In the gram-negative pathogen *Providencia stuartii*, the AarA protein is a member of the rhomboid family of intramembrane serine proteases that are widely distributed in prokaryotes and eukaryotes (6, 7, 13, 15, 19, 20). AarA is required for the production of an extracellular signaling molecule that regulates cellular functions including peptidoglycan acetylation, methionine transport, and cysteine biosynthesis. Additional *aarA*-dependent phenotypes include (i) loss of an extracellular yellow pigment, (ii) inability to grow on MacConkey agar, and (iii) abnormal cell division. Since these phenotypes are easily assayed, the *P. stuartii aarA*-mutant serves as a useful host system to investigate rhomboid function. The *Escherichia coli* GlpG protein was shown to be functionally similar to AarA and rescued the above *aarA*-dependent phenotypes in *P. stuartii*. GlpG proteins containing single alanine substitutions at the highly conserved catalytic triad of asparagine (N154A), serine (S201A), or histidine (H254A) residues were nonfunctional. The *P. stuartii* *aarA* mutant was also used as a biosensor to demonstrate that proteins from a variety of diverse sources exhibited rhomboid activity. In an effort to further investigate the role of a rhomboid protein in cell physiology, a *glp* mutant of *E. coli* was constructed. In phenotype microarray experiments, the *glp* mutant exhibited a slight increase in resistance to the β-lactam antibiotic cepotaxime.

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The GlpG protein of *E. coli* can functionally replace AarA in *P. stuartii*. To determine whether GlpG or another *E. coli* protein could function as a rhomboid protein and substitute for AarA, the *P. stuartii* strain XD37.A (*cma37::lacZ AaarA*) was electroporated with an *E. coli* (PB103) genomic library of 2- to 5-kb partial Sau3A fragments in pET21a that was obtained from P. deBoer (Case Western Reserve University). Plasmids that complemented the *aarA* mutation were identified as colonies with restored production of an extracellular yellow pigment. One class of inserts contained overlapping fragments of the *glp* region of the chromosome. One plasmid, pLibG, was used for further studies and contained a 3.8-kb insert with the entire *glp* gene. A library of random Tn7Cm insertions in pLibG was constructed in vitro using a Tn7Cm transposon contained in the GPS-LS Linker Scanning System (New England Biolabs). A pool of random insertions was prepared in *E. coli*, and the resulting plasmid DNA was used to transform *P. stuartii* XD37.A (*ΔaarA*) by electroporation. Plasmids containing Tn7Cm insertions that inhibited the ability of pLibG to complement the *aarA* allele in *P. stuartii* XD37.A (*ΔaarA*) were identified as transformants that failed to produce pigment. Three noncomplementing plasmids were isolated.
and DNA sequence analysis using primers that read outward from the end of Tn7Cm indicated that the transposon had inserted into the glpG coding sequence in all three plasmids.

The ability of glpG to complement several additional aarA-dependent phenotypes was investigated. First, the production of extracellular activating signal was tested using an aarA-dependent cma37::lacZ reporter gene fusion (Fig. 1). Conditioned medium was prepared from P. stuartii strain XD37.A containing pET21a derivatives encoding the wild-type GlpG protein or various GlpG mutant proteins with single alanine substitutions. Each preparation of conditioned medium was tested for activation of the cma37::lacZ fusion in P. stuartii cells at a low density ($A_{600} = 0.35$) as described previously (15, 18). Values represent the activation ($n$-fold) by conditioned medium from GlpG-containing strains relative to the values obtained with conditioned medium from XD37.A containing the vector control pET21a. Values were calculated from quadruplicate samples obtained from two independent experiments.

The chain-forming phenotype of P. stuartii XD37.A was also examined. The chain-forming phenotype of P. stuartii XD37.A was rescued by the glpG gene, as was pigment production and the ability of P. stuartii XD37.A to grow on MacConkey medium (Table 1).

**Mutagenesis of glpG.** The rhomboid family of proteins contains three highly conserved residues that are proposed to form a catalytic triad for protease activity (19). In GlpG, these residues are Asn154, Ser201, and His254. Single alanine substitutions were made at these residues in the GlpG protein using plasmid pLibG as a template and the QuikChange mutagenesis system (Stratagene, La Jolla, CA). DNA sequence analysis of the entire glpG gene confirmed that only the desired change was present in each mutant.

Each of the glpG mutant constructs containing N154A, S201A, and H254A was unable to restore signal production to the aarA mutant based on activation of the cma37::lacZ reporter gene fusion (Fig. 1). Each of the mutant glpG genes was also unable to rescue the cell division defect, growth on MacConkey plates, and the lack of pigment production in aarA mutant P. stuartii (Table 1).

**Use of a P. stuartii aarA mutant to test rhomboid activity from diverse organisms.** Previous studies have demonstrated that expression of various prokaryotic rhomboids in eukaryotic CHO cells resulted in cleavage of the Drosophila rhomboid substrates Spitz, Gurken, and Keren (20). However, the ability of these rhomboid proteins to function in a prokaryote has not been tested. The use of the P. stuartii aarA mutant provides a powerful screening approach to identify proteins from other bacteria that have rhomboid-like activity. Rhomboid genes from *Pseudomonas aeruginosa* (NP251776), *Bacillus subtilis* (NP390367), *Aquifex aeolicus* (NP213910), *Methanococcus jannaschii* (NP247593), *Pyrococcus horikoshii* (NP143361), *Streptococcus pyogenes* (NP268586), and RHBDL2, a human rhomboid (NM017821), were introduced into P. stuartii XD37.A (∆aarA) and tested for rhomboid activity by restored production of the AarA-dependent extracellular activating signal. Table 2 shows the ability of conditioned medium from P. stuartii XD37.A containing various plasmid-encoded rhomboids to activate the cma37::lacZ reporter gene fusion. All the proteins examined exhibited various degrees of rhomboid activity based on restoration of aarA mutant phenotypes, with the *P. aeruginosa* rhomboid exhibiting the strongest activity based on the 14-fold activation of the cma37::lacZ reporter gene fusion.

**Table 1. E. coli glpG can complement an aarA mutation in P. stuartii**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pigment production</th>
<th>Growth on MacConkey agar[a]</th>
<th>Cell morphology[a]</th>
</tr>
</thead>
<tbody>
<tr>
<td>XD37 wild type</td>
<td>+ + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>XD37.A ΔaarA</td>
<td>--</td>
<td>--</td>
<td>Chain</td>
</tr>
<tr>
<td>XD37.A/pET21a</td>
<td>--</td>
<td>--</td>
<td>Chain</td>
</tr>
<tr>
<td>XD37.A/pLibG (GlpG wild type)</td>
<td>++</td>
<td>++</td>
<td>Rod</td>
</tr>
<tr>
<td>XD37.A/pLibG (GlpG-NA)</td>
<td>--</td>
<td>--</td>
<td>Chain</td>
</tr>
<tr>
<td>XD37.A/pLibG (GlpG-SA)</td>
<td>--</td>
<td>--</td>
<td>Chain</td>
</tr>
<tr>
<td>XD37.A/pLibG (GlpG-HA)</td>
<td>--</td>
<td>--</td>
<td>Chain</td>
</tr>
</tbody>
</table>

[a] Assayed on LB agar plates. +++, wild-type pigment level; ++, moderate pigment level; --, no visible pigment.
[b] +, normal growth; --, no growth.
[c] Determined by phase-contrast microscopy.

**FIG. 1.** Ability of GlpG and various mutants to restore signal production to a P. stuartii aarA mutant. Conditioned medium was prepared at an optical density of $A_{600} = 1.0$ from P. stuartii strain XD37 wild type and XD37.A (∆aarA) containing pET21a derivatives encoding the wild-type GlpG protein or various GlpG mutant proteins with single alanine substitutions. Each preparation of conditioned medium was tested for activation of the cma37::lacZ fusion in P. stuartii cells at a low density ($A_{600} = 0.35$) as described previously (15, 18). Values represent the activation ($n$-fold) by conditioned medium from GlpG-containing strains relative to the values obtained with conditioned medium from XD37.A containing the vector control pET21a. Values were calculated from quadruplicate samples obtained from two independent experiments.
TABLE 2. Rhomboid activity from diverse organisms

<table>
<thead>
<tr>
<th>Rhomboid source</th>
<th>Pigment production</th>
<th>Growth on MacConkey agar</th>
<th>Fold activation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>+++</td>
<td>+</td>
<td>14</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>+</td>
<td>+/−</td>
<td>6</td>
</tr>
<tr>
<td>Human</td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td><em>Aquifex aeolicus</em></td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td><em>Methanococcus jannaschii</em></td>
<td>+/−</td>
<td>−</td>
<td>3</td>
</tr>
<tr>
<td><em>Pyrococcus horikoshii</em></td>
<td>+/−</td>
<td>−</td>
<td>3</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
</tbody>
</table>

* With the exception of *S. pyogenes*, all rhomboid genes were present in the vector pCDNA3.1 (Invitrogen) and are described in reference 20. The *S. pyogenes* rhomboid was amplified by PCR and cloned under control of the lac promoter in pBCSK (Stratagene).

* Assayed on LB agar plates. +++: wild-type pigment level; +: moderate pigment; +/−: weakly visible pigment; −: no pigment.

* +, normal growth; +/−, inability to form single colonies when restreaked; −, no growth.

* Determined by the expression of *cma37::lacZ* with conditioned medium from XD37ΔaarA with various rhomboids divided by the expression with XD37ΔaarA-conditioned medium containing the control vector without insert. Values represent the averages of duplicate experiments with four independent data points.

(Table 2). Next, the rhomboid protein YgP (GluP) from *Bacillus subtilis* in XD37Δ restored signal production, as evidenced by the sixfold activation of *cma37::lacZ*. Interestingly, the human rhomboid RHBDL2 exhibited significant activity, with a fivefold activation of *cma37::lacZ* by conditioned medium from this strain. The ability of these rhomboid proteins to restore the other AarA-dependent phenotypes, such as loss of pigment and growth on MacConkey agar, was also examined. Pigment production was restored to various degrees and correlated well with rhomboid activity based on extracellular signal production (Table 2). However, the rescue of growth on MacConkey agar was observed only with rhomboid proteins from *P. aeruginosa* and *B. subtilis*, both of which appeared to have the strongest activity based on restoration of signal activity and pigment production (Table 2).

**Phenotype microarray analysis of a glpG mutant.** To address the role of *glpG* in *E. coli*, the PCR-mediated allelic replacement procedure of Datsenko and Wanner was used to construct a *glpG::cat* allele that disrupted *glpG* at position 67 of the 276-amino-acid protein (3). This insertion point was chosen because it was known to inactivate function based on Tn7 insertions that disrupted *glpG* function on a plasmid, and it was upstream of the two promoters within *glpG* that transcribe the downstream *glpR* gene and would not be polar (23). The resulting PCR fragment was used to electroporate *E. coli* MG1655/pKD46 carrying genes for an arabinose-inducible, lambda *red* recombination system (3). Transformants were selected on LB medium containing chloramphenicol (25 μg/ml). The *glpG::cat* allele was verified by PCR using the *glpG* ORFmers (Sigma-Genosys) and also by Southern blot analysis.

The role of GlpG in *E. coli* was investigated by the use of phenotype MicroArrays (Biolog, Hayward, CA). These experiments were conducted by Michael Zimon at Biolog. A comprehensive set of 20 plates was used to test metabolic differences with respect to growth under a variety of conditions and sensitivities to a variety of compounds. The only parameter that was verified by our lab as consistently different in the *glpG* mutant was an increased resistance to cefotaxime, a β-lactam antibiotic. These results were followed up by testing the levels of resistance by Kirby-Bauer disk diffusion assays according to CLSI (formerly NCCLS) guidelines (2). These experiments were repeated in triplicate with all experiments exhibiting the pattern of resistance. The cefotaxime zone diameter for wild-type *E. coli* MG1655 was 32.5 ± 0.7 mm, and for the *glpG::cat* mutant it was 30.5 ± 0.7 mm. The zone diameters for ampicillin and ceftriaxone (additional β-lactams) and for the structurally unrelated antibiotics ciprofloxacin (a fluoroquinolone) and amikacin (an aminoglycoside) were the same for both wild-type *E. coli* MG1655 and the *glpG* mutant.

We could find no additional phenotypes resulting from the *glpG::cat* mutation with respect to colony morphology, growth at 30 to 42°C, and growth on minimal medium with either glucose or glycerol.

**glpG is not required for extracellular signal production in *E. coli*.** In a previous study, the ability of *E. coli* to produce a factor biologically similar to that of *P. stuartii* was demonstrated by the ability of conditioned medium from *E. coli* to activate the *aarA*-dependent *cma37::lacZ* fusion in *P. stuartii* (18). Signal production was examined in conditioned medium prepared from six cultures each from an independent *glpG::cat* mutant and six colonies of wild-type MG1655. The degree of *cma37::lacZ* activation in the *P. stuartii* biosensor varied from 18- to 29-fold with wild-type MG1655, with an average of 24-fold (±4-fold). For the *glpG::cat* mutants, the degree of activation ranged from 15- to 39-fold (average, 27-fold ± 10-fold). The basis for the high variability is unknown. However, the *glpG::cat* allele does not appear to significantly alter signal production. Moreover, the frequencies of transduction of the *glpG::cat* allele into *E. coli* MG1655/pET21a or MG1655/pLibG were similar, and these transductants exhibited the same variability in signal production (data not shown).

To rule out the possibility that the inability to detect a role for *glpG* in signal production was due to its lack of expression under laboratory growth conditions, we performed Northern blot analysis of *glpG* mRNA in cells at mid-log phase in LB-only medium and LB supplemented with 0.4% glycerol (Fig. 2). The *glpG* mRNA was clearly detectable, and the levels were similar in LB with and without glycerol. The accumulation of *glpG* mRNA was also examined in M9 salts containing glucose (0.2%) or glycerol (0.4%). The levels of *glpG* mRNA were similar under each condition, although the absolute levels were lower than in cells grown in LB (Fig. 2). As a control, the levels of mRNA for the housekeeping gene *secD* did not significantly vary under the growth conditions tested (Fig. 2).

The effect of glycerol on signal activity in conditioned medium was examined from cells grown in LB containing either glucose (0.2%) or glycerol (0.4%). For these experiments, conditioned medium was harvested at an optical density at 600 nm (OD600) of 1.0. In addition, cells grown in conditioned medium were harvested at an OD600 of 0.4, a point where the optimal response to the extracellular signal has been observed previously (18). Signal production was examined in conditioned medium prepared from LB plus 0.4% glycerol was 1.3-fold (±0.03-fold) above the levels seen in LB-only-grown cells (data not shown). The addition of glucose had no effect. To rule out the possibility that residual glycerol from conditioned medium stimulated expression, 0.4% glycerol was added to LB-only
blot analysis. The top panel represents the ethidium bromide-stained by capillary action. Probes were generated by PCR using the ORFmer 1.2% formaldehyde agarose gels, and transferred to a nylon membrane vested using the MasterPure RNA purification kit, electrophoresed in plus 0.2% glucose, and M9 plus 0.4% glycerol. Total RNA was har-
tained under the following growth conditions: LB, LB plus 0.4% glycerol, M9 

for the rhomboid family of proteins has been identi-

FIG. 2. Accumulation of glpG mRNA under various growth condi-
tions. Cells of MG1655 were grown to mid-log phase (OD₆₀₀ = 0.6) under the following growth conditions: LB, LB plus 0.4% glycerol, M9 plus 0.2% glucose, and M9 plus 0.4% glycerol. Total RNA was har-

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utes to glucose export. BMC Microbiol. 4:1–16.

medium and β-galactosidase expression from cma37::lacZ was examined in cells grown to an OD₆₀₀ of 0.4. There was no effect of glycerol on cma37::lacZ expression (data not shown).

Concluding remarks. The results from this study provide fur-
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GlpG in E. coli. Interestingly, in eukaryotic CHO cells, GlpG N254A was also nonfunctional (20); however, the purified GlpG N254A was active in vitro (8, 10).

In E. coli, a chromosomal glpG::cat null allele resulted in only one detectable phenotype, a slightly increased resistance to cefotaxime. Presently, there are only two examples where a function for the rhomboid family of proteins has been identified in prokaryotes. In addition to the AarA-dependent phe-

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In summary, this study demonstrates the utility of the P. stuartii aarA mutant as a biosensor strain to assess rhomboid activity and conduct structure-function studies. This strain will be useful in identifying or verifying new rhomboids from both prokaryotic and eukaryotic genomes. For example, genomic libraries can be introduced into a P. stuartii XD37.A aarA cma37::lacZ strain and plasmids encoding strong rhomboid activity can be directly selected by growth on MacConkey plates. Plas-
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FIG. 1. (Left) Northern analysis of glpG expression in P. stuartii. Hybridization was performed with a digoxigenin-labeled DNA probe specific to a 303-bp region of the 1,724-bp cma37::lacZ construction. Lane 1: MG1655 under nitrate conditions. Lane 2: MG1655 under nitrate conditions followed by growth on a 2% glucose solid medium. Lane 3: MG1655 under nitrate conditions followed by growth on a 2% glucose solid medium and then screening for cma37::lacZ expression (Table 1 and Fig. 1). This result differs from that reported by Meagawa at al., where GlpG containing an N254A substitution still exhibited activity (10). This difference may reflect the use of different substrates and organ-
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