TATC2 IS IMPORTANT FOR GROWTH OF *ACINETOBACTER BAYLYI* UNDER STRESS CONDITIONS

Justin M. Langro[1]
Megan M. Chamberland[2]
Celena M. Gwin[3]
Natalia Prakash[4]
Danielle T. Velez[5]
Nathan W. Rigel[6]

1.Department of Biology, Hofstra University, Long Island, New York City, USA

3. Present address: Department of Microbial Pathogenesis, Yale University, New Haven, CT, USA

5. Present Address: Regeneraon Pharmaceuticals, Tarrytown, NY, USA

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CORRESPONDING AUTHORS

Department of Biology
Hofstra University
318A Gittleson Hall
Hempstead, NY 11549
nathan.w.rigel@hofstra.edu
Phone: 516-463-6542
Fax: 516-463-5112

KEYWORDS

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ABSTRACT

Protein export pathways are important for bacterial physiology among pathogens and non-pathogens alike. This includes the Twin-Arginine Translocation (Tat) pathway, which transports fully folded proteins across the bacterial cytoplasmic membrane. Some Tat substrates are virulence factors, while others are important for cellular processes like peptidoglycan remodeling. Some bacteria encode more than one copy of each Tat component, including the Gram-negative soil isolate *Acinetobacter baylyi*. One of these Tat pathways is essential for growth, while the other is not. We constructed a loss-of-function mutation to disrupt the non-essential *tatC2* gene and assessed its contribution to cell growth under different environmental conditions. While the *tatC2* mutant grew well under standard laboratory conditions, it displayed a growth defect and an aberrant cellular morphology when subjected to high temperature stress including an aberrant cellular morphology. Furthermore, increased sensitivities to detergent suggested a compromised cell envelope. Lastly, using an *in vitro* co-culture system, we demonstrate that the non-essential Tat pathway provides a growth advantage. The findings of this study establish the importance of the non-essential Tat pathway for optimal growth of *A. baylyi* in stressful environmental conditions.

INTRODUCTION

Transport of proteins between membrane-bound compartments is a fundamental biological problem faced by all cellular organisms, including bacteria. Protein transport is carried out by multi-subunit, membrane-spanning molecular machines (12, 23). In Gram-negative bacteria, most exported proteins cross the inner membrane *via* the General Secretion (Sec) pathway. Proteins that take this path must remain unfolded in order to pass through the SecYEG inner membrane translocase. However, some proteins are exported in a fully folded state by using a separate machine called the Twin-Arginine Translocase (Tat) (15). Proteins destined for export *via* Sec or Tat contain a recognizable tripartite signal peptide composed of a positively charged region at the N-terminus, followed by a hydrophobic region in the middle and a polar region at the end (30). However, there are several key differences between Sec and Tat signal peptides; among them the charged region of canonical Tat signal peptides contains an arginine dipeptide within a SRRXFLK sequence motif (6, 8). Signal peptides are recognized by the Sec and Tat machinery during the initial stages of protein translocation, and are cleaved by periplasmic signal peptidases upon export (29).

The Tat machinery is composed of *TatA*, *TatB*, and *TatC* proteins; some bacteria lack *TatB*, but in these cases the role of this protein is performed by *TatA* (17, 22, 45). Other organisms may also encode TatE, which acts as a functional homolog of *TatA* (39). To achieve substrate transport, the *TatB* and *TatC* proteins form a subcomplex in the inner membrane that recognizes the Tat-specific signal peptide (1). Recent evidence suggests that *TatA* protomers are part of the *TatB*C receptor complex and that after binding signal peptide, additional *TatA* subunits are recruited to form the translocase (2, 18). The active translocase requires the proton motive force for translocation of the substrate across the inner membrane. Upon export of the substrate, the *TatA* protomers dissociate from the recognition complex and the cycle can begin again.

To date, most studies of this protein transporter have been performed using *Escherichia coli* as the model. Recent analyses of bacterial and archaeal genomes reveals that in some organisms, multiple tat homologs are present (29). In fact, there are reports of Gram-negative bacteria with two distinct Tat translocases (24, 33). Similarly, there are two distinct Tat translocases in the Gram-positive bacterium *Bacillus subtilis*, though in this case only *TatA* and *TatC* components are found. In some cases, these extra Tat components are functionally redundant (4), while in other cases they are used to export specific substrates (13). There are few studies of protein transport in organisms with multiple Tat components, and thus the significance of possessing two distinct Tat pathways is not fully appreciated. In order to better characterize the importance of dual Tat pathways, we used the Gram-negative soil bacterium *Acinetobacter baylyi* as a model. Scanning the genome of *A. baylyi* reveals the presence of two distinct Tat pathways.

Remarkably, comprehensive mutagenesis of *A. baylyi* suggests that the components of one Tat machine are essential for growth, while the components of the other machine are dispensable (11). For clarity, henceforth we refer to the essential Tat genes as *tatA1*, *tatB1*, and *tatC1* and the non-essential Tat genes as *tatA2*, *tatB2*, and *tatC2*. In this report, we describe experiments characterizing the non-essential Tat pathway of *A. baylyi*. Specifically, we sought to understand how Tat-dependent protein export enables the growth of *A. baylyi* under different environmental conditions. We uncovered a role for the non-essential Tat pathway in maintaining normal cell envelope integrity and cellular morphology under stress conditions. Using co-culture experiments, we found that the non-essential Tat pathway provides a growth advantage to *A. baylyi* in competition with Pseudomonads that also possess two Tat machines. Taken together, our findings provide insight into the function of the non-essential Tat pathway of a model soil microbe.

MATERIALS & METHODS

Strains and growth conditions. Unless otherwise noted, growth of *A. baylyi* wild-type strain ADP1 (strain 33305 from the American Type Culture Collection; Manassas, VA) was performed at 30 degrees Celsius. For routine culturing, all bacterial strains used in this study were grown in LB Lennox (10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl per liter; Fisher Scientific). For growth curve experiments, each strain was grown overnight in LB and then diluted 1:100 in fresh media. These cultures were incubated with intermittent shaking at 200 rpm and optical density (OD) measurements (at 600 nm) were taken every 30 minutes for 8-10 hours. All strains were grown in triplicate.

Construction of an *A. baylyi tatC2*::kan insertion-deletion mutant. The *A. baylyi tatC2* mutant was constructed using an overlap-extension PCR strategy described previously (3). Briefly, upstream and downstream sequences flanking *tatC2* (ACIAD0521) were amplified by colony PCR from the *A. baylyi* wild-type strain ADP1 (Fig. 1A). The upstream flanking sequence was amplified using primers A (5'-TGGAG*TATA*TAAAAATGGC-3') and B (5'-ATTGTTTTAGTACCGAGCTCCTTG-GGCAGGCATGATGTC-3'). The downstream flanking sequence was amplified using primers C (5'-GCCATTTATTATTTCCTTCGATCCTC-GAAAAACGTAG-3') and D (5'-GATTACCTTTG-GCATCAAC-3'). A 795 bp kanamycin-resistance cassette was amplified from plasmid pIM1445 (gift from Ichiro Matsumura) (28) using primers E (5'-GAGCTCGGTACTAAAACAAT-3') and F (5'-GAAGGAAATAATAAATGGC-3'). These three products were mixed together in equal molar ratios in a new tube and joined together in a final PCR using the outer-most upstream and downstream flanking primers (A and D), resulting in the ::kan insertion-deletion allele. This PCR product was purified and then used to transform naturally competent wild-type *A. baylyi* (14). Incorporation of the insertion-deletion allele was confirmed by PCR analysis and DNA sequencing.

Construction of *pTatC2* expression plasmids. To perform complementation experiments on the *tatC2*::kan mutant, the wild-type *tatC* gene was cloned into the BamHI site of pWH1266 by custom gene synthesis (GenScript, Piscataway, NJ) using sequences from the *A. baylyi* genome (www.biocyc.org). Plasmids were transformed into *A. baylyi* as follows. From an overnight culture of wild-type strain ADP1, 0.1 ml of cells was gently mixed with 100 ng of plasmid DNA. The mixture was allowed to incubate at room temperature for 1 hour. To allow outgrowth of any transformed cells, 0.9 ml of LB was added to the mixture followed by overnight incubation at 30°C while shaking. The next day, 0.1 ml of the transformation mixture was plated onto LB-ampicillin agar and grown overnight at 30°C to select transformants.

Detergent sensitivity assay by Efficiency of Plating method. Cultures of wild-type, *tatC2* mutant, and complemented *tatC2* mutant bacteria were grown overnight in LB broth, then diluted in series in fresh LB in a 96 well plate. Using a multi-prong metal replicator, approximately 2 °l of each dilution was transferred to the surface LB agar or LB agar supplemented with 2% sodium dodecyl sulfate (SDS) and 1 mM ethylenediaminetetraacetic acid (EDTA). Plates were incubated overnight at 30°C and growth on the plate was assessed the next day.

Microscopic examination of cellular morphology. Wild-type and *tatC2* mutant starter cultures were grown at 30°C overnight. The next day, each strain was diluted 1:100 in fresh LB broth in duplicate. One set of strains was grown at 30°C while the duplicates were grown at 42°C until late exponential phase. Ten microliters of each culture was spotted onto a glass slide and the smears were allowed to air dry. Following heat fixation, the smears were stained with crystal violet and visualized using an Olympus BX41 microscope under the 100X objective with immersion oil. Images shown are a representative field from at least three independent experiments (Figure 2).

Competitive co-culture experiments. Overnight cultures were used to inoculate flasks containing 50 ml of fresh LB broth for competition assays. The optical density (OD600 nm) of each culture was normalized to obtain a final OD of 0.1 in the flask. Flasks were incubated at 200 rpm on a platform shaker at 30°C for 24 hours. One hundred microliter aliquots were taken from the initial inoculum and at

the 24-hour time point, diluted in 10-fold series to a final dilution of 10-7. To enumerate colony forming units (CFUs), 0.1 ml aliquots were plated from the dilutions to obtain well-isolated colonies. Individual competition assays were performed in triplicate. Input and output CFU counts were used to determine the competitive index (CI) for each *A. baylyi* strain using the following formula: CI = (Af/Ai)/(Bf/Bi), where A represents the *A. baylyi* strain and B represents the competitor strain, with f and i denoting the final and initial CFU counts, respectively. CI values were subsequently analyzed using one-way ANOVA.

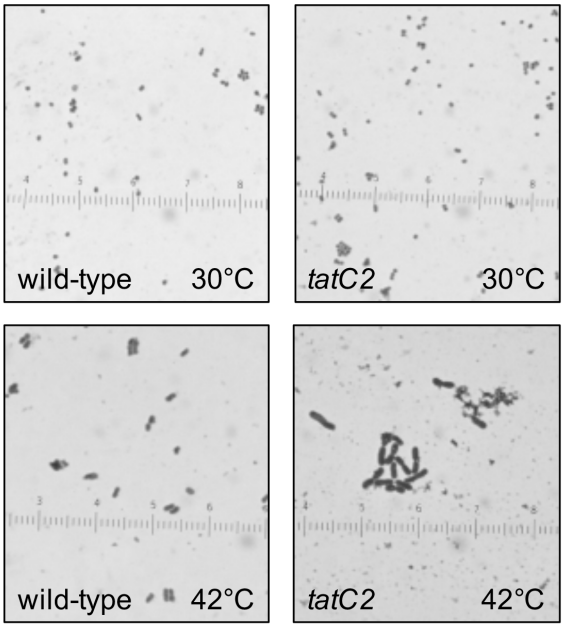
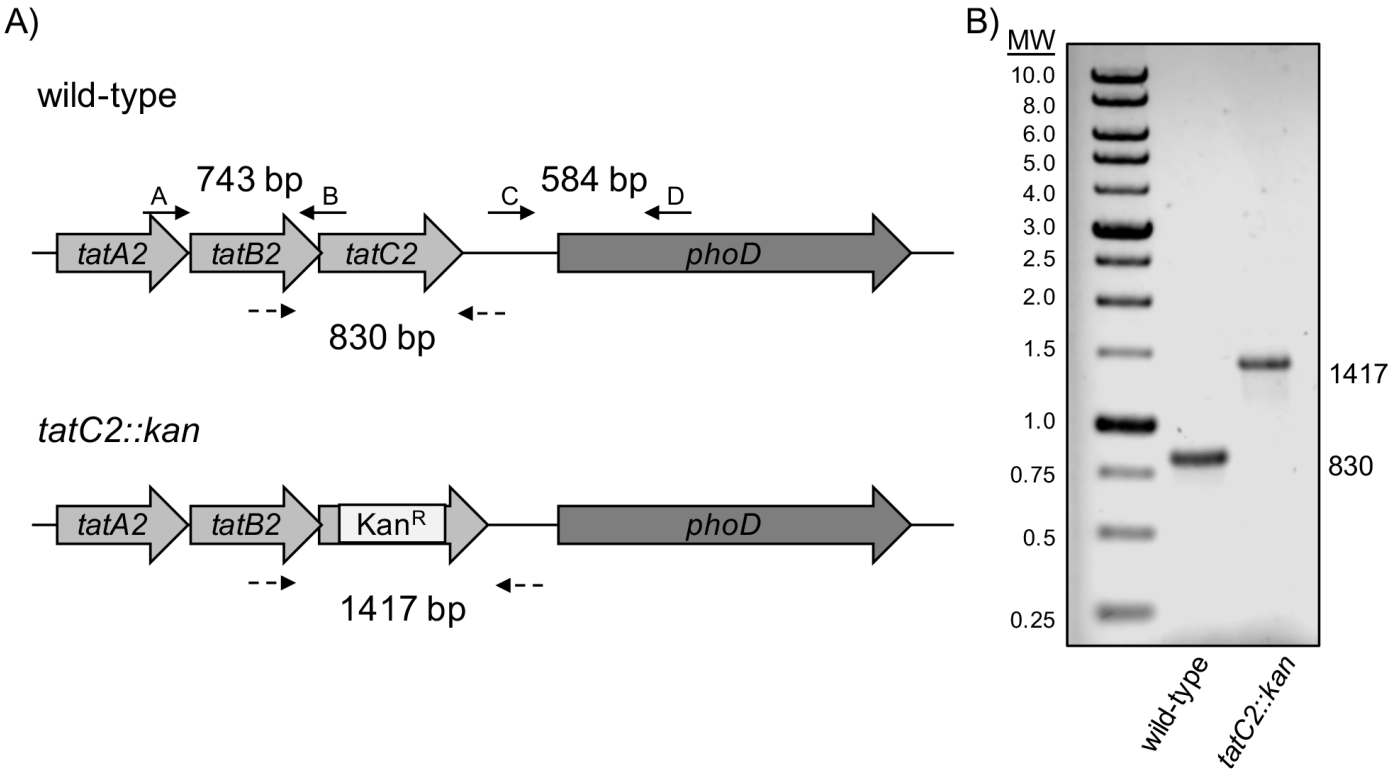


Figure 2. The *tatC2* mutant displays abnormal cellular morphology under high temperature stress conditions. Overnight cultures of wild-type (left column) and *tatC2* mutant (right column) bacteria were diluted 1::100 into fresh LB broth and grown to late exponential phase at 30°C (top row) or 42°C (bottom row). Aliquots from each culture were heat-fixed to a glass slide and stained with crystal violet. Images were captured using the 100X objective. Each tick in the scale bar equals 1 um.

Figure 1. Strategy to construct a *tatC2*::kan insertion-deletion mutant. A) Primers used to generate the upstream (A and B) and downstream (C and D) regions flanking *tatC2* are indicated by solid arrows. Primers used to for PCR analysis of KanR recombinants are indicated by dashed arrows. B) Colony PCR analysis of wild-type and *tatC2*::kan strains. PCR products were resolved on a 0.7% agarose gel and visualized by staining with ethidium bromide. Molecular weight (MW) size standards are shown (in kb) for comparison.



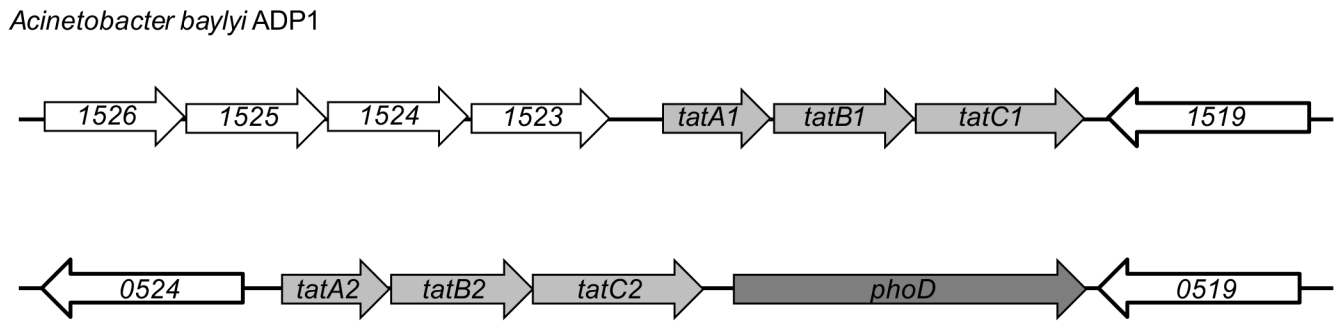


Figure 3. Genome architecture of the tat genes of *Acinetobacter* spp. Homologs of *tatA*, *tatB*, and *tatC* have light shading. Putative alkaline phosphatase *phoD* has dark shading. Flanking genes of unknown function are labeled with ACIAD genome reference numbers.

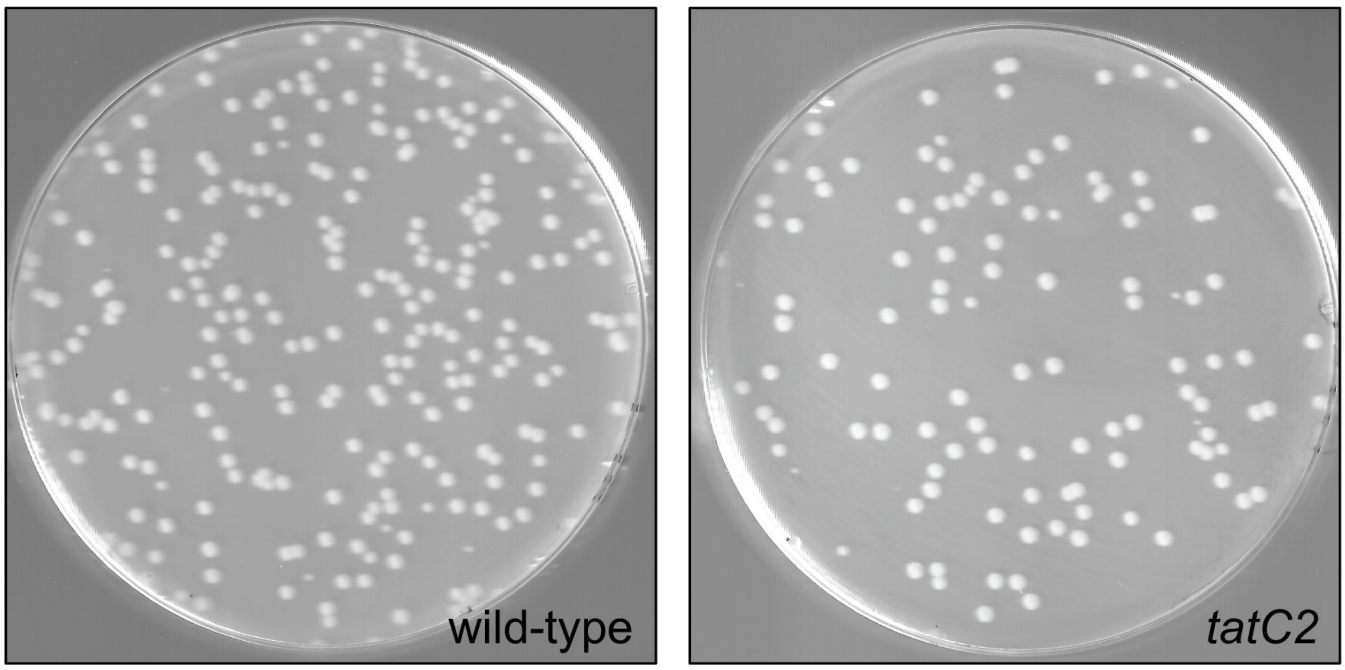


Figure 3. Colony morphology of wild-type and *A. baylyi tatC2* mutant strains. Wild-type (left) and *tatC2* mutant (right) *A. baylyi* strains were grown overnight in LB broth cultures at 30°C. Then serial dilutions of each strain were plated onto LB agar. The plates were incubated overnight at 30°C prior to imaging.

RESULTS

The genome of *Acinetobacter baylyi* encodes homologs of the Twin-arginine translocation (Tat) pathway. The genus *Acinetobacter* has recently drawn increased attention due to the rise of drug resistant isolates of *Acinetobacter baumannii* (31). In addition, *Acinetobacter baylyi* is of growing interest as a model Gram-negative organism due to its ease of genetic manipulation and diverse metabolic capabilities (14). While exploring the genome of *A. baylyi* for evidence of known protein export machines, we located homologs of the Tat pathway. Interestingly, when we examined the *A. baylyi* genome, we noted two homologs each of *tatA*, *tatB*, and *tatC* (Fig. 3). We found that the *TatA* homologs have the highest sequence identity to each other at 86%, while the sequence identity of the *TatB* and *TatC* homologs is lower scoring 62.3% and 70.8%, respectively (Table 1). In addition to the differences in amino acid sequence, there was also a reported difference in function between these two putative Tat pathways. De Berardinis and colleagues reported that the genes encoding one Tat pathway were essential for growth (*tatA1B1C1*), while genes encoding the other (*tatA2B2C2*) were dispensable for growth (11). This finding suggests the possibility that each of these Tat pathways has a different function in secreting proteins out of the cytoplasm. In particular, it could be the case that some Tat substrates are specifically routed through one Tat pathway rather than the other. As precedent, the dual Tat pathways of *Bacillus subtilis* are known to display substrate specificity (32). To clarify the role played by each Tat pathway in *A. baylyi* physiology, we focused on the non-essential Tat machine. Given the established role of *TatC* homologs in substrate recognition (19, 20, 35), we started our analysis by creating a loss-of-function *tatC2* mutant strain.

Construction and phenotypic analysis of a *tatC2* mutant. We constructed an insertion-deletion *tatC2* mutant using a previously described method (3). The *tatC2*::kan mutant allele was transformed into the wild-type strain of *A. baylyi* ADP1, and kanamycin-resistant colonies were selected on LB agar supplemented with kanamycin. Recombination of each mutant allele into the bacterial chromosome was confirmed by PCR and DNA sequence analysis (Fig. 1). We obtained numerous transformants for the *tatC2*::kan allele, suggesting that this gene is dispensable for growth under standard laboratory conditions, in agreement with previous findings (11). To begin characterizing these mutants, the mutant cultures were assessed for their growth properties. Colonies of the *tatC2* mutant are indistinguishable from wild-type *A. baylyi* when grown on LB plates at 30°C (Fig. 4). When grown in LB broth at 30°C, the *tatC2* mutant exhibited similar growth kinetics as wild-type *A. baylyi* (Fig. 5A). A similar growth trend was observed when we plated for viable counts (data not shown). In some organisms, loss of a functional Tat pathway has been associated with defects in virulence, while in other organisms tat mutants exhibit aberrant morphological and physiological phenotypes under *in vitro* stress conditions, including when subject to high salt or detergent concentrations (10, 42, 44). We next tested the ability of the *tatC2* mutant to grow when challenged with various environmental stresses.

The *tatC2* mutant is sensitive to detergents and growth at high temperature.

Defects in bacterial protein export machines, including the Tat pathway, are often accompanied by increased sensitivity to toxic small molecules (21, 25, 26, 36). In *E. coli*, the Tat pathway exports proteins that help maintain the structural integrity of the cell envelope. Failure to export these proteins weakens the cell envelope, thereby increasing its permeability. To determine if the non-essential Tat pathway is important for cell envelope integrity in *A. baylyi*, we tested the ability of the *tatC2* mutant to grow in the presence of the detergent SDS. Using an efficiency of plating assay, we found that growth of the *tatC2* mutant is impaired by treatment with detergent (Fig. 6). Importantly, we observed that complementation of the detergent sensitivity phenotype of the *tatC2* mutant could be achieved by constitutive expression of *tatC2* on a plasmid.

Given the increased cell envelope permeability associated with the *tatC2* mutant, we wondered if the non-essential Tat machine was important to resist temperature stress. Although the *tatC2* mutant grew as well as wild-type at 30°C, its growth was impaired at 42°C (Fig. 5B). At later time points, growth of the *tatC2* mutant slowed as compared to wild-type cultures grown at 42°C. This finding is consistent with the the function of Tat in other organisms and suggests that the non-essential Tat pathway of *A. baylyi* plays a role in cell envelope biogenesis (10). Given the established role of Tat in maintaining normal cellular morphology (7), we next examined the cellular morphology of the *tatC2* mutant by microscopy (Fig. 2). We found that the *tatC2* mutant cells grown at

30°C were indistinguishable from the wild-type cells. Both strains appeared as small coccobacilli, which is characteristic of the *Acinetobacter* genus (41). At 42°C, cells of wild-type *A. baylyi* were slightly larger than those grown at 30°C. Cells of the *tatC2* mutant were longer and thicker when grown at 42°C. Thus *TatC2* is important for maintaining normal cellular morphology of *A. baylyi* under high temperature stress conditions.

The non-essential Tat pathway provides a competitive advantage to *A. baylyi* grown in co-culture.

We showed that *TatC2* is important for maintaining normal cellular morphology under stressful growth conditions in the laboratory. This led us to wonder how important the non-essential Tat machine is for *A. baylyi* to grow in the environment. The soil is home to complex microbial communities; these microbes are often in competition with each other for scarce nutrients. In such an environment, some protein secretion systems are important for nutrient acquisition (5, 27, 37), while others are used to directly inhibit growth of microbial competitors (16, 38, 43). With this in mind, we tested whether the non-essential Tat pathway, and *TatC2* in particular, was important for the growth of *A. baylyi* in a competitive co-culture system. *Pseudomonas* species are closely related to *Acinetobacter* species, and often share similar environmental niches. Therefore, we selected a subset of *Pseudomonas* species for the co-culture experiments. As shown by the competitive index (CI) values in Table 2, all three *Pseudomonas* strains tested grew significantly better than both wild-type and *tatC2* mutant strains of *A. baylyi*. However, when comparing the relative CI values between wild-type and the *tatC2* mutant, we noticed that the *tatC2* mutant was significantly impaired when grown in competition with *P. putida*. In competitions with both *P. aeruginosa* and *P. fluorescens*, there was no significant difference in CI between wild-type and the *tatC2* mutant. The difference in CI does not appear to be due to a secreted toxin as *P. putida* grown in spent media from either wild-type *A. baylyi* or the *tatC2* mutant showed no difference in growth compared to control (data not shown).

Table 1. Similarity between *A. baylyi* Tat proteins and their homologs.

Tat Protein[a]	Accession no.	% similarity	
		Tat1	Tat2
Acinetobacter baylyi TatA1	WP_004925257	100.0	86.0
Acinetobacter baylyi TatA2	CAG67449.1	86.0	100.0
Acinetobacter baylyi TatB1	WP_004925259	100.0	62.3
Acinetobacter baylyi TatB2	WP_004920102	62.3	100.0
Acinetobacter baylyi TatC1	WP_004925261	100.0	70.8
Acinetobacter baylyi TatC2	WP_004920104	70.8	100.0
Escherichia coli TatA	WP_001295260	69.5	66.2
Escherichia coli TatB	WP_053879116	54.2	53.3
Escherichia coli TatC	WP_072859488	66.4	64.8
Escherichia coli TatE[b]	WP_047613167	77.3	76.9

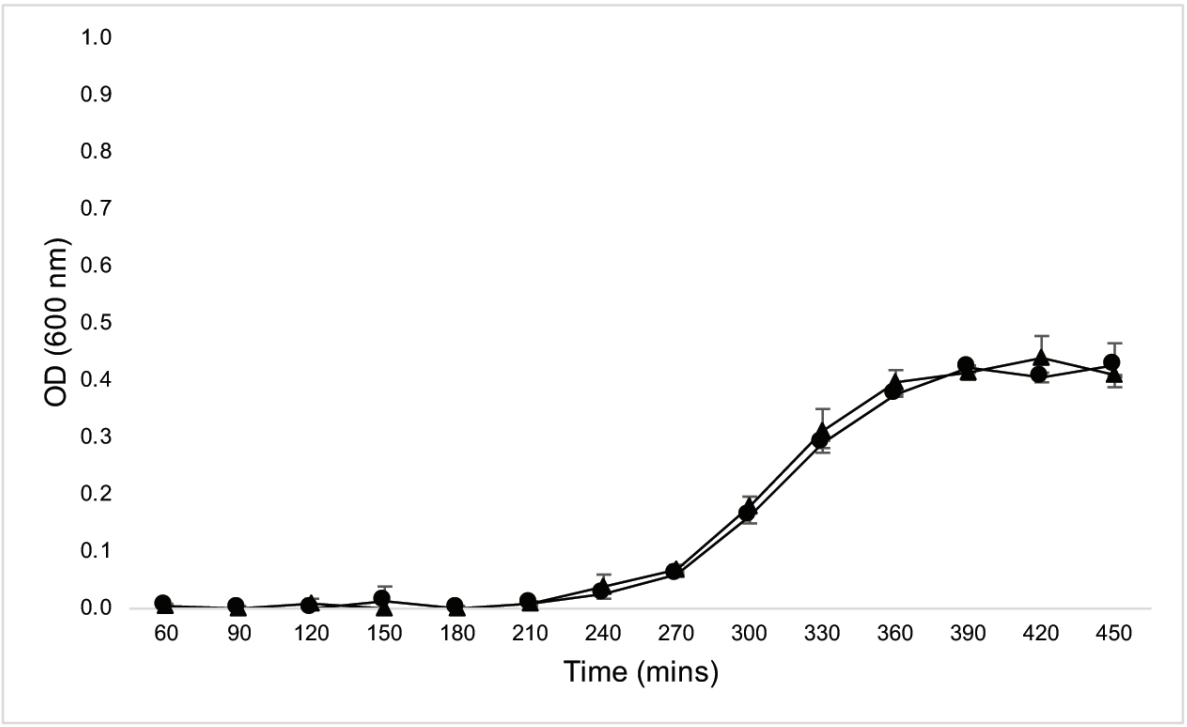
[a] Protein sequences were obtained from NCBI for *A. baylyi* ADP1 and *E. coli* MG1655 and were analyzed by pairwise BLAST. [b] Similarity as compared to *A. baylyi* TatA1 or TatA2.

Table 2. Competition between *A. baylyi* and *Pseudomonas* spp.

A. baylyi strain	*Pseudomonas* strain	Mean CI[a]	SD[b]
wild-type	*P. aeruginosa*	0.086	0.014
tatC2	*P. aeruginosa*	0.074	0.013
wild-type	*P. fluorescens*	0.133	0.023
tatC2	*P. fluorescens*	0.146	0.021
wild-type	*P. putida*	0.148[c]	0.040
tatC2	*P. putida*	0.043[c]	0.011

[a] $CI = (A_f/A_i)/(B_f/B_i)$; A represents the *A. baylyi* strain and B represents the *Pseudomonas* strain, with *f* and *i* denoting the final and initial CFU counts, respectively. [b] Standard deviation (SD) calculated from the mean of triplicate experiments. [c] $P < 0.05$ as calculated by ANOVA.

A)



B)

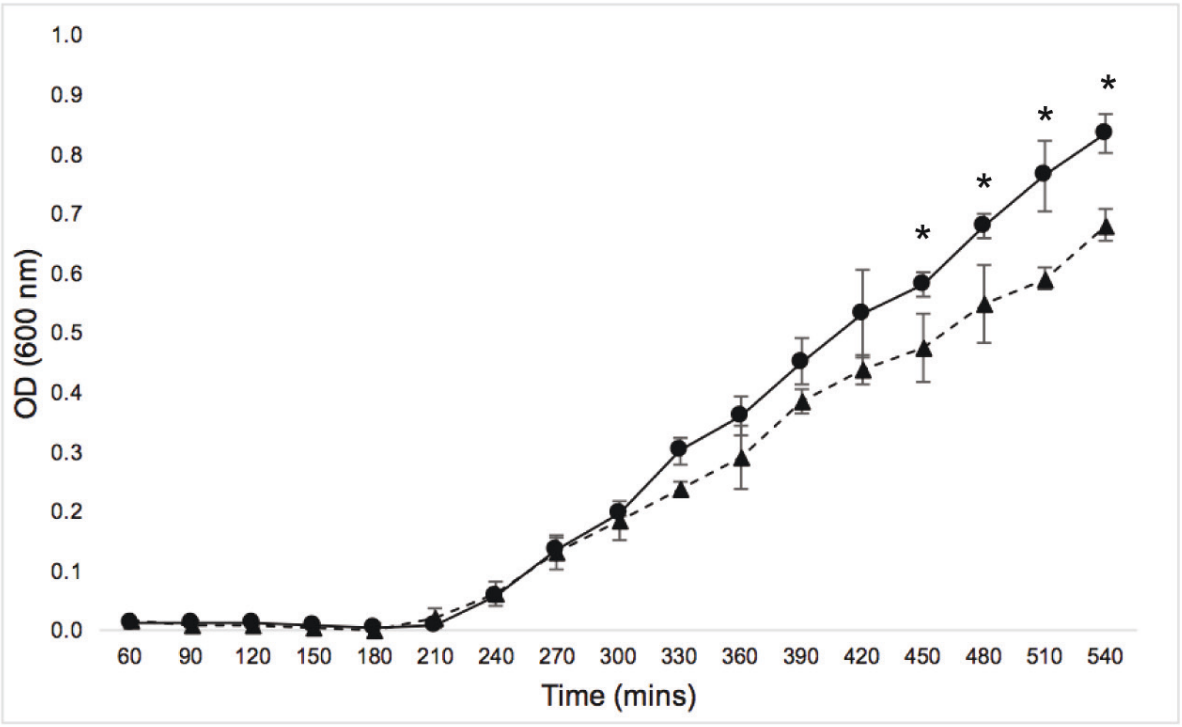


Figure 5. Growth of wild-type and *A. baylyi tatC2* mutant strains in liquid culture. Wild-type (●) and *tatC2* mutant (▲) *A. baylyi* strains were grown at 30°C (A) or 42°C (B) in LB broth cultures. Overnight cultures of each strain were diluted 1:100 in fresh LB at the start of the experiment. Optical density (OD600 nm) was measured periodically. Each data point represents the mean of triplicate experiments. Error bars indicate standard deviation from the mean. ⋆, $P < 0.05$ by one-way ANOVA.

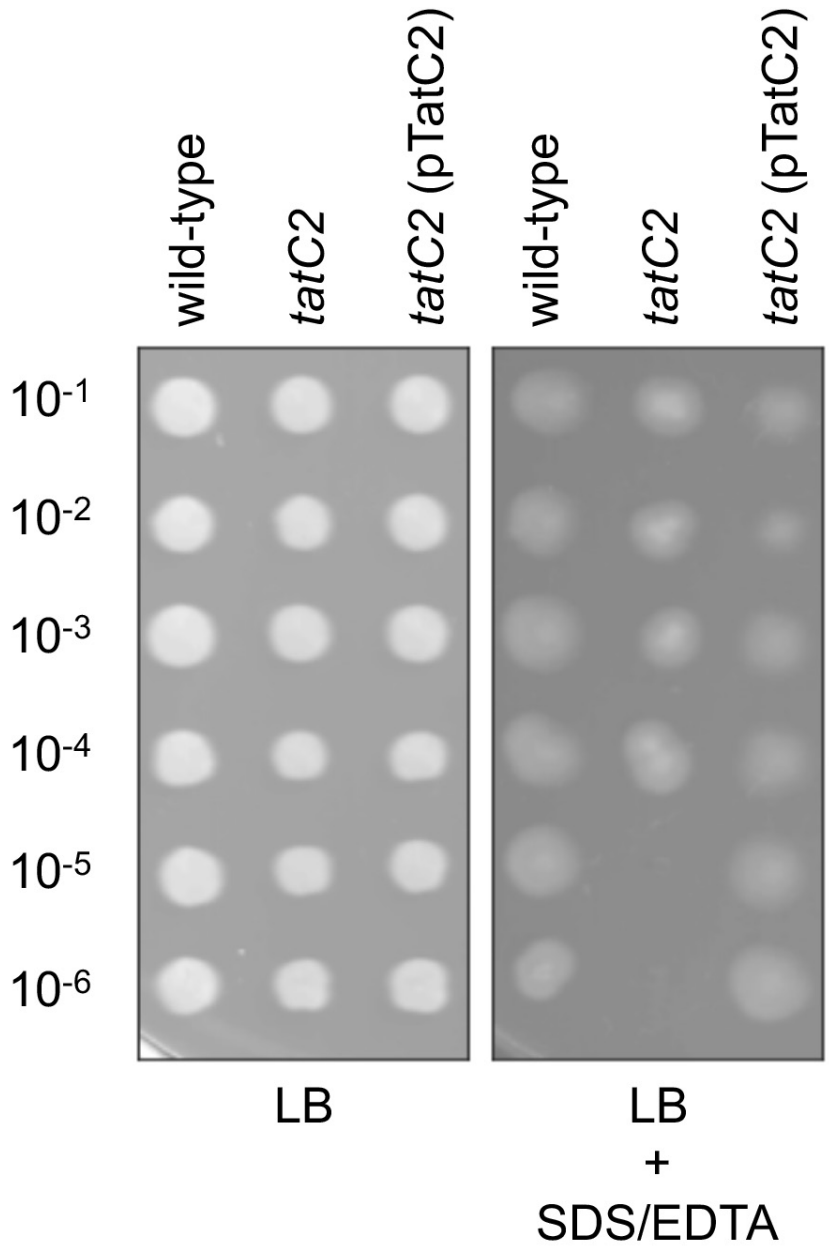


Figure 6. The *A. baylyi tatC2* mutant is sensitive to detergents. The indicated strains were tested for detergent sensitivity using an efficiency of plating assay. Overnight cultures of each strain serially diluted and plated onto LB agar or LB agar supplemented with 2% SDS + 1 mM EDTA. Each plate was incubated overnight at 30°C prior to imaging. Shown is a representative experiment from triplicate trials.

DISCUSSION

Despite the fundamental nature of protein export systems, the important contributions of these pathways to bacterial physiology and growth is often underappreciated. Since its discovery, much has been learned about the structural features and mechanistic details of how the Tat pathway transports proteins. Yet there are still key questions to be addressed. Specifically, how do organisms with two Tat pathways sort their cargo to the correct translocase? What is the benefit to having two Tat pathways? Using *A. baylyi* as a model system, we have started exploring these questions in detail.

By disrupting the non-essential Tat pathway by mutation of *tatC2*, we uncovered a role for this machine during stress conditions. When exposed to detergents, viability of the *tatC2* mutant was decreased as compared to wild-type. This finding supports a role for the non-essential Tat pathway in maintaining integrity of the bacterial cell envelope. We also found that the *A. baylyi tatC2* mutant displays aberrant cellular morphology under high temperature stress. Similar phenotypes have been observed in tat mutants of other bacterial species; notably, cells of *E. coli* tat mutants form long chains because the cell wall amidases important for proper cell division are Tat substrates (7). Our search of the *A. baylyi* genome did not reveal homologs to these particular amidases. Given that *E. coli* only possesses a single Tat machine, it is possible that there is a more diverse collection of Tat substrates in organisms with two Tat machines like *A. baylyi*. We believe that biochemical methods will be needed to identify the full suite of Tat proteins in *A. baylyi*.

While the role of protein export systems in bacterial pathogenesis has been well-studied, the importance of these machines with respect to growth in polymicrobial communities is poorly understood. By using a simple co-culture assay, we uncovered a role for the non-essential Tat pathway when grown in competition with other bacterial species. Interestingly, this phenotype was only evident when *A. baylyi* was grown in competition with *P. putida*, an organism that also encodes two distinct Tat translocases. To explain this difference in CI among the *Pseudomonas* strains, we considered the basic physiology of all three organisms. All three grow at a similar rate and under similar conditions. *P. aeruginosa* is a human pathogen but can also be isolated in environmental samples (40). Neither *P. fluorescens* nor *P. putida* are human pathogens, however, both are associated with the plant root microbiome (9, 34). Interestingly, when we examined the genomes of each species for tat gene homologs, we found that *P. aeruginosa* and *P. fluorescens* possess genes encoding a single Tat pathway, while *P. putida* encodes genes for two separate and distinct Tat pathways. Perhaps the non-essential Tat pathway of *A. baylyi* is important when facing competition from other dual-Tat pathway bacteria, particularly when growing in complex microbial communities in soil. More experiments are needed to rigorously test this possibility.

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