The twin arginine translocation system contributes to symbiotic colonization of Euprymna scolopes by Vibrio fischeri
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Abstract
In many bacteria, the twin arginine translocation (Tat) system transports folded proteins across the cytoplasmic membrane, and these proteins can play a role in symbiotic or pathogenic infections. A role for the Vibrio fischeri Tat system was identified during symbiotic colonization of its host Euprymna scolopes, demonstrating a function for the Tat system in host colonization by a member of the Vibrionaceae. Using bioinformatics, mutant analyses, and green fluorescent protein fusions, a set of Tat-targeted proteins in V. fischeri was identified.

Introduction
The twin arginine translocation (Tat) system is widespread in bacteria and transports folded proteins across the cytoplasmic membrane (Palmer et al., 2005; Lee et al., 2006), including symbiosis and pathogenicity determinants (Ding & Christie, 2003; Meloni et al., 2003; Caldelari et al., 2006; Lavander et al., 2006; Gonzalez et al., 2007). With the exception of a study of the role of the Tat system in laboratory growth of Vibrio cholerae (Zhang et al., 2002) and the prediction of Tat-targeted proteins in V. cholerae (Dilk et al., 2003), little is known about the importance of this system in pathogenic and symbiotic Vibrio species. The goal of this study was to determine whether the Tat system contributes to the ability of Vibrio fischeri to symbiotically colonize the Hawaiian bobtail squid, Euprymna scolopes (Visick & Ruby, 2006), and to identify potential Tat-targeted proteins in V. fischeri. The results demonstrate a function for the Tat system in host colonization by a member of the Vibrionaceae.

Materials and methods
Bacterial strains and plasmids
Bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli was grown in Luria–Bertani (LB) medium (Miller, 1992) with 20 μg mL⁻¹ chloramphenicol or 40 μg mL⁻¹ kanamycin where noted. Vibrio fischeri was grown in either Luria–Bertani with added salt (LBS) medium (Stabb et al., 2001) or mineral salts medium with N-acetylglucosamine (NAG) as a carbon and nitrogen source (per liter): 1.0 g NAG, 378 mg L⁻¹ M NaPO₄ (pH 7.5), 50 mL L⁻¹ M Tris (pH 8.0), 6 mg FeSO₄·7H₂O, 13.6 g MgSO₄·7H₂O, 0.83 g KCl, 19.5 g NaCl, 1.62 g CaCl₂·2H₂O, with 2 μg mL⁻¹ chloramphenicol or 100 μg mL⁻¹ kanamycin added where noted. When added to media, trimethylamine N-oxide (TMAO) or sodium nitrate was used at a final concentration of 40 mM.

Plasmids were constructed using standard cloning procedures in E. coli strains DH5α (Hanahan, 1983) and DH5αλpir (Dunn et al., 2005). Constructs were verified by sequencing at the University of Michigan DNA Sequencing Core Facility and introduced into V. fischeri via triparental mating (Stabb & Ruby, 2002). For mutant construction, allelic exchange was performed as described previously (Bose et al., 2007).

Putative V. fischeri tat genes were identified based on similarity to E. coli K12 TatABC (E. coli accession numbers AAC76839, YP_026270, and NP_418282) using the BLAST algorithm (Altschul et al., 1990). The putative V. fischeri tatABC operon along with c. 200 bp of upstream sequence was PCR-amplified (forward primer:
Plasmids pCR®-BluntII-TOPO®: Plasmid in Zero Blunt® TOPO® PCR cloning kit, KnR
pTOPtatABC: pCR®-BluntII-TOPO® containing V. fischeri tatABC
pTOPtatABC.1: pTOPtatABC digested with SpeI and religated to delete part of tatABC
pSV105: E. coli–V. fischeri shuttle vector, CmR
pSV105tatABC: pSV105 containing V. fischeri tatABC, CmR
pAKD600: Δtat allele for generating AKD600, EmR, KnR
pAKD601: Contains lacIΔ and an IPTG-inducible promoter with KpnI and NheI restriction sites downstream for generating signal sequence-GFP fusions, KnR
pEVS104: Conjugative helper plasmid, KnR

*cmR, chloramphenicol resistance; knR, kanamycin resistance; emR, erythromycin resistance.

To generate a Tat-deficient strain of V. fischeri, plasmid pAKD600 was used for allelic exchange. pAKD600 consists of an c. 1.6-kb DNA fragment upstream of the translational start of tatA (forward primer: 5′-TAGATGAGCCTGTACCTGAACATGCGT; reverse primer: 5′-AACGCTAGCAATAACCACCGATATTATTTTCCTAGC) fused to an c. 1.6-kb DNA fragment downstream of the translational end of tatC (forward primer: 5′-AATGTGACGTGGAAAGATGGAACATATGCTTATG; reverse primer: 5′-TGTTGGAGTCAATCGACGGCG) using a NheI site engineered into the primers. Exchange of this mutant allele onto the chromosome resulted in the generation of an in-frame deletion mutant where the coding sequence of tatABC was replaced with a NheI site.

### Complementation assays of tat mutants

Plasmids pTOPtatABC and pTOPtatABC.1 were transformed (Miller, 1992) into E. coli strains MC4100 (Casadaban, 1976) and DADE (Wexler et al., 2000), a tat deletion mutant. A single colony of each strain was inoculated into 3 mL of LB containing kanamycin in an 18-mm tube and the cultures were grown aerobically for c. 16 h at 37°C with shaking. Cultures were then diluted 100-fold into 3 mL of LB, LB supplemented with TMAO, or LB supplemented with nitrate in sealed 18-mm anaerobic tubes. Anaerobic tubes were prepared by sparging sealed tubes with a gas mixture containing 5% CO₂, 10% H₂ and 85% N₂, followed by autoclaving. The OD₅₉₅ nm was determined after incubation at 37°C for 16 h. Each experiment consisted of three independent cultures of each strain, and the averaged data of one representative experiment is shown.

Similarly, V. fischeri ES114 and the tat mutant AKD600 containing pSV105 or pSV105tatABC were grown overnight in LBS containing chloramphenicol and diluted 1000-fold into sealed anaerobic tubes containing LBS, LBS supplemented with TMAO, or LBS supplemented with nitrate. Tubes were prepared as described above, and the OD₅₉₅ nm was determined after incubation at 24°C for 14 h. Each experiment consisted of three independent cultures of each strain, and the averaged data from one representative experiment is shown.

### Assays for symbiotic competence

Aposymbiotic Euprymna scolopes hatchlings were inoculated with a c. 1:1 mix of AKD600 and wild type, as described previously (Dunn et al., 2006). After 72 h, individual

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**Table 1. Strains and plasmids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Sources</th>
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</thead>
<tbody>
<tr>
<td><em>Vibrio fischeri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES114</td>
<td>Wild-type V. fischeri</td>
<td>Boettcher &amp; Ruby (1990)</td>
</tr>
<tr>
<td>AKD600</td>
<td>ES114 ΔtatABC (allele exchanged from pAKD600)</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F′ lacI Δ pir (φ 80d λacZ)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>DH5α pir</td>
<td>λpir derivative of DH5α</td>
<td>Dunn et al. (2005)</td>
</tr>
<tr>
<td>MC4100</td>
<td>F- araD139 M(lacF-jac)U169 rpsL150 relA1 deoC1 rbsR FthDS301 fruA25</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>DADE</td>
<td>MC4100 ΔtatABCD ΔtatE</td>
<td>Wexler et al. (2000)</td>
</tr>
</tbody>
</table>

**Plasmids**

- pCR®-BluntII-TOPO®: Plasmid in Zero Blunt® TOPO® PCR cloning kit, KnR
- pTOPtatABC: pCR®-BluntII-TOPO® containing V. fischeri tatABC
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- pEVS104: Conjugative helper plasmid, KnR

*cmR, chloramphenicol resistance; knR, kanamycin resistance; emR, erythromycin resistance.
animals were homogenized and dilution plated. Plates were incubated aerobically at 28°C overnight, and individual colonies were patched to plates containing TMAO and incubated anaerobically in GasPak™ jars (BD, Franklin Lakes, NJ) at 28°C for 16–20 h to determine the ratio of AKD600 (V. fischeri patches that did not grow) to wild type. The ability of AKD600 to grow after patching was verified in a subset of the samples by incubation of replicate plates aerobically. The anaerobic growth data are presented as a relative competitiveness index (RCI), which is the ratio of AKD600 to wild type in the host divided by the ratio of these strains in the inoculum. A RCI >1 indicates AKD600 dominates, whereas a RCI <1 indicates wild type dominates. Statistical significance was determined using a Student’s t-test on log-transformed data.

### Mixed-culture competition assays

The competitiveness of AKD600 relative to wild type was assayed in mixed cultures, as described previously (Dunn et al., 2006). The RCI was calculated similarly to the squid assays and equals the ratio of AKD600 to wild type after coculturing divided by the ratio of these strains at the start of the experiment (generation 0). Each experiment consisted of three independent mixed cultures and the averaged data from three experiments is reported. Statistical significance was determined using a Student’s t-test on log-transformed data.

### Bioinformatic prediction of Tat-targeted proteins

All ORFs present in the V. fischeri genome sequence (Ruby et al., 2005) were analyzed using the default settings for TATP (Bendtsen et al., 2005) and TATFIND version 1.4 (Rose et al., 2002). PSORTB v.2.0 (Gardy et al., 2005) was used to predict the localization of each identified protein.

### Green fluorescent protein (GFP) fusion assay for verifying Tat-targeting

To determine Tat-specific targeting of proteins identified by TATP – and/or TATFIND, the putative signal sequence, including five to eight codons past the predicted cleavage site, was PCR-amplified (primer sequences are available in the supplementary material) and cloned into plasmid pAKD601, translationaly fusing each signal sequence to a gene encoding GFP, and placing the fusion under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (Bose et al., unpublished data). pAKD601 was constructed by inserting a KpnI–NotI DNA fragment containing lacU and an IPTG-inducible promoter (Bose et al., unpublished data) into KpnI and NotI-digested pVSV33 (Dunn et al., 2006), followed by removal of the promoterless chloramphenicol resistance gene and ATG start codon of gfp by digesting with NheI and religating the plasmid. Signal sequences were cloned into the KpnI and NheI restriction sites in pAKD601, using KpnI and NotI sites engineered into the PCR primers.

To visualize localization, V. fischeri ES114 or AKD600 harboring the fusion plasmids were grown overnight at 28°C in LBS containing kanamycin and 2 mM IPTG, pelleted and resuspended in fresh LBS, and incubated at 28°C for 45 min. Cultures were diluted 10-fold in filter-sterilized artificial seawater (Instant Ocean®, Aquarium Systems, Inc., Mentor, OH), placed on Superfrost Gold slides (Fisher Scientific, Pittsburgh, PA), and visualized using a Nikon (Melville, NY) Eclipse E600 epifluorescence microscope outfitted with a Nikon Chroma 41017 Endow GFP filter cube and a Nikon Coolpix 5000 camera.

### Results and discussion

#### Identification of the Tat operon in V. fischeri and demonstration of function

Sequence similarity to the E. coli Tat proteins suggested that the V. fischeri ORFs VF0049-51 could be assigned as TatA (59% identity over 88 of the 89 amino acids in E. coli TatA), TatB (51% identity over 104 of 171 amino acids), and TatC (73% identity over 236 of 258 amino acids), respectively. To determine whether these genes encoded a functional Tat system, they were introduced on pTOPtatABC into an E. coli Tat-deficient strain, DADE (Wexler et al., 2000). This strain displays several phenotypic differences from its parent MC4100, including a defect in anaerobic growth with TMAO or nitrate as terminal electron acceptors due to improper localization of the Tat-targeted TMAO and nitrate reductases (Weiner et al., 1998), and the formation of long chains in culture, apparently due to improper localization of cell wall amidases (Bernhardt & de Boer, 2003; Ize et al., 2003). The tatABC operon from V. fischeri complemented the chaining phenotype of DADE (data not shown), and restored its ability to grow anaerobically in the presence of TMAO and nitrate (Fig. 1a), indicating tatABC encodes a Tat system functionally similar to that in E. coli.

To determine the function of the Tat system in V. fischeri, an in-frame tatABC deletion mutant, AKD600, was constructed. Similar to E. coli DADE, AKD600 displayed a growth defect relative to wild type under anaerobic conditions in LBS containing TMAO or nitrate, and these phenotypes could be complemented by introduction of pVSV105tatABC (Fig. 1b). However, AKD600 does not display the DADE chaining phenotype. Unlike E. coli, V. fischeri ES114 has only one predicted amidase (VF2326), and it does not have a recognizable Tat signal sequence, suggesting that although the Tat system of V. fischeri is
functionally similar to that of *E. coli*, the Tat-targeted proteins may differ between the organisms.

**Tat system plays a role in symbiotic colonization**

Because Tat-targeted proteins can include symbiosis and pathogenicity determinants (Ding & Christie, 2003; Meloni *et al*., 2003; Caldelari *et al*., 2006; Lavander *et al*., 2006; Gonzalez *et al*., 2007), the ability of AKD600 to colonize the *Euprymna scolopes* light organ was tested. In single inoculum experiments AKD600 infected *Euprymna scolopes*, and the onset of bioluminescence and colonization levels were similar to that of wild type-infected animals (data not shown). However, in some mutants, symbiotic defects are only apparent when they are forced to compete with wild type (Visick & Ruby, 1998; Stabb & Ruby, 2003). When squid were inoculated with a 1:1 mixture of AKD600 and wild type, the mutant was outcompeted approximately twofold by wild type 72 h postinoculation, as evidenced by an RCI value of 0.49 for three combined experiments (Fig. 2a). These data demonstrate a host-associated role for the Tat system in the *Vibrionaceae*.

**Tat system plays a role during aerobic growth in laboratory culture**

To determine whether the Tat system was also important during aerobic growth of *V. fischeri* in liquid culture, competition assays were performed by coculturing AKD600 and wild type in both rich (LBS) and defined media. Interestingly, the results suggest that the Tat system contributes to the aerobic growth of *V. fischeri* in LBS, but not in

![Fig. 1. Complementation of anaerobic growth defects of Tat-deficient bacterial strains by introduction of *Vibrio fischeri* tatABC. (a) Anaerobic end-point growth assays for *Escherichia coli* strain MC4100 (wild type) containing pTOPtatABC.1 (white bars), *E. coli* strain DADE (ΔtatABCΔtatE) containing pTOPtatABC.1 (light grey bars), and DADE containing pTOPtatABC (dark grey bars), in LB, LB containing 40 mM TMAO, and LB containing 40 mM sodium nitrate. (b) Anaerobic end-point growth assays for *V. fischeri* strain ES114 containing pSV105 (white bars), *V. fischeri* AKD600 (ΔtatABC) containing pSV105 (light grey bars) and AKD600 containing pSV105tatABC (dark grey bars), in LBS, LBS containing 40 mM TMAO, and LBS containing 40 mM sodium nitrate.](image)

![Fig. 2. Competition of the ΔtatABC mutant AKD600 vs. its wild-type *Vibrio fischeri* parent, ES114. (a) Competition in the host 72 h postinoculation. The RCI was calculated by dividing the ratio of AKD600 to wild type in individual animals by the inoculum ratio. The average RCI for the indicated number of animals is presented. Student's *t*-tests were used to determine if the RCI in each experiment was significantly different from 1 (a RCI of 1 indicates the strains compete equally well). (b) In-culture competition in mineral salts medium (white bar) and LBS medium (grey bar). Data is the average of three experiments each with three independent cultures. The RCI was calculated by dividing the ratio of AKD600 to wild type at 0 generations by the ratio at 20 or 40 generations. The asterisk indicates an average RCI that was significantly different from 1 (Student's *t*-test, *P* < 0.05). Error bars represent 95% confidence intervals.](image)
Important, the lack of any discernable attenuation of AKD600 in the defined minimal medium shows that the negative effects of deleting tatABC are specific to certain conditions and not a generalized property of AKD600. On the other hand, the competitive disadvantage of AKD600 in LBS suggests that growth in this medium may more closely mimic growth in the host.

### Bioinformatic identification of potential Tat-targeted proteins

The characterization of AKD600 in culture and in its host indicated that the Tat-targeted proteins in *V. fischeri* may belong to several potentially overlapping classes, including anaerobic respiratory proteins, symbiosis determinants, and those important for aerobic growth in rich medium. To identify potential Tat-targeted proteins in the *V. fischeri* ES114 genome (Ruby *et al.*, 2005), TATP (Bendtsen *et al.*, 2005) and TATFIND 1.4 were used (Rose *et al.*, 2002). Thirteen ORFs contained a twin arginine signal motif characteristic of Tat-targeted proteins and 11 ORFs were identified by TATP as containing likely signal sequences and cleavage sites, but no identifiable twin-arginine motif (Table 2, columns 2 and 3). Consistent with the anaerobic growth studies (Fig. 1b), putative TMAO reductases (VFA0082, VFA0188, and VFA0299) and nitrate reductase (VF1905) were predicted to be Tat-targeted. In addition, several putative or hypothetical proteins were identified that may be linked to nutrient acquisition (VFA0899, VFA1007, VFA1057) or transport (VF0660, VF1105, VF1177, VFA1062).

To predict whether these proteins would localize to the cytoplasmic membrane or periplasmic space (as expected for Tat-targeted proteins), PSORTB v.2.0 was used (Gardy *et al.*, 2005). In many cases PSORTB indicated that the identified ORFs were likely localized to the cytoplasmic membrane.

### Table 2. Bioinformatics-predicted Tat-targeted proteins in *Vibrio fischeri* ES114

<table>
<thead>
<tr>
<th>Chromosome 1</th>
<th>Annotation</th>
<th>TATP predicted signal motif</th>
<th>TATFIND predicted signal motif</th>
<th>Tat-targeting GFP assay</th>
<th>Predicted localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>VF0660</td>
<td>Putative symporter</td>
<td>n.t.m.</td>
<td>None</td>
<td>Nonspecific</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>VF0901</td>
<td>CydD</td>
<td>n.t.m.</td>
<td>None</td>
<td>No</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>VF1105</td>
<td>Transporter</td>
<td>None</td>
<td>KRRTIL</td>
<td>ND</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>VF1158</td>
<td>Hypothetical protein</td>
<td>n.t.m.</td>
<td>None</td>
<td>Not visible</td>
<td>ND</td>
</tr>
<tr>
<td>VF1177</td>
<td>Putative transporter</td>
<td>n.t.m.</td>
<td>None</td>
<td>ND</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>VF1360</td>
<td>Formate dehydrogenase α chain</td>
<td>TRRMMFM</td>
<td>None</td>
<td>No</td>
<td>Periplasm</td>
</tr>
<tr>
<td>VF1361</td>
<td>Hypothetical</td>
<td>NRRNLL</td>
<td>None</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>VF1552</td>
<td>NfC</td>
<td>SRRNFL</td>
<td>SRRNFL</td>
<td>No</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>VF1640</td>
<td>Hypothetical</td>
<td>None</td>
<td>KRRNFN</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>VF1641</td>
<td>DNA topoisomerase III</td>
<td>n.t.m.</td>
<td>None</td>
<td>No</td>
<td>ND</td>
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<tr>
<td>VF1905</td>
<td>NapA</td>
<td>TRRAFV</td>
<td>TRRAFV</td>
<td>No</td>
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<td>VF1981</td>
<td>Hypothetical protein</td>
<td>n.t.m.</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>VF2007</td>
<td>Hypothetical protein</td>
<td>n.t.m.</td>
<td>None</td>
<td>No</td>
<td>ND</td>
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<tr>
<td>VF2220</td>
<td>Ubiquinol-cytochrome C reductase subunit</td>
<td>GRRRFL</td>
<td>GRRRFL</td>
<td>Yes</td>
<td>Cytoplasmic membrane</td>
</tr>
<tr>
<td>VF2469</td>
<td>General secretion pathway protein I</td>
<td>n.t.m.</td>
<td>None</td>
<td>Not visible</td>
<td>ND</td>
</tr>
</tbody>
</table>

Chromosome 2

| VF00082 | DmsA | SRRSFV | SRRSFV | Nonspecific | Periplasm |
| VF0188 | TorZ | SRRGFL | SRRGFL | Yes | Periplasm |
| VF0299 | TorA | SRRSFL | SRRSFL | Yes | Periplasm |
| VF0725 | Choloylglycine hydrolase family protein | n.t.m. | None | Nonspecific | ND |
| VF0899 | Putative sulfatase | n.t.m. | None | Nonspecific | ND |
| VF0990 | BisC | SRRTFL | SRRTFL | Yes | Periplasm |
| VF1007 | Putative sulfatase | TRRDIL | TRRDIL | No | Cytoplasm |
| VF1057 | Putative phosphatase | SRRGFL | SRRGFL | Nonspecific | ND |
| VF1062 | Putative transporter | n.t.m. | None | No | Cytoplasmic membrane |

*As determined by the program TATP (Bendtsen *et al.*, 2005), see ‘Materials and methods’. n.t.m., ORF contains a putative signal peptide sequence but a conserved twin-arginine motif was not detected.

w As determined by the program TATFIND 1.4 (Rose *et al.*, 2002), see ‘Materials and methods’.

z Targeting to the Tat system was determined by fusing the putative signal sequence for each ORF to *gfp* and determining localization of the fusion protein using epifluorescence microscopy (see ‘Materials and methods’).

‰ Predicted by PSORTB v.2.0 (Gardy *et al.*, 2005). Program was not able to predict localization. None, no detectable signal sequence, ND, not determined.?
membrane or periplasmic space, although localization could not always be predicted using this program (Table 2).

**Testing of Tat-targeting using GFP fusions**

To further test the bioinformatic predictions, GFP-fusion assays for signal sequence targeting were used, which have been used previously to positively identify Tat signal sequences (Thomas *et al.*, 2001). This approach was adapted for use in *V. fischeri* using pAKD601 to translationally fuse the signal sequences for specific ORFs to GFP, and placing the fusions under transcriptional control of an IPTG-inducible promoter to reduce variability associated with differential regulation of the native promoters. The plasmid constructs were introduced into both wild-type *V. fischeri* and AKD600 to determine whether GFP would be periplasmically targeted, and if so, whether this was Tat-dependent.

If GFP was periplasmically located in wild type, but not AKD600, the ORF was designated Tat-targeted (e.g., Fig. 3a and b). If the GFP was periplasmic in both strains, the ORF was designated nonspecific for targeting. In several cases no periplasmatic targeting was observed (designated as not targeted; e.g., Fig. 3c), or the fusions resulted in no detectable GFP production (designated as not visible) (Table 2).

As predicted, the putative TMAO reductase TorA (VFA0299) signal was Tat-targeted (Table 2) as evidenced by the localization of the GFP signal to the periplasmic space in the wild type (Fig. 3a) but not in AKD600 (Fig. 3b). Three other identified ORFs were also positive in this assay, including signal peptides for putative respiratory chain components TorZ (VFA0188) and an ubiquinol-cytochrome c reductase subunit (VF2220), and a nonrespiratory putative biotin sulfoxide reductase BisC (VFA0990). All of the ORFs predicted by TATP as having signal peptide sequences, but lacking a twin-arginine motif were not specifically Tat-targeted (Table 2; Fig. 3c). Interestingly, some signal sequences appeared to target GFP to the periphery of the cells in both the wild type and AKD600 (VF0660, VFA0082, VFA0725, VFA0899, and VFA1057). This result is puzzling considering that the GFP assays were originally developed based on the premise that GFP can only correctly fold in the cytoplasm and not in the periplasm (Thomas *et al.*, 2001). Therefore, any GFP located periplasmically is presumed to have folded in the cytoplasm and been transported as a folded protein across the cytoplasmic membrane by the Tat system. However, these results suggest that either the signal sequences localize GFP fusions peripherally to the cytoplasmic membrane and appear periplasmically located, or that the signals are targeting the folded GFP through another pathway.

Although the signal sequence-GFP fusion assays did identify certain proteins as probable Tat targets, other likely candidates with twin arginine signal motifs did not appear to be Tat-targeted, indicating limitations to this assay or bioinformatic predictions. A possible source of misidentification using these fusions could be linked to the signal sequence fusion affecting GFP folding and therefore detection. Another possibility could be related to variation in expression levels. Although all fusion constructs were placed under the control of an IPTG-inducible promoter, several constructs resulted in lower than average GFP expression levels (VF1360, VF1552, and VF1905; data not shown), and in certain cases (VF1158 and VF2469), GFP could not be detected. Low levels of GFP expression can make it more difficult to visualize targeting, and possibly lead to a false negative result. For example, *V. fischeri* containing the NapA

![Fig. 3. Localization of signal sequence–GFP fusions in *Vibrio fischeri* via epifluorescence microscopy. Negative images are presented, with darker areas representing GFP localization. Images were color reversed in Adobe Photoshop 7.0 for Macintosh, with identical treatment of each image. (a) *Vibrio fischeri* ES114 cells harboring pAKD601 containing the TorA (VFA0299) signal sequence, demonstrating localization of GFP to the periplasm. (b) *Vibrio fischeri* AKD600 (lacking tatABC) cells harboring pAKD601 containing the TorA signal sequence, demonstrating a lack of periplasmic GFP localization. (c) *Vibrio fischeri* ES114 cells harboring pAKD601 containing the hypothetical protein (VF2007) signal sequence, which was scored as not Tat-targeted. Scale bars represent c. 1 μm.](image-url)
(VF1905) fusion construct displayed lower fluorescence levels, and GFP did not appear to be Tat-targeted. However, AKD600 does display a growth defect during anaerobic growth in the presence of nitrate (Fig. 2b), suggesting that NapA is Tat-targeted. Therefore, proteins predicted to have twin arginine signal motifs by TATP and/or TATFIND 1.4 that were not Tat-positive in the GFP fusion assays may in fact be Tat-targeted.

Limitations in the use of single reporter molecules have been reported previously (Tullman-Ercek et al., 2007), and the most definitive approach for determining the symbiotic importance of each potential Tat-targeted protein will be construction of V. fischeri strains lacking each protein(s). However, the results of the GFP assay have confirmed the presence of four Tat-targeted proteins in V. fischeri and suggest that the twin arginine motif is necessary for targeting.

**Summary**

These data indicate that the V. fischeri Tat system contributes to symbiotic competence and to growth under specific laboratory culture conditions. Using TATP and TATFIND, potential protein(s) responsible for the observed colonization and growth phenotypes were identified that include anaerobic respiratory proteins and proteins potentially linked to nutrient acquisition. These proteins may be important during host-associated growth or laboratory growth in nutrient-rich medium, conditions where cells are most likely exposed to lower oxygen concentrations and several carbon and nitrogen sources. For example, two of the four proteins identified as Tat-targeted in the GFP fusion assay are likely TMAO reductases, and TMAO reductase activity has been measured in squid light organ tissue colonized by V. fischeri (Proctor & Gunsalus, 2000). Therefore, it is possible that utilization of TMAO is important during squid colonization and that improper localization of the TMAO reductases in AKD600 is responsible for the symbiotic colonization defect observed in this strain. However, the possibility that other currently unidentified proteins may also be Tat-targeted and contribute to the observed phenotypic differences between AKD600 and wild-type cells cannot be ruled out. In conclusion, the results of this study further the knowledge of the role of the Tat system in Vibrio species and symbiotic associations.

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**References**


**Supplementary material**

The following supplementary material is available for this article:

**Table S1.** PCR primers used to amplify putative Tat-targeted ORF signal sequences for cloning into pAKD601. This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.01043.x. (This link will take you to the article abstract.)

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