were used for the construction of strains by P1 transduction (38). P1*vir*, used to generate P1 lysates for transductions, was from our laboratory collection. The strains used in this study are shown in Table 1. Plasmid transformations were performed using a Bio-Rad (Hercules, Calif.) *E. coli* Gene Pulser according to the manufacturer's recommendations.

Maltose sensitivity disk assays. For maltose sensitivity assays, strains were grown to saturation in 5 ml of liquid minimal medium at 30°C with aeration. The cells were pelleted at 4,000 rpm and resuspended in 2.5 ml of fresh M63 medium. Fifty microliters of the cell suspension was added to 3 ml of molten F-Top agar (38) (cooled to 50°C) and plated on prewarmed minimal-glycerol agar plates. Two sterile 7-mm-diameter filter paper disks were placed on each plate, 10 μl of 10% maltose was pipetted onto the first disk, and 10 μl of 2.5% maltose was pipetted onto the second disk. The plates were incubated at 30°C overnight. The width of the zone of clearing around each disk was determined by averaging three measurements of the outer diameter of the zone of clearing, subtracting the diameter of the disk, and dividing the resulting value by two.

Identification of transposon insertion locations. The insertion points of the mini-Tn*cam* transposon from λNK1324 (24) TnCam candidate suppressors were identified using the anchored random-PCR strategy described by Gibson and Silhavy (17).

Protein analysis. Sample buffer was prepared either as described by Silhavy et al. (with β-mercaptoethanol as a reducing agent) (38) or as described by Sambrook et al. (with dithiothreitol as a reducing agent) (37).

Crude whole-cell lysates were prepared for steady-state protein analysis by subculturing overnight cultures 1:100 into fresh media, growing the cells to an appropriate density, and harvesting them by centrifugation. The cell pellet was

TABLE 1. Strains used in this study

Name	Genotype	Reference
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	10
NJH101	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)]	This study
NJH102	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *prlF1*	This study
NJH138	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *prlF*::*kan* (StuI)	This study
NJH234	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *prlF1 lon*::Tn*10 non*	This study
NJH350	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *prlF1 lon*::Tn*10 non ygdP*::*cm18*	This study
NJH351	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *ygdP*::*cm18*	This study
NJH402	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *ygdP*::*cm18 lon*::Tn*10*	This study
NJH445	MC4100 λInCh TrcFLAG-YgdP	This study
NJH445.1	MC4100 λInCh TrcFLAG-YgdP *lon*::Tn*10*	This study
NJH446	MC4100 λInCh TrcFLAG-YgdP *prlF1*	This study
NJH446.1	MC4100 λInCh TrcFLAG-YgdP *prlF1 lon*::Tn*10*	This study
NJH455	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP	This study
NJH456	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP *prlF1*	This study
NJH463	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcYgdP(E51AE52A) *ygdP*::*cm18*	This study
NJH464	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP *lon*::Tn*10*	This study
NJH465	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP *prlF1 lon*::Tn*10*	This study
NJH466	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP *ygdP*::*cm18 lon*::Tn*10*	This study
NJH495	MC4100 ϕ(*lamB-lacZ*) Hyb42-1 [λpl(209)] *ygdP*(E51AE52A)	This study
NJH522	MC4100 ϕ(*lamB-lacZ*X90) Hyb10-1 [λpl(209)] λInCh TrcFLAG-YgdP	This study
NJH523	MC4100 ϕ(*lamB-lacZ*X90) Hyb10-1 [λpl(209)] *prlF1* λInCh TrcFLAG-YgdP	This study
NJH524	MC4100 ϕ(*lamB-lacZ* S60) Hyb42-1 [λpl(209)] *prlF1*	This study
NJH525	MC4100 ϕ(*lamB-lacZ* S60) Hyb42-1 [λpl(209)] λInCh TrcFLAG-YgdP	This study
NJH526	MC4100 ϕ(*lamB-lacZ* S60) Hyb42-1 [λpl(209)] *prlF1* λInCh TrcFLAG-YgdP	This study

resuspended in 1/10 volume of 1× sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min, and 5 to 10 μl was loaded on denaturing SDS-polyacrylamide gels (10 or 15% acrylamide, depending on the size of the protein of interest).

Samples were separated on SDS-polyacrylamide gels according to the technique of Laemmli (26). A Bio-Rad Mini Trans-Blot apparatus was used for SDS-polyacrylamide gel electrophoresis and for electrotransfer to Protran 0.2-μm-pore-size nitrocellulose membranes, according to the manufacturer's recommendations. The transferred proteins were visualized by staining them with 0.5% (wt/vol in 5% acetic acid) Ponceau S and destained by washing them several times with deionized water and then Western Wash Solution (10 mM Tris · HCl [pH 7.5], 0.9% NaCl, 0.2% Tween 20). The membranes were blocked by shaking them in blocking solution (10 mM Tris · HCl [pH 7.5], 0.9% NaCl, 0.2% Tween 20, 2.5% powdered milk, 0.5% bovine serum albumin) either at 4°C overnight or at room temperature for 1 h. The membranes were then incubated with primary antibodies in fresh blocking solution (1:8,000 for LacZ, LamB, and MalE antisera and 1:1,000 for FLAG mouse monoclonal antiserum) with shaking, either at 4°C overnight or at room temperature for 2 h. The membranes were washed for at least 1 h at room temperature, with shaking, in Western Wash Solution. The membranes were incubated with secondary antibody (1:8,000 in blocking solution) for 45 min at room temperature and then washed as described above. ECL reagents and Kodak X-AR film were used for chemiluminescence detection of peroxidase activity according to the manufacturer's instructions. The relative intensities of the bands were determined using the ImageQuant program (Molecular Dynamics). For the FLAG Western blots, expression of the chromosomal Trc-FLAG-YgdP construct was induced by the addition of 2 mM IPTG to the culture medium.

Plasmid construction. Plasmid pCRScript-YgdP was constructed using DNA amplified from MC4100 by PCR with primers NH4GDP5F (5′-AACTCTGCA CATAACTGTG-3′) and NH4GDP3R (5′-ATTGGCGTGACTTAACCTC-3′). The resulting plasmid contained DNA sequence corresponding to nucleotides (nt) 9494 to 10162 of GenBank entry AE000366. The cloned DNA is inserted at nt 728 of pPCRScript-Amp such that the orientation of the YgdP ORF is the same as that of the LacZα fragment (nt 9494 of the insert abuts nt 728 of the vector, and nt 10162 of the insert abuts nt 729 of the vector).

Plasmid pTrc-YgdP$^+$ was constructed as follows. DNA was amplified from MC4100 by PCR, using primers NH4GDP11 (5′-TGAGGTGAATTCGTGATT GATGACGATGGCT-AC-3′) and NH4GDP12 (5′-CGCAGGAAGCTTAGCA TAATTGGCGTGACTTAAC-3′). Recognition sequences for restriction enzymes used in the plasmid construction are underlined.

The purified PCR product was cut with *Eco*RI and *Hin*dIII and cloned into *Eco*RI-*Hin*dIII-cut pTrc99a. The resulting plasmid, pTrc-YgdP$^+$, carries the DNA sequence corresponding to nt 10035 to 9489 of GenBank entry AE000366, replacing nt 276 to 320 of pTrc99a (GenBank entry U13872).

Plasmid pTrc-FLAGYgdP was constructed using primers NHFL5YGD (5′-A ACAGACCATGGACTACAAGGACGATGATGACAAGATTGATGACGA TGGCTAC-3′) and NH4GDP12 (5′-CGCAGGAAGCTTAGCATAATTGGC GTGACTTAAC-3′). The resulting plasmid, pTrc-FLAGYgdP, attaches the DNA sequence encoding the FLAG epitope, with a start codon, to nt 10032 to 9489 of GenBank entry AE000366 (the *ygdP* ORF minus the start codon). The resulting DNA sequence replaces nt 271 to 320 of pTrc99a (GenBank entry U13872).

Generation of a chromosomal ygdPE56AE57A mutation by allelic exchange. The mutagenic primers NHFMUTRA (5′-CAGGCGATGTACCGTGAATTG TTTGCTGCAGTAGGATTAAGCCGCAAAGACGTTC-3′) and NHRMUTRA (5′-GAACGTCTTTGCGGCTTAATCCTACTGCAGCAAACAATTCACGG TACATCGCCTG-3′) were used to generate mutations in the conserved Nudix motif of *ygdP*. The mutated derivatives of pCRScript-YgdP$^+$ and pTrc-YgdP$^+$ contain the double mutation E56AE57A and were designated pCRScript-YgdP$^-$ and pTrc-YgdP$^-$, respectively. Mutation of the native *ygdP* chromosomal locus was accomplished by subcloning the insert from pCRScript-YgdP$^-$ into pGS284 (40) in strain S17λ*pir*. The parental plasmid carries the counterselectable *sacB* gene from *Bacillus amyloliquefaciens*. The mutated *ygdP* was introduced into strain NJH351 by conjugal transfer, and exconjugants were selected on LB agar containing low levels (25 μg/ml) of ampicillin and chloramphenicol. Since pGS284 has an R6K origin of replication, the plasmid cannot replicate in the recipient strain (which lacks λ*pir*). Selection for ampicillin resistance results in the formation of a plasmid cointegrate at the chromosomal *ygdP* locus. The resolution of the cointegrate was selected by plating it on LB agar without salt, with 5% sucrose, to counterselect the plasmid-borne *sacB* gene. The sucrose-resistant colonies were screened for chloramphenicol sensitivity (indicating loss of the *ygdP*::*cm18* allele). The presence of the desired *ygdP*E56AE57A allele was screened by colony PCR followed by *Pst*I restriction digestion and was confirmed by DNA sequencing.

Construction of single-copy gene expression strains. The plasmids pTrc-YgdP$^+$, pTrc-YgdP$^-$, and pTrc-FLAGYgdP were targeted to the chromosome as stable single-copy lysogens using the λInCh procedure as described by Boyd et al. (8).

Targeted disruption of the *ygdP* and *ptsP* genes. The chromosomal disruptions of the *ygdP* and *ptsP* ORFs were made according to the procedure of Datsenko and Wanner (13).

The linear DNA used for the disruption of the ORFs was produced by amplifying the kanamycin resistance cassette from the pUC4K plasmid with primers NHYGDPKF (5′-TATCCACCCCTTCCTCTGTTTATAACTCTGCACATAA

CTGTGAGTTATAAGAATTCTGATTAGAAAAACTC-3′) and NHYGDPKR (5′-AACCAGTTACGCGTTGAAGCAAGGATTCGAACGTCTTTGCGGCT TAATCCGGATTCTTCAACTCAGCAAAAG-3′) (in the case of *ygdP*) and with primers NHPTSPKF (5′-CCACAAAACGCATCTGCTTATCGACGTAA AAGAGGTTAAGTCACAGAATTCTGATTAGAAAAACTCATCGAG-3′) and NHPTSPKR (5′-TAGTAACAACGTCGATCATGATCGGCCAGGTAG ACCGAACAGACCTCGGTTAGGATTCTTCAACTCAGCAAAAG-3′) (in the case of *ptsP*). Disruption of the ORFs was confirmed by PCR.

RESULTS

Rationale. The *prlF1* mutation confers a Lac-down, maltose-resistant (Lac$^-$ Mal^r) phenotype in a strain carrying the toxic, maltose-inducible LamB-LacZ hybrid protein (Hyb42-1). In an effort to further understand the mechanism by which *prlF1* suppresses hybrid jamming, we sought mutants with *prlF1*-like phenotypes. Since we knew that *lon* null mutations suppress all known phenotypes of *prlF1*, we used as a parent strain NJH234 (Hyb42-1 *prlF1 lon*::Tn*10 non*), hoping to identify genes functioning downstream or independently of *lon*.

Null mutations in *lon* produce a mucoid phenotype due to the overproduction of capsular polysaccharide (18, 45). The *non* (for "nonmucoid") mutation maps to the *cps* (for "capsular polysaccharide synthesis") gene cluster at 45.7 min and suppresses mucoidy (33). We therefore used a strain with an uncharacterized *non* mutation for practical reasons: it is difficult to pick individual colonies, and to score their phenotypes, from crowded plates of mucoid strains.

Isolation of *lon* suppressors. A pooled P1*vir* lysate representing ~40,000 independent mini-TnCam insertions in an MC4100 strain was prepared as described previously (24). Random insertions from this pool were introduced into strain NJH234 (Hyb42-1 *prlF1 lon*::Tn*10 non*) by P1 transduction, and the transductants were plated on LB agar containing X-Gal and chloramphenicol. Approximately 30,000 independent transductants were screened, representing a theoretical coverage of the genome at an average density of one insertion every 133 bp. The parent strain, NJH234, produces dark-blue colonies on LB agar containing X-Gal. Transductants with decreased Lac activity, which produced light-blue colonies, were colony purified for further study. This initial screen yielded 67 mutants with stable Lac-down phenotypes.

In addition to identifying transposon insertions with novel effects on the hybrid phenotypes, we expected to obtain Lac-down transductants from this due to the cotransduction of the wild-type *lon*$^+$ allele from the MC4100 strain used to generate the TnCam pooled P1 lysate. In the latter case, the Lac phenotype would simply be due to the (unsuppressed) *prlF1* mutation. In order to distinguish the two classes, each of the candidate Lac suppressors was tested for the presence of the *lon*::Tn*10* allele by screening for the ability to grow on LB agar containing tetracycline. Of the 67 Lac-down TnCam insertions with stable phenotypes, 44 proved to be sensitive to tetracycline and were thus identified as *lon*$^+$ transductants. These transductants were not subjected to further study.

The insertion points of the remaining 23 candidate suppressors were identified using the degenerate PCR method described by Gibson and Silhavy (17). In most cases, the reduced β-galactosidase activity of the LamB-LacZ gene fusion caused by the transposon insertions could be explained by decreased expression of the maltose regulon. The phenotypes of these strains were likely due either to nonspecific effects on protein synthesis (*spoT tnaA*) or on carbohydrate metabolism (*gatZ uhpC cya*) or to specific effects on the maltose regulon (*malI*). However, two strains, cm18 and cm43, contained independent TnCam insertions in the *ygdP* ORF (Fig. 1), the effects of which could not easily be explained in terms of a known phenotype of the gene.

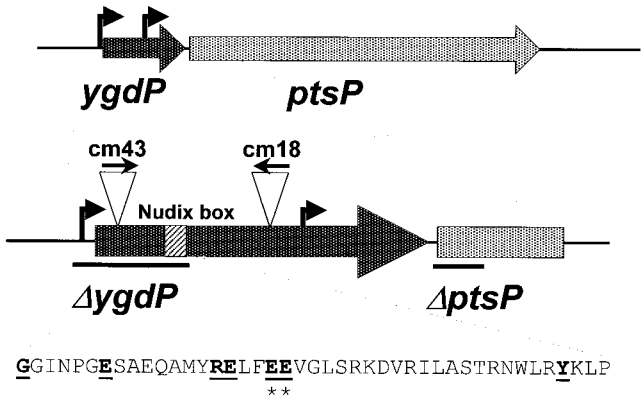

FIG. 1. Positions of the TnCam transposon insertions in the *ygdP* ORF. The arrows above the insertions indicate the direction of the chloramphenicol acetyltransferase promoter. The bars indicate the regions deleted in the *ygdP*(Δ5′)::*kan* and *ptsP*(Δ5′)::*kan* alleles. The amino acid sequence corresponding to the Nudix box is shown, with the invariant signature residues underlined. The asterisks identify the residues mutated in the *ygdP*E56AE57A allele.

The decrease in Lac activity of the hybrid protein is secretion dependent. The *ygdP* insertions do not decrease the activity of a derivative of the *lamB-lacZ* Hyb42-1 gene fusion (BZR60) in which the signal sequence is disrupted (data not shown). In contrast, mutations that decrease the expression of the maltose regulon produce a Lac-down phenotype in strains with this cytoplasmically localized LamB-LacZ hybrid. This indicates that, like the *prlF1* mutation, the decrease in the Lac activity of LamB-LacZ conferred by the *ygdP* mutation depends on proper targeting of the hybrid to the translocator.

Two independent insertions in *ygdP* act as antisuppressors of *lon*. The *ygdP* insertions in an NJH234 (Hyb42-1 *prlF1 lon*::Tn*10 non*) background produce a maltose-resistant phenotype that is indistinguishable from that of a *prlF1 lon*$^+$ strain (Table 2).

TABLE 2. Hybrid protein phenotypes

Name	Relevant genotype	Phenotype	
		Lac[a]	Mal[b]
NJH101	Hyb42-1	+	S
NJH102	Hyb42-1 *prlF1*	−	R
NJH234	Hyb42-1 *prlF1 lon*::Tn*10*	+	S
NJH350	Hyb42-1 *prlF1 lon*::Tn*10 ygdP*::*cm18*	−	R
NJH351	Hyb42-1 *ygdP*::*cm18*	+/−	R/S
NJH402	Hyb42-1 *ygdP*::*cm18 lon*::Tn*10*	−	R
NJH495	Hyb42-1 *ygdP*E51AE52A	+/−	R/S
NJH463	Hyb42-1 *ydsP*::*cm18* {λInCh Trc YgdP(E51AE52A)}	+/−	R/S

[a] +, positive; −, negative; +/−, intermediate.
[b] S, sensitive; R, resistant; R/S, partial resistance.

In addition to the effects on the Lac and Mal phenotypes of the Hyb42-1 protein, the *prlF1* mutation confers a cold-sensitive phenotype that is suppressed in *prlF1 lon* double-mutant strains. Both of the *ygdP* insertions resulted in a cold-sensitive growth defect at 18°C that was more severe than that of a *prlF1* single-mutant strain. Furthermore, when the *ygdP*::*cm* insertions were introduced into a *degP*::Tn*10* strain, they increased the efficiency of plating of the strain at 42°C by 2 to 3 log units relative to an isogenic *ygdP*$^+$ strain. As noted previously, the *prlF1* mutation was isolated independently as a suppressor of the temperature-sensitive phenotype of a null mutant of *htrA* (*degP*). Taken together, these results showed that the *ygdP*::*cm* insertions affected all of the phenotypes known to be affected by *prlF1* and initially suggested to us that *ygdP* acts in the same genetic pathway as *prlF1*, downstream of *lon*. Both insertions in *ygdP* behaved identically with respect to all of the phenotypes assayed.

A 5′ deletion of the *ygdP* gene phenocopies the *ygdP*::*cm* insertions. To eliminate the possibility that the effects of *ygdP*::*cm* might be due to an effect on the expression of the downstream gene *ptsP*, we independently constructed defined null alleles of *ygdP* and *ptsP* using the single-step PCR strategy of Datsenko and Wanner (13). In the case of the *ygdP*(Δ5′)::*kan* allele, the promoter region and approximately one-third of the *ygdP* ORF were replaced by a kanamycin resistance cassette. The deleted region of *ptsP*(Δ5′)::*kan* replaces 150 bp of the 5′ end of the *ptsP* gene with a kanamycin resistance cassette, thus removing the start codon of *ptsP*, and several potential alternative start codons in the 5′ region of the gene, without affecting the *ygdP* ORF (Fig. 1). When transduced into the NJH234 (Hyb42-1 *prlF1 lon*::Tn*10 non*) strain, the *ygdP*::*kan* allele was indistinguishable from the *ygdP*::*cm* insertions while the *ptsP*::*kan* allele had no effect on either the Lac or Mal phenotype of the parent strain (data not shown). This indicated that the original *ygdP*::*cm* insertions were nulls and that the phenotypes associated with them were due to loss of *ygdP* function and not to an effect on *ptsP*.

The *ygdP* gene product is a member of the conserved family of Nudix proteins. Sequence homology identifies the *ygdP* gene product as a member of the Nudix family of proteins (5). Members of the Nudix family are cytoplasmic proteins that possess the highly conserved signature sequence GX_5EX_7RE UXEEXGU, where X is any amino acid and U represents a bulky hydrophobic amino acid (isoleucine, leucine, or valine). The acronym Nudix is derived from the observation that the substrates of these proteins are nucleoside diphosphates linked to an additional component, X. To test if mutation of the Nudix motif could produce an antisuppression phenotype, we generated a site-directed *ygdP*E56AE57A mutant allele on a plasmid. This allele was transferred to the native locus in the chromosome by allelic exchange. In the strain thus formed, NJH495 (Hyb42-1 *ygdP*E51AE52A), the mutation of the Nudix motif resulted in a Lac-down, maltose-resistant phenotype indistinguishable from that of the *ygdP* null alleles. Furthermore, the expression of a single copy of *ygdP*E51AE52A (at the λ attachment site) driven by the IPTG-inducible pTrc promoter fails to complement a chromosomal *ygdP* null in strain NJH463 [Hyb42-1 λInCh TrcYgdP(E51AE52A) ygdP::cm18].

The *ygdP* single mutants do not fully phenocopy *prlF1*. In the simplest case, if *ygdP* were in a genetic pathway downstream of *lon*, then disruptions of the gene should produce *prlF1*-like phenotypes in a *prlF*$^+$ *lon*$^+$ background. We therefore transduced the insertions into a strain containing the *lamB-lacZ* Hyb42-1 fusion in an otherwise wild-type background and tested both the maltose resistance and the Lac phenotypes on lactose MacConkey agar. The results of the tests are shown in Table 2.

The maltose sensitivity and Lac phenotypes of the two *ygdP* transposon insertions are indistinguishable from one another but clearly intermediate between the wild type and *prlF1*. Both insertions produce a more severe cold-sensitive growth defect at 18°C than *prlF1*. Since protein export is an inherently cold-sensitive process (32), a tantalizing possibility is that the cold sensitivities of both the *prlF1* allele and the *ygdP*::*cm* mutants reflect a perturbation of the secretion machinery. Consistent with this notion, the presence of the LamB-LacZ hybrid protein enhances the cold sensitivity of the *ygdP*::*cm* strains, even in the absence of maltose induction, as does growth on (lactose) MacConkey agar. These growth conditions place additional stress on the cell by the presence of the slowly exported hybrid protein or due to the bile salts in the MacConkey agar.

A *lon* null allele enhances the effects of *ygdP*::*cm* on Hyb42-1. The strains NJH350 (Hyb42-1 *prlF1 lon*::Tn*10 non ygdP*::*cm18*) and NJH102 (Hyb42-1 *prlF1*) behave identically with respect to the Lac and Mal phenotypes associated with the hybrid protein. In contrast, NJH351 (Hyb42-1 *ygdP*::*cm18*) has higher Lac activity and is less maltose resistant (exhibiting partial sensitivity in a maltose disk assay) than either NJH350 or NJH102. Remarkably, when the *lon*::Tn*10* allele was introduced into NJH351, the Lac activity was decreased and the maltose resistance was increased (Table 2). Thus, while the *ygdP* mutations behaved as antisuppressors of *lon* when introduced into NJH234, the *lon* null mutant enhanced the partial suppression due to the *ygdP* mutation.

Insertions in *ygdP* relieve hybrid jamming. Maltose induction of the hybrid protein in a wild-type strain leads to jamming of the secretion apparatus, which results in a general cytoplasmic accumulation of unprocessed precursors of envelope proteins. The *prlF1* mutation relieves hybrid jamming. To test whether the *ygdP*::*cm* insertions could also relieve hybrid jamming, we assayed the accumulation of unprocessed MalE in strains containing the LamB-LacZ hybrid, Hyb42-1, with a *prlF*$^+$, *prlF1*, *prlF*::*kan*, or *ygdP*::*cm18* mutation (Fig. 2).

No suppression of jamming occurred in the wild-type and *prlF*::*kan* (null) strains. In contrast, jamming was relieved in the *prlF1* strain. A dramatic reduction in jamming occurred in the *ygdP*::*cm* strain; however, there is still detectable precursor MalE present. This intermediate amount of precursor MalE observed in the *ygdP*::*cm* strain is consistent with the intermediate effects of the mutation on both the Lac activity and the maltose sensitivity of the hybrid protein in an otherwise wild-type strain. Furthermore, as with the *prlF1* mutation, Western analysis indicated that the levels of the Hyb42-1 hybrid protein were not significantly altered by the *ygdP* mutations. Thus, the relief of jamming is not due to the degradation of the hybrid protein.

Levels of epitope-tagged YgdP do not correlate with the allele of *prlF*. Given that *prlF1* causes a hyperactivation of Lon (44), a major cytoplasmic protease, and since degradation of the hybrid is clearly not responsible for the suppression of

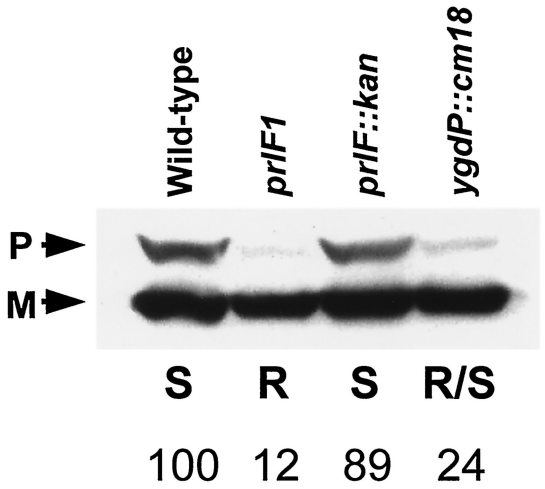

FIG. 2. Disruption of *ygdP* relieves hybrid jamming. Western analysis of whole-cell lysates of strains containing the LamB-LacZ hybrid protein Hyb42-1 was performed after 30 min of maltose induction. The analysis was performed using antibody against the maltose-binding protein, MalE. The presence of unprocessed precursor MalE (P) is a consequence of hybrid jamming. The position of the mature MalE protein (M) is indicated. No accumulation of precursor is observed in the *prlF1* strain, while the jamming phenotype is partially relieved by the *ygdP*::*cm18* null mutation. The strains are (from left to right) NJH101, NJH102, NJH138, and NJH351. The maltose phenotypes of the strains are indicated: S, sensitive; R, resistant; R/S, partial resistance. The numbers indicate the intensity of the pMalE band (corrected for loading using the lower, mature MalE band) expressed relative to the level seen in the wild-type strain.

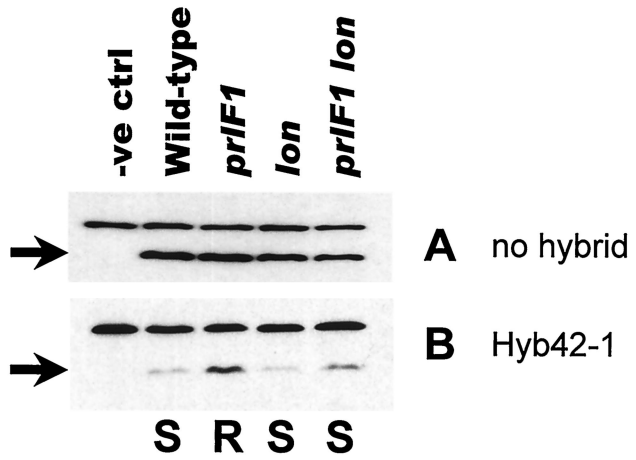

FIG. 3. Levels of Flg-YgdP are lower in the presence of the LamB-LacZ hybrid protein. Western analysis was performed using a monoclonal antibody against the FLAG epitope. The arrows indicate Flg-YgdP protein; the upper cross-reacting band serves as an internal loading control. The strains in panels A and B are isogenic, except that the strains in panel B carry the *lamb-lacZ* Hyb42-1 fusion. (A) Strains (from left to right): MC4100, NJH445, NJH446, NJH445.1, and NJH446.1. (B) Strains (from left to right): NJH101, NJH455, NJH456, NJH464, and NJH465. The maltose sensitivity phenotypes (S, sensitive; R, resistant), where relevant, are indicated below panel B. MC4100 and NJH101 do not carry Flg-YgdP and serve as negative controls (−ve ctrl).

hybrid jamming, we had originally reasoned that YgdP might be the relevant Lon substrate. If this were true, then we would expect significant degradation of YgdP in *prlF1 lon*$^+$ strains. To test this, we generated a FLAG-tagged derivative of *ygdP* by PCR and cloned the epitope-tagged gene into pTrc99a. To eliminate the possibility of artifactual effects of the presence of the epitope-tagged protein in high copy numbers, we transferred the construct to the λ attachment site in single copy, using the λInCh procedure (8). We compared the properties of pTrc-Flg-YgdP in single copy to a corresponding untagged pTrc-YgdP$^+$ construct at the λ attachment site to address the possibility that the presence of the epitope tag might alter the properties of the YgdP protein. Our results show that in *trans* to a *ygdP*::*cm* allele at the chromosomal locus, the epitope-tagged YgdP behaves identically to the wild-type construct. Both constructs complement the *ygdP* null mutant, restoring the phenotypes associated with the LamB-LacZ hybrid protein to wild type (Lac$^+$ Mal^s). Based on this observation, we concluded that the Flg-YgdP protein was functional.

Whole-cell protein extracts were prepared from wild-type, *prlF1*, *lon*::Tn*10*, and *prlF1 lon*::Tn*10* strains carrying the tagged construct on the chromosome, and steady-state levels of the Flg-YgdP protein were examined by Western analysis. The results are shown in Fig. 3A. The inclusion of the MC4100 control sample (in which there is no Flg-YgdP construct) identified the upper band as a cross-reacting band which serves as an internal loading control. No significant difference was found among the levels of Flg-YgdP in the strains tested. We repeated this experiment using isogenic strains containing the toxic *lamB-lacZ* gene fusion under conditions of maltose induction. Surprisingly, while there were differences in the levels of Flg-YgdP, the alteration in the levels of the tagged protein was opposite to that anticipated. Rather than seeing enhanced degradation of Flg-YgdP in the activated Lon (*prlF1*) strain, we saw an increased steady-state level of the protein compared to the corresponding *prlF*$^+$ strain (Fig. 3B). Contrary to our expectation, the decrease in the Flg-YgdP levels correlated, not with the allele of *prlF* present, but with the Mal phenotype, and by extension, with the state of cellular stress. The level of Flg-YgdP was lower in the maltose-sensitive strains than in the maltose-resistant *prlF1* strain. Similarly, strains carrying the LamB-LacZ hybrid protein have low levels of Flg-YgdP relative to the corresponding strain lacking the hybrid (compare Fig. 3B to A), except in the case of the *prlF1* mutants.

Decreased levels of epitope-tagged YgdP correlate with cellular stress. To test if the higher level of Flg-YgdP in strain NJH456 relative to that in NJH455 might reflect lower cellular stress in the *prlF1* strain relative to its *prlF*$^+$ counterpart, we took advantage of two alleles of the *lamB-lacZ* gene fusion. The *lamB*S60 mutation deletes part of the signal sequence of the LamB domain. Thus, the BZR60 derivative of the hybrid (carrying the S60 mutation in the LamB domain) is not targeted for export and therefore causes neither hybrid jamming nor maltose sensitivity. The *prlF1* allele has no effect on the Lac phenotype of BZR60. The X90 allele of *lamB-lacZ* results in an eight-residue truncation of the carboxy terminus of the LacZ domain of the hybrid protein. The carboxy terminus of LacZ is apparently important for efficient protein folding, since this small alteration relieves jamming of the ZX90 derivative of the hybrid protein. Instead, the mutant hybrid protein is secreted into the periplasm. As noted previously, the presence of

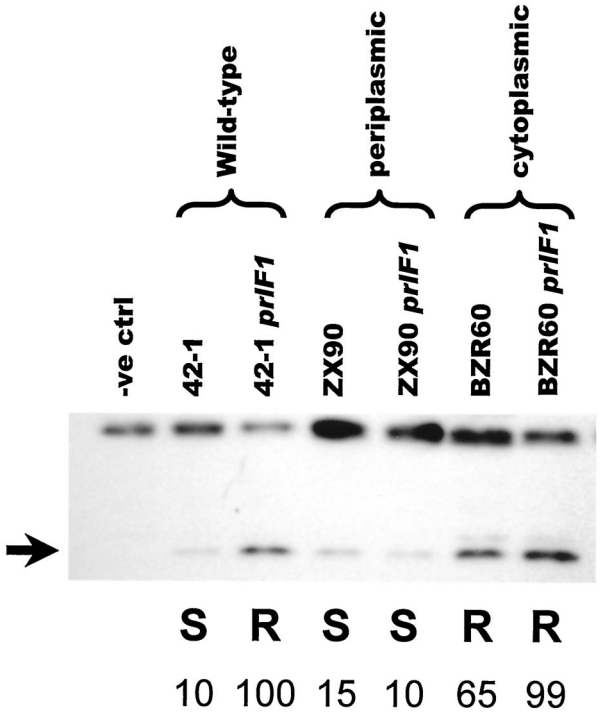

FIG. 4. Flg-YgdP levels correlate with cellular stress, not with the location of the hybrid protein. We used three alleles of the hybrid protein to test if the location of the LamB-LacZ hybrid affected the levels of Flg-YgdP. The wild-type 42-1 fusion allele is toxic but is suppressed by the *prlF1* mutation. The ZX90 allele of the *lamB-lacZ* fusion is efficiently secreted into the periplasm, where the hybrid protein is toxic. In contrast, the BZR60 allele is not efficiently targeted for export due to a signal sequence mutation. Western analysis was performed using the anti-FLAG antibody. Low Flg-YgdP levels in the jammed 42-1 strain, and in both ZX90 strains, indicate that the smaller amounts of Flg-YgdP reflect cellular stress rather than localization of the hybrid protein. Strains (from left to right): NJH101, NJH455, NJH456, NJH522, NJH523, NJH525, and NJH526. The maltose sensitivity phenotypes (S, sensitive; R, resistant) are indicated. The arrow indicates the Flg-YgdP band. Strain NJH101 does not carry Flg-YgdP and serves as a negative control (−ve ctrl). The numbers indicate the intensities of the Flg-YgdP band (corrected for loading using the upper cross-reacting band) expressed relative to the level seen in the Hyb42-1 *prlF1* strain.

the cysteine-rich LacZ domain in the periplasm is toxic. Thus, the ZX90 hybrid has a Mal^s phenotype that is not suppressed by *prlF1*.

Figure 4 shows Western analysis of strains containing the chromosomal *flg-ygdP* construct and either wild-type LamB-LacZ, cytoplasmic LamB-LacZ (nontoxic), or periplasmic LamB-LacZ (toxic). A comparison of the levels of Flg-YgdP relative to the cross-reacting higher-molecular-weight band again suggests a correlation with the Mal phenotype rather than with the *prlF* allele. Thus, levels of Flg-YgdP are higher in the Mal^r BZR60 strains than in the Mal^s ZX90 strains. We consistently see a subtle difference between the levels of Flg-YgdP in the *prlF*$^+$ and *prlF1* BZR60 strains (NJH525 and NJH526). This difference likely reflects some leakiness of the S60 signal sequence mutation such that in strain NJH525 a small proportion of the mutant hybrid protein causes a minor perturbation of the export machinery.

DISCUSSION

We present here the identification of a novel suppressor of LamB-LacZ hybrid jamming, *ygdP*. The screen that identified this gene was intended to elucidate the mechanism by which the *prlF1* mutation suppresses hybrid jamming. Since *prlF1* increases the activity of the Lon protease, we postulated that the degradation of some unknown protein might be responsible for the effects of the *prlF1* allele. We hoped to identify this hypothetical Lon substrate and thus gain insight into *prlF1* suppression. We have instead discovered a *prlF*-independent mode of suppression of hybrid jamming. The phenotypes associated with this new gene are the same as those of *prlF1*, namely, cold-sensitive growth, relief of hybrid jamming, and suppression of the temperature sensitivity of the *degP* null strain.

The fact that two independent suppressors, the *prlF1* allele and *ygdP* null mutations, affect three phenotypes in common suggests that these phenotypes may be fundamentally linked. Although the nature of the link among the phenotypes is unclear, it is tempting to speculate that they all relate to aspects of translocator function. While it is known that secretion is a fundamentally cold-sensitive process (32) and that induction of the LamB-LacZ hybrid causes a severe general protein export defect, the connection between *degP* and secretion is less clear. Previous models explaining the temperature sensitivity of the *degP* null strain suggested that the DegP requirement is for the proteolysis of unfolded periplasmic proteins. This conclusion is based on studies implicating DegP in the degradation of unchaperoned pilin subunits (20) and of heterologous proteins targeted to the periplasm (12), as well as on the synthetic phenotypes of *degP* null mutants with some periplasmic protein folding factors (36). We favor the view that the lethal stress caused by the absence of DegP at 42°C is due to the misfolding of newly synthesized proteins rather than the unfolding of folded periplasmic proteins. If this is the case, at higher temperatures misfolded translocating proteins may remain associated with the translocator in the absence of DegP, impairing secretion in a manner that can be relieved either by *prlF1* or by *ygdP* null mutations.

The connection among *prlF1*, *ygdP*, and *degP* hints at a general role for DegP in secretion. It may be that DegP assists in the clearance of exported proteins from the translocator. This could reflect an involvement of the DegP chaperone function in the translocation of many substrates, analogous to that of Kar2p (9, 25, 46) in yeast, or a more specific secretion-related stress function, in which the protease activity degrades misfolded, partially translocated polypeptides that might otherwise impair secretion.

Although for technical reasons we cannot satisfactorily address the question of the subcellular location of the hybrid protein, our data suggest that in the *prlF1* and *ygdP* mutant strains the hybrid is most likely released from jammed transloctors into the inner membrane. It may be that these suppressors cause an increase in an innate property of the SecYEG pore, namely, lateral release. Lateral opening of the translocator is required for the integration of transmembrane segments into the inner membrane. Altering the properties of the translocator itself might result in cold sensitivity, and increasing lateral release rates would be likely to have the profoundest

effect on slowly translocating or stalled translocation substrates.

The *E. coli* gene encoding the Nudix protein YgdP has recently been cloned, overexpressed, and purified (6). Analysis has shown that this protein catalyzes the hydrolysis of diadenosine oligophosphates in vitro (tetra-, penta-, and hexaphosphates), with a preference for diadenosine pentaphosphate. YgdP catalyzes the hydrolysis of Ap_5A into ATP and ADP. YgdP has no detectable activity for other typical substrates of the Nudix family of hydrolases: NADH, modified sugars (ADP-, GDP-, and UDP-sugars), and nucleotide triphosphates and their derivatives (6). Collectively, the Nudix hydrolases share the property of recycling of small, undesirable molecules, and for this reason they are sometimes referred to as "housecleaning" proteins (5).

Dinucleoside oligophosphates were first detected as byproducts of tRNA charging aminoacyl tRNA synthetase reactions (47). These compounds have since been found to be involved in a wide variety of cellular responses in both prokaryotes and eukaryotes (reviewed in references 3 and 23) and are believed to constitute a novel class of signaling molecules. In bacteria, concentrations of diadenosine oligophosphates have been shown to increase >100-fold over the endogenous level in response to heat or oxidative stress, and for this reason these molecules are collectively called alarmones (7, 19, 27, 28).

We have shown that mutagenesis of the signature Nudix box in *ygdP* results in phenotypes similar to those caused by the *prlF1* mutation. Our results suggest that YgdP mediates an alarmone response with an effect on protein secretion. The likely role of YgdP is to purge the cell of an alarmone, allowing it to exit a stress response state. In the absence of YgdP function, a stress-adapted state would exist, enabling cells to tolerate conditions such as hybrid jamming and growth at 42°C in the absence of DegP. We propose that the normal function of YgdP is to turn over the alarmone and dampen this response. Thus, in a wild-type strain containing the LamB-LacZ fusion, levels of the alarmone are low and jamming occurs. However, in a *ygdP* null mutant strain, the higher endogenous level of the alarmone partially phenocopies the *prlF1* mutation.

In a *ygdP* mutant strain, the higher endogenous levels of alarmone may lead to increased activity of a protease. This might reflect an increase in either the expression or the activity of the protease. Overlapping substrate specificities between this putative protease and Lon would explain the similar phenotypes of *ygdP* and *prlF1*. Support for this hypothesis comes from the work of Fuge and Farr (16), who characterized a disruption of the *apaH* gene. While ApaH is unrelated to YgdP at the primary sequence level, it is also a hydrolase and has been shown to have overlapping substrate specificity with YgdP in vitro. The disrupted copy of *apaH* was found to enhance a heat shock-stimulated proteolytic activity in the absence of Lon. Specifically, degradation of abnormal proteins (produced by puromycin treatment) following heat shock was 40% higher in an *apaH lon* mutant strain than in a *lon* single mutant. If a similar effect results from the disruption of *ygdP*, then the *ygdP* suppression phenotypes may be due to increased activity by a protease other than Lon. Since the presence of the LamB-LacZ hybrid protein induces the heat shock response (although not to levels sufficient to suppress its toxicity), disruption of *ygdP* may cause an alarmone-mediated increase in the activity of a heat shock protease. Thus, the effect of the *ygdP* null mutations would be convergent with that of the *prlF1* mutation, with both circumstances leading to the activation of proteases. Since neither the *prlF1* allele nor the *ygdP* null mutations result in detectable degradation of the hybrid protein, it seems likely that the substrate(s) of the proteases has yet to be identified.

Homology between the PrlF protein sequence and that of VAT-Nn, a domain of the *T. acidophilum* thermosome (11) that assists in the unfolding of proteins prior to degradation, hints that PrlF itself may have a chaperone function. This, coupled with the genetic evidence implicating the Lon protease in the phenotypes associated with the *prlF1* mutation (44), suggests that PrlF may act as a Lon-associated chaperone. If this is the case, presumably the mutant PrlF1 protein has higher-than-wild-type chaperone activity, resulting in the observed posttranslational hyperactivation of Lon. Interestingly, while the mechanisms by which alarmones exert their pleiotropic effects in bacteria are unclear, it has been shown that the alarmone AppppA (diadenosine tetraphosphate) binds to a number of proteins, including the cytoplasmic chaperones, DnaK, GroEL, and ClpB (16, 19). Thus, it may be that alarmones modulate the activities of protease-associated chaperones, thus increasing the activity of the proteases.

Rendering the localization of the hybrid protein independent of the *prlF* allele by using mutations in the *lamB-lacZ* gene fusion suppresses the allele-specific stabilization of the tagged Flg-YgdP protein. This indicates that it is the suppression of stress that is responsible for the higher level of Flg-YgdP in the Hyb42-1 *prlF1* strain and not the localization of the hybrid protein per se. In the experiments involving Flg-YgdP, all of the sequence elements necessary for the transcription and translation of the tagged protein originate from the vector sequences in the parent construct. It seems clear, therefore, that the differences in the steady-state level of the protein are achieved by the differential regulation of the proteolysis of the tagged protein. Either the proteolysis of Flg-YgdP is enhanced under stressed conditions or proteolysis is decreased in the absence of cellular stress. Whichever is the case, the identity of the protease responsible, the mechanism by which the difference between the strains is sensed, and how this leads to differences in the levels of Flg-YgdP all remain issues to be resolved. Regulation of the levels of an alarmone hydrolase would allow negative feedback on an alarmone-mediated response, thus making the response self-limiting. Such a feedback mechanism is consistent with other secretion-related stress-responsive pathways (34, 35).

Consistent with the notion that the *prlF1* and *ygdP* suppression phenotypes occur via convergent pathways, Phenotypic MicroArray (Biolog, Inc., Hayward, Calif.) analysis of the effects of *prlF1* and *ygdP*::*cm* in strain MG1655 indicate that while the effects of *prlF1* are very subtle (in the absence of the hybrid protein), the *ygdP* mutant has more pleiotropic effects (X. Lei and B. Bochner, personal communication). This indicates that while the response mediated by *ygdP* overlaps with the effects of the *prlF1* mutation, its effects are likely not limited to secretion.

Homologues of YgdP appear to be associated with invasion of cellular tissues. For example, the *Bartonella bacilliformis*

homologue, IalA, is required for the invasion of human erythrocytes in vitro, and transformation of a minimally invasive *E. coli* strain with the *Bartonella ialA* operon increases the invasiveness of the strain (30). Moreover, in *E. coli* K1 (the primary gram-negative bacterium responsible for neonatal meningitis), the expression of *ygdP* was found to be elevated in bacterial cells isolated from brain microvascular endothelial cells (1), and introduction of a disrupted copy of *ygdP* rendered the resulting strain noninvasive. Finally, of the 16 closest homologues of *E. coli* YgdP, 13 are from known invasive pathogens. Thus, the alarmone-mediated response regulated by *ygdP* appears to play a physiologically significant role in the survival of bacteria invading host tissue. Interestingly, in the context of the role in pathogenesis, wild-type *ygdP* strains have an advantage. While this may reflect a general selective disadvantage of the stress-adapted state, based on our results, it is tempting to speculate that the loss of invasiveness of the *ygdP* mutants reflects an alarmone-mediated perturbation of secretion in these strains.

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